Biological properties of blueberries and their effects on breast cancer in DMBA-induced mammary tumorigenesis rat model

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Abstract

Breast cancer is the most common form of cancer found in women. Approximately 75% of breast cancer patients are diagnosed with estrogen receptor positive (ER+) breast cancer. The standard clinical treatments for breast cancer include surgery, chemotherapy and radiation; however, dietary bioactive compounds from various plants have also been proposed to have chemopreventive or therapeutic effects on breast cancer. Blueberries have been reported to contribute to several health benefits including anti-tumour activity. Blueberry pomace, a by-product of the blueberry juice industry having high fibre content, may also have health benefits but has not been tested for efficacy against breast cancer previously. Therefore, the primary objective of this thesis was to investigate the effects of selected rabbiteye blueberries grown in New Zealand and blueberry pomace on their potential for managing mammary tumorigenesis induced by 7,12-dimethylbenz[a]anthracene (DMBA).

Five rabbiteye blueberry *(Vaccinium ashei)* cultivars ('Centurion', 'Maru', 'Rahi', 'Ono' and 'Tifblue') were initially characterised by measuring total phenolic concentration (TPC) using a Folin-Ciocalteu procedure, total flavonoid concentration (TFC), and anthocyanin profiles and chlorogenic acid concentration by HPLC. Further experiments were then carried out to investigate whether these rabbiteye blueberries possessed bioactivity that may affect breast cancer growth and development such as antioxidant capacity, prebiotic (*Lactobacillus* spp.) and antimicrobial activities (*Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus*) and anti-angiogenic activity using chicken chorioallantoic membrane (CAM) assay. Finally, the effects of selected rabbiteye blueberry extracts or highbush blueberry pomace supplemented diet consumption on DMBA-induced mammary tumorigenesis, oxidative stress biomarkers, serum estrogen level, populations of intestinal microflora and caecal β-glucuronidase enzyme activity were assessed in a rat model.

The five rabbiteye blueberry cultivars were found to contain sufficient polyphenolics, flavonoids, total anthocyanins and chlorogenic acid to exert bioactive effects, even in a water extract of freeze-dried material. The 'Tifblue' cultivar contained the highest TPC, TFC, total anthocyanins and chlorogenic acid of the studied cultivars. Blueberry pomace also contained high concentrations of polyphenolic compounds. Total polyphenolic concentration of blueberry pomace in this study ranged from 0.74 - 1.20 mg GAE/g frozen berries. The blueberry extracts both from fruits and pomace possessed antioxidant activity

as measured by ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays. Some evidence of prebiotic activities of blueberry extracts was shown *in vitro* (ca. 0.6-0.9 log CFU/mL increases for *Lactobacillus. rhamnosus* and *Lactobacillus. acidophilus* respectively). However, the blueberry extracts in this study did not exhibit anti-microbial activity. The water extracts of 'Maru', 'Centurion' and 'Tifblue' demonstrated more than 50% inhibition of angiogenesis compared to controls in CAM assay. Total polyphenolic concentration and chlorogenic acid concentrations were strongly correlated with antioxidant activity while total anthocyanins showed a strong relationship with anti-angiogenic activity. An animal trial was conducted with 100 female Sprague-Dawley rats (*Rattus norvegicus*) and assigned equally in five treatment groups; negative control (no DMBA with normal feed and normal water), positive control (DMBA with normal feed and normal water), 'Centurion' (DMBA with normal feed and 'Centurion' extract), 'Maru' (DMBA with normal feed and 'Maru' extract) and pomace (DMBA with 5% blueberry pomace supplemented diet and normal water). Seven week old rats were gavaged with DMBA and, starting shortly after (ca. 2 h), their diets were supplemented with 25% blueberry juice in feeding water or 5% blueberry pomace in solid diet. The major effects of blueberry extracts or pomace consumption were inhibition of the number of tumours and slower tumour progression from adenoma to carcinoma. A total of 35 tumours were found from animals in a positive control group (without blueberry treatment), while animals that received blueberry supplementation had fewer than 15 tumours per group ($\chi^2 = 22.1$, $P < 0.01$). In addition, approximately 85% of tumours found in animals without blueberry treatment were carcinomas while less than 50% of tumours in all blueberry-treated animals were carcinomas. Blueberry consumption in both extract and pomace forms restored levels of oxidative stress in serum from DMBA treated rats to normal levels. Consumption of blueberry water extracts did not alter the level of circulating estrogen in animal blood serum but pomace-supplemented diet significantly reduced circulating estrogen. Even though blueberry consumption did not show any effects on measured components of intestinal bacteria population (*Lactobacillus* spp., *Bifidobacterium* spp. and *E. coli*). β-glucuronidase enzyme activity was reduced in caeca of animals that received pomace-supplemented diet. A positive correlation was also found between serum estrogen levels and β-glucuronidase enzyme activity. Blueberry consumption has therefore been shown to be a promising strategy to reduce progression of mammary tumours in a DMBA treated rat model. This study suggests that including fibre with polyphenolic compounds in the food matrix leads to improved bioefficacy.

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Abbreviations

16α-OHE1	16α-hydroxyestrone
2-MeOE2	2-methoxyestradiol
2-OHE2	Hydroxyestradiol
4-OHE2	4-hydroxyestradiol
5%FA	5% aqueous formic acid
AAPH	2,2’ azobis(2-methylpropionamidine)dihydrochloride
AB	Alveolar bud
ACY	Total anthocyanins
AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AV	Alveoli
BMD	Bone mineral density
BMI	Body mass index
CAM	Chicken chorioallantoic membrane
CAT	Catalase
CD	Cluster of differentiation
CE	Catechin equivalent
CFU	Colony forming units
CGA	Chlorogenic acid concentration
CRC	Colorectal cancer
C_T	Threshold cycle
CYP1A1	Cytochrome P450 1A1
CYP1B1	Cytochrome P450 1B1
DAGDL	2,5-di-O-acetyl-D-glucaro-l,4:6,3-dilactone
DCIS	Ductal carcinoma in situ
DMBA	7,12-dimethylbenz[a]anthracene
E1	Estrone
E2	17β-estradiol
E3	Estriol
EC	Endothelial cell
EIA	Enzyme immunoassay
ER	Estrogen receptor
FB	Frozen berries
$FeCl_3$	Ferric chloride
$FeSO_4$	Iron (II) sulfate
FISH	Fluorescence in situ hybridization
FL	Fluorescein
FOS	Fructooligosaccharides
FRAP	Ferric reducing antioxidant power

GAE	Gallic acid equivalent
GPx	Glutathione peroxidase
H&E	Heamatoxylin and eosin
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HER2	Human epidermal growth factor receptor 2
HIF-1	Hypoxia-inducible factor-1
HPFs	High power fields
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cell
ICR	Imprinting controlled region
LCIS	Lobular carcinoma in situ
LB	Lobule
LPS	Lipopolysaccharide
MAM-A	Mammaglobin-A
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MQ	MilliQ water
MRS	Man-Rogosa-Sharpe
MVD	Microvessel density
Na2CO3	Sodium carbonate
NDOs	Non-digestible oligosaccharides
NK cells	Natural killer cells
NMU	Nitrosomethylurea
ORAC	Oxygen radical absorbance capacity
PAHs	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PC	Principle component
PCA	Principle component analysis
PCNA	Proliferating cell nuclear antigen
PDA	Photo-diode array
PFS	Progression-free survival
PPB	Phosphate buffer
PR	Progesterone receptor
QE	Quercetin equivalent
qPCR	Quantitative polymerase chain reaction
RFS	Relapse-free survival
ROS	Reactive oxygen species
SAPU	Small Animal Production Unit
SCFAs	Short chain fatty acids
SD	Sprague-Dawley
SOD	Superoxide dismutase

TAC	Total antioxidant capacity
TBA	Thiobarbituric acid
TE	Trolox equivalent
TEB	Terminal end bud
TFC	Total flavonoid concentration
TPC	Total phenolic concentration
TPTZ	2,4,6-tripyridyl-s-triazine
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TSB	Tryptic soy broth
VEGF	Vascular endothelial growth factors
WHO	World Health Organization
XREs	Xenobiotic response elements

Introduction

Breast cancer is the most common form of cancer found in women. Approximately 75% of breast cancer patients are diagnosed with estrogen receptor positive (ER+) breast cancer where estrogen exposure can promote the growth and progression of the tumours (Ratini, 2015). There are several risk factors associated with the incidence of breast cancer. Non-modifiable risk factors include, but are not limited to, a family history of breast cancer, genetic predisposition, endogenous hormone levels; while diet, alcohol consumption and physical activity are considered modifiable risk factors (American Cancer Society, 2013). In New Zealand breast cancer accounts for more than 20% of all registered cancers and has become the most expensive cancer to treat with more than 80 million NZD spent annually (Ministry of Health, 2011). Moreover, the public health service also has to cover diagnostic costs. The cost is estimated to rise more than 20% in the next decade (Ministry of Health, 2011). For this reason, research into breast cancer prevention and alternative therapeutic approaches has become an important task for the public health care system.

A link between diet and breast cancer have been well documented. Low fat and high fiber diet consumption resulted in lowering serum estrone levels in premenopausal subjects compared to high fat low fiber diet consumption (Bagga et al., 1995). Consumption of one litre soymilk every day for approximately 30 days reduced levels of circulatory 17β-estradiol by 15% compared to the control subjects without soymilk consumption (Lu et al., 2000). The reduction of circulatory estrogen could be a strategy for decreasing breast cancer risk. Therefore, a study of dietary bioactive compounds from plants is an area of research interest for chemopreventive and alternative therapy.

Research suggests that several fruit polyphenols possess anti-tumour activity including against breast cancer (Wang et al., 2012a). Mice that received grape skin extract showed an increased survival rate by 40% compared to control mice in a metastasis-specific mouse mammary carcinoma 4T1 model (Sun et al., 2012). Like grapes, blueberries (*Vaccinium* spp.) contain various polyphenols, the most numerous being anthocyanins. The fruits are high in antioxidant activity and have been shown to exhibit various health benefits including reduce risk of breast cancer (Smith et al., 2000; Neto, 2007; Howell, 2009; Adams et al., 2010; Jeyabalan et al., 2014; Lacombe et al., 2013). Rabbiteye blueberries are the second most cultivated in New Zealand. The production of rabbiteye blueberries is increasing as the fruit crops later (Feb-April; summer - autumn) in the season and requires fewer chilling hours than other varieties (“Blueberry,” 2012). Even though

several studies have reported the chemopreventive and therapeutic effect of blueberry (*Vaccinium* spp.) consumption on breast cancer (Aiyer & Gupta, 2010; Jeyabalan et al., 2014), little data is available on the role of rabbiteye blueberries (*Vaccinium ashei*) on ER+ breast cancer chemoprevention. Moreover, a by-product from blueberry juice industry, blueberry pomace, has been shown to contain high level of anthocyanins (Bener et al., 2013; Li et al., 2013) and also exhibits several health benefits (Gil, 2013; Håkansson et al., 2012) however its effect on breast cancer is currently unknown. Therefore, the main objective of the present study was to investigate whether selected cultivars of rabbiteye blueberries and blueberry pomace have a chemopreventive effect and delay tumour progression on 7,12-dimethylbenz[a]anthracene (DMBA) induced ER+ mammary tumorigenesis. In this thesis, *in vitro* studies were carried out to evaluate polyphenol levels in blueberry fruits and pomace and their biological activities including antioxidant activities and prebiotic and antimicrobial properties. Anti-angiogenic activities of blueberry fruits and pomace were evaluated *in ovo* using the chicken chorioallantoic membrane (CAM) assay. The studies of blueberry polyphenols and their biological activities were used to select the most suitable blueberry cultivars for *in vivo* study. The *in vivo* study was carried out to investigate the effect of selected rabbiteye blueberry extracts and diet supplemented with blueberry pomace on rodent mammary tumorigenesis chemoprevention. Oxidative stress, circulatory estrogen concentrations and intestinal microflora were also investigated to confirm the effect of blueberry intervention. This study provides more information and understanding on the role of polyphenols and other dietary components found in blueberries on mammary tumour chemoprevention.

CHAPTER 2

Literature Review

2.1 Breast cancer statistics

In 2012, almost 1.7 million new cases of breast cancer were diagnosed worldwide which accounted for 25% of all cancers. Breast cancer affects women worldwide regardless of the region. However, breast cancer incidence rates are higher in more developed regions such as Western Europe (96 cases per 100,000) than in less developed regions such as Middle Africa and Eastern Asia (27 cases per 100,000). The lower rate of breast cancer in less developed region might result from poor diagnosis or could related to lifestyle factors including diet. Even though the incidence rates in more developed regions were higher, mortality rates in these regions were similar to the mortality rates in the less developed regions (fewer than 20 cases per 100,000 annually) (Ferlay et al., 2013).

New Zealand has been ranked in the top ten countries with the highest breast cancer incidence around the world ("Breast Cancer: Estimated Incidence, Mortality and Prevalence Worldwide in 2012," 2015). In New Zealand, female breast cancer is the most frequently registered form of cancer and accounts for 28% of all female cancer registrations in 2010 (figure 2-1). Breast cancer has been diagnosed in New Zealand women from the age of 25 and approximately half of all registered cases fall within the 45 – 64 age groups. Even though breast cancer incidence is the most frequent cancer among New Zealand women, its mortality rate is the second after lung cancer. Ten years' data collection has revealed that registration rates of breast cancer are stable while the mortality rate has dropped slightly. The free breast screening campaign for women age 45 to 69 in New Zealand may have influenced the decrease in mortality rate by early detection of breast cancer incidence (Ministry of Health, 2013).

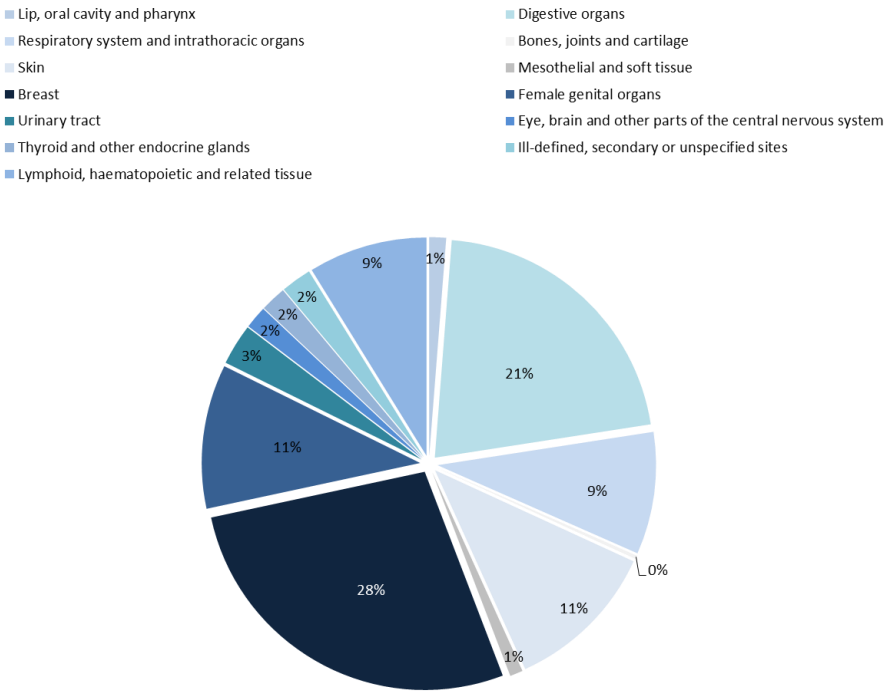


Figure 2-1 Numbers of female cancer registrations in 2010, by organ site. Data derived from Ministry of Health, New Zealand 2013.

2.2 Breast development and breast cancer

2.2.1 Normal mammary gland development

The human breast is an organ responsible for milk production which is important for providing nutrients for the newborn. The breast houses mammary glands which do not fully function at birth. Development of the mammary gland is divided into two distinct phases; development and differentiation. Development starts in the foetus and includes nipple epithelium and lobule formation. Primitive structures of the mammary gland, ductules and terminal ducts, are found in newborns. The growth of the mammary gland during childhood and the pre-puberty period is hormone-independent. The gland structure branches and grows moderately until the onset of puberty. Terminal end buds (TEBs) are the dominant mammary gland structure in pre-pubertal period. The major change or differentiation stage starts when girls enter puberty. The gland will be fully mature after the first full term pregnancy. Mammary gland structures differentiate to lobule type 1 (LB 1) under the control of ovarian hormones. Lobules of type 2 (LB 2) and lobules of type 3 (LB 3) are also found in virgin females which result from hormonal effects during the menstruation cycle however the percentage of LB 1 is approximately three times higher

than LB 2 in nulliparous (has not borne offspring) women. A human mammary gland diagram is presented in figure 2-2. LB 1 gradually transforms into LB 2 and LB 2 to LB 3 by the process of new alveolar bud (AB) formation. The numbers of AB increase from 11 in LB 1 to 47 in LB 2 and LB 3 is found with approximately 80 ABs. In parous (having given birth to one or more viable offspring) women, LB 3 is the predominant structure (Russo & Russo, 2004).

