Phytochemical-rich potato extracts and potential for risk reduction in tamoxifen treatment of breast cancer

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Abstract

Existing data suggest an inverse correlation between breast cancer risk and vegetable consumption, and the anticancer effects of vegetables are attributed to the diversity and abundance of phytochemicals. Standard endocrine therapies for breast cancer are associated with significant side effects and not always effective. Undoubtedly, there is a need for improved treatment of breast cancer. In the quest for better breast cancer treatments with fewer side effects, food and nutrition represent a logical strategy to explore. Potato (*Solanum tuberosum* L.) was chosen for the present project as the target vegetable for investigation. Pigmented potato has recently attracted research attention because of its potential health benefits. Two potato extracts were prepared from a white and purple variety ('Urenika') and referred to as WPE and PPE respectively. Tamoxifen and estradiol exhibited paradoxical effects: each of them was inhibitory at high doses but stimulatory at low to moderate doses, on proliferation of two breast cancer cell lines, MCF-7 and T-47D. In contrast, both PPE and WPE inhibited cell proliferation in a dose-dependent manner without paradoxical effects. The potato extracts also blocked estradiol- or tamoxifen-induced cell proliferation of these two cell lines. These findings imply that both potato extracts may have a role to play in prevention of breast cancer, or complementing tamoxifen to achieve desirable treatment efficacy. Since both PPE and WPE were equivalent in efficacy, one (PPE) was selected for further study, given the intention of developing a nutraceutical or therapeutic product of New Zealand proprietary value. Phytochemical compositions of the potato extracts were identified and quantified using ultra high performance liquid chromatography-mass spectrometry, many of which were reported for the first time in variety 'Urenika'. Several compounds were found at doses which have been reported individually to exert bioactive effects against cancer. It is possible the antiproliferative effects of potato extracts resulted from more than one of these bioactive compounds working together. Dose-dependent apoptotic effects of PPE were observed in T-47D culture, and a combined effect seems to exist between PPE and tamoxifen in modulating the S and G2/M phase. In summary, the key contributions and significance of current thesis are: (1) demonstration

of the "risk" zone for tamoxifen (10^{-8} to 10^{-6} M) and estradiol (10^{-10} to 10^{-8} M) concentrations which may stimulate breast cancer cell growth. Note that these concentrations of tamoxifen or estradiol are physiologically achievable. Furthermore, one key novel finding is regarding the estradiol dependency of tamoxifen action. Specifically, at low to moderate doses (10^{-9} to 10^{-8} M) of tamoxifen, there is a threshold of estradiol (> 10^{-8} M) which allows a significant inhibitory action to occur. The stimulatory action of tamoxifen and complex interaction between tamoxifen and estradiol observed *in vitro* may partially explain the failure of tamoxifen treatment in some patients. Owing to the vast differences between cell culture experiments and the human body, a more systematic *in vivo* investigation of clinical effects of tamoxifen over a range of different doses under various estradiol concentrations is warranted; (2) pioneering data on the efficacy of 'Urenika' extract against breast cancer *in vitro*; (3) a metastatic breast cancer animal model which successfully generated metastasis to distant sites (lymph nodes, lungs, livers and spleens), mimicking advanced stage of breast cancer in humans. This model could be used in future testing of the effect of PPE and the combined treatments (PPE with tamoxifen) on establishment and metastasis; and (4) a 'refined' non-invasive feeding methodology, which is more ethical than oral gavages, for tamoxifen administration in mice was developed and results obtained were comparable to the method of intraperitoneal injection. Using this model and the non-invasive feeding method, a dose-dependent stimulatory effect of tamoxifen on growth of 4T1 tumours was observed in mice. The current thesis has derived a new hypothesis which may be worth clinical investigation: tamoxifen may induce excessive leukocytosis which contributes to tumour invasiveness and growth. This thesis also represents a significant contribution to the potential use of potato extracts in reducing the risk of tamoxifen in stimulating cancer growth.

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Last but not least, I wish to acknowledge my God for constantly renewing my strength, so I could run and not be weary, during this PhD journey, perhaps the most exhausting yet amazing chapter of my life.

I dedicate this thesis especially to my mother and father,
who work extremely hard for my education,
who encourage me to pursue my dreams,
who catch me when I fall,
whose love sustains me till the end.

I also wish to dedicate this thesis to Irene who encourages me throughout my PhD journey.

Lastly, in loving memory, I dedicate this thesis to my late grandmothers and aunt. My aunt was another breast cancer patient whose bravery in battling the disease inspired me to contribute to cancer research.

Table of Contents

List of Figures

List of Tables

Abbreviations

AC	Adriamycin + cyclophosphamide
ACY	Anthocyanins
AI	Aromatase inhibitor
AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
AP-1	Activation protein-1
ATCC	American Type Culture Collection
BMI	Body mass index
BPC	Base peak chromatogram
BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2
CA	Caffeic acid
CChA	Cryptochlorogenic acid
CFBS	Charcoal-treated fetal bovine serum
CFCS	Charcol-treated fetal calf serum
ChA	Chlorogenic acid
CMF	Cyclophosphamide + methotrexate + fluorouracil
CO_2	Carbon dioxide
CT	Computerized Tomography
DAB	p-dimethylaminoazobenzene
DMEM	Dulbecco's minimal essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E2	Estradiol
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMH	Extramedullary hematopoiesis
EPIC	European Prospective Investigation Into Cancer and Nutrition Italy Study
ER	Estrogen receptor
ERE	Estrogen responsive element
ER-	Estrogen receptor-negative
ER+	Estrogen receptor-positive
ESI	Electrospray ionization
FAC	Fluorouracil + adriamycin + cyclophosphamide
FAO	Food and Agriculture Organization
FCS	Fetal calf serum
FSC	Forward-angle light scatter
GC	Gas chromatography

H&E	Hematoxylin and eosin
HCA	Hydroxycinnamic acids
HER2	Human epidermal growth factor receptor type 2
IC_{50}	Concentration of an agent which shows 50% inhibition of the response measured (e.g. cell proliferation)
IGF	Insulin growth factor
IGFR	Insulin growth factor receptor
IMEM	Improved minimum essential medium
IMEM-ZO	Improved minimum essential medium, zinc option
IP	Intraperitoneal injection
LHRH	Luteinizing hormone releasing hormone
m/z	Mass-to-charge ratio
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium
MF	Methotrexate + fluorouracil
µg/mL	Microgram per Litre
µM	Micromole per Litre
MISS	Membrane-initiated steroid signalling
MLN	Mediastinal lymph node
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NASH	Nonalcoholic steatohepatitis
NChA	Neochlorogenic acid
OA	Ovarian ablation
PBS	Phosphate buffered saline
PI	Propidium iodide
PI3K	Phophoinositide 3-kinase
PPE	Purple potato extract
RNase	Ribonuclease
RPMI	Roswell Park Memorial Institute
SEM	Standard error of mean
SSC	Side-angle light scatter
Tam	Tamoxifen
TD	Diameter
TOF	Time-of-flight
UHPLC	Ultra high performance liquid chromatography
WCRF	World Cancer Research Fund
WPE	White potato extract

Chapter 1 Literature review: current issues with endocrine treatments, and directions for the present thesis

1.1 Breast cancer epidemiology: magnitude of the problem

About one tenth of all new cases of cancer registered globally are cancer of the female breast. Breast cancer accounts for 23% of cancer diagnoses in women and is by far the most common form of female cancer in all regions (135 countries) (Bray et al., 2012). A total of 1.4 million new breast cancer cases were diagnosed and 0.5 million women died of this disease in 2008 (Jemal et al., 2011). One of the determinants of prognosis and survival is the stage of cancer at diagnosis, which takes into account tumour size and whether there is lymph node involvement and/or distant metastasis. Despite treatment, some patients with primary breast cancer eventually suffer a relapse (i.e. 24-30% among patients with node-negative disease and 40-80% of those with node-positive disease) (Adelson et al., 2011). Patients have merely 1.5 to 3 years of median survival if metastasis occurs.

Breast cancer incidence or incidence rate, defined as the number of new cancer cases occurring in a defined population within a specified period of time, has continued to rise globally since the 1960s (Figure 1.1) (Bray et al., 2004; Kamangar et al., 2006; Parkin & Fernandez, 2006). Geographical variation in breast cancer prevalence is evident. Prevalence is defined the number and/or the proportion of people with past or present diagnosis of disease, within a well-defined population at fixed point in time (Verdecchia et al., 2002). Incidence rates are high in the United States, Western and Northern Europe, Australia and New Zealand (> 80 per 100000 females) and comparatively lower in Asia and Africa (< 30 per 100000) (Jemal et al., 2011). The international divergence in breast cancer incidence has attracted a great deal of research. For instance, it has been reported that Asian women who migrated to the U.S. had a higher risk (possibility of suffering the disease) than women in their countries of origin (Ziegler et al., 1993). The authors also found that migrants who lived

there for 10 years or more had 80% higher risk of breast cancer compared to recent migrants. Location of residence was also found to be a determinant; migrants living in urban areas have 30% higher risk than migrants in rural areas. A similar study showed breast cancer risk among Asian-Americans who migrated from China or Japan was approaching that of native Americans, and was two-fold higher compared to that among women living in Asia (Stanford et al., 1995). Consistently, a shift to a higher breast cancer risk was associated with migration of women to Australia from Italy, a country with low breast cancer incidence (McMichael & Giles, 1988). Furthermore, breast cancer mortality rate among these migrants approached that of the Australian-born population as the duration of residence increased. Overall, migration studies have provided a strong body of evidence suggesting that environmental changes, with diet being one of the major contributing factors, are responsible for the geographical variation of breast cancer incidence (Bray et al., 2004; McPherson et al., 2000).

The majority of breast cancer cases are diagnosed among elderly, with the average age at diagnosis of 55 years (Blamey et al., 2010; Grube & Giuliano, 2007). Therefore, it is plausible that a longer life expectancy in developed countries has contributed to a higher breast cancer incidence compared to developing countries. For instance, USA, Australia and New Zealand, with female life expectancy of >80 years, have a higher breast cancer incidence (80-100 per 100,000), compared to South Africa and India (<40 per 100,000), where female life expectancy is only 50-60 years (Jemal et al., 2011; Vaupel et al., 2011). Nevertheless, this notion is not valid for countries such as Japan, which has a very high life expectancy (87 years), but yet has considerably lower breast cancer incidence than other developed countries (<30 per 100,000, Figure 1.1) (Vaupel et al., 2011). Some influences, including reproductive factors, use of hormonal therapy, and nutritional determinants (consumption of calorie-dense food, obesity, physical inactivity) are more recognized as contributing factors than life expectancy to the risk and incidence of breast cancer, as reviewed in the following sections.

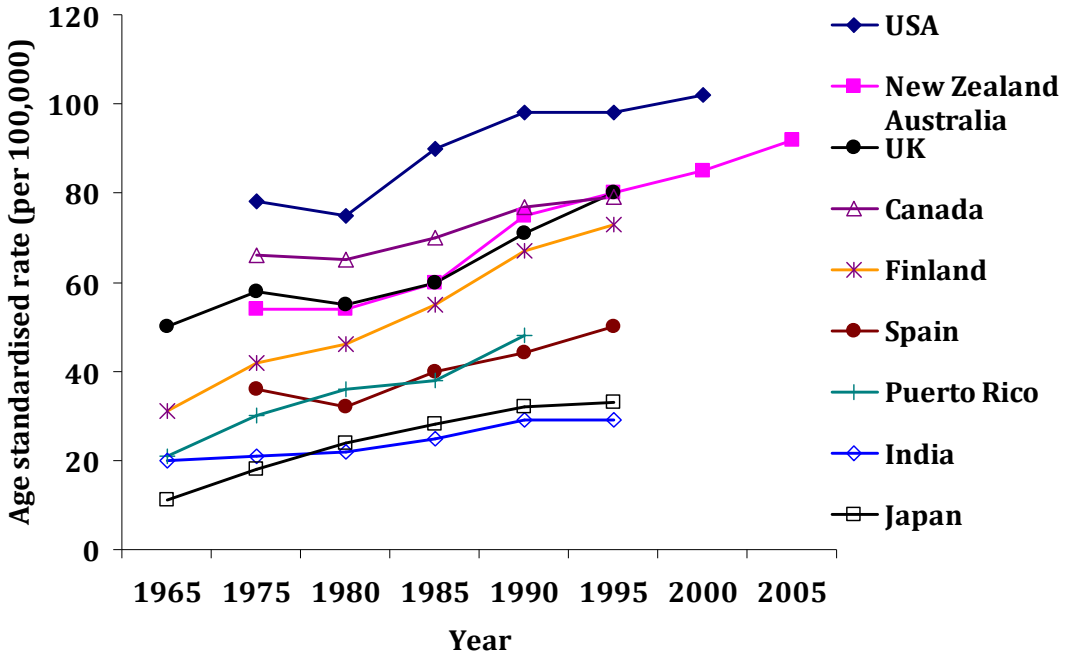


Figure 1.1 Breast cancer incidence rate between 1965 and 2005 in many countries.

(Source of information: Bray et al., 2004; New Zealand Health Information Service, 2007).

1.2 Breast cancer risk factors

1.2.1 Reproductive and nutritional factors

Several reproductive characteristics are recognized as risk factors for breast cancer. Early onset of menarche (≤ 11 years), regular ovulation, and late age at menopause (≥ 55 years) increase the risk of breast cancer. To the contrary, lactation is a convincing protective factor against breast cancer (Calhoun & Giuliano, 2007; Key et al., 2001). Body fatness is now an established breast cancer risk factor in postmenopausal women, and probably a risk factor for premenopausal women as well (World Cancer Research Fund / American Institute for Cancer Research, 2007). A large study, Women's Health Initiative Observational Study (n = 85,917; age 50-79) confirmed that obesity increased the risk of breast cancer (relative risk = 2.52 comparing BMI > 31.1 *vs.* BMI ≤ 22.6) (Morimoto et al., 2002). The international variation of breast cancer

prevalence may also be partly explained by the variation of obesity prevalence from one country to another. This notion was briefly examined based on some data on obesity prevalence and breast cancer incidence rate in the literature (Bray et al., 2004; Jemal et al., 2011; Low et al., 2009). Notably, countries which have a high breast cancer incidence rate also tend to have a higher national obesity prevalence ($R^2 = 0.8959$) (Figure 1.2).

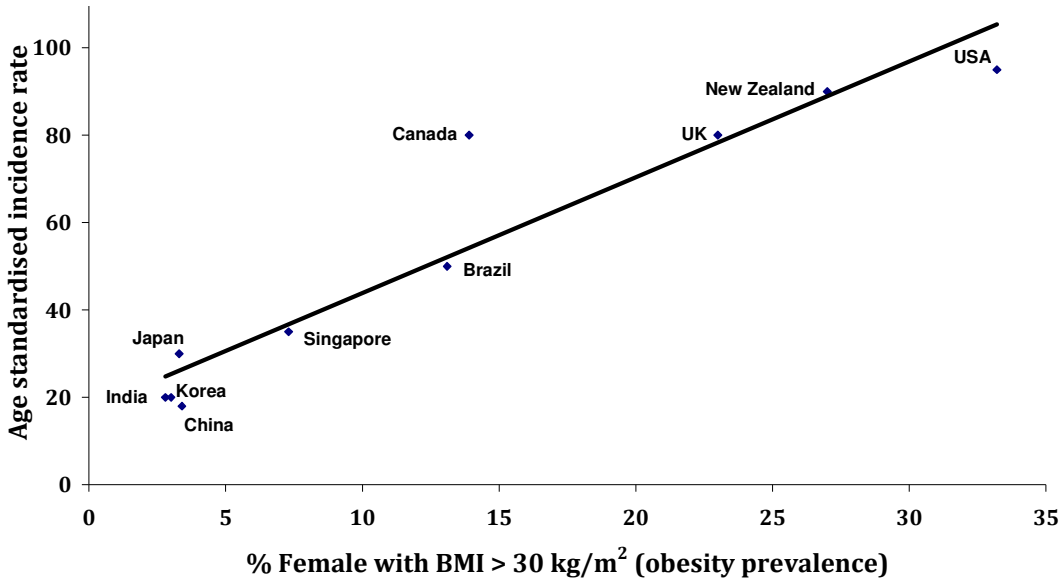


Figure 1.2 Breast cancer incidence versus obesity prevalence in many countries.

(Source of data: Bray et al., 2004; Jemal et al., 2011, Low et al., 2009).

1.2.2 Alcohol consumption

Alcohol consumption increases breast cancer risk, as suggested by a strong body of evidence (World Cancer Research Fund / American Institute for Cancer Research, 2007). Alcohol use alone may be responsible for 4% of the breast cancer incidence in the developed countries, based on a meta-analysis of 53 epidemiological studies (58,515 breast cancer patients, 95,067 controls), which represents over 80% of worldwide evidence (Hamajima et al., 2002). Recently, a large prospective observational study (n=105,986) showed even a moderate alcohol intake (5 to 9.9 g alcohol per day), earlier or later in adult life, could

increase the risk significantly (Chen et al., 2011). The drinking culture and trend of increasing alcohol intake among women over the past decades may possibly have played a part in the increasingly high breast cancer incidence rate in New Zealand (McEwan, 2010; McPherson et al., 2004).

1.2.3 Animal products: dairy, meat and eggs

It has been hypothesized that animal products, particularly dairy products and meat could significantly increase the risk of breast cancer, since these foods are commonly consumed in the Western countries where breast cancer incidence rates are high. A pooled analysis of 20 cohort studies found no significant association between intakes of total meat, red meat, white meat, dairy products and risk of breast cancer (Missmer et al., 2002). Another meta-analysis of cohort studies found a direct correlation between intake of total dairy food, but not milk, and risk of breast cancer (Dong et al., 2011). A large scale prospective cohort study, the European Prospective Investigation into Cancer and Nutrition (EPIC), which obtained data from 10 countries and 319,826 subjects, found no consistent relation between consumptions of meat, eggs, or dairy products (Pala et al., 2009). Another recent large scale study, the Black Women's Health Study, involving 52,062 subjects, also found no significant evidence to support the hypothesis that total dairy or meat intakes increases the risk breast cancer (Genkinger et al., 2013).

Nonetheless, it is noteworthy that intakes of specific group of dairy or meat products have been shown to have an association with breast cancer risk. Specifically, the EPIC study found butter consumption may contribute to breast cancer risk. This finding agrees well with another study, Nurses' Health Study II (n = 90655 premenopausal women), which has revealed intake of animal fat, mainly from red meat and high-fat dairy foods could increase the risk of breast cancer (Cho et al., 2003). The EPIC study also found significant association between high intakes of processed meat, which agrees well with another study, the UK Women's Cohort study (n = 35,372) (Taylor et al., 2007). Processed meat is high in nitroso compounds, a known carcinogen, and usually high in saturated

fat as well. Furthermore, intake of meat cooked at high temperature was also found to increase breast cancer risk (Pala et al., 2009). Meat cooked at high temperature is the source of established mammary carcinogens, heterocyclic amines and polyaromatic hydrocarbons (Linos & Willett, 2009). Altogether, it appears that it is not the diary food or meat as a whole, but rather the fat content of these foods and the preparation method which are the major concerns or risk factors in relation to breast cancer.

1.2.4 Exposure to biochemical and physical carcinogens

Use of oral contraceptives or hormone replacement therapy have also been associated with increased breast cancer risk (Weiderpass et al., 2011). Diethylstilbestrol, a synthetic estrogen, used clinically as estrogen supplementation for prevention of miscarriage, was implicated to increase breast cancer risk (Bamigboye & Morris, 2003; Weiderpass et al., 2011). Exposure to X-radiation and gamma-radiation is now recognized as a breast cancer risk factor, whereas it is unclear for electromagnetic field exposure (Weiderpass et al., 2011). Cigarette smoking, including exposure to passive smoking, is found to weakly associate with breast cancer risk (Khuder et al., 2001; Luo et al., 2011).

1.2.5 Emotional factors: stress & depression

The idea of a possible link between emotion or mind and cancer has begun since the 18th century (Priestman et al., 1985). Psychoneuroimmunology is a popular tool used to study the link between psychological features, specifically stress and depression, and cancer. Animal and human studies have shown chronic stress and depression resulted in compromised immune system, such as decreased cytotoxic T-cell and natural-killer-cell activities, that may contribute to initiation and progression of cancer (Reiche et al., 2004). On the other hand, recent meta-analyses of clinical studies, which investigated the relationship between stress or stressful events and breast cancer, have found no significant evidence to support this hypothetical link (Duijts et al., 2003; Santos et al.,

2009). However, these meta-analyses have concluded that high-intensity stress cannot be ruled out as a possible risk factor for breast cancer.

1.2.6 Genetic factor

In 1914, Theodor Boveri proposed that genetic abnormality is fundamental to cancer based on the work of David von Hansemann and Bover's own investigations (Boveri, 2008; Hansemann, 1890; Knudson, 2001). Subsequent studies by different groups have supported this concept to be correct (Berger et al., 2011). Analysis conducted by Alfred Knudson provided the evidence that as few as two mutations can initiate tumorigenesis (Knudson, 1971). It was proposed that in the inherited form of cancer, one mutant allele (first 'hit') is inherited via the germinal cells, and the other (second 'hit') is generated somatically or via the somatic cells.

Around five to 10 percent of breast cancers are associated with genetic predisposition. Forty percent of these cases are caused by inherited mutations of one of the two genes, breast cancer susceptibility gene 1 (BRCA1) and 2 (BRCA2) (Eeles & Powles, 2000; King et al., 2003). Carriers of these two gene defects are susceptible to a markedly increased risk of breast cancer, having a lifetime risk of 35 to 85% (Calhoun & Giuliano, 2007; King et al., 2003). Inherited mutations occuring in other genes such as CHEK2, ATM, TP53 and PTEN also influence the risk of breast cancer (Osborne et al., 2004; Walsh et al., 2006). For instance, CHEK2 (cell cyle checkpoint kinase 2) activates DNA damage repair as well as regulation of cell cycle progression after DNA damage, thus playing a central role in maintaing genomic integrity (Robson, 2010). Mutations occuring in CHEK2 has been asociated with an increased risk of breast cancer. The ataxia telangiectasia mutated (ATM) kinase senses DNA-strand breaks induced by ionizing radiation and initiates cell cycle checkpoint arrest, DNA repair or apoptosis by phophorylating a large number of proteins including CHEK2, BRCA1 and BRCA2 (Bernstein et al., 2010). Mutations in ATM have been associated with increased breast cancer risk particularly in the context of radiation exposure (Bernstein et al., 2010).

1.3 Estrogens: the link between risk factors and breast cancer

Estrogen is a generic term for a group of structurally similar substances that function endogenously as hormones (Ruggiero & Likis, 2002). Three most potent naturally occurring estrogens in the female body are estradiol, also known as 17β-estradiol (E2), followed by estrone (E1) and estriol (E3) (Coelingh Bennink, 2004). In premenopausal woman, E2, mainly produced by the ovary, is present at the largest quantity. In postmenopausal women, E1 is the main form of estrogen, produced by enzymatic conversion of androgen (androstenedione) by aromatase in the adipose tissue. E1 can also be converted into E2 by 17β-hydroxysteroid dehydrogenase type 1 (Pasqualini & Chetrite, 2005). E3 is only produced in significant amount during pregnancy, since it is made by the placenta from an androgen steroid (16-hydroxydehydroepiandrosterone sulfate). E2 is the most potent in causing biologic activity because it has the highest affinity to the estrogen receptors among the three forms of estrogens (Coelingh Bennink, 2004; Ruggiero & Likis, 2002).

Although the breast cancer risk factors discussed in section 1.2.1 & 1.2.2) are apparently different entities, most of them share some similarity. These risk factors are known to either modulate the levels of estrogen or duration of exposure to estrogen (Kelsey & Bernstein, 1996). Long menstrual history (early menarche, late menopause) increases the duration of lifetime exposure to endogenous estrogen. In contrast, lactation delays the restoration of ovulation (i.e. menstruation reduces or stops), which in turns leads to lower cumulative exposure to estrogen. Greater adiposity (body fatness) in postmenopausal women leads to higher endogenous estrogen levels. This is because estrogen biosynthesis mainly occurs in adipose tissue after menopause (Kelsey & Bernstein, 1996; Morimoto et al., 2002). Alcohol consumption (0.1-28 drinks per week) caused significant elevation of estrogen levels (Gavaler et al., 1993; Onland-Moret et al., 2005). One possible mechanism is that alcohol stimulates the adrenal gland to produce androgen, which can be converted to estradiol by

aromatase in the peripheral tissue (Onland-Moret et al., 2005). Therefore, it appears that several risk factors may mediate effects associated with estrogen.

To examine whether estrogen is indeed closely related to breast cancer risk, the Endogenous Hormones and Breast Cancer Collaborative Group (2002) reanalyzed 9 prospective studies. Their analysis concluded there was a strong correlation between the levels of endogenous estrogen and androgen and the risk of breast cancer in postmenopausal women. Among the various sex hormones involved, the strongest association was found for estradiol (Endogenous Hormones and Breast Cancer Collaborative Group, 2002). A few subsequent case-control studies revealed similar results (Farhat et al., 2011; Kaaks et al., 2005; Missmer et al., 2004). Likewise, in premenopausal women, the levels of endogenous estradiol were also shown to be directly related to breast cancer risk, based on a meta-analysis of 7 prospective studies (Walker et al., 2011). Collectively, all these studies support the hypothesis that estrogen, particularly estradiol, plays a pivotal role in the etiology of breast cancer.

1.4 Mechanism of estrogen action in breast cancer: Estrogen receptor signaling

Various forms of estrogen exist intracellularly, with the predominant one being estradiol. Estradiol promotes normal mammary epithelial cell proliferation, but also the proliferation of neoplastic breast epithelium (Russo et al., 2000). The involvement of estrogen receptor (ER) in estrogen-related cellular effects was first reported in the 1960s (Jensen & Jacobson, 1962). A second ER was discovered in 1995 and designated as ERβ; the first reported ER was named ERα (Cheskis et al., 2007). Most of the existing knowledge about estrogen signalling in breast cancer was derived from studies of ERα action (Stein & McDonnell, 2006). Normally, ERα is expressed at low levels in the nucleus of epithelial cells in the ducts and lobules. This normal low ERα-expressing, non-proliferating mammary cell phenotype changes to a high ERα-expressing, proliferating phenotype in high risk lesions. Overexpression of ERα has been commonly observed in early stage breast cancer (Hayashi et al., 2003). As much

as 75% of breast carcinomas upon diagnosis are ER-positive (Abe et al., 2005; Blamey et al., 2010; Clarke et al., 1998).

While there are various possible mechanisms of action responsible for the carcinogenicity of estrogen, the most widely recognized mechanism is the ER-mediated signaling that leads to increased cellular proliferation and decreased apoptosis in mammary tissue (Russo et al., 2000; Yager & Davidson, 2006a). Following is an overview of three major pathways involved in ER signaling, as depicted in Figure 1.3.

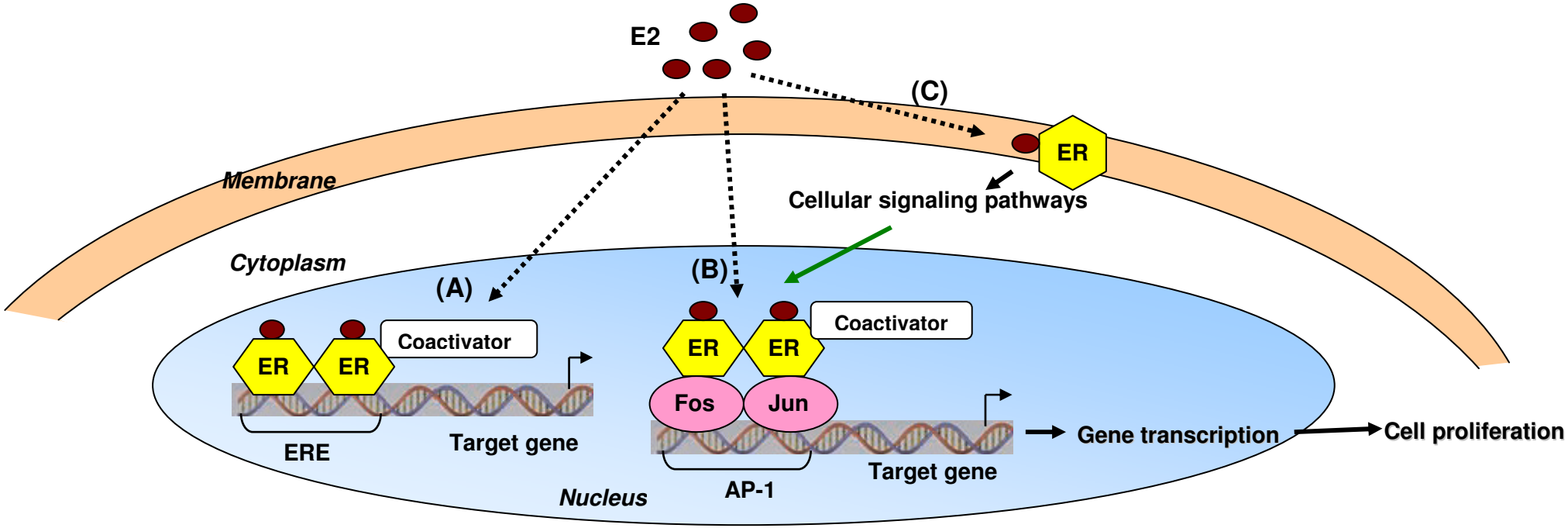


Figure 1.3 Estrogen receptor signalling.

There are at least 3 possible pathways by which estradiol (E2) may trigger estradiol signalling, causing transcription of the gene and cell proliferation. (A) Classical genomic pathway: E2-bound estrogen receptor (ER) recruits transcriptional coactivator, and then binds to estrogen response element (ERE) in the promoter region of target gene; (B) Non-classical genomic pathway: E2-ER-coactivator complex bound to transcriptional factors (Fos, Jun), which bind to activator protein-1 (AP-1) responsive element in the promoter region of target gene; (C) E2 binds to membrane ER, inducing cellular signalling pathways such as mitogen-activated protein kinase, which enhances the activity of ER and coactivator thus nuclear ER signalling.

(Keys: **--->** entering into cell membrane or nucleus; → activates; → enhances nuclear ER signalling)

1) Classical genomic pathway: Estrogen binds to ER. The activated ER forms a complex with transcriptional coactivators, then bind to the estrogen response elements (ERE), resulting in transcription of estrogen-regulated genes. The majority of these genes, including cyclin-D1, c-Myc, vascular endothelial growth factor (VEGF), growth factor-α (TGFα), insulin-like growth factor 1 (IGF1) are essential to cell proliferation and suppression of programmed cell death (Osborne & Schiff, 2005).

2) Non-classical genomic pathway: In the non-classical genomic pathway, the activated estradiol-ER complex binds indirectly to a target gene, via tethering with transcription factors Fos and Jun, at activator protein-1 (AP-1) response elements, i.e. the promoter region of target gene, thus amplifying the transcription of gene and subsequent cell proliferation (Zilli et al., 2009).

3) Membrane-initiated steroid signalling pathway (MISS): Estradiol binds to ER, activating cellular signalling pathways, which in turn up-regulate cell proliferation and down-regulate apoptosis (Osborne & Schiff, 2005; Schiff et al., 2003).

1.5 Tamoxifen: standard but imperfect treatment

Tamoxifen is currently the first-line endocrine medicine for treating both early and metastatic ER-positive breast cancer (Early Breast Cancer Trialists' Collaborative, 2011). However, tamoxifen is not the perfect solution for breast cancer. When used to treat patients with ER-positive breast cancer, treatment for 5 years resulted in 47% reduction in recurrence and only a 29% decrease in mortality during year 0-4 (Early Breast Cancer Trialists' Collaborative, 2011). This implies that 53% of patients with ER-positive breast cancer did not respond to tamoxifen. Moreover, some patients who initially responded to tamoxifen treatment eventually developed some form of *de novo* or acquired drug resistance, and tamoxifen administration correlated with stimulation of tumour growth in some cases (Clarke et al., 2001; Criscitiello et al., 2011; Osborne & Schiff, 2005).

Tamoxifen is a partial estrogen antagonist with agonist activity as well. This endocrine medicine is classified as a selective estrogen receptor modulator (SERM), which exerts tissue-specific estrogenic effects. Specifically, tamoxifen is predominantly antiestrogenic in the breast cells, but estrogenic in the uterus, liver and bone cells (Osborne & Schiff, 2005). These estrogenic effects have been associated with two- to three fold increased risk of endometrial cancer in postmenopausal women treated with tamoxifen (Fisher et al., 2005; Mourits et al., 2001). Other notable side effects include hot flushes, increased risk of pulmonary embolism, deep-vein thrombosis, stroke and cataracts (Table 1.1), as observed by a major trial, the 7-year National Surgical Adjuvant Breast and Bowel Project P-1 Study, which involved 13,388 women (Fisher et al., 2005). There are animal data and case reports that suggest an increased risk of abnormalities in the genital tracts in fetus and new born babies with tamoxifen exposure during pregnancy (Barthelmes & Gateley, 2004).

In the breast, the triphenylethylene drug tamoxifen predominantly exerts antiproliferative activity by competing with estradiol for binding to ER, blocking the ER signaling (Figure 1.3) that triggers cell proliferation (Bardon et al., 1984; Osborne, 1998). Nevertheless, recent studies have revealed there are at least 3 possible pathways by which tamoxifen (Tam) may trigger estradiol signalling, causing transcription of the gene and cell proliferation. In the classical genomic pathway, like estradiol, tamoxifen-bound estrogen receptor could recruit transcriptional coactivator, and then bind to estrogen response element (ERE) in the promoter region of target gene, causing transcription of the genes and cell proliferation (Osborne & Schiff, 2005). In the non-classical genomic pathway, tamoxifen-ER-coactivator complex could bind to transcriptional factors (Fos, Jun), which bind to activator protein-1 (AP-1) responsive element in the promoter region of target gene, causing gene transcription hence cell proliferation (Dahlman-Wright et al., 2012). In the third possible pathway, tamoxifen could bind to membrane ER, inducing cellular signalling pathways such as mitogen-activated protein kinase, which enhances the activity of ER and coactivator thus nuclear ER signalling (Osborne & Schiff, 2003, 2005).

Table 1.1 Endocrine therapies for estrogen receptor-positive breast cancer: rationale, efficacy & side effects

Treatment	Mechanism of action	Efficacy	Side effects
Tamoxifen	Competing with estrogen for binding to ER to block estrogen-activated ER signaling involved in cell proliferation and growth	5-year treatment: reduction in recurrence rate by 47%, mortality rate by 29% in primary BC (Early Breast Cancer Trialists' Collaborative, 2011). RR: 17-30% as first-line treatment in metastatic BC (Canney et al., 1987; Mouridsen et al., 2001; Paridaens et al., 2008).	Hot flushes, menstrual irregularity, increased risk of endometrial cancer, pulmonary embolism, deep-vein thrombosis, stroke, cataracts, tumour flare
Ovarian ablation	Removal of ovarian source of estrogen by surgical oophorectomy or suppression of ovarian biosynthesis of estrogen by medicine (LHRH agonists)	RR: 25-30% in primary and metastatic BC (Prowell & Davidson, 2004). 50% risk reduction of BRCA1 BRCA2 related breast cancer (Rebbeck et al., 2002).	Permanent loss of fertility, premature menopause, an increased risk of coronary heart disease and osteoporosis
Aromatase inhibitors	Suppression of aromatase activity thus inhibiting peripheral estrogen biosynthesis	RR: 20-46% as first-line endocrine treatment in primary and metastatic BC (Brodie, 2002; Mouridsen et al., 2001; Paridaens et al., 2008).	Joint disorders (arthritis, arthralgia), osteoporitic fracture, asthenia, nausea, vomiting, headache, pharyngitis, anorexia, hot flushes
Fulvestrant	Pure antagonist to block ER pathway	RR: 32% as first line in metastatic BC (Howell et al., 2004). RR: 17-20% as second-line treatment in patients whose disease progressed after tamoxifen treatment (Howell et al., 2002; Osborne et al., 2002)	Joint disorders (arthritis, arthralgia), asthenia, nausea, pain, vomiting, headache, pharyngitis, bone pain, anorexia, hot flushes
High-dose estrogen	Less well-known. It has been propose high-dose estrogen can induce apoptosis	RR: 30-40% as first-line treatment in metastatic BC (Ingle et al., 1981)	Nausea, vomiting, diarrhea, pain, hypercalcemia, thrombosis, stroke, pleural effusion

[Abbreviation: BC: breast cancer, RR: Response rate, defined as proportion of patients with complete response or partial response among all per-protocol patients (Therasse, 2002)]

1.5.1 Tamoxifen-flare

Another side-effect of tamoxifen, called tamoxifen flare, has been observed in some patients (Arnold et al., 1979; Clarke et al., 2001; Clarysse, 1985; Criscitiello et al., 2011; Hartley et al., 1987; McIntosh & Thynne, 1977; Plotkin et al., 1978). Clinical signs of tamoxifen flare include rapid progression of metastatic disease, increased bone pain and hypercalcemia. Plotkin et al. (1978) reported 6 out of 45 (13%) patients treated with tamoxifen experienced tamoxifen flare 2 to 21 days after receiving the medicine. Following this report, another medical team investigated the effect of tamoxifen in 68 postmenopausal women with metastatic breast cancer (Arnold et al., 1979). Six patients experienced the flare reaction. Three of these six patients eventually gained some benefits from tamoxifen. However, two of them had progressive disease while being treated with tamoxifen. In one of these patients, tamoxifen was discontinued due to the severity of flare reaction (Arnold et al., 1979). In premenopausal patients with metastatic disease, it has been shown that 50% of those initially responding to tamoxifen had disease progression subsequently (Pritchard et al., 1980). Cases of tamoxifen flare were also reported by others (Hartley et al., 1987; McIntosh & Thynne, 1977). Tamoxifen flare is possibly attributed to the estrogen agonistic activity of tamoxifen (Hartley et al., 1987). Nevertheless, the mechanisms of tamoxifen flare are yet to be elucidated. There is a lack of both *in vivo* and *in vitro* data regarding tamoxifen concentration in relation to tamoxifen flare.

1.6 Other endocrine treatments for ER-positive breast cancer

The intent of this section is to provide some basic and key information about some other options of endocrine treatments available for ER+ breast cancer.

1.6.1 Ovarian ablation

Ovarian ablation (OA), typically achieved by surgical oophorectomy, is an approach to remove the major source of estrogen to treat breast cancer in

premenopausal women. Oophorectomy was first performed in 1896 to treat premenopausal women with metastatic disease and multiple studies have been performed since then to evaluate the efficacy of OA (Beatson, 1896). This treatment produced a response rate of 25-30% in women with primary or metastatic breast cancer (Table 1.1) (Davidson, 2001; Prowell & Davidson, 2004). In women with BRCA1 and BRCA2 gene defects, surgical oophorectomy reduced breast cancer incidence by 50% (Rebbeck et al., 2002). OA can also be achieved by therapeutic radiation, or pharmacologically using luteinizing hormone releasing hormone (LHRH) agonists, which suppress the ovarian biosynthesis of estrogen. However, routine use of OA is not recommended, and OA should only be considered in women who refuse or cannot tolerate systemic therapy (e.g. chemotherapy), according to the latest Cancer Care Ontario Practice Guideline (Griggs et al., 2011). It remains unclear whether combined treatment of tamoxifen and OA is superior to either treatment alone.

1.6.2 Aromatase inhibitor

Aromatase is an enzyme which catalyzes the conversion of androstenedione into estrogen, the main source of estrogen during postmenopause (Brodie, 2002). Aromatase inhibitor (AI) works by inhibiting aromatase activity, thus lowering the levels of circulating estrogen. The response rates for patients treated with AI range from 20 to 42% (Brodie, 2002). A recent meta-analysis of randomised controlled trials (n = 28632 patients) concluded that first-line treatment with AI is as effective as tamoxifen in treating early-stage breast cancer in postmenopausal women (Josefsson & Leinster, 2010). Furthermore, their analysis also revealed that switching to AI after 2 to 3 years of tamoxifen improved survival, compared to 5 years continuous therapy with tamoxifen. In terms of treating metastatic breast cancer, a meta-analysis (n = 8504 patients) found improved survival with third generation AIs (e.g. letrozole, anastrazole) versus other hormonal therapies including tamoxifen (Mauri et al., 2006). A higher response rate was also obtained with AI than tamoxifen (33 *vs.* 25%) in postmenopausal patients with advanced disease, as shown by six randomised trials which compared AI and tamoxifen as treatment arms (Xu et al., 2011).

Overall, the existing data indicate that AI is superior to tamoxifen in treating postmenopausal breast cancer in both primary and metastatic settings. Nevertheless, the choice between AI and tamoxifen as first-line endocrine medicine is further complicated by their side effects. Tamoxifen has been shown to reduce the risk of osteoporotic fracture (Fisher et al., 2005). To the contrary, AI treatment has been frequently associated with osteoporotic fracture. Furthermore, arthralgia (joint pain) and myalgia (muscle pain) have been reported in up to 50% of patients treated with AI (Dunn & Ryan, 2009). On the other hand, AI has fewer gynaecological side effects and thromboembolic events than tamoxifen. Overall AI has a more favourable risk-benefit profile than tamoxifen but the musculoskeletal side effects pose a considerable challenge (Dunn & Ryan, 2009). Refer to Table 1.1 for more information on side effects of AI.

1.6.3 Fulvestrant

Fulvestrant, a pure antiestrogen devoid of known estrogen agonist activity, was developed to replace tamoxifen (partial agonist) and AI (Valachis et al., 2010). Fulvestrant is a new endocrine medicine with some clinical data emerging over the past decade. At least 6 clinical trials have been conducted to compare the efficacy of fulvestrant versus tamoxifen (1 trial) or AI (5 trials) (Flemming et al., 2009; Valachis et al., 2010). The fulvestrant *vs.* tamoxifen trial involved postmenopausal patients with advanced or metastatic breast cancer with no prior endocrine or cytotoxic treatment. This trial found similar response rate (i.e. not significantly different) between fulvestrant and tamoxifen (32 *vs.* 34%) as a first-line treatment in postmenopausal patients with advanced breast cancer (Howell et al., 2004). The fulvestrant *vs.* AI trials compared the efficacy of these two treatments in postmenopausal women with metastatic disease progressing after prior endocrine treatments. These trials showed that fulvestrant is similar to AI (anastrozole, exemestane) in efficacy as second-line treatments (Flemming et al., 2009; Valachis et al., 2010). In patients whose primary or metastatic disease had progressed after prior tamoxifen treatment, the subsequent fulvestrant treatment gave a response rate of 17-20%, based on

two randomized controlled trials (Howell et al., 2002; Osborne et al., 2002). However, these studies also reported several undesirable side effects in both fulvestrant and AI groups, including asthenia, nausea, vomiting, joint disorders (pain, inflammation), headache, pharyngitis and anorexia (Table 1.1) (Howell et al., 2002; Osborne et al., 2002).

1.6.4 High-dose estrogen

Prior to the advent of tamoxifen in 1970, estrogen was the mainstream endocrine medicine used for breast cancer (Adelson et al., 2011; Muss, 1992). Back in 1944, in view of the lack of success with surgery and radiation for advanced breast cancer, Dr Aleaxander Haddow bravely attempted treatment with estrogen, a hormone which had been known to stimulate mammary tumour growth, to treat his patients (Haddow et al., 1944). He observed significant benefit in 10 out of 22 patients (46%) who were treated with a synthetic estrogen, called triphenylchlorethylene, though the tumour regression achieved was only partial or temporary. Similar result was obtained for 5 out of 14 patients (36%) who received the treatment with stilboestrol, another synthetic estrogen. Dr Haddow's trial was actually inspired by his laboratory observations that some estrogenic polycyclic hydrocarbons which were carcinogenic also posed anti-tumour property. He hypothesized that estrogen though shown to be carcinogenic might also retard the growth of tumours (Haddow et al., 1944). Another study showed high-dose estrogen treatment (using another synthetic estrogen, diethylstilbestrol) gave similar response rate as tamoxifen (30-40%) (Ingle et al., 1981). Nevertheless, estrogen therapy was associated with more adverse reactions compared to tamoxifen. Side effects of estrogen therapy include nausea, vomiting, diarrhea, arthralgia, uterine bleeding and even thrombophlebitis and heart failure in some patients (Table 1.1). Therefore, estrogen therapy has been gradually abandoned since the advent of tamoxifen. Recently, estrogen therapy has been revisited mainly because of the need for more treatment options for patients who have failed first- and second-line treatments (e.g. tamoxifen, AI) (Lønning et al., 2001).

1.7 Treatment for estrogen receptor-negative breast cancer

Endocrine therapy, of which the target is ER, has no or little effect on ER-negative breast cancer. Chemotherapy is therefore of particular interest concerning the treatment for ER-negative breast cancer (Berry et al., 2006). Since the late 1980s, some major clinical data have begun to emerge, indicating the efficacy of chemotherapy in both premenopausal and postmenopausal women with ER-negative breast tumours. Chemotherapy found to be effective includes methotrexate + fluorouracil (MF), cyclophosphamide (C) + MF (CMF), doxorubicin (also called adriamycin) + cyclophosphamide (AC), and fluorouracil + adriamycin + cyclophosphamide (FAC) (Fisher et al., 2001; Fisher et al., 1996; Fisher et al., 1989). The Early Breast Cancer Trialists' Collaborative Group (EBCTCG) (2008) conducted a meta-analysis to address the existing evidence, covering the data of 20,000 patients with ER-negative breast cancer, involved in 96 trials (began 1972-1996). Chemotherapy was found to be effective, but worked slightly better in younger than older patients (50-69 years) in terms of reducing recurrence rate (by 27 *vs.* 18%), and mortality rate (by 27 *vs.* 14%) (Early Breast Cancer Trialists' Collaborative, 2008). Addition of tamoxifen to chemotherapy did not significantly affect the overall outcome. One major disadvantage of chemotherapy is that, while eradicating cancerous cells, the drugs also attack normal fast-dividing cells including those in the hair follicles, lining of the intestine and mouth and bone marrow. Side effects include hair loss (alopecia), fatigue, loss of appetite, nausea and vomiting (Lemieux et al., 2008; Partridge et al., 2001).

1.8 Treatment for HER2-positive breast cancer: Trastuzumab

In addition to ER, human epidermal growth factor receptor 2 (HER2), also known as HER2/neu or ErB-2, has begun to emerge as another important receptor in breast cancer etiology since 1980s (Hudis, 2007). HER2, a transmembrane tyrosine kinase receptor, is involved in regulating cellular responses including cell survival, growth and adhesion. Over-expression of HER2 was detected in 20-30% of breast cancer carcinomas. Studies using *in*

vitro and animal models have demonstrated the potential efficacy of anti-HER2 treatment in suppressing breast cancer cell proliferation and have led to the clinical development of several anti-HER2 drugs (Dahabreh et al., 2008). Trastuzumab (Herceptin) is a monoclonal antibody which binds to the extracellular domain of the HER2 receptor and blocks HER2-regulated signalling pathways involved in cell proliferation and survival. However, trastuzumab monotherapy results only in 30% regression (Singer et al., 2008). Cardiotoxicity is one of the key adverse effects associated with trastuzumab. About 10% patients treated with trastuzumab monotherapy experienced cardiac dysfunction (Singer et al., 2008). Trastuzumab in combination with chemotherapy provides higher efficay compared to trastuzumab alone, however, a higher incidence of cardiac events (28%) was a major drawback. Trastuzumab treatment has also been associated with development of central nervous system metastasis and disease (Dahabreh et al., 2008; Yau et al., 2006). Other side effects associated with trastuzumab include pulmonary toxicity, hypersensitivity and infusion reaction (Hansel et al., 2010). Furthermore, trastuzumab is also extremely costly. One-year trastuzumab therapy, which is the standard treatment for HER-positive breast cancer patients, could cost US$40,000 (Goldhirsch et al., 2011; Singer et al., 2008).

1.9 The role of plant-based food in breast cancer chemoprevention and treatment

Concerning the causes for high cancer rate in the USA, a landmark paper provided a remarkable message of the potential role of diet in relation to cancer (Doll & Peto, 1981). Subsequently, the WRCF report suggested appropriate diet, exercise and prevention of obesity could reduce 30-40% of all cancer incidences globally, which means 3 to 4 million cancer cases could be prevented each year by these environmental and lifestyle factors (World Cancer Research Fund / American Institute for Cancer Research, 1997). In particular, a number of studies have reported the favorable effect of vegetables and fruits in combating cancer. An earlier meta-analysis of studies (n = 26 studies, published 1982-1997) has reported an inverse association between vegetable intake and breast

cancer risk (Gandini et al., 2000). The International Agency for Research on Cancer working group evaluated the diet-breast cancer relationship and concluded that vegetable and fruit intake could reduce the risk of breast cancer (International Agency for Research on Cancer World Health Organization, 2003). More recently, after 11 years of follow-up, the European Prospective Investigation into Cancer and Nutrition Italy study (EPIC) (n=31,000 women, aged 36-64 years) has revealed a strong inverse relation between intake of all types of vegetables and breast cancer; vegetable intake of more than 264.8 g/day has been found to reduce breast cancer risk by 35%, when compared to vegetable intake of less than 107.8 g/day (Masala et al., 2012).

Vegetables and fruits are abundant in dietary fiber, vitamins and minerals. In agreement with the evidence from case-control studies, a meta-analysis of 16 propective studies revealed an inverse relation between high level of dietary fiber intake (> 25 g/day) and breast cancer risk (Aune et al., 2012; Howe et al., 1990). High dietary fiber diet has been shown to reduce serum estrogen concentration, possibly by binding to estrogen thus reducing the reabsorption of estrogen in the colon (Goldin et al., 1994; Rose et al., 1991). Minerals specifically selenium and potassium, as well as vitamins particularly vitamin C and B vitamins may confer chemoprotection against breast cancer (Howe et al., 1990; World Cancer Research Fund / American Institute for Cancer Research, 2007; Zhang et al., 1999). Plant-based foods are also rich in phytochemicals. The word '*phyto*' means 'plant' in Greek, so phytochemicals refer to substances of plant-origin. Over recent decades, data suggesting the beneficial effects of phytochemicals in combating human chronic diseases such as cancers have accumulated (Amiot, 2009; World Cancer Research Fund / American Institute for Cancer Research, 2007).

The term 'chemoprevention' was first coined by Sporn et al. (1976, referred in Mehta et al. 2010), and referred to the application of natural compounds such as phytochemicals or their synthetic analogs in the prevention of cancer by inhibiting or reversing the progression of cancer. In addition to the role as chemopreventive agents, natural compounds could also have potential

chemotherapeutic efficacy (da Rocha et al., 2001; Mehta et al., 2010; Taraphdar et al., 2001). Farnsworth and Morris (1976, reference in Cragg et al. 1997) conducted a National Prescription Audit and found over 25% of medicines dispensed in 1973 were derived from plants. The group later reported at least 119 compounds derived from 90 plant species are important medicines used in one or more countries (Farnsworth et al., 1985). Further analysis conducted by another team at Division of Cancer Treatment (National Cancer Institute, U.S.) revealed more than 60% of drugs approved or candidates applied for drug approval are of natural origin (Cragg et al., 1997). Their analysis reached an important conclusion: *'This review highlights the invaluable role that natural products have played, and continue to play, in the drug discovery process, particularly in the areas of cancer and infectious diseases.'* This conclusion was further supported by their more recent and expanded analysis of data from 1940 to 2007 (Newman & Cragg, 2007). Mechanistic studies have demonstrated the possible mechanims of anticancer action of natural compounds, including antiproliferative effects, inducing apoptosis and cell cycle control (Mehta et al., 2010; Taraphdar et al., 2001; Yang et al., 2001). As an example, Paclitaxel (also known as Taxol), derived from a plant called *Taxus brevifolia,* showed cytotoxic and cell cycle arrest activity against several forms of cancers including ovarian, breast, lung, head and neck (Rowinsky & Donehower, 1995).

Altogether, the existing epidemiological evidence, laboratory data and the history of natural product in cancer treatment have pointed to an important direction and thus adopted for this thesis: exploration of plant materials as a source of efficacious chemotherapeutic agents against breast cancer.

1.9.1 Potato: a possible target for research

Potato ranks as one of the world's top staple plant foods; its consumption continues to grow globally. In North America and Europe, at least 10% of dietary energy is provided by potato (World Cancer Research Fund / American Institute for Cancer Research, 2007). In New Zealand, potato is the most commonly consumed type of vegetable (FAO, 2008).