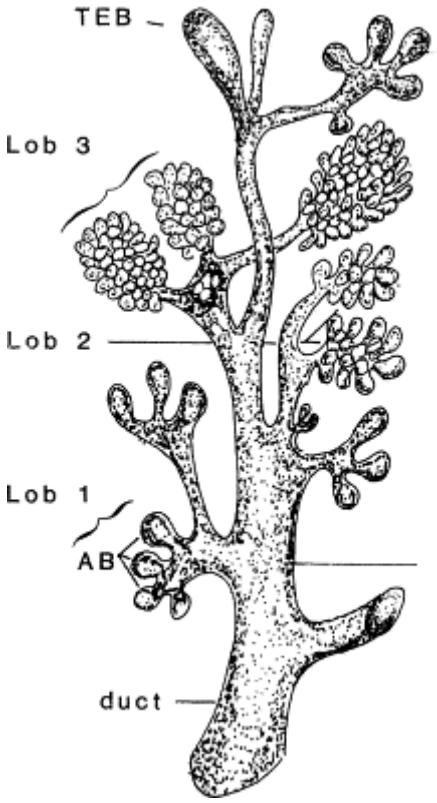


Figure 2-2 Diagrammatic representation of the lobular structures of the human breast (Source: Russo & Russo, 2004, reprinted by permission of Elsevier)

Understanding mammary gland structure is an important aspect for studying hormone positive breast cancer. A study showed that cells that are highly proliferative are more likely to attract chemical carcinogenesis (Russo & Russo, 1994). Not only do LB 1 have a higher proliferative index than LB 2 and LB 3, but LB 1 in nulliparous women show a higher proliferative index than LB 1 in parous women. Interestingly, prolonged estrogen exposure is one of the causes of breast cancer by direct stimulation of cell proliferation. Lobule type 1 contains more estrogen receptors than LB 2 and 3 (Russo & Russo, 2004).

2.2.2 Breast cancer

Cancer is a chronic disease resulting from an uncontrollable growth of cells and different kinds of cancer are named after the tissues in which they originate. Breast cancer, therefore, refers to a cancer started from the breast tissue in a milk production unit (lobules) or milk delivery unit (ducts). In the past, breast cancers have been detected by palpation which often resulted in late stage detection and poor prognosis. Nowadays, breast cancer can be detected earlier using mammogram screening before lumps or masses can be felt (Tabár et al., 2014). Early breast cancer detection coupled with suitable treatments therefore have contributed to a lower death rate (Ministry of Health, 2013). However, breast abnormalities detected by mammogram are mostly benign. Even though the benign breast condition is not cancerous, some types of benign breast conditions can lead to a higher risk of developing malignant breast cancer later in life ("Non-cancerous Breast Conditions," 2015). Based on mammogram and other breast exam techniques, masses or lumps that are suspected to be cancers will need to be microscopically analyzed to characterize the type of breast cancer cell and define the extent of tumour spread. Generally, there are two types of breast cancers, non-invasive and invasive breast cancers. There are two types of non-invasive carcinoma based on their origins, ductal carcinoma in situ (DCIS) where the abnormalities start from the cells lining the breast duct and lobular carcinoma in situ (LCIS) where it originates from cells in lobules. Both are considered non-invasive because the abnormal cells have grown but still remain inside the layer of their original cells. Approximately 83% of non-invasive cancers are DCIS and 33% of those may progress into invasive breast cancer (American Cancer Soceity, 2013). The progression of DCIS to invasive carcinoma is presented in figure 2-3.

Breast cancer often occurs in the upper outer quadrant of the breast (Toombs & Kalisher, 1977). Blood vessels, nerves and lymphatic systems of the breast provide channels for cancerous cells to spread throughout the body. The earliest symptom that could be recognized is a painless mass at the breast area. The other symptoms such as breast pain, skin irritation, changing in size and contours of the breast and nipple abnormalities such as erosion, inversion and spontaneous discharge are likely to occur when the cancers progress. Therefore, familiarisation with your own breast will help detect abnormalities at an early stage (American Cancer Society, 2013).

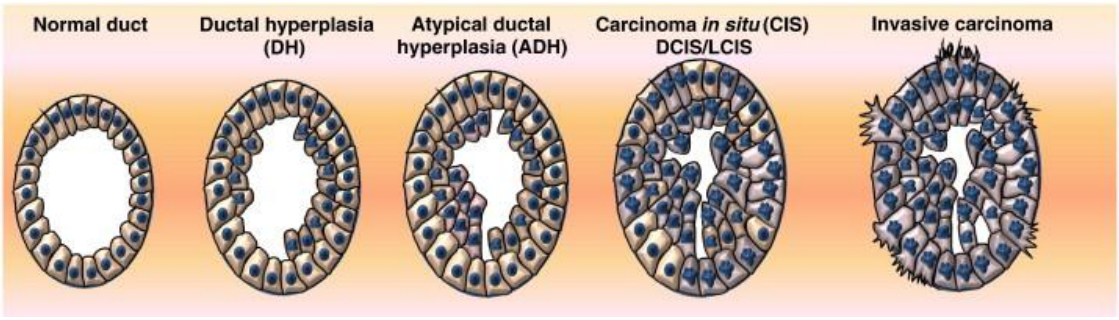


Figure 2-3 Progression of breast ductal carcinoma

(Source: Mukhopadhyay et al., 2011, reprinted by permission of Elsevier)

The stage of breast cancer when it is first diagnosed strongly impacts the outcome of the disease. In a clinical setting, the grading system which is commonly used is the TNM classification: this system requires information on tumour size (T), the extent of cancer spread to nearby lymph nodes (N) and metastasis (M) to assign stages of the cancer. The combination of T, N and M information helps clinicians diagnose stages of breast cancer. Stages of breast cancer start from stage 0 (carcinoma in situ), stage I (an early stage of invasive carcinoma), stage II (tumours spread to 1-3 lymph nodes), stage III (tumours spread to more than four lymph nodes but no metastasis) and stage IV (metastasis) ("How is breast cancer staged?", 2015).

2.2.3 Classification of breast cancer types

Breast cancers can be classified based upon their characteristic appearance under the light microscope (histopathological classification). The most common type of breast cancer is invasive ductal carcinoma which comprises approximately 70 – 80% of all breast cancers. The invasive ductal carcinoma originated from ducts of mammary glands similar to ductal carcinoma in-situ. While both originate from malignant epithelial cells, invasive ductal carcinoma spreads to nearby lymph nodes while ductal carcinoma in situ is confined to mammary ducts. Another type of breast cancers is invasive lobular carcinoma which is uncommon. It is found in only 5 – 10 % of breast cancers. The invasive lobular carcinoma originates from lobules (Liu, 2001). For research and therapeutic purposes, the molecular subtypes of breast cancers have been identified using the presence and absence of estrogen and progesterone receptors as biological markers. Estrogen is responsible for proliferation and progesterone causes differentiation in normal breast development (Russo & Russo, 2004). Some types of breast cancer respond to hormonal treatment but others do

not. It depends on the presence of hormonal receptors and hormone susceptibility of the cancer cells. Breast cancers are classified into three different types.

First, hormone receptor positive breast cancer is a type of cancer that responds to endocrine therapy (both estrogen and progesterone). Approximately 75% of all breast cancers respond to estrogen (ER+), about 60% of all breast cancers react to progesterone (PR-positive) and about 50% of all breast cancers react to both hormones (ER/PR-positive). In these types of breast cancer, hormone receptors (estrogen or progesterone) are found abundantly in the tumours. These types of breast cancer are more likely to respond to endocrine therapy in comparison with other breast cancer types. Secondly, around 20% of all breast cancers are found with excess human epidermal growth factor receptor 2 (HER2) proteins. They are more aggressive and faster growing than other breast cancer types and they are less sensitive to endocrine therapy. However, several treatments have been developed for treatment of HER2-positive breast cancer such as Herceptin, Tykerb and Perjeta. These cancers respond well to treatments. The third type of breast cancers account for only 10% of all breast cancers. They are less likely to respond to endocrine therapy (Ratini, 2015). The breast tumours lack estrogen and progesterone receptors and also do not overexpress HER2 protein therefore they are known as “triple-negative breast cancer”. This type of breast cancer associates to breast cancer gene (BRCA1 and BRCA2) mutation. There is no targeted therapy available for this type of cancer and they have a poor prognosis. However, they respond well to additional therapy after surgery (adjuvant therapy) (Movva, 2015).

Both histopathological and molecular classifications help physicians and patients obtain useful information on the nature of the cancer that has been found and to determine the most suitable treatment.

2.2.4 Estrogen and breast cancer

Estrogen is the predominant circulating steroid sex hormone in pre-menopausal adult females and the most biologically active ovarian steroid. In contrast to other hormones, estrogen is a group of hormones consisting of nine estrogens but the three major ones are 17β-estradiol (E2), estrone (E1), and estriol (E3) (Okoh et al., 2011).

The estrogens are synthesized primarily in the ovaries while androgen conversion in peripheral tissues serve as a secondary source of estrogen production in premenopausal women (Simpson, 2003). Estrogens are also synthesized in males but the level in peripheral

blood is lower (average 69 pg/ml) than that in the blood of non-ovulating women (average 199 pg/ml) (Jiang et al., 1973). In addition to estrogens produced by endogenous sources, the level of circulating estrogen might be increased by the use of synthetic medicinal estrogens (Okoh et al., 2011).

The role of estrogen in breast carcinogenesis is widely recognized. The World Health Organization (WHO) lists estrogens (both endogenous and exogenous) as carcinogenic in humans. Prolonged exposure to estrogen in one's lifetime is considered a major risk factor for breast cancer, and many known risk factors for the disease are related to a woman's reproductive history such as early menarche, nulliparity or late pregnancy, late menopause, prolonged oral contraceptive use and hormone replacement therapy (American Cancer Society, 2013).

Estrogens cause breast cancer through two different pathways: production of reactive oxygen species from estrogen metabolites causing DNA damage and mutation; and binding with estrogen receptor causing uncontrolled cell proliferation. Oxidative metabolism in steroidal estrogen pathways is summarised in figure 2-4. Estrogen metabolites can cause oxidative stress and damage which leads to carcinogenesis. E2 is metabolized to catechol estrogens 2-hydroxyestradiol (2-OHE2) and 4-hydroxyestradiol (4-OHE2) by cytochrome P450 1A1 (CYP1A1) and cytochrome P450 1B1 (CYP1B1), respectively (Cavalieri & Rogan, 2004). 4-OHE2 is highly genotoxic while 2-OHE2 and 2-methoxyestradiol (2-MeOE2) exhibit chemoprotective effects (Mense et al., 2009). Quinone metabolites derived from oxidative metabolism of estrogen are able to bind covalently to DNA causing covalent DNA adducts and resulting in DNA damage. Superoxide radicals may be formed by the reaction between semiquinone intermediates and oxygen which also contributes to oxidative stress and DNA damage.

Breast cancer patients showed higher levels of oxidative stress than healthy controls (Tas et al., 2005). Estrogen-induced breast cancer showed a similar pattern of oxidative stress modulation where levels of the lipid oxidation marker (8-isoprostane) were higher in the breast tissues of rats treated with E2 compared to their age-matched controls. Moreover, antioxidant enzymes, superoxide dismutase and glutathione peroxidase, were up-regulated in animals treated with E2, which may have resulted from higher production of reactive oxygen species (Mense et al., 2009).

Another pathway of estrogen in causing breast cancer relates to the estrogen receptor. The function of estrogens in ER+ breast cancer are similar to their function in breast development where the hormones bind with ER resulting in cell proliferation. In

addition, the number of ERs increases in breast cancer tissues compared to normal mammary tissues (Miyoshi et al., 2010). 16α-hydroxyestrone (16α-OHE1) has been found to be correlated with mammary tumour incidence. This metabolite was found to stimulate proliferation by binding with estrogen receptor and DNA of mouse mammary epithelial cells *in vitro* (Yager & Davidson, 2006). An *in vivo* study using female ACI rat model showed that proliferative change of the mammary gland was observed after 7 days exposure to E2 and within 120 days after exposure, atypical ductal proliferation was found. After 240 days of exposure, *in situ* and invasive carcinomas were found in rats treated with E2 (Mense et al., 2009).

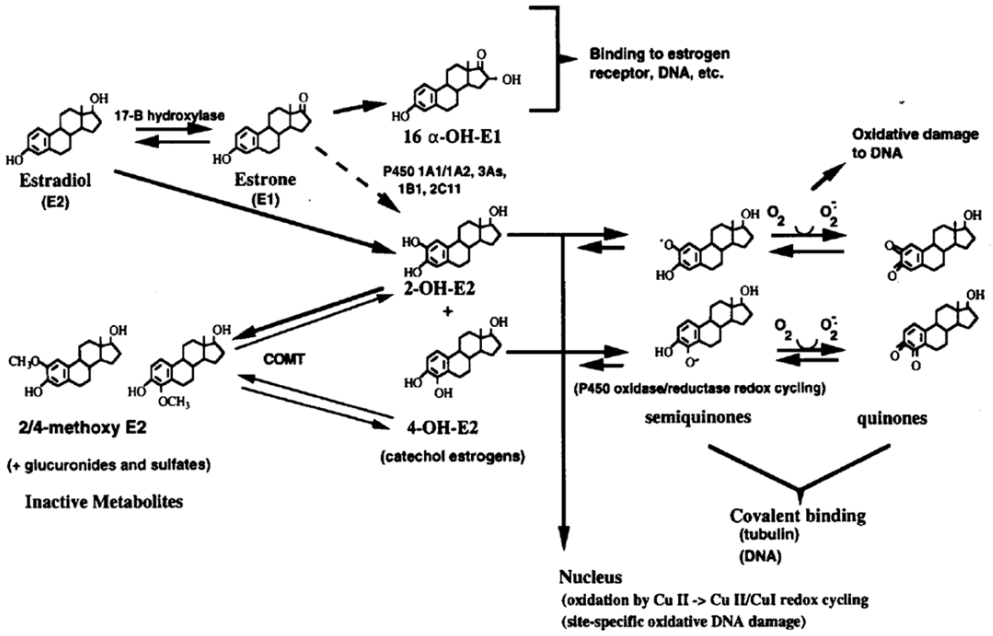


Figure 2-4 Oxidative metabolisms of steroidal estrogens. (Permission not required, Source: Yager & Liehr, 1996)

2.2.5 Immune system and breast cancer

The immune system is important in organisms as it protects the organisms against disease. In breast cancers, the immune response is triggered in response to cancer progression and inflammation (Standish et al., 2008). Several immune markers have been studied in breast cancers such as natural killer cells (NK cells), T lymphocytes (T-cell) (Strayer et al., 1984; Ben-Eliyahu et al., 1999; Campbell et al., 2005). NK cells are part of an innate immune system. A major function of the cells is to destroy cells that presented with abnormally lower level of major histocompatibility complex I (MHC I) (a cell-surface marker) such as tumour cells. The level of NK cell activity could be used to predict the risk

of breast cancer where people with family history of breast cancer have significantly ($P \leq 0.05$) lower NK cell activity than people from families with lower incidence of breast cancer (Strayer et al., 1984).

However, a comparative study between 30 women with breast cancer and healthy controls revealed that the NK cells activity of breast cancer patients were lower than healthy controls. The author suggested that stress caused by tumour growth might depress NK cell activity (Tsavaris et al., 2002). T cells are part of the adaptive immune system where the cells are activated and secrete cytokine to regulate or assist immune response. A study found that in breast cancer patients, the percentage of T cells (CD4+ and CD8+) was significantly lower when compared with healthy controls (Campbell et al., 2005). The study also showed a positive correlation between T-cell cytokine level and micrometastases in bone marrow and lymph nodes.

A recent study demonstrated that an immune-targeted vaccine effectively improved progression-free survival (PFS) in breast cancer patients with stable metastases compared to non-vaccinated patients. The vaccine targeted the immune response of mammaglobin-A (MAM-A), a secretory protein that is overexpressed in 80 % of primary and metastatic human breast cancers (Tiriveedhi et al., 2013). After vaccination, the frequency of MAM-A–specific CD8 T cells and the number of MAM-A–specific IFNγ-secreting T cells increased significantly ($P < 0.001$) (Tiriveedhi et al., 2014).

Even though immune-based therapies are of interest, HER2+ breast cancer receives the most benefit from the immune response modulation therapy. The main focus of our study is hormone-dependent breast cancer. Therefore the immune function and immune response in this study is not assessed.

2.2.6 Factors that influence breast cancer risks

In order to treat or prevent breast cancer, it is important to know the cancer risk factors that show a high statistical correlation with the occurrence of the disease. However, correlation does not mean that risk factors are necessarily a true or direct cause of breast cancer (American Cancer Soceity, 2013). Breast cancer risk factors can be classified into two major groups: non-modifiable and modifiable risk factors. Breast cancer risk factors are summarised in figure 2-5.

Non-modifiable risk factors

Most of the non-modifiable risk factors relate to endogenous estrogen exposure during one's life time. Family history of multiple-case breast cancers has been found to relate to mutations in two major breast cancer susceptibility genes, BRCA1 and BRCA2 (Miki et al., 1994). The breast cancer risk is 1.8 times higher in women who have one first degree relative with breast cancer, moreover the risk increases to nearly three times in women who have two first degree relatives with breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 2001). Personal history of breast abnormalities such as

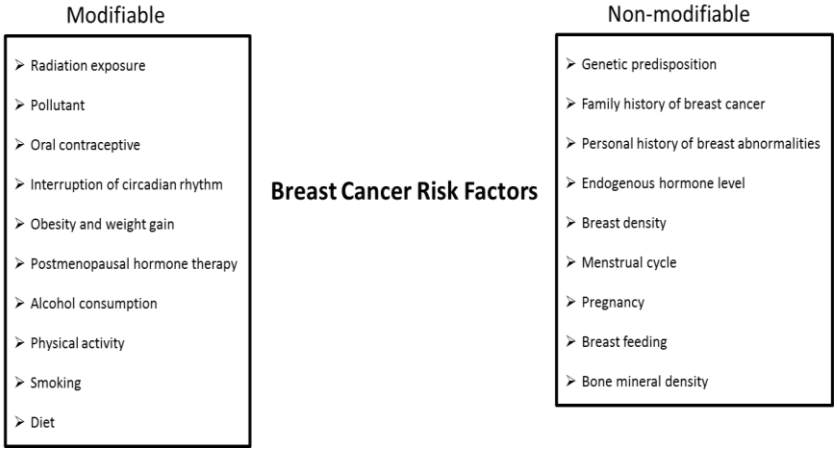


Figure 2-5 Breast cancer risk factors (Source: modified from American Cancer Society, 2013)

proliferative lesions are also related to the risk of breast cancer later in life. Women found with proliferative lesions without atypia have a 1.5 to 2-fold higher breast cancer risk and up to 5 times higher if they have had proliferative lesions with atypia compared to women who have never been diagnosed with any lesions (London et al., 1992; Hartmann et al., 2005; Kabat et al., 2010). The risk for women with LCIS is 7 - 12 times higher than for women without LCIS (Kilbride & Newman, 2010). Breast cancer survivors aged under 40 have a 4.5 times higher chance of getting a second breast cancer than women without a history of breast cancers (American Cancer Soceity, 2013).

Breast density (amount of breast and connective tissue relative to fatty tissue in the breast) also exhibits a strong, independent and positive relationship with breast cancer development (Tamimi et al., 2007). The risk increases 4-6 times in women with high breast density, therefore additional screening processes should be considered (Brower, 2013). Early menarche (before age 12) and late menopause (after age 55) contribute to a higher breast cancer risk (Kelsey et al., 1981) which is attributed to prolonged exposure to

estrogen. Pregnancy and breast feeding contribute to lowering breast cancer risk. The benefit of pregnancy is achieved only in the first full term pregnancy especially at an age under 30 (Kelsey et al., 1993; Lambe et al., 1994). A collaborative reanalysis of individual data from 47 epidemiological studies in 30 countries, including 50,302 women with breast cancer and 96,973 women without the disease, showed that every 12 months of prolonged breast feeding resulted in lowering the cancer risk by 4.3 % (Beral et al., 2004). Mammary gland maturation only presents after full term pregnancy and results in transformation of highly proliferative and susceptible structures (LB 1) to less proliferative and susceptible structures (LB 2, 3) (Russo & Russo, 2004). Moreover, breast-feeding also maintains the maturity of gland structure therefore reducing breast cancer risk.

Bone mineral density (BMD) is observed as a marker for cumulative estrogen exposure. Therefore, high estrogen exposure results in high bone mineral density which subsequently leads to higher breast cancer risk. A recent meta-analysis showed that high BMD in the hip and spine are associated with an increased risk of breast cancer by 62% and 82%, respectively (Qu et al., 2013). However, high BMD is also observed in women after weight training exercise, yet regular exercise was shown to reduce breast cancer risk (Thune et al., 1997). Even though high BMD may relate to breast cancer risk, the BMD should not be used as the sole information for a prediction of breast cancer risk. Other modifiable risk factors should also be taken into consideration.

Modifiable risk factors

Modifiable risk factors are mainly lifestyle risk factors such as working hours or working conditions, physical activities, and diets (figure 2-5). Working irregular hours (night shift) after extended periods led to moderate increase in breast cancer risk. Data from a 10 year follow up study showed that the breast cancer risk of women who worked at least three rotating night shifts for 1 – 29 years showed a relative risk of breast cancer of 1.08 while the relative risk of breast cancer in women who worked night shift for more than 30 years increased moderately to 1.36 (Schernhammer et al., 2001).

Women who have a history of exposure to radiation (atomic bomb survivors or high-radiation treatment history) were found to have an increased breast cancer risk, particularly if exposure began at an early age (Preston et al., 2002). As mentioned previously (Non-modifiable risk factors), prolonged exposure to endogenous sex hormones resulted in higher breast cancer risk, similarly using synthetic hormones (progestin with

estrogen) in post-menopausal hormone therapy also increased breast cancer risk. High-dose estrogen oral contraceptive increases breast cancer risk by 10% to 30% but the effect is diminished when contraceptive use stops (Collaborative Group on Hormonal Factors in Breast Cancer, 1996).

The effect of physical activity on breast cancer risk is clear in premenopausal women. The risk was reduced in women who exercised at least 4 hours per week. Women who had higher activity at work were also found to have a lower risk of developing breast cancer (Thune et al., 1997). An equivalent of 1.25 - 2.5 hours of brisk walking per week reduced breast cancer risk by 18% in postmenopausal women. The risk reduction was observed in women who were in the low to middle tertile of body mass index (< 28.4), interestingly the effect of exercise was greater in leaner women (BMI < 24) (McTiernan et al., 2003). A study showed that average walking for 3-5 hours per week also benefits women who have been diagnosed with breast cancer by reducing the rate of death from breast cancer (Holmes et al., 2005). Biological mechanisms that might explain the reduction of the breast cancer risk by increasing physical activities have been proposed. The mechanisms were: alteration of endogenous sexual hormones and growth factors, changing immune function and lowering central adiposity and obesity (Friedenreich & Orenstein, 2002).

An effect of obesity on breast cancer risk depends on menopausal status. In postmenopausal women, it is clear that overweight women and obese women had a 1.5 and 2 times higher breast cancer risk than average body weight women, respectively (La Vecchia et al., 2011). Alcohol consumption was found to associate with an increase of breast cancer risk with the plausible mechanisms included increasing endogenous estrogen and androgen, increasing mammary gland susceptibility to carcinogenesis and induction of mammary gland DNA damage (Singletary & Gapstur, 2001).

One of the most important modifiable breast cancer risk factors is diet. Several studies have been carried out to seek a relationship between diet and breast cancer risk (Bagga et al., 1995; Aubertin-leheudre et al., 2008). A study on low fat and high fibre diets on blood estrogen were carried out in a group of 12 premenopausal women. The subjects consumed normal diet (30% energy from fat, 15-25 g fibre / day) for one month then the diet was changed to low fat and high fibre diet (10% energy from fat, 25-35 g fibre) for another two months. The results showed that estrone and estradiol levels in the serum decreased significantly without any interference in ovulation of the subjects. Since estrogen

is a progesterone receptor inducer which affects breast cell proliferation rates and breast cancer risk, decreasing the circulating estrogen level may result in lowering the chance of getting breast cancer (Bagga et al., 1995). Aubertin-leheudre et al. (2008) investigated the link between dietary fat: fibre ratio and estrogen metabolism in order to explain the association between diet and breast cancer risk. In this study, half of the participating healthy women were put on a high-fat, low-fibre diet and the other half were given a low-fat, high-fibre diet. The results of this study showed that the women on the high-fat, low-fibre diet had significantly increased total estrogens measured in the urine. The study also showed that total fat intake correlated significantly with plasma concentrations of specific forms of estrogens including estrone, estradiol, urinary 2-hydroxyestrone, 2-hydroxyestradiol, 2-hydroxyestrone:4-hydroxyestrone ratio and total urinary estrogens. The authors concluded that dietary fat affects estrogen metabolism more than fibre intake, and that one mechanism resulting in high estrogen values is an increased reabsorption of estrogens into enterohepatic circulation. Milk and dairy products are highly consumed in New Zealand and can contribute to saturated fatty acid (SFA) intake, which has a positive association with breast cancer risk (Khodarahmi & Azadbakht, 2014).

Bioactive compounds found in plants called phytochemicals are believed to play a key role in modifying the risk of cancer. Phytochemicals such as terpenes, carotenoids, flavones and tannins have been found to have anti-carcinogenic properties (Dillard & German, 2000). However, a significant finding from the enormous European Prospective Investigation into Cancer and Nutrition (EPIC; monitoring 500,000 individuals over eight year (1992-2000) showed that the inverse correlation between total fruit and vegetable intake and overall cancer risk is small (Boffetta et al., 2010). Nevertheless the lack of a strong overall correlation does not rule out specific fruit and vegetables being associated with reduced risks of particular cancers. For example, a more recent EPIC finding has indicated that colorectal cancer showed an inverse relationship with total fruit and vegetable intake and total fibre intake (Bradbury et al., 2014). Total fruit and vegetable intake might not have a direct effect on breast cancer, however, various mechanisms have been proposed such as induction of apoptosis (Mannal et al., 2010), modification of gut microflora (Burns & Rowland, 2005) and/or scavenging estrogen metabolites (Yuan et al., 1999) such as quinones and free radicals which are formed through catechol estrogen metabolism. Various dietary plants and their phytochemicals have been shown to exert a chemopreventive effect in breast cancer (Wang et al., 2012a). Evidence of phytochemical effects on breast cancer chemoprevention is shown in table 2-1.

Table 2-1 Effect of major phytochemical classes from various plants on breast cancer

Plants	Phytochemicals	Effect of phytochemicals on breast cancer molecular biomarkers	References
Soybean	Phytoestrogens: Genistein, daidzein	Tumour weight in rats that consumed soy significantly lower ($P < 0.05$) than control rats in DMBA-induced mammary tumorigenesis in rat model.	Kang et al., 2009
Green tea	Epigallocatechin-3-O-gallate (EGCG)	Mice that received green tea extract had lower tumour weight by 34.8% compared to water treatment control in a metastasis-specific mouse mammary carcinoma 4T1 model	Luo et al., 2014
Grapes	Resveratrol, anthocyanins, catechin, epicatechin, procyanidinB2, quercetin	A metastasis-specific mouse mammary carcinoma 4T1 model was used. Mice that received grape skin extract showed 40 % higher survival rate than water treated control mice. Lung metastasis was also inhibited in mice fed grape skin extract.	Sun et al., 2012
Apples	Triterpenoids	In DMBA-induced mammary tumorigenesis rat model, rats that received apple triterpenoids had lower total cumulative tumour weight than DMBA-treated rats without apple triterpenoids.	He et al., 2012

As shown in table 2-1, these concepts of diet and their relation to breast cancer are of interest. However, few studies have investigated possible links between blueberry consumption and breast cancer development. Therefore blueberries (which are of growing commercial importance in New Zealand) were chosen for the project. Their relation to breast cancer is explored in greater detail in the following sections.

2.3 Intestinal microflora

The human digestive tract contains a complex ecosystem of intestinal microflora. There are approximately 100 trillion microorganisms in human intestine with bacteria making up most of the population (Sekirov at al., 2010). Gut microflora colonize a baby's gastrointestinal tract after birth (O'Hara & Shanahan, 2006). The colonisation pattern in each individual is affected by the type of delivery, type of diet and other environmental factors such as country of origin (Guarner & Malagelada, 2003). A relationship between humans and their beneficial gut microflora is a mutualistic relationship (where each benefits from the relationship).

It is now generally accepted that the composition of the human intestinal microbiota has an important role in health and disease. An extensive review by Sekirov et al. (2010) showed that beneficial gut microflora benefit their host's health in various ways. Some bacteria, such as lactobacilli and bifidobacteria, are necessary for optimal health and provide numerous physiological functions such as improving the barrier function of the intestinal wall, competing with and suppressing pathogenic bacteria and yeasts, modulating or stimulating the immune response, reducing inflammation and playing a role in nutrient and enzyme synthesis and absorption (Schrezenmeir & de Vrese, 2001; Penner et al., 2005; Medellin-Peña & Griffiths, 2009). The possible benefits of gut microflora on host health are summarised in table 2-2.

On the other hand, an imbalance of enteric bacteria may result in pathogenic conditions. Several bacteria such as *E. coli* and *Clostridium spp.* produced β-glucuronidase enzyme which generates carcinogens, mutagens, and tumour-promoting agents in the large bowel (Goldin, 1990). Moreover, the population of *Clostridium* spp., *Bacteroides* spp., and *Bifidobacterium* spp. increased in colorectal cancer patients (Scanlan et al., 2008). Interestingly, diet can manipulate enteric bacteria composition. Consumption of western diet resulting in weight gain also modulated the intestinal microflora composition *in vivo*, and a reverse effect was achieved when a diet to limit weight gain was consumed (Turnbaugh et al., 2008).

Table 2-2 Possible beneficial function of intestinal microflora

(Source: modified from Sekirov et al., 2010).

Beneficial functions of intestinal microflora	**Intestinal bacteria**
Immunomodulation	*Lactobacillus* spp.
Gastrointestinal tract maturation and function	*Bacteroides thetaiotaomicron*
Modification of stress response	*Bifidobacterium infantis*
Provide nutrients	*Bifidobacterium* spp. and *Clostridium spp.*
Xenobiotic metabolism	*Bifidobacterium* spp., *Clostridium* spp., and *Oxalobacter formigenes*

Impact of gut microflora on breast cancer risk

It has been found that increased estrogen exposure is a risk factor for the development of breast cancer and the changes in intestinal flora may alter either estrogen metabolism or carcinogen exposure (Byrne, 2009). Dietary components, pharmaceuticals, and steroid sex hormones with hydrophobic properties are transported to the liver, where they undergo oxidation, resulting in the corresponding hydroxyl compounds. The latter are subsequently conjugated with either glucuronic acid or sulfate to increase their water solubility and facilitate their urinary or biliary excretion. Compounds excreted in bile reach the large intestinal tract, where they undergo deconjugation by the bacterial hydrolytic enzymes such as β-glucuronidase and sulfatase (Mcbain & Macfarlane, 1998). These bacterial enzymes catalyse the cleavage of terminal glucuronic acid or sulphate, releasing the harmful metabolites including some steroid sex hormones. Consequently, the deconjugated metabolites may be reabsorbed and enter the enterohepatic circulation. Detoxification process and enterohepatic circulation is presented in figure 2-6.

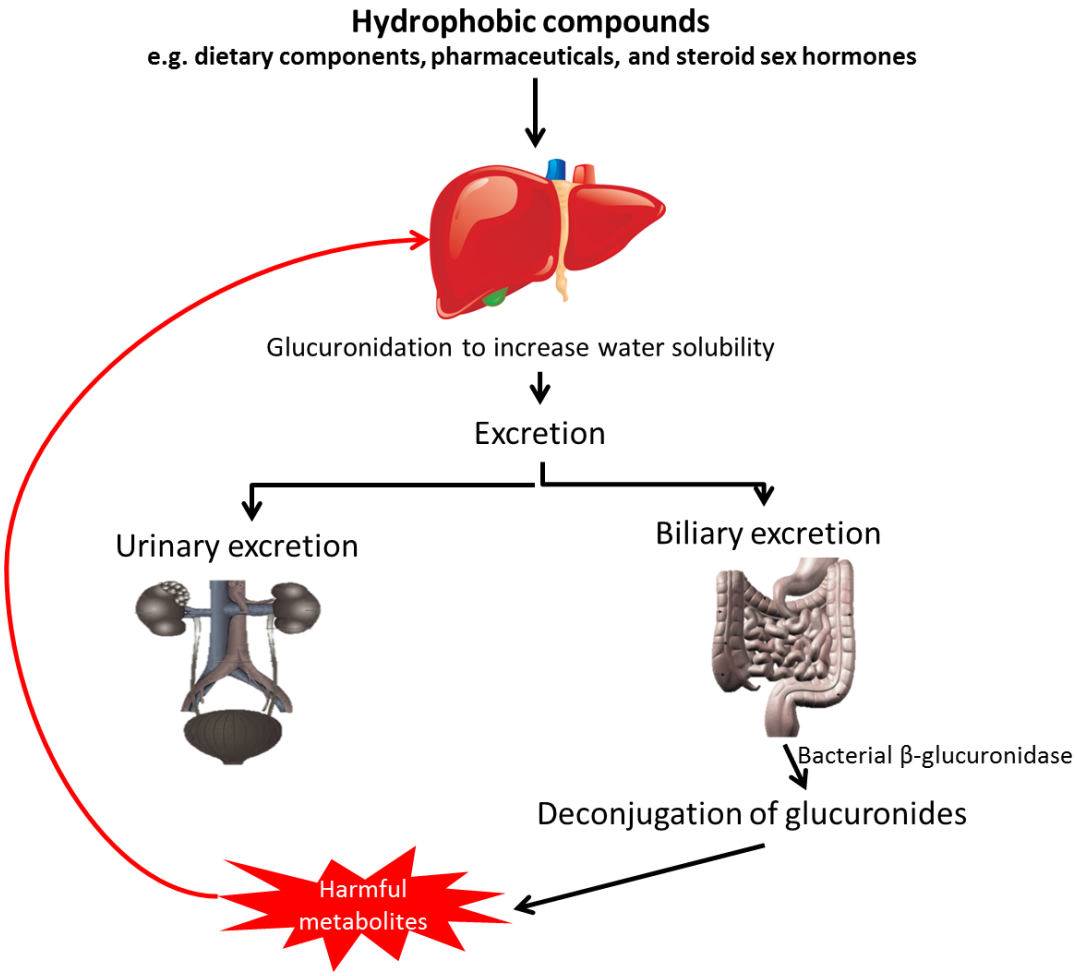


Figure 2-6 Detoxification mechanism and enterohepatic circulation as affected by intestinal microflora.