While potato has been cultivated for over 10,000 years for food, its potential therapeutic value in chronic disease such as cancer has only begun to emerge in recent decades (Camire et al., 2009). Hirose et al. (1995) conducted a large scale hospital based study to investigate the risk factors for breast cancer among 36,944 premenopausal and postmenopausal women in Japan. After a 5-year follow-up, the researchers found a reduced risk of breast cancer associated with the intake of bean curd, green-yellow vegetables, potato and fish (Hirose et al., 1995). The chemoprotective effects of potato may be attributed to its nutritious components, such as vitamin C, B vitamins, amino acids, minerals, dietary fiber and phytochemicals (Brown, 2005; Camire et al., 2009; Lachman & Hamouz, 2005).

Potato contains a diverse range of phytochemicals. Several classes of phenolic compounds are present in potato tuber, among which hydroxycinnamic acids are the most abundant namely chlorogenic acid, caffeic acid, followed by flavonoids such as anthocyanins in pigmented potatoes and flavonols that include the derivatives of kaempferol and quercetin (Im et al., 2008; Lewis et al., 1998). Recently, pigmented potatoes such as the purple potatoes have attracted research interest because of their anthocyanin (ACY) content that may contribute to consumers' health, including cancer prevention and therapy. ACY or ACY-rich extracts made from colourful vegetables and fruits were shown to have anti-proliferative activity against various types of cancers in vitro, including breast cancer (Hou, 2003; Katsube et al., 2002; Leardkamolkarn et al., 2011; Nguyen et al., 2010; Noratto et al., 2009; Olsson et al., 2004; Reddivari et al., 2007; Sun & Liu, 2006; Thomasset et al., 2009; Wang & Stoner, 2008; Zhang et al., 2005). Potato ACY, in particular, was shown to inhibit cell proliferation and up-regulate apoptosis in stomach cancer cells, prostate cancer cells and leukaemia cells in vitro (Zhao et al., 2009).

Of the other compounds identified in the potato extracts, phenolic acids (hydroxycinnamic acids) have the potential to be biologically active. Chlorogenic acid (ChA), the most abundant phenolic acid of potato, accounts for

65 and 70% of total phenolic acids in PPE and WPE respectively. Like potato ACY, ChA has begun to attract some research interest in recent years in relation to its potential therapeutic value in cancer treatment. Antiproliferative activity of ChA has been reported in MCF-7 cells (Leo et al., 2008), and in animal models of large intestine, liver and tongue cancers (Mori et al., 1999). Some data also exist for another phenolic acid of potato, caffeic acid (CA), in relation to its effect on breast cancer cell proliferation. A study investigated the effects of 6 phenolic acids on T-47D cell proliferation using the MTT assay. Among all the phenolic acids tested, CA was the most potent antiproliferative agent (Kampa et al., 2004). Chlorogenic acid and CA were also shown to have pro-apoptotic activity in breast cancer cells MCF-7 and T-47D in vitro (Kampa et al., 2004; Leo et al., 2008). Another group of phytochemicals present in potato, glycoalkaloids, were also found to have antiproliferative activity against several cancer cell lines (breast, lung stomach and skin) (Chataing et al., 2009; Nakamura et al., 1996). Glycoalkaloids exert cytotoxic effect possibly via stimulating apoptosis (Ji et al., 2008).

It is questionable, though, if potato truly has a protective or therapeutic effect against breast cancer, should it not already be having a tremendous impact on global health given its abundance in the diet of different world populations? One possible explanation could be related to the form of potato commonly consumed. In parallel with accumulating data suggesting the potential anticancer effects of phytochemicals, research interest in bioactive composition changes during food preparation is growing. Method of cooking has been shown to affect the phenolic content of potatoes. Oven-baking destroyed all chlorogenic acid content of potatoes, while boiling and microwave-baking caused 65% and 45% loss of the original amount of chlorogenic acid (Friedman, 1997). Commercially prepared French-fried potatoes, mashed potato flakes, potato skins were found to contain no chlorogenic acid (Friedman, 1997). Oven-baking and frying also lead to more than 50% loss of ACY in pigmented potatoes (Brown et al., 2008). There is, however, not yet an unequivocal evidence how phytochemical profile of potato changes during cooking and which phytochemicals are truly physiologically significant. Fried potato products,

which are commonly consumed, are typically high in fat and salt, which could potentially increase the risk of chronic diseases including cancer and coronary heart disease. French fries and potato chips are also a major source of acrylamide, a compound formed between amino acids, primarily asparagine, and reducing sugar at high temperature. Acrylamide has been classified as probable human carcinogen by IARC, although evidence regarding the carcinogenic role of acrylamide are equivocal (Hogervorst et al., 2007; Mucci et al., 2005; Pelucchi et al., 2011; Tareke et al., 2002). Taken together, both commercial and domestic processing methods for potatoes need to be optimised in order to retain potentially health-promoting effects of potatoes.

1.9.2 Characterization of plant material for research

Laboratory studies suggest the anticancer activities of vegetables and fruits result from the synergistic or additive effect of several phytochemical compounds (e.g. phenolic acids, flavonols and anthocyanins), rather than from a single substance (Boivin et al., 2009; Lee et al., 2004; Liu, 2004, 2003; Russo, 2007; World Cancer Research Fund / American Institute for Cancer Research, 2007). Therefore, the approach of using a whole plant extract is an appropriate starting point for *in vitro* studies, in the pursuit of a strategy to prevent breast cancer, and/or to complement standard breast cancer treatments (e.g. drugs, radiation). However, equally important is that whole plant extracts used for anticancer research should be fully characterised, i.e. composition of phytochemicals identified. In particular, potato extracts can vary substantially due to difference in varieties, cultivation, seasonal changes and the processing involved.

1.10 Directions and significance of the current thesis

1.10.1 To determine dose-response effects of estradiol & tamoxifen on breast cancer cell proliferation in vitro

Apparently, tamoxifen does not always work in inhibiting breast cancer cell proliferation, and the supposedly causal agent estradiol in breast cancer, is not always stimulatory to breast cancer cell proliferation. However, it is not clear in the literature under which dose range or doses these paradoxical effects may occur. Effects of tamoxifen or estradiol on cell proliferation have been previously reported in the literature, but the doses tested varied from one study to another and were typically within a narrow range (Brünner et al., 1989; Cover et al., 1999; Duda et al., 1999; Katzenellenbogen et al., 1987; Kim et al., 2004; Mai et al., 2007; Osborne et al., 1983; Perry et al., 1995; Pratt & Pollak, 1993; Seeger et al., 2006). The accuracy of extrapolating the overall dose-response profile of tamoxifen using the data provided by different laboratories is questionable given the variability in several aspects, especially experimental conditions, procedures and individuals conducting the assays. Experimental work in Chapter 2 used a single set of cell proliferation procedures, conditions and equipment, to define the dose-response profiles of tamoxifen or estradiol. Another key objective was to investigate the interaction between tamoxifen and estrogen in relation to dose, which is also unclear in the literature.

1.10.2 To prepare potato extracts & perform characterisation by ultra high performance liquid chromatography mass spectrometry

Given potential effects of several classes of phytochemicals in potato, unselective extraction and subsequent characterisation were performed. As mentioned earlier, common cooking methods for potatoes may have destroyed some of the phytochemicals. Current thesis prepared potato extracts from raw potato, intended to retain as much as potato phytochemicals as possible, particularly optimized for phenolic compounds. Previous studies on the effects of potato extract on breast cancer cell proliferation have used methanol or

phosphate buffer saline (PBS) as the solvent for making the potato extracts (Leo et al., 2008; Stushnoff et al., 2008). The current thesis has used ethanol for extraction, which is a more safer and commonly used solvent compared to methanol or PBS in the food or pharmaceutical industry. In Chapter 3, potato extracts were made from white potato and 'Urenika', a purple potato variety (*Solanum tuberosum* L.) that was traditionally consumed by Maori in New Zealand. Cultivar 'Urenika' was chosen because of the intention to develop a nutraceutical or therapeutic product of New Zealand proprietary value. For 'Urenika', there are no prior data on composition by LCMS nor have the effects of this potato variety been tested in relation to breast cancer. The ultra high performance liquid chromatography mass spectrometry (UHPLC-MS) technique was performed in Chapter 3 to determine the metabolomic profile or composition of the potato extracts produced.

1.10.3 To determine the effects of potato extracts on breast cancer cell proliferation & cell cycle parameters *in vitro*

Data are scarce regarding the effect of potatoes or extracts made from fresh potato in relation to breast cancer (Leo et al., 2008; Stushnoff et al., 2008). There is no prior data regarding the effect of 'Urenika' on breast cancer cell. Breast cancer cell lines such as MCF-7 and T-47D are the main tool used for the investigations performed in this study. In Chapter 4, experiments were conducted to determine the effect of potato extracts alone on breast cancer cell proliferation. In addition, a series of experiments were conducted to determine any potential interaction between potato extracts and tamoxifen or estradiol. The focus of this project moved to 'Urenika' purple potato extract (PPE), given the intention of developing a nutraceutical or therapeutic product of New Zealand proprietary value. Subsequently, flow cyctometry experiments were conducted to determine whether PPE alone, or the combined treatments with this potato extract and tamoxifen or estradiol, had significant effects on cell cycle control and apoptosis of breast cancer cells. Apoptosis is a vital homeostatic mechanism of regression, counteracting aberrant cell proliferation, maintaining proper cell population in tissues and thus tissue

integrity. Both cell cycle control and apoptosis are well recognised as important targets in cancer therapy and chemoprevention. The effects of potato on cell cycle control or apoptosis have not been reported previously.

1.10.4 To establish a metastatic mouse model & test the effects of tamoxifen *in vivo*

The last major goal of the current thesis was to develop a metastatic breast cancer animal study model that could be used to test the effect of tamoxifen *in vivo*, and for future investigations of PPE or combined treatments of interest using the animal model. Tamoxifen is the first-line endocrine treatment for metastatic breast cancer, but only provides a response rate of 17 to 30% among metastatic breast cancer patients (Table 1.1). There is a need to know more about the effects of tamoxifen in the metastatic setting. Tamoxifen has been previously studied using models induced by administration of carcinogens, typically *N*-methylnitrosourea (NMU), or 1,2-dimethylbenz (a)-anthracene (DMBA) (Gottardis & Jordan, 1987; Jordan, 1976; Osborne et al., 1992). These models have major drawbacks such as variations in tumour multiplicity and its ability to metastasize (Persě et al., 2009). Animal model involving transplantation of human breast cancer cell MCF-7 was also used to test the effect of tamoxifen (Gottardis & Jordan, 1988). Nonetheless, the main drawback of using human breast cancer cell line is the difficulty and low success rate in tumour development, as well as a lack of involvement of immune system since nude mice or severe immunodeficiency mice are needed for this model (Kim et al., 2003). Mouse breast cancer cell line 4T1 can be transplanted into immunocompetent mice and are able to metastasize to various organs similar to stage IV human breast cancer, making this cell line highly relevant for metastatic breast cancer modelling, hence 4T1 cell line was chosen for the current project (Heppner et al., 2000).

Chapter 2 Paradoxical dose-response effects of tamoxifen and estradiol on MCF-7 cell proliferation

2.1 Introduction

Experimental work of this chapter was conducted to determine the dose-response profiles of tamoxifen or estradiol. Another key objective was to investigate the interaction between tamoxifen and estrogen in relation to dose, which is also unclear in the literature.

2.1.1 Tamoxifen dose-response effects

Tamoxifen has been the standard adjuvant endocrine medicine for more than 30 years; however, tamoxifen treatment only works on some occasions (Chapter 1). In addition, tamoxifen often produces undesirable side effects, including an increased risk of endometrial cancer (Hirsimäki et al., 2002) and the occurrence of flares in some patients due to its partial agonist property (Arnold et al., 1979; Clarke et al., 2001; Clarysse, 1985; Criscitiello et al., 2011; Hartley et al., 1987; McIntosh & Thynne, 1977; Plotkin et al., 1978). The effect of tamoxifen on MCF-7 cell growth and survival has previously been investigated, but typically over a limited range of doses, i.e. 10^{-6} and 10^{-5} M (Brünner et al., 1989; Cover et al., 1999; Duda et al., 1999; Kim et al., 2004; Mai et al., 2007; Osborne et al., 1983; Perry et al., 1995; Pratt & Pollak, 1993). A few studies covered a dose range of 10^{-7} to 10^{-5} M (Karami-Tehrani, 2003; Reddel et al., 1985; Stuart et al., 1992; Tari et al., 2005), while studies which include doses below 10^{-8} M are scarce (deGraffenried et al., 2003; Roos et al., 1982). The intent of the current study was therefore to investigate the effect of tamoxifen on MCF-7 cell proliferation over a broad range of concentrations to construct a dose-response curve, covering the range of 10^{-9} to 10^{-5} M, which encompassed the serum concentrations of tamoxifen in patients receiving this treatment (Lien et al., 1991). The experiments conducted in this chapter used MCF-7 cell line because majority of the dose response studies for tamxifen and estradiol *in vitro*

have used MCF-7, a well established and recognised breast cancer cell line for investigating drug efficacy in hormone-responsive breast cancer (Levenson & Jordan, 1997). Data obtained from the experiments done in this chapter were compared to these studies (Table 2.1 & 2.2) which used MCF-7 cell lines.

2.1.2 Estradiol dose-response effect

Estrogen is generally considered as a key player in growth and development of breast cancer (Henderson et al., 1988; Yager & Davidson, 2006b). Several animal studies demonstrated estrogen to be a carcinogen in breast tissue, as reviewed by Yager & Davidson (2006). In parallel with *in vivo* data, several *in vitro* studies have consistently shown that estradiol, the predominant form of intracellular estrogen, stimulates cell proliferation or growth at doses of 10^{-8} to 10^{-10} M (Brünner et al., 1989; Duda et al., 1999; Katzenellenbogen et al., 1987; Kim et al., 2004; Pratt & Pollak, 1993; Seeger et al., 2006). Thus, the experiments described in the current chapter were designed to define the dose where estrogen is stimulatory on cell proliferation.

Different cell culture conditions have a significant effect on the overall result of tamoxifen treatment (2.4.1). Studies have reported contradictory results most likely due to the estrogen content in their media (non-charcoal-treated or higher concentration of serum). Thus the intent of the current chapter is to test the following hypothesis:

- Tamoxifen doses previously shown to be stimulatory could be inhibitory in the presence of estrogen.

2.2 Methods and materials

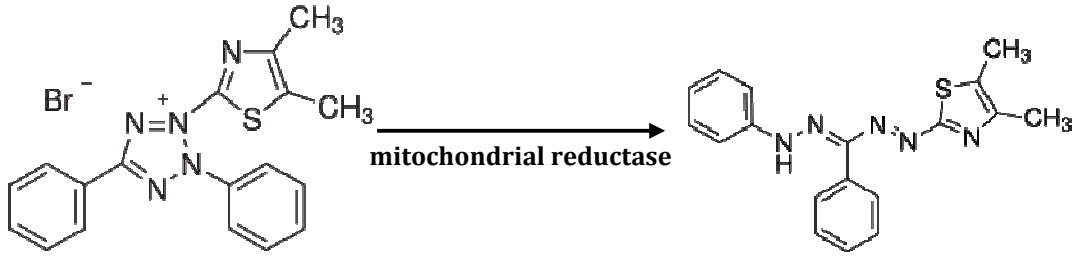
2.2.1 Cell line and cell culture conditions

The breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in 75 cm^2 cell culture flasks (T-75) containing complete medium consisting of Minimum Essential Medium (MEM), 10% fetal calf serum (FCS), 10 µg/mL bovine insulin, plus 1% penicillin-streptomycin-glutamine mixture (i.e. 10000 units of penicillin, 10000 µg of streptomycin, 29.2 mg/mL of L-glutamine in a 10 mM citrate buffer to maintain penicillin potency). All tissue culture reagents were purchased from Gibco Invitrogen Corporation, Grand Island, NY. Cell culture flasks were incubated under a humidified atmosphere with 5% CO_2 and 95% air at 37℃. Subculturing procedures were performed when cells reached 80% confluence, as follows:

i) Cells were washed twice with 20 mL of phosphate buffered saline (PBS).
ii) Four mL of 0.25% (w/v) Trypsin-0.53 mM EDTA was added to the cells and left for 3 to 5 minutes until cells detached.
iii) Once the cells began to detach, 6 mL of complete medium was added to the flask and cells were aspirated by gentle pipetting.
iv) An appropriate aliquot of cell suspension was then added to a new flask to give a final cell concentration of 10^5 cells/mL in a 75 cm^2 flask (20mL total volume).
v) Cells were subcultured every 3 to 5 days when these became fully confluent and routinely maintained in the same culture conditions. MCF-7 cells between passages 5 and 10 were used in the experiments conducted in this chapter.

2.2.2 Cell proliferation measurements using MTT assay

Cell survival and proliferation was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983). This assay measured the ability of viable cells, which have active mitochondrial dehydrogenase, to reduce the yellow tetrazolium salt to a purple formazan precipitate (Figure 2.1). An organic solvent (dimethyl sulfoxide, DMSO) was added to the formazan which was then quantified colorimetrically at 550 nm. The resulting absorbance value is proportional to the number of viable cells in the test sample.



MTT (yellow) **Formazan (Purple)**

Figure 2.1 Conversion of MTT.
Yellow tetrazolium salt is converted to purple formazan by mitochondrial reductase

Optimization experiments were carried out to determine the optimal cell number for seeding, amount of fetal calf serum required and the MTT incubation time. As a result, the protocol for the MTT assay was set up as follows:

1) On day 1, cells were harvested, counted using haemocytometer and seeded onto a 96-well plate, with a cell density of 5000 cells per well (100 µL) in medium consisting of Minimum Essential Medium (MEM), 10 µg/mL bovine insulin, 1% penicillin-streptomycin-glutamine mixture, and 5% charcoal-stripped FCS.

2) Twenty-four hours later, the growth medium on the 96-well plate was changed to treatment medium containing test agent, i.e. tamoxifen or estradiol. Tamoxifen and estradiol were purchased from Sigma, St. Louis. Stock solutions

of tamoxifen (10^{-2} M in ethanol) or estradiol (10^{-1} M in ethanol) were prepared and diluted with MEM to the required concentrations (final ethanol concentration <0.1%). Each treatment was carried out in at least 6 wells per experiment.

3) Seventy-two hours post-treatment, 10 µL of MTT (Sigma, St. Louis, MO) solution (5 mg/mL in PBS, filtered with 0.45 µm filter) was added to each well and incubated for 4 to 5 h at 37°C under 5% CO_2 atmosphere.

4) The medium was then removed, followed by the addition of 100 µL of DMSO (Merck, Darmstadt, Germany) to dissolve the formazan.

5) The absorbance (which is proportional to the number of viable cells) of the coloured solution formed was measured with a microplate scanning spectrophotometer (Ultra microplate reader, ELX 808, Bio-Tek Instruments Inc.) at 550 nm. The reading of each test well was normalised to the mean reading of the control wells (containing cells and vehicle, with no test agent, tamoxifen or estradiol), which were set at 100%.

2.3 Results

2.3.1 Tamoxifen

MCF-7 cells were treated with tamoxifen over a wide range of concentrations (10^{-9} to 10^{-5} M). Results from 2 to 8 independent MTT experiments were pooled and a bell-shaped dose-response graph was obtained (Figure 2.2). At 10^{-9} M, a significant antiproliferative effect (40% reduction) was observed ($P<0.001$). Interestingly, at 10^{-8}, 10^{-7}, and 10^{-6} M, tamoxifen stimulated cell proliferation significantly by 16% ($P < 0.01$), 18% ($P < 0.05$) and 30% ($P < 0.01$) respectively. As the dose increased to 10^{-5} M, significant inhibition of cell growth and survival was again observed (65%) ($P < 0.001$).

2.3.2 Estradiol & its combined treatments with tamoxifen

As shown in Figure 2.3, estradiol stimulated MCF-7 cell proliferation significantly at doses of 10^{-10} to 10^{-8} M by 18 to 27% ($P < 0.001$). At 10^{-7} and 10^{-6} M, no significant effect was observed. However, significant inhibition (34%) was observed with estradiol treatment of 10^{-11} M ($P < 0.001$).

The combined effects of tamoxifen of various doses with estradiol 10^{-9}, 10^{-8}, or 10^{-7} M were presented in Figure 2.4. Instead of being stimulatory (Figure 2.2), 10^{-6} M tamoxifen exerts the opposite effect in the presence of estradiol at all three concentrations tested. At 10^{-7} and 10^{-8} M tamoxifen also become inhibitory in the presence of 10^{-8} M estradiol. On the other hand, the addition of estradiol may have partially blocked or rescued the inhibition caused by 10^{-9} M tamoxifen and to a lesser extent in the case of 10^{-5} M tamoxifen.

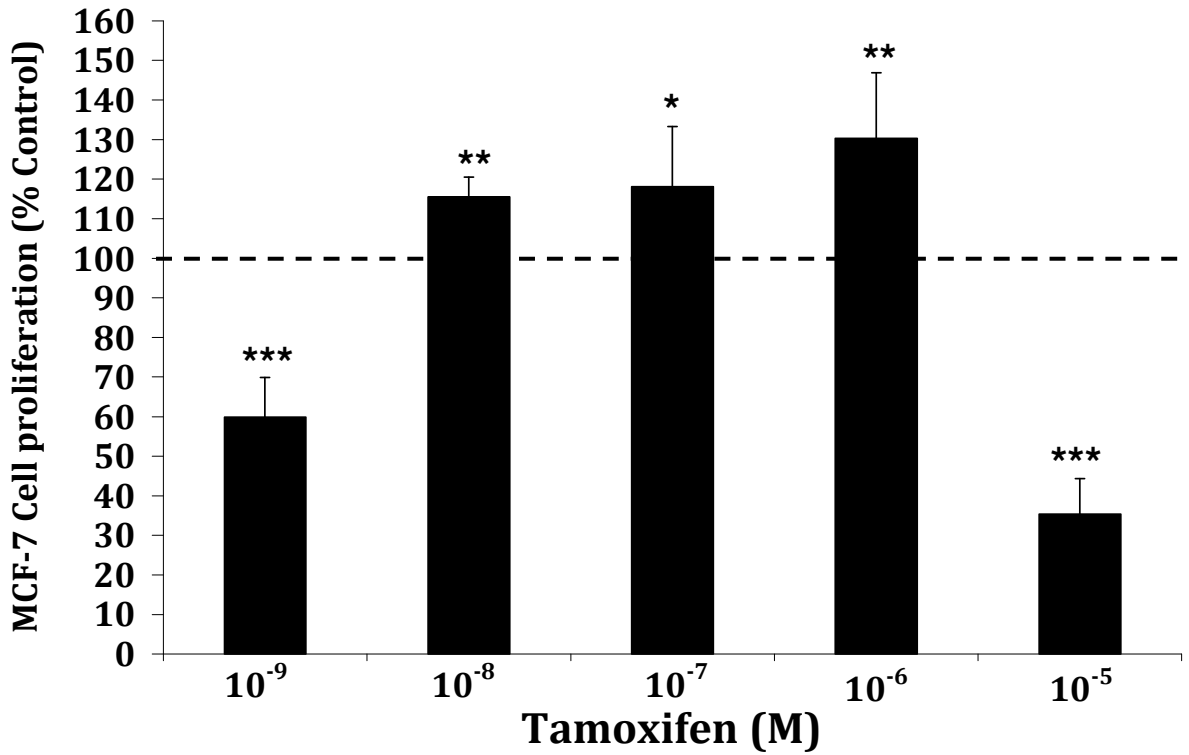


Figure 2.2 MCF-7 cells were treated with tamoxifen at various doses.

Cell proliferation was measured at 3 days post-treatment by MTT assay and the results normalized to the no-treatment control (100%). Results were pooled from 2 to 8 independent experiments, each carried out with $N \geq 6$ wells per treatment, and expressed as mean ± SEM (Asterisks denote the treatments are statistically different from the control with no tamoxifen: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ by ANOVA, using Sigma Plot statistical software).

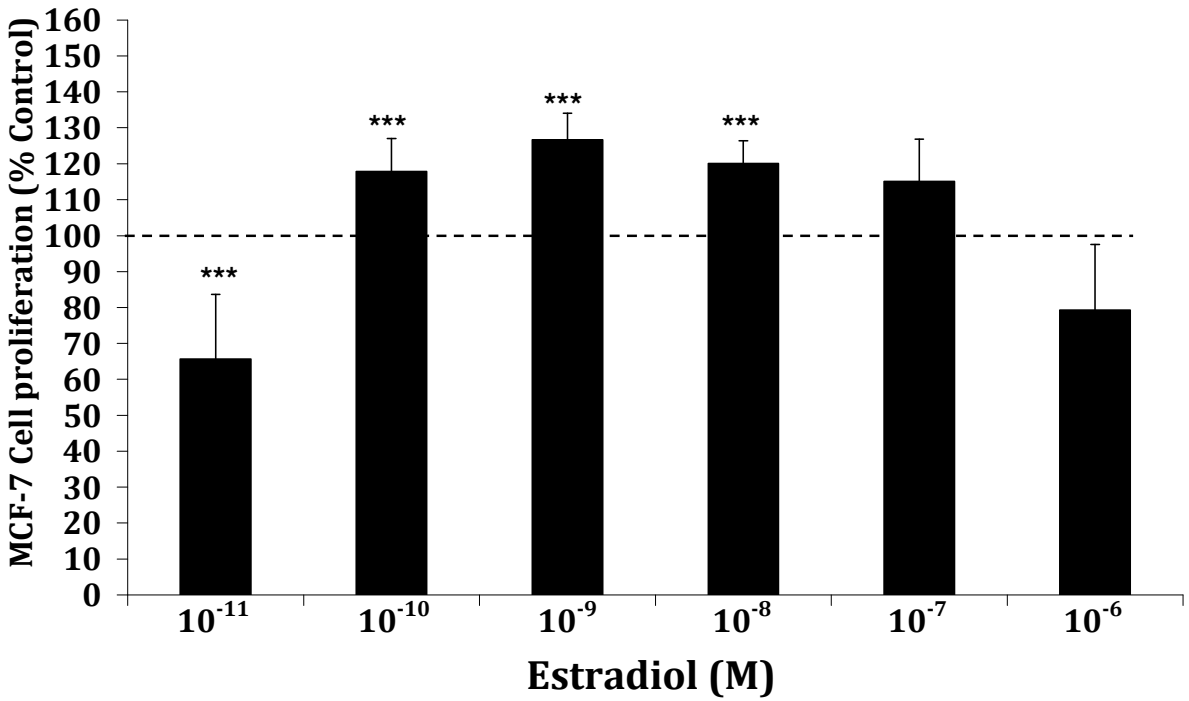


Figure 2.3 MCF-7 cells were treated with estradiol at various doses.

Cell proliferation was measured at 3 days post-treatment by MTT assay and the results normalized to the no-treatment control (100%). Results were pooled from 2 to 8 independent experiments, each carried out with $N \geq 6$ wells per treatment, and expressed as mean ± SEM (*** statistically different from control, $P < 0.001$, by ANOVA using Sigma Plot statistical software).

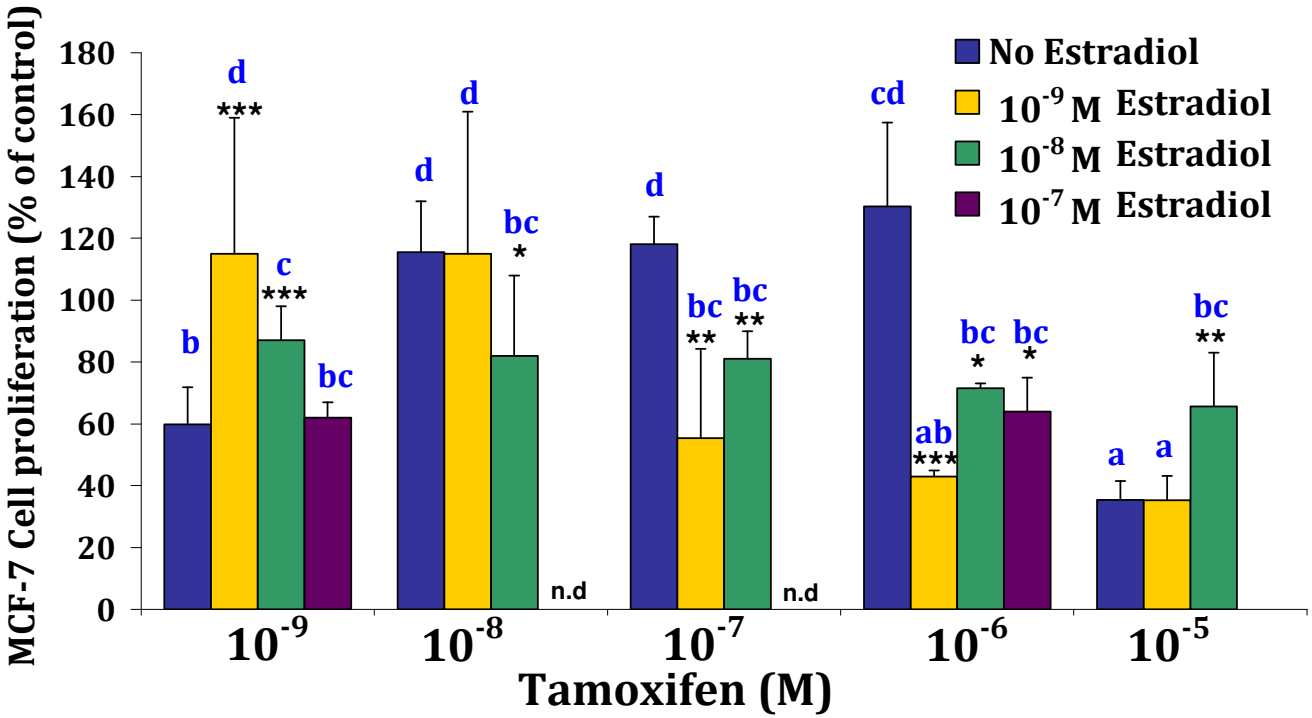


Figure 2.4 MCF-7 cells were treated with tamoxifen and estradiol at various doses.
For the ease of reference, data from Figure 2.2 were repeated in blue bars. Cell proliferation was measured at 3 days post-treatment by MTT assay and the results were normalized to the no-treatment control, i.e. no exposure to estradiol or tamoxifen (100%). Results were pooled from a total of 10 independent experiments, each carried out with N ≥ 6 wells per treatment, and expressed as mean ± SEM. Combinations of estradiol at 10^{-7} M with tamoxifen at 10^{-7} or 10^{-8} M were not done (n.d.). Asterisk denotes that the treatment is significantly different from tamoxifen treatments with no estradiol (blue bars): *P < 0.05, **P<0.01, ***P<0.001. Alphabetical labels on bars denote significantly different treatment groups, based on Tukey's Honestly Significant Difference test, 95% confidence level, using Minitab software.

2.4 Discussion

2.4.1 Tamoxifen dose-response effects

MCF-7 cells were originally isolated from the pleural effusion of a 69 year-old breast cancer patient with metastatic breast cancer disease, and established as an *in vitro* ER-positive breast cancer model (Brooks et al., 1973; Soule et al., 1973). In humans, the majority (70-80%) of breast carcinomas are ER+ which makes this cell line a relevant and important model to use in breast cancer research (Blamey et al., 2010; Early Breast Cancer Trialists' Collaborative Group, 1998, 2005; Osborne, 1998). MCF-7 cells used for the experiments conducted in this chapter were in cultures less than 2.5 months to minimize genetic and phenotypic variation (Freshney, 2005).

The current study reports the response of MCF-7 cells 3 days post-treatment of tamoxifen at doses ranging from 10^{-5} to 10^{-9} M. The effect of tamoxifen on MCF-7 cell proliferation was found to be dose-dependent (Figure 2.2). At a tamoxifen treatment of 10^{-9} M, cell growth and survival were significantly reduced. It has been suggested however that tamoxifen at 10^{-9} M is cytostastic only and this inhibitory action is reversible by mitogens such as estrogen (Mandlekar & Kong, 2001). Significant inhibition (66%) was again observed at 10^{-5} M tamoxifen treatment as observed consistently by others (Cheah, 2007; Duda et al., 1999; Kim et al., 2004; Mai et al., 2007; Perry et al., 1995; Reddel et al., 1985; Stuart et al., 1992; Tari et al., 2005). The dose of tamoxifen required to cause 50% inhibition of MCF-7 cell proliferation (IC_{50}) *in vitro* has been reported to range from 6 x 10^{-6} to 10^{-5} M, which agrees well with the current findings (Kim et al., 2004; Mai et al., 2007; Stuart et al., 1992). It is well known that tamoxifen works by competing with estrogen for binding to the ER (Clarke et al., 2001). Estrogen-activated ER recruits coactivators, causing enhanced transcription of estrogen-responsive genes and promoting tumour growth. In contrast, tamoxifen-bound ER typically recruits co-repressors which causes cell cycle arrest and suppresses tumour growth. Specifically, it has been shown that tamoxifen at doses above 5 x 10^{-6} M induces programmed cell death, cell cycle arrest at

G0/G1, and a simultaneous decrease in the percentage of S phase cells, altogether resulting in a strong and inhibitory effect on growth and survival (Etienne et al., 1989; Perry et al., 1995; Sutherland et al., 1983).

As shown in Figure 2.2, tamoxifen stimulated cell growth significantly by 16 to 30% between 10^{-8} M and 10^{-6} M. Roos and others (1982) also observed significant stimulation of MCF-7 cell proliferation with 10^{-7} and 10^{-6} M tamoxifen. Clinically, tamoxifen treatment has occasionally been associated with an acceleration of tumour growth rate and increase in size of tumour mass in patients (Belani et al., 1989; Legault-Poisson et al., 1979). Furthermore, subsequent discontinuation of tamoxifen administration has brought about tumour regression, indicating that the tumour growth is tamoxifen-dependent. Simon et al. showed *in vitro* tamoxifen-stimulated growth of breast cancer cells, derived from patients who have relapsed after tamoxifen treatment (Simon et al., 1984). Similar observations of tamoxifen-dependent growth of tumours have also been reported in an animal study (Fendl & Zimniski, 1992). The stimulatory action of tamoxifen observed in the present study may also correlate with the clinical phenomenon termed tamoxifen flare (i.e. exacerbation of disease characterised by increased pain, rapid progression of disease, and hypercalcemia) experienced in 4 to 20% of postmenopausal patients during the beginning of tamoxifen treatment (Arnold et al., 1979; Clarysse, 1985; Criscitiello et al., 2011; McIntosh & Thynne, 1977; Plotkin et al., 1978). It is noteworthy that tamoxifen concentrations found in postmenopausal patients' serum after 1 to 3 weeks treatment range from 5 x 10^{-8} M to 5 x 10^{-7} M (Etienne et al., 1989; Lien et al., 1989; Lien et al., 1991). These tamoxifen serum concentrations fall within the dose range where stimulation of cell proliferation was observed.

As summarized in Table 2.1, some studies have reported the opposite findings, i.e. tamoxifen has been shown by some authors to inhibit MCF-7 cell proliferation or growth at doses of 10^{-8} to 10^{-6} M (Cheah, 2007; Cover et al., 1999; deGraffenried et al., 2003; deGraffenried et al., 2004; Mai et al., 2007; Reddel et al., 1985; Stuart et al., 1992). The methodology and treatment

duration of these studies (24 to 96 hours) are comparable to the present study. The discrepancy between these and the present studies could be attributed to the cell culture conditions involved. In particular, detection of stimulatory activity of tamoxifen *in vitro* has been hindered by the interference caused by estrogenic contaminants present in cell culture systems (Thompson et al., 1988). For instance, phenol red, a pH indicator commonly present in standard culture media, has been implicated as an estrogen analogue that can interfere with sensitivity of MCF-7 cells to estrogen or tamoxifen (Berthois et al., 1986). A series of experiments conducted by Moreno-Cuevas and Sirbasku (2000) have concluded however that phenol red at the concentration in standard medium was not sufficient to cause estrogenic activity that could interfere with the responsiveness of MCF-7 to tamoxifen (Moreno-Cuevas & Sirbasku, 2000). Interestingly, both the aforementioned studies (Cheah, 2007; Cover et al., 1999; deGraffenried et al., 2003; deGraffenried et al., 2004; Reddel et al., 1985; Stuart et al., 1992) and the present study have used phenol-red containing media, suggesting that the discrepancy arises from factors other than phenol red. The most significant difference between cell culture conditions of the current study and these studies, that can be identified, is serum. It is important to note that all the experiments described in this thesis have used charcoal-stripped serum (5%) whereas the published studies used non-charcoal treated serum (at 5 or 10%). Similar to the present study, Perry et al. (1995), Roos and others (1992) observed stimulatory effects of tamoxifen in MCF-7 in their studies using charcoal-stripped serum. Reddel and Sutherland (1984) demonstrated that when charcoal-treated serum was substituted with standard serum, the stimulatory effect of tamoxifen on breast cancer cell proliferation diminished.

Table 2.1 Effects of tamoxifen treatment on MCF-7 cell proliferation reported in the literature

Literature/study	Tamoxifen dose (M)						Medium	Phenol red (Yes:+, No : -)	Standard Serum	Charcoal-treated serum
	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}				
(Roos et al., 1982)	↔	↔	↔	↑	↑	↔	IMEM-ZO	+	-	5%
(Perry et al., 1995)					↑	↓	MEM	-	-	5%
(Reddel et al., 1985)				↓	↓	↓	RPMI	+	5%	–
(Stuart et al., 1992)				↓	↓	↓	RPMI	+	10%	-
(Cover et al., 1999)					↓		DMEM	+	10%	-
(deGraffenried et al., 2003)		↓	↓	↓	↓		IMEM	+	10%	
(deGraffenried et al., 2004)		↓	↓	↓	↓		IMEM	+	10%	
(Cheah, 2007)			↓	↓	↓	↓	DMEM	+	5%	-
(Mai et al., 2007)					↓	↓	DMEM	+	10%	-

(Key: ↓ Inhibitory, ↑ stimulatory, ↔ no significant effect)

The rationale is that serum contains a stimulatory amount of estrogen or estrogen analogue which can interfere with the response of MCF-7 to tamoxifen and thus compromise the accuracy of the assay (Butler et al., 1981; Wiese et al., 1992). It has been shown that a medium containing non-charcoal-stripped 5% serum has an equivalent of 2 x 10^{-10} M estrogen (Spink et al., 2003). As demonstrated in the estrogen dose-response study of current chapter (Figure 2.3), estrogen doses of this magnitude were found to be stimulatory to cell growth. Charcoal treatment has been applied to reduce estrogen content of serum significantly (90 to 99% reduction) and was effective in improving the sensitivity of the assay (Butler et al., 1981; Taylor et al., 1984; Wiese et al., 1992). Taken together, it is most likely that the use of non-charcoal treated and/or a higher concentration of serum has masked the agonistic or growth-stimulatory effect of tamoxifen in these previous studies.

Such discrepancies could also carry a clinically relevant message and suggest the hypothesis that tamoxifen at 10^{-8}, 10^{-7} and 10^{-6} M is stimulatory in the absence of estrogen, but inhibitory in the presence of estrogen. This hypothesis was tested and the results were discussed in the following section (2.4.3).

2.4.2 Estradiol dose-response effects

Estrogen is vital for many traditional roles including maintaining healthy bone mass, regulation of ovulation, normal cell proliferation and differentiation in the mammary gland (Koos, 2011; Nelson & Bulun, 2001). Nevertheless, as the case raised in the introduction of this chapter, estrogen is also thought to contribute to breast cancer (Russo et al., 2000; Yager & Davidson, 2006b). Experiments investigating the response of MCF-7 to estradiol have typically involved a narrow range of doses, i.e. 10^{-10}, 10^{-9} and 10^{-8} M (Table 2.2). Consistently, these studies have observed significant stimulation of breast cancer cell proliferation induced by estradiol (Bezwoda & Meyer, 1990; Brünner et al., 1989; Chujo et al., 2003; Duda et al., 1999; Huynh et al., 1996; Katzenellenbogen et al., 1987; Kim et al., 2004; Pratt & Pollak, 1993; Seeger et al., 2006). In line with the findings of these researchers, the present study has

observed significant stimulation in this dose range (18 to 27%), with maximal stimulation at 10^{-9} M (Figure 2.3). At 10^{-7} M, a non-significant increase in cell proliferation versus control took place, similar to the findings of two previous studies (Chujo et al., 2003; Seeger et al., 2006).

Paradoxically, estradiol treatment at 10^{-11} M conferred a significant inhibition of MCF-7 cell proliferation by 34% ($P < 0.001$) (Figure 2.3). This finding is different from the limited studies covering this low dose of estradiol. Specifically, Bezwoda et al. (1990) and Huynh et al. (1996) have found a stimulation instead of inhibition. Treatment duration was 4 and 2 days for these two studies respectively, which is comparable to the current study (3 days). The discrepancy is most likely due to the different cell culture conditions used. The experiments described in this chapter have used 5% charcoal-treated fetal calf serum (CFCS), a serum level commonly reported by similar studies (Table 2.2). In contrast, Huynh et al. (1996) and Bezwoda et al. (1990) have used serum-free culture medium (0% serum) and 2% CFCS for their experiments respectively. This means their cell culture media contains comparatively fewer growth factors for the cells. Therefore, it is possible that as a result of estradiol and growth-factor deprivation, the MCF-7 cells may have become hypersensitive to the proliferative effect of estradiol. Furthermore, in the study conducted by Bezwoda and others (1990), MCF-7 cells were also maintained in estrogen-depleted medium (5% CFCS) prior to the actual estradiol dose-response study. It has been reported that estradiol-deprivation resulted in slower basal cell proliferation rate of MCF-7 while estradiol treatment markedly increased the growth rate (Katzenellenbogen et al., 1987; Masamura et al., 1995; Santen et al., 2008).

Table 2.2 Effect of estradiol treatment in MCF-7 cell culture

Literature	Estradiol (M)						Medium	Phenol red (Yes: +, No : -)	% CFCS or CFBS
	10^{-11}	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}			
(Katzenellenbogen et al., 1987)			↑				MEM	+	5
(Duda et al., 1999)			↑				DMEM	-	5
(Kim et al., 2004)			↑				DMEM	+	5
(Pratt & Pollak, 1993)		↑					DMEM	-	5
(Brünner et al., 1989)				↑			MEM	+	5
(Bezwoda & Meyer, 1990)	↑	↑	↑	↑			KSMLS	-	2
(Huynh et al., 1996)	↑	↑	↑	↑			MEM	-	(0) Serum free
(Seeger et al., 2006)		↑	↑	↑	↑		DMEM	+	5

(Denotation: CFCS or CFBS: dextran-coated charcoal-treated fetal calf or bovine serum, ↓ - Inhibitory, ↑ - stimulatory, blanks – concentrations were not tested)

Interestingly, the current observations together with others (Bezwoda & Meyer, 1990; Huynh et al., 1996) may have clinical significance in view of the *in vivo* findings in premenopausal women with breast cancer treated with oophorectomy (surgical removal of ovary). Based on his observation that postmenopausal women experienced better prognosis than premenopausal women, Albert Schnizinger rationalized that oophorectomy might be effective in treating breast cancer in premenopausal patients (Love & Philips, 2002). As early as 1896, George Thomas Beaston reported a beneficial effect of oophorectomy in premenopausal women with breast cancer, as reviewed by Love et al. (2002). A more recent meta-analysis conducted by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG), which evaluates 15-year effects of various breast cancer treatments, has found significant benefit of oophorectomy in reducing breast cancer recurrence and mortality in premenopausal women (Abe et al., 2005). Pharmacologic oophorectomy, using lutenizing hormone releasing hormone (LHRH) agonists instead of surgery, was also shown to be effective with about 40% objective tumour response rate being reported (Klijn et al., 1985; Muss, 1992). Both surgical and pharmacological oophorectomy reduce estradiol level from 200 pg/ml (i.e. 7.3 x 10^{-10} M) to 10 pg/ml (i.e. 3.7 x 10^{-11} M) (Santen et al., 2008). In the present study, MCF-7 cell cultures were maintained in medium containing 10% fetal calf serum (FCS) prior to the dose-response experiments, which may reflect the premenopausal condition especially in terms of estradiol level (Fan et al., 2009). When MCF-7 cells were treated with estradiol at 10^{-11} M, similar to the serum estradiol level found in women after oophorectomy, significant inhibition (34%, $P<0.001$) in cell proliferation was observed. These *in vitro* data could provide an explanation for how oophorectomy works clinically.

A high dose of 10^{-6} M, estradiol caused a non-statistically significant change of MCF-7 cell proliferation based on the pooled results of 4 independent experiments. Prior to the introduction of tamoxifen in 1970s, estrogen or estrogen analogues were the mainstream endocrine medicine used for breast cancer (Adelson et al., 2011; Muss, 1992). A number of earlier studies reported regression in 30-40% patients treated with estrogen (Haddow et al., 1944).

These authors suggested that estrogen, although shown to be carcinogenic, might also retard the growth of . The current dose-response effect for estradiol (Figure 2.3) favours this hypothesis.

2.4.3 Estradiol dependency of tamoxifen action

The second hypothesis to address was that tamoxifen at doses shown to be stimulatory (10^{-8} and 10^{-7} M) (Figure 2.2) becomes inhibitory in the presence of estradiol. Cell proliferation data obtained for combined treatment of estradiol and tamoxifen were graphically represented in Figure 2.4. A tamoxifen dose of 10^{-5} M, which suppressed MCF-7 cell proliferation by 65% by itself, still remained inhibitory (35 and 66% of control) even in the presence of estradiol at stimulatory doses. This finding suggests that the inhibition contributed by this dose is less reversible by physiological concentrations of estradiol. At 10^{-6} M and 10^{-7} M tamoxifen, the combination with tested concentrations of estradiol (10^{-9} to 10^{-7} M) was always significantly inhibitory (Figure 2.4). Roos and others (1982) also observed 10^{-7} and 10^{-6} M tamoxifen alone were stimulatory to MCF-7 cell proliferation in the absence of estradiol but became inhibitory in the presence of 10^{-9} M estradiol, a finding consistent with the current study. However, at 10^{-9} M and 10^{-8} M tamoxifen, the effects become variable in the presence of estradiol. In essence, the data indicate that at moderate doses of tamoxifen (10^{-9} and 10^{-8} M), there is a threshold of estradiol which allows a significant inhibitory action to occur.

2.5 Conclusions

The present study has demonstrated a dose-dependent action of tamoxifen on MCF-7 cell proliferation with a paradoxical pattern: inhibitory at very low and high concentrations and stimulatory at intermediate concentrations. All of the concentrations are within the expected physiological range that would be found in patients undergoing tamoxifen treatment. Low dose stimulatory and high dose inhibitory effects of tamoxifen have been reported in another ER+ cell line, T-47D (Reddel & Sutherland, 1984). These authors also mentioned a similar

biphasic effect of tamoxifen in MCF-7 cells. The stimulatory action of tamoxifen in breast cancer cell culture observed *in vitro* could correlate with the clinical phenomenon of 'tumour flare' as discussed and may have clinical implications. Nonetheless, undoubtedly there are major differences between cell culture experiments and the human body. The current study highlights the need for a more systematic *in vivo* investigation of clinical effects of tamoxifen treatment over a range of different doses.

Estrogen at very low doses (10^{-11} M) was inhibitory to MCF-7 cell proliferation. This could partially explain the efficacy of oophorectomy in treatment of breast cancer. At high dose (e.g. $\geq 10^{-6}$ M), estrogen may be of therapeutic value as it can inhibit breast cancer cell proliferation. Estradiol alone at 10^{-10} to 10^{-8} M, normal physiological estradiol concentrations in women, showed a stimulatory activity to breast cancer cell proliferation and hence could be a risk factor for breast cancer. It is not well appreciated that estrogen alone can be either stimulatory or inhibitory, depending on the doses and sensitivity of MCF-7 cells to estrogen, which could arise from different cell culture conditions or cellular environment. In addition, the data reported here are consistent with the hypothesis raised at the beginning of the chapter: that tamoxifen doses previously shown to be stimulatory may become inhibitory in the presence of estrogen. These data suggest that the effects of tamoxifen are modulated by estrogen. A practical question raised here is: could the various issues discussed in this chapter, i.e. serum concentration of estrogen and tamoxifen, and sensitivity of breast cancer cells to estrogen, partially explain why tamoxifen therapy is effective in less than 50% of the cases? This question may warrant a clinical investigation.

Chapter 3 Metabolite profiling and quantification of compounds in potato extracts

3.1 Introduction

As presented in Chapter 2, tamoxifen and estrogen have shown efficacy in breast cancer treatment, but their actions appear to be paradoxical. Epidemiological data support that a diet rich in vegetables and fruits may contribute to a decreased risk of cancer including breast cancer (Block et al., 1992; Gandini et al., 2000). Knowledge about the role of potato in human diet has evolved, not only as a major source of energy, but also with potential value in chemoprevention and management of chronic diseases such as cancer (Camire et al., 2009). Purple potato 'Urenika' has been chosen for the current thesis because of its New Zealand proprietary value. In relation to breast cancer, there is a lack of data on the effect of 'Urenika'. There is also very limited published information regarding the metabolite profile of *Solanum tuberosum* L. 'Urenika'.

Owing to the vast diversity of metabolites in the plant kingdom and variation in growing season and conditions (e.g. soil, water, nutrients), profiling of plant materials is tedious and challenging with traditional chemical analysis. Furthermore, great variability in the concentrations of plant metabolites makes identification and quantification difficult. Hence, deciphering the chemical composition of plant material and extracts is traditionally one of the greatest bottlenecks in plant related research. Nuclear magnetic resonance is a powerful tool for identification of bulk metabolites, however, it is less sensitive than mass spectrometry (MS) for measuring compounds present at minute concentrations (Allwood & Goodacre, 2009; De Vos et al., 2007). Gas chromatography (GC) coupled with MS is useful for analysis of volatile organic compounds and primary metabolites; prior derivatisation is normally required (Allwood & Goodacre, 2009). Plant metabolites also embrace a rich diversity of secondary metabolites including phenolic acids, anthocyanins, flavonols, glycoalkaloids

and polyamine derivatives which are semi-polar. These compounds are found to be best detected and analysed by LC-MS (Allwood & Goodacre, 2009; De Vos et al., 2007). The advent of ultra high performance liquid chromatography (UHPLC) and MS based technology allows a better separation of compounds and precision of mass measurements.

There are two key aims for the current chapter of work:

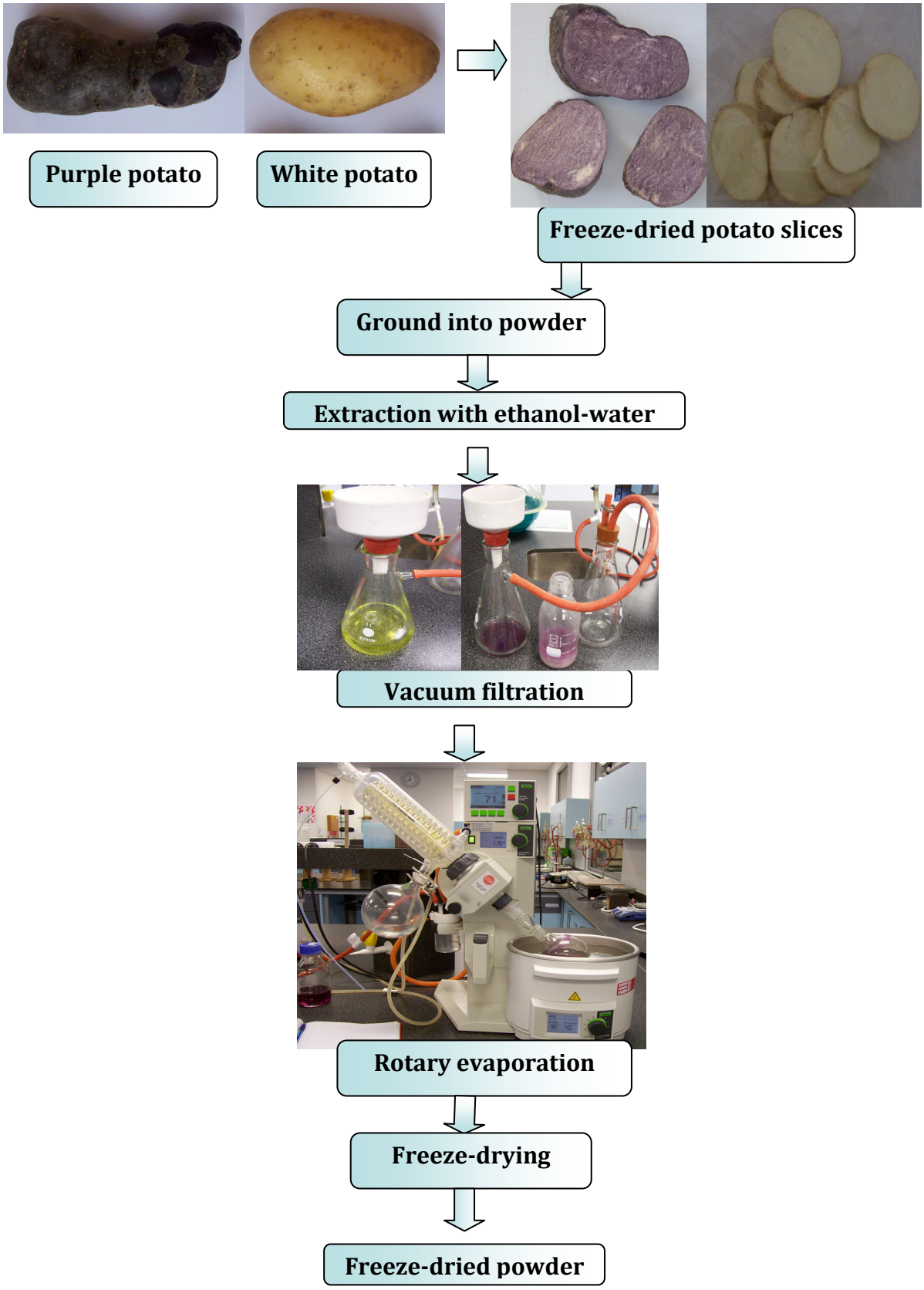
1) To produce a phytochemical-rich potato extract which could be used for cell proliferation study and cell cycle analysis (Chapter 4). Both pigmented, purple potato *S. tuberosum* L. 'Urenika' and white potato were selected for making two individual extracts – purple potato extract (PPE) and white potato extract (WPE). Single batch of each extract was used throughout all the experiments presented in the current thesis.