It has been shown that the natural estrogens, estrone (E1) and 17β-estradiol (E2), are excreted from human bodies through urine or via bile as conjugated forms, estrogen glucuronides (E2-3-β-D-glucuronide) or estrogen sulfonides, and conjugated estrogen did not show estrogenicity (Tanaka et al., 2009). Glucuronidation together with sulfation are the most useful forms of conjugation observed during metabolic processes in mammals and also represent the major pathway for detoxification mechanisms which occur in the liver (Beaud et al., 2006). About 50% of the estrogen metabolites appear in bile in conjugated forms, but only 7% of the metabolites appear in the feces, while the rest are hydrolysed to the free hormone and reabsorbed in the intestinal tract (Adlercreutz et al., 1976). The study also showed that the administration of antibiotics causes a significant increase in the fecal excretion of estrogen conjugates which means intestinal flora played an important role in estrogen metabolism (Adlercreutz et al., 1976). However, using antibiotics in order to increase excretion of conjugated estrogen is not recommended. A

breast cancer animal study was done using germ-free rats in comparison with gnotobiotic rats, with both receiving a high lignan diet. The study showed that tumour number per tumour-bearing rat, tumour size and tumour cell proliferation was reduced in gnotobiotic rats. Lignan in food matrix was transformed to enterolignans enterodiol and enterolactone which did not occur in germ-free rats (Mabrok et a., 2012). Therefore the presence of intestinal microflora benefits host's health.

In addition, incubation of estradiol-3-glucuronide at low concentrations with fecal bacteria from rats and humans resulted in rapid hydrolysis to free estradiol and it has been concluded that multiple androgen and estrogen conversions can occur in the large bowel of both animals and humans, and these conversions are affected by diet, antibiotics, and the makeup of the resident microflora (Eriksson & Gustafsson, 1970). One study found that supplementation with probiotic lactobacilli in rats suppressed the reabsorption of bile acids into the enterohepatic circulation and enhanced the excretion of acidic steroid hormones in the faeces (Hosono, 2000) and it has been concluded that if gut bacteria can reduce the reabsorption of estrogens by promoting bile excretion from the body, it would reduce excess estrogens associated with increased breast cancer risk.

2.4 Bioactive compounds in blueberries and blueberry pomace

Blueberries are produced by a number of species of plant in the genus *Vaccinium*. Other plants in this genus are the cranberry (*V. macrocarpon*), ligonberry (*V. vitis-idaea*) and bilberry (*V. myrtillus*). According to agricultural importance, there are three main species of blueberries, lowbush, highbush and rabbiteye. Most blueberries are grown in loam and acidic soil (pH 4.5 to 5.5) with good drainage. Lowbush blueberries (*V. angustifolium* and *V. myrtilloides*) are named so because of the height of the plant (30 to 45 cm), whilst another type which can reach 1.8 m is called highbush blueberry (*V. corymbosum*) (Gough, 1994). Rabbiteye blueberry (*V. ashei*) fruits are similar to highbush blueberry except for their thicker skin and the gritty texture of the seed (figure 2-7). Moreover, rabbiteye blueberries suit warmer climates since they require only 400 to 500 chilling hours per year (Trehane, 2004). In New Zealand, blueberry production is based mainly on the highbush type followed by rabbiteye each of these species has many commercial cultivars. Although rabbiteye blueberries cannot tolerate extremely low temperature, they are suitable for cultivation throughout New Zealand. Because rabbiteye

blueberries have a long flowering to ripening period, their fruits ripen later than highbush (Feb – April), therefore rabbiteye blueberry production in New Zealand has increased ("Blueberry," 2012).

Figure 2-7 Rabbiteye blueberries (*Vaccinium ashei*).

(Source: Photo by Manuel; https://www.flickr.com/photos/martius/14244643729)

A by-product from blueberry juice processing, blueberry pomace, consists of skin, seed and pulp of the fruit (Goni & Hervert-Hernandez, 2011). Blueberry pomace has been used for landfill, fertilizer or animal feed. Blueberry pomace is low in sugar (< 2.05 g/100 g) and high in dietary fibre (more than 70%) ("Blueberry Fibre," 2015). The pomace is good for the addition of fibre to food products and can also be used for preparation of gluten-free products (Mišan et al., 2014). Additionally, blueberry pomace is a good source of blueberry polyphenols specifically anthocyanins as the blueberry skin contains more than 180 mg total anthocyanins in 100 g of berry skins (Lee & Wrolstad, 2004). The high polyphenol content has been associated with several biological activities including anti-inflammation, antioxidants, lowering cholesterol and prebiotic properties (Kalt et al., 2000; Kim et al., 2010; Gil, 2013; Li et al., 2014; Salaheen et al., 2015).

2.4.1 Total polyphenolic concentration

Blueberry polyphenols can be divided into two classes based on their chemical structure. The first class, flavonoids, consists of six major subclasses: anthocyanidins

(cyanidin, delphinidin), flavanols (catechin, epicatechin), flavanones (hesperitin, narigenin), flavones (luteolin, tangeritin), flavonol (myricetin, quercetin), and isoflavonoids (daidzein, genistein). Non-flavonoid class contains phenolic acids (benzoic acid and cinnamic acid), stilbene (resveratrol, curcuminoids) and lignan (precursor of enterodiol and enterolactone) (figure 2-8) (Manach et al., 2004; Seeram, 2006; Etienne-Selloum & Dandache, 2013). Anthocyanins or glycosylated anthocyanidins are the major polyphenols presented in blueberries. Chlorogenic acid is a non-flavonoid polyphenol which is also found abundantly in blueberries (Kalt et al., 2008).

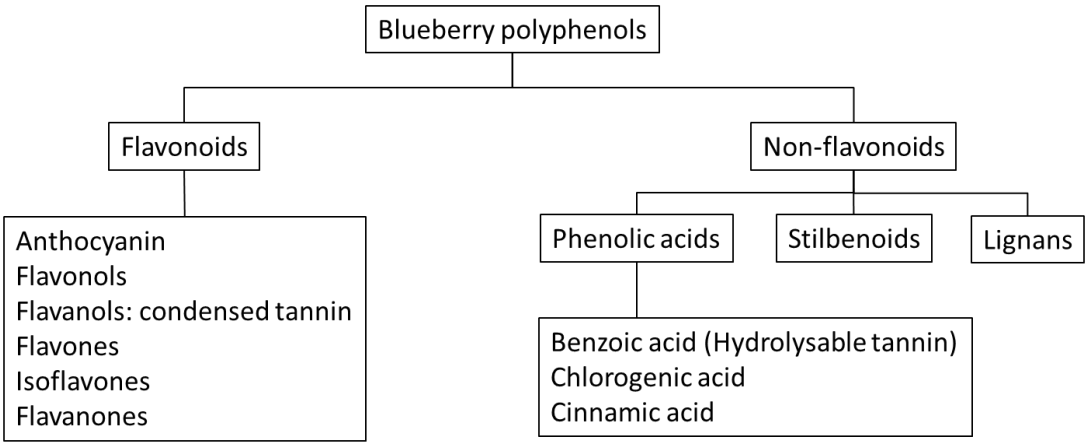


Figure 2-8 Polyphenols classification in blueberries

(Source: modified from Manach et al., 2004; Seeram, 2006; Etienne-Selloum & Dandache, 2013)

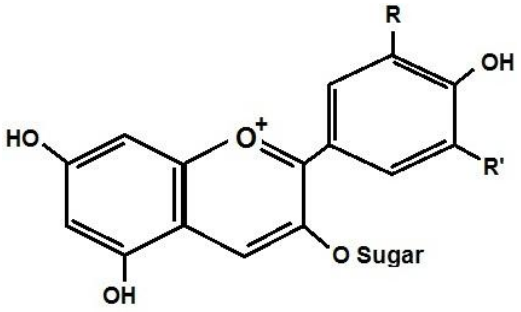
The total phenolic concentration (TPC) of blueberries reported previously for genotypes grown in different countries varied widely. It is difficult to compare total polyphenolic contents of blueberries from different studies. Factors impacting on TPC includes genotype, growing conditions, heat treatment, storage time and temperature and experimental conditions especially extracting solvents. TPC values reported in literature are summarized in table 2-3. TPC values from rabbiteye blueberry extracted by different solvents ranged from 230 to 930 mg/100 g berries (Prior, 1998; Connor et al., 2002; Moyer et al., 2002; Sellappan et al., 2002; Rodrigues et al., 2011; You et al., 2011; Li et al., 2013) while highbush ranged from 274 to 833 mg/100 g berries (Lee & Wrolstad, 2004; Reyes et al., 2011). A report showed that growing system (organic and conventional) affected TPC values where organic blueberries showed higher TPC than blueberries grown conventionally (You et al., 2011). Blueberry pomace also contained high polyphenolic contents approximately 32 to 41 mg GAE/g dried weight (Kitchen, 2013; Li et al., 2013). Li's

study showed that blueberry pomace extract contained higher TPC than the fruit extract. Kalt et al. (2000) also reported that flavonoid anthocyanins and chlorogenic acid are two major contributors of TPC of blueberries.

Total polyphenolic content of blueberries were found to correlate well with their antioxidant activities (Dragović-Uzelac et al., 2010; You et al., 2011). This may lead to an assumption that blueberries with high TPC will demonstrate high antioxidant activity.

2.4.2 Anthocyanins

Anthocyanins are abundant in berry fruits. They belong to a flavonoid subclass. Since the name "anthocyanin" is derived from two Greek words "anthos"= flower and "kyanos" = blue, they contribute to the colours red, blue and purple of plants. Basic anthocyanin structure contains two benzene rings (A and B) which are joined by a heterocyclic ring C. The anthocyanin structure is presented in figure 2-9. Six anthocyanins found in nature result from different substitutions at 3 and 5 position in the B ring.



Anthocyanin

	R	R'
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH_3	OCH_3
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Pelargonidin	H	H

Figure 2-9 Structure of anthocyanidins commonly found in blueberries (Source: modified from Lee et al., 2005)

Table 2-3 Total phenolic concentration of blueberries and blueberry pomace.

Blueberry types	Cultivars	Solvent	Total phenolics (mg/100 g fruit)	References
Rabbiteye	Powder blue, Climax, Tifblue, Woodward	Methanol/water/acetic acid	300-489	You et al., 2011
Rabbiteye	Bluegem	Acetone/water	717	Moyer et al., 2002
Rabbiteye	N/A	Acidified methanolic extract	574-831	Connor et al., 2002
Rabbiteye	Austin, Brightblue, Brightwell, Climax, Tifblue	Methanol/HCl	270–929	Sellappan et al., 2002
Rabbiteye	Brightwell (fruit)	Methanol/water/glacial acetic acid	269	Li et al., 2013
	Brightwell (pomace)		412	
Rabbiteye	Climax, Brightwell, Tifblue, Little Giant	Acetonitrile/ acetic acid	230.8-457.5	Prior, 1998
Rabbiteye	Brightwell, Tifblue (Pomace)	Acetone/water/ HCl	324	Kitchen, 2013
Rabbiteye	Delite, Woodard, Climax, Bluegem, Florida M, Powderblue, Briteblue, Bluebelle, Tifblue	Methanol	288 to 694	Rodrigues et al., 2011
Highbush	Duke	Methanol	832.9	Reyes et al., 2011
Highbush	Rubel (whole berries)	Acetone/water	737.3	Lee & Wrolstad, 2004
	Rubel (skin)		300.4	

Of the six major anthocyanidins found naturally in the food matrix (cyanidin, delphinidin, malvidin, petunidin, peonidin and pelargonidin), blueberries have been found to contain five, (all except pelargonidin); this is more diversity than is found in most species (Hamamatsu et al., 2004; Wang et al., 2012b; Li et al., 2013). Structure and color of anthocyanins depend on their environmental pH. The color of anthocyanins at low pH (pH1) is red and the anthocyanins are in the flavylium cation form. The structure changes to either the carbinol base or chalcone when the environmental pH is higher (around pH 4.5). At this stage the anthocyanins are colourless. The anthocyanins turn blue when the environment becomes more basic (above pH 7) because of quinoidal base formation (Giusti & Wrolstad, 2001).

Total anthocyanin concentration varies on the studies. Types of blueberries, cultivars, growing conditions and experimental conditions affected total anthocyanin concentration and their composition. Table 2-4 presents a range of total anthocyanins reported previously where the content ranged from 70 to 378.3 mg/ 100 g fruit and 120 to 436 mg/100 g pomace in blueberry fruits and pomace, respectively (Connor et al., 2002; Sellappan et al., 2002; Hamamatsu et al., 2004; Lee & Wrolstad, 2004; Rodrigues et al., 2011; You et al., 2011; Rodríguez-mateos et al., 2012; Wang et al., 2012b; Bener et al., 2013;Gil,2013).

In blueberries, anthocyanidins are found in the glycosylated form. Three major sugar moieties found conjugated with anthocyanidins form glucosides, galactosides and arabinosides. Glycosylated anthocyanins found in blueberries depend on blueberry genotypes, extraction methods and analysis methods. Individual anthocyanins found in blueberry fruits and pomace are listed in table 2-5. There were nine to fifteen anthocyanins present in blueberry fruits and four to nine anthocyanins were found in blueberry pomace (Hamamatsu et al., 2004; Wang et al., 2012b Bener et al., 2013; Gil, 2013; Li et al., 2013). Pomace derived from different processes showed variation of individual anthocyanins presented.

Table 2-4 Total anthocyanins of blueberries and blueberry pomace.

Blueberry types	Cultivars	Solvents	Total anthocyanin (mg/100 g berries)	References
Rabbiteye	Powder blue (organic) Climax Tifblue (organic) Woodward (conventional)	Methanol/ water/acetic acid	116-224	You et al., 2011
Rabbiteye	Delite, Woodard, Climax, Bluegem, Florida M, Powderblue, Briteblue, Bluebelle, Tifblue	Methanol	70 – 378.3	Rodrigues et al., 2011
Highbush/ Lowbush	N/A	Methanol/HCl	128.1/187.3	Rodríguez-mateos et al.,2012
Rabbiteye	N/A	Acidified metanolic extract	168-313	Connor et al., 2002
Rabbiteye	Austin, Brightblue, Brightwell, Climax, Tifblue	Methanol/HCl	12.7 – 197	Sellappan et al., 2002
Rabbiteye	Tifblue	Methanol/formic acid	129	Hamamatsu et al., 2004
Rabbiteye	Tifblue	Acetone/ water/ formic acid	230	Wang et al., 2012b
Highbush	Rubel (Whole berries)	Acetone/ water	230	Lee & Wrolstad, 2004
	Rubel (Skin)		188.5	
Blueberry pomace	N/A	Methanol/HCl	120	Gil, 2013
Blueberry pomace	N/A	Water/HCl	436	Bener et al., 2013

Table 2-5 Individual anthocyanins present in blueberry fruits and pomace

Anthocyanins	Hamamatsu et al. (2004)	Li et al. (2013)	Wang et al. (2012b)	Gil (2013)	Li et al. (2013)	Bener et al. (2013)
Blueberry types	**Rabbiteye**	**Rabbiteye**	**Rabbiteye**	**N/A (pomace)**	**Rabbiteye (pomace)**	**N/A (pomace)**
Extraction	**Methanol/ formic acid**				**Methanol/ water/glacial acetic acid**	**Water/HCl**
Delphinidin-3-galactoside	Present	Present	Present	Present	Present	Present
Delphinidin-3-glucoside	Present	Present	Present	Present		Present
Cyanidin-3-galactoside	Present	Present	Present	Present	Present	Present
Delphinidin-3-arabinoside	Present		Present			Present
Cyanidin-3-glucoside	Present		Present	Present		
Petunidin-3-galactoside	Present	Present	Present	Present		Present
Cyanidin-3-arabinoside	Present	Present		Present		Present
Petunidin-3-glucoside	Present	Present	Present	Present		Present
Peonidin-3-galactoside	Present	Present		Present	Present	
Petunidin-3-arabinoside	Present		Present			
Peonidin-3-glucoside	Present					Present
Malvidin-3-galactoside	Present	Present	Present	Present		Present
Peonidin-3-arabinoside	Present					
Malvidin-3-glucoside	Present	Present	Present	Present		
Malvidin-3-arabinoside	Present	Present	Present	Present	Present	

As mentioned previously on page 23 five anthocyanidins are found in blueberries, however the percentage compositions of anthocyanidins from different blueberry types vary. Delphinidin is the predominant anthocyanidin in highbush blueberries (Rodríguez-mateos et al., 2012; Pranprawit, 2013). However, more than 50% of the anthocyanidins found in rabbiteye blueberries consisted of malvidin (table 2-6) (Hamamatsu et al., 2004; Wu et al., 2006; Rodríguez-Mateos et al., 2012; Wang et al., 2012b; Gil, 2013). Percentage distribution of sugar moieties in different blueberry types were also investigated. Galactosides accounted for more than 60% of sugar moieties in highbush blueberries but they were found only up to 50% in rabbiteye blueberries.

Table 2-6 Percentage composition of anthocyanins in blueberry fruits

Anthocyanins	Hamamatsu et al. (2004)	Wang et al. (2012b)	Gil (2013)	Rodriguez-mateos et al. (2012)	Wu et al. (2006)
Blueberry types	**Rabbiteye**	**Rabbiteye**	**N/A**	**Highbush**	**N/A**
Malvidin	50.9	51.7	57.6	30.5	34
Delphinidin	6.4	11.1	5.4	39.3	31.2
Cyanidin	18	17.7	21.5	9.5	7.4
Petunidin	10.2	25.1	10.7	18.9	18.6
Peonidin	14.5	0	4.9	1.8	8.8

2.4.3 Phenolic acids

Phenolic acid is a large subclass of non-flavonoid polyphenolic compounds. In blueberries, the hydroxybenzoic acids were found in gallic acid and ellagic acid form (Samappito & Butkhup, 2010). However, the hydroxybenzoic acids do not receive great nutritional interest because there are only few edible plants present with the hydroxybenzoic acids. The hydroxycinnamic acids gain more attention since they are more common. Caffeic acid, ferulic acid and chlorogenic acid are examples of the hydroxycinnamic acids found in blueberries. Highbush and lowbush blueberries contained approximately 50 – 100 mg/100 g fruit weight of chlorogenic acid (Gao & Mazza, 1994). A comparative study among fruits in *Vaccinium* spp. (blueberries, cranberries, and ligonberries) showed that only blueberries contained chlorogenic acid (65 mg/100 g fruit weight) (Zheng & Wang, 2003). Wang et al. (2012b) reported that chlorogenic acid present in rabbiteye blueberries ranged from 10.5 to 68.6 mg/100 g fruit weight. Presence of chlorogenic acid in blueberries increased colour intensity of anthocyanin as a result of copigmentation (Mazza & Brouillard, 1999). However, to our knowledge there is no report on chlorogenic acid in blueberry pomace.