2) To conduct comprehensive characterisation or composition profiling using Ultra high performance liquid chromatography-electrospray ionisation-time of flight-mass spectrometry (UHPLC-ESI-TOF-MS).

3.2 Methods & materials

3.2.1 Preparation of potato extracts

Purple and white potatoes were obtained from a fresh produce market and farms in the Manawatu region of New Zealand. Whole potatoes were washed, sliced thinly, freeze-dried and ground to powder prior to extraction of phenolic compounds. Ethanol was chosen as the solvent for extraction (Shahidi & Naczk, 2004). The potato powder was mixed with absolute ethanol (Sigma, St Louis) and water (4:1) for 2 h. The mixture was filtered through filter paper (11 µm) and ethanol present in the filtrate was removed under vacuum using a rotary evaporator (Rotavapor R-215, Buchi, Switzerland). The concentrated extract liquid was freeze-dried (Figure 3.1). The resulting solvent-free purple and white potato extracts were stored as powder at -20°C for further analysis including UHPLC-MS profiling.



Fgure 3.1 Process of making potato extracts

3.2.2 Standards

Pure standards were used for qualitative and quantitative analysis (Table 3.1). All chemicals were purchased from Sigma (St. Louis), except for citric acid (Merk, Darmstadt), L-tyrosine (BDH Laboratory Supplies, Poole) and α-solanine (Phenomenex, Auckland).

Table 3.1 Standards for identification and quantification of unknowns

Pure compounds
Ascorbic acid
Caffeic acid
Catechin
Chlorogenic acid
Citric acid
p-coumaric quinic acid
Cyanidin-3-galactoside
Cyanidin-3-glucoside
Malvidin-3-glucoside
Epicatechin
Kaempferol-3-glucoside
Kaempferol-3-rutinoside
α-solanine
Procyanidin B2
L-phenylalanine
Quercetin
Quercetin-3-rutinoside
Quercetin-3-galactoside
Quercetin-3-glucoside
Quercetin-3-rhamnoside
Quinic acid
Spermidine
Spermine
L-tyrosine
L-tryptophan

3.2.3 Ultra high performance liquid chromatography - mass spectrometry : Tentative identification and quantification of compounds present in potato extracts

Ultra high performance liquid chromatography-electrospray ionisation-time of flight-mass spectrometry (UHPLC-ESI-TOF-MS) was used to measure the compounds present in the potato extracts. Potato extract was reconstituted in methanol/water/formic acid (50:50:1 v/v/v). The UHPLC system was a Dionex Ultimate 3000 Rapid Separation LC system (McGhie et al., 2012). The chromatographic column was a Zorbax SB-C18 2.1 x 100 mm, 1.8 µm (Agilent, Melbourne, Australia), maintained at 50 °C and operated under gradient mode. Mobile phases A and B consisted of 90% of methanol in water (v/v) and 0.5% formic acid respectively. Column flow rate was set at 400 µL/min. The solvent gradient was as follows: 95% B, 0-0.5 min; linear gradient to 60% B, 0.5-8 min; linear gradient to 25% B, 8-11 min; linear gradient to 100% A, 11-12 min; composition held at 100% A, 12-14 min; linear gradient to 95% B, 14-14.2 min; return to initial conditions before another sample injection at 16.5 min. Samples and standards contained in HPLC vials were placed in the sample compartment and uploaded automatically by the LC system. The injection volume was 2 µL. The UHPLC system was connected to a micrOTOF Q II time-of-flight (ESI-TOF) mass spectrometer (Bruker Daltonik, Bremen, Germany) fitted with an electrospray ionization source. Samples were analysed under both positive and negative ionisation modes. Capillary voltages of -4000 V and +3500 V were applied for positive and negative modes respectively. Nebulizer gas (N_2) with a pressure of 1.5 bars, and dry gas (200 ℃) flow of 8 L/min was applied. The mass analyser measures mass (m) to charge (z) ratio (m/z) of the ions, ranging from 80 to 1500 Da, at a speed of 2 acquisitions per second.

Identities of compounds were deduced based on a combination of information generated by the LC-MS system, i.e. retention time, order of elution, mass-to-charge (*m/z*) ratio, and chemical formula. Table A1 also displays two important quality control parameters: Error and mSigma values. Error (mDa) is the deviation between the measured mass and theoretical mass. Error alone is

insufficient to ensure a high accuracy in molecular formula assignment. Another quality parameter, isotopic pattern fit factor (mSigma), is a powerful approach to reduce the number of false candidates, thus improving the accuracy of formula assignment substantially (Pelander et al., 2011). In essence, the smaller the 'error' and 'mSigma' values, the better the fit between the theoretical and assigned formula. An error of 4 mDa was set as the limit in the current study. The mSigma value of 50 was used as an additional criterion (Pelander et al., 2011).

For quantification or estimation of concentrations of these compounds detected, external calibration curves were used for 25 authentic standards, measured at 5 to 6 µg/mL, which was in the middle of their linear concentration range (r^2 = 0.9998 to 1). Hence, the regression equations were used to calculate the amount of corresponding compounds detected. In the case where standard was not commcercially available, the compound in question was quantified with the standard compound with similar structure (Table 3.2, i.e. caffeoyl quinic acid derivatives were quantified by reference to chlorogenic acid; polyamine derivatives by reference to spermine; anthocyanins by reference to malvidin-3-glucose).

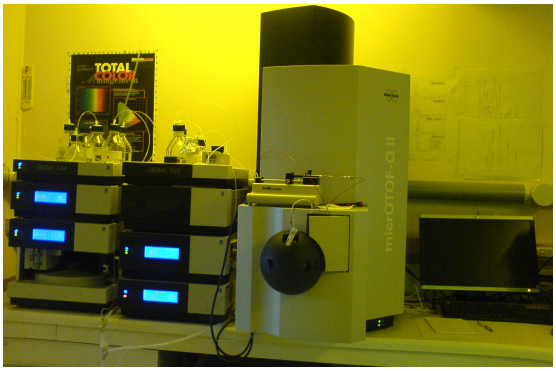


Figure 3.2A UHPL-MS system.

UHPLC (left) was combined with micrOTOF-Q II mass spectrometry (middle), connected to a computer system (right) built-in with software used for running the LC-MS system and data analysis.

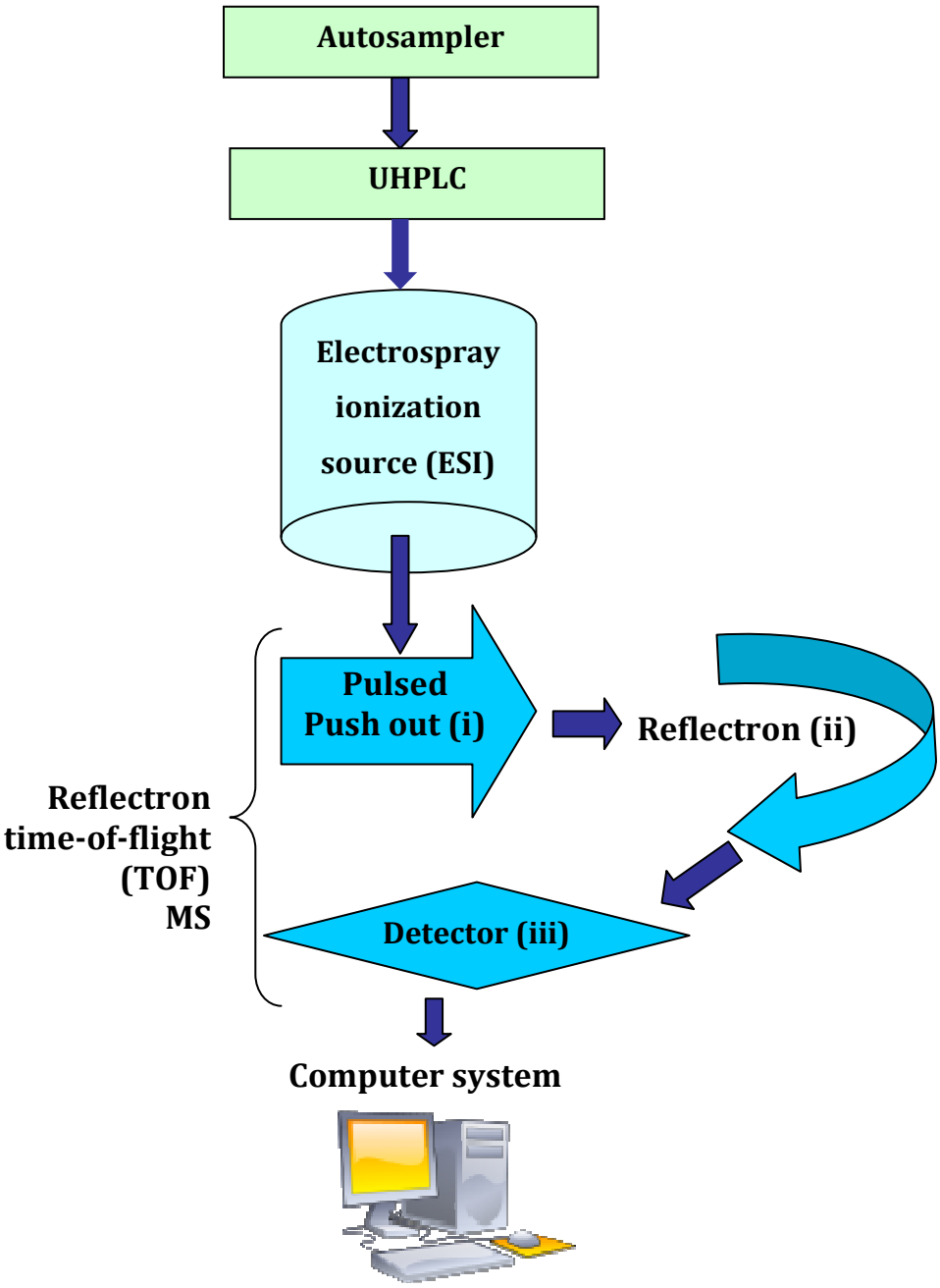


Figure 3.2B Steps involved in LC-MS analysis.

Sample was injected automatically into the HPLC system, and then eluted and ionised by the ESI source. Charged droplets were focused toward the TOF MS and reflected by the reflectron. Components were detected; signals or data were collected and retrievable with the specialised computer programmes built-in for the LC-MS analysis. Figure created after information given by Korfmacher (2005).

3.3 Results

A number of compounds were identified for the first time in 'Urenika' purple potato extract. In the current study, seven and six classes of metabolites have been identified for the purple and white potato extracts respectively (Figure 3.3 & 3.4). Standard profiles, as shown from Figure A2 to A7 (Appendix), were used for confirmation of the peak assignment as well as quantification (Table 3.2).

A total of 31 compounds, as indicated in the base peak chromatogram (BPC) (Figure 3.3 & 3.4), have been identified and tabulated in Table A1 (Appendix). In the current study, the average of error was 0.2 mDa (26 formulae with <2 mDa, and 6 formulae: 2 to 4 mDa). All mSigma values were well below the limit of 50, with the majority being ≤ 20 with an average of 12.7. Therefore, formulae and names of compounds in the potato extracts have been assigned with a high level of confidence and accuracy. Chemical structures of compounds detected are illustrated in Figure A9 to A16 (Appendix) for reference.

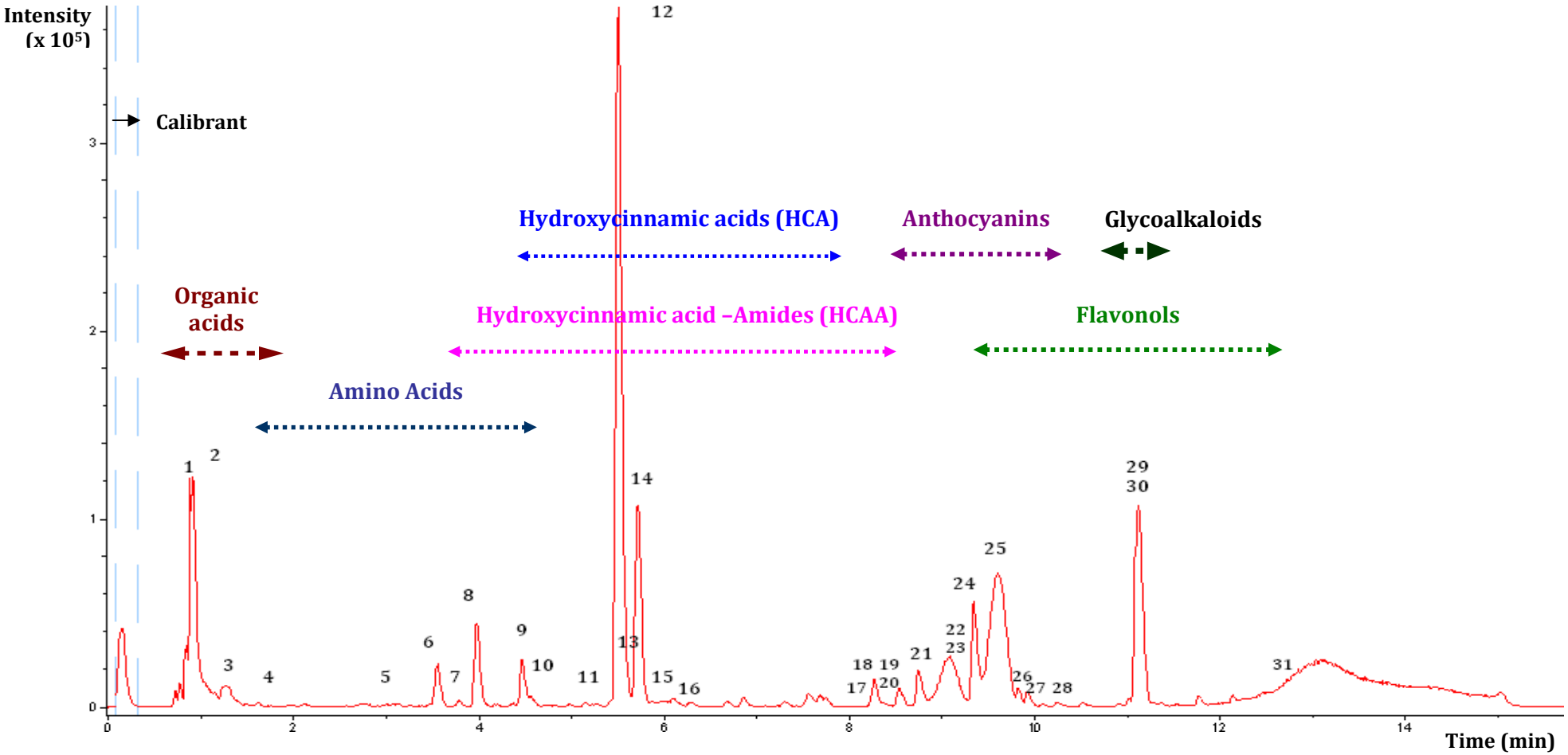


Figure 3.3 Base peak chromatogram (BPC) of purple potato extract obtained by UHPLC-ESI-TOF-MS.
The numbers correspond to the different compounds detected as tabulated in Table A1 (Appendix). Classes of compounds and elution time range were indicated.

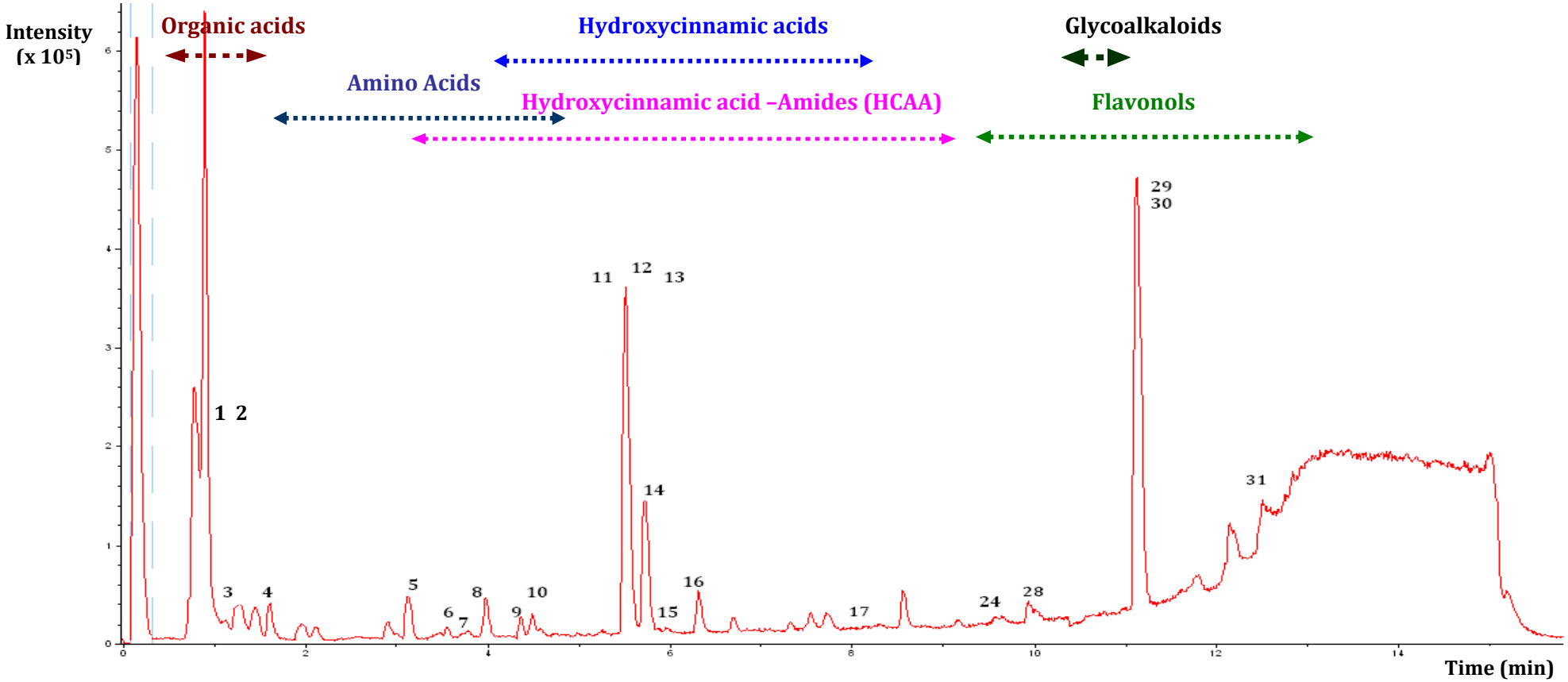


Figure 3.4 BPC of white potato extract obtained by UHPLC-ESI-TOF-MS.
The numbers correspond to the different compounds detected as tabulated in Table A1. Classes of compounds and elution time range were indicated.

Table 3.2 Quantity of each compound detected in the potato extracts using UHPLC-ESI-TOF-MS

Peak #	Compound	Quantified with standard	PPE*	WPE*	Fold (WPE/PPE)
1	Quinic acid	Quinic acid	1.8	3.3	2.4
2	Ascorbic acid	Ascorbic acid	0.1	5.3	53
3	Citric acid	Citric acid	64.8	46.1	0.7
4	Tyrosine	Tyrosine	6.3	18.3	2.9
5	Phenylalanine	Phenylalanine	1.8	10	5.5
6	Caffeoyl putrescine	Spermine	72.9	20.4	0.3
7	Caffeoyl spermine	Spermine	2.8	5.4	1.9
8	Neochlorogenic acid	Chlorogenic acid	1.7	0.5	0.3
9	Tryptophan	Tryptophan	0.7	1.6	2.4
10	3-Caffeoyl-5-feruloylquinic acid	Chlorogenic acid	1.0	0.3	0.4
11	Caffeoyl-D-glucose	Chlorogenic acid	0.3	0	0
12	Chlorogenic acid	Chlorogenic acid	25.2	11.8	0.5
13	Caffeic acid	Caffeic acid	2.7	1.3	0.5
14	Crytochlorogenic acid	Chlorogenic acid	6.1	2.6	0.4
15	Dehydrophaseic acid hexose	No standard	-	-	1.2
16	Bis(dihydrocaffeoyl) spermidine	Caffeic acid	9.4	101.5	11
17	5-feruloylquinic acid	Chlorogenic acid	1.7	0.4	0.3
18	Malvidin-3-rutinoside	Malvidin-3-glucose	0.03	0	0
19	Petunidin-3-caffeoylrutinoside-5-glucoside	Malvidin-3-glucose	0.025	0	0
20	Delphinidin-3-p-coumaroylrutinoside-5-glucoside	Malvidin-3-glucose	0.4	0	0

Abbreviation: PPE – Purple potato extract, WPE – White potato extract, unit: mg/g dry weight)

Table 3.2 *(Continued)*

Peak #	Compound	Quantified with standard	PPE*	WPE*	Fold (WPE/PPE)
21	N^1,N^4,N^{12}-tris(dihydrocaffeoyl) spermine	Caffeic acid	18.2	29.5	1.6
22	Petunidin-3-p-coumaroylrutinoside-5-glucoside	Malvidin-3-glucose	5.4	0	0
23	Petunidin-3-feruloylrutinoside-5-glucoside	Malvidin-3-glucose	0.3	0	0
24	Quercetin-3-rutinoside	Quercetin-3-rutinoside	0.015	0.12	8.3
25	Malvidin-3-p-coumaroylrutinoside-5-glucoside	Malvidin-3-glucose	8.1	0	0
26	Malvidin-3-feruloylrutinoside-5-glucoside	Malvidin-3-glucose	0.92	0	0
27	Petunidin-3-p-coumaroylrutinoside	Malvidin-3-glucose	0.1	0	0
28	Kaempferol-3-rutinoside	Kaempferol-3-rutinoside	0.038	0.0049	0.1
29	Chaconine	Solanine	24.5	36.5	1.5
30	Solanine	Solanine	3.5	3.2	1.2
31	Quercetin dimethyl ether	Quercetin	0.25	0.12	0.5

3.4 Discussion

Both purple and white potato extracts were freeze dried and ground into powder to increase surface area for maximum extraction efficiency. An unselective extraction protocol was used with ethanol and water as solvent, in order to extract the majority of metabolites expected in potato. Ethanol was chosen since it is one of the most commonly used and efficient solvents for extracting both polar and semi-polar metabolites from plant materials (Shahidi & Naczk, 2004). In addition, compared to other solvents such as methanol and chloroform, ethanol is more favourable in terms of toxicity and food safety, so that if the potato extract was to be developed into a nutraceutical or pharmaceutical product, it could be done safely (Lapornik et al., 2005). The extraction method resulted in an extract containing various classes of compounds as evidenced in Table 3.2. The potential functional role of compounds identified will be discussed in relation to cancer in Chapter 4.

3.4.1 Organic acids

This is the first report of the presence and quantification of quinic acid and ascorbic acid (vitamin C) in 'Urenika'. Quinic acid derivatives have been previously reported in a white potato cultivar but not quantified (Shakya & Navarre, 2006). Citric acid, quinic acid, and vitamin C detected in 'Urenika', were quantified with the use of standard compounds (Figure A5 & A6, Appendix).

3.4.2 Amino acids

Three amino acids, namely tyrosine, phenyalanine and tryptophan, were detected in both potato extracts, confirmed and quantified with corresponding standards (A5 & A6, Appendix). Amino acids ionise more efficiently in positive ion mode than negative ion mode. As an example, Table 3.3 shows that the intensity of the tryptophan molecular ion detected by positive ion mode is 4-fold that in negative ion mode for both potato extracts. Therefore, the

quantification of the amino acids was based on the data obtained with positive ion mode.

Table 3.3 Intensity of tryptophan molecular ion obtained with positive and negative ionisation modes – a comparison

Potato extracts	Signal intensity		Fold (P/N)
	Positive ion mode (P)	Negative ion mode (N)	
PPE	28958	6723	3.3
WPE	48707	13195	3.7

Tyrosine was found to be the most abundant among the three amino acids, in agreement with a LC-MS study conducted by Shakya and Navarre for a potato extract (Shakya & Navarre, 2006). The present study showed that white potato extract has more tyrosine and phenylalanine than the purple potato extract.

3.4.3 Phenolic acids: hydroxycinnamic acids and its derivatives

Apart from chlorogenic acid, the presence and quantity of all the other phenolic acids identified, i.e. cryptochlorogenic acid, neochlorogenic acid, 3-caffeoyl-5-feruloylquinic acid, caffeoyl-D-glucose and 5-feruloylquinic acid are thus far reported for the first time for 'Urenika' (Table 3.2). Multiple forms of hydroxycinnamic acids (HCA) exist in the plant kingdom; the most abundant form is caffeic acid, i.e. 3,4-dihydroxycinnamic acid (Clifford, 2000). In potato tubers, the most common form of HCA is caffeic acid (Lachman & Hamouz, 2005). Other forms of HCA of less abundance in potato tuber include ferulic acid, *p*-coumaric acid, sinapic acid, salicylic acid (Figure A11, Appendix). However, it has been suggested that HCA rarely exists as free acid, instead, it is usually present as a conjugate in nature (Clifford, 2000). Indeed, as revealed in the present study, only caffeic acid was detected, but not the other free forms of HCA. Caffeic acid was confirmed and quantified based on data obtained for a

standard (Figure A3, Appendix). One ferulic acid derivative was detected, i.e. 5-feruloylquinic acid, but not the free form of ferulic acid.

It has been reported that purple potato contained 2-fold higher total phenolic acids than non-pigmented potato (Lewis et al., 1998). The current study observed the same phenomenon; the purple potato extract was found to contain 38.4 mg total phenolic acids / g of extract, which was twice as high as white potato extract (16.9 mg/g extract). Chlorogenic acid was the predominant form of phenolic acid in both potato extracts, followed by two isomers i.e. cryptochlorogenic acid and neochlorogenic acid (Figure A12, Appendix), in agreement with others (Table 3.4).

Table 3.4 Proportion of each chlorogenic acid isomer reported for potato tubers

CHA isomers	% Total chlorogenic acid isomers (CHA+CCHA+NCHA)			
	Current Study		(Vinson et al., 2012)	(Navarre et al., 2011)
	PPE	WPE	Purple Majesty	Average of 50 cultivars
ChA	76	79	84	83
CChA	19	18	12	12
NChA	5	8	5	5

(Abbreviations: PPE – purple potato extract, WPE – white potato extract, ChA – chlorogenic acid, CChA – cryptochlorogenic acid, NChA – neochlorogenic acid)

3.4.4 Anthocyanins

The present study detected 5 anthocyanin compounds which have not been reported previously in cultivar 'Urenika', namely petunidin-3-caffeoylrutinoside-5-glucoside, delphinidin-3-*p*-coumaroylrutinoside-5-glucoside, petunidin-3-feruloylrutinoside-5-glucoside, malvidin-3-feruloylrutinoside-5-glucoside and petunidin-3-*p*-coumaroylrutinoside. All the

anthocyanins detected are in the form of acylated glucosides, which agree well with the notion that more than 98% of anthocyanins in potato are acylated anthocyanins (Lachman & Hamouz, 2005). These anthocyanins contain a hydroxycinnamic acid component, for instance, coumaric acid is contained in malvidin-3-p-coumaroylrutinoside-5-glucoside; caffeic acid is contained in petunidin-3-caffeoylrutinoside-5-glucoside; ferulic acid is contained in petunidin-3-feruloylrutinoside-5-glucoside (Figure A13, Appendix). The purple potato extract was found to contain 15.3 mg anthocyanins/g of extract whereas there was none detected in the white potato extract.

It has been suggested that the predominant form of anthocyanins in purple potato varieties are malvidin-3-*p*-coumaroylrutinoside-5-glucoside and petunidin-3-*p*-coumaroylrutinoside-5-glucoside (Brown, 2005; Lewis et al., 1998). Indeed, malvidin-3-*p*-coumaroylrutinoside-5-glucoside (peak **25**) was found to be the most abundant anthocyanin in the purple potato extract, accounting for 53% of the total anthocyanin content, followed by petunidin-3-*p*-coumaroylrutinoside-5-glucoside (35%) and malvidin-3-feruloylrutinoside-5-glucoside (6%). The other 5 anthocyanins made up the balance of 6%. Note that all these 8 anthocyanins eluted within a very short span of time of 2 min, yet the UHPLC and methodology adopted were able to produce good separation (Figure 3.3). Several compounds co-eluted at the same time, i.e. 5-feruloylquinic acid (peak **17**) and malvidin-3-rutinoside (peak **18**); petunidin-3-*p*-coumaroylrutinoside-5-glucoside (peak **22**) and petunidin-3-feruloylrutinoside-5-glucoside (peak **23).** Petunidin-3-caffeoylrutinoside-5-glucoside (peak **19**) and delphinidin-3-*p*-coumaroylrutinoside-5-glucoside (peak **20**) also co-eluted at the same time. The identification of potato anthocyanins has been challenging due to the unavailability of standards of these unusual anthocyanins. Nevertheless, these anthocynanin compounds have been confidently identified, on the basis of formula detected, measured *m/z* values for the molecular ion, and reference to existing relevant literature (Eichhorn & Winterhalter, 2005; Hillebrand et al., 2009).

3.4.5 Flavonols

Three types of flavonols were detected in both potato extracts using the negative ionisation mode. The first flavonol eluted at 9.57 min with *m/z* 609 (peak **24**) was assigned as quercetin-3-rutinoside, also known as rutin. The second flavonol eluted at 10.2 min with *m/z* 593 (peak **28**) was assigned as kaempferol-3-rutinoside. With the inclusion of the pure standards in the current study, the identity and quantity could be confidently confirmed. The identities and quantities detected for these two flavonols by the present study are in good agreement with the findings of Navarre et al. (2011) (Table 3.5). The third flavonol detected in both potato extracts was quercetin dimethyl ether, based on its chemical formula, retention time and *m/z* 329 (Shakya & Navarre, 2006). Overall, a low flavonol concentration (0.3 mg/g) was present in the purple potato extract, accounting for only 0.6% of the total of phenolic compounds discussed (phenolic acids + anthocyanins + flavonols). Similar amounts of flavonols, 0.25 mg/g, were detected in the white potato extract, which is about 1.5% of the phenolic acids content of the extract. Previous studies have also reported minute presence of flavonols, ranked the least among various phenolic groups detected in potato tubers (Lewis et al., 1998; Navarre et al., 2011; Shakya & Navarre, 2006).

Table 3.5 Quantity of each flanovols estimated in potato tubers

Flavonol	Present study (mg / 100g DW)		(Navarre et al., 2011) (mg / 100g DW)
	Purple potato tuber	White potato tuber	50 cultivars
Quercetin-3-rutinoside	0.15	1.2	0 to 14
Kaempferol-3-rutinoside	0.38	0.049	0 to 43
Quercetin dimethyl ether	2.5	1.2	Not reported

3.4.6 Hydroxycinnamic acid amides

Four forms of hydroxycinnamic acid amides or polyamines were reported for the first time in 'Urenika', namely caffeoyl putrescine, caffeoyl spermine, bis(dihydrocaffeoyl) spermidine and N^1,N^4,N^{12}-tris(dihydrocaffeoyl) spermine. At least 3 isomers of bis(dihydrocaffeoyl) spermidine have been reported (Figure A15, Appendix) (Gancel et al., 2008; Parr et al., 2005). As an authentic standard was not available, the specific isomer could not be determined. Caffeoyl amides including caffeoyl putrescine and caffeoyl spermine were found in many plants (Martin-Tanguy et al., 1978). Dihydrocaffeoyl polyamines also called kukoamines were first found in a Chinese medicinal plant *Lycium chinense* (Funayama et al., 1980; Funayama et al., 1995). Subsequently, these compounds were also found in other solanaceous species including *Solanum quitoense* Lam (naranjilla fruit) and *Solanum lycopersicum* (tomato) (Gancel et al., 2008; Parr et al., 2005).

3.4.7 Glycoalkaloids

Two compounds (peak **29**, **30**) co-eluted at 11.1 min, the same retention time as the authentic standard α-solanine (Figure A3 Appendix). One of them with *m/z* 868.5072 (positive ionisation mode) was unambiguously confirmed as α-solanine. The second compound with *m/z* 852.5136 and chemical formula $C_{45}H_{73}NO_{14}$ was identified as α-chaconine. Both compounds were quantified as α-solanine equivalent, given their similarity in chemical structures and behaviour in LC-MS analysis. Both compounds contain the same aglycone unit, solanidine, and a glycosidic unit which distinguishes them (Figure A16, Appendix).

Alpha-chaconine and α-solanine are the commonest glycoalkaloids found in *S. tuberosum* species, comprising 95% of the total potato glycoalkaloid content (Sotelo & Serrano, 2000). Consistently, the present study together with several other studies have demonstrated that these two forms of glycoalkaloids are the

readily detectable glycoalkaloids in a number of potato cultivars (Kozukue et al., 2008; Navarre et al., 2011; Shakya & Navarre, 2006; Sotelo & Serrano, 2000). The white potato extract contained a slightly higher amount of glycoalkaloids than the purple potato extract (40.7 mg versus 28 mg / g extract).

3.5 Conclusions

The objectives set for the present chapter have been successfully accomplished: 1) Purple and white potato ethanol extracts were successfully produced; 2) Using UHPLC-ESI-TOF-MS, the potato extracts were comprehensively characterised. For the purple potato (Urenika), the presence and quantity of majority of the compounds were reported for the first time (e.g. bis(dihydrocaffeoyl) spermidine, tris(dihydrocaffeoly) spermine, several forms of anthocyanins and hydroxycinnamic acid derivatives). In conclusion, the unknown plant metabolites in both potato extracts have been assigned with molecular formulae and identified with high confidence. Quantification for the individual metabolites is also accurate; a number of relevant standards were included in the LC-MS analysis. The phytochemical composition knowledge obtained from this chapter helps in understanding the potential biological acivity of the potato extracts. These exact concentrations each phytochemical identified, however, are specific to these single batch of extracts and may not be generalised beyond that.

Chapter 4 Purple and white potato extracts alone inhibit cell proliferation of four breast cancer cell lines (MCF-7, T-47D, MDA-MB-231 and 4T1) and work synergistically with estradiol or tamoxifen

4.1 Introduction

In the quest to prevent and combat breast cancer with higher success rates and fewer side effects, food and nutrition represent a logical strategy to explore, given the proven link between cancer and diet (World Cancer Research Fund / American Institute for Cancer Research, 2007). Research pertaining to the potential anticancer effects of vegetables and fruits has suggested the health benefits of a diverse array of phytochemicals (Amiot, 2009). Furthermore, it is becoming increasingly appreciated that potential chemopreventive or anticancer activity of certain plant foods result from the combined or synergistic effects of numerous compounds such as phytochemicals present in these foods (Boivin et al., 2009; Lee et al., 2004; Liu, 2003, 2004; Russo, 2007; World Cancer Research Fund / American Institute for Cancer Research, 2007). Therefore, the use of a whole plant extract is a meaningful approach to assess the anticancer properties of a particular vegetable or fruit, since such an extract contains many bioactive compounds, and when compared to a single compound or class of compounds, a whole extract is more representative of the original food consumed by human beings. Predigestion of potato extracts was not performed prior to cell culture experiments; future *in vivo* efficacy study would be more meaningful to investigate the potential physiological effects upon digestion of these extracts (e.g. metabolism, toxicity). As shown in the previous chapter, purple potato extract (PPE) and white potato extract (WPE) contained a wide range of phytochemicals including chlorogenic acid, caffeic acid, anthoacyanins which have been reported to have anticancer biogical effects (Kampa et al., 2004; Leo et al., 2008; Zhao et al., 2009). This observation forms the fundamental rationale for the use of whole potato extract throughout the work presented in the current chapter.

4.1.1 Cell proliferation

Data were presented in Chapter 2 that suggested estradiol or tamoxifen over a certain dose range can be stimulatory to cancer cell growth. The overall effect on cell proliferation when potato extract was administered at the same time as estradiol or tamoxifen had not been examined to date. Hence, in this chapter, MCF-7 cells were treated with potato extracts in the presence of various physiological concentrations of estradiol to identify any potential interaction. Another estrogen receptor positive (ER+) breast cancer cell line, T-47D, was also treated with several doses of estradiol or tamoxifen to examine the effect of these agents on cell proliferation. Furthermore, in the presence of either estradiol or tamoxifen, T-47D cells were treated with several concentrations of potato extract to determine potential interactions between the potato extract and these agents. Two aggressive cell lines, MDA-MB-231 and 4T1, were also included and treated with potato extract and tested in a cell proliferation assay. These experiments were designed to test the following hypotheses:

- Hypothesis 1: the potato extracts described in Chapter 3 are inhibitory to MCF-7, T-47D, MDA-MB-231 and 4T1 breast cancer cell proliferation.
- Hypothesis 2: the potato extracts attenuate cell proliferation in the presence of physiological concentrations of estradiol.
- Hypothesis 3: the potato extract works in complement with tamoxifen to suppress breast cancer cell proliferation.

4.1.2 Apoptosis & cell cycle modulation

Cell proliferation is characterised by cells actively cycling continuously in different cycle stages (Williams & Stoeber, 2012). The cell cycle in a eukaryotic cell is divided into five sequential phases (Figure 4.1). The synthesis of DNA and replication of the genome occurs during the S phase. Prior to S phase, the cells can either be at G0 (quiescence) or G1. Following the G2 phase, the cells enter the M (mitosis) phase, whereby the separation of chromosomes and division of the cytoplasm is taking place, giving rise to two new daughter cells (Garrett,

2001). Deregulation of cell cycle control leads to aberrant cell proliferation, a common feature of malignant disease (Garrett, 2001; Williams & Stoeber, 2012).

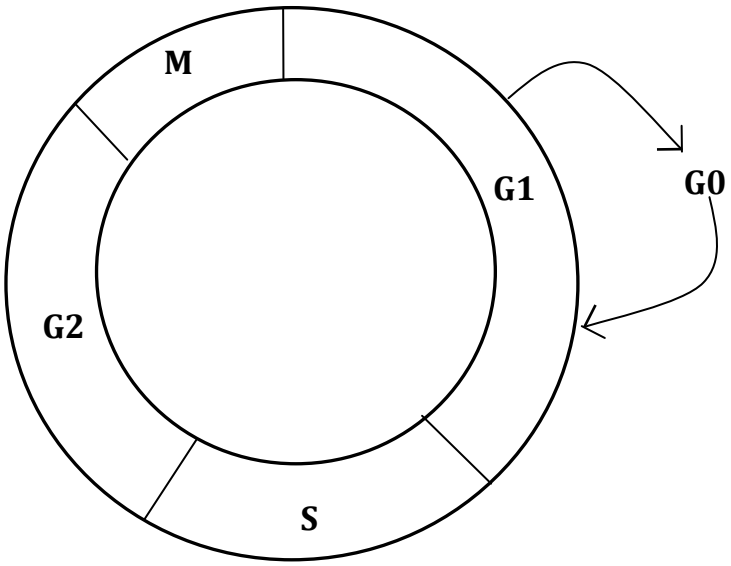


Figure 4.1 The cell cycle

Cancer cells can also possess the ability to suppress apoptosis or "programmed cell death", the counterpart to mitosis. Apoptosis is a vital homeostatic mechanism of regression, counteracting aberrant cell proliferation, maintaining proper cell population in tissues and thus tissue integrity. Deregulation of apoptosis has been closely related to many forms of cancers (Elmore, 2007). Therefore, both cell cycle control and apoptosis are well recognised as important targets in cancer therapy and chemoprevention.

PPE and WPE exerted similar inhibitory effects against breast cancer cell proliferation (4.3.1). Additive or synergistic effects were also observed with the combined treatments of PPE or WPE with estradiol or tamoxifen in T-47D ER+ human breast cancer cell line (4.3.1). As discussed, cell proliferation, cell cycle and apoptosis are biological processes that are closely associated. Furthermore, the antiproliferative effect of tamoxifen and potato extracts (WPE and PPE) could be partially explained by the mechanisms of apoptosis and cell cycle modulation. The following hypotheses were tested using T-47D human breast cancer cell line:

1) Treatment with PPE induces apoptosis and/or modulates cell cycle parameters.
2) Treatment with tamoxifen induces apoptosis and modulates cell cycle parameters.
3) Combined treatment with PPE and tamoxifen induces apoptosis and modulates cell cycle parameters.
4) Combined treatment with PPE and estradiol induces apoptosis and/or modulates cell cycle parameters.

4.2 Methods & materials

4.2.1 Cell proliferation experiments

Cell culture conditions and MTT assay methodology are detailed in Chapter 2. The cell lines used in the current study require different media for growth. Basic or incomplete media used for each of the cell lines are listed as follows:

- MCF-7: Minimum Essential Medium
- T-47D: RPMI-1640 medium
- 4T1: RPMI-1640 medium
- MDA-MB-231: Dulbecco's Modified Eagles Medium
- To make complete growth medium, 10% fetal calf serum (FCS) and 1% penicillin-streptomycin-glutamine mixture (i.e. 10000 units of penicillin, 10000 µg of streptomycin, 29.2 mg/mL of L-glutamine) were added to the basic medium. For MCF-7 and T-47D, supplementation of bovine insulin (10 µg/mL) was required. All four cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells between passages 5 and 10 were used in the experiments conducted in this chapter. All tissue culture reagents mentioned were purchased from Gibco Invitrogen Corporation, Grand Island, NY.

4.2.2 Apoptosis & cell cycle

Flow cytometry (FC) was chosen for the current study because of several significant advantages. FC offers the rapidity and statistical strength of measurement; it can evaluate a large cell population in a single run over a very short period of time (up to 50,000 – 100,000 cells/sec) (Robinson, 2004; Sklar et al., 2007). In addition, FC allows measurements of cell cycle parameters and apoptosis simultaneously (Bruno et al., 2002; Vermes et al., 2000).

4.2.2.1 Basic principle of flow cytometry

The FC protocol of the current study differentiates cells of various stages of cell cycle, apoptotic and necrotic cells by measuring propidium iodide (PI) uptake (i.e. DNA content), and light scattering properties of the cells relating to cell morphology. Prior to FC analysis, samples were treated with PI, which has become one of the most widely used procedures in FC (Riccardi & Nicoletti, 2006). PI is a fluorogenic compound, which binds stoichiometrically to DNA. The resultant cellular fluorescence intensity, subsequently detected by the flow cytometer, is proportional to the DNA content of the cells. Cells in the G2/M phase have twice as much DNA as G0/G1 cells, thus will have 2-fold fluorescence intensity accordingly. Apoptotic or necrotic cells have fragmented or degraded DNA, hence less fluorescence intensity compared to cells in other cell cycle phases. Apoptotic cells appear as a broad hypodiploid, sub-G0/G1 peak in the histogram (Krysko et al., 2008; Nicoletti et al., 1991). In addition, the FC system was equipped with forward-angle light scatter (FSC) and side-angle light scatter (SSC) that relate to cell diameter and inner cellular structures respectively. Necrotic cells yield high FSC signals due to cell swelling, whereas apoptotic cells give low FSC signals due to cell shrinkage. Both late apoptotic and necrotic cells have low SSC due to loss of normal inner cellular structure (Vermes et al., 2000).

4.2.2.2 Cell cycle analysis protocol

T-47D cells were seeded in a 12-well plate at 2.4 x 10^5 cells per well with 1 mL medium and incubated at 37°C. The medium was replaced the following day with fresh medium containing no test agents (control), tamoxifen, estradiol, PPE, combinations of PPE and estradiol (10^{-8} M) or tamoxifen (10^{-7} M), or combination of all three test agents. Each treatment was carried out in triplicate for 40 hours. Cells were subsequently washed with PBS, trypsinised, collected into flow cytometry tubes and centrifuged at 300 x g for 10 minutes. The supernatant was decanted; tubes were vortexed to loosen the cell pellet. Cells were resuspended and fixed in ice-cold 70% ethanol at 4°C for 1-2 hours. The fixed cells were then washed with PBS, centrifuged to remove the PBS, followed by incubation with ribonuclease (RNase) at 40 µg/mL for 20 minutes in 37°C water bath. Propidium iodide (PI) was added to the cell suspension to a final concentration of 12 µg/mL. All tissue culture reagents mentioned were purchased from Gibco Invitrogen Corporation, Grand Island, NY. Chemicals tamoxifen, estradiol, PBS, RNase and propidium iodide were purchased from Sigma, St. Louis. Samples were analysed by a flow cytometer (FACS Calibur system, BD Biosciences). Histograms (Figure A17, Appendix) and percentages of cells of each stage of cell cycle were obtained using the CellQuest software (BD Biosciences) as described by others (Bruno et al., 2002; Nicoletti et al., 1991). Note that standard thresholds (PI intensity) were used for assessing spread of counts (i.e. each stage of cell cycle) as shown in Figure A17.

4.3 Results

4.3.1 Cell proliferation

A total of 37 experiments were carried out and the data are presented in this chapter. Statistical analysis software (SigmaPlot and Minitab) and Multiple Comparison Procedures (Tukey Honestly Significant Difference and Holm-Sidak methods) were used to compute P-values and identify statistical differences among various treatments. The results are graphically represented from Figure

4.2 to 4.9. PPE and WPE reduced MCF-7 cell proliferation in a dose-dependent manner, by 32 to 72% at concentrations from 50 to 500 µg of dry extract/mL (Figure 4.2). Both the potato extracts tested are statistically similar in terms of effectiveness. Subsequent experiments using another ER+ cell line, T-47D, demonstrated a similar active dose range of PPE, with 30% inhibition at 50 µg/mL (Figure 4.3). An inhibitory effect of WPE or PPE was found for some lower concentrations (2 to 10 µg/mL) during the course of the experiments. However, the inhibition of MCF-7 and T-47D cell proliferation was statistically significant across all experiments when the cells were treated with doses equal to or greater than 50 µg/mL. PPE at 30-40 µg/mL inhibited T-47D proliferation even when tamoxifen was present at stimulatory doses (10^{-8} and 10^{-7} M). Furthermore, low PPE concentration (0.1 µg/mL) became inhibitory once tamoxifen concentration reached 10^{-7} M. PPE at 250 µg/mL drastically inhibits MDA-MB-231 cell proliferation (22% of control) (Figure 4.8). When 4T1 cells were treated with 250 µg/mL PPE, a small but significant inhibition of cell proliferation was also observed (Figure 4.9). Both MDA-MB-231 and 4T1 are highly invasive breast cancer cell lines.

4.3.2 Cell cycle analysis

The results are graphically represented from Figure 4.10 to 4.13. In brief, all the treatment groups tested showed a similar degree of necrosis (Figure 4.10). Treatments with PPE alone ≥ 30 µg/mL, tamoxifen alone, and in combination resulted in a significant increase in apoptotic cells compared to the no-treatment control (Figure 4.11). Among all treatment groups, only tamoxifen (10^{-5} M) caused a significant increase in the cell population in the G0/G1 phase cell population (Figure 4.12). PPE (≥ 30 µg/mL), tamoxifen (10^{-5} M) and the combined treatments of these two test agents significantly reduced the number of cells from S or G2/M phase (Figure 4.13).

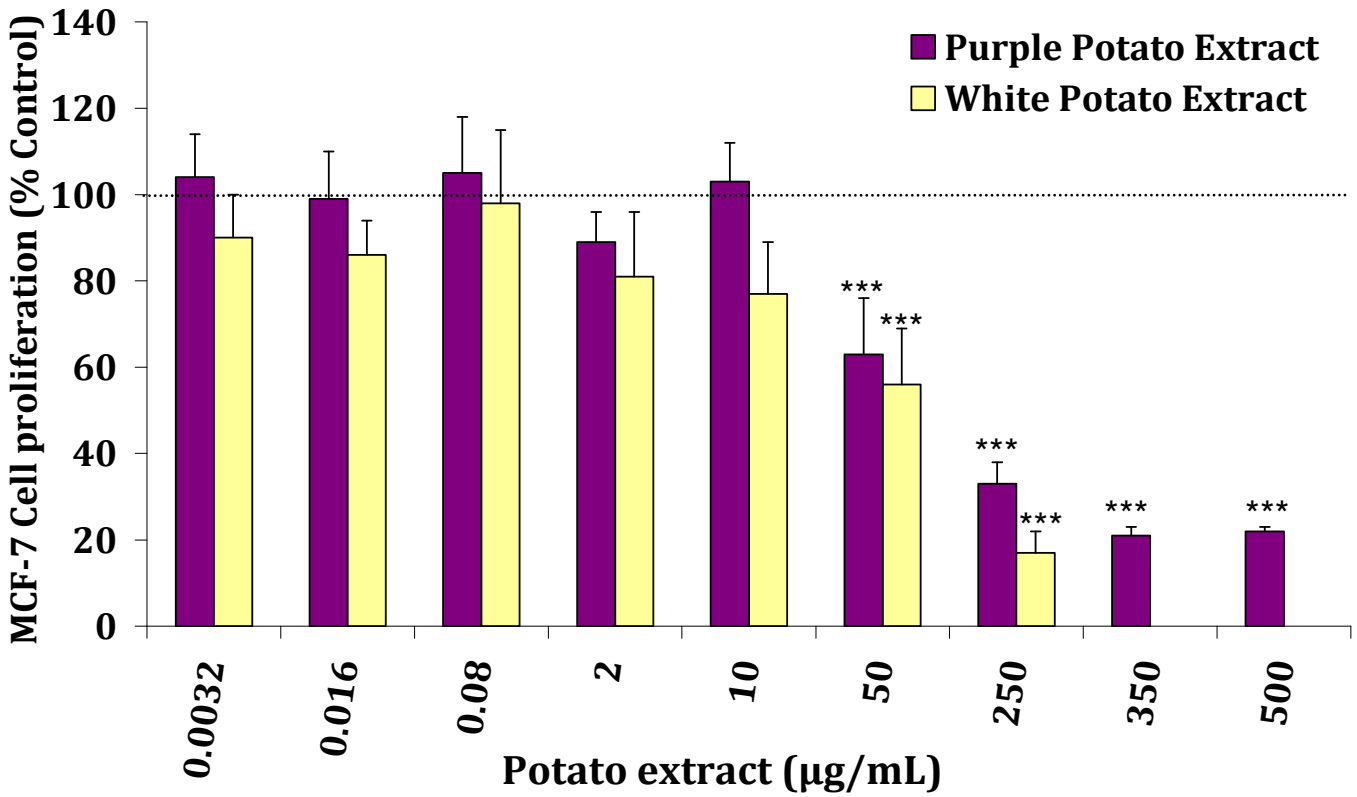


Figure 4.2 MCF-7 cells were treated with PPE or WPE at various doses.

Cell proliferation was measured at 3 days post-treatment by MTT assay and results normalized to the no-treatment control (100%). Results were pooled from 6 independent experiments, each carried out with $N \geq 6$ wells per treatment, expressed as mean ± SEM. Asterisks denote treatments are statistically significant from the no-treatment controls: *** $P<0.001$.

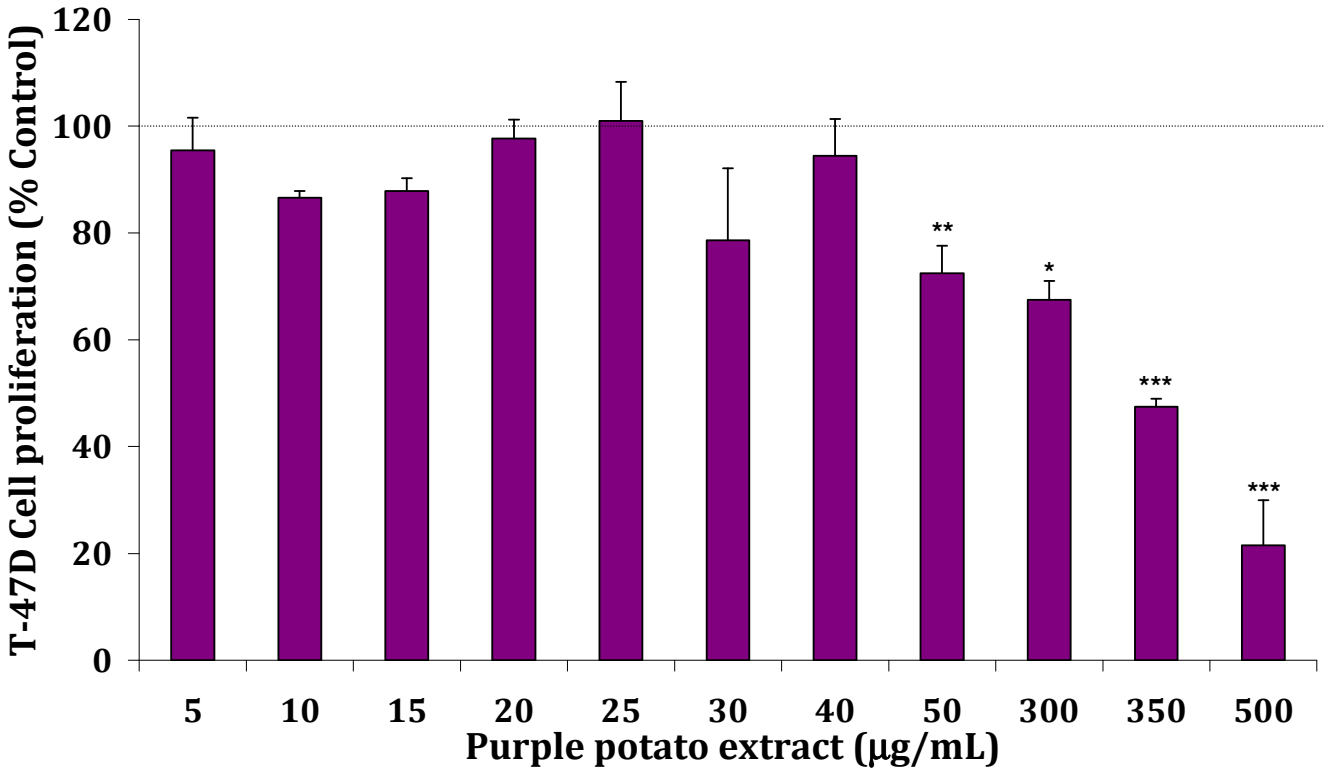


Figure 4.3 T-47D cells were treated with various doses of PPE.
Cell proliferation results were pooled from 9 independent experiments, each carried out with N ≥ 6 per treatment, expressed as mean ± SEM. Asterisks denote treatments are significantly different from the no-treatment controls: * P<0.05, ** P<0.01, *** P<0.001.

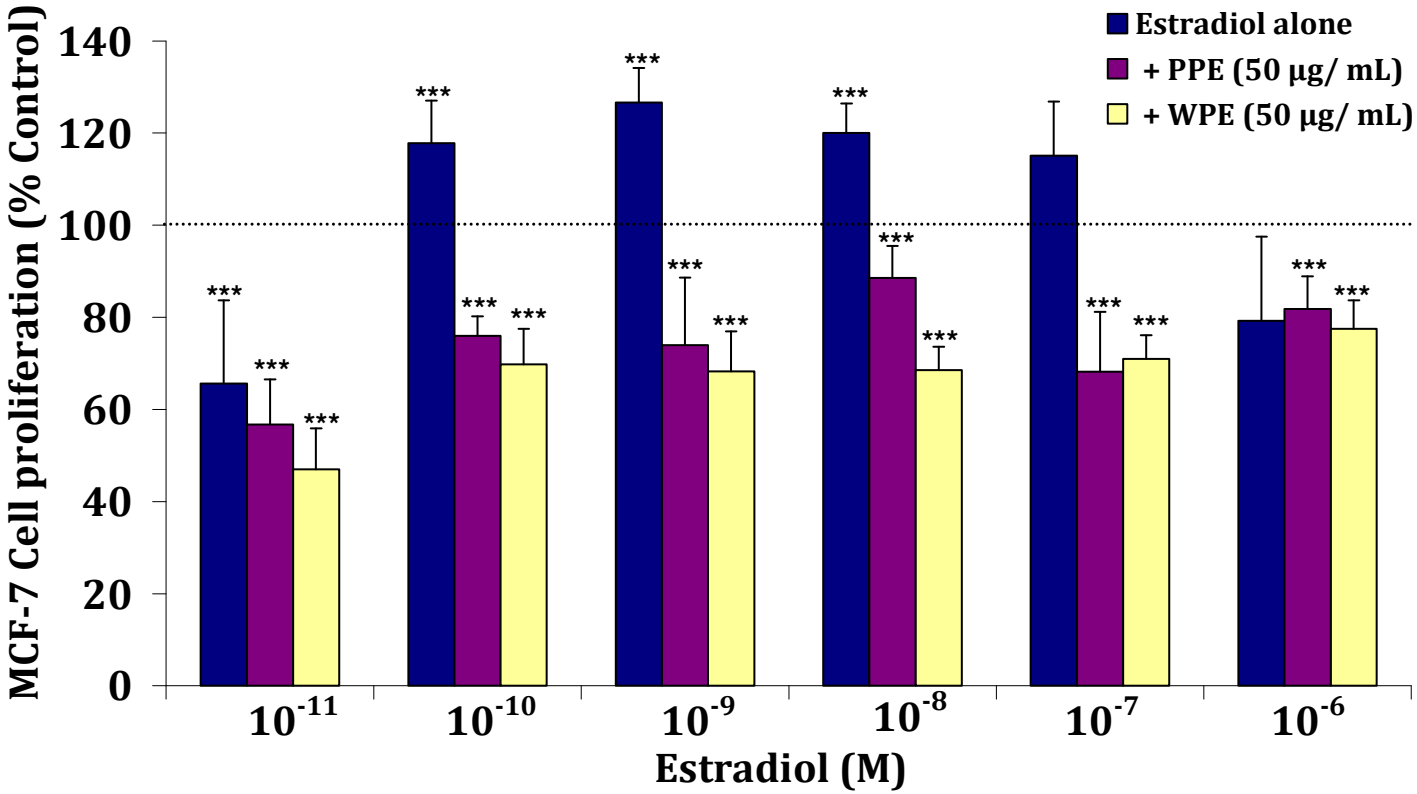


Figure 4.4 MCF-7 cells were treated with or without PPE in the presence of various doses of estradiol.

Results presented by blue bar were presented earlier (Chapter 3) and are shown in this figure for reference. The results for combined treatment of PPE and estradiol (co-treatment) were pooled from 2 independent experiments, each conducted with N≥6 per treatment, expressed as mean ± SEM. Asterisks denote treatments are statistically significant from the no-treatment controls: *** P<0.001.

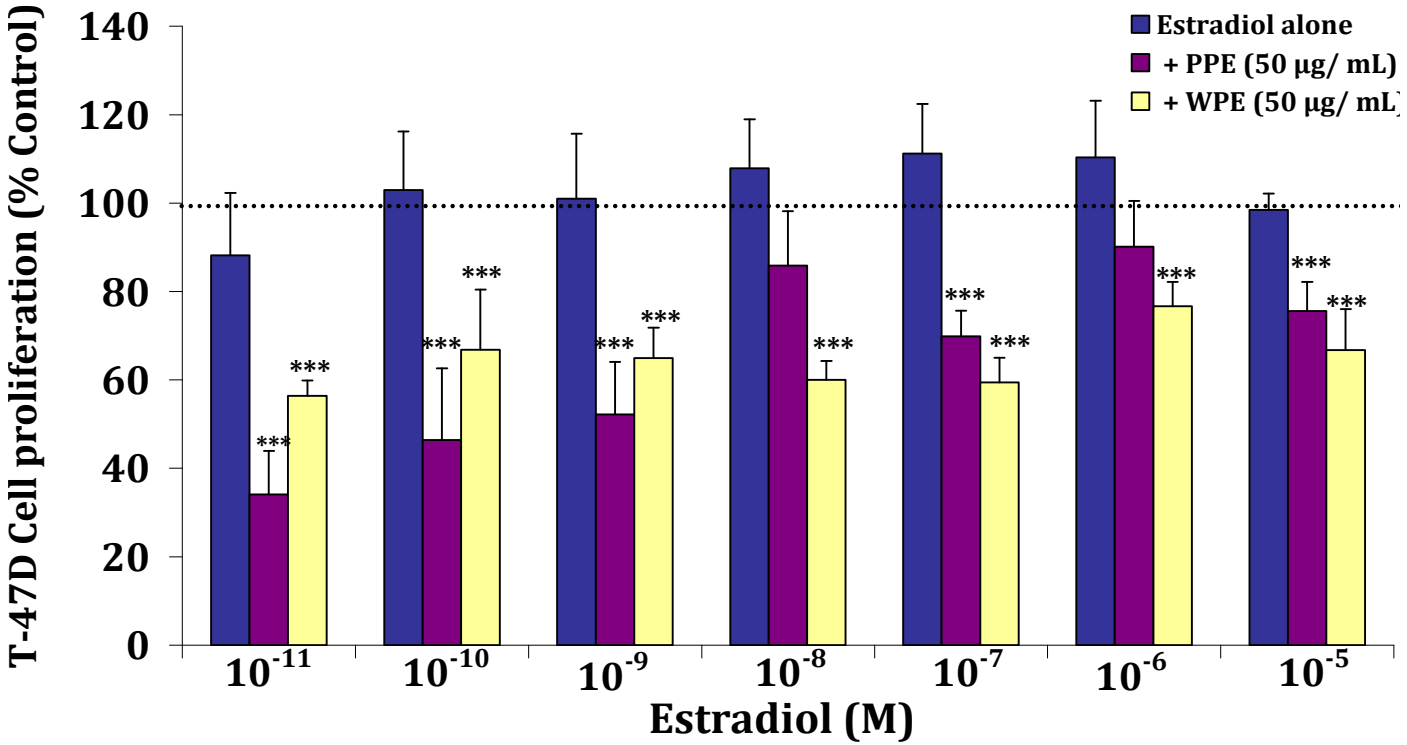


Figure 4.5 T-47D cells were treated with or without PPE in combination with various doses of estradiol.

The results for combined treatment of PPE and estradiol were pooled from 4 independent experiments, each conducted with N≥6 per treatment, expressed as mean ± SEM. Asterisks denote treatments are statistically significant from the no-treatment controls: *** P<0.001.

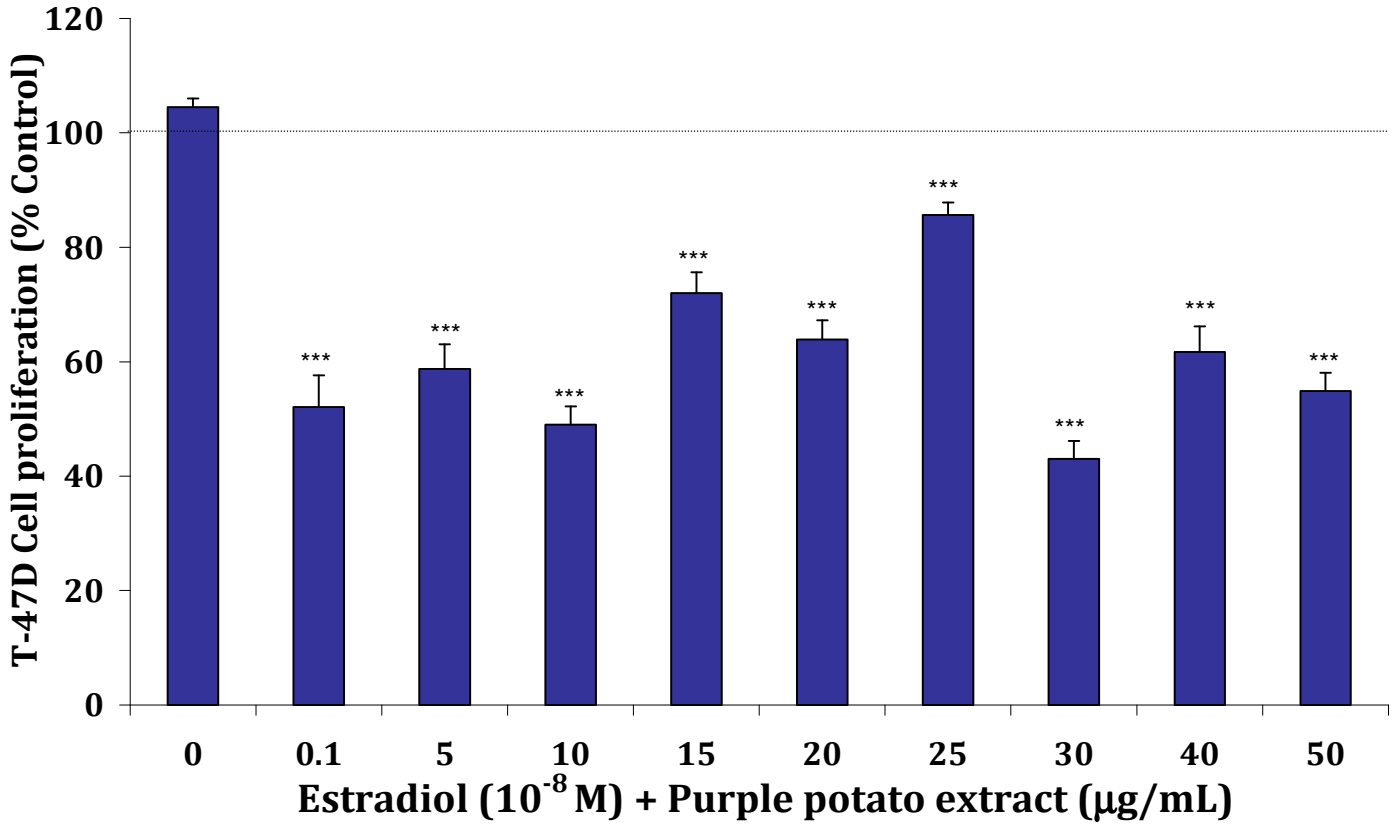


Figure 4.6 T-47D cells were treated with or without estradiol in combination with various doses of PPE.

Cell proliferation results were pooled from 2 independent experiments, each conducted with N ≥ 6 per treatment, expressed as mean ± SEM. Asterisks denote treatments are statistically significant from the no-treatment controls: *** P<0.001.

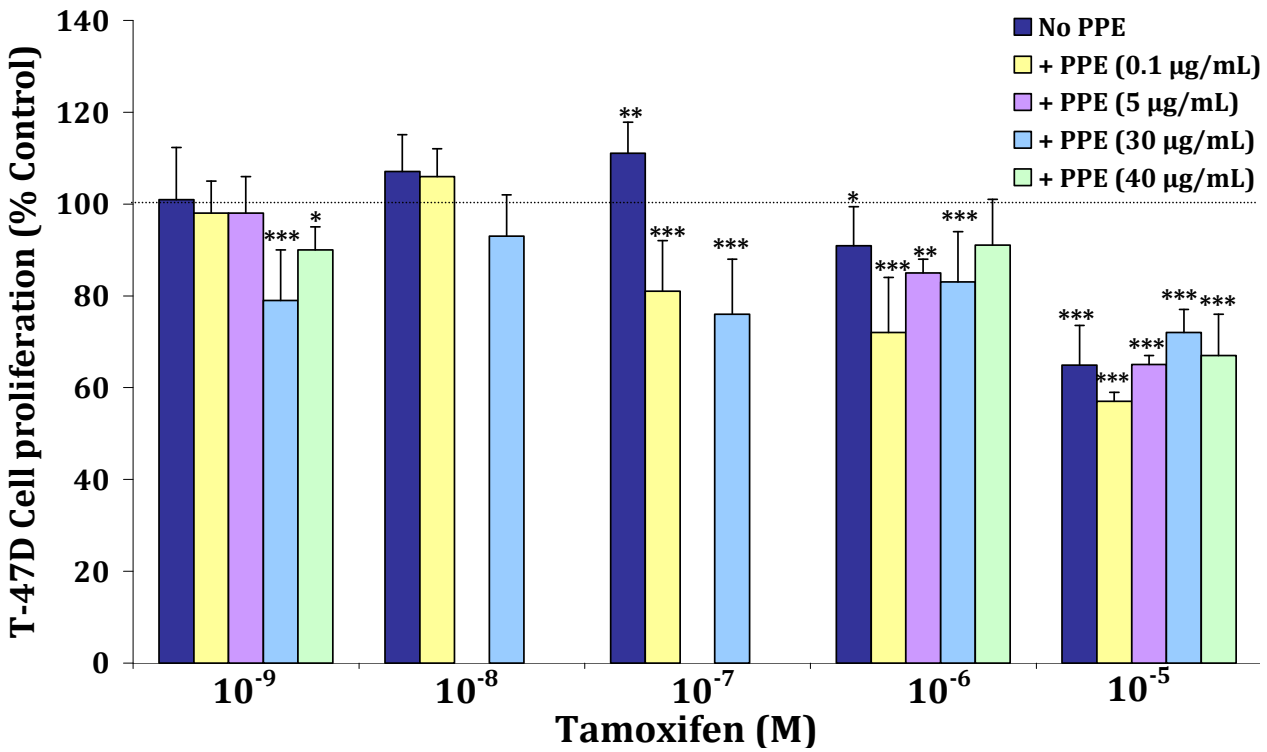


Figure 4.7 T-47D cells were treated with different doses of tamoxifen, in combination with PPE of various doses. Results were pooled from 2 independent experiments, each conducted with N ≥ 6 wells per treatment. Asterisks denote treatments are statistically significant from the no-treatment controls: * P<0.05, ** P<0.01, *** P<0.001.

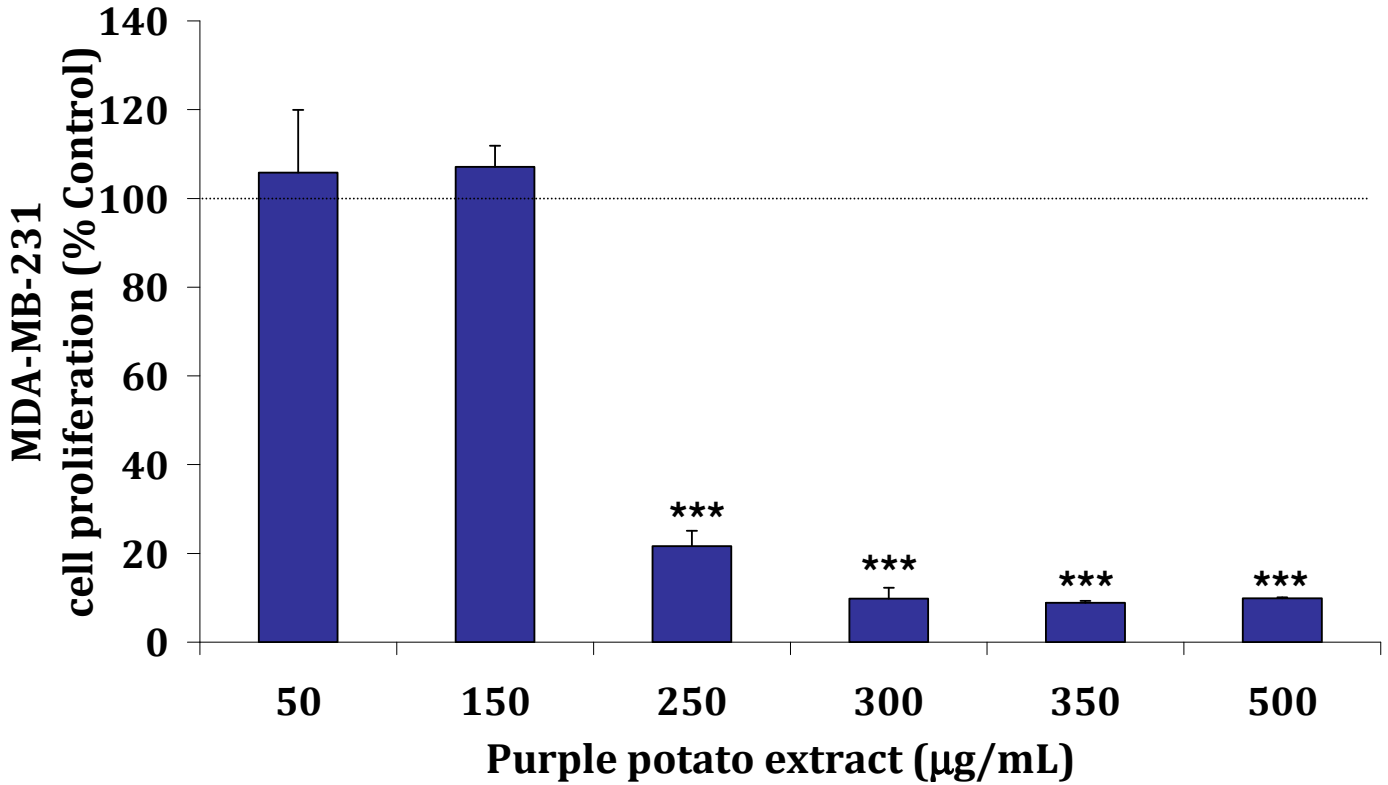


Figure 4.8 MDA-MB-231 cells were treated with PPE at various doses.

Results were pooled from 2 independent experiments, each conducted with N ≥ 6 wells per treatment. Asterisks denote treatments are statistically significant from the no-treatment controls: *** P<0.001.

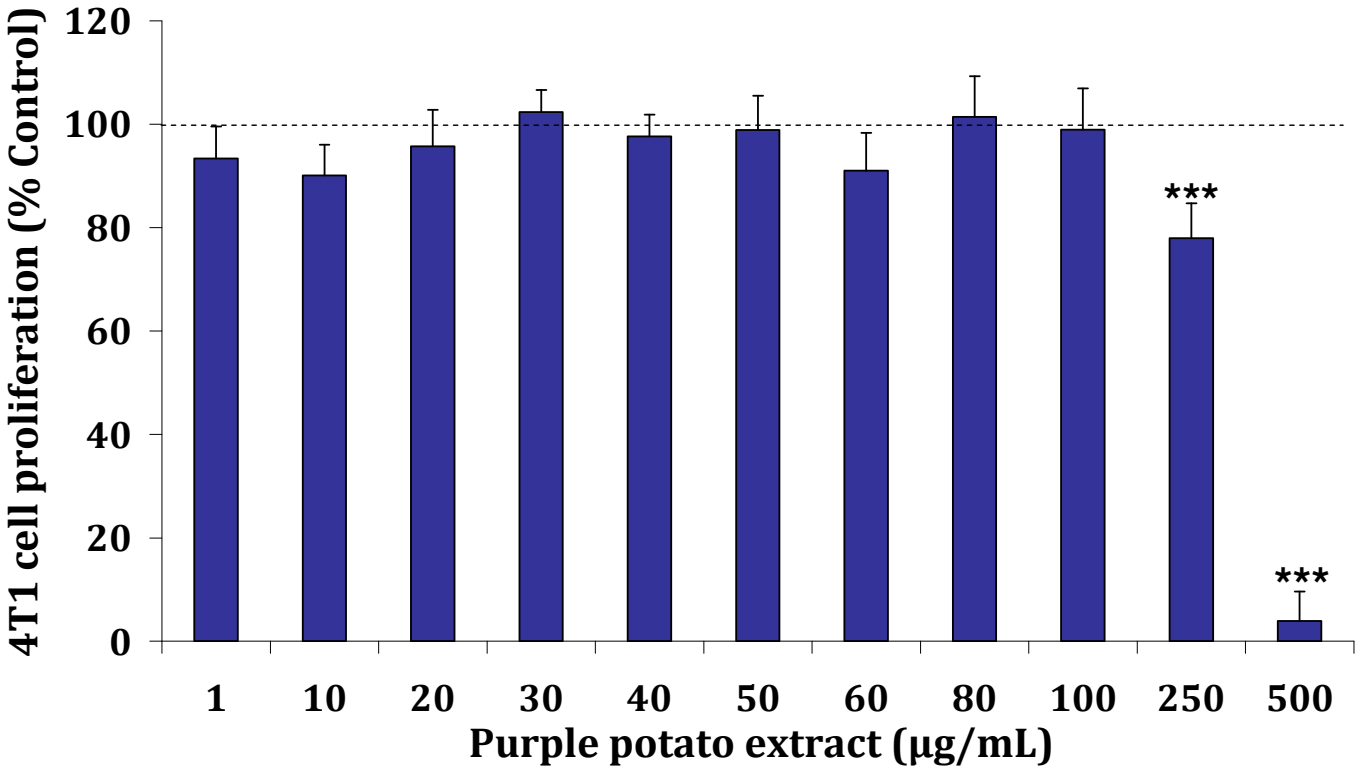


Figure 4.9 4T1 cells were treated with PPE at various doses.

Results were pooled from 2 independent experiments, each conducted with N ≥ 6 wells per treatment. Asterisks denote treatments are statistically significant from the no-treatment controls: *** P<0.001.

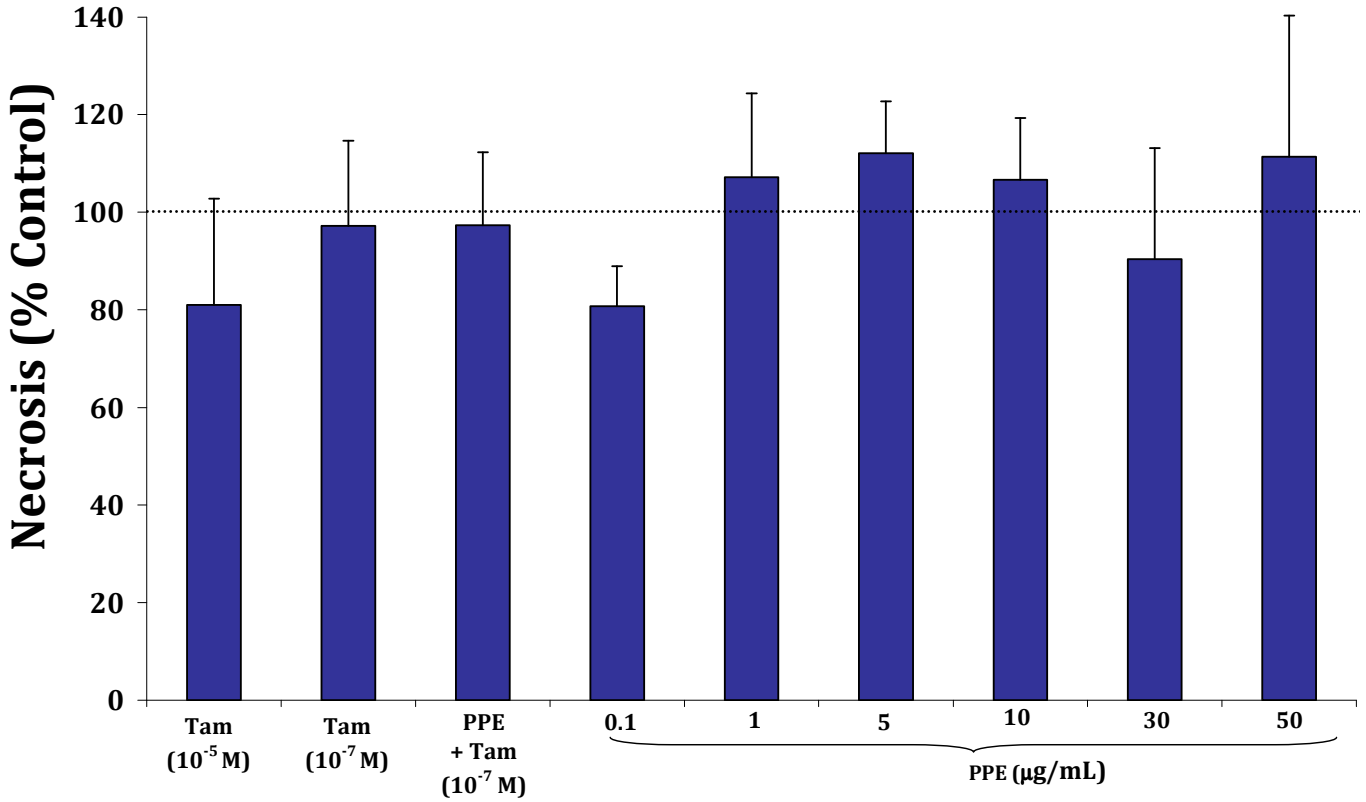


Figure 4.10 Nerosis results: Tam *vs.* PPE *vs.* PPE+Tam.

Necrotic phase cell population 40 hrs post-treatment of T-47D cells with PPE or tamoxifen (Tam) alone, or combined treatments of PPE (30 µg/mL) plus Tam (10-7 M). Results were normalized to the no-treatment control (100%), and pooled from 2 to 5 independent experiments, each carried out with N=3 per treatment, and expressed as mean ± SEM. There is no statistical significance among the various treatment groups.

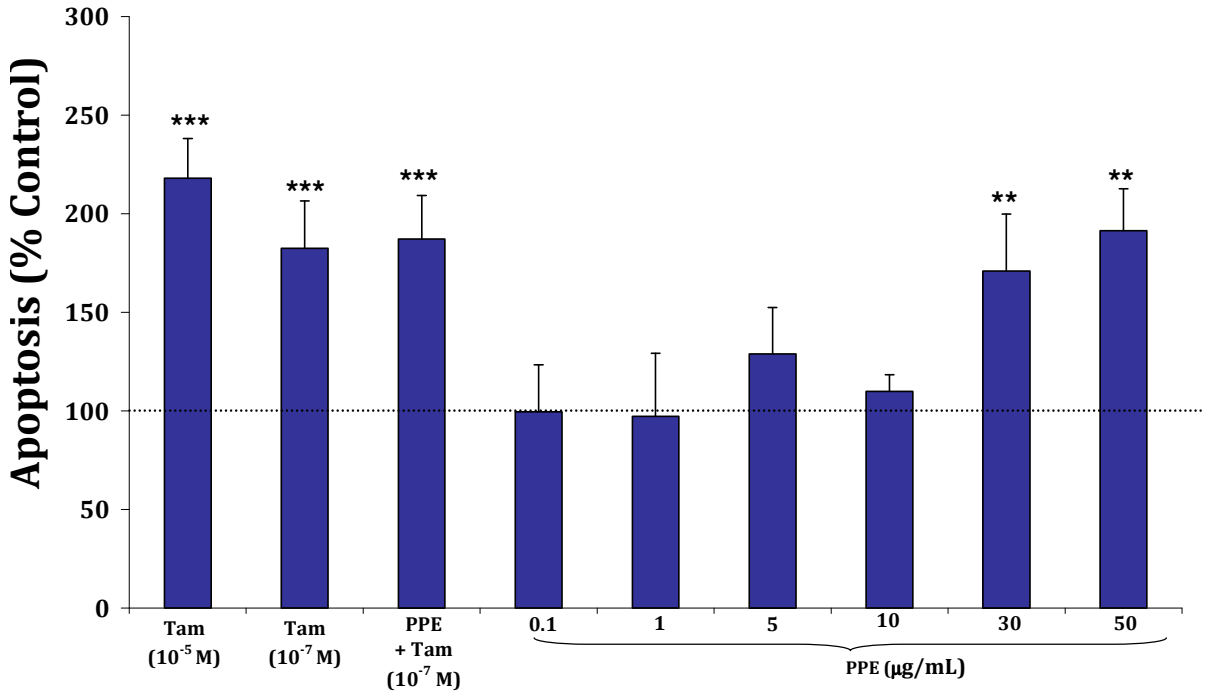


Figure 4.11 Apoptosis results: Tam *vs.* PPE *vs.* PPE+Tam.

Apoptotic phase cell population 40 hrs post-treatment of T-47D cells with PPE or tamoxifen (Tam) alone, or combined treatments of PPE (30 µg/mL) plus Tam (10-7 M). Results were normalized to the no-treatment control (100%), and pooled from 2 to 5 independent experiments, each carried out with N=3 per treatment, and expressed as mean ± SEM. Asterisks denote treatments are significantly different from the no-treatment controls: ** P<0.01, *** P<0.001

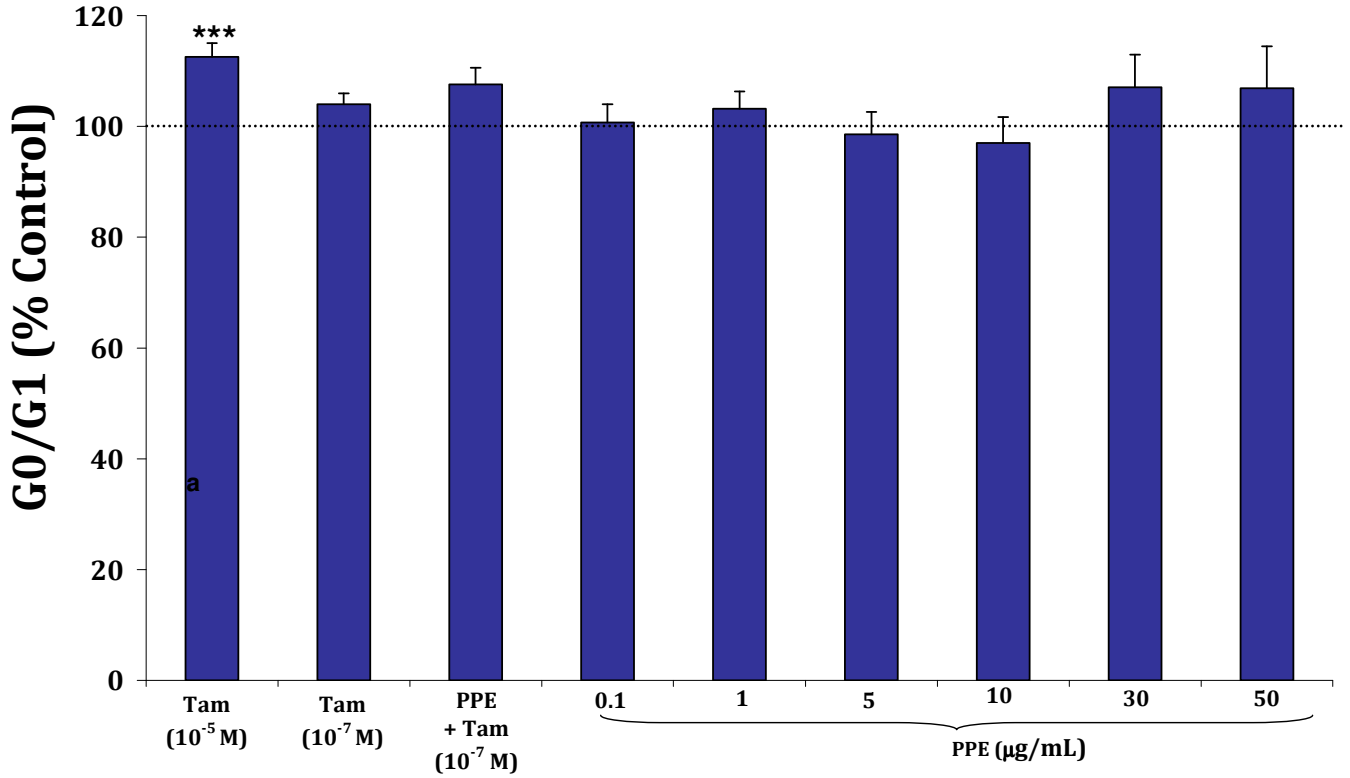


Figure 4.12 Go/G1 results: Tam *vs.* PPE *vs.* PPE+Tam.

G0/G1 phase cell population 40 hrs post-treatment of T-47D cells with PPE or tamoxifen (Tam) alone, or combined treatments of PPE (30 µg/mL) plus Tam (10-7 M). Results were normalized to the no-treatment control (100%), and pooled from 2 to 5 independent experiments, each carried out with N=3 per treatment, and expressed as mean ± SEM. Asterisks denote treatments are significantly different from the no-treatment controls: *** P<0.001

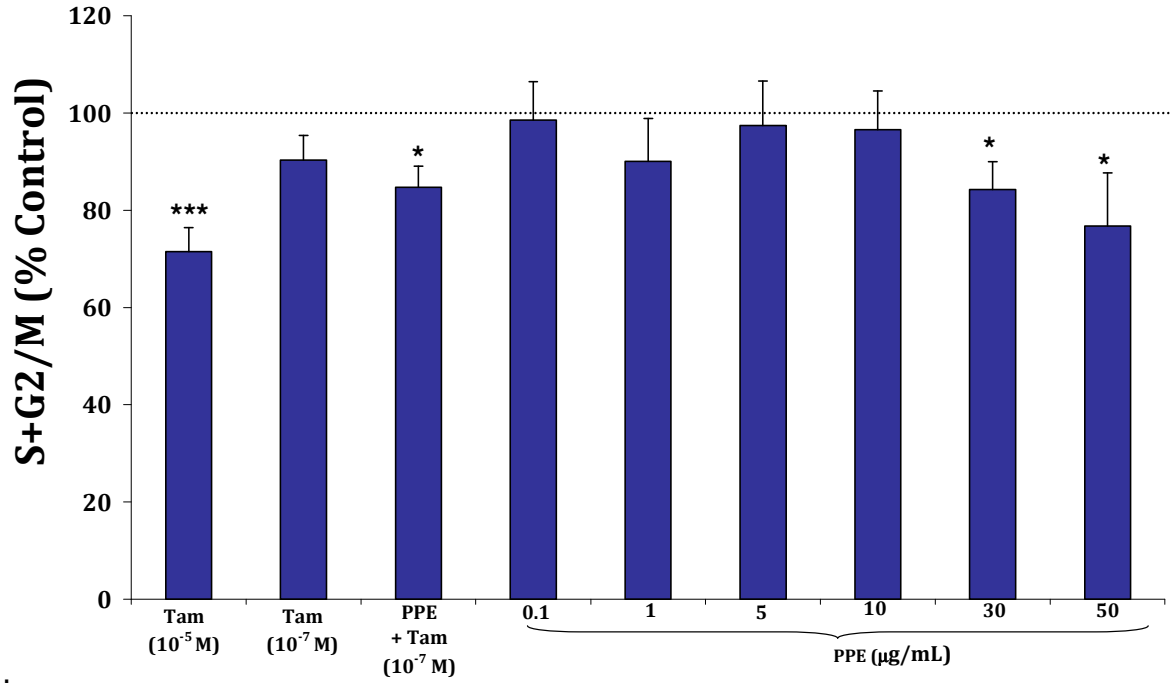


Figure 4.13 S+G2M results: Tam *vs.* PPE *vs.* PPE+Tam.

S+G2/M phase cell population 40 hrs post-treatment of T-47D cells with PPE or tamoxifen (Tam) alone, or combined treatments of PPE (30 µg/mL) plus Tam (10-7 M). Results were normalized to the no-treatment control (100%), and pooled from 2 to 5 independent experiments, each carried out with N=3 per treatment, and expressed as mean ± SEM. Asterisks denote treatments are significantly different from the no-treatment controls: * $P<0.05$, *** $P<0.001$.

4.4 Discussion

4.4.1 Cell proliferation

Since estradiol provided a major stimulus to MCF-7 proliferation, as shown in Chapter 2, in subsequent experiments, MCF-7 cells were co-treated with varied concentrations of estradiol in the presence of a constant concentration of PPE or WPE (Figure 4.4). In this case, the effective dose of 50 µg/mL was chosen. As shown in Figure 4.4, MCF-7 cell proliferation was significantly reduced by treatment with 50 µg/mL potato extracts regardless of estradiol concentration between 10^{-10} to 10^{-7} M. This suggests that the effect of PPE and WPE on inhibition of proliferation of MCF-7 cells is seen even at estradiol concentrations that would normally have stimulated cell proliferation. When experiments were repeated with T-47D, estradiol alone did not appear to influence cell proliferation (Figure 4.5). This observation agrees with others that estradiol responses differ between MCF-7 and T-47D cells, and a cell-type specific effect may exist (Gupta et al., 1998; Zampieri et al., 2002). Despite this, the addition of 50 µg/mL PPE or WPE significantly reduced cell proliferation at every estradiol concentration with up to 52% reduction versus estradiol treatments alone. In essence, the experiments with MCF-7 and T-47D discussed so far (Figure 4.2 to 4.5) have demonstrated that:

1) PPE or WPE alone at 50 to 500 µg/mL consistently showed a small but significant inhibitory effect;
2) PPE or WPE (50 µg/mL) can attenuate cell proliferation stimulated by estradiol in MCF-7.

The next question was whether the potato extract at non-effective doses would inhibit breast cancer cell proliferation in the presence of estradiol. Note that PPE, but not WPE, was chosen for further investigations (Figure 4.6 to 4.13) because of the limitation of time and resources as well as the intention to focus on 'Urenika' which has New Zealand proprietary value. As depicted in Figure 4.6, PPE doses found to be ineffective earlier (Figure 4.3) were strongly

inhibitory in the presence of estradiol (10^{-8} M). On average, the combined treatments of PPE (0.1 to 50 μg/mL) and estradiol led to 45% inhibition versus control. This series of experiments suggest that PPE could be effective at doses lower than 50 μg/mL in the presence of physiologically achievable concentrations of estradiol.

Since tamoxifen is the gold standard of treatment for breast cancer, it is of great interest whether the combined treatment of PPE and tamoxifen would be inhibitory to cell proliferation. As shown in Figure 4.7, PPE at 30-40 μg/mL inhibited T-47D proliferation even when tamoxifen was present at stimulatory doses (10^{-8} and 10^{-7} M). Furthermore, low PPE concentration (0.1 μg/mL) became inhibitory once tamoxifen concentration reached 10^{-7} M. The effectiveness of 10^{-6} M tamoxifen was also further slightly enhanced by 0.1, 5 and 30 μg/mL PPE. In summary, these experiments suggest a combined effect of PPE and tamoxifen. Furthermore, PPE may have a beneficial role to play in avoiding tamoxifen-induced stimulation of cell proliferation as discussed in Chapter 2.

To date, only limited *in vivo* and *in vitro* data exist regarding any potential anticancer effect of potatoes or extracts made from potato. A few *in vitro* studies have reported the effects of potato extracts in relation to breast cancer cell proliferation; these studies were carried out using MCF-7 cells (Boivin et al., 2009; Leo et al., 2008; Stushnoff et al., 2008). In one of the studies, potato juice (166 mg raw vegetable/mL cell culture) was found to be more effective (50% inhibition of cell proliferation) in MCF-7 and PC-3 (prostate cancer) than for other cancer cells tested in the study, namely AGS (stomach), Panc-1 (pancrease), A-549 (lung), Caki-2 (kidney), Daoy (medulloblastoma) and U-87 (giloblastoma) (Boivin et al., 2009). The effective dose of PPE or WPE in the current study required to achieve 50% inhibition was shown to be 250 μg extract/mL in MCF-7 (i.e. 25 mg raw potato/mL), which is 6.6 times lower than the effective dose reported by Boivin et al. (2009). Both studies used comparable cell proliferation methods. The difference in effectiveness of potato samples observed between the current study and Boivin et al. is likely to be

attributed to the different sample preparation processes employed. PPE and WPE were produced by a series of steps such as ethanol extraction and rotary evaporation, as detailed in Chapter 3, that enable dissolving, extracting and concentrating a number of bound or unbound bioactives. The potato juice used in Boivin et al. (2009) was produced by passing the potato through a juice extractor, without involving solvent extraction or concentrations steps. While the composition data of their potato juice were not available for quantitative comparison, it is possible that the potato juice sample they used had fewer bioactives. For instance, use of solvents such as ethanol is essential to extract polyphenols (Shahidi & Naczk, 2004). Therefore, it is conceivable that the different polyphenol profiles and content may partially explain why one potato sample was more effective than the other in inhibiting breast cancer cells proliferation (Yang et al., 2001). It is important to consider which of the PPE or WPE components may have contributed to the cell proliferation modulation activity observed. The data contained in this chapter coupled with the characterisation of potato extracts (Chapter 3), as well as analysis of the literature, allows tentative inferences to be made pertaining to the potential active factors of PPE and WPE.

4.4.1.1 Anthocyanins

Anthocyanin (ACY) gives a purple or red appearance to pigmented potatoes and potentially an anticancer effect. ACY of potato and other plant origin have been shown to have an anticancer effect in MCF-7 cell culture (Table 4.1). For instance, a rice bran extract reduced MCF-7 cell proliferation by 50% at ACY dose of 413 µg/mL (Leardkamolkarn et al., 2011). A cranberry extract, containing at least 300 µg/mL of ACY, was shown to be antiproliferative in MCF-7 cell culture (Sun & Liu, 2006). Zhang and colleagues examined the effect of ACY of fountain grass leaves and fruits (*Cornus mas*) and found that ACY at concentrations from 12.5 to 200 µg/mL were not inhibitory to cell proliferation of MCF-7 and other cell lines tested in their study (Zhang et al., 2005). Taken together, these studies suggest that, if ACY is a key active factor for the inhibitory effect observed, the minimum concentration required for ACY would

be at least 300 µg/mL. The ACY content of PPE at its effective dose of 250 µg/mL (for 50% inhibition) is 3.8 µg/mL, which is almost 100 times lower than the effective dose of ACY reflected in the literature.

More importantly, WPE, which has no ACY, was as effective as PPE in inhibiting cell proliferation of the breast cancer cell lines tested. Therefore, the active factor in potato extracts is likely to be some other bioactive compound present in both PPE and WPE. This deduction is further supported by the literature; at least two studies have demonstrated non-pigmented potato cultivars with little or no ACY were highly effective in suppressing MCF-7 cell growth (Leo et al., 2008; Stushnoff et al., 2008).

Table 4.1 Phytochemical concentration in PPE & WPE compared with effective doses reported in the literature.

Class of compounds	**Concentration in PPE or WPE at 250 µg/mL** ***(µg/mL)***		**Effective doses against breast cancer proliferation *in vitro* *(µg/mL)/***	**Breast cancer cell lines or other cancer cell lines**	**References**	**Fold** ***(Concentration in PPE or WPE divided by the effective concentration reported in the references)***	
	PPE	**WPE**				**PPE**	**WPE**
Anthocyanins	3.8	0	500	MCF-7	(Hayashi et al., 2006)	0.0076	0
			38	MCF-7	(Stushnoff et al., 2008)	0.1	0
			413	MCF-7	(Leardkamolkarn et al., 2011)	0.0092	0
			>200	MCF-7	(Zhang et al., 2005)	0.019	0
			>300	MCF-7	(Sun & Liu, 2006)	0.013	0
Hydroxycinnamic acids:							
Chlorogenic acid	6.3	2.9	0.05 to 0.5	MCF-7	(Leo et al., 2008)	13 to 126	6 to 58
Caffeic acid	0.7	0.3	0.03	MCF-7	(Hudson et al., 2000)	23	10
			0.0018	T-47D	(Kampa et al., 2004)	389	167
			0.03	MDA-MB-231	(Hudson et al., 2000)	23	10
Flavonols:							
Quercetin derivative	0.004	0.03	5.2 to 15	MCF-7	(So et al., 1997)	0.0003 to 0.0008	0.002 to 0.006
Kaempferol derivative	0.01	0.001	14	MDA-MB-231	(Le Bail et al., 1998)	0.0007	0.00007

Table 4.1 ***(continued)***

Class of compounds	**Concentration in PPE or WPE at 250 µg/mL** ***(µg/mL)***		**Effective doses against breast cancer proliferation** ***in vitro*** ***(µg/mL)/***	**Breast cancer cell lines or other cancer cell lines**	**References**	**Fold** ***(Concentration in PPE or WPE divided by the effective concentration reported in the references)***	
Glycoalkaloids							
α-chaconine	6	9	1.54	MCF-7	(Nakamura et al., 1996)	4	6
			1.83	PC-6 (lung)		3	5
			1.46	SW620 (colon)		4	6
			1.43	NUGC-3 (stomach)		4	6
			1.58	P388 (leukaemia)		4	6
			2	A375 (skin)	(Chataing et al., 2009)	3	4.5
α-solanine	0.9	1.2	8.29	P388	(Nakamura et al., 1996)	0.1	0.1
			15.7	PC-6	(Nakamura et al., 1996)	0.06	0.08
			14.47	$HepG_2$ (liver)	(Ji et al., 2008)	0.06	0.08
			> 50	SGC-7901 (gastric)	(Ji et al., 2008)	0.018	0.024
			> 50	LS-174	(Ji et al., 2008)	0.018	0.024

4.4.1.2 Phenolic acids

Of the other compounds identified in PPE and WPE, phenolic acids (hydroxycinnamic acids) have the potential to be biologically active. Chlorogenic acid (ChA), the most abundant phenolic acid of potato, accounts for 65 and 70% of total phenolic acids in PPE and WPE respectively. Like potato ACY, ChA has begun to attract some research interest in recent years in relation to its potential therapeutic value in cancer treatment. Antiproliferative activity of ChA has been reported in MCF-7 cells (Leo et al., 2008), and in animal models of large intestine, liver and tongue cancers (Mori et al., 1999). Using an MCF-7 model, Leo and colleagues demonstrated that treatment of cells with purified ChA (Sigma-Aldrich) at concentrations of 0.05 to 0.5 µg/mL inhibited cell proliferation by 20% (Leo et al., 2008). IC_{50} for ChA in PPE and WPE were 6.3 and 2.9 µg/mL respectively, which were 126- to 58-fold higher than the effective dose reported by the authors (Table 4.1). Leo et al. (2008) also observed strong antiproliferative effects of methanol-water extracts made from two non-pigmented potato cultivars, Nicola and ISCi 67. The ChA contents of these two cultivars at IC_{50} were 0.6 and 0.4 µg/mL respectively. Their experimental methods, particularly methanol-water extraction and MTS cell proliferation assay, are comparable with the present study (ethanol-water extraction, MTT cell proliferation assay). The findings of Leo et al. (2008) support the possibility that ChA could be an important active factor in PPE and WPE.

Some data also exist for another phenolic acid of potato, caffeic acid (CA), in relation to its effect on breast cancer cell proliferation (Table 4.1). A study investigated the effects of 6 phenolic acids on T-47D cell proliferation using the MTT assay. Among all the phenolic acids tested, CA was the most potent antiproliferative agent (Kampa et al., 2004). Treatment of T-47D cells at 10 nM (i.e. 0.0018 µg/mL) CA for 3 days caused 70% inhibition of cell proliferation (Kampa et al., 2004). In another study, a phenolic acid mixture containing CA (0.03 µg/mL) was effective in inhibiting MCF-7 and the ER- breast cancer cell line MDA-MB-468 cell proliferation by about 60% (Hudson et al., 2000). The CA

content of potato extracts at IC_{50} (T-47D, MCF-7 and MDA-MB-231) was about 0.7 and 0.3 µg/mL in PPE and WPE respectively. Comparing these doses with the data of the two studies discussed, it is possible that CA could be a potential active constituent for both potato extracts used in the current study. Overall, it is conceivable that phenolic acids, particularly CA and ChA, may contribute significantly to the inhibitory effect observed for PPE and WPE in the experiments described here. Nonetheless, it is still questionable if this class of compounds can be taken as the sole active factor, because if this is the case, PPE, which has more phenolic acids (2.3-fold) than WPE, should exert higher potency than WPE, which was not the case. This finding agrees well with the findings of Stushnoff and others (2008). In their study, pigmented potatoes varieties, Purple Majesty (PM) and Mountain Rose (MR), have 3-fold higher phenolic acid content than the non-pigmented cultivar, Rio Grande Russet (RGR). Yet, PM and MR extracts were found to have significantly lower antiproliferative activity in MCF-7 and MDA-MB-468 breast cancer cells than the RGR extract.

4.4.1.3 Flavonols

Both PPE and WPE contain trace amount of flavonols, i.e. quercetin and kaempferol derivatives. For quercetin, an IC_{50} of 5.2 to 15 µg/mL has been reported in a MCF-7 cell proliferation study (So et al., 1997). This active dose was 150 and 1500 times higher than the concentration of quercetin derivative (quercetin-3-rutinoside) in WPE (0.03 µg/mL) and PPE (0.004 µg/mL) at IC_{50} respectively. Kaempferol at 14 µg/mL was inhibitory to MDA-MB-231 but not inhibitory to MCF-7 growth (Le Bail et al., 1998). The concentrations of kaempferol derivative present in PPE and WPE at IC_{50} were 0.01 µg/mL and 0.001 µg/mL respectively. Therefore, it is likely that both quercetin and kaempferol derivatives in the potato extracts are not important active factors.