Chlorogenic acid exhibits several biological activities which might contribute to health benefits such as antioxidant activity, protection against DNA damage, anti-angiogenesis, and induction of phase II detoxification enzymes (Szaefer et al., 2004; Xu et al., 2012; Park et al., 2015).

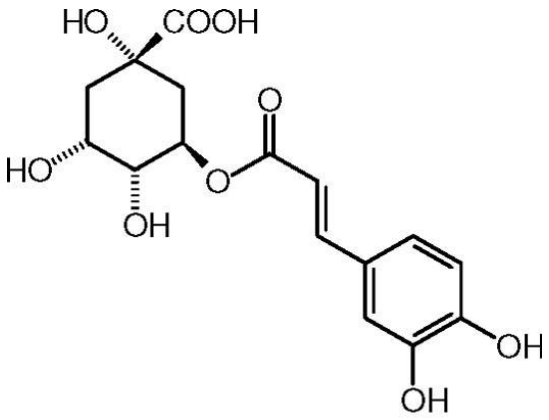


Figure 2-10 Structure of chlorogenic acid (Source: modified from Wu & Kang, 2012)

2.4.4 Blueberry dietary fibre

Dietary fibre exhibits many health benefits including but not limited to regularity promotion, enhancing weight loss, reducing risk of coronary heart disease, reducing blood pressure and serum cholesterol level, improving glycaemia and insulin sensitivity and reducing the risk of gastrointestinal disease (Anderson et al., 2009). Dietary fibre can be separated into two categories, soluble fibres that can be fermented in colon and insoluble fibres that possess bulking effect and have limited fermentation in colon. Recommended dietary fibre consumption is 28 and 36 g/day in women and men, respectively.

Blueberries contain both soluble and insoluble fibre. According to New Zealand food composition database, blueberries contain approximately 3.5 g dietary fibre /100 g fruit weight (Sivakumaran et al., 2015). One serving size of fresh blueberries contains 5 g dietary fibre, which is approximately 17% and 14% of recommended fibre consumption per day in women and men, respectively ("Blueberries: Frequently asked questions," 2002). A study showed that lowbush blueberries contained approximately 3.7 to 5 g/100 g fruit weight dietary fibre and pectin accounted for 7 to 10 % of their total dietary fibre content (Chen & Camier, 1997). Blueberry pomace consists of seeds, pulp and skin therefore is found with greater amount of dietary fibre. Bränning et al. (2009) reported that dietary fibre accounted for approximately 40% of blueberry pomace weight.

2.5 Biological properties of blueberries

2.5.1 Antioxidant activity

Consumption of fruit and vegetables has been proposed to reduce the risk of some chronic diseases, including cardiovascular disease and cancers (Hung et al., 2004). Besides vitamin C which contributes to antioxidant activity, fruits pigments such as xanthophyll, carotenoids and anthocyanins exhibit antioxidant activity as well (Wang et al., 1996). Blueberries exhibit antioxidant activity. A study among *Vaccinium* spp. showed that their antioxidant activities as measured by oxygen radical absorbance capacity (ORAC) ranged from 17 to 37 µmol Trolox equivalents (TE)/g of fresh berries, 13.9 to 37.8 µmol TE/g of fresh berries and 27.8 to 45.9 µmol TE/g of fresh berries in highbush, rabbiteye and lowbush, respectively (Prior et al., 1998). Moyer (2002) broadened the study by comparing fruits from *Vaccinium* spp. to other small fruits in *Rubus* spp. and *Ribes* spp. The fruits from *Vaccinium* spp. showed the highest overall mean ORAC values (62.5 µmol TE/g of fresh berries) followed by *Ribes* spp. (53.9 µmol TE/g of fresh berries) and *Rubus* spp. (52.4 µmol TE/g of fresh berries) (Zheng & Wang, 2003). Since blueberry antioxidants are based upon polyphenols and anthocyanins in the fruit, they may vary by species, cultivation systems and experimental conditions (Howard et al., 2003; Ju & Howard, 2003)..

The antioxidant activity of blueberries has been attributed to different classes of polyphenols as reported by Zheng & Wang (2003). By measuring ORAC values of HPLC fractions collected from each chromatographic peak, they found that anthocyanins accounted for more than 50% of the ORAC values while chlorogenic acid and other flavonols were responsible for 23% and 21%, respectively. In addition, ORAC values of blueberries were found to correlate positively with total polyphenolics or total anthocyanins where a stronger correlation was found with polyphenols than anthocyanins (Ehlenfeldt & Prior, 2001; Moyer et al., 2002; Zheng & Wang, 2003).

One study suggested that antioxidant activity of phenolic compounds depended on their ability to donate hydrogen atom (Rice-Evans et al., 1996). The study showed that the higher number of hydroxyl groups in anthocyanidins was associated with higher antioxidant activity. For example, malvidin, which contains four hydroxyl groups, has a lower antioxidant activity than delphinidin which contains six hydroxyl groups. The study also revealed that saturation of the C-ring in flavonoids (catechin) resulted in lower antioxidant activity compared to other flavonoids that contain an unsaturated C-ring (quercetin). Moreover, glycosylation of anthocyanidins also diminished their antioxidant activities. This

finding could explain the weaker correlation of total anthocyanins and ORAC because in nature anthocyanidins are generally found in glycosylated form.

Therefore, consumption of blueberries which present high *in vitro* antioxidant activity might lead to a protection of cells against free radicals. However, anthocyanins found in blueberries are poorly absorbed; in one study, anthocyanin was detected in human blood serum from 0.5 - 4 hours after consumption at very low concentration (1.4 to 592 nmol/L) (Kay, 2006). Regardless of their poor absorption, blood serum antioxidants of subjects consuming blueberries were up regulated in several studies (Kay & Holub, 2002; Mazza et al., 2002; Molan et al., 2008). The consumption of a high fat meal supplemented with 100 g of wild blueberry powder resulted in increasing $ORAC_{total}$ by 15% value in participants' blood serum in a single-blinded crossover study (Kay & Holub, 2002). In addition, 19 anthocyanins were detected in participants' blood serum samples out of 25 anthocyanins following consumption of a wild blueberry powder. The authors concluded that anthocyanins found in blood serum contributed to the higher ORAC values (Mazza et al., 2002). An ability of rabbiteye blueberry extracts in enhancing blood serum antioxidant *in vivo* was also documented (Molan et al., 2008). The study revealed that animals that received 1 mL of blueberry extracts ('Centurion' or 'Maru') for 6 consecutive days showed higher serum total antioxidant activity as measured by ferric reducing antioxidant power (FRAP) compared to animals receiving only water.

In vivo oxidative stress is a result of an imbalance between free radicals generated and antioxidant molecules in cells (Reuter et al., 2010). A high free radical environment can severely damage macromolecules such as protein, lipid and DNA. The damage to macromolecules is one major cause of breast cancer (Hakkak et al., 2013). Strong reactive oxygen species such as superoxide anions and hydrogen peroxide (H_2O_2), which can convert to hydroxyl radicals, are elevated in breast cancer patients' blood serum compared to their age-matched healthy controls. Similarly, malondialdehyde (MDA) level, a biomarker for lipid peroxidation, was higher in breast cancer patients than in healthy controls. With the high production of reactive oxygen metabolites, catalase (CAT) enzyme activity, which decomposes H_2O_2 into water and oxygen, decreased in breast cancer patients' blood serum (Ray et al., 2000). Total antioxidant status was evaluated and found to be lower in breast cancer patients' blood serum than their controls. A comparison between breast cancer tissues and non-malignant tissues revealed that MDA levels were higher while CAT activities were lower in breast cancer tissues than non-malignant tissues (Tas et al., 2005).

Similar to the previous report, the MDA level from the blood serum of breast cancer patients was also higher than the controls (Sener et al., 2007).

The *in vitro* free radical scavenging activity of blueberry extracts, also exhibit CAT activity (Samad et al., 2014). Freeze-dried blueberry extract was mixed with a standard rat chow and given to an animal model of hypertension related to renal injury. The study showed that catalase activity of animals that received blueberry-enriched diet increased markedly (Elks et al., 2011). Three weeks' consumption of standard feed supplemented with 10% freeze-dried whole blueberries did not show any alteration in lipid peroxidation levels in young, healthy, male Sprague-Dawley rats (Dulebohn et al., 2008). However, lipid peroxidation levels in a rat model of hypobaric hypoxia induced an oxidative stress decrease in animals given blueberry extract intraperitoneally compared to controls (Zepeda et al., 2012). Interestingly, this study also showed that there was no significant difference in lipid peroxidation between animals that received blueberry extract and control animals under normal condition. Taken together, these studies suggest that the effect of blueberries in modulating lipid peroxidation is minimal in healthy rats but significant under pathogenic conditions.

2.5.2 Prebiotic and antimicrobial activity

Gibson and Roberfroid (1995) defined a prebiotic as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that have the potential to improve host health". The most widely known prebiotics are semi-digestible carbohydrates such as fibre and resistant starches (Silvi et al., 1999) and non-digestible oligosaccharides (NDOs) (Swennen et al., 2006). Since NDOs are hardly digested in the small intestine, the molecules therefore reach the large intestine and become substrates for the gut microflora. The compounds specifically deactivate bacteroides, clostridia and *E. coli* while enhancing the growth of lactobacilli and bifidobacteria.

Prebiotics, such as fructooligosaccharides (FOS), are converted in the gut to short chain fatty acids (SCFAs) by intestinal bacteria. SCFAs, particularly butyrate, provide several beneficial functions such as providing energy for the cells that line the colon, promoting mucosal cell restoration, protecting the mucosal lining from damaging intestinal contents and stimulating mucous production that is an important part of the intestinal mucosal barrier (D'Argenio & Mazzacca, 1999; Shimotoyodome et al., 2000; Cherbut et al., 2003).

Furthermore, research suggests that butyrate may inhibit tumour formation in animal models of breast cancer. One study showed that rats fed a high-fat diet supplemented with butyrate and exposed to nitrosomethylurea (NMU) to induce breast cancer showed a decrease in tumour incidence by 20 - 52 percent compared to the NMU treated rats fed the high-fat diet alone (Belobrajdic & McIntosh, 2000).

Blueberry extracts contain phytochemicals, some of which are in glycosylated forms (McGhie et al., 2003). These compounds might be responsible for the prebiotic properties of the extracts. The glycosylated anthocyanidins might be hydrolysed by β-glucosidase enzyme and released a sugar moiety which could serve as an energy source for bacteria (Ávila et al., 2009). Blueberry extracts using acidified water (pH 2) were reported to enhance the growth of *L. rhamnosus*, *L. reuteri* and *B. lactis in vitro* (Sutherland et al., 2009). Blueberry extracts of two rabbiteye genotypes ('Centurion' and 'Maru') were studied for their prebiotic activity on *L. rhamnosus* and *B. breve*. *In vitro* assays were done using pure bacterial cultures incubated with blueberry extract at 10% and 25% concentration for five days (Molan et al., 2009b). The numbers of both species significantly increased after five days of incubation in comparison with the control incubation (without blueberry extracts). Prebiotic activities of blueberry extracts were also examined *in vivo*. A significant effect on the population size of lactobacilli and bifidobacteria was observed after administration of water extracts from the same genotypes to rats daily for 6 days in comparison with the control rats (Molan et al., 2009b). The numbers of bacteria from rat ceca were evaluated by fluorescence in situ hybridization (FISH). The results showed that in rats gavaged orally with blueberry extracts, the population of lactobacilli and bifidobacteria increased significantly. The authors concluded that the blueberry extracts could modify the bacterial profile by increasing the numbers of beneficial bacteria and thereby improving gut health.

Research has also been conducted to evaluate the prebiotic properties of wild blueberry powder drink on *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteriodes* spp., *Prevotella* spp., *Enterococcus* spp., and *Clostridium coccoides* in humans (Vendrame et al., 2011). The results showed that the number of *Bifidobacterium* spp. increased significantly in the participants who consumed wild blueberry powdered drink when compared with the placebo groups. Interestingly, *Lactobacillus acidophilus* was found to increase in both the blueberry drink and the placebo drink and this might result from glucose and fructose in the placebo drink which may serve as prebiotic. For *Bacteriodes* spp., *Prevotella* spp.,

Enterococcus spp., and *Clostridium cocoides*, no significant difference was found after consumption of the wild blueberry drink. The authors concluded that phytochemical compounds in the wild blueberry drink contributed to the higher populations of *Bifidobacterium* spp. but did not have any effects on other microorganisms (Vendrame et al., 2011).

Apart from the prebiotic activity of blueberries, blueberries also exhibit antimicrobial activity in several pathogenic bacteria. Breast cancer patients were found to have a different pattern of gut microflora compared to healthy controls (Minelli et al., 1990). The *E. coli* population in breast cancer patients was approximately 10,000 times higher in fresh faeces than in controls. A reduction in pathogenic bacteria may lower breast cancer risk; therefore, the antimicrobial activity of blueberries is also of interest. The growth of foodborne pathogenic bacteria *Salmonella enteritidis* was reduced approximately 1,000 times by a phenolic acid-rich extract from blueberries (Park et al., 2011). Lowbush blueberry crude extract had weaker antimicrobial activity than an anthocyanin and proanthocyanidin fraction against *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium* (Lacombe et al., 2012).

The mechanisms by which blueberries exert their antimicrobial effect appear to be diverse. Outer membrane disintegration as measured by lipopolysaccharide (LPS) release was found in *S. sv. typhimurium* and *S. sv. infantis* treated with blueberry phenolic extract (Nohynek et al., 2006). Tannin, a blueberry polyphenol, showed antimicrobial activity via several mechanisms including inhibition of bacterial extracellular enzymes, inhibition of oxidative stress phosphorylation and deprivation of substrates necessary for bacterial growth (Scalbert, 1991). Other flavonoids, kaempferol and quercetin, found in blueberries demonstrated antimicrobial effects against both gram-positive and gram-negative pathogenic bacteria where their mechanisms of action were inhibition of nucleic acid synthesis, cytoplasmic membrane function and energy metabolisms (Cushnie & Lamb, 2005; Hendra et al., 2011). *In vivo* studies also support antimicrobial activity of blueberry extracts (Molan et al., 2010). Rats gavaged with blueberry crude extracts had lower numbers of bacteroides and clostridia compared to rats gavaged with water.

As discussed in Section 2.3, β-glucuronidase enzyme, produced by intestinal microflora such as *E. coli*, *Bacteroides fragilis* and *Clostridium perfringens* (Skar et al., 1986) plays an important role in enterohepatic circulation of xenobiotic compounds and steroid sex hormones (figure 2-6). The enzyme favours estrogen reabsorption by cleaving a

glucuronide bond. The estrogen reabsorption also improves the likeliness of mammary glands to be exposed to estrogen. Therefore inhibition of β-glucuronidase enzyme producing bacteria should improve detoxification in humans. In addition to antimicrobial activity of blueberry extracts *in vivo,* Molan and colleagues also demonstrated that β-glucuronidase enzyme activity was reduced in rats that had received blueberry extracts for 4 weeks (Molan et al., 2010).

An *in vivo* effect of blueberries and their polyphenols on prebiotic and antimicrobial activities and consequential effects on breast cancer have not yet been explored.

2.5.3 Anti-angiogenesis

Angiogenesis or the sprouting of micro-blood vessels from the existing vessels is a part of neovascularization (the growth of the vascular system) (Veeramani & Veni, 2010). Although angiogenesis plays an important role in normal conditions such as embryogenesis and menstruation, excessive angiogenesis also serves as a key process for several pathogenic conditions such as cancer growth and metastasis, age-related macular degeneration, obesity and asthma (Fan et al., 2006).

In general, by the time palpable tumours are detected they have already been neovascularised. Tumours are supplied with nutrients and oxygen via the new blood vessels moreover, the vessels serve as a route for metastasis (Folkman & Hanahan, 1991). Fan et al. (2006) summarised ten sequential steps of tumour angiogenesis (figure 2-11). The first step starts from tumour cells releasing angiogenic factors such as vascular endothelial growth factors (VEGF) in response to deprivation of oxygen and nutrients. Then angiogenic factors bind to receptors on endothelial cells (ECs) resulting in EC activation. Protease enzymes are then released causing the basement membrane to dissolve, followed by migration and proliferation of ECs. The next step occurs when adhesion molecules facilitate sprouting of blood vessels toward the tumour mass. After that, remodelling of new blood vessels is initiated by matrix metalloproteinase (MMP) enzymes. Next, Tie2-angiopoietin system and EphB-ephrinB systems modulate tubule and loop formation, respectively. Incorporation of pericytes present at the end of the process stabilise the newly formed vessels. Collectively, inhibition or modulation at each step of tumour angiogenesis might serve as a promising strategy in cancer prevention and therapy.