The next question is whether there is a relationship between total phenolic content (Phenolic acids, plus flavonoids including ACY, quercetin and

kaempferol derivatives) and antiproliferative activity. PPE has about 3 times the total polyphenol as WPE, yet, WPE is not less effective than PPE. Leo et al. (2008) tested 8 different potato cultivars, and the relationship of total phenolic content and antiproliferative activity was only found for two of the cultivars. Stushnoff et al. (2008) demonstrated that all the 3 potato cultivars, which have lower total phenolic content, exerted higher antiproliferative activity in MCF-7 than the other 3 potato cultivars with higher total phenolic content. The present work and these two studies suggest that antiproliferative activity of the potato extracts is not solely contributed by phenols, but rather some other constituents play an important role.

4.4.1.4 Glycoalkaloids

PPE and WPE also contain non-phenolic compounds including glycoalkaloids α-chaconine and α-solanine. One study showed that the IC_{50} value for glycoalkaloid (α-chaconine) in MCF-7 was 1.54 µg/mL, after 72 hours of treatment (Nakamura et al., 1996). The other 4 cancer cell lines tested in the same study, SW620 (colon), NUGC-3 (stomach), PC-6 (lung), and P388 (leukaemia), showed similar sensitivity as MCF-7 to α-chaconine (IC_{50}: 1.4 to 2 µg/mL). Alpha-solanine was found to be less effective than α-chaconine as an inhibitory agent (IC_{50} of 8.29 and 15.70 µg/mL in P388 and PC-6 respectively) (Nakamura et al., 1996). Another study showed 72 hours treatment of α-chaconine significantly inhibited the cell proliferation of human melanoma cells A375 with an IC_{50} value of about 2 µg/mL (Chataing et al., 2009). PPE contains 6 and 0.9 µg/mL of α-chaconine and α-solanine respectively; WPE contains slightly more α-chaconine (9 µg/mL) and α-solanine (1.2 µg/mL). As these compounds are present in PPE and WPE within the effective range, they could have a role in the inhibition of MCF-7 cell growth (Table 4.1). Overall, it is likely that more than one active factor in PPE and WPE, acting in concert or antagonistically, may have resulted in the antiproliferative effects observed in the current study.

4.4.2 Apoptosis and cell cycle analysis

4.4.2.1 Necrosis and apoptosis

Necrosis and apoptosis are two important and distinct mechanisms of cell death. Apoptosis is an indispensible and normal physiological mechanism to control cell proliferation as part of homeostatic control. Multicellular organisms utilise apoptosis as a tool to remove excess, redundant, damaged (e.g. DNA damaged cells) or potentially deleterious cells such as malignant cells (Kroemer et al., 1998; Sun et al., 2004). Therefore, apoptosis is a beneficial and vital physiological process essential for health and disease control.

Alternatively, cells could also die as passive victims due to extreme injury caused by various factors, including physicochemical stress such as heat, osmotic shock, toxins, or extreme shortage of nutrients (e.g. glucose). Cells undergoing apoptosis show well-defined morphological changes such as cell shrinkage, chromatin condensation, nuclear segmentation and DNA fragmentation (Krysko et al., 2008). On the contrary, necrotic cells are characterized by rapid swelling of the entire cytoplasm (oncosis), mitochondrial swelling, and eventually the cells culminate in plasma membrane rupture and organelle breakdown. Such morphological differences between apoptotic and necrotic cells allow them to be distinguished by light or electron microscopy. However, evaluation and interpretation of dying cell morphology may be complex and challenging. Furthermore, microscopic techniques are not ideal for quantification purposes (e.g. limited capacity for number of cells). Comparatively, FC is a superior technique because: 1) it distinguishes necrotic and apoptotic cells by their different light scattering and fluorescence properties (due to their different morphologies and DNA content); 2) quantification of necrotic or apoptotic cells is based on analysis of a high throughput of cells in the FC system (Krysko et al., 2008; Ormerod et al., 1993; Sklar et al., 2007).

Nevertheless, the limitation of the current study is that flow cytometric assay alone is not definitive of apoptosis; additional distinct assays are needed for confirmation of apoptosis and could be done in future studies. For instance, the DNA laddering technique and terminal dUTP-Nick End-Labeling (TUNEL) assay could be used to measure DNA fragmentation occurring during apoptosis. Apoptosis can also be determined by measuring membrane alterations, using Annexin V which binds to cell membrane phosphatidylserine residues exposed during apoptosis (Elmore, 2007).

Necrosis is typically recognised as a "nonspecific" form of cell death and is often associated with undesirable loss of cells in human pathologies (Kasibhatla & Tseng, 2003; Krysko et al., 2008). Necrosis could also lead to local inflammation (Kroemer et al., 1998). In contrast, apoptosis is a tightly regulated or "programmed" mode of cell death. Resistance to apoptosis has been associated with cancer due to superfluous mutated or cancerous cells (Elmore, 2007; Kroemer et al., 1998). Therefore, apoptosis has been increasingly recognised as the most favourable and target mechanism of cell death in cancer treatment as well as chemoprevention (Ghobrial et al., 2005; Kasibhatla & Tseng, 2003; Ocker & Höpfner, 2012; Sun et al., 2004).

The current study showed that treatment of T-47D cells with PPE (0.1 to 50 μg/mL) resulted in the same number of necrotic cells as the no-treatment controls, indicating that treatment with PPE had no effect on necrosis. The same observation applies to tamoxifen and combined treatments of PPE (30 μg/mL) and tamoxifen and/or estradiol (Figure 4.10). Tamoxifen treatments resulted in a significant increase in apoptosis (Figure 4.11). In particularly, tamoxifen induced 118% increases in apoptosis of T-47D cells at 10^{-5} M. Moriai et al. (2009) also found a significant increase in apoptosis of MCF-7 cells 72 hours post-treatment with tamoxifen at 10^{-5} M. Previous studies made similar observations that tamoxifen at 5×10^{-6} M caused significant induction of apoptosis in MCF-7 culture (Mandlekar et al., 2000; Mandlekar & Kong, 2001). As shown in Chapter 1 and this chapter, tamoxifen at 10^{-5} M inhibits 66 and 35% of cell proliferation in MCF-7 and T-47D culture respectively. Apoptosis

data obtained for tamoxifen in the current study adds to the evidence that apoptosis is involved as one of the mechanisms by which tamoxifen suppresses cell proliferation and growth of breast cancer cells (Moriai et al., 2009).

PPE showed a dose-dependent increase in apoptosis in T-47D culture 40 hours post-treatment (Figure 4.11). At 30 and 50 μg/mL, PPE significantly increased apoptosis by 71% and 91% respectively, compared to the no-treatment control. PPE at 30 μg/mL caused some degree of inhibition of T-47D cell proliferation (79% of control, Figure 4.3), but the effect was not statistically significant. As the dose of PPE increased from 30 to 50 μg/mL, the antiproliferative effect became significant (72% of control, Figure 4.3). It is possible that although there is significant induction of apoptosis at 30 μg/mL, the effect was not strong enough to counteract cell proliferation. It is plausible that, as PPE dose increased (50 μg/mL), PPE-induced apoptosis increased and was able to contribute significantly to the antiproliferative effect observed. Note that PPE at this concentration brought about apoptosis to a similar extent as 10^{-5} M tamoxifen (Figure 4.11).

Some of the active factors postulated for the PPE-induced inhibitory effect on cell proliferation (4.4.1), have been reported to exert an apoptotic effect in breast cancer cells. Caffeic acid at 0.018 μg/mL significantly induced apoptosis of T-47D cells (Kampa et al., 2004). PPE at 30 and 50 μg/mL, where significant increases in apoptosis were observed, contained 0.084 and 0.14 μg/mL of caffeic acid, which is above the active doses reported in the literature. Hence, caffeic acid may contribute partly to the apoptotic effect of PPE observed.

The glycoalkaloid, α-solanine, from 0.08 to 10 μg/mL, was shown to be effective in stimulating apoptosis in human hepatocarcinoma cells 48 hours post-treatment (Ji et al., 2008). There is a lack of published data on the effect of glycoalkaloids on apoptosis in breast cancer cells. A study demonstrated that α-chaconine and α-solanine, purified from potato cultivar "Dejima", induced apoptosis in human colon cells HT-29, at the concentrations of 5 and 10 μg/mL

respectively (Yang et al., 2006). PPE at 50 µg/mL contained 1.2 and 0.2 µg/mL of α-chaconine and α-solanine respectively, which were reasonably close to the doses of glycoalkaloids effective in modulating apoptosis. The limited data may indicate a possible role of α-chaconine and α-solanine in contributing to the apoptotic effect of PPE observed in T-47D breast cancer cells. Potato anthocyanins (ACY) have also been shown to give an apoptotic effect in the KATO III stomach cancer cell line, but at a high concentration of 2.5 mg/mL, which is a few thousand-fold higher than the ACY content in PPE at its apoptosis-inducing dose (Hayashi et al., 2006). Therefore, ACY is unlikely to play an important role in causing apoptosis, which supports the deduction earlier that ACY is unlikely to play a part in the antiproliferative activity of PPE.

4.4.2.2 G0/G1 phase

Tamoxifen (10^{-5} M) caused a significant increase of cells in the G0/G1 phase (Figure 4.12). This observation was consistent with the findings of cell cycle studies conducted by others. Sutherland et al. found that tamoxifen at 5 µM caused cell cycle arrest at the G0/G1 phase (Sutherland et al., 1983). Another study also showed the accumulation of G1 cells post-treatment of tamoxifen in MCF-7 culture (Lykkesfeldt et al., 1984). To the contrary, no significant cell cycle arrest at G0/G1 phase was observed with 10^{-7} M tamoxifen, PPE and other treatments tested (Figure 4.12).

4.4.2.3 S & G2/M phase

In cancer prevention and therapy, it is desirable to prevent and reduce the synthesis of mutated DNA and mitosis, whereby the cancer cells have successfully progressed to produce new cancer cells. Hence, it is considered that the most crucial phases of the cell cycle in controlling the progression of malignant disease are S and G2/M phases (Garrett, 2001; Williams & Stoeber, 2012). In the current study, tamoxifen at high dose (10^{-5} M) was shown to reduce the number of S and G2/M phase cells significantly (Figure 4.13), which agrees well with findings reported by others (Lykkesfeldt et al., 1984;

Sutherland et al., 1983; Watts et al., 2009). It appears that tamoxifen reduced the S and G2/M phase cells, at least, by arresting the cells in the preceding phases (G0/G1) (Figure 4.12), i.e. blocking the progression or entry of cells into the S and G2/M phase. Tamoxifen at a moderate dose (10^{-7} M) was ineffective in inducing S and G2/M phase arrest. PPE showed a dose-dependent decrease in S and G2/M phase cells; the extent of reduction induced by PPE (at 50 µg/mL) was comparable to 10^{-5} M tamoxifen (77 versus 71% of control respectively, Figure 4.13).

4.5 Conclusions

In conclusion, PPE or WPE alone were demonstrated to be effective in inhibiting proliferation of several breast cancer cell lines (MCF-7, T-47D, MDA-MB-231 and 4T1) *in vitro*. Since these breast cancer cell lines are different in their ER status and invasiveness, this finding may imply PPE or WPE alone would be effective against different types of breast cancer (ER+, ER-, highly or less invasive forms). Estradiol concentration varies physiogically between 10^{-11} to 10^{-6} M. PPE or WPE remained effective regardless of estradiol concentration, unlike tamoxifen which was shown to have estradiol dependency for its action (Chapter 3). Moreover, PPE also seemed to prevent the potentially stimulatory effect of low-dose tamoxifen. This is the first report on the effect of an extract made from purple potato cv. Urenika in breast cancer cells *in vitro*, and the first report of the effect of combination treatment of potato and tamoxifen or estradiol on breast cancer cell proliferation *in vitro*. In essence, Hypothesis 1 to 3 set forth for this chapter (Section 4.1.1) were demonstrated to be valid.

Both PPE and WPE displayed comparable efficacy. These two potato extracts were not identical in their chemical profiles. Multiple active factors exist that may work together, contributing to the antiproliferative effect of PPE and WPE reported in the current chapter. PPE treatment did not appear to induce necrosis, a form of cell death often related to cell injuries. Instead, PPE showed dose-dependent effects on apoptosis in T-47D culture. Furthermore, a combined effect was observed among PPE and tamoxifen in modulating the S

and G2/M phase. Tamoxifen reduced the proportion of S and G2/M phase cells, partly by arresting cells at the G0/G1 phase. While PPE (50 μg/mL) also reduced the proportion of S and G2/M phase cells, no significant arrest of G0/G1 cells was observed. Hence, it appears that PPE accelerates apoptosis resulting in a lower proportion of cells entering into S- and G2/M phases. Based on a review and analysis of the existing literature, it is possible that phenolic acids and glycoalkaloids contributed to the PPE-inducing apoptotic effect observed. It is noteworthy that the dose-dependent effect of PPE as well as the combined effect observed among PPE and tamoxifen in modulation of cell cycle and apoptosis, agree reasonably well with the corresponding cell proliferation data. This finding is consistent with the existing knowledge about the central role of cell cycle and apoptosis in controlling cell proliferation. Overall, PPE alone, or in combination with physiological concentrations of estradiol or tamoxifen, inhibited cell proliferation, possibly partially through mechanisms of inducing apoptosis and cell cycle arrest at DNA synthesis, G2 and mitosis. Hypotheses 1) to 4) set at the beginning of the chapter (4.1.2) were tested and shown to be valid.

Chapter 5 Metastatic mouse breast cancer model 4T1 & tamoxifen dose-response study

5.1 Introduction

Metastasis of a primary tumour to distant sites, disrupting normal functions of the vital organs, accounts for the majority of breast cancer mortality (Hagemeister et al., 1980). A medicine or agent with high anti-metastatic efficacy and fewer side effects than current treatments is therefore highly desirable in breast cancer treatment. Metastasis comprises a series of biological events including evasion of the immune system, local invasion, intravasation, survival in the lymph and blood vascular system, proliferation and colonization in distant organs, and formation of new blood vessels (Céspedes et al., 2006). It is impossible to reproduce all the complex events occurring in metastasis using *in vitro* models (Kim & Price, 2005). Therefore, the work described in this chapter aimed to establish a metastatic breast cancer animal model that could be used for testing the effect of tamoxifen, and future investigations of the *in vivo* effect of PPE on establishment and metastasis. As shown in Chapter 4, PPE may have potential to reduce the stumulatory effect of tamoxifen at low dose (10^{-8} to 10^{-7} M). Therefore, this animal study protocol could be useful to investigate and verify the effect of combined treatments of PPE and tamoxifen observed *in vitro* in the current thesis.

Two possible directions have been considered regarding metastatic animal breast cancer modelling:

(1) Human xenograft models: breast cancer cells of human origin (e.g. MCF-7, T-47D, MDA-MB-231) are transplanted or introduced into nude mice which are immuno-compromised to induce metastasis. This approach, however, has several major limitations. Specifically, it is difficult to transplant human breast cancer cells into rodents; a success rate of only 7

to 20% has been suggested (Kim et al., 2003). Furthermore, there is a lack of immune response for all xenograft models.

(2) Murine breast cancer models: cells of mouse origin are inoculated into immuno-competent mice to generate a metastatic breast cancer model. In contrast with human xenograft models (1), this second approach is superior since it involves animals with a normal immune system, given the recognised role of immune functions in cancer development and progression (Céspedes et al., 2006; Fantozzi & Christofori, 2006; Kim et al., 2003).

The 4T1 murine mammary carcinoma cell line is able to metastasize to various organs similar to stage IV human breast cancer (Heppner et al., 2000), making this cell line highly relevant for metastatic breast cancer modelling and it was thus chosen for the current thesis The dose effect of tamoxifen has not yet been well studied or reported in this model, hence tamoxifen was included in this animal trial. The objectives of this chapter were:

1) To establish an animal model of breast cancer metastasis using the 4T1 cell line;
2) To determine the effect of this model on animal health;
3) To conduct a tamoxifen dose-response study (i.e. effect on animal health, establishment and metastasis).

5.2 Methods & materials

5.2.1 Optimization: formulation of tamoxifen solution for intraperitoneal (IP) injection and oral administration

Tamoxifen is relatively insoluble in water or other aqueous solutions. Therefore, there are challenges incorporating the drug in saline for IP injection. Drinking water is an unfavourable oral route for administration of tamoxifen, because its dosage would be limited by its inadequate aqueous solubility, and also dependent on the drinking behaviour of the animal. Hence, experiments

were designed to optimize administration of tamoxifen in mice and consisted of the following:

1) Creating a tamoxifen liquid preparation satisfactory for IP injection procedure.
2) Formulating a food that allows administration of test agent to mice. Gel or jelly was chosen as the mode of delivery.
3) Conducting a mouse trial to test: a) if the mice would eat the jelly, and b) how well the feeding methodology works
4) Conditioning the mice to be used in a breast cancer study, to the jelly-feeding regime.

5.2.2 Cell line and cell culture conditions

4T1 cells were grown in 75 cm^2 cell culture flasks (T-75) containing complete medium consisting of RPMI-1640 medium, 10% fetal calf serum (FCS), plus 1% penicillin-streptomycin-glutamine mixture. All tissue culture reagents were purchased from Gibco Invitrogen Corporation (Grand Island, NY). Cell culture flasks were incubated under a humidified atmosphere with 5% CO_2 and 95% air at 37℃. Subculturing procedures were performed when cells reached estimated 80% confluence at a subcultivation ratio of 1:8 (refer to Chapter 2 for more details).

5.2.3 Metastatic breast cancer model - animal trial

Full approval for this animal trial (e.g. animal care, treatments administered) was attained from Massey University Animal Ethics Committee prior to the study. The 4T1 cell line was handled in accordance with Ministry of Agriculture and Forestry requirements. Briefly, 4T1 cells were placed into a test tube, then sealed in a plastic bag which was then kept in a lidded box with 'biohazard' label during the course of transport from PC-2 laboratory to the animal study facility. The box also contained sufficient paper towel and disinfectant to render cells

non-viable should a spill occur. Female BALB/c mice were obtained from Animal Resources Centre (Western Australia) and bred in the Massey University animal research facility. Age-matched mice (n=25) of 12 to 13 weeks old, which had been previously conditioned to jelly-feeding (Section 5.2.1), were used for this trial. All mice were individually caged, housed under standard conditions (22 °C, 12 hour light/dark cycle), and fed water and chow *ad libitum*. Baseline food intake data were collected daily for 1 week prior to the trial. Baseline body weights were measured 3 days before the trial.

On Day 1 of the trial, 4T1 cells (passage number: 24) were seeded and suspended in sterile phosphate-buffered saline (PBS) at cell concentration of 100,000 cells per mL; cell counting was performed using haemocytometer. Each mouse was inoculated with 50 µL of the cell suspension containing 5000 cells, via subcutaneous injection in the mammary gland. The mice were divided into 5 groups (n = 5 each group, as determined by power analysis) and subjected to 5 different treatment regimes from Day 1, as detailed in Table 5.1. All mice were fed with jelly containing tamoxifen (Tam-75, Tam-200 and Tam-600 groups), or no tamoxifen (Control and Tam-IP groups). Tam-IP mice were gently injected with IP tamoxifen; Control mice were not injected with IP solution. Body weight and chow intake were measured twice weekly. Jelly intake was measured 24 hours after each administration. During the trial, animal health conditions and clinical signs of sickness were observed including: loss of responsiveness and inquisitive behaviour, inactiveness, hunched posture, rough coat, laboured breathing, loss of balance or gait and moribund. Once a clinical sign of sickness was observed, a score was given (i.e. normal as 0, score of 1 to 3 for an increase in severity). The total health score for each mouse was the sum of the scores recorded for the various clinical signs observed.

The growth of primary tumours was detected by visual observation and palpation. Once palpable, dimensions of the tumour (length and width) were measured with vernier callipers every day. Primary tumour volume was calculated using the following formula (Smith et al., 2008): 0.52 x length x width^2. Upon termination of the trial, animals were euthanased. Primary

tumours, lungs, livers and spleens were excised from animals, formalin-fixed, and stained with hematoxylin and eosin (H&E). The H&E-stained slides were analysed using an Olympus research microscope (BX53). Photomicrographs were taken at 100-, 400- and 1000-fold total magnification.

Table 5.1 Treatment regimes for tamoxifen dose-response study

Group	No. of mice	Treatment for each mouse	Route of drug delivery
Control	5	0 mg/kg/day	-
Tam-IP	5	2 mg/kg/day	Intraperitoneal injection (100 µL per mouse)
Tam-75	5	75 mg/kg/day	Oral (jelly)
Tam-200	5	200 mg/kg/day	Oral (jelly)
Tam-600	5	600 mg/kg/day	Oral (jelly)

5.3 Results

5.3.1 Optimization: formulation of tamoxifen solution for intraperitoneal (IP) injection and oral administration

A total of 9 formulations were prepared for tamoxifen solvation (Appendix, Table A2). Tamoxifen was found to be the most soluble in formulation 9 (i.e. clear solution, no precipitate of undissolved compounds) and hence was chosen for the metastatic breast cancer model study, i.e. 2 mg tamoxifen in 0.25 mL ethanol, 0.15 mL Tween 80 and 3.6 mL PBS. A total of 16 jelly formulations were prepared and tested (Appendix, Table A3). Formulation 15 (i.e. 3%

gelatine, 3% sodium alginate, 30% sucrose) was found to be the best formulation that fulfils all the criteria (described in Table A3), and therefore was chosen for the mouse trial. Three trials were conducted to test if the mice would eat the jelly, also how well the device or set-up worked in the cages. In brief, the jelly formulation was proved to be successful if the mice (n = 12) ate all the jelly provided. In addition, taste and colour were tested for any preference. In the metastatic breast cancer study, there would be multiple oral doses of tamoxifen to be administered, hence jelly was colour-coded by treatment to avoid confusion, reducing intensive labelling, and facilitate the work of distributing jelly in the mouse husbandry. No specific colour preference in mice was observed. Overall, mice ate all the jellies fed regardless of the colour of the jelly (Appendix, Table A4). One mouse (# 2) ate only half of the jelly on Day 1 but subsequently ate all jelly provided.

5.3.2 Conditioning of jelly-eating behaviour

Twenty-six individually caged mice were fed jelly for 24 hours over 3 consecutive days (Appendix, Figure A18 & A19). As shown in Figure A19 (Appendix), mice 2 to 26 displayed a similar degree of acceptance to eating the jelly during the 3-day conditioning procedure, based on both observations and quantitative measurement of jelly intake (eaten >95% of jelly fed). There was no statistically significant difference among mouse 2 to mouse 26 in terms of jelly intake, based on Multiple Comparison statistical analysis (Tukey Honestly Significant Difference ANOVA, CI:95%). Mouse 1 consumed significantly less jelly than the other mice, hence it was excluded from the breast cancer animal trial.

5.3.3 Metastatic breast cancer model - animal trial

Primary tumour diameter, body weight, food intake (chow and jelly) and health score results were obtained (Table 5.2, Figure 5.1 to 5.5). Apart from jelly intake, individual animal data were presented because of variability of data. Furthermore, small sample size (n=5 per treatment group) may also limit the

reliability of analysis of variance, this suggests more animals should have been used per group. Visible metastatic tumours were found attached to spleen, livers, mesenteric lymph node, lungs, diaphragm, stomach, and small intestine. Specimens of primary tumours, lung, spleen and liver were collected for H&E processing (Figure 5.6). For some animals, however, histological preparation was not possible due to unexpected death or immediate need for euthanasia. The Tam-75 group was excluded from histologic analysis, since it was possible to collect only one mouse's organs from this group and therefore would not be meaningful for analysis and interpretation of results. Hence, organs excised from Control, Tam-IP, Tam-200 and Tam-600 (3 to 5 mice per group) were included for spleen weight analysis (Figure 5.7), and histological examination for 4T1 metastasis and hepatosteatosis (fatty liver) (Figure 5.8 to 5.18).

Table 5.2 Tumour development & visible secondary tumours in mice.
Tumour development in female BALB/c mice inoculated with 4T1 cells, and treated with or without tamoxifen

Group	No. of mice developed primary tumour	Primary tumour detection day (Day)	Mean primary tumour volume at termination of trial/death (mm^3)	Mean primary tumour weight at termination of trial/death (g)	No. of mice with visible secondary tumours /metastasis upon dissection
Control	5/5	12 to 14	321	0.7	2
Tam-IP	5/5	12 to 13	293	0.4	3
Tam-75	5/5	12 to 14	259	0.4	4
Tam-200	5/5	12 to 13	433	0.7	3
Tam-600	5/5	12 to 15	293	0.5	4

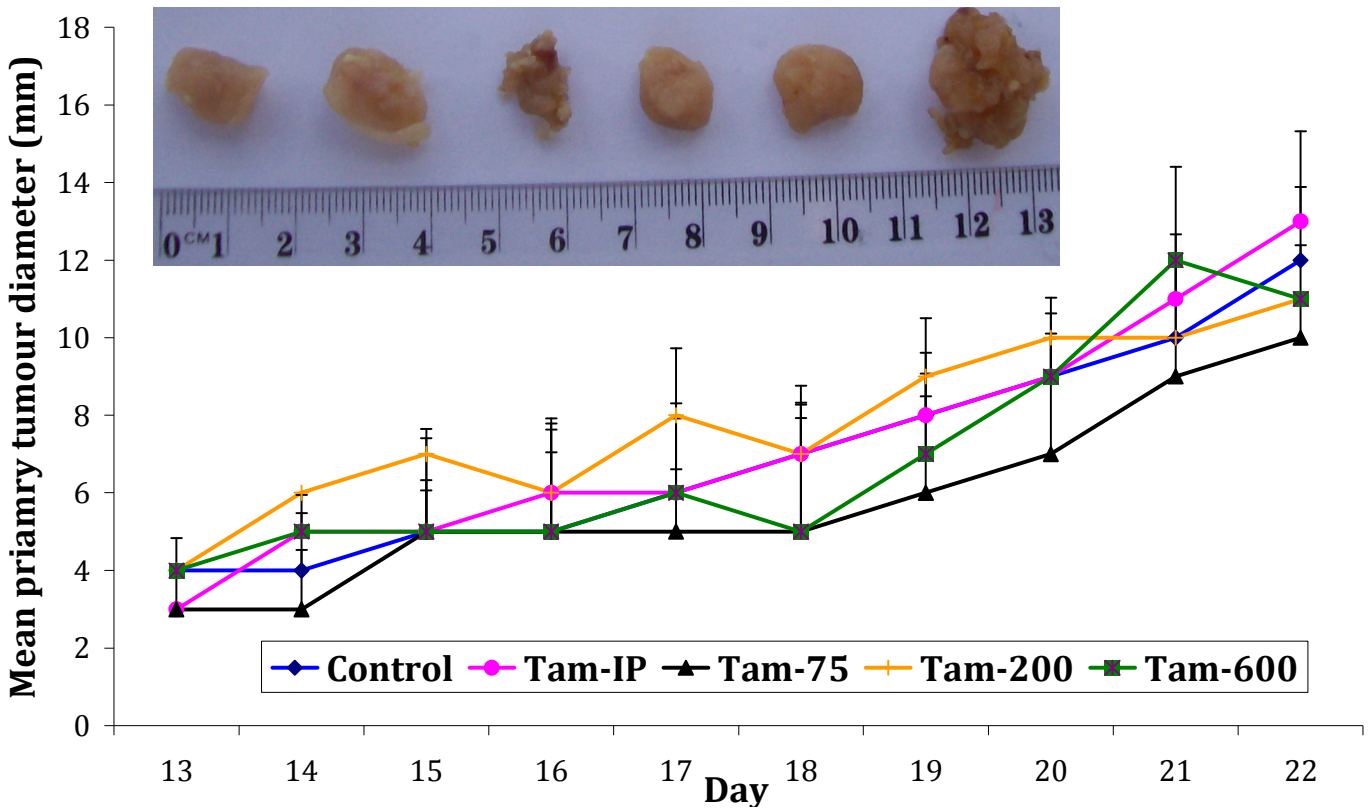


Figure 5.1 Primary tumour growth: diameter results.

Primary tumour diameters were measured daily from the first day of detection till the end of trial. Results were expressed as the mean of each experimental group ± SD (error bars). Images shown represent some primary tumours dissected (Day 22). There was no significant difference among the treatment groups at any time of measurement.

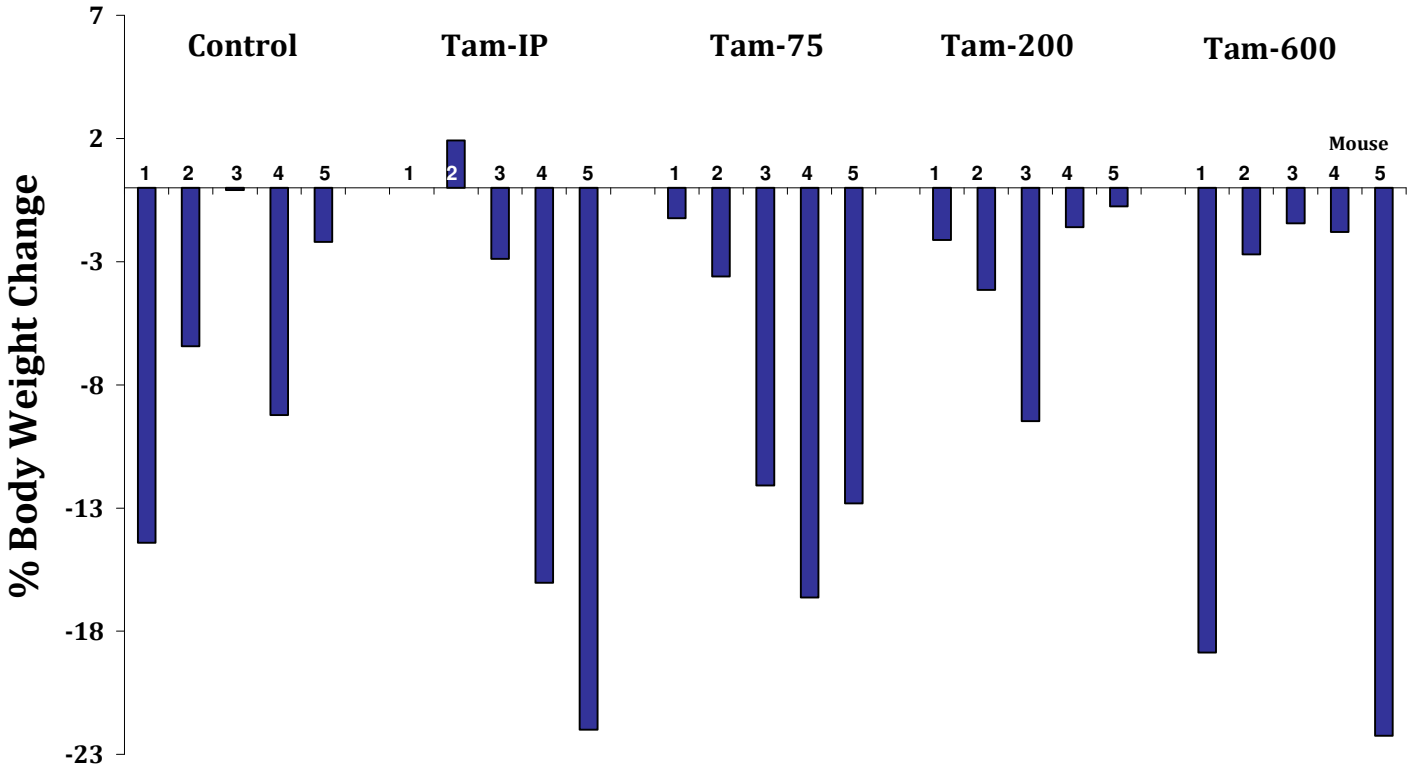


Figure 5.2 % Body weight change in mice during week 3.

Initial loss of body weight was evident at Week 3. Several tamoxifen-treated mice showed more weight loss than controls (i.e. Tam-IP group mice 4 & 5; Tam-600 group mice 1 & 5).

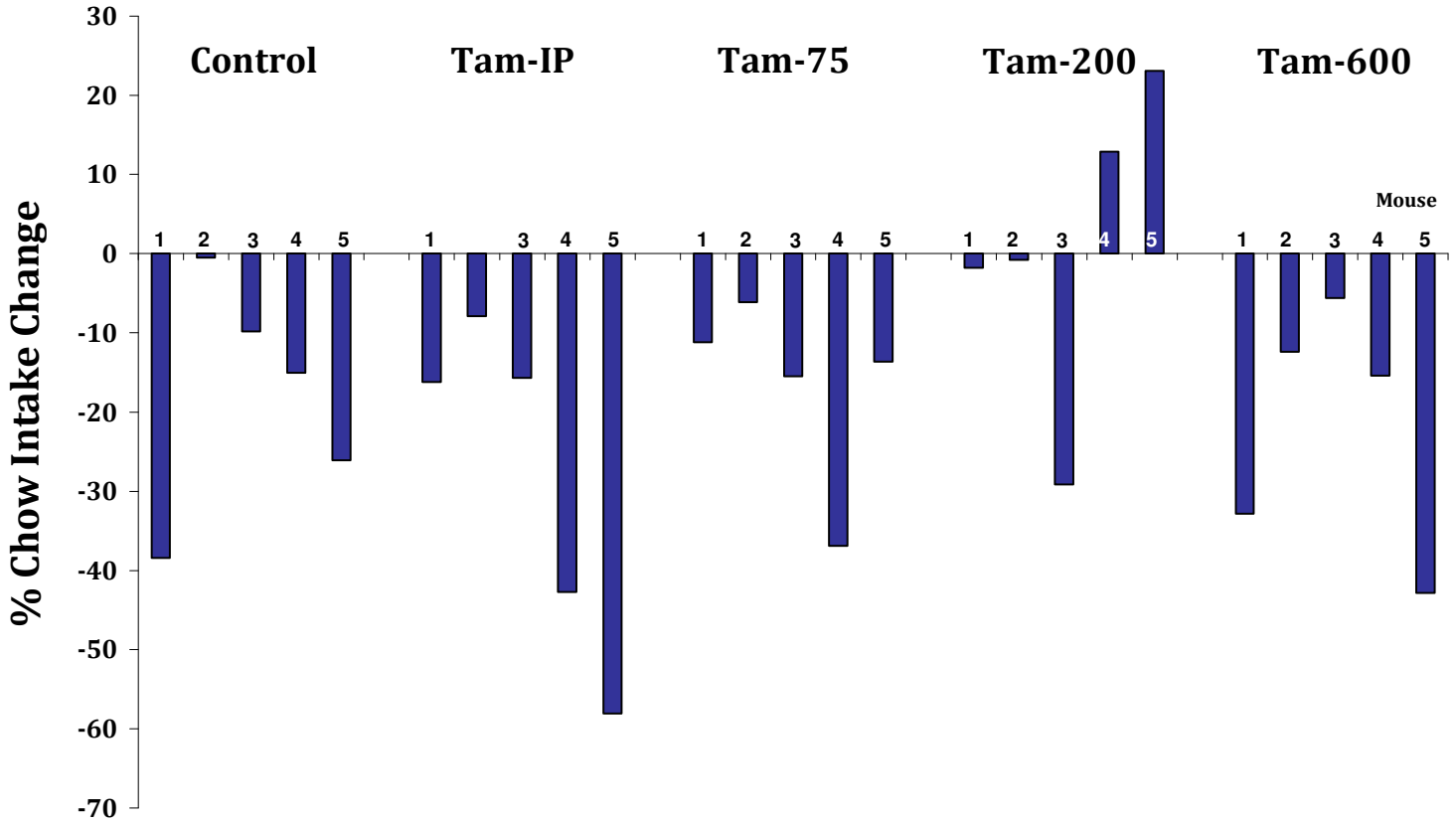


Figure 5.3 Chow intake changes of each mouse during week 3.

A pattern of greater chow intake reduction was observed in Tam-IP (-8 to -58%), and Tam-600 (-6 to -43%) groups compared to controls (-1 to -38%).

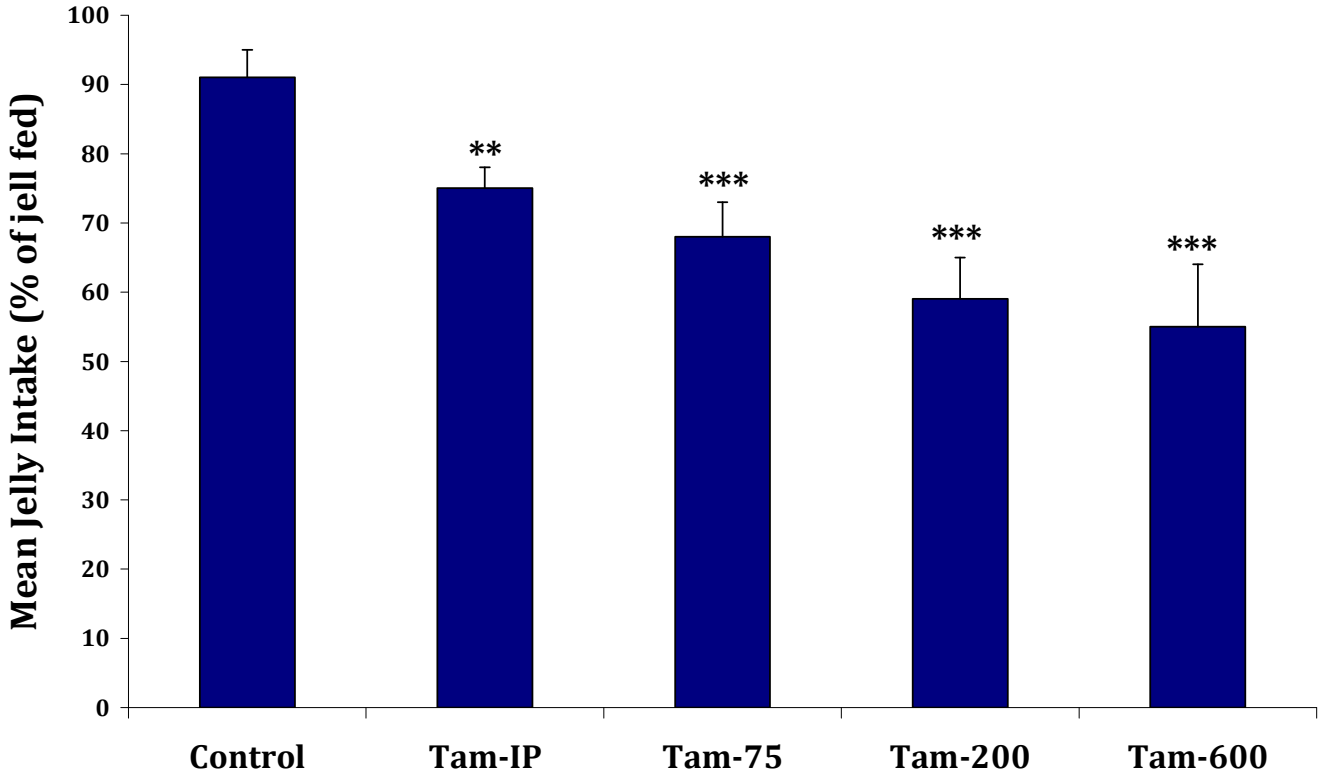


Figure 5.4 Group mean jelly intake during the trial.

Tamoxifen treatments (IP & oral) resulted in significantly less jelly intake than controls. Asterisk denotes the treatments are statistically different from control: ** P< 0.01, *** P<0.001.

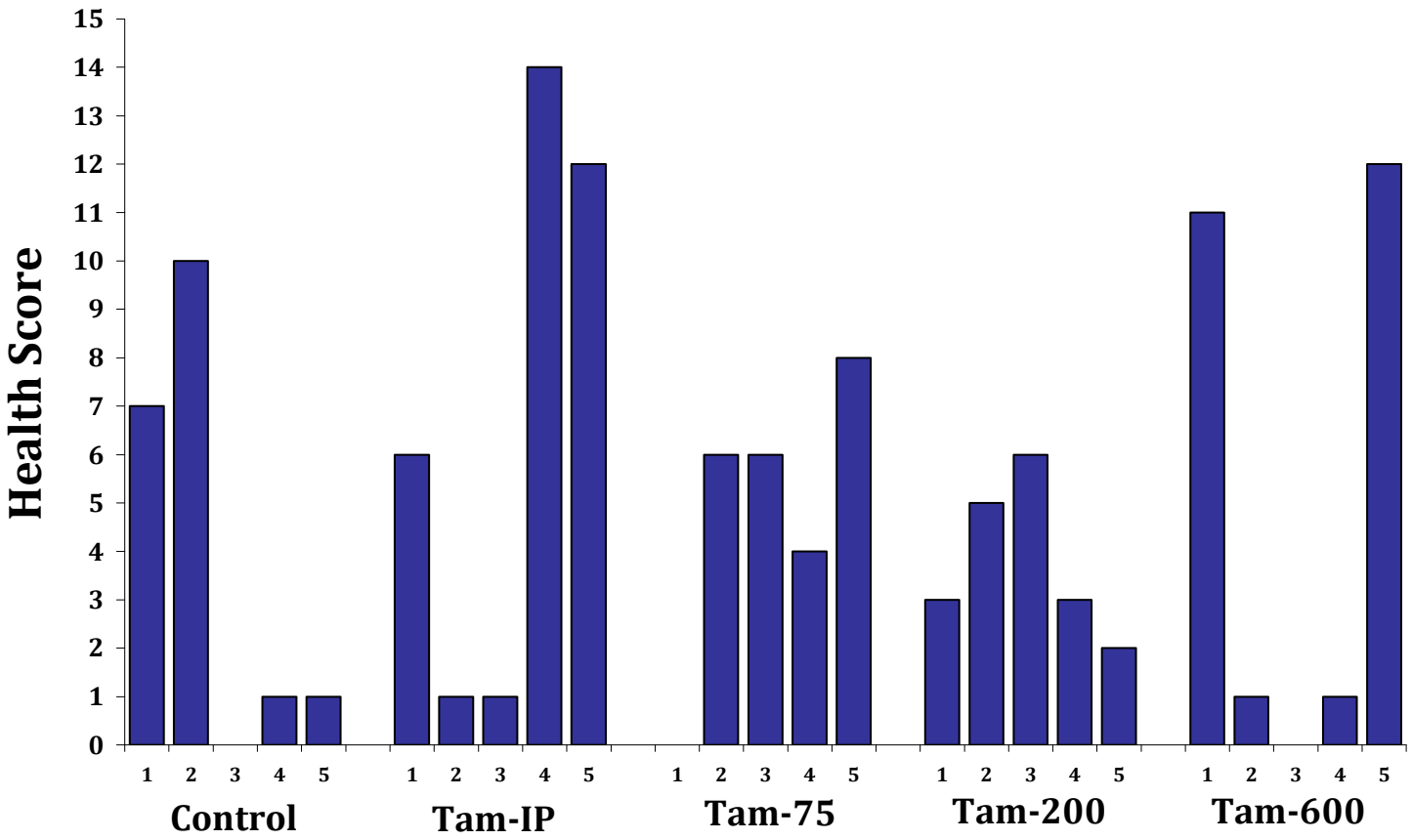


Figure 5.5 Health score for each of the mouse at the end of the trial.

Health of animals was monitored closely daily. Each abnormality observed was given a minimum score of '1'; the score increases as the severity increases. Tamoxifen treatments exacerbated the health score (as high as 14) versus the control group (highest score 10).

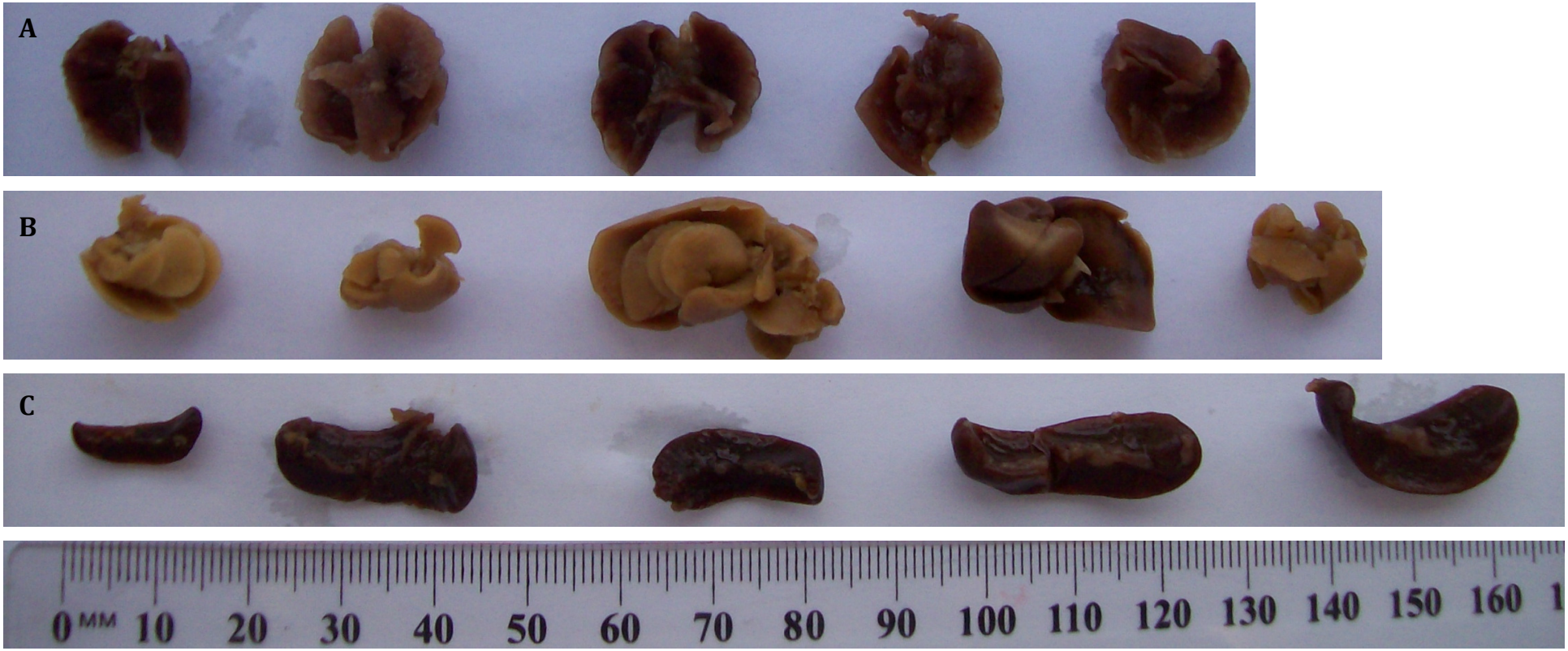


Figure 5.6 Organ specimens excised at the end of trial.

Examples of organ specimens: lungs (A), livers (B) and spleens (C) excised from normal mice, 4T1 Control, Tam-IP, Tam-200, and Tam-600 groups (Left to right). Note the spleens from all experimental groups were strikingly larger than normal spleen. Hypertrophy was also evident in some of the liver samples.

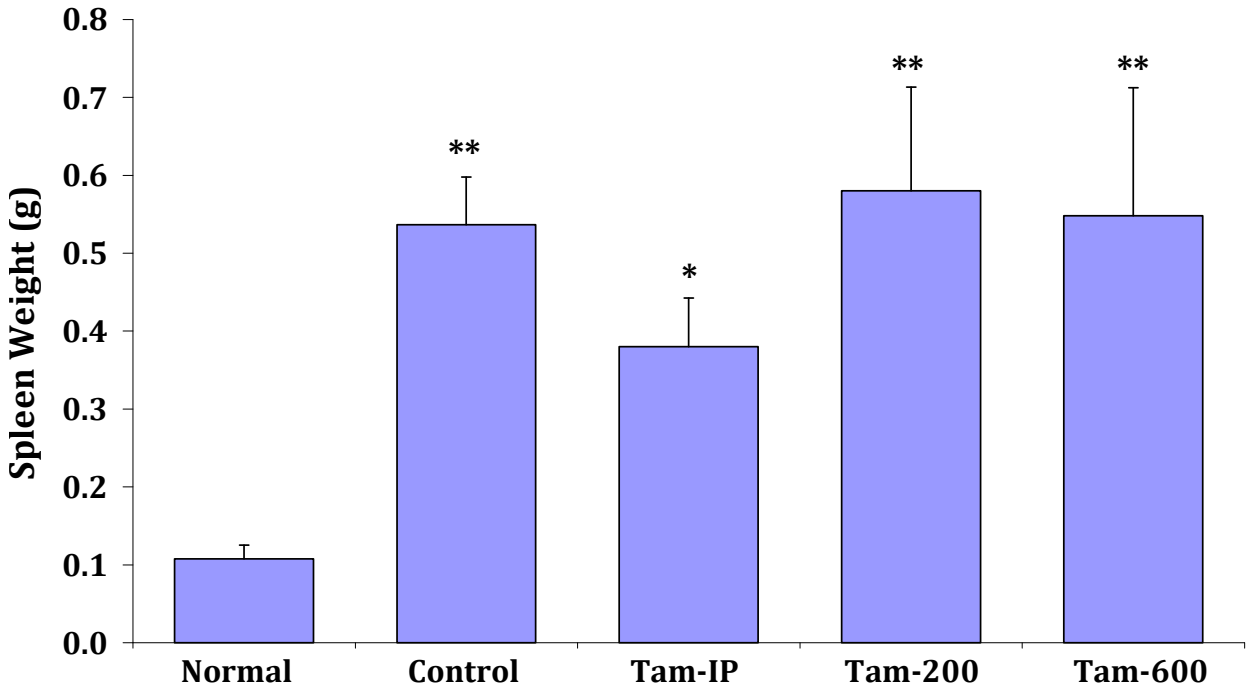


Figure 5.7 Spleen weight *vs.* various treatments.

Weights of spleens collected from normal healthy mice, 4T1 Control, Tam-IP, Tam-200 and Tam-600 experimental groups. Asterisks denotes treatments are significantly different from the Normal group (n=3): *P < 0.05, **P < 0.01.

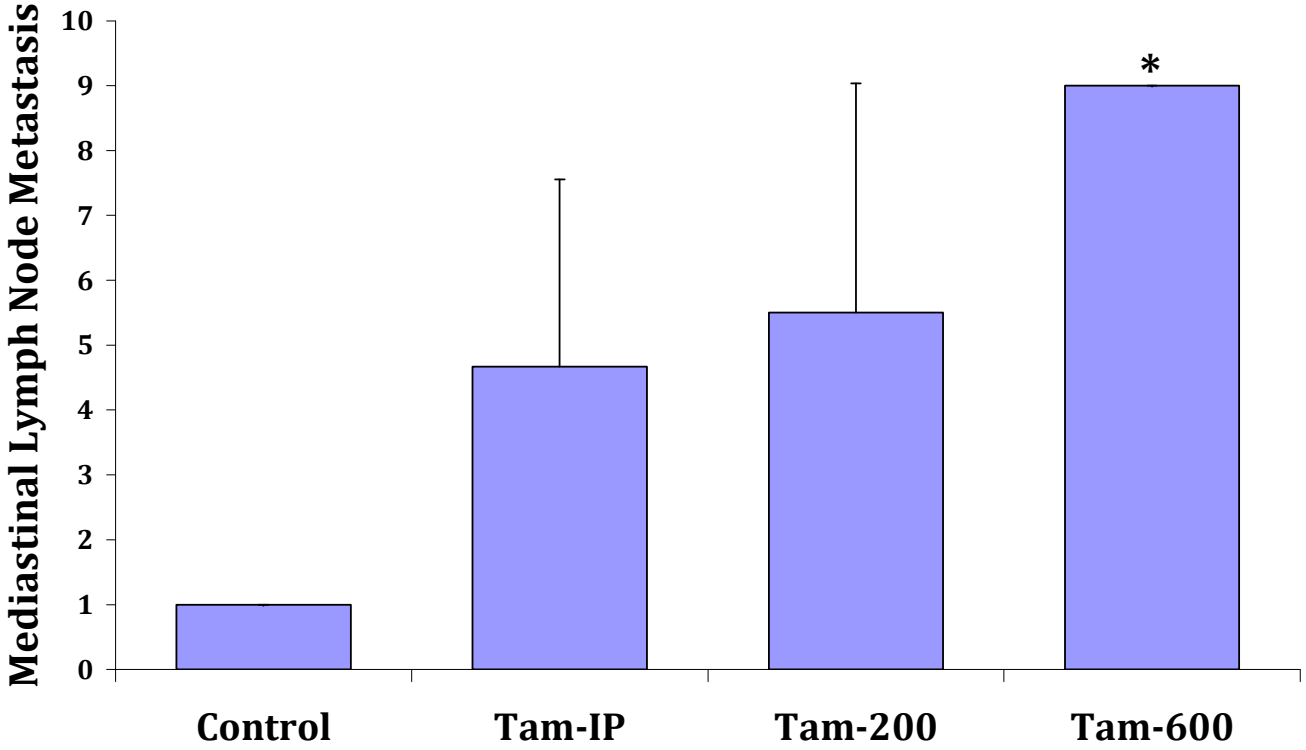


Figure 5.8 Mediastinal lymph node (MLN) 4T1 metastasis *vs.* various treatments.

4T1 metastasis in MLN was assessed by histological examination and scored on a scale of 10 for each of the treatment groups (N = 2 -3 mice per group). Results were expressed as mean ± SD. Asterisks denote the treatment is statistically different from Control (* $P < 0.05$).

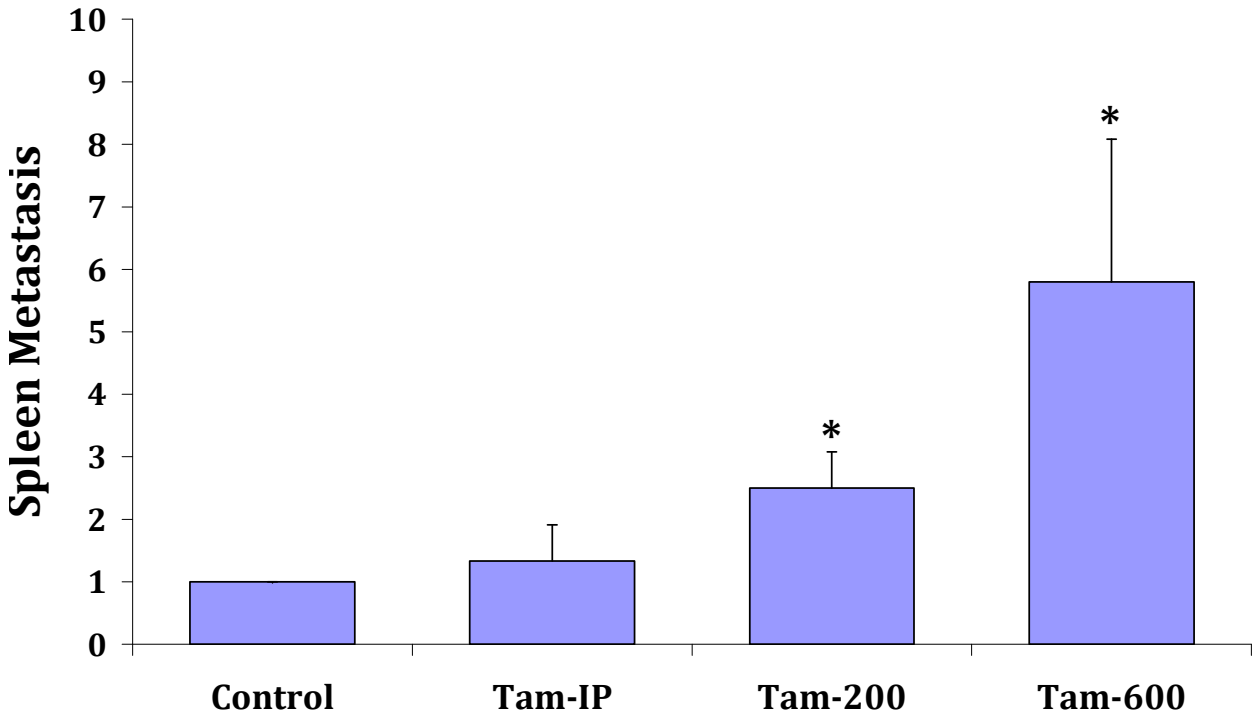


Figure 5.9 Spleen 4T1 metastasis vs. various treatments.

4T1 metastasis in spleen was assessed by histological examination and scored on a scale of 10 for each of the treatment groups (N = 3 - 5 mice per group). Results were expressed as mean ± SD. Asterisks denote the treatment is statistically different from Control (* $P < 0.05$)

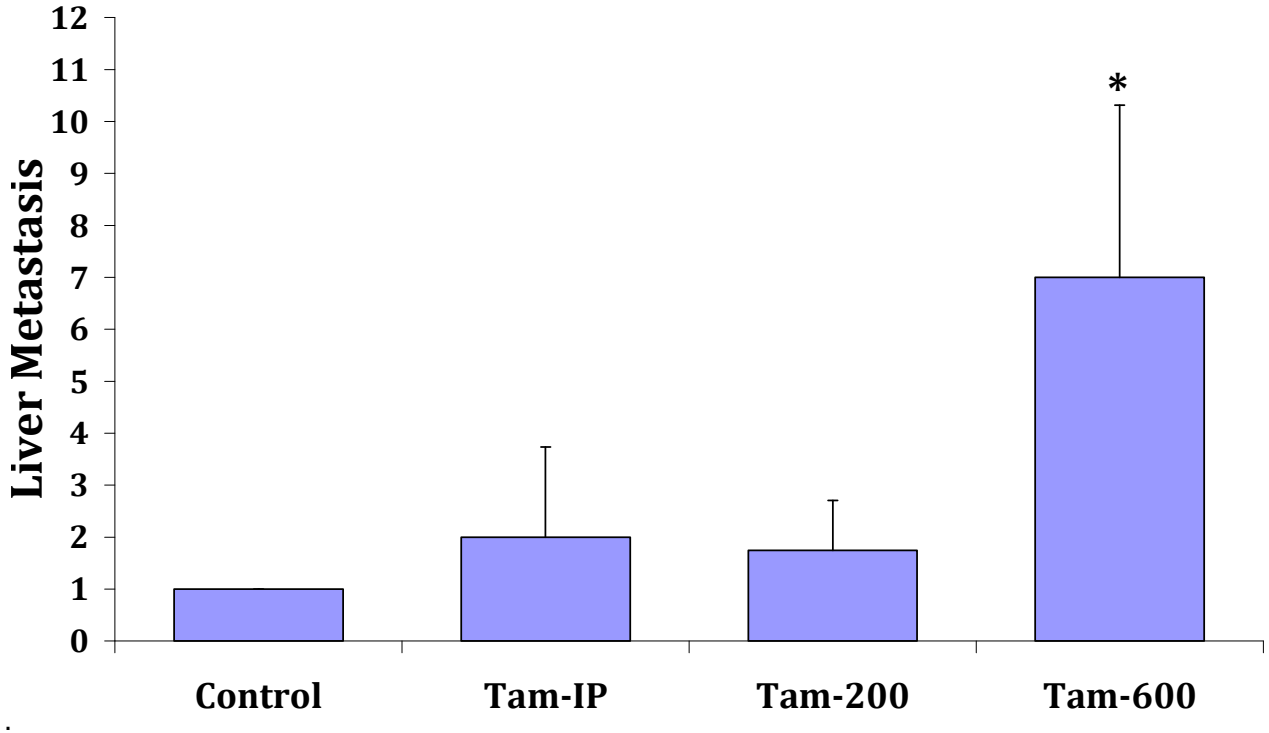


Figure 5.10 Liver 4T1 metastasis *vs.* various treatments.

4T1 metastasis in liver was assessed by histological examination and scored on a scale of 10 for each of the treatment groups (N = 3 - 5 mice per group). Results were expressed as mean ± SD. Asterisks denote the treatment is statistically different from Control (* $P < 0.05$).

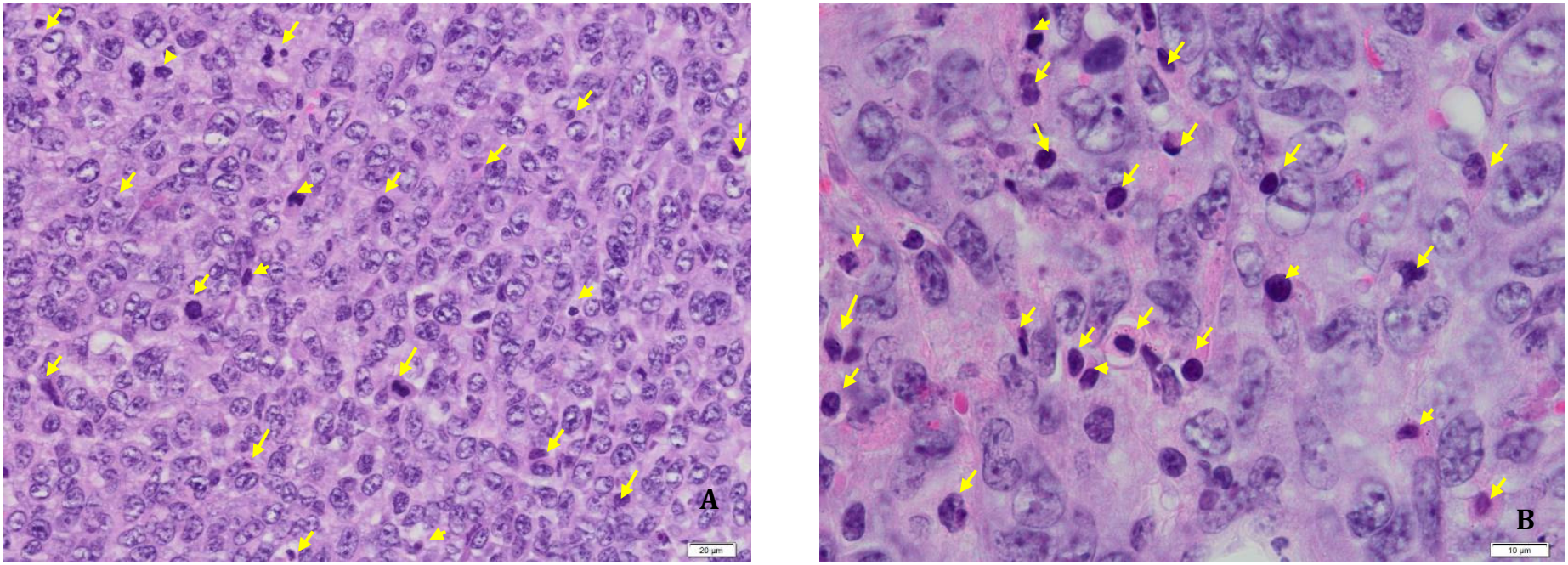


Figure 5.11 Histological view of 4T1 primary tumours resulted from 4T1 cell transplantation into mice.

Cells morphologically resembling myeloid precursor cells were prominent (Yellow arrows show some examples). Scale bar: (A) 20 µm; (B) 10 µM.

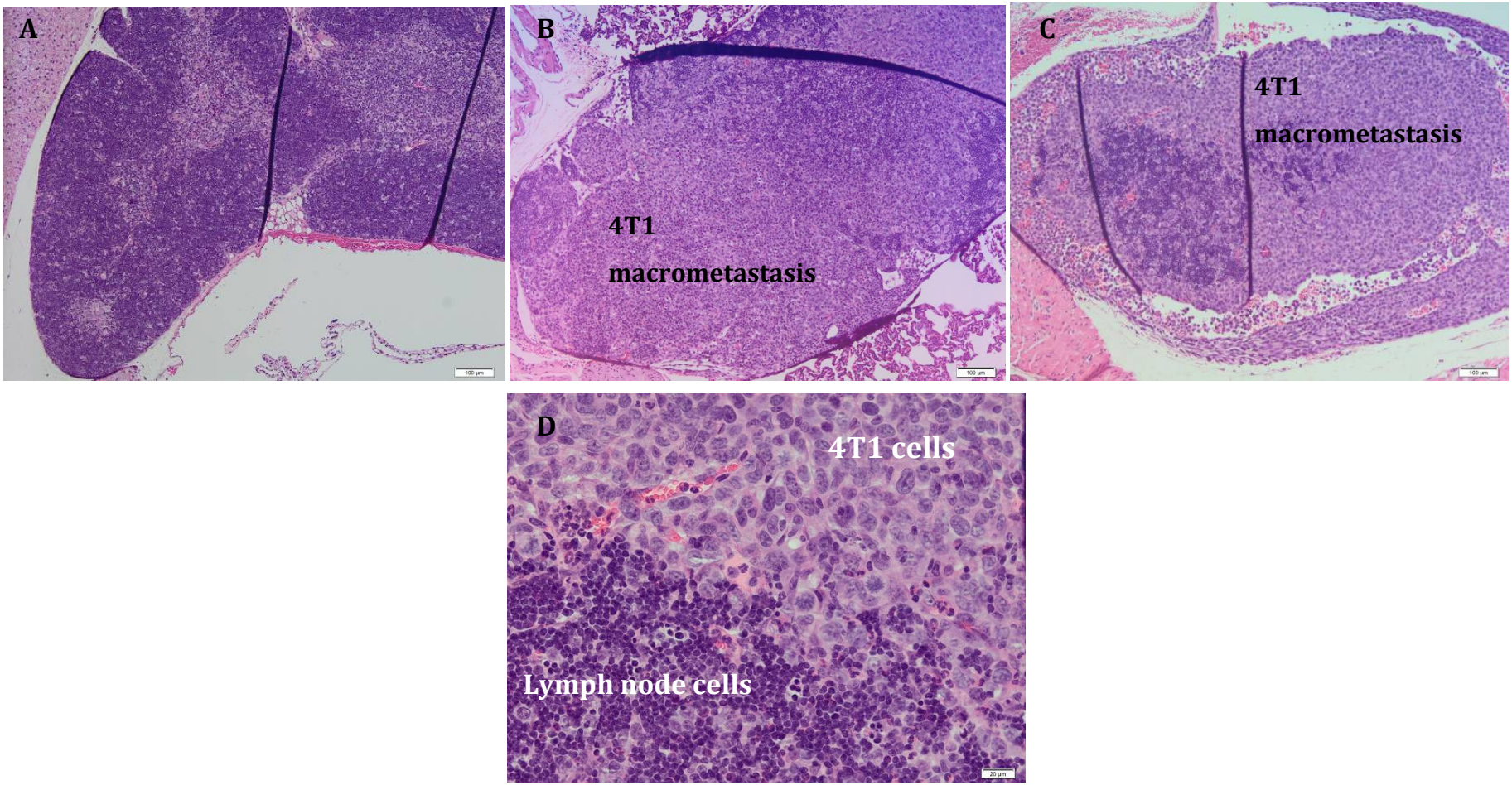


Figure 5.12 Histological view of metastasis to mediastinal lymph node.
Compared to control (A), more severe 4T1 metastasis was evident in some of the mediastinal lymph nodes of tamoxifen-treated mice (B: Tam-IP, C & D: Tam-600). Invasion and replacement of lymph node parenchyma cells by 4T1 cells. Scale bar: A-C, 100 µM; D, 10 µM.

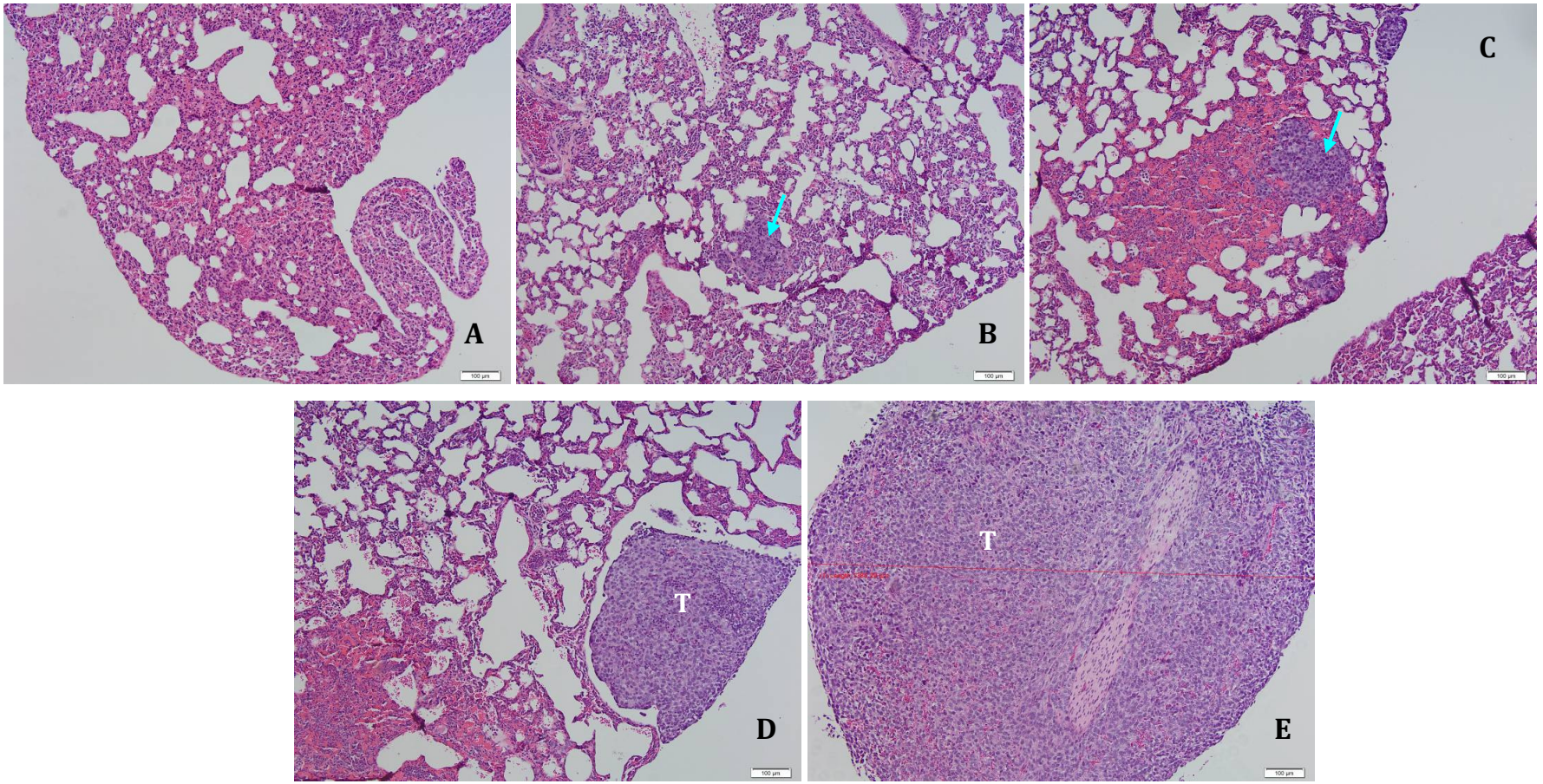


Figure 5.13 Histological view of metastasis to lungs.

A: normal lung. B: 4T1 control lung. 4T1 nodule (blue arrows) in a tamoxifen-treated mouse (Tam-IP group) within lung (C) and 2 tumour masses (T) were attached to this lung specimen (D & E). Scale bar: A – E, 100 µM.

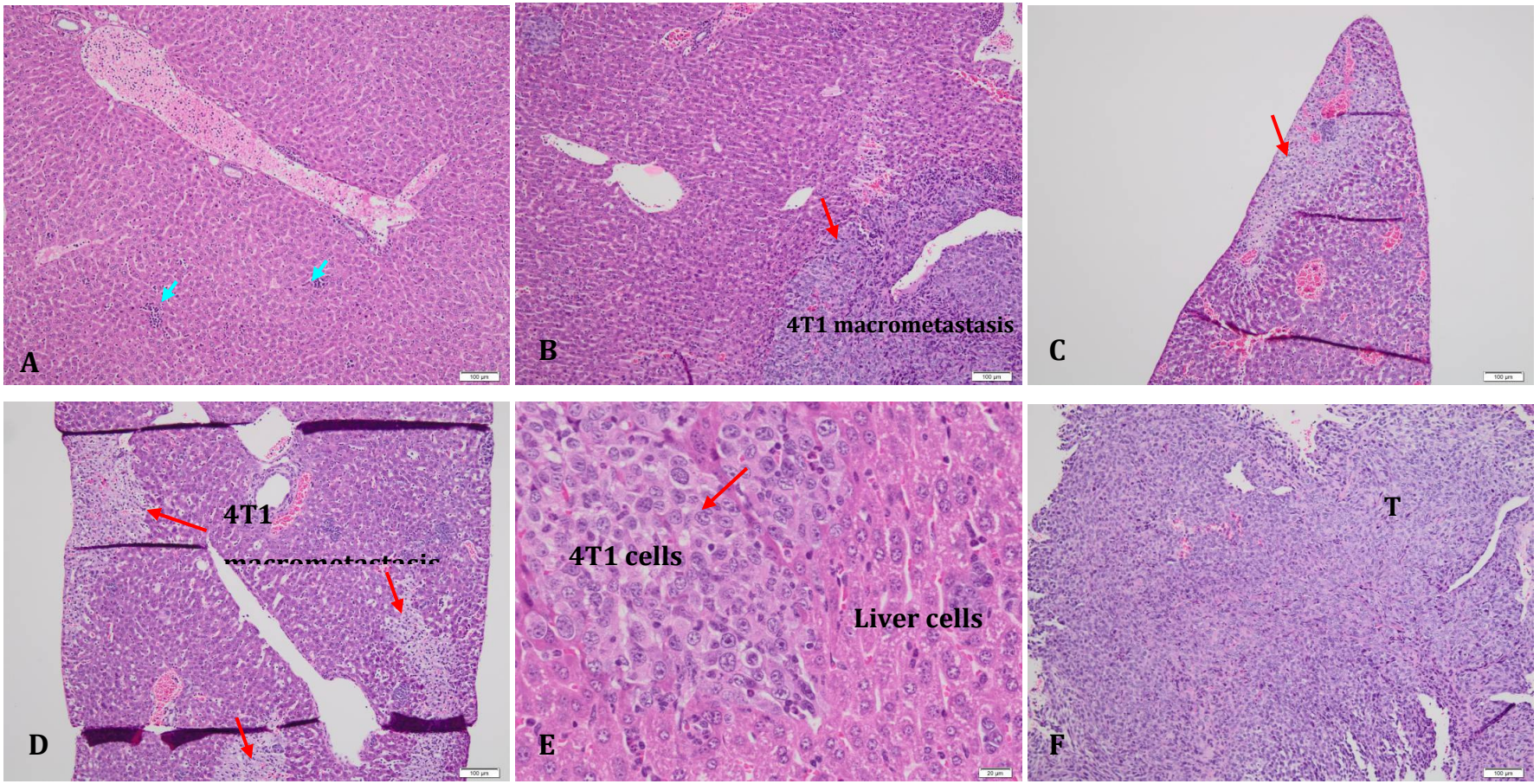


Figure 5.14 Histological view of metastasis to livers.

Micrometastasis (blue arrows) was observed in 4T1 controls (A). More severe 4T1 macrometastasis (red arrows) was evident in some mice from 3 tamoxifen groups (B, C, D). Invasion and replacement of liver parenchyma cells by 4T1 cells (E: 400x magnification). 4T1 tumour mass attached to two liver specimens of Tam-600 group (F as an example). Scale bar: all 100µM, except E, 20 µM.

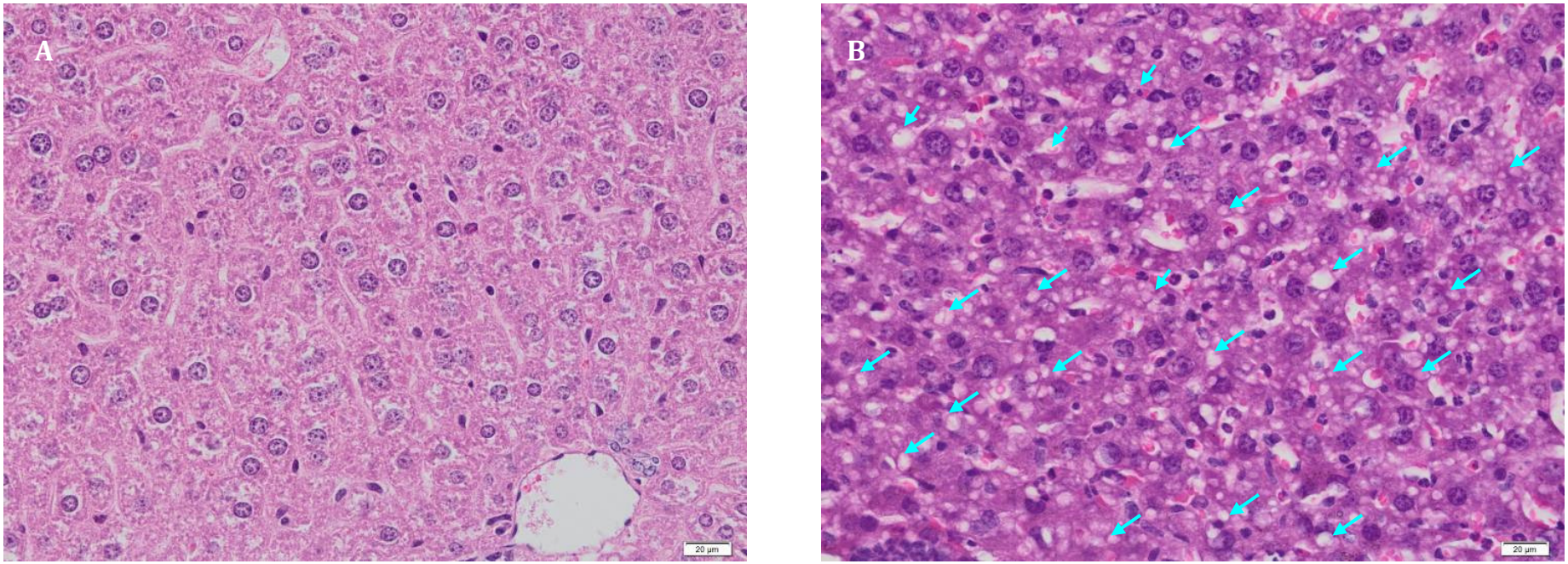


Figure 5.15 Histological view of hepatic steatosis (fatty liver).

A: Control liver. Fat droplets (blue arrows) were omnipresent in the liver specimens of some tamoxifen-treated mice, indicating the occurrence of fatty changes (example B: Tam-IP).

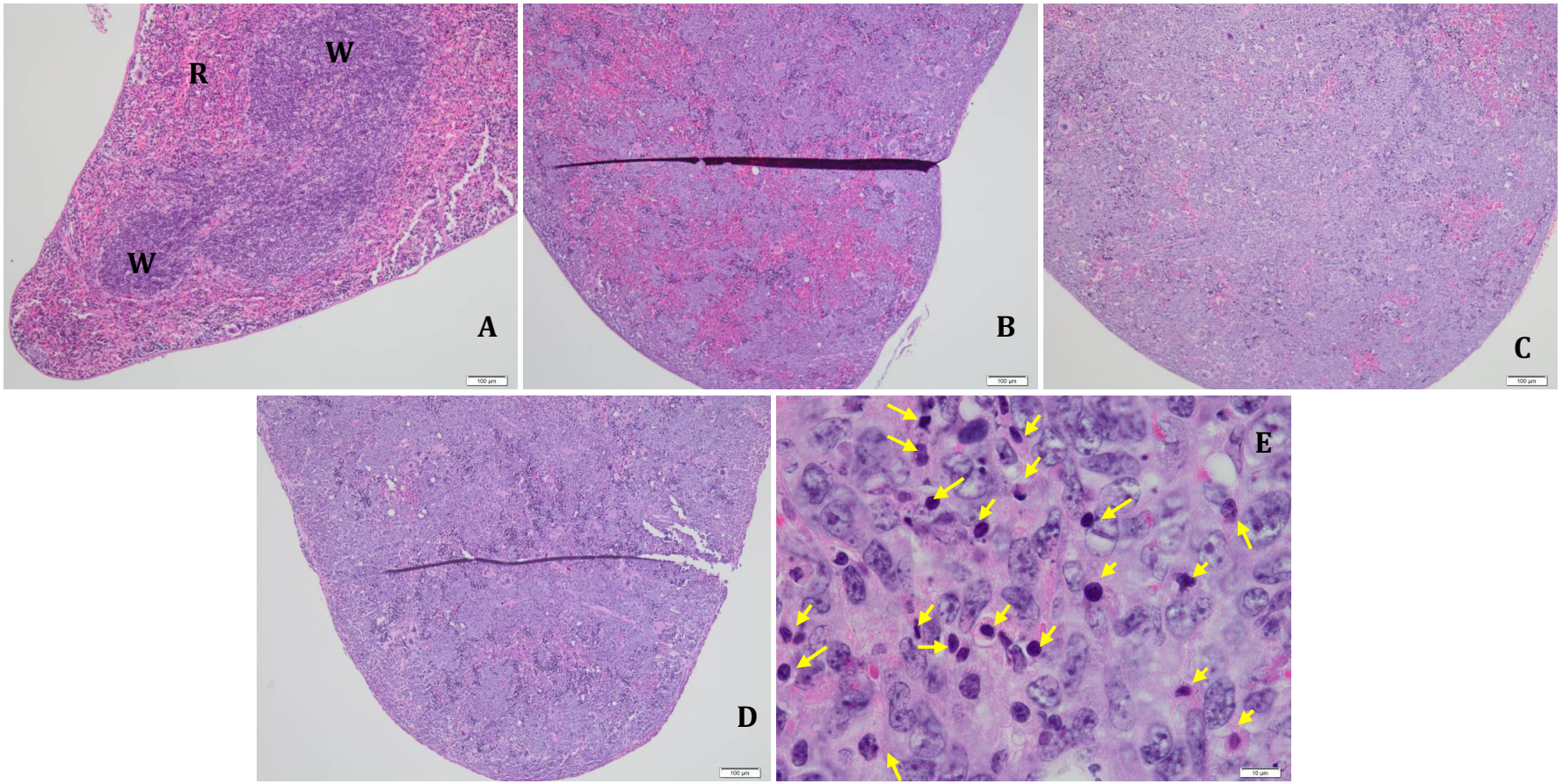


Figure 5.16 Spleen metastasis and extramedullary hematopoiesis (EMH).
In the spleens of tumour-bearing mice (B, C, D), white pulp (W) area diminished and myeloid cell-rich red pulp (R) area (i.e. a major site of EMH) expanded versus a normal spleen (A). Compared to 4T1 control (B), more severe metastasis of 4T1 cells and EMH was notable in the spleens of tamoxifen-treated mice from Tam-200 (C) and Tam-600 (D; E at 1000x). Yellow arrows: myeloid precursor cells.

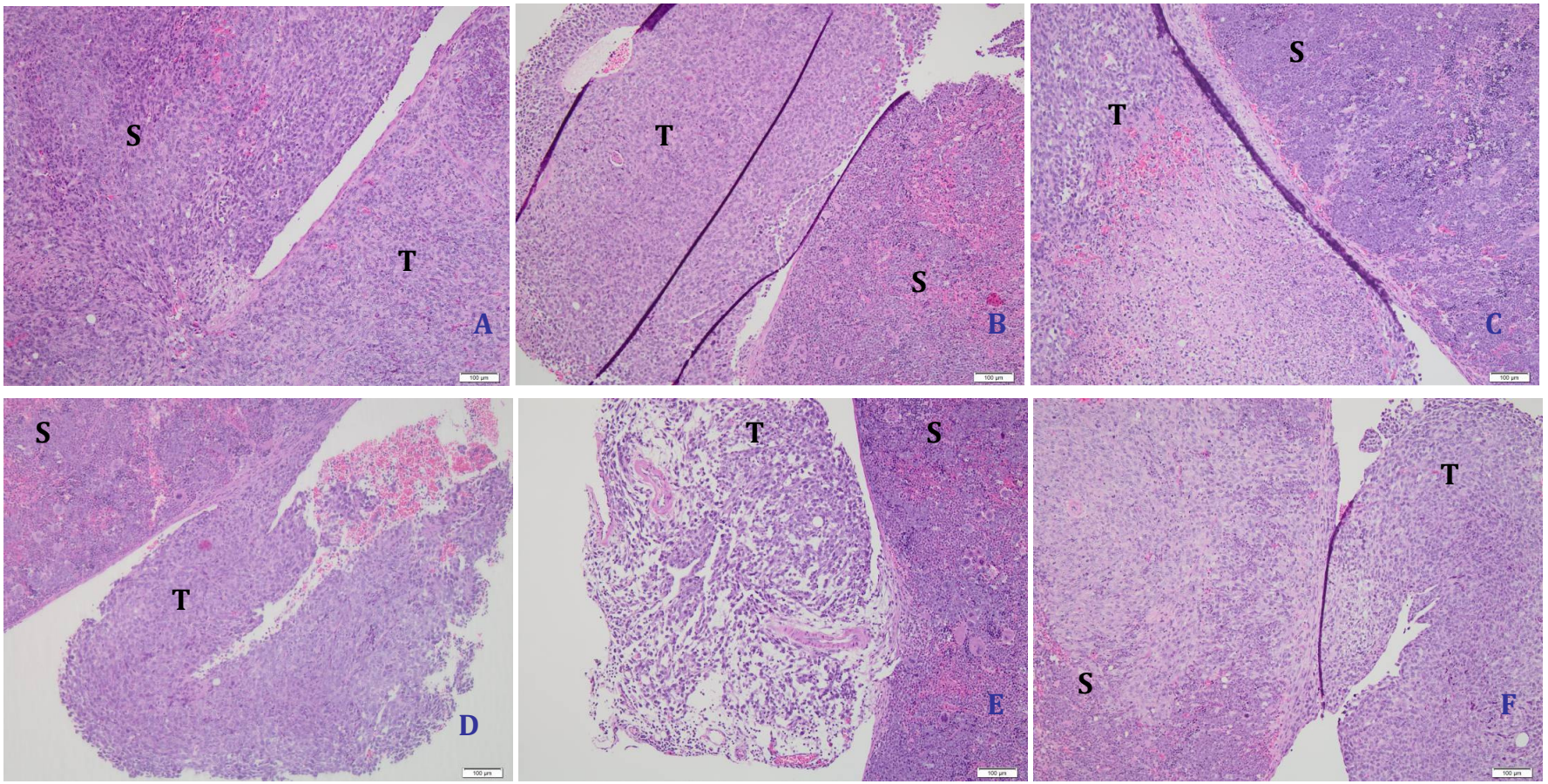


Figure 5.17 4T1 Metastatic tumours attached to spleens.
4T1 metastatic tumours (T) attached to spleens (S) of mice from Tam-IP (A), Tam-200 (B), Tam-600 (E-F) groups. Scale bar: 100 µm.

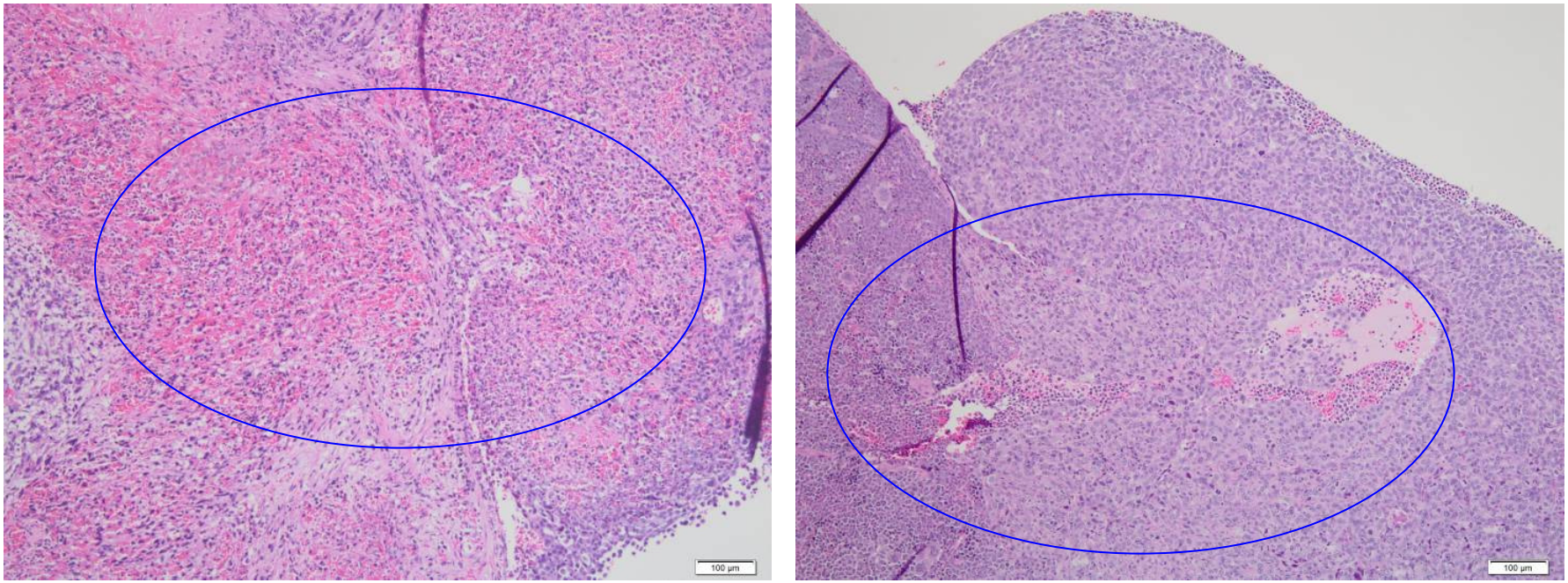


Figure 5.18 Potential role of myeloid precursors cells in 4T1 metastasis.

Cells morphorlogically resembling myeloid cells (blue circle area) were omnipresent in the tumour masses and spleen, as well as in the blood vessels and connecting tissues connecting the tumours attached and the spleen (circles) of mice treated with tamoxifen (A, from Tam-600; B, from Tam-200 as examples)

5.4 Discussion

The research protocol successfully produced the first palpable primary tumours in all the animals 11 to 14 days after 4T1 implantation (Table 5.2). One study injected the same number of 4T1 cells (5000 per mouse) as the current study into the mammary gland of BALB/c mice; the authors observed a palpable tumour in all animals 11 to 26 days post-injection (Pulaski & Ostrand-Rosenberg, 1998). Others have observed a shorter latency period with a higher cell load. For instance, mice injected with 1 x 10^5 cells developed palpable tumours 7 days after injection (Smith et al., 2008). No significant difference in latency period was observed among all treatment groups in this study. Post-mortem dissection revealed 3-4 mice in each of the tamoxifen-treated groups had metastatic tumours in their visceral area, versus 2 mice in the Control group (Table 5.2). Others have also reported metastasis to distant organs including lung, liver, lymph node and blood 22 days after inoculation of 4T1 cells into mice mammary gland (Pulaski & Ostrand-Rosenberg, 1998). 4T1 cells are highly metastatic which mimics aggressive human breast cancer and are known to metastasize spontaneously to distant organs (Eckhardt et al., 2012; Kim & Price, 2005). While the occurrence of metastasis observed in the current thesis was possibly a result of systemic inoculation of the 4T1 cancer cells, it is more likely that real metastasis has taken place given the highly aggressive nature of 4T1 cells and there was sufficient time for primary tumour to develop and metastasize (current trial ended on Day 22).

The growth rate, based on the tumour diameter (TD) measured, was found to be similar among all treatment groups (Figure 5.1). By Day 22, TD reached 8 to 16 mm. The trial was terminated on this day, in accordance with animal ethics, i.e. animal should be euthanized when primary tumours reach 14 to 16 mm. Primary tumours were excised, weighed and volumes were calculated (Section 5.2.3). There was no significant difference in primary tumour volumes or weights across all treatment groups (Table 5.2). Overall, it was found that both the IP and oral tamoxifen treatments administered were not effective in preventing or delaying the growth of the 4T1 primary tumour. Limited data

exist regarding the effect of tamoxifen in the 4T1 model. Two studies involving daily parenteral administration of tamoxifen, at 0.25 and 0.6 mg/kg respectively, found no significant effect of tamoxifen on induction time and weight (Goel & Majumdar, 2009; Xanthopoulos et al., 2005). A higher dose of tamoxifen might be required to exert a significant anti-tumour effect in this model. A minimum 1 mg/kg/day injection was shown to produce serum tamoxifen concentrations in BALB/c mice similar to those observed in patients receiving standard tamoxifen treatment (Osborne et al., 1985). Therefore, the dose of 2 mg/kg/day per mouse (50 μg) was chosen for tamoxifen IP administration in this study, which was also shown to be ineffective.

Body weight of mice was monitored closely during the trial. Normal body weight gain occurred during the first two weeks in 84% of all the animals used in the study. However, drastic loss of body weight emerged notably across all the treatment groups during week 3 (Figure 5.2). It was observed that 8 out of 25 mice had lost more than 10% of body weight; the group mean body weight loss ranged from 4 to 9%. The trial was terminated at the end of week 3, to be in compliance with animal ethics, which stipulates initial body weight loss of 10% as a humane endpoint of the study.

This is the first detailed report of the dose-effect of tamoxifen on body weight and food intake in this advanced breast cancer animal model. Compared to controls, tamoxifen-treated mice in Tam-IP, Tam-75 and Tam-600 groups experienced the most severe weight loss (Figure 5.2). Specifically, the Tam-75 group contained the highest number of mice with more than 10% weight loss among all treatment groups (3 out 5; 12 to 17% weight loss). The greatest magnitude of weight loss was observed in the highest dose Tam-600 and Tam-IP groups (16 to 22% weight loss). Therefore, tamoxifen was found to reduce body weight compared to controls in the current study. This finding may be partially explained by the food intake data obtained concurrently (Figure 5.3 & 5.4). Specifically, mice which had the most reduction in chow intake suffered the most severe body weight loss (e.g. mouse 1 and 5 in Tam-600 group; mouse 3 in Tam-200 group; mouse 4 in Tam-75 group; mouse 4 and 5 and Tam-IP

group, mouse 1 in Control group). Hence, a good correlation appeared to exist between the food intake and body weight data. Overall, a pattern of greater chow intake reduction was observed in Tam-IP (-8 to -58%), and Tam-600 (-6 to -43%) groups compared to controls (-1 to -38%). Similarly, in terms of jelly intake, mice treated with tamoxifen showed reduced jelly intake compared to controls. This raised the possibility that the taste of tamoxifen might have contributed solely to the observation. However, this notion is most unlikely to be true because significant reduction of jelly intake (25%) was also observed in the Tam-IP group, which was fed with jelly without tamoxifen. Indeed, tamoxifen administration, either orally or parenterally, had been shown to reduce food intake and body weight in rodents (Bowman et al., 1983; Wade & Heller, 1993; Wallen et al., 2001). Tamoxifen, a partial estrogen agonist, may modulate feeding behaviour similarly to estradiol. López and others showed that tamoxifen induced an anorexic response by inhibiting fatty acid synthase and accumulation of malonyl-CoA in the hypothalamus in rodent models (López et al., 2006). Based on the amount of tamoxifen-containing jelly consumed (Figure 5.4), the dosages of tamoxifen received by Tam-75, Tam-200 and Tam-600 groups would equate to 51, 118 and 330 mg/kg/day respectively. An oral dose of tamoxifen at 200 mg/kg/day has been shown to result in serum tamoxifen levels in mice similar to those observed in patients receiving standard tamoxifen treatment (Larosche et al., 2007). Therefore, the oral tamoxifen dose range tested in this study covered the dose applied clinically, although the mice did not eat all the jelly provided.

Health of animals was monitored closely daily. Each abnormality observed was given a minimum score of '1'; the score increases as the severity increases (Figure 5.5). On Day 17 and 18, two of the Tam-IP mice (mouse no. 1 & 5) appeared to be very ill and thus were euthanized immediately in accordance with ethical practice. On Day 20, another Tam-IP mouse (no. 4) and two of the Tam-600 mice (no.1 & 5) were also found moribund and euthanized accordingly. During the following day, two other tamoxifen-treated mice, i.e. mouse no. 4 (Tam-75) and 3 (Tam-200), were found dead in the cages. Three other Tam-75 mice and two Control mice were also euthanized on the same day

because of their moribund condition. The rest of the mice were euthanized on Day 22 and 23 when their primary tumour diameters were approaching 14-16 mm (i.e. humane end point). Overall, it was found that tamoxifen treatments exacerbated the health score (as high as 14) versus the control group (highest score 10) (Figure 5.5), including unexpected death of animals. Clinical signs of illness or abnormality observed include weakness, loss of responsiveness, becoming inactive, lethargy, loss of appetite, rough coat, hunched posture, impaired mobility, and laboured breathing. A good correlation was observed between the health score and the severity of metastasis observed in the animals. For instance, mouse 1 and 5 in Tam-600 group, mouse 4 and 5 in Tam-IP group had the most severe metastasis in spleen, liver and lung; these mice were scored the highest (worst) for their health conditions.

Mediastinal lymph nodes (MLN) obtained from both Control and tamoxifen groups (2 to 3 MLN each group), showed metastasis of 4T1 cells. However, more severe MLN metastasis was observed for tamoxifen groups when compared to the Control (Figure 5.8 & 5.12). Tamoxifen appeared to have a dose-dependent effect; the highest dose Tam-600 group had almost all LN invaded by 4T1 tumour mass (Figure 5.12). Metastasis of MLN for Tam-600 group was significantly more severe than the other treatment groups (Figure 5.8). The presence of LN metastasis has been associated with poor prognosis in cancer patients and is one of the key factors in staging of lung cancer (De Leyn et al., 2007; Ercan et al., 2004). None of the Control lung sections showed attachment of 4T1 tumour mass. To the contrary, notable tumour masses were found attached to 2 lung specimens obtained from Tam-IP and Tam-600 groups respectively (Figure 5.13). There was no significant difference in metastasis of 4T1 cells in the lungs among the various treatments (Figure A20, Appendix).

Histological examination revealed a remarkable stimulatory effect of tamoxifen on the growth of 4T1 tumours in the livers, which has not been reported in the literature. Compared to the Control mice, 50% of the tamoxifen-treated mice showed notably more severe liver metastasis in terms of number or size of nodules (Figure 5.10). Strikingly, two out of five of the highest dose group

(Tam-600) had large tumour masses attached to the livers (Figure 5.14). Metastasis of 4T1 in the Tam-600 group was significantly higher than the other groups (Figure 5.10). Tamoxifen may stimulate tumour growth in a dose-dependent manner. Indeed, tamoxifen has been shown to be a strong inducer of hepatocellular carcinomas *in vivo*. A few carcinogenicity studies demonstrated that tamoxifen induced tumours in the livers of healthy female rats in relation to dose, with the highest dose group suffering the greatest incidence, severity, and drug-related mortality (Gary et al., 1993; Greaves et al., 1993; Hirsimäki et al., 1993). The tumour-promoting effect of tamoxifen in the livers of rodents could be partly attributed to its genotoxicity. Studies have shown tamoxifen treatment caused a dose-related increase in DNA adducts in rat liver (Divi et al., 2001; Li et al., 1997). Consistently, in a mouse model, tamoxifen administered orally or subcutaneously for 7 days resulted in DNA adducts in the livers (Hellmann-Blumberg et al., 2000). These authors found that the level of DNA adducts formed was dependent on the dose of tamoxifen tested, 50, 100 and 200 mg/kg; these doses coincide with the tamoxifen dose range tested in the current study. A study investigated the effect of tamoxifen in *p*-dimethylaminoazobenzene (DAB) induced hepatocarcinogenesis in mice (Caballero et al., 2001). It was found tamoxifen treatment in addition to DAB resulted in solid tumour growth whereas the control treatment (DAB) only showed hyperplasia of liver cells. Similarly, the current study also found a tumour-promoting activity of tamoxifen in some mice inoculated with 4T1 cells.

Furthermore, when compared to livers of normal mice and Control mice, 5 out of 12 livers obtained from tamoxifen-treated mice (Tam-IP, Tam-200 and Tam-600) showed abnormal fatty changes. This finding is in good agreement with one study, which reported that oral administration of tamoxifen by gastric intubation at 200 mg/kg daily resulted in hepatic steatosis on Day 12 and 28 (Larosche et al., 2007). As shown in Figure 5.15 and Figure A21 (Appendix), histological examination revealed remarkable accumulation of globular fat droplets (i.e. steatosis), as well as prominent infiltration of leukocytes in the hepatic lobules, indicating a condition known as nonalcoholic steatohepatitis

(NASH). Mouse 5 in the Tam-600 group (highest dose tested in the present study) exhibited the most severe NASH as compared to the other tamoxifen treatment groups; a dose-dependent effect of tamoxifen may exist. Clinically, tamoxifen has been implicated in causing lipemia (Brun et al., 1986). Ogawa's group reported 36.4% of patients (n = 66) undergoing or had undergone tamoxifen treatment showed fatty liver after 3 to 5 years according to their computed-tomography (CT) examination (Ogawa et al., 1998). Later, another medical group reported a similar finding that 43.2% of patients (n = 67) developed a fatty liver condition during the first 2 years of tamoxifen therapy (Nishino et al., 2003). Cases of tamoxifen-related cirrhosis were also reported (Oien et al., 1999). The underlying mechanisms of tamoxifen-induced hepatic steatosis may be related to tamoxifen's predominant antiestrogenic activity. In particular, estrogen was shown to up-regulate gene expression and enzyme activity required for hepatic lipid β-oxidation in mice (Nemoto et al., 2000). Hepatic steatosis in mice was improved by estrogen treatment (Nemoto et al., 2000). Since tamoxifen could block the estrogen action predominantly, it could also impair hepatic lipid β-oxidation. Furthermore, tamoxifen was also shown to inhibit the secretion of triglyceride from liver, contributing to hepatic steatosis (Larosche et al., 2007).

Spleens of 4T1 tumour-bearing mice were strikingly larger than normal spleens (Figure 5.6), and this visual observation parallels the spleen weight results (Figure 5.7). Examination of the spleen H&E sections of these 4T1 tumour-bearing mice revealed a notable increase in immune myeloid progenitors (i.e. precursors of myeloid cells) (Figure 5.16). Furthermore, the red pulp area which was rich in myeloid cells was also expanded. The red pulp is the site of hematopoiesis in mice (Cesta, 2006). Together, these observations indicate the presence of extramedullary hematopoiesis (EMH), specifically excessive production of leucocytes, known as leukemoid reaction. A group of researchers have also reported EMH in the 4T1 model and demonstrated a strong correlation between spleenogaly (enlargement of spleen) and leukemoid reaction induced by 4T1 cells (DuPré & Hunter, 2007). Histological examination of 4T1 primary tumours in the current study also revealed a

notable presence of cells morphorlogically resembling immature myeloid cells, while immunohistochemical tests would be required for confirmation (Figure 5.11). The stimulation of leukemoid reaction by 4T1 cells was associated with the ability of these cells in producing myeloid colony-stimulating factors (DuPré & Hunter, 2007). Clinically, leukemoid reaction occurs in a variety of cancers and has been implicated with a poor prognosis. A recent study, involving 758 patients with solid tumours including breast cancer, has demonstrated that patients with paraneoplastic leukocytosis tend to have poor clinical outcome (Granger & Kontoyiannis, 2009). Accumulation of myeloid cells has been associated with immune dysfunction in patients with considerable load (Ostrand-Rosenberg & Sinha, 2009). Indeed, the immune system has been implicated as a double-sided sword in cancer. Supposedly, leucocytes play a protective role by eliminating cancerous cells. Unfortunately, multiple lines of evidence point to the existence of the opposite action of immune cells (Ostrand-Rosenberg & Sinha, 2009; Qian & Pollard, 2010). For instance, immature myeloid cells are reported as potent inhibitors of T-cell-mediated anti-tumour immunity in patients and animal models.

One of the key observations of this study, which had not been reported previously, concerned a stimulatory effect of tamoxifen on spleen metastasis of breast cancer cell. It is noteworthy that compared to Control, tamoxifen-treated mice had more severe 4T1 metastasis in spleens (Figure 5.9, 5.16 & 5.17). Furthermore, a dose-dependent effect of tamoxifen exists: Tam-600 showed notably greater severity of spleen metastasis than the Tam-200 and Tam-IP groups (Figure 5.9 & 5.16). Spleen sections of Control mice showed metastasis, however, there was no tumour mass found attached to the control spleens. In contrast, several tumour masses were found attached to spleens of 1 to 4 mice of the tamoxifen treatment groups (Figure 5.17). The Tam-600 group (4/5) had a higher incidence of tumours attached to spleens, compared to the Tam-200 (2/4) and Tam-IP (1/3) groups. Moreover, the size of attached tumours was larger in the Tam-600 group than that of the Tam-200 group. Overall, it was found that the group receiving the highest dose suffered the greatest severity of spleen metastasis, indicating a potential dose-dependent, stimulatory effect of

tamoxifen on the metastasis of 4T1 cells to the spleen. The majority of tamoxifen-treated mice (9 out of 12) exhibited more severe metastasis (within the spleens and/or having tumour masses attached to its). Five out of twelve tamoxifen-treated mice also showed more severe spleenogaly (Figure 5.6, spleen hypertrophy) than 4T1 controls, which could suggest greater severity of leukemoid reaction.