Tumour angiogenesis plays an important role in breast cancer growth and progression; moreover, the angiogenesis can be used to predict the outcome of breast

cancer patients. The microvessel density (MVD) of 328 breast cancer patients was evaluated using immunocytochemical staining for factor VIII-related antigen. The patients

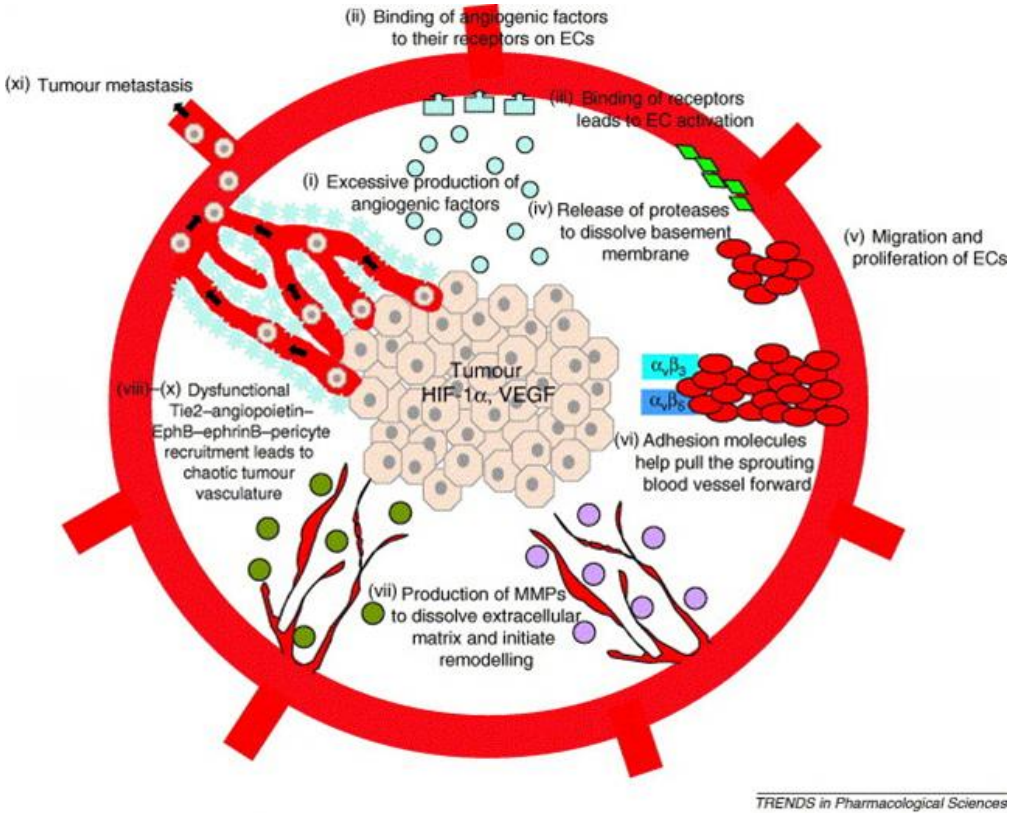


Figure 2-11 Sequential steps of tumour angiogenesis and metastases.

(Source: Fan et al., 2006, reprinted with permission from Elsevier).

with over 100 microvessels/mm^2 in a microscopic field had poorer relapse-free survival (RFS) rate than the patients with fewer than 100 microvessels/mm^2 in a microscopic field (Toi et al., 1995). Vascular endothelial growth factors (VEGF) expression was found to be higher in mammary tumours of 7,12-dimethybenzanthracene treated rats than the VEGF level in mammary gland of untreated rats. The authors suggested that higher VEGF expression was a result of higher protein transcription in tumour led to angiogenesis facilitation (Nakamura et al., 1996).

Six berry extracts (strawberry, raspberry seed, elderberry, cranberry, wild blueberry and bilberry), two formulas of six berry mixtures and grape seed extract were investigated for their antioxidant and anti-angiogenic activities (Roy et al., 2002). Antioxidant activity of

the test samples were measured by ORAC assay. Anti-angiogenic activity was evaluated by measuring (VEGF) expression of human HeCaT keratinocytes and tube formation assay using CHEMICON anti-angiogenesis kit. Wild blueberry, wild bilberry, berry mix 1, berry mix 2 and grape seed extract exhibited the highest ORAC values among all tested samples. All of the extracts with high ORAC values were able to inhibit VEGF expression except grape seed extract. Therefore, antioxidant properties alone may not be responsible for anti-angiogenic property. The anti-angiogenic property of the berry extracts was confirmed by the *in vitro* tube formation assay. Human microvascular endothelial cells incubated with the berry extracts could not develop tube-like structures in comparison with control samples. In order to identify the bioactive compound which exhibited this anti-angiogenic activity, the researchers also studied the effects of pure flavonoids (ferrulic acid, catechin and rutin) and tocopherol (found in grape seed extract) on VEGF expression. The pure flavonoids, especially rutin, showed anti-angiogenic properties via VEGF suppression. It was concluded that the anti-angiogenic properties of edible berries resulted from their high concentration of flavonoids (Roy et al., 2002).

The major advantage of *in vitro* assays is that the experimental conditions can be tightly controlled. However, the complicated metabolism *in vivo* could affect the pro- or anti-angiogenic activity of the test substances; therefore, the compounds that are highly effective *in vitro* might have different effects under *in vivo* conditions (Veeramani & Veni, 2010). The chick chorioallantoic membrane (CAM) assay is a screening method for both pro- and anti-angiogenic agents (Ribatti, 2010). The shells of fertilized chicken eggs are opened and the test substances are applied to the CAM. Eggs are then incubated for several days while new blood vessels on the CAM are observed. The details such as age of embryos, time of incubation and volume of test agents vary in each experiment (Ozgurtas et al., 2008; Ribatti, 2010; Lin et al., 2011).

A bilberry (*Vaccinium mytillus* L.) solution, rich in anthocyanins and flavonoids, was studied for anti-angiogenic activity using the CAM assay (Ozgurtas et al., 2008). 50 µL of 100 µg /mL or 100 ng /mL bilberry solution was placed on the 4 day-old CAM. The result was observed after 48 hours incubation. The CAM treated with 100 ng solution showed thinner vessels and less branching than the control CAM (without bilberry extract), the higher concentration (100 µg) showed similar result to the lower concentration solution. The researchers concluded that bilberry might be a useful treatment for angiogenesis-dependent human diseases.

2.5.4 Estrogenic/anti-estrogenic activities

Levels of serum estradiol have been found to positively correlate with breast cancer risk in both premenopausal and postmenopausal women (Key, 1999). Several studies measured serum estradiol levels in mammary tumorigenesis animal models; but the results were varied (Bhat & Pezzuto, 2002; Whitsett et al., 2006). Resveratrol, a blueberry polyphenol in the stilbenoid class, exhibits both anti-estrogenic and estrogenic activity depending on experimental conditions (Bhat & Pezzuto, 2002). The polyphenol was used in a 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumorigenesis study (Whitsett et al., 2006). 0.1% resveratrol supplemented diet was given to animals from birth and the carcinogen was administered when animals reached 50 days old. Even though the animals receiving resveratrol-supplemented diet showed lower tumour multiplicity and longer tumour latency compared to the control animals receiving DMBA alone, levels of serum estradiol in both groups were not significantly different. Freeze-dried whole blueberries as a supplement to a normal diet were used in an *in vivo* study of estrogen-induced mammary tumorigenesis. Levels of serum estradiol were not affected by blueberries in animals without estradiol implant; however, animals implanted with estradiol and receiving the blueberry-supplemented diet had lower serum estradiol levels than animals treated with estradiol without blueberry diet (Ravoori et al., 2012). Even though evidence showed that blueberry consumption in animal trials reduced circulating estrogen levels, the mechanism of estrogen excretion is still unknown. Possible mechanisms for blueberries lowering circulating estradiol may be as an aromatase inhibitor that inhibits estrogen formation or dietary fiber, presented in blueberries may enhance estrogen excretion (Bagga et al., 1995).

2.6 Blueberries and breast cancer

Blueberries possess possible biological activities that might affect different stages of breast cancers and these have been reviewed in the previous section. In this section, effects of blueberries and their polyphenols in the modulation of breast cancer both *in vitro* and *in vivo* will be summarised.

Many studies on breast cancer have been carried out however; there are few studies about the effect of blueberry phytochemicals on breast cancer. MDA-MB-231 (ER-) and MCF 7 (ER+) are breast cancer cell lines used widely for breast cancer studies. They were used to study the effects of an anthocyanin extract from blueberry and an anthocyanin-

pyruvic acid adduct extract against breast cancer. The anthocyanin-pyruvic acid adducted extract showed a stronger effect than the pure anthocyanin blueberry extracts, but both extracts inhibited cell proliferation of both cell lines at physiological concentrations (concentration that can be achieve *in vivo*) (Faria et al., 2010).

Extracts of highbush blueberry fruit, extracted with three different solvents (water, methanol and ethyl acetate), were tested for their efficacy on four breast cancer cell lines (HCC38, HCC1937, MDA-MB-231 AND MCF-10A). All extracts decreased cell proliferation in all cell lines except the untransformed cell line (MCF-10A) (Adams et al., 2010). The metastatic potential of the most aggressive cell line (MDA-MB-231) was lower when treated with blueberry extracts. It was also found that among the three extracts, the water extract was the most active against metastatic potential of the breast cancer cell line regardless of polyphenolic content. This may be due to the phytochemical mixture present in the water extract; or simply the loss of active components during fractionation.

Even though blueberries might be able to inhibit mammary tumorigenesis via different pathways, the evidence is not strong enough to claim a proven ability of blueberries in breast cancer prevention and therapy. Therefore, *in vivo* experiments are required to explore a physiological health benefit of blueberries. In breast cancer research, several animal models have been used, such as cancer cell induction, carcinogen induction or estrogen induction, which result in different types of breast cancers and also different outcomes.

7,12-Dimethylbenz[a]anthracene is one of the most widely used carcinogens for breast cancer studies *in vivo* (Russo & Russo, 1994). Carcinogenesis in rat models starts primarily at terminal end buds (TEB) which equate to a structure called the terminal ductal/lobular unit in human mammary glands. Moreover, histopathological comparison between DMBA-induced breast cancer in rat models and human breast cancer have shown that they share some similarities including mitotic activity, pattern grade, criteria of malignancy and various degrees of cytologic atypia (Irmgard et al., 2002). The carcinogen always causes estrogen receptor positive (ER+) breast cancer which means exogenous and endogenous estrogen played an important role in cancer promotion and progression (Hakkak et al., 2007).

7,12-Dimethylbenz[a]anthracene-induced mammary tumours allow the study of chemopreventive agents in every step of tumour development. It is a pre-carcinogen that requires metabolic activation to be transformed into its active form (7-hydroxyl DMBA). The metabolite is a highly reactive carbonium ion which leads to cellular chain reaction

initiation and the formation of DMBA-DNA adduct resulting in DNA mutation and cancer (Flesher & Sydnor, 1971). There is no direct research on prevention of DMBA-DNA adduct formation by blueberry consumption; however, a study using commercial Tahitian noni (a fruit of *Morinda citrifolia*) juice (TNJ, a mixture of noni juice, blueberry and grape juice) prevented DMBA-DNA adduct formation in animals receiving 10% TNJ two weeks prior to DMBA administration compared to animals that received solely DMBA (Wang et al., 2013). Purple grape juice, rich in anthocyanins, reduced DMBA-DNA adduct formation in mammary glands of rats receiving the extracts compared to animals without the extract (Jung et al., 2006). Prevention of DMBA-DNA adduct formation would subsequently prevent the initiation stage of breast cancer.

The effects of blueberries and their polyphenols in the prevention of estrogen-dependent breast cancer have been studied (Verma et al., 1988; Whitsett et al., 2006; Aiyer & Gupta, 2010; Ravoori et al., 2012; Jeyabalan et al., 2014). The chemopreventive and chemotherapeutic effects of blueberries and their polyphenols in *in vivo* mammary tumorigenesis are summarized in table 2-7.

Blueberries and their polyphenols have been studied for their anti-mammary tumorigenesis activity *in vivo*. In 1988, the flavonol quercetin was studied for its capacity to inhibit breast cancer caused by DMBA. Quercetin was mixed with the diet and the quercetin supplemented diet was introduced to Sprague-Dawley rats one week prior to the carcinogen. The rats were fed with DMBA coupled with an injection of *N*-nitrosomethylurea to induce breast cancer. The rats on the quercetin diet had a 40% lower tumour incidence than controls (Verma et al., 1988).

Animals that consumed approximately 100 mg resveratrol/ kg body weight/day from birth showed significantly lower tumour multiplicity and also increased tumour latency compared to control animals (Whitsett et al., 2006). Resveratrol was detected in the blood serum of animals receiving resveratrol treatment however the level of E2, progesterone and prolactin were not affected. Mammary gland morphology changed in resveratrol treated animals. They were found to develop a mammary gland structure less susceptible to carcinogenesis, which led to lower tumour multiplicity.

Two concentrations (1% and 2.5% (w/w)) of whole blueberry powder mixed with standard diet were used in a separate estrogen dependent mammary tumorigenesis study (Aiyer & Gupta, 2010). Animals received the blueberry diets 2 weeks prior to E2

implantation (9 mg per rat). The experiment was terminated 26 weeks after E2 implantation. The 1% blueberry diet did not affect tumour incidence and tumour multiplicity compared to controls (animals without blueberries in the diet); however tumour volume was reduced by 41%. Higher concentrations of blueberry resulted in modulation in all three tumour indices. Reductions of tumour incidence, tumour multiplicity and tumour volume compared to the controls were 31%, 38% and 59%, respectively (Aiyer & Gupta, 2010). Another group of researchers from the same laboratory also investigated a higher dose of blueberry and blackberry supplemented diet (5% w/w) on the same mammary tumorigenesis animal model (Ravoori et al., 2012). The results showed that tumour latency was delayed by 24 and 39 days compared to their controls in blueberry fed and blackberry fed animals, respectively. Tumour multiplicity and serum estrogen levels were not significantly different between the two berry-fed groups but significantly lower than their controls. Blueberry and blackberry fed animals showed different effects at the molecular level. Blueberry fed animals showed lower CYP1A1 expression while blackberry fed rats expressed lower ERα than their control group. The authors concluded that the different mechanisms of action in both berries might come from the differences in their polyphenolic profile. The same laboratory also examined therapeutic activity of dietary blueberry using an estrogen-mediated breast cancer model (Jeyabalan et al., 2014). Animals were implanted with E2 and received normal diet for 14 weeks before changing to 5% blueberry supplemented diet. The study was terminated 25 weeks after E2 implantation. Tumour volume and tumour multiplicity of animals receiving blueberry diet were significantly lower than their control. CYP1A1 and ERα expression were also down-regulated in animals fed blueberry diet compared to the controls. According to the study, a blueberry-supplemented diet could be used after the detection of tumours and still provide benefits against breast cancer.

Table 2-7 Effects of blueberries or their polyphenols in *in vivo* mammary tumorigenesis rat model.

Carcinogens	Blueberries/ Blueberry polyphenols	Experimental design	Effect of diet supplemented with blueberries/blueberry polyphenols on tumour indices and molecular biomarkers	References
DMBA	Quercetin	- Female Sprague-Dawley rats received quercetin (2% and 5%) supplemented diet one week prior to DMBA administration	2% quercetin supplemented diet - 25% less tumour incidence - 39% less average number of tumours per rat 5% quercetin supplemented diet - 42% less tumour incidence - 48% less average number of tumours per rat	Verma et al., 1988
DMBA	Resveratrol	- Female Sprague-Dawley rats received resveratrol (1mg/g diet) supplemented diet started at birth - DMBA was gavaged at 50 days postpartum - The resveratrol diet was continued until the end of the study	- Mammary gland morphology of animals received resveratrol diet was less susceptible to carcinogenesis. - Cell proliferation index significantly decreased in rats received resveratrol diet - Tumour latency was delayed by 19 days in rats received resveratrol diet - Tumour multiplicity decreased compared to animals received DMBA without resveratrol diet. - No significant different between the control and resveratrol treated group in level of serum estradiol, progesterone and prolactin	Whitsett et al., 2006

Table 2-7 Effects of blueberries or their polyphenols in *in vivo* mammary tumorigenesis rat model (cont.).

Carcinogens	Blueberries/ Blueberry polyphenols	Experimental design	Effect of diet supplemented with blueberries/blueberry polyphenols on tumour indices and molecular biomarkers	References
17β-estradiol (E2)	Dehydrated powdered blueberry	- Female ACI rats received blueberry supplemented diet (1% or 2.5%) two weeks prior to E2 implantation - E2 (9 mg) was implanted two weeks after the induction of experimental diet.	1% blueberry supplemented diet - Reduction of tumour volume - No significant change in tumour incidence and tumour multiplicity 2.5% blueberry supplemented diet - Tumour incidence was reduced by 31% - Tumour multiplicity decreased by 38% - Tumour volume was lower by 59%	Aiyer & Gupta, 2010
17β-estradiol (E2)	Dehydrated powdered blueberry	- Female ACI rats received blueberry supplemented diet (5%) two weeks prior to E2 implantation - E2 (9 mg) was implanted two weeks after the induction of experimental diet.	- Tumour incidence was reduced by 16% - Tumour multiplicity decreased by 36.2% - Tumour volume was lower by 50.7% - Levels of serum estrogen and prolactin were significantly decreased in E2 treated animals with 5% blueberry diet - Proliferation index was reduced by 37.4% - Level of CYP1A1 expression was down-regulated	Ravoori et al., 2012

Table 2-7 Effects of blueberries or their polyphenols in *in vivo* mammary tumorigenesis rat model (cont.).