It is possible that tamoxifen stimulated growth of 4T1 cells which produce a greater leukemoid reaction in the spleen. Conversely, it is also possible that tamoxifen stimulated the production of myeloid cells, which subsequently enhance the metastatic capacity of 4T1 cells. This notion is supported by histological examination in the current study. Specifically, myeloid cells were found abundantly in the blood vessels which connect the spleen and mass attached to it, as well as in the blood vessels of mass within the spleen (Figure 5.18). In parallel, in liver specimens in which tumour-promoting activity of tamoxifen was observed, there was also an omnipresence of myeloid cells in the liver blood vessels and sinusoids, as well as in the tumour mass attached to the organ (Figure A22, Appendix). In severely invaded mediatinal lymph nodes obtained from tamoxifen-treated mice, hematopoietic stem cells (myeloid or lymphatic origin) were also found abundant in the lymph node. Note the abundance of myeloid cells in the subcapsular sinus (Figure A22, Appendix), a main channel by which cells migrate into the organ. These observations might suggest that myeloid precursor cells could have played an important role in intravasation and survival of 4T1 cells in lymph and blood vascular system. In the literature, a preliminary study has suggested that leucocytes could work as a carrier for cells to migrate through the endothelial barrier into the blood stream (Wu et al., 2001). Recent studies have demonstrated various possible mechanisms by which immune cells can potentiate metastasis of cancer cells, including producing proteases matrix MMP, growth factors that turn cancer cells into more invasive phenotypes, and increasing survival of cancer cells by suppressing immune functions (Erez & Coussens, 2011; Kusmartsev & Gabrilovich, 2002; Ostrand-Rosenberg & Sinha, 2009). Therefore, it is possible that these tamoxifen-stimulated myeloid cells have stimulated metastasis and

growth of 4T1 tumours observed in the current study. It is noteworthy that tamoxifen has stimulated more growth in the major sites of leukemoid reactions, i.e. spleen, liver and lymph node, compared to lung, which is a less common site for extramedullary hematopoiesis (Wang & Darvishian, 2006). This observation again supported the notion that leukemoid reaction or specifically myeloid cells may be a key mechanism by which tamoxifen enhances metastasis and tumour growth in some mice. Future animal study could also examine bone marrow as another tissue to test the hypothesis of the role of myeloid cells and leukocytosis in metastatic capacity.

5.5 Conclusions

A highly metastatic breast cancer model was successfully developed involving injection of 4T1 cells into BALB/c mice. Overall, tamoxifen treatment of these mice resulted in worse health scores in some experimental mice, and increased severity of 4T1 metastasis, based on the histological examinations of their lymph nodes, lungs, spleens and livers. This thesis is the first report of stimulatory effects of tamoxifen in spleen, and mediastinal lymph node *in vivo*. The tumour-promoting activity of tamoxifen demonstrated in the current study may correlate with the clinically observed tumour flare discussed earlier (Chapter 2). The current work also delivers an important message: tamoxifen's failure in treatment may be partly contributed by leukemoid reaction, which was induced by the drug. This is a new hypothesis arising from this thesis that has not been reported in the literature.

Chapter 6 Overall discussion and future directions

6.1 Paradoxical effects of tamoxifen on MCF-7 and T-47D cell proliferation; estradiol dependency of tamoxifen action

6.1.1 Key findings & future direction

Tamoxifen was inhibitory to MCF-7 cell proliferation at high dose (10^{-5} M) but stimulatory at 10^{-8} to 10^{-6} M. Further experiments described in Chapter 2 demonstrated that the antiproliferative action of tamoxifen was dependent on the dose of tamoxifen and estradiol. For instance, the effect of tamoxifen at doses $<10^{-7}$ M is variable when estradiol is present at $<10^{-8}$ M. This could be of clinical significance since physiological estradiol concentrations in women are normally $\leq 10^{-8}$ M (Fan et al., 2009). The most widely prescribed tamoxifen treatments of 20 to 40 mg daily result in a serum tamoxifen level ranging from 10^{-8} to 10^{-6} M (Decensi et al., 2003; Etienne et al., 1989; Lien et al., 1989; Lien et al., 1991). These *in vitro* observations may correlate with the clinically observed side effect, tumour flare (increase in tumour size or numbers, worsening of disease), which occurs occasionally in some patients.

Nevertheless, data and conclusion reached in current study, regarding the estradiol dependency of tamoxifen, may be limited by a lack of full range of estradiol concentrations tested in combination with tamoxifen. Moreover, there are major differences between cell culture and *in vivo* systems; extrapolation of *in vitro* data to clinical observations may not be appropriate. Further systematic *in vivo* investigations could be conducted to test the following hypothesis developed from findings of Chapter 2:

- **H_{new1}**: Interaction between tamoxifen dose and physiological estradiol concentration determines treatment outcome.

In order to test this hypothesis, there could be two strategies:

1) To conduct animal studies involving a range of different tamoxifen doses. Parameters to be measured may include circulating concentrations of tamoxifen and estradiol, and treatment outcomes (i.e. remission, resistance, stimulation of tumours, disease progression).

2) To conduct a prospective study over a 5-year period to obtain relevant data from breast cancer patients treated with standard tamoxifen regimes, i.e. circulating estradiol and tamoxifen levels would need to be measured and correlated with treatment outcomes as above to investigate the effects of tamoxifen and potential interaction between estradiol and tamoxifen.

Women with ovarian ablation (OA) have a very low serum estradiol concentration (10^{-11} M). If tamoxifen action is truly dependent on estradiol concentration *in vivo*, then a combination of OA and tamoxifen may be deleterious. A randomised trial revealed oophorectomy alone gave a better response rate compared to the treatment of combining oophorectomy and tamoxifen (46.6% vs. 11.1%) (Boccardo et al., 1994). Various guidelines regarding whether oophorectomy should be combined with tamoxifen are equivocal and confusing to readers (Goldhirsch et al., 2011; Griggs et al., 2011). Results described in the current thesis suggest that more research is required to investigate the effect of combined treatment of oophorectomy (i.e. very low estradiol levels) and tamoxifen versus either treatment alone. One strategy could be using animal models that mimic the OA condition in women (e.g. using ovarectomised rodents). The study should compare the effects of OA alone *vs.* tamoxifen alone (several doses) *vs.* combined treatment of OA and tamoxifen in the treatment of breast cancer. The parameters and outcome measures include circulating concentrations of tamoxifen, estradiol, and treatment outcomes (e.g. remission, stimulation of tumours).

6.1.2 Individualised therapy: Use of MTT to study preclinical drug sensitivity or resistance

A panel of leading breast cancer researchers conducted a gap analysis to determine which areas need to be targeted in order to produce the greatest impact on patients (Thompson et al., 2008). One of the major problems or gaps they identified is to determine which patients will benefit from a particular treatment in order to provide individualised therapy. Prescription and optimisation of treatment is still based on a trial and error approach. Using the MTT assay, the current thesis achieved new data as well as data consistent with the literature for tamoxifen and estradiol. This raises a question: apart from being a useful research tool, could MTT also be a practical approach to test preclinical drug sensitivity in hospitals? Indeed, this notion is supported by the work published by two medical groups. A team of medical researchers at Pembury Hospital (UK) who have worked in the field of drug resistance reported their experience and results in tailoring chemotherapy to their cancer patients (Sargent, 2003). Prior to administration of treatment to the patients, they commenced MTT assay, involving incubation of fresh tumour cells of patients with certain drugs for 48 to 96 hours followed by re-incubation with MTT for 4 hours (similar to protocol used in the current thesis). Encouragingly, the researchers have found a highly significant correlation ($P < 0.0001$) between *in vitro* and *in vivo* outcomes for ovarian cancer and acute myeloid leukaemia with predictive accuracy of 80 to 90%. Five-year survival rate is higher when patients were treated with drugs found to be effective in MTT compared to the drugs found not effective in MTT (Sargent, 2003).

Another group of medical researchers investigated the potential use of MTT in predicting the response of breast cancer patients to chemotherapy drugs (Xu et al., 1998). Their protocol involved 72 hours incubation of samples with drugs. When patients were treated with drugs found to be effective *in vitro*, 77% of them (56/73) responded to the medicine, similar to the finding of Sargent et al. (2003) for ovarian cancer. These two medical teams have concluded that MTT is a technique of significant clinical relevance for testing preclinical

chemosensitivity of anti-tumour drugs, which would help clinicians choose or customise the right drug for individual patients. A future study could be conducted to determine how well the MTT results correlate with clinical outcomes for breast cancer drugs (endocrine therapy or chemotherapy). A lack of standards for the use of MTT partly explains why this assay has yet to be adapted in routine clinical application (Hatok et al., 2009). Hence, inter-laboratory efforts could be needed in the future to establish approriate standards (MTT procedures and criteria to determine drug responses). MTT is a reproducible, simple and economical assay to adopt, and if it is proven useful by more studies in predicting clinical outcomes, it could have a significant impact globally in individualised treatment of breast cancer, i.e. higher response rates to treatments.

When testing the preclinical drug sensitivity for tamoxifen, the serum estradiol concentration of the patient should also be taken into account because of the estradiol dependency of tamoxifen (Chapter 2). Serum estradiol concentration in the patient should be measured, which would then be incorporated into the MTT assay (i.e. adding estradiol to test cell culture) for testing the effectiveness of tamoxifen over a range of doses *in vitro*. The most effective tamoxifen dose could then be administered to patients. This future work may be useful to test the hypotheses::

- H_{new2}: MTT data correlate well with the clinical outcome for tamoxifen

6.2 PPE/WPE inhibits MCF-7 and T-47D cell proliferation without paradoxical effects and estradiol dependency, and works in synergy with tamoxifen

Unlike tamoxifen, PPE and WPE were inhibitory to MCF and T-47D cell growth regardless of estradiol concentration (10^{-11} to 10^{-6} M), i.e. no estradiol dependency was observed (Chapter 4). Furthermore, PPE (30-40 µg/mL) treatment attenuated stimulation induced by tamoxifen at 10^{-7} M, resulting in overall inhibitory activity (Figure 4.7). Consistently, cell-cycle analysis revealed

a potential combined effect between PPE (30 μg/mL) and tamoxifen (10^{-7} M) in modulating the S and G2/M phase. PPE above 250 μg/mL was inhibitory to two highly aggressive breast cancer cell lines, MDA-MB-231 and 4T1. In summary, the effects of PPE differ from tamoxifen and estradiol in two important aspects:

1) Tamoxifen or estradiol alone exhibited paradoxical effects in MCF and T-47D cell proliferation, whereas PPE did not;
2) Tamoxifen exhibited estradiol dependency for its antiproliferative action, whereas PPE did not.

Nonetheless, the current findings are limited by a lack of toxicity study regarding the effect of PPE or WPE on normal cells. Therefore, future work could include an MTT assay with normal breast cancer cell lines or *in vitro* genotoxicity test, for instance using comet assay, which measures the extent of DNA strand breaks (damage) in eukaryotic cells given a treatment. A short-term toxicity study using rodents, involving several doses of PPE and duration of 4 weeks, could be carried out to determine any toxic effects of PPE *in vivo*.

If PPE is proven to have a favourable toxicity profile, then it could be worth testing the dose-response effect of PPE in prevention or treatment of breast cancer using the 4T1 model developed in Chapter 5. Specifically, the outcome measures should include: tumour development, observation of health conditions and organ-specific evaluation (measurement of weight and size, and determination of metastasis by histological examination). This study would be an approach to test the following hypothesis developed from the *in vitro* findings of Chapter 4:

- **H_{new3}**: PPE alone may have a preventive or therapeutic role in breast cancer, or when used in combination with tamoxifen may achieve desirable treatment efficacy and reduce the risk of tamoxifen-induced stimulation.

Using UHPLC-MS analysis, a total of 31 compounds have been identified and quantified in PPE and many of these appear to be present in the concentration range known to induce physiological benefits. As mentioned in Literature Review (1.9.1), method of preparation could affect phytochemical profile of potatoes. Current study produced PPE from fresh raw potatoes. Future studies could include extract samples made from potatoes subjected to various cooking methods (e.g. baking, frying) to compare potential difference in phytochemical profiles and bioactivities (e.g. cell growth).

6.3 Directions to study molecular actions of PPE: mechanisms involved in tamoxifen-induced stimulation or resistance

PPE alone (50 – 500 μg dry extract/mL) was inhibitory to cell proliferation of both ER-positive and ER-negative breast cancer cells (Chapter 4). The combined treatments with PPE and estradiol or tamoxifen were always inhibitory, even when the latter test agents were present at stimulatory doses in ER-positive breast cancer cells. The molecular mechanisms underlying these observations are unclear. The current knowledge about estradiol actions and tamoxifen resistance in the ER signalling pathway may provide some potential future research directions.

Classical genomic pathway

In the classical genomic pathway, estradiol binds to ER in the nucleus. The activated ER forms a complex with transcriptional coactivators. This complex then binds directly to the promoter region of target genes at estrogen-response elements (ERE), which leads to the transcription of estrogen-regulated genes. One of the known mechanisms of tamoxifen resistance is that tamoxifen also recruits coactivators, which activate genes that upregulate cell proliferation, as depicted in Figure 6.1 (Osborne & Schiff, 2005).

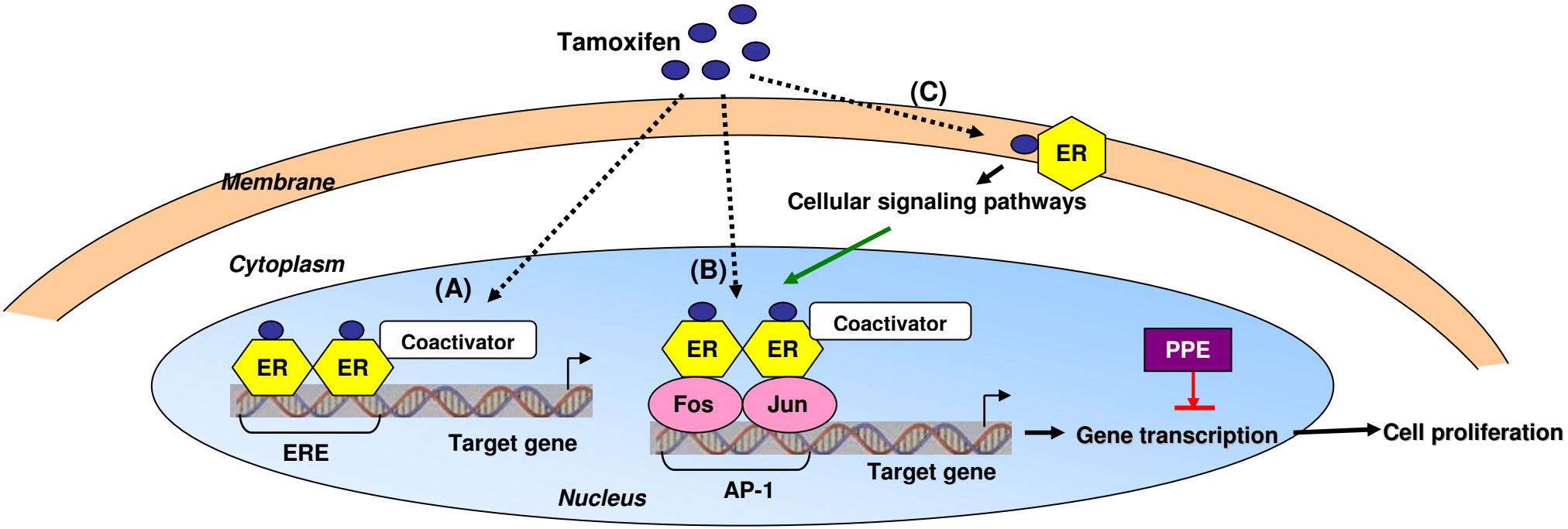


Figure 6.1 **Mechanisms of tamoxifen resistance in estrogen receptor signalling.**
There are at least 3 possible pathways by which tamoxifen (Tam) may trigger estradiol signalling, causing transcription of the gene and cell proliferation (Osborne & Schiff, 2005; Schiff et al., 2003). (A) Classical genomic pathway: Tam-bound estrogen receptor (ER) recruits transcriptional coactivator, and then binds to estrogen response element (ERE) in the promoter region of target gene; (B) Non-classical genomic pathway: Tam-ER-coactivator complex bound to transcriptional factors (Fos, Jun), which bind to activator protein-1 (AP-1) responsive element in the promoter region of target gene; (C) Tam binds to membrane ER, inducing cellular signalling pathways such as mitogen-activated protein kinase, which enhances the activity of ER and coactivator thus nuclear ER signalling. It is hypothesized that purple potato extract (PPE) may block gene transcription by interfering with one or more of these pathways, thus inhibiting the proliferation of breast cancer cells.
(Keys: **--->** entering into cell membrane or nucleus; → activates; → enhances nuclear ER signalling; → down-regulate)

Non-classical genomic pathway (ERE independent)

In the non-classical genomic pathway, the activated estradiol-ER complex binds indirectly to a target gene, via tethering with transcription factors Fos and Jun, at activator protein-1 (AP-1) response elements, i.e. the promoter region of the gene, which induces gene transcription (Figure 6.1). Studies on clinical samples of breast tumours from patients with tamoxifen resistance revealed an elevated AP-1 transcriptional activity (Johnston et al., 1999; Schiff et al., 2000). In MCF-7 cell culture, tamoxifen resistance has been associated with up-regulated AP-1 activity (Dahlman-Wright et al., 2012).

Membrane-initiated signalling pathway

Data suggesting the localization of a small pool of ER in the cell membrane has accumulated (Levin, 2009; Osborne & Schiff, 2005; Pedram et al., 2006; Yager & Davidson, 2006a). A study identified estradiol-binding ER isolated from MCF-7 cell membrane as classical ERα, the same as the nuclear ER isolated from the same cell (Pedram et al., 2006). Estradiol signalling can also be mediated by membrane ER, called the membrane-initiated steroid signalling pathway (MISS) (Osborne & Schiff, 2005). In this pathway, like estradiol, tamoxifen can also bind to ER, activating cellular signalling pathways, which in turn up-regulates cell proliferation and down-regulates apoptosis (Figure 6.1) (Osborne & Schiff, 2005; Schiff et al., 2003). The agonist effect of tamoxifen in the MISS pathway is more prominent when there is an over-expression of cell-surface receptors (Osborne & Schiff, 2005).

Overall, estradiol and tamoxifen actions in the ER signalling pathway are extremely complex and yet to be fully elucidated. Figure 6.2 summarizes some key knowledge derived from the literature. A hypothesis was derived based on the literature and findings of the current thesis, which might be worth future investigation:

- **H_{new4}**: Tamoxifen activates estradiol signalling and expression of genes involved in MCF-7 cell proliferation at doses of 10^{-8} M to 10^{-6} M, in the absence of estradiol or when estradiol is present at < 10^{-8} M, whereas PPE (50 – 500 µg/mL) downregulates the expression of these genes, independent of estradiol or ER status of the breast cancer cells.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis could be conducted to measure gene expression in MCF-7 cells after 72 hrs treatment with tamoxifen, and/or PPE, and/or estradiol. Target genes may include insulin-like growth factor receptor 1, c-Myc, cyclins D1 and cyclin E (Butt et al., 2005).

6.4 Insights from 4T1 metastatic breast cancer study

The current thesis successfully established an animal model of breast cancer metastasis using the mouse 4T1 mammary carcinoma cells. The tumour cells metastasised to distant sites including livers, lungs and spleen, mimicking the advanced stage of breast cancer. Several parameters, however, were not measured in this study, including serum concentrations of tamoxifen and estradiol in mice. ER status of 4T1 primary and metastatic tumours was unclear. These parameters should be included in a future study focussed on similar objectives to determine potential interactions among them. Since it was a pilot trial and involved a highly aggressive form of cancer, it was not ethical to use a large number of animals. Therefore, only 5 mice were used per treatment. This small sample size was not substantial for statistical significance analysis, which was a limitation of the current study. Nevertheless, there are some insights gained from the 4T1 breast cancer study and new hypotheses evolved accordingly, which may be worth future clinical investigation.

6.4.1 Leukocytosis as a useful marker for metastasis and monitoring disease progression

Currently, risk assessment for metastasis and treatment decisions are based on tumour size, tumour burden, lymph node status, hormonal receptors and human epidermal growth factor receptor type 2 (HER2) status, detailed physical examination for detecting metastasis (e.g. magnetic resonance imaging, Computerized Tomography, ultrasound, X-ray), and patient-related factors (e.g. menopause, history of disease, response to treatment). Though the risk assessment seems comprehensive, it is still far from being satisfactory. To quote what is stated by Thompson *et al.* (2008), one of the major gaps in breast cancer therapy is *"We cannot determine who goes on to develop advanced disease"*. There is an urgent need to establish new and precise prognostic markers, to identify those with high propensity to metastastic disease and allow more intensive disease monitoring (e.g. follow-up) and individualised treatment, right from the primary stage of cancer.

One key observation of Chapter 5 is the remarkable increased production of myeloid cells or leukocytosis in organs affected by 4T1 metastasis. It was found that the more severe the metastasis, the more severe leukocytosis appeared to be. Clinically, severe leukocytosis occurs in breast cancer and has been implicated with poor prognosis (Granger & Kontoyiannis, 2009). A study investigated leukocytosis in a variety of non-hemotological malignancies and has revealed a direct association between leukocytosis and incidence of metastasis as well as shorter survival time (Shoenfeld et al., 1986). The mechanisms by which leukocytes promote metastasis have been reviewed in detailed elsewhere (Erez & Coussens, 2011). Recently, it has been hypothesized that cancer is a natural wound healing-related process (Meng et al., 2012). Wound healing process involves recruitment of leukocytes and other inflammatory factors. It is now known that leukocytes could promote growth and metastasis by several mechanisms such as: i) activation of epidermal growth factor activity which is essential for growth and cell dissemination; ii) secretion of proteolytic enzymes (matrix metalloproteinase, serine protease)

which could enhance the capacity of malignant cells to move through the basement membranes, thus increasing the motility and invasive capacity of the cells, and iii) suppression of anti-tumour immunity (T-cell mediated response). Based on the animal study described here and in the literature, the following hypothesis was developed, which warrants future research:

- **H_{new5}**: Leukocytosis is a useful marker to assess the risk of breast cancer metastasis and monitor the prognosis of patients throughout the course of disease. Total white blood count would be measured in order to detect and monitor leukocytosis and correlate with prognosis.

6.4.2 Insights into risks associated with tamoxifen

6.4.2.1 A possible mechanism of tamoxifen resistance: leukocyctosis

The 4T1 model could be an excellent model to study the mechanisms of tamoxifen failure or resistance in advanced breast cancer. In particular, it was found that compared to control, tamoxifen treatments resulted in more severe leukocytosis concomitant with severe distant metastasis to various organs in some of the animals (Chapter 5). Serious leukocytosis associated with tamoxifen treatment has also been observed in other animal species (Lupu, 2000). Future research may be warranted to test the following hypothesis:

- **H_{new6}**: Tamoxifen fails (drug resistance or stimulation of tumour growth) in some patients because tamoxifen induces excessive leukocytosis which contributes to metastasis. Leukocytosis can be measured by white blood count and diagnostic imaging techniques such as MRI, CT and X-ray are commonly used to detect metastasis. Serum tamoxifen and estradiol concentration need to be measured as well to determine how these parameters may affect leukocytosis and/or metastasis.

6.4.2.2 Second cancer associated with tamoxifen: is there an increased risk of acute myeloid leukaemia and spleen cancers?

Data on the effect of tamoxifen on spleen is scarce. In Chapter 5, tamoxifen was shown to stimulate severe tumour growth in spleens of mice. Spleen health may need to be monitored during and after tamoxifen therapy in routine clinical practice or clinical trials, in order to assess whether spleen cancer could be induced by tamoxifen treatment.

Acute myeloid leukaemia (AML) has been reported in patients during tamoxifen therapy (Yalçın et al., 1997). By analysing 420,076 patients' data, sourced from the Surveillance, Epidemiology, and End Results (SEER) population-based comprehensive cancer database in the United States (http://seer.cancer.gov), a study revealed a marked increased risk of subsequent development of AML among breast cancer survivors (RR:4.14, <50 yrs; RR:2.19, 50-64 yrs) (Martin et al., 2009). The authors commented that the increased risk of AML is attributed to chemotherapy and genetic predisposition. However, it is possible that tamoxifen treatment *per se* may also contribute to the increased risk of AML reported by Martin et al. (2009). Re-analysis of the SEER data may be warranted to stratify the effect of tamoxifen treatment alone on the risk of developing subsequent AML.

6.4.2.3 Hepatic steatosis associated with tamoxifen treatment

Some of liver specimens (42%) obtained from tamoxifen-treated mice showed abnormal fatty changes (Chapter 5). This finding adds to the existing evidence that tamoxifen treatment increases the risk of hepatic steatosis (Larosche et al., 2007; Nemoto et al., 2000; Nishino et al., 2003). It may be useful to regularly assess hepatic steatosis as one of the potential side effects for patients treated with tamoxifen.

6.5 Concluding remarks

Current *in vitro* studies showed that tamoxifen and estradiol had paradoxical effects in ER+ breast cancer cell lines, MCF-7 and T-47D, and the action of tamoxifen might be dependent on estradiol. To the contrary, PPE was always inhibitory to cancer cell growth even in the presence of a stimulatory dose of tamoxifen or estradiol. Animal models could be useful to further test the estradiol dependency of tamoxifen and efficacy of PPE against breast cell proliferation observed in the current thesis. Further animal or clinical studies may be warranted to determine the role of leukocytosis in breast cancer progression, furthermore, to investigate how tamoxifen may cause undesirable stimulation via the induction of leukocytosis.

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Appendices

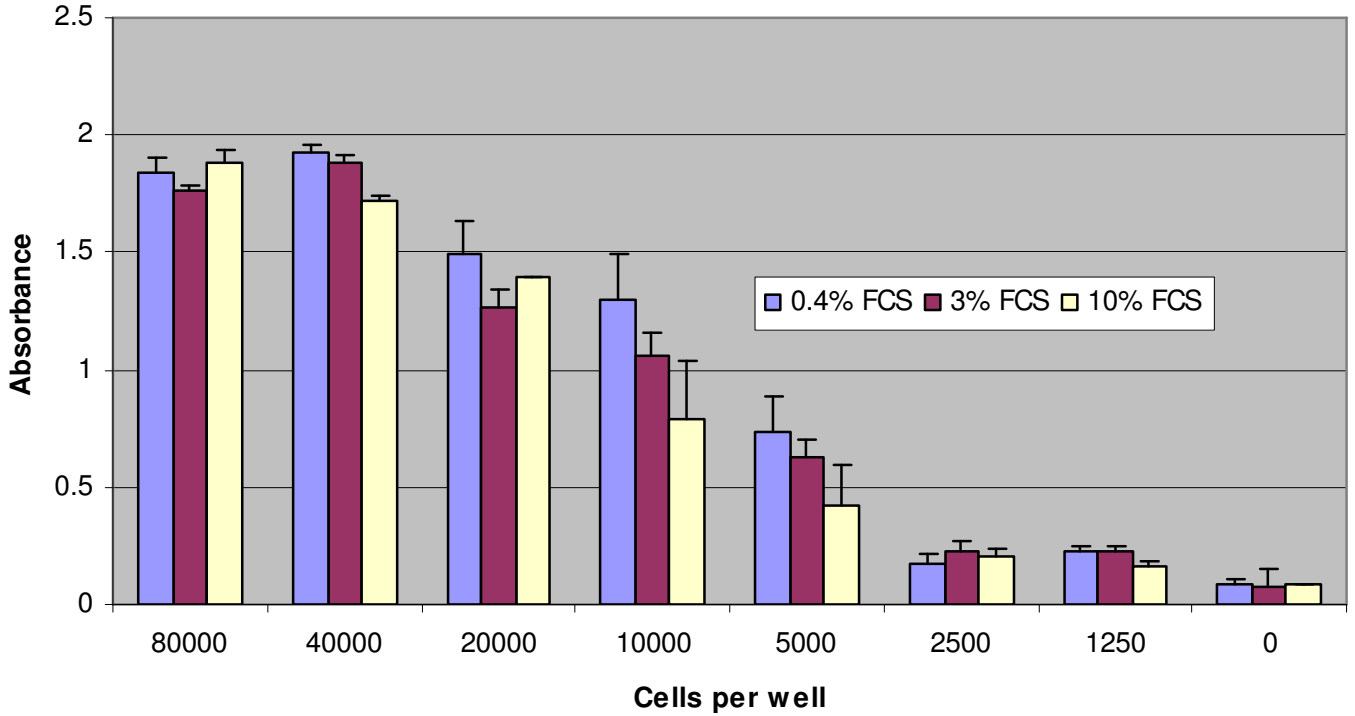


Figure A1 Optimisation of cell number to be plated for MTT assay using MCF-7 cell line

Among the various cell numbers tested (X-axis), 5000 cells per well was found to be the optimum since it was the first cell density that showed differential effect when given different treatments. Hence, 5000 cells per well was chosen as the cell density plated onto the 96-well plates for the MTT procedures conducted.

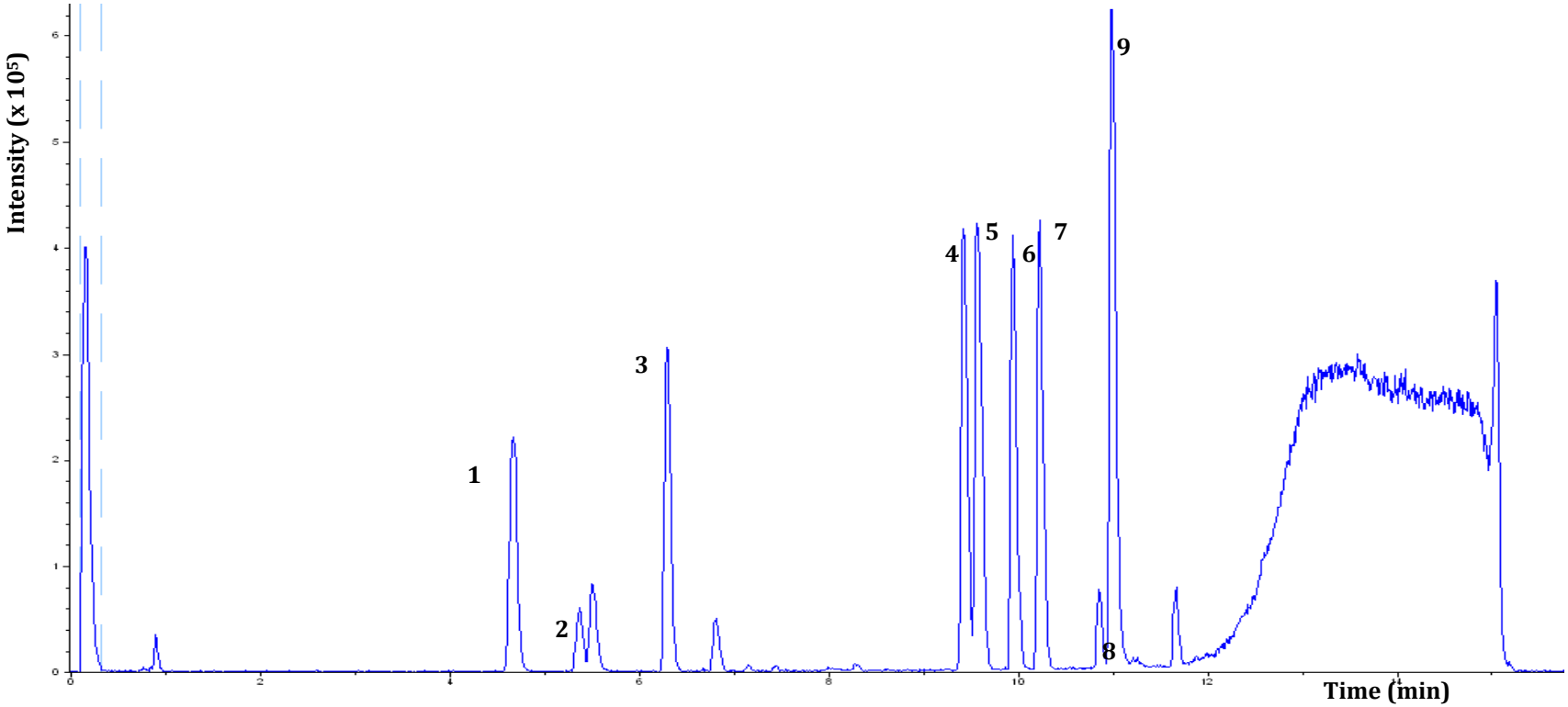


Figure A2 **Base peak chromatogram (BPC) obtained by UHPLC-ESI-TOF-MS for phenolics standards.**
(1) Catechin (2) Chlorogenic acid (3) Epicatechin (4) Quercetin-3-galatoside (5) Quercetin-3-rutinoside (6) Quercetin-3-glucoside (7) Quercetin-3-xyloside (8) Quercetin-3-rhamnoside (9) Quercetin.

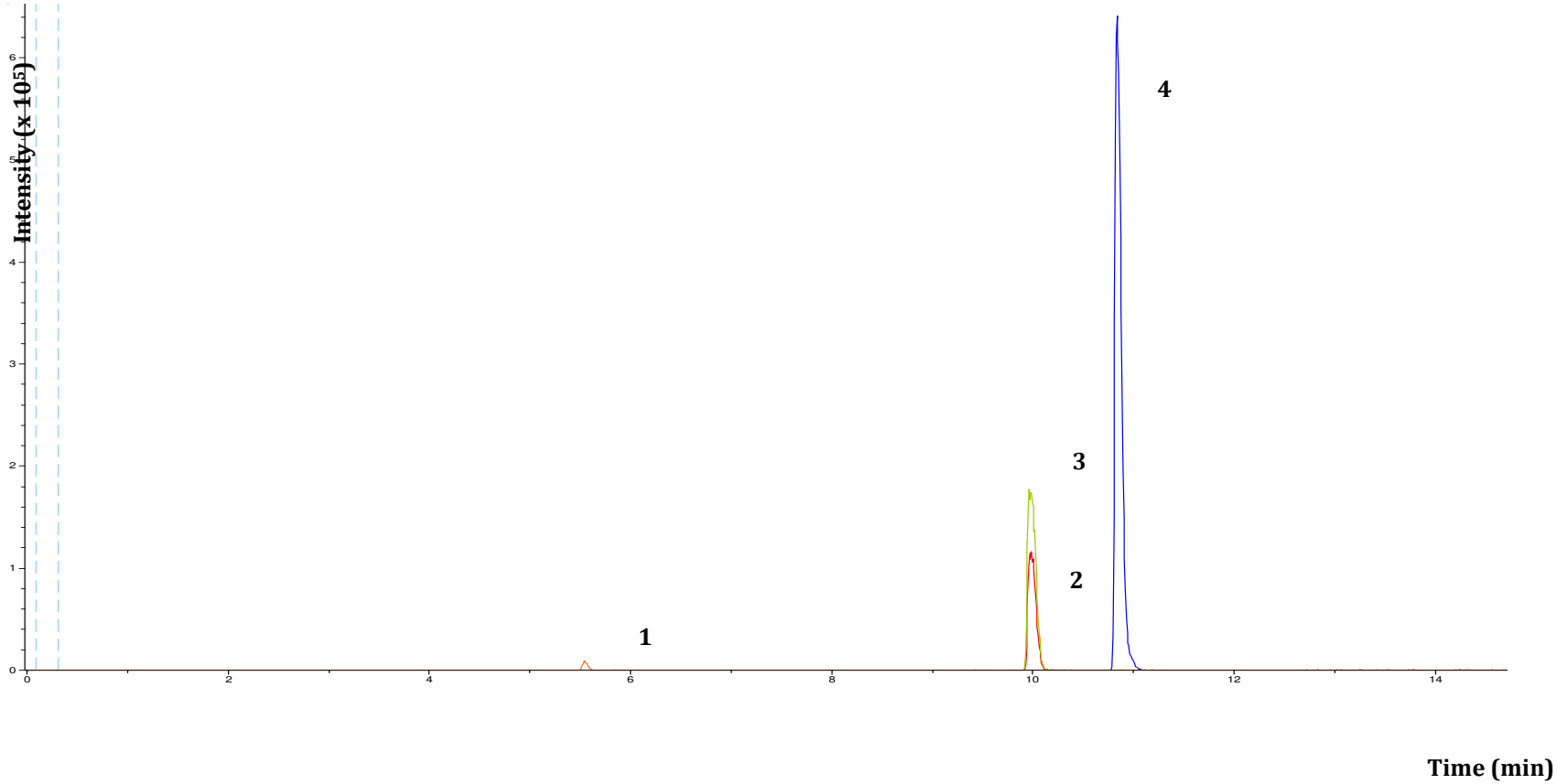


Figure A3 **BPC obtained by UHPLC-ESI-TOF-MS for phenolics and glycoalkaloid standards.**
(1) Caffeic acid (2) Kaempferol-3-rutinoside (3) Kaempferol-3-glucoside (4) α-solanine.

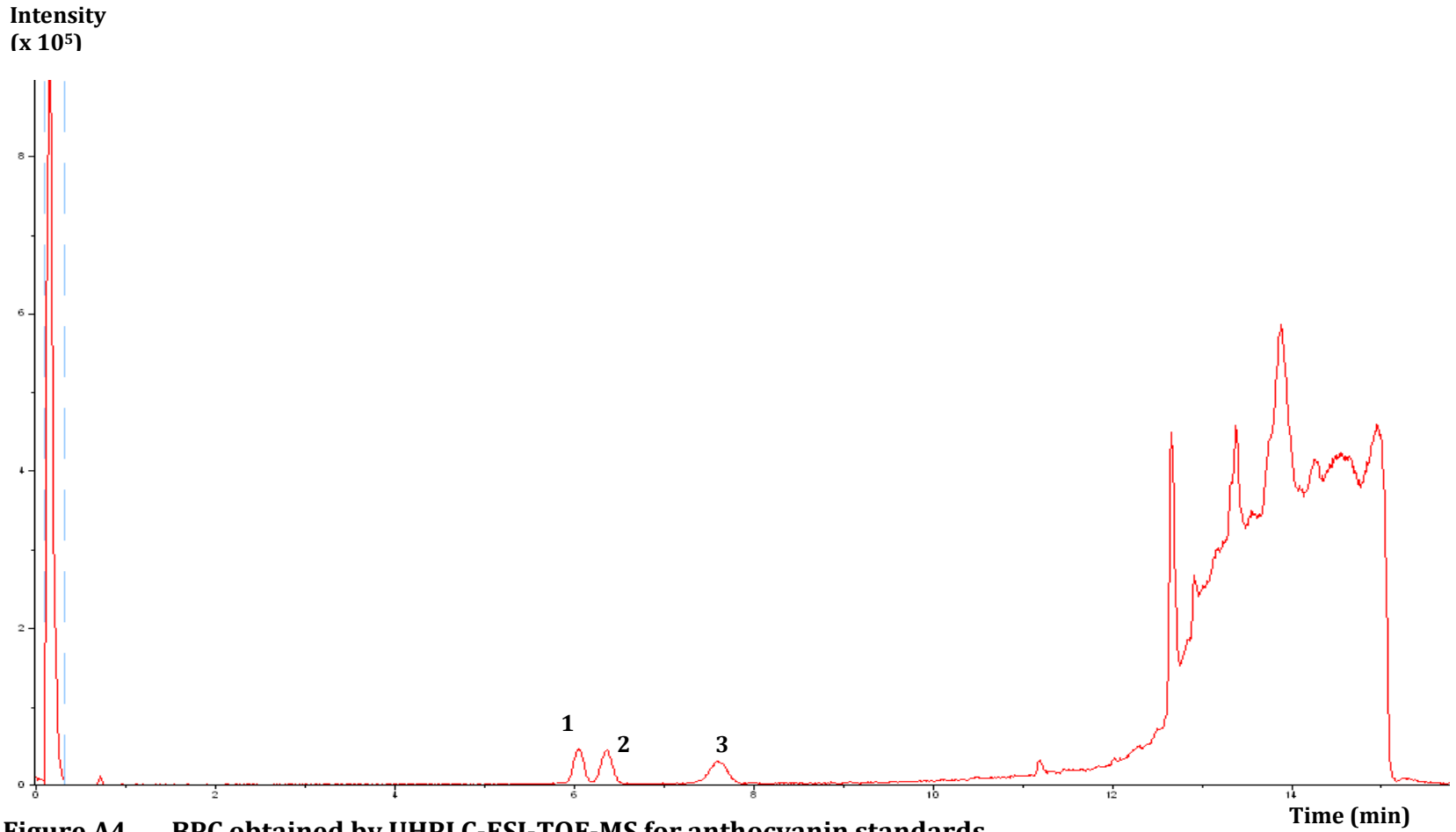


Figure A4 **BPC obtained by UHPLC-ESI-TOF-MS for anthocyanin standards.**

(1) Cyanidin-3-galatoside (2) Cyanidin-3-glucoside (3) Malvidin-3-glucoside.

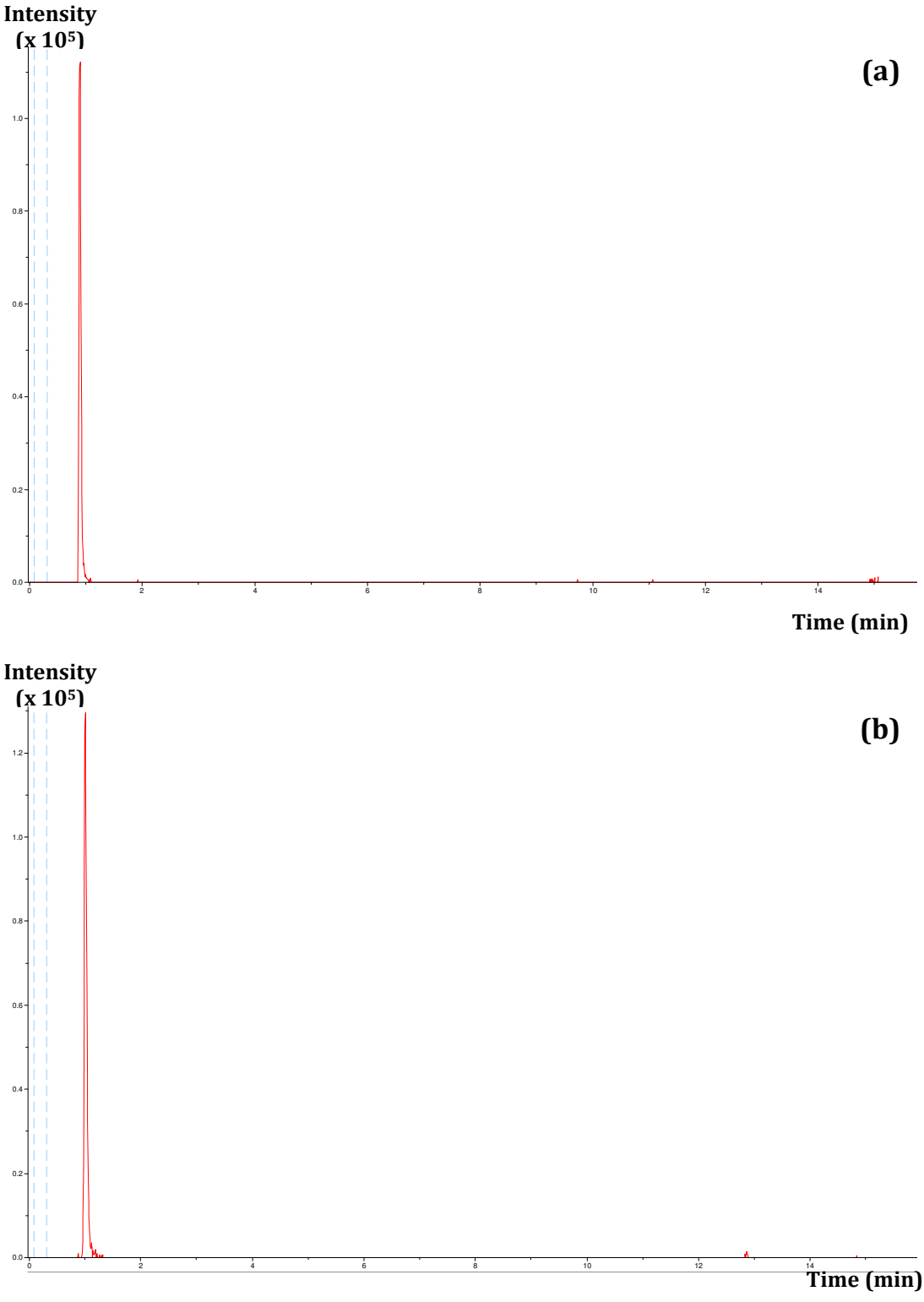
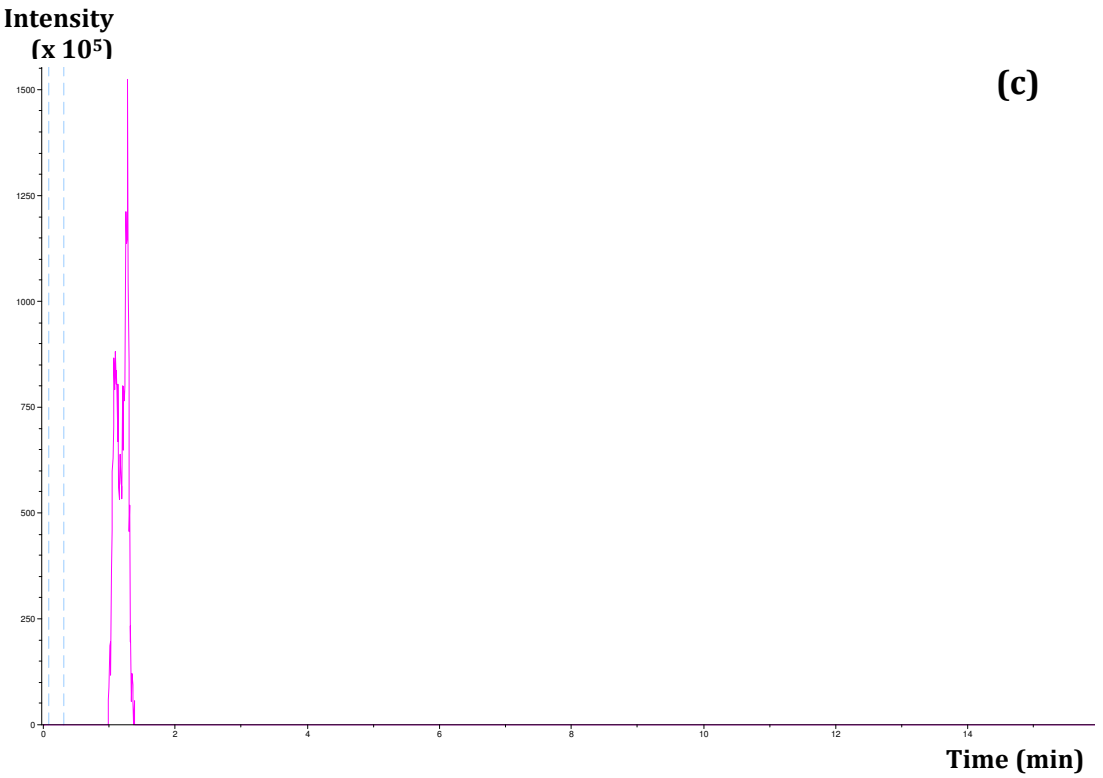


Figure A5 BPC for standards: (a) Quinic acid (b) Ascorbic acid.



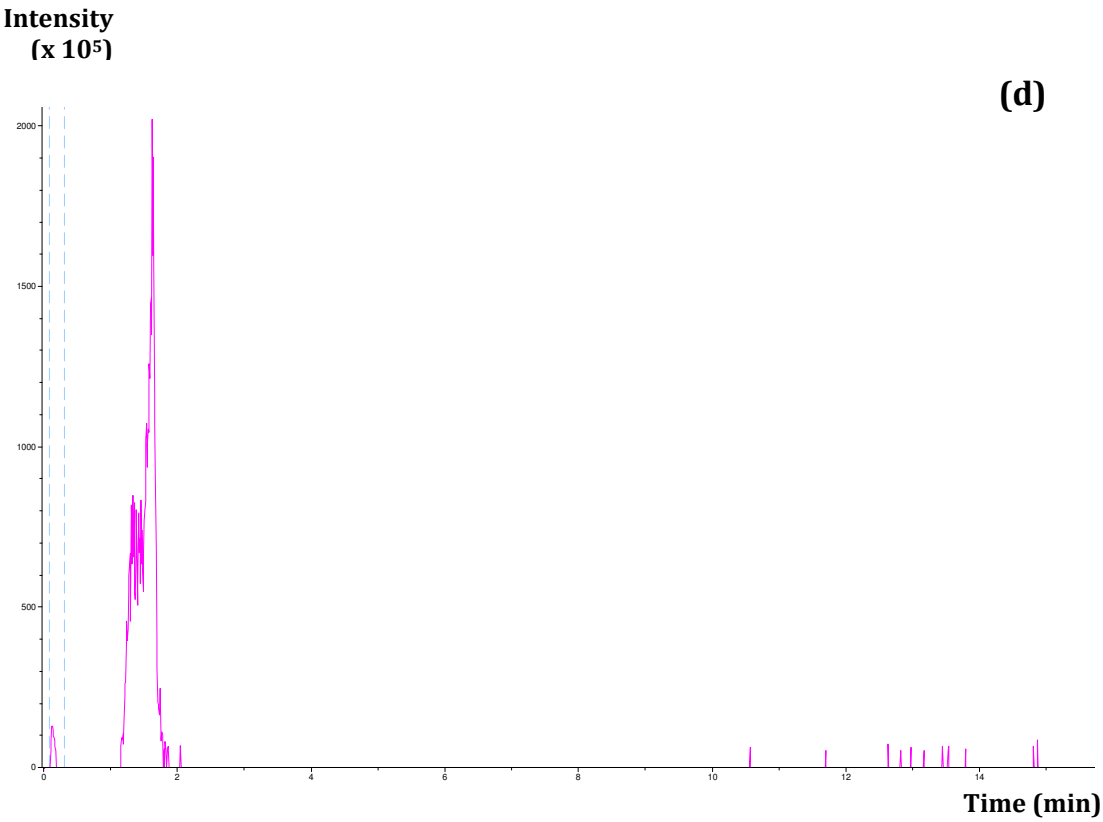


Figure A6 BPC for standards: (c) Citric acid (d) Tyrosine.