Carcinogens	**Blueberries/ Blueberry polyphenols**	**Experimental design**	**Effect of diet supplemented with blueberries/blueberry polyphenols on tumour indices and molecular biomarkers**	**References**
17β-estradiol (E2)	- Freeze-dried whole blueberry powder (Tifblue : Rubel blend)	- 5 – 6 weeks old ACI rats received normal diet for 2 weeks then E2 (9 mg) was implanted. - The rats continued with the normal diet until the first tumour appeared (12 weeks after E2 implantation). - After the first tumour appeared, animals received 5% blueberry supplemented diet and continued with the blueberry diet until the end of the experiment.	- Tumour incidence decreased by 41%. - Tumour volume was reduced by 46%. - Tumour multiplicity decreased by 42%. - Level of plasma prolactin was reduced by 28%. - Expression of estrogen-specific genes, CYP1a1 and ERα, were down-regulated by 46% and 43.6%, respectively	Jeyabalan et al., 2014

The combined effect of blueberry consumption along with common breast cancer therapy has not yet been elucidated. However pterostilbene, a compound found in blueberries, was examined for its additional effect when paired with Tamoxifen. Tamoxifen, a prescription medicine, is a widely used breast cancer treatment. ER+ breast cancer cell lines (MCF-7) were pre-treated with pterostilbene for 24 hours then Tamoxifen at 5 µmol/L was applied. The results showed that when Tamoxifen and pterostilbene were used in combination, percentage viability of the cancer cells was lower by 7% in comparison with the cells treated with only Tamoxifen. However, there was no difference between pterostilbene alone and the combination in MDA-MB-231 (estrogen receptor-negative cell line). The apoptotic effect of all cell lines was also examined using the Cell Death Detection ELISA^{PLUS}. When the combination of pterostilbene and Tamoxifen was used, the apoptotic effect in MCF 7 and ZR-75-1 cell lines was increased 4 and 2.6 times, respectively (Mannal et al., 2010).

According to the previous study, it is possible to examine the effect of blueberries on breast cancer in addition to conventional therapies such as estrogen receptor modulator medicine or chemotherapy.

2.7 Aim and research objectives

Based on the previously literature published, rabbiteye blueberries contain a high concentration of polyphenols such as flavonoids, phenolic compounds and chlorogenic acid. The high content is not only found in berry fruits; but also in the juicing by-product, blueberry pomace, which contains high levels of the polyphenols as well. According to the literature, both blueberry fruits and pomace demonstrated several biological activities related to breast cancer prevention including antioxidant, prebiotic, antimicrobial, anti-angiogenic, estrogenic and anti-estrogenic activities. However, to our knowledge, an effect of New Zealand grown blueberries on ER+ breast cancer has not been investigated *in vivo*. The effects of blueberry consumption, either fruits or pomace, on several oxidative stress biomarkers in relation to breast cancer initiation, promotion and progression such as catalase enzyme and malondialdehyde (lipid peroxidation) have not yet been reported.

Even though the relationship between host health and intestinal microflora has been widely studied, there is no study reporting a direct relationship between intestinal

microflora in ER+ breast cancer. Moreover, a relationship between blueberry consumption, prebiotic and antimicrobial activity and breast cancer incidence is of interest.

The question is raised whether blueberry consumption as a blueberry juice extract which equates to approximately two cups of whole blueberry consumption per person per day or consumption of diet supplemented with blueberry pomace which enhances dietary fiber intake in animal model will have a similar effect on ER+ breast cancer.

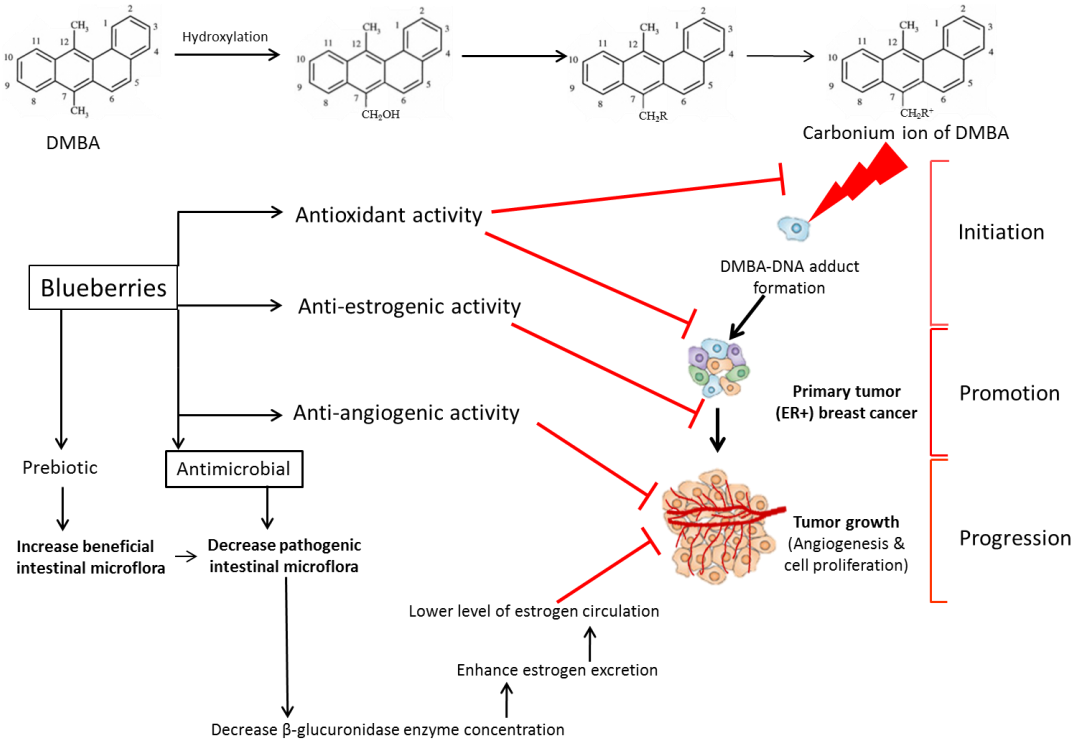


Figure 2-12 Possible activities of blueberries and blueberries pomace in the prevention and modulation of DMBA-induced breast cancer in animal model.

It has been previously published that blueberries and their polyphenols possess several activities that would benefit breast cancer prevention as discussed earlier. The information is summarised in figure 2-12.

By using DMBA as a cancer initiator where the free radical active metabolite is generated, the metabolite might be scavenged by antioxidant activity of blueberries. Blueberries can reduce oxidative stress by increasing the level of catalase enzyme which facilitates molecular detoxification system. The modification of oxidative stress in the cells might result in slowing the growth of primary tumours during tumour promotion. Angiogenesis is very important for tumour growth, blueberries and their polyphenols showed lowering VEGF expression might subsequently inhibit tumour angiogenesis and

delay tumour progression and metastases. Prebiotic and antimicrobial activity of blueberries can modulate population and types of intestinal bacteria. Some bacteria produce the enzyme β-glucuronidase which can cause enterohepatic reabsorption of xenobiotic compounds and steroid sex hormones eg. estrogen. The reabsorption of estrogen into the bloodstream increases the chance of mammary gland and mammary tumour exposure to the hormone and a possible increase in cell proliferation which, in the case of tumours, can lead to tumour growth. Collectively, blueberry consumption might indirectly facilitate estrogen excretion by modulation of intestinal microflora.

Therefore, to investigate the effects of blueberry consumption on breast cancer prevention and modulation and to determine whether blueberry pomace consumption would have similar benefit to blueberry juice consumption, a series of *in vitro* assays and *in vivo* animal trials was established. More specifically, this proposed research addresses the following four objectives:

1. To measure levels of total anthocyanins, total phenolic concentration, total flavonoid concentration and chlorogenic acid concentration of five cultivars of rabbiteye blueberries grown in New Zealand (Chapter 3).

2. To determine the antioxidant, prebiotic and antimicrobial activity of the five cultivars of rabbiteye blueberries using *in vitro* methods and anti-angiogenesis using an *in ovo* method (Chapter 4).

3. To examine the effect of rabbiteye blueberry extracts or diet supplemented with highbush pomace consumption on tumour incidence using 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumorigenesis in Sprague-Dawley rats (Chapter 5).

4. To examine the effect of rabbiteye blueberry extracts or diet supplemented with highbush pomace consumption on mammary gland morphology, histopathological examination of mammary tumours, level of oxidative stress, level of estrogen in animal blood serum, intestinal microflora and β-glucuronidase enzyme activity using 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumorigenesis in a Sprague-Dawley rat model (Chapter 6).

CHAPTER 3

Polyphenols of rabbiteye blueberries and effect of freeze-drying and extraction solvents

Abstract:

Blueberries have been shown to offer human health benefits. Research has shown that the health benefits of blueberries result from their biological activities such as antioxidant, anti-inflammatory, prebiotic and antimicrobial activities. This activity is likely to be attributable to different polyphenols in blueberries therefore, total phenolic concentration (TPC), total flavonoid concentration (TFC), anthocyanin profiles and chlorogenic acid concentrations of five cultivars of rabbiteye blueberry (*Vaccinium ashei*) ('Centurion', 'Maru', 'Rahi', Ono' and 'Tifblue') fruit and pomace were measured. Blueberry samples were extracted with either MilliQ water (MQ) or 5% aqueous formic acid (5%FA). Effects of solvent acidification and freeze-drying treatment were also studied. The results showed that the 'Tifblue' cultivar contained the highest TPC, TFC, anthocyanins and chlorogenic acid of the studied cultivars. Total phenolic concentration of the selected frozen rabbiteye blueberries in this study ranged from 1.33 – 2.95 mg gallic acid equivalent (GAE)/g frozen berries (FB). Total flavonoid concentration ranged from 0.41 – 0.91 mg catechin equivalent (CE)/g FB. Total anthocyanin and chlorogenic acid ranged from 0.2 – 0.61 mg/g FB and 184 – 482 µg/g FB, respectively. Total phenolic concentration and total flavonoid concentration of frozen blueberry pomace ranged from 0.74-1.20 mg GAE/g FB and 0.2-0.38 mg CE/g FB, respectively. Anthocyanins and chlorogenic acid were also present in blueberry pomace. Total anthocyanins were 0.09 – 0.17 mg/g FB and chlorogenic acid concentration was 3.8-24.8 µg/g FB. The blueberry samples extracted with 5%FA had higher TPC, TFA, total anthocyanins and chlorogenic acid concentration than the samples extracted with MQ. 5%FA might damage plant cell wall and release more polyphenols. Moreover, anthocyanins are also more stable in acidic conditions. TPC, TFC and total anthocyanins were higher in freeze dried samples compared to non-freeze dried. However, freeze drying treatment decreased chlorogenic acid concentrations.

Material from this chapter is included in papers: a) Vuthijumnonk, J., Molan, A. L., & Heyes, J. A., (2013). Effect of freeze-drying and extraction solvents on the total phenolic contents, total flavonoids and antioxidant activity of different Rabbiteye blueberry genotypes grown in New Zealand , *IOSR Journal of Pharmacy and Biological Sciences (IORS-JPBS), 8*(1), 42-48.
b) Vuthijumnonk, J., Heyes, J. A., & Molan, A. L. (2016). Total anthocyanins, chlorogenic acid concentration, antioxidant and *in ovo* anti-angiogenic activities of rabbiteye blueberries, *International Food Research Journal, 23*(2), 515-520.

Anthocyanidins from rabbiteye blueberry consisted of malvidin, delphinidin, cyanidin, petunidin and peonidin. MilliQ water extraction showed malvidin and cyanidin as the major anthocyanidins but delphinidin was one of the major anthocyanins instead of cyanidins in 5%FA extracts. Anthocyanidins were found glycosylated with sugar moieties which are glucoside, galactoside and arabinoside. Galactoside was the major sugar followed by glucoside and arabinoside. The pattern of sugar distribution was not altered by freeze-drying treatment or solvent acidification. Principal component analysis in frozen berries extracted with water showed that 'Maru' and 'Tifblue' extracts formed one group and 'Ono', 'Centurion' and 'Rahi' another; and the groups were largely distinguished by the higher delphinidin concentration in 'Maru' and 'Tifblue'.

Introduction:

Rabbiteye blueberries *(Vaccinium ashei*) are the second most common species of blueberry grown in New Zealand. They gain more attention especially because they crop late in the season (January – April) and require less winter chilling than highbush blueberries (*Vaccinium corymbosum*), (less than 700 h), which makes them suitable for warmer climates. Moreover, a study showed that rabbiteye blueberries exhibit higher antioxidant activity than highbush blueberries (Moyer et al., 2002) but they can taste a bit 'gritty' and are generally smaller than highbush blueberries, which are therefore preferred particularly for fresh consumption.

Anthocyanins are the major polyphenols found in blueberries. Several studies showed that anthocyanins from blueberries provided human health benefits (Faria et al., 2010; He & Giusti, 2010; Norberto et al., 2013). Anthocyanins derive from anthocyanidins glycosylated with sugar molecules such as glucose, galactose or arabinose (Lohachoompol et al., 2008). Blueberries are well known for their complex anthocyanin pattern. While more than 90% of anthocyanin in black raspberries is cyanidin, anthocyanin pattern in blueberries consists of cyanidin, malvidin, delphinidin, peonidin and petunidin glycosylated with 3-monogalactoside, 3-monoglucoside and 3-monoarabinoside (Ravoori et al., 2012) as well as many acylated forms. The ratio of anthocyanins is different among cultivars.

Anthocyanins in blueberries can be evaluated using different assays such as pH-differential assay (Prior et al., 1998; Moyer et al., 2002) and high performance liquid chromatography (HPLC). The pH-differential method is fast and inexpensive however the assay can only provide the total anthocyanin concentrations without differentiation of individual anthocyanins. HPLC is time consuming but provides data on each individual

anthocyanin. Total anthocyanin concentration of blueberries varies widely. Parameters that affect the variation are genotypes, growing conditions, maturity, storage conditions and experimental conditions (Howard et al., 2003; Ju & Howard, 2003). Solvents such as acetone, acetonitrile, methanol and ethanol or their mixtures have been used widely for anthocyanin or polyphenol extraction (Moyer et al., 2002; Lee & Wrolstad, 2004; You et al., 2011; Li et al., 2013); acidification of extracting solvent with organic acids such as formic acid or acetic acid enhanced the solvent extractability and anthocyanin stability (Wilhelmina Kalt et al., 2008). However, toxic solvents used for anthocyanin extraction are not permitted for food use. Therefore in this study, MilliQ water was chosen for anthocyanin extraction. Chlorogenic acid, found abundantly in blueberries, is gaining more attention because it, like other polyphenols, appears to exhibits various biological activities such as antioxidant, anti-angiogenesis and DNA-protective activities (Wu, 2007; Xu et al., 2012; Park et al., 2015).

Apart from blueberry whole fruits, blueberry pomace is also of interest. It is a by-product from blueberry juice processing and contains the skins, pulp residues and seeds (Bener et al., 2013). It is generally used for animal feed or fertilizer. Blueberry pomace contained high concentration of anthocyanins and phenolic compounds (Lee & Wrolstad, 2004; Kitchen, 2013). Consumption of blueberry pomace has also been associated with several health benefits against pathological conditions such as colorectal inflammation, liver injuries (Håkansson et al., 2012) and lower plasma cholesterol (Kim et al., 2010).

As blueberries are seasonal and highly perishable, freezing or drying treatment has been used for extending storage life of the fruits. Anthocyanins and antioxidant activity of blueberries decreased in fruits dried using high temperature drying treatment (Lohachoompol et al., 2004; Sablani et al., 2011). Thawing also caused polyphenol loss by increasing polyphenoloxidase enzyme activity (Vámos-Vigyázó & Haard, 1981). Even though freeze drying or lyophilisation is not the most cost effective drying treatment, total anthocyanins and antioxidant activity of blueberries were retained or sometimes increased by using this low temperature drying method (Reyes et al., 2011; Sablani et al., 2011).

As mentioned earlier, rabbiteye blueberries are the second most commonly cultivated blueberry species in New Zealand, but little research has been published on their bioactive compounds (Scalzo et al., 2008). In order to further investigate their anti-carcinogenic potential *in vivo*, it is important to determine their polyphenols *in vitro*. Moreover, various factors impact on their polyphenol concentration such as variety, species

harvest maturity and growing conditions (You et al., 2011). This study aimed to examine total phenolic concentration, total flavonoid concentration, total anthocyanins, percentage distribution of individual anthocyanidins and chlorogenic acid concentration of five New Zealand grown rabbiteye blueberry cultivars. The blueberry cultivars used in this study were 'Centurion', 'Maru', 'Rahi', 'Ono' and 'Tifblue'. Milli Q water (MQ) was used as an extracting solvent instead of organic solvents because of the toxicity of organic solvents, which might not be suitable for using *in vivo*. 5% aqueous formic acid (5%FA) was also used as another extracting solvent which may enhance extraction yield compare to MQ. Blueberry pomace from all cultivars was extracted and determined for their polyphenolic concentrations. The effect of freeze drying treatment was also investigated in this study.

Materials and Methods:

Chemicals and standards

Cyanidin-3-glucoside chloride, chlorogenic acid, gallic acid, and ferrous sulphate ($FeSO_4$), quercetin, Folin-Ciocalteu's phenol reagent, sodium carbonate (Na_2CO_3), 2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride ($FeCl_3$), fluorescein, AAPH (2,2' azobis(2-methylpropionamidine) dihydrochloride, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), sodium acetate, and hydrochloric acid were purchased from Sigma-Aldrich Chemical Co. (Auckland, New Zealand). HPLC-grade methanol and formic acid were purchased from Thermo Fisher Scientific (Palmerston North, New Zealand). Ultrapure water obtained from a Milli-Q system (Millipore, Milford, MA) was used for the preparation of the reagents and blueberry extracts.