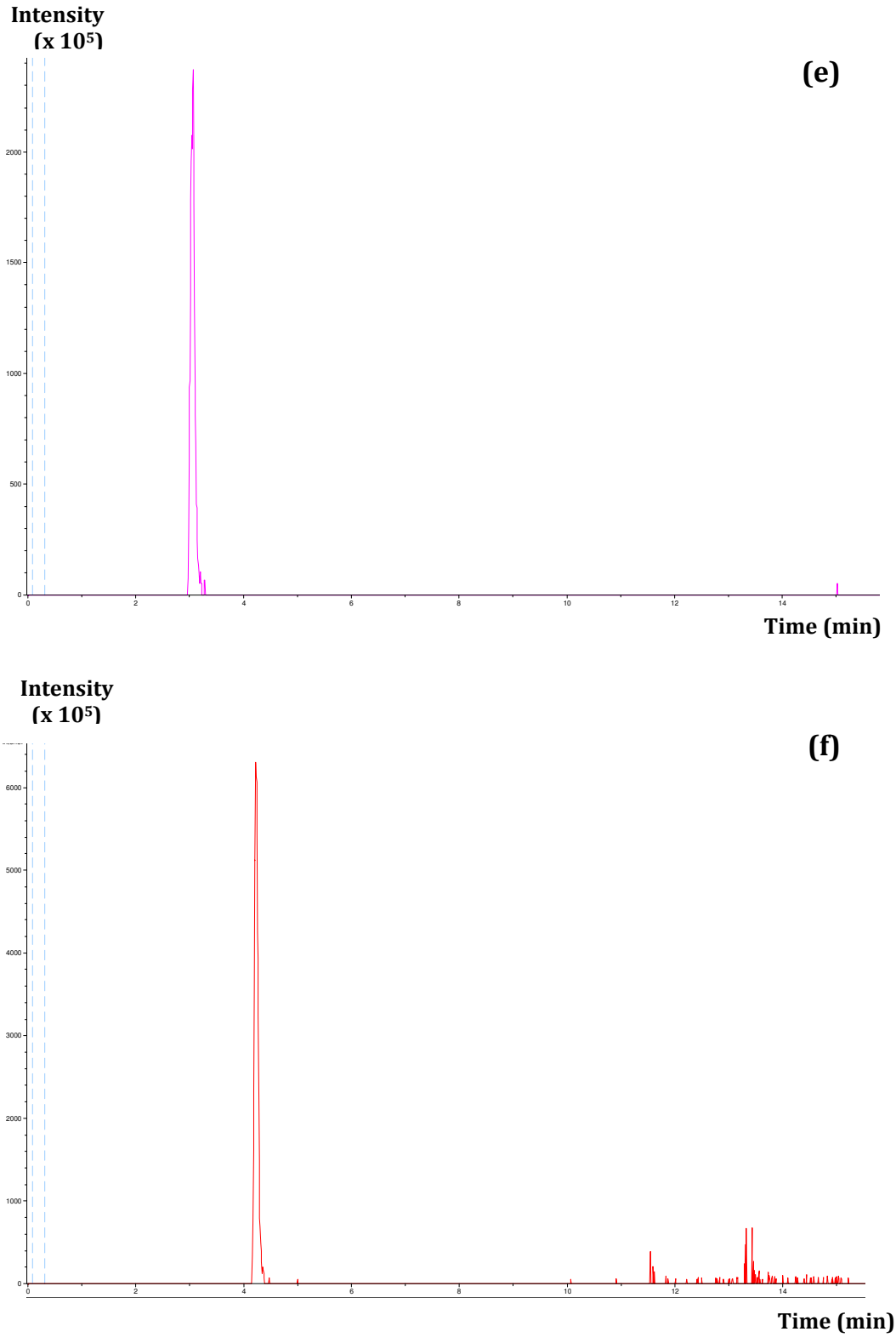
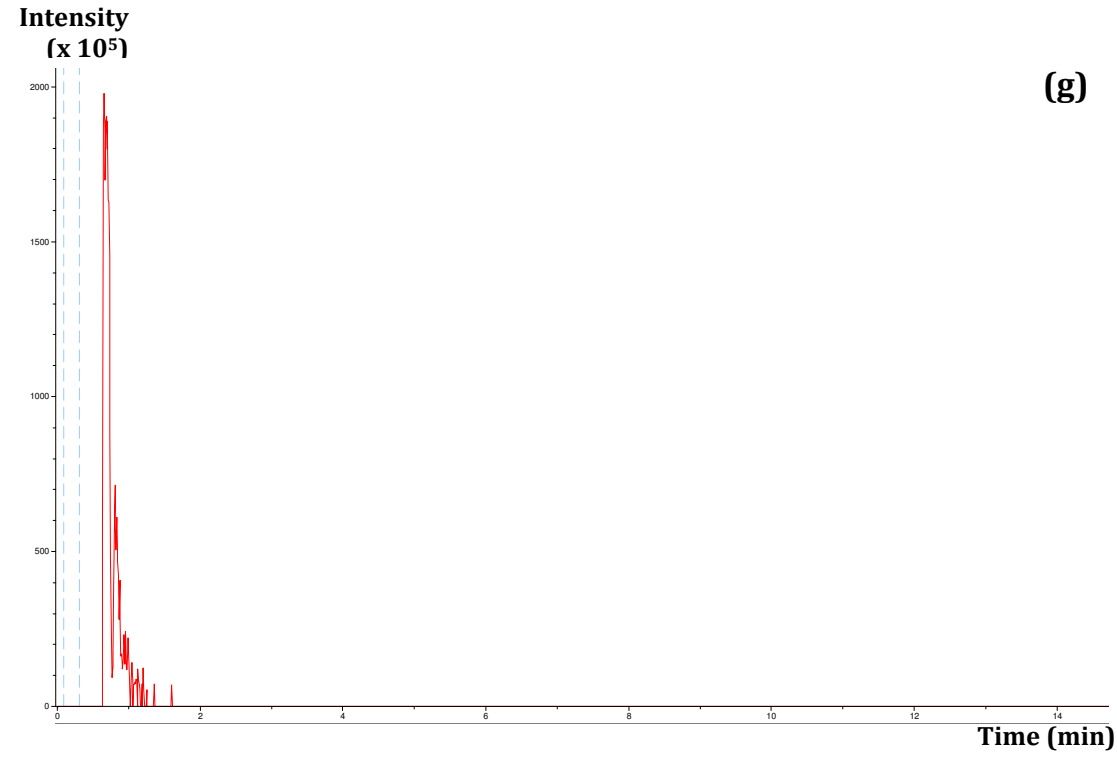


Figure A7 BPC for standards: (e) Phenylalanine (f) Tryptophan.



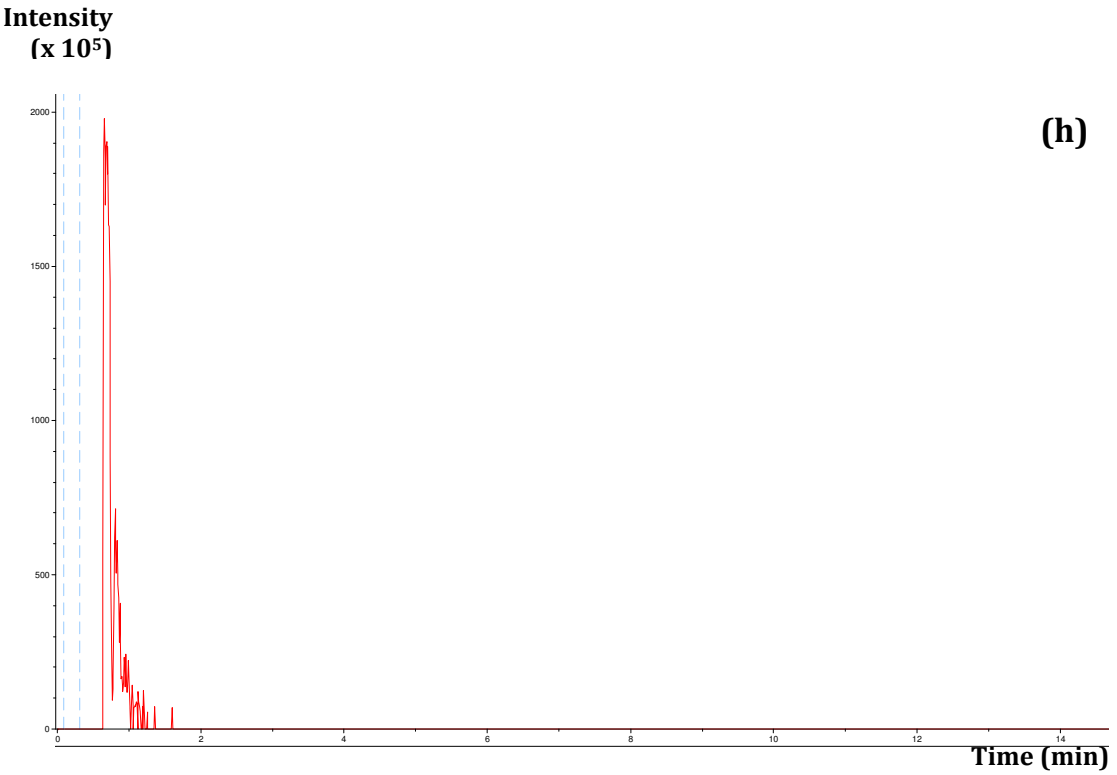


Figure A8 BPC for standards: (g) Spermine (h) Spermidine.

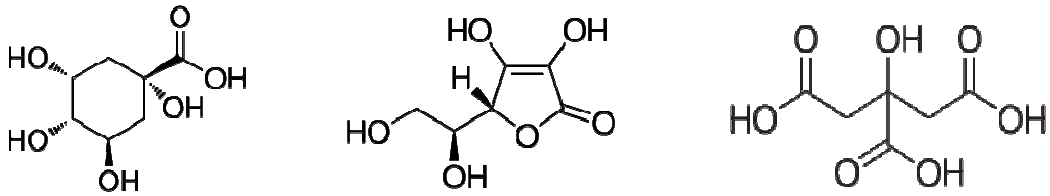
Table A1 Characterization of unknown compounds in PPE & WPE with UHPLC-ESI-TOF-MS.

Peak assignment of the potato extracts (Figure 3.3 & 3.4), UHPLC retention time (RT), ESI-TOF mass-to-charge ratio, chemical formula and quality control parameters (Err & mSigma)

Peak #	Assignment Tentative identification	LC RT ±0.2 min	Measured Mass-to-charge ratio (Negative ion mode: -) (Positive ion mode: +) m/z ±0.01	Formula for the detected molecular ion		Error (mDa)	mSigma
				Formula	Theoretical m/z		
1	Quinic acid	0.93	191.0565 (-)	$C_7H_{11}O_6$	191.0561	-0.4	3.6
2	Ascorbic acid	0.96	175.0253 (-)	$C_6H_7O_6$	175.0248	-0.5	7.2
3	Citric acid	1.27	215.0158 (+)	$C_6H_8NaO_7$	215.0162	0.4	7.5
4	Tyrosine	1.64	182.0803 (+)	$C_9H_{12}NO_3$	182.0812	0.9	7.9
5	Phenylalanine	3.08	166.0859 (+)	$C_9H_{12}NO_2$	166.0863	0.4	12.1
6	Caffeoyl putrescine	3.46	251.1377 (+)	$C_{13}H_{19}N_2O_3$	251.1390	1.4	6.5
7	Caffeoyl spermine	3.64	614.1715 (+)	$C_{26}H_{32}NO_{16}$	614.1716	0.1	15.6
8	Neochlorogenic acid	3.85	377.0828 (+)	$C_{16}H_{18}NaO_9$	377.0843	1.5	21.4
9	Tryptophan	4.25	205.0957 (+)	$C_{11}H_{13}N_2O_2$	205.0972	1.4	12.9
10	3-Caffeoyl-5-feruloylquinic acid	4.36	531.3175 (+)	$C_{27}H_{47}O_{10}$	531.3164	-1.1	6.9
11	Caffeoyl hexose	5.40	341.0874 (-)	$C_{15}H_{17}O_9$	341.0878	0.4	28
12	Chlorogenic acid	5.50	377.0830 (+)	$C_{16}H_{18}NaO_9$	377.0843	1.3	2.4
13	Caffeic acid	5.60	179.0358 (-)	$C_9H_7O_4$	179.0350	-0.8	12.7
14	Crytochlorogenic acid	5.50	377.0843 (+)	$C_{16}H_{18}NaO_9$	377.0843	0.0	17.6
15	Dehydrophaseic acid hexose	5.91	443.1928 (-)	$C_{21}H_{31}O_{10}$	443.1923	-0.5	8

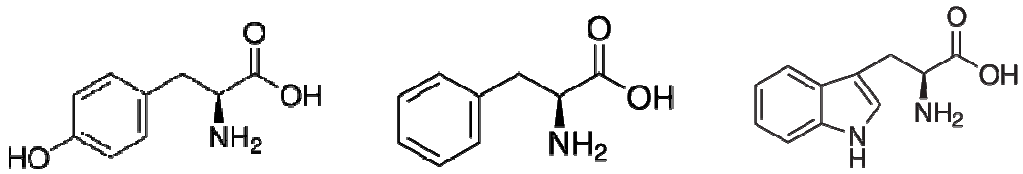
Table A1 ***(continued)***

Peak #	Assignment Tentative identification	RT ±0.2 min	Measured Mass-to-charge ratio (Negative ion mode: -) (Positive ion mode: +) m/z ±0.01	Formula for the detected molecular ion		Error (mDa)	mSigma
				Formula	Theoretical m/z		
16	Bis(dihydrocaffeoyl) spermidine	6.08	474.2569 (+)	$C_{25}H_{36}N_3O_6$	474.2599	3.0	30.0
17	5-feruloylquinic acid	8.27	367.1032 (-)	$C_{17}H_{19}O_9$	367.1035	0.2	0.3
18	Malvidin-3-rutinoside	8.30	640.1998 (+)	$C_{29}H_{35}O_{16}$	640.2000	0.2	3
19	Petunidin-3-caffeoylrutinoside-5-glucoside	8.38	949.2608 (+)	$C_{43}H_{49}O_{24}$	949.2608	-0.9	21.6
20	Delphinidin-3-*p*-coumaroylrutinoside-5-glucoside	8.38	919.2542 (+)	$C_{42}H_{47}O_{23}$	919.2503	-3.9	16.2
21	N^1,N^4,N^{12}-tris(dihydrocaffeoyl) spermine	8.23	695.3642 (+)	$C_{37}H_{51}N_4O_9$	695.3651	0.8	37.1
22	Petunidin-3-*p*-coumaroylrutinoside-5-glucoside	8.70	933.2684 (+)	$C_{43}H_{49}O_{23}$	933.2659	-2.5	4.1
23	Petunidin-3-feruloylrutinoside-5-glucoside	9.01	963.2789 (+)	$C_{44}H_{51}O_{24}$	963.2765	-1.3	6.6
24	Quercetin-3-rutinoside	9.57	609.1466 (-)	$C_{27}H_{29}O_{16}$	609.1461	-0.5	6.4
25	Malvidin-3-*p*-coumaroylrutinoside-5-glucoside	9.60	947.2814 (+)	$C_{44}H_{51}O_{23}$	947.2816	0.1	3.9
26	Malvidin-3-feruloylrutinoside-5-glucoside	9.70	977.2900 (+)	$C_{45}H_{53}O_{24}$	977.2921	2.1	35
27	Petunidin-3-*p*-coumaroylrutinoside	9.97	771.2144 (+)	$C_{37}H_{39}O_{18}$	771.2131	-1.3	11.5
28	Kaempferol-3-rutinoside	10.2	593.1538 (-)	$C_{27}H_{29}O_{15}$	593.1512	-2.6	20.8
29	Chaconine	11.1	852.5136 (+)	$C_{45}H_{74}NO_{14}$	852.5104	-3.2	3.2
30	Solanine	11.1	868.5072 (+)	$C_{45}H_{74}NO_{15}$	868.5053	-1.9	5.5
31	Quercetin dimethyl ether	12.7	329.2322 (-)	$C_{18}H_{33}O_5$	329.2333	1.2	16.9



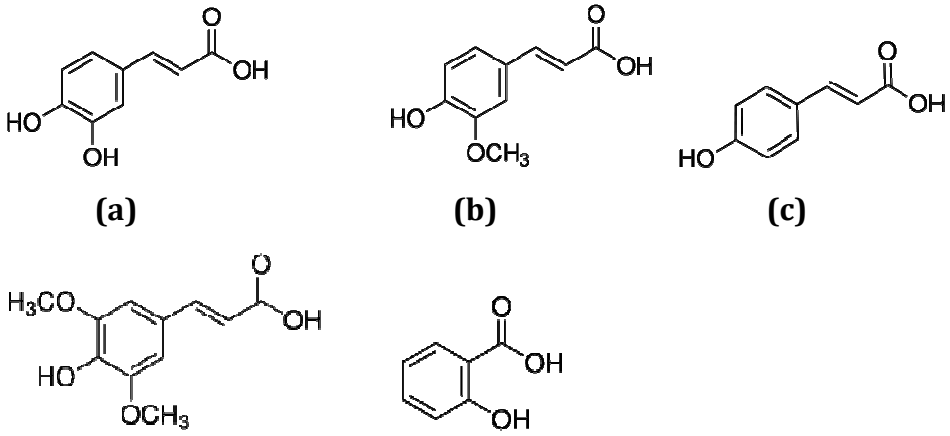
(a) Quinic acid **(b) Ascorbic acid** **(c) Citric acid**

Figure A9 Organic acids detected in potato extracts.



(a) Tyrosine **(b) Phenyalanine** **(c) Tryptophan**

Figure A10 Amino acids detected in potato extracts.

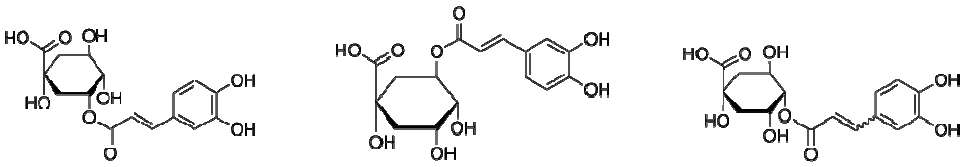


(a) **(b)** **(c)**

(d) **(e)**

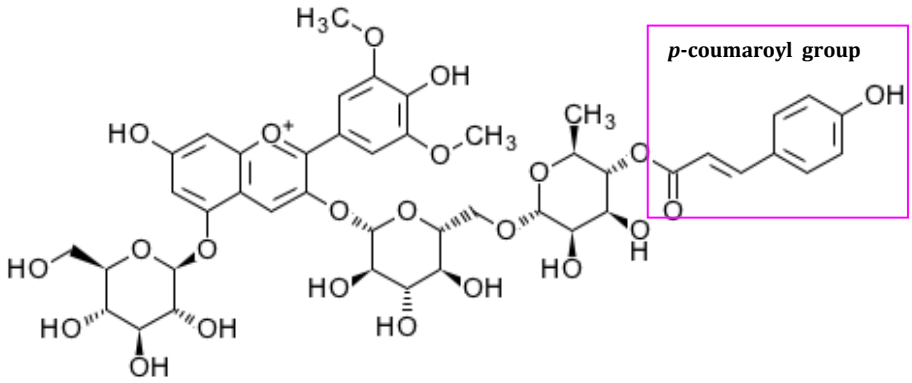
Figure A11 Structures of some phenolic acids.

(a) caffeic acid, (b) ferulic acid, (c) *p*-coumaric acid, (d) sinapic acid, (e) salicylic acid.

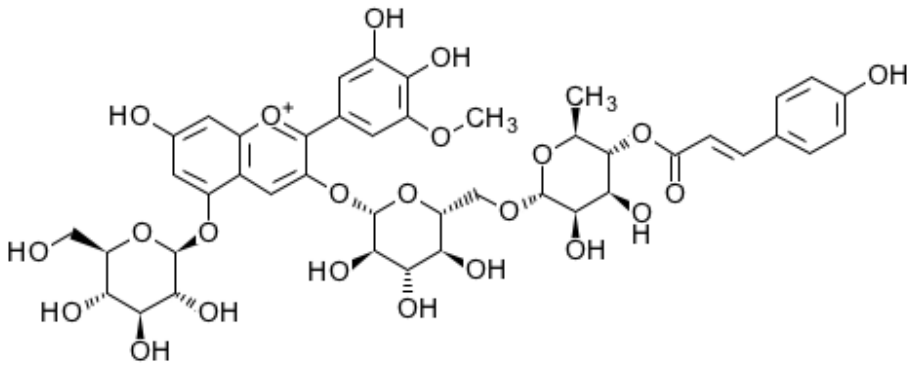


(a) Neochlorogenic acid (b) Chlorogenic acid (c) Cryptochlorogenic acid

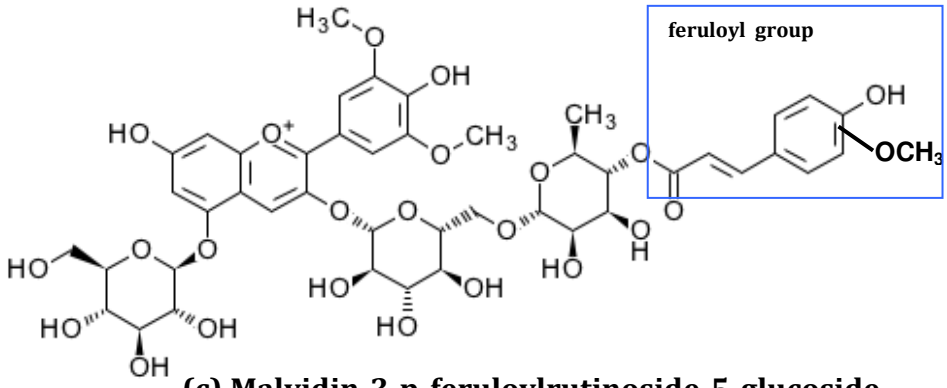
Figure A12 Chlorogenic acid isomers detected in potato extracts.



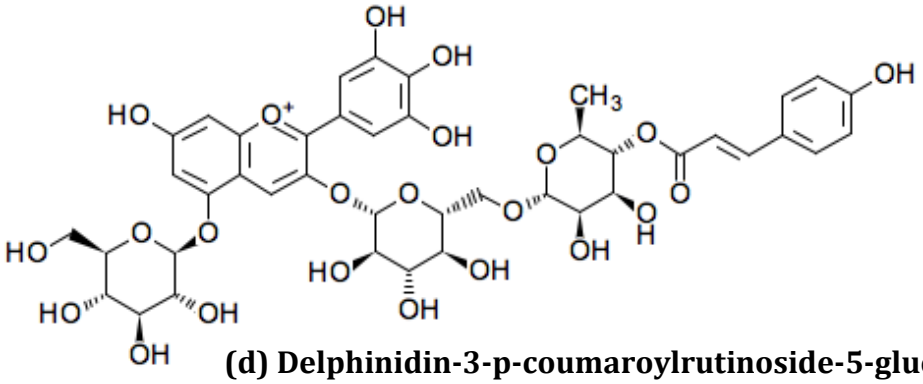
(a) Malvidin-3-p-coumaroylrutinoside-5-glucoside



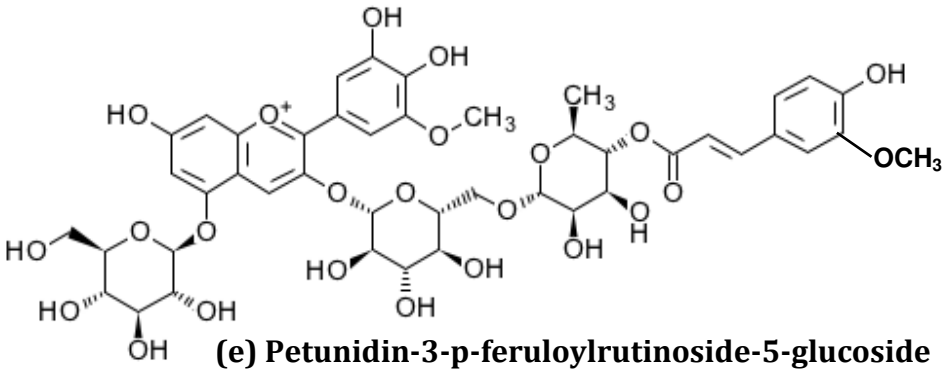
(b) Petunidin-3-p-coumaroylrutinoside-5-glucoside



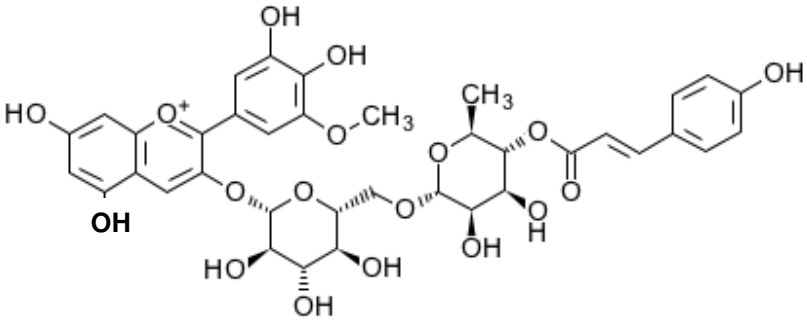
(c) Malvidin-3-p-feruloylrutinoside-5-glucoside



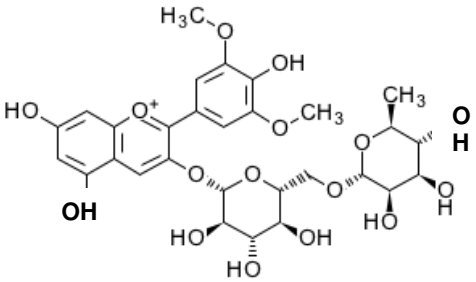
(d) Delphinidin-3-p-coumaroylrutinoside-5-glucoside



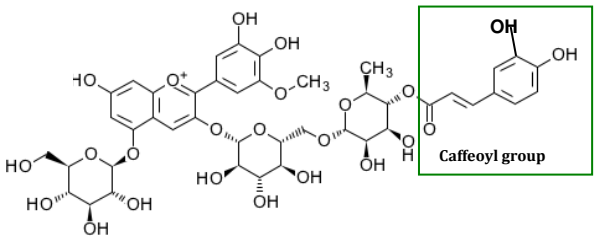
(e) Petunidin-3-p-feruloylrutinoside-5-glucoside



(f) Petunidin-3-p-coumaroylrutinoside



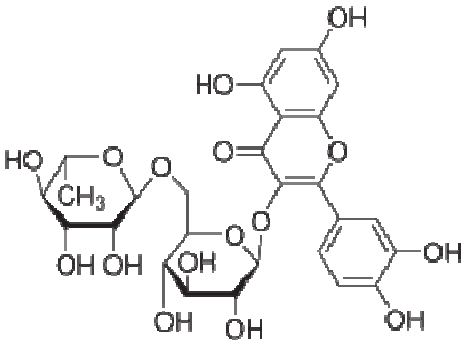
(g) Malvidin-3-rutinoside



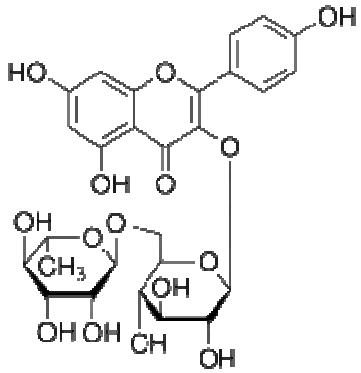
(h) Petunidin-3-p-caffeoylrutinoside-5-glucoside

Figure A13 Anthocyanins found in PPE.

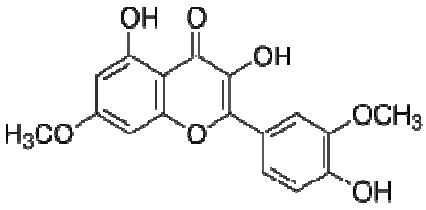
Compounds were presented in the order of abundance.



Quercetin-3-rutinoside



Kaempferol-3-rutinoside



Quercetin dimethyl ether

Figure A14 Flavonols detected in the potato extracts.

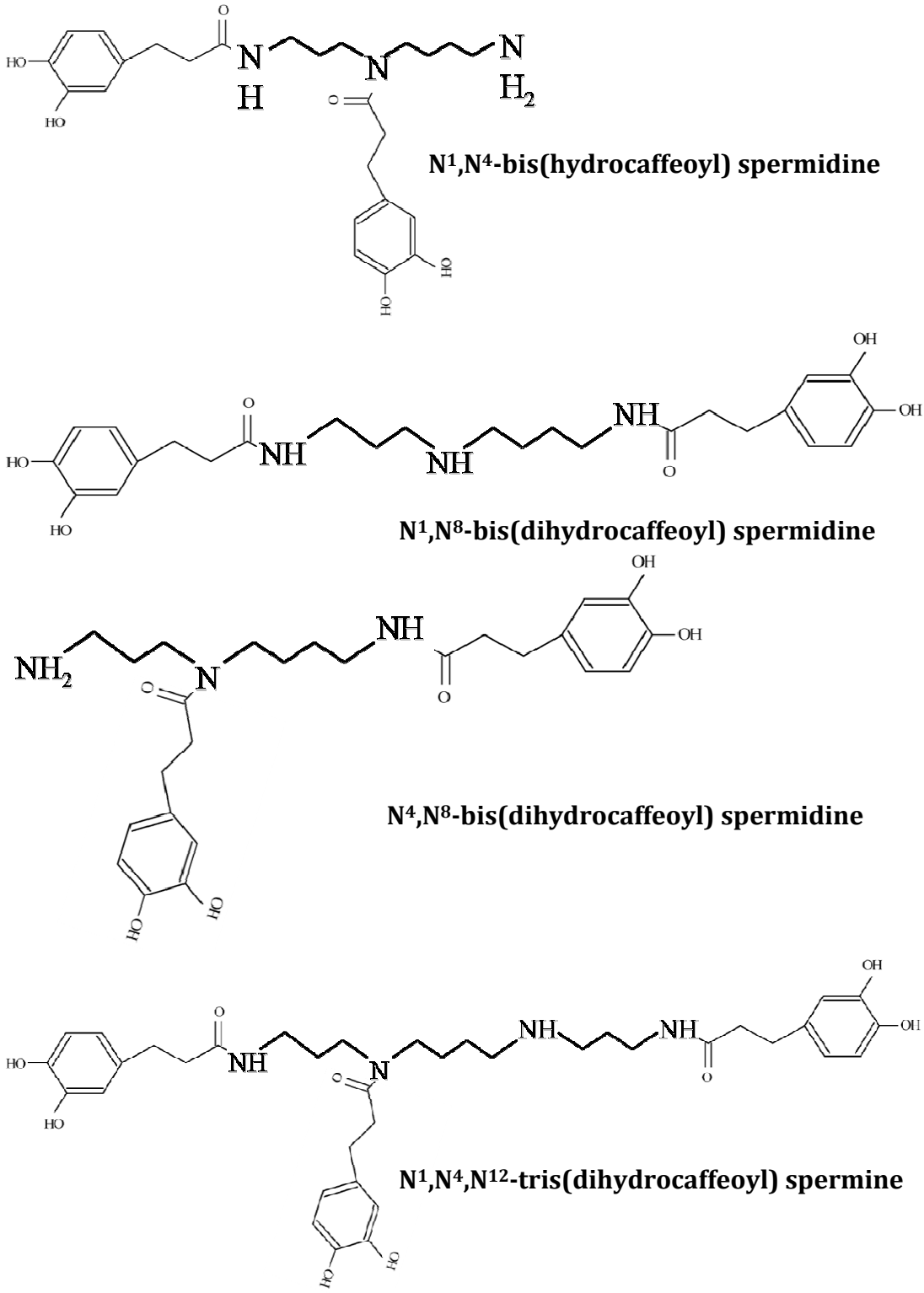
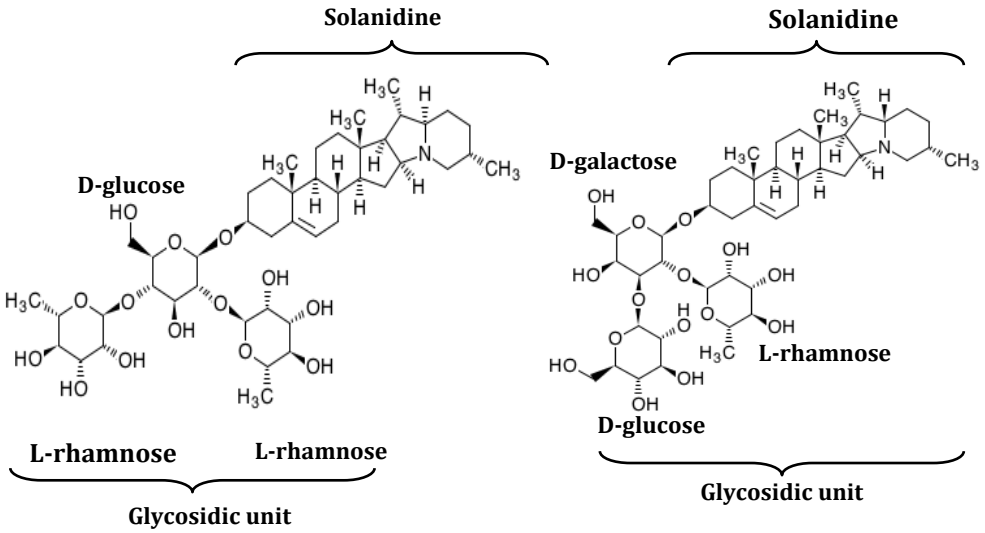


Figure A15 Bis(dihydrocaffeoyl) spermidine isomers & N^1,N^4,N^{12}-tris(dihydrocaffeoyl) spermine.



(a) α-chaconine (b) α-solanine

Figure A16 Structures of glycoalkaloids detected in potato extracts.

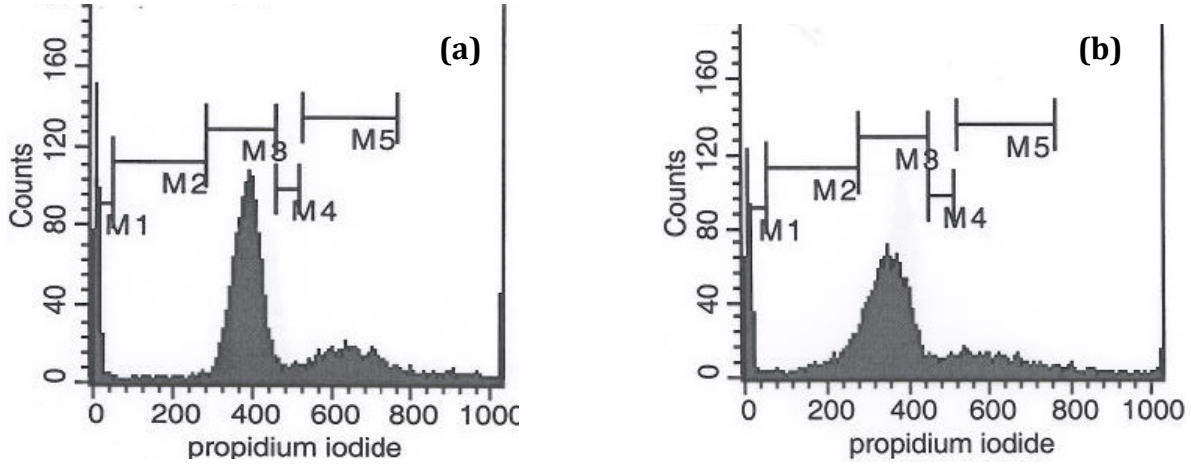


Figure A17 Cell cycle distribution histogram.

Standard thresholds were used for assessing each phase of cell cycle (a): M1, necrosis; M2, apoptosis; M3, G0/G1; M4, Synthesis (S); M5, G2 & mitosis (G2/M). As an example, histogram (b) illustrates an increase in apoptotic cell count and reduction in G0/G1 and S + G2/M phase cell count compared to histogram (a).

Table A2 Tamoxifen (Tam) IP solution formulations tested and observations

Test	Target [Tam] (mg/mL)	Formulation	Was Tam dissolved? (Yes: √; No: X)	Remarks	Applicable? (Yes: √; No: X)
1	1	1 mg Tam 1 mL PBS	X	Tam crystals were visible	X
2	0.5	0.5 mg Tam 1 mL PBS	X	Tam crystals were visible	X
3	0.1	0.1 mg Tam 1 mL PBS	X	Less Tam crystals were observed	X
4	0.5	3 mg Tam 5.4 mL PBS 0.6 mL Ethanol	X	Milky solution formed. Heating at 37 ℃, or sonication were not able to improve solubility.	X
5	0.5	2.5 mg Tam 4.5 mL PBS 0.313 mL Ethanol 0.188 mL Corn oil	X	Milky solution formed, but less milky than Test 4 solution.	X
6	0.5	0.5 mg Tam 0.10 mL Ethanol 0.90 mL Corn oil	√	Clear solution. Clarity remained after 1 & 2 days storage at 4 °C.	√
7	1	1.5 mg Tam 0.15 mL Ethanol 1.35 mL Corn oil	√	Same as Test 6	√
8	0.5	2 mg Tam 0.52 mg Citric acid 0.25 mL Ethanol 0.15 mL Tween 80 (polysorbate 80) 3.6 mL PBS	√	Clear solution but it was foamy. Bubbles disappeared after 1 day storage at 4 ℃. Measured pH=6.83	√
9	0.5	2 mg Tam 0.25 mL Ethanol 0.15 mL Tween 80 (polysorbate 80) 3.6 mL PBS	√	The clearest solution observed. No bubbles or foams formed. Clarity remained after 1 and 2 days of storage at 4 ℃. Measured pH=7.23	√
10	0.5	Formulation Test 9 repeated	√	Same observations as Test 9. Measured pH=7.21	√

Table A3 Jelly formulations created and observations.

#	Form.	State [a]	Ease of processing [b]	Feasibility [c]	Gel strength, storage stability [d]	Applicable? (Yes: √; No: X)
Calcium-alginate gel formulations						
1	1 % CC 2 % SA 30 % S	Phase separation (aqueous layer & lumps)	X	X Phase separation	X	X
2	0.1 % CC 2 % SA 30 % S	Solid gel	√	X Solid gel	√	X
3	0.1 % CC 1.5 % SA 30 % S	Solid gel	√	X Solid gel	X	X
4	0.1 % CC 1 % SA 30 % S	Solid gel	√	X Solid gel	X	X
5	0.1 % CC 0.5 % SA 30% S	Phase seperation	√	X Phase separation	X	X
6	0.1% CC 0.8 % SA 30% S	Viscous, not runny	√	√	X	X
7	0.1 % CC 1 % SA 20 % S	Viscous, not runny	√	√	X	X
8	0.1 % CC 0.8 % SA 0.8 % G 30 % S	Viscous but runny	√	√	X	X
9	0.05 % CC 0.5 % SA 0.8 % G 30 % S	More viscous than Test 8	√	√	X	X

Table A3 (continued)

#	Form.	State	Ease of processing	Feasibility	Gel strength, storage stability ***	Applicable? (Yes: √; Best: √√; No: X)
Gelatin-alginate gel formulations						
10	1 % G 1 % SA 30 % S	Viscous	√√	√	X	X
11	1.5 % G 1.5 % SA 30 % S	More viscous than Test 10	√√	√	X	X
12	2 % G 1 % SA 30 % S	Viscous, similar to Test 11	√√	√	√	√
13	2 % G 1.5 % SA 30 % S	More viscous than Test 12	√√	√	√	√
14	2 % G 2 % SA 30 % S	More viscous than Test 13	√√	√	√	√
15	3 % G 3 % SA 30 % S	Very viscous	√√	√	√√	√√
16	4 % G 4 % SA	More viscous than Test 15	√√	√	√√√	√

(Abbreviations: CC – calcium chloride, SA – sodium alginate, S – sucrose, G – gelatine)

[a] After being mixed and heated in water bath at 90 °C

[b] Refers to procedure of mixing and heating at 90 ℃ to achieve good consistency (√: easy, √√: easier, X: difficult)

[c] Feasibility of mixing in medicine when the mixture is not hot (<37 °C) (√: Yes, X: No); no destruction to gel matrix or final gel strength

[d] Jelly stayed firm after it had been stored overnight (4 ℃), and remaining firm after being removed from the fridge and kept at room temperature (√: strong; √√: stronger, √√√: Strongest, X: Weak).

Table A4 Experiments to determine if there is colour preference for jelly in mouse feeding trial

Mice	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***	***11***	***12***
Day 1	R	R	B	B	Y	Y	G	G	O	O	P	P
	√	x	√	√	√	√	√	√	√	√	√	√
Day 2	Y	Y	G	G	P	P	O	O	B	B	R	R
	√	√	√	√	√	√	√	√	√	√	√	√
Day 3	G	G	R	R	B	B	Y	Y	P	P	O	O
	√	√	√	√	√	√	√	√	√	√	√	√

Formulation of jelly: 3% gelatine, 3% sodium alginate, 30% sucrose, colour.
Abbreviations: R: red, B: blue, Y: yellow, G: green, O: orange, P: purple, √: jelly was completely eaten, x: jelly was partially eaten)

(A)

(B)

Figure A18 Conditioning mice to eating the jelly. (A) Day 1 (B) After 24-hours feeding.

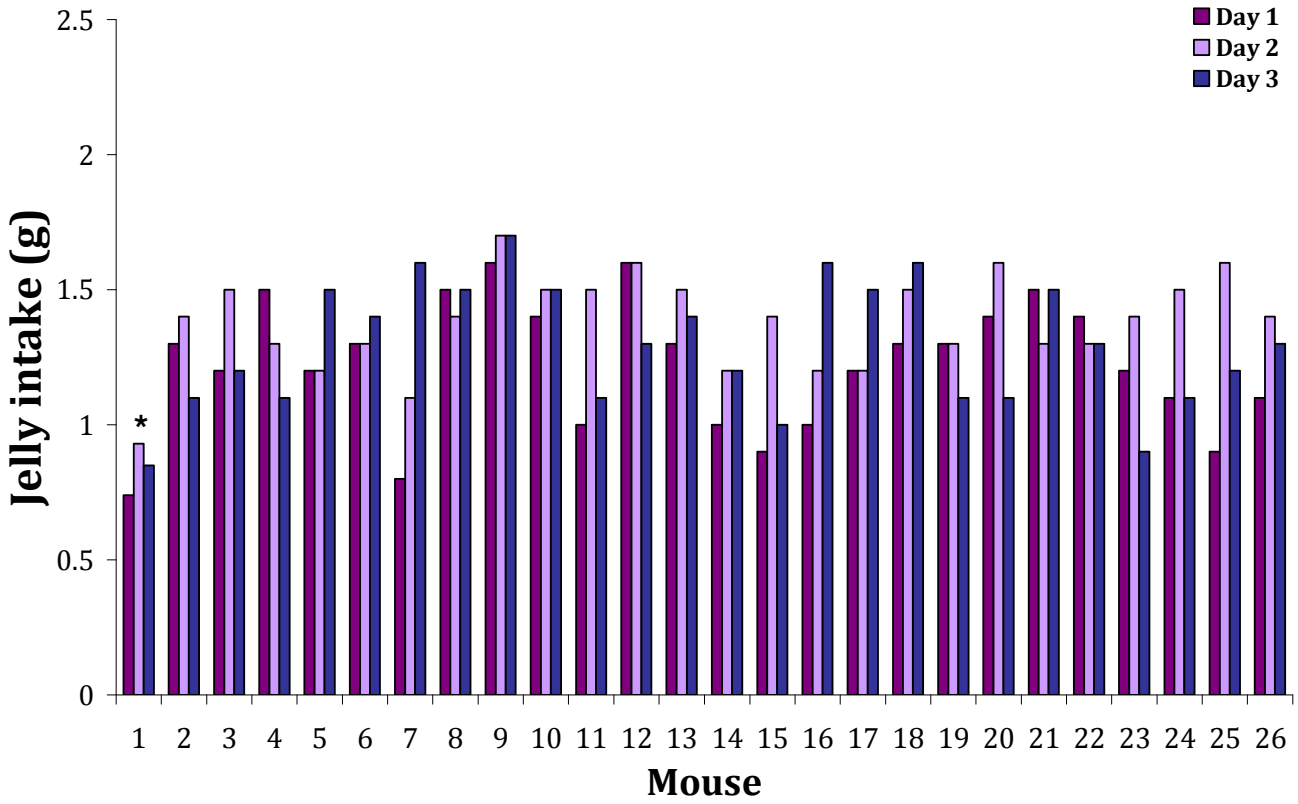


Figure A19 Three-day jelly intake of mice subjected to conditioning procedure.

Asterisk denotes jelly intake was significantly different from the others ($P < 0.05$). The same formulation of jelly was used throughout the 3-day trial, i.e. 3% gelatine, 3% sodium alginate, 30% sucrose, colouring (orange).

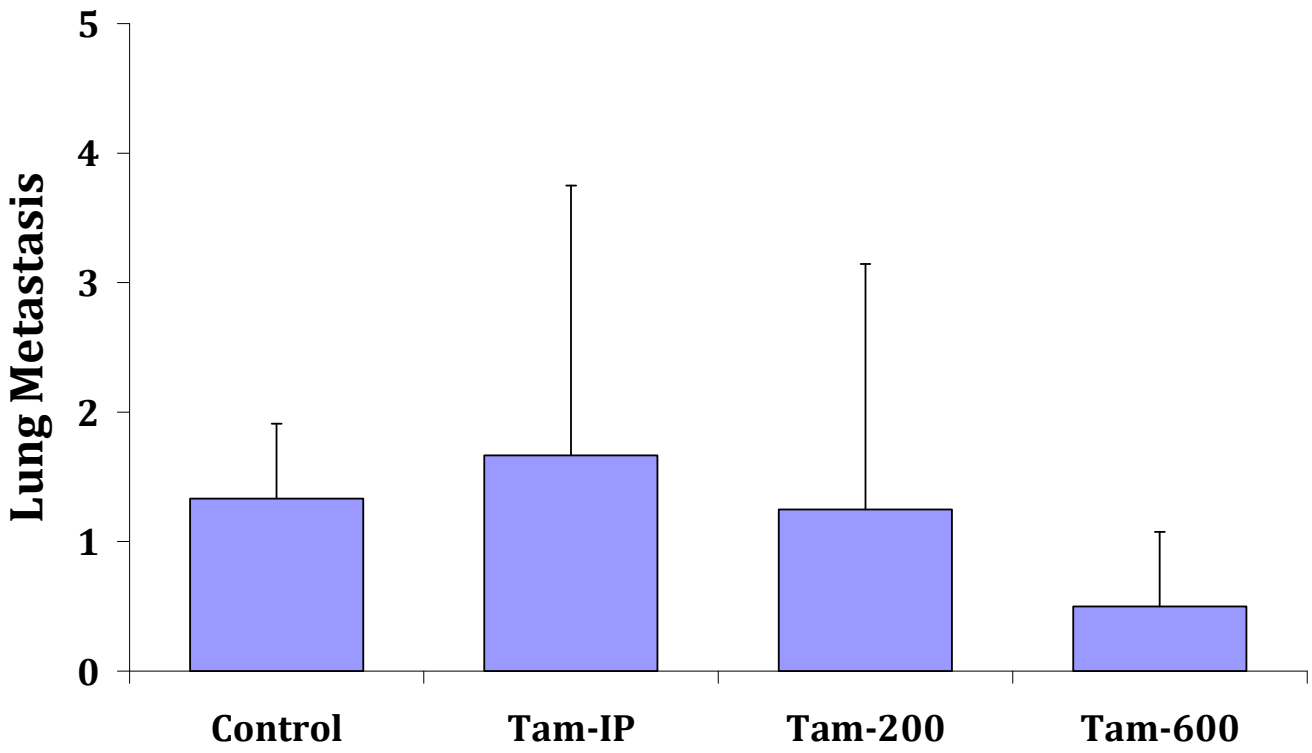


Figure A20 Lung 4T1 metastasis versus various treatment groups.

4T1 metastasis in lung was assessed by histological examination and scored on a scale of 10 for each of the treatment groups (N = 3-5 mice per group). Results were expressed as mean ± SD. There was no significant difference among the treatments.

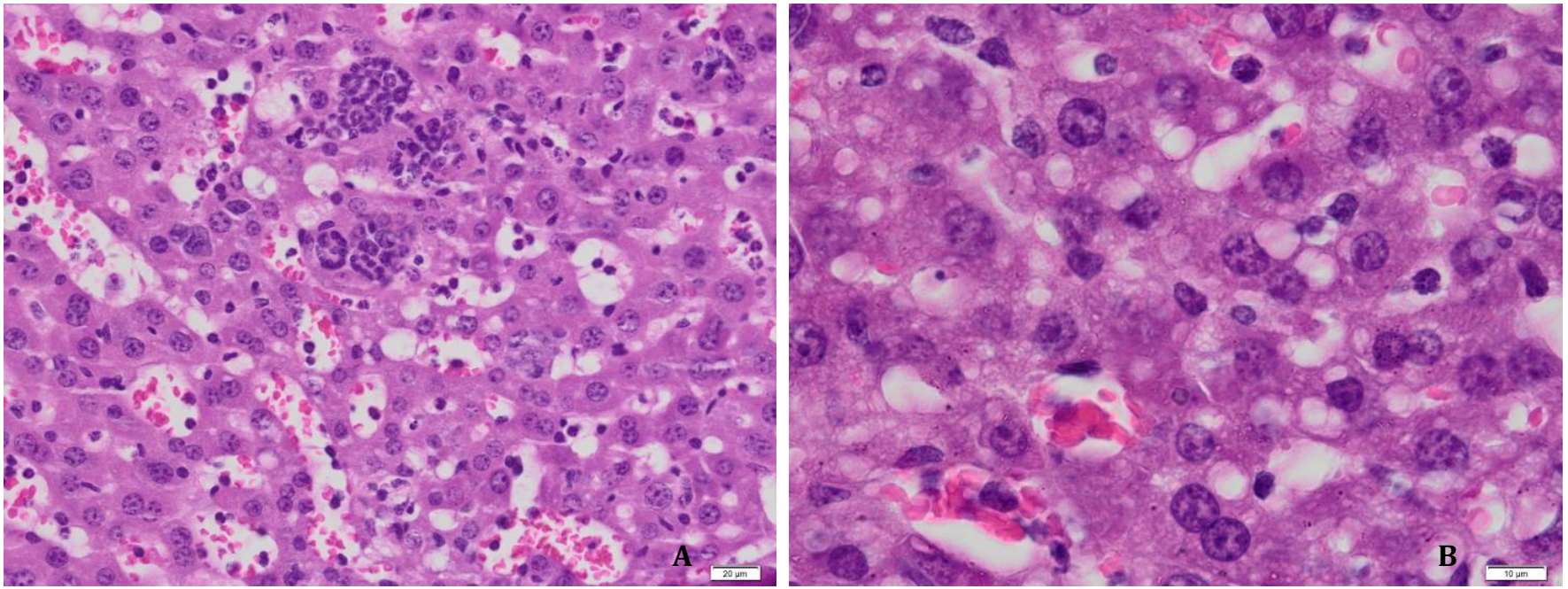


Figure A21 Hepatic steatosis (fatty liver).

Fat droplets were omnipresent in the liver specimens of some tamoxifen-treated mice, which suggest the presence of fatty changes in the liver (H&E staining, scale bar: (A) 20 µm, (B) 10 µm).

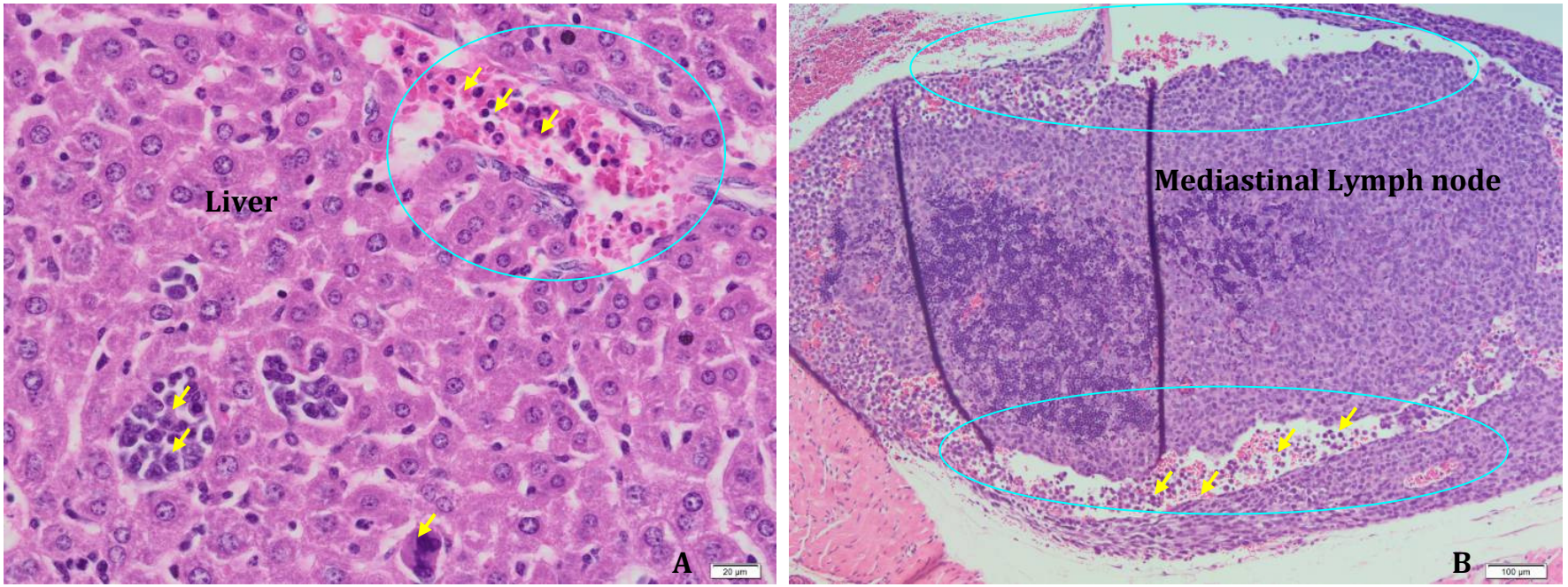


Figure A22 Potential role of myeloid precursor cells in 4T1 metastasis.

Myeloid precursor cells (yellow arrows, blue circles) were abundant in the blood vessels and sinusoids in the liver, and subcapsular sinus of mediastinal lymph node severely invaded by 4T1 cell, hence myeloid precursor cells may play a potential role in intravasation and survival of 4T1 cells in lymph and blood vascular system (H&E staining, scale bar: (A) 20 µm, (B) 100 µm).