Raw materials

Ripe berries of five rabbiteye blueberry (*Vaccinium ashei*) cultivars ('Centurion', 'Maru', 'Rahi', 'Ono' and 'Tifblue') were purchased from the Beaches Blueberries Ltd, Hamilton, New Zealand. All samples were grown under the same environmental conditions. They were all harvested at commercial maturity and stored at -20°C until used.

Crude extract

One hundred grams of frozen berry fruits were mixed with 100 mL MilliQ water or 5% formic acid and blended using a stand mixer (Sunbeam, SM6200). The fruit mixture was transferred to centrifuge tubes and centrifuged at 10 000 *g* for 5 minutes. The supernatant was collected and frozen at -20°C until analysis.

Pomace extract

The pomace (10 g) left from the first extraction was re-suspended again by 10 mL MilliQ water or 5% aqueous formic acid. The mixture was vortexed for 1 minute and centrifuged at 10 000 *g* for 5 minutes. The supernatant was collected and kept at -20°C until analysis.

Freeze-dried samples

One hundred grams of ripe frozen berries were placed in a plastic bag, crushed and frozen at -20°C overnight. Similarly, the pomace from the first extraction was also frozen under the same conditions. The lyophilisation process was carried out using a freeze-dryer model FD18LT (Cuddon Ltd., New Zealand). The products were cooled to -35°C. The temperature was maintained at -35°C with a pressure of 2 mbar for 3 hours. Then the temperature was increased up to -20°C and maintained at this condition until the product dried. The dried residue was weighed, ground into a fine powder using coffee grinder (Breville, CG2B) and then stored dry at -20°C until further extraction. For the bioassays, one gram of the freeze-dried berries was extracted by mixing with 10 mL of either MilliQ water or 5% aqueous formic acid. The mixture was then centrifuged at 10 000 *g* for 5 minutes and the supernatant was kept at -20°C until analysis.

The supernatants used for anthocyanins and chlorogenic acid determination were filtered through 0.22 µm PVDF filter (Phenomenex, USA) and transferred into HPLC vials.

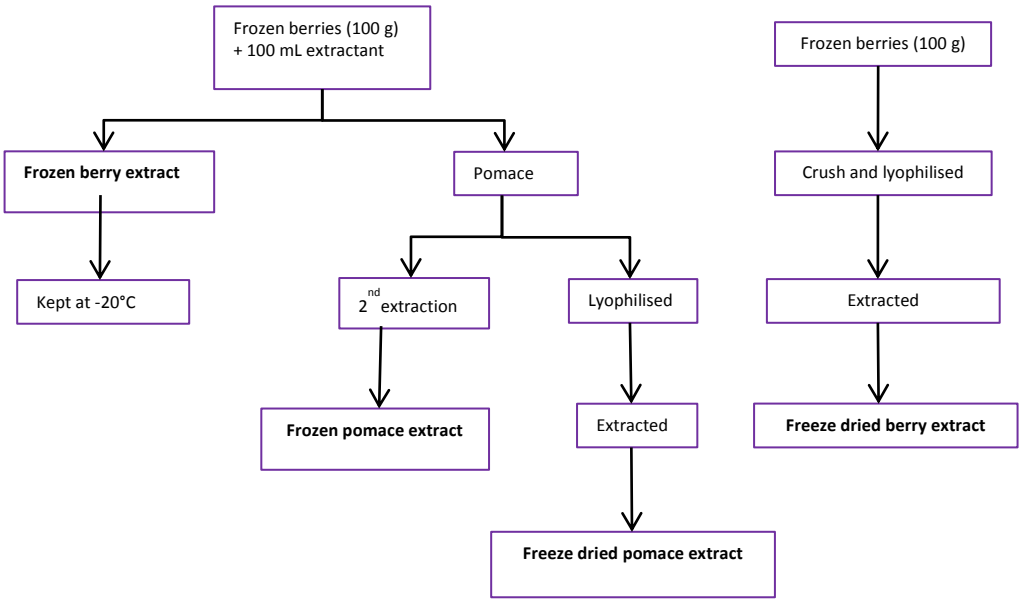


Figure 3-1 Blueberry extraction scheme

Total of 4 extracts were obtained from one blueberry cultivar extracted by one solvent.

Determination of total phenolic concentration (TPC)

Total phenolic concentration (TPC) were measured using Folin-Ciocalteu reagent as described previously (Molan et al., 2009a). Briefly, an aliquot of 12.5 µL of extract was mixed with 250 µL of 2% Na_2CO_3 in 96-well microplates, the reaction was left for 5 minutes at room temperature and then an aliquot of 12.5 µL of 50% Folin-Ciocalteu reagent was added to the mixture. The mixture was allowed to react at ambient temperature for 30 minutes. Then the absorbance was measured at 650 nm using microplate reader (ELX 808 Ultra microplate reader, BIO-TEK instrument) with KC4 software. TPC of the blueberry samples was expressed as mg gallic acid equivalent (GAE)/g frozen weight, using a gallic acid standard curve which was linear from 0-100 mg/mL gallic acid. Each sample was analysed in triplicate.

Determination of total flavonoid concentration (TFC)

Total flavonoid concentration in blueberry extracts was determined using the aluminium chloride colorimetric assay (Kumar et al., 2008) with some modifications. In 96-wells plates, 25 uL of blueberry samples were mixed with 100 µL Milli Q water and 5% $NaNO_2$ (7 µL). The plate was left at room temperature for 5 minutes. Then 7 µL of 10% $AlCl_3$ was added to the mixture and allowed to react at ambient temperature for 5 minutes and then 1 M NaOH (50 µL) and Milli Q water (60 µL) were added to the mixture. The absorbance of the mixture was measured at 490 nm. The TFC in blueberry samples was expressed as mg catechin equivalent (CE) per gram frozen berries, using a catechin standard curve which was linear from 0-100 mg/mL catechin. Each sample was analysed in triplicate.

Anthocyanin and chlorogenic acid analysis

Anthocyanin and chlorogenic acid analysis was carried out using a modified high performance liquid chromatography (HPLC) method (Wang et al., 2000). The HPLC system consisted of a Shimadzu HPLC CTO-20A (Shimadzu Corp., Kyoto, Japan) coupled with an auto-sampler (SIL-20AC) and a photo-diode array (PDA) detector SPD-M20A. Phenomenex Luna C18 (2) 150 × 4.6 mm (5 µm) reverse phase column (Phenomenex, North Shore City, NZ) was used for anthocyanin separation. Mobile phase consisted of 5% (v/v) aqueous formic acid (solvent A) and 100% HPLC grade methanol (solvent B). A linear gradient profile

containing solvent A with the following proportions (v/v) of solvent B: 0-1 min, 14% B; 1-10.24 min, 14-17% B; 10.24-35.28 min, 17-23% B; 35.28-64.59 min, 23-47% B; 64.59-66.59 min, 47-14% B. Total running time was 70 min with 1 mL/min flow rate. Cyanidin 3-O-glucoside chloride was used as the anthocyanin standard. Peak areas of the anthocyanin were quantified at 520 nm. Chlorogenic acid was determined at 280 nm in comparison with the peak area of chlorogenic acid standard. The determination of anthocyanin and chlorogenic acid was done in triplicate for each extract.

Statistical analysis

Total phenolic concentration, total flavonoid concentration, total anthocyanins and chlorogenic acid concentration were analysed for mean comparisons using one-way analysis of variance (one-way ANOVA) to assess significant treatment effects, and then Tukey's test was used as a posthoc comparison. The differences were considered statistically significant at $P \leq 0.05$. All statistical analysis was performed on Minitab software (version 15; Minitab Inc., Pennsylvania, USA).

Results and discussion:

Total phenolic concentration (TPC)

In frozen berries, TPC ranged from 1.33 – 2.95 mg GAE/ g frozen berries (FB) and 2.16 – 3.66 mg GAE/ g FB when extracted with MQ water and 5% aqueous formic acid, respectively (figure 3-2A). In freeze-dried frozen berries, TPC ranged from 2.68 – 4.74 mg GAE/ g FB and 7.42 – 11.56 when extracted with MQ water and 5% aqueous formic acid, respectively (figure 3-2B). Total phenolic concentration in frozen blueberry pomace was lower than TPC in frozen berries. The values ranged from 0.74 – 1.20 mg GAE/ g FB when extracted with MQ water and from 2.14 – 3.16 mg GAE/ g FB when extracted with 5% aqueous formic acid (figure 3-2C). Freeze-dried pomace also showed higher TPC than non-freeze-dried pomace where the values ranged from 2.52 – 3.39 mg GAE/ g FB and 5.12 – 11.25 mg GAE/ g FB when extracted with MQ water and 5% aqueous formic acid respectively (figure 3-2D). All data from freeze dried samples were corrected for water loss and expressed as concentration per g of frozen berries.

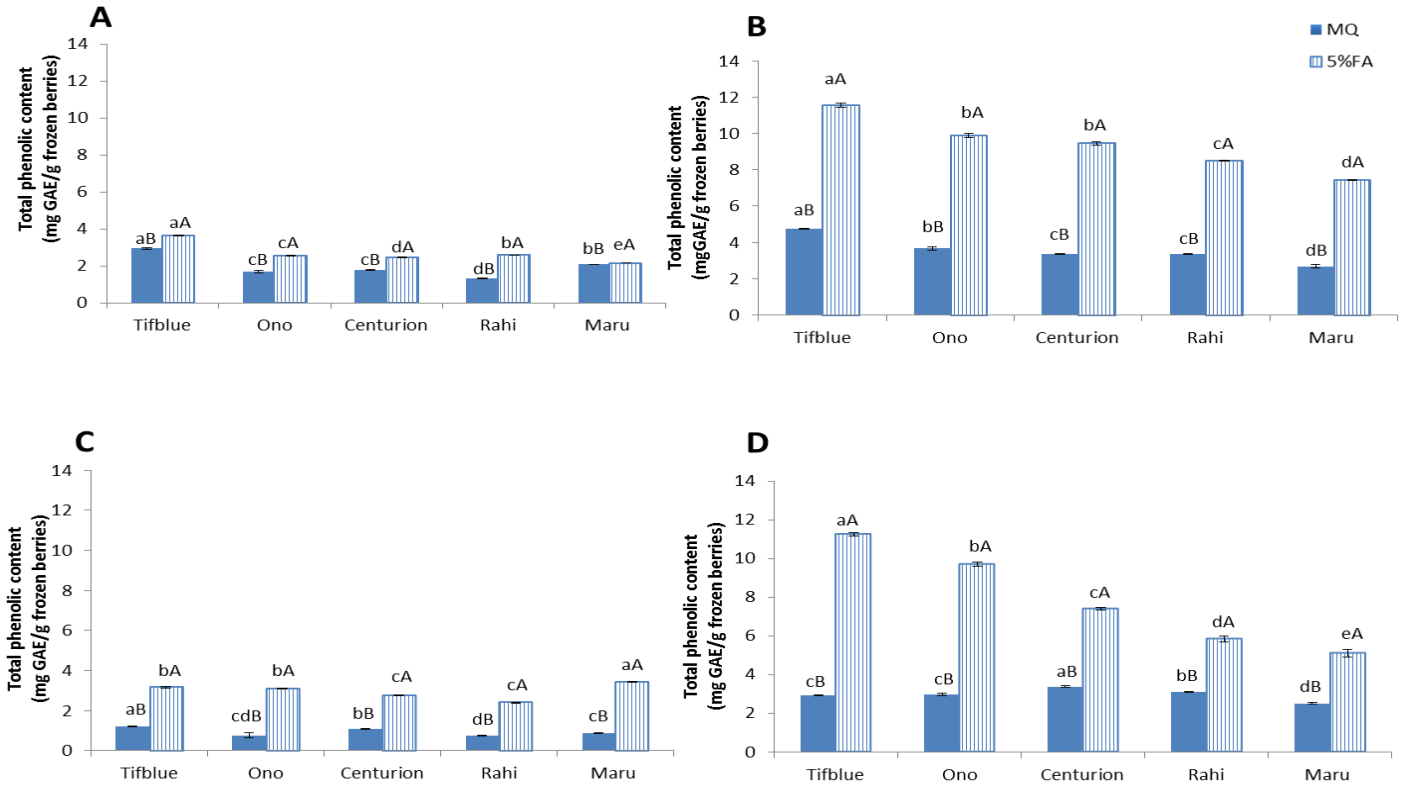


Figure 3-2 Total phenolic concentration (TPC) of A) frozen berries, B) freeze-dried berries, C) frozen pomace and D) freeze-dried pomace of five rabbiteye blueberry cultivars extracted by MilliQ water or water plus 5% formic acid. The TPC data are expressed as mg gallic acid equivalent (GAE)/ g frozen berries. Data expressed as means ± SEM. Different lower-case letters indicate significant differences ($P \leq 0.05$) between blueberry cultivars within each solvent type. Different capital letters indicate significant differences ($P \leq 0.05$) between solvents for each cultivar.

Blueberry pomace also contained a significant amount of TPC. Previous studies showed that TPC of blueberry pomace ranged from 3.0 to 4.1 mg GAE/ g berries (Lee & Wrolstad, 2004; Kitchen, 2013; Li et al., 2013). Total phenolic concentration in pomace was lower than TPC of the whole berries even though anthocyanins and other polyphenols were located mainly in the blueberry skin. Polyphenols in the pomace are not the only part of polyphenols presented in the fruit. A study showed that some polyphenols such as cinnamic acid and chlorogenic acid are found mainly in the flesh of blueberries (Gao & Mazza, 1994: Lee & Wrolstad, 2004).

Total phenolic concentration of blueberries from previous studies varied depending on various factors (Prior et al., 1998; Moyer et al., 2002; Howard et al., 2003; You et al., 2011). Differences in blueberry cultivars affected TPC values. Frozen 'Tifblue' whole berries extracted with MQ had the highest TPC values while the lowest was found in frozen 'Rahi'. The TPC values from the study fall within the ranges (0.44 to 9.52 mg GAE/ g FB) reported previously for blueberry genotypes grown in different countries using different solvents such as methanol and acetone for extraction. Howard et al. (2003) reported that the TPC of blueberry genotypes varied over the growing seasons, suggesting that environmental growing conditions can affect levels of phenolic compounds in blueberries.

It was obvious that freeze drying treatment increased TPC in both fruits and pomace. Studies showed that TPC was higher in freeze dried but lower in high temperature treated samples (Lohachoompol et al., 2004; Sablani et al., 2011). High temperature treatment affected polyphenolic compounds and may have caused degradation. However, the compounds are more stable when drying under low temperature. Moreover, Sablani and co-worker (2011) also reported TPC increasing in freeze-dried blueberries in comparison with fresh fruits. The increase of TPC may have resulted from ice crystal formation in plant cells which led to cell rupture and released phenolic compounds from the plant cell matrix (Keinanen & Julkunen-Titto, 1996); and to more complete extraction from the freeze-dried samples which were ground into a fine powder.

In extracts prepared in 5% formic acid, TPC values were significantly higher ($P \leq 0.05$) than extracts prepared in water only. The effect of different solvents on TPC from plants has been well documented (Ju & Howard, 2003; Chirinos et al., 2007; Koffi et al., 2010; Michiels et al., 2012). Solvents such as ethanol and methanol are more efficient than water for polyphenol extraction. The addition of organic acid into extracting solvents also helps enhance polyphenolic extraction. The weak organic acid present in extracting solvent also

led to cell wall deconstruction which subsequently resulted in higher polyphenol extraction (Dai & Mumper, 2010). This finding showed higher TPC in 5%FA extracts than MQ extracts.

Total flavonoid concentration (TFC)

Anthocyanins belong to the flavonoid class of phytochemicals. Total flavonoid concentration in frozen berries extracted by MQ water were the highest in 'Tifblue' genotypes at 0.91 mg CE/g FB. The lowest TFC was found in 'Rahi' and 'Maru' at 0.41 mg CE/g FB (figure 3-3A). Similar to TPC, TFCs from pomace extracts were lower by 40-60% of the TFCs from frozen berries. TFC in pomace ranged from 0.20 - 0.38 mg CE/g FB where 'Tifblue' was the highest and 'Rahi' was the lowest (figure 3-3C). Freeze-drying increased TFC in both berry and pomace extracts (figure 3-3B and 3-3D). The range of TFC were 1.02 – 2.60 mg CE/g FB and 0.55 – 1.14 mg CE/g FB in FD berry extracts and FD pomace extracts, respectively. 5%FA extraction gave higher yield than their water extracted counterparts.

The TFC of freeze-dried blueberries extracted in 80% aqueous methanol has been reported by Marinova et al. (2005) to be 1.90 mg CE/g FB. Total flavonoid concentration of fresh highbush blueberry juice varied widely as it ranged from 0 – 2.90 mg CE/g fruit weight (Sinelli et al., 2009). Total flavonoid concentration in our study were within range of those from previous studies. Acidified ethanolic extract of rabbiteye blueberry fruits and pomace has been measured for TFC. Total flavonoid concentration of the fruits was 14.39 mg quercetin equivalent (QE)/g dried fruit weight which was significantly lower than TFC from pomace (21.65 mg QE/g dried pomace weight) (Li et al., 2013). However, the results from this study may not be comparable with our study due to different standard used (quercetin and catechin). Moreover, TFC from blueberry pomace in their study contained higher TFC than blueberries which was at odds with our findings. However, the blueberry pomace from Li's study derived from a wine-making process which may cause degradation of plant cells and using acidified 70% ethanol as an extracting solvent. The combination led to better extractability than blueberry pomace from our study. An effect of extracting solvent was also significant in TFC where 5%FA extracts had higher TFCs than MQ extracts. Similar to TPC extraction, weak organic acid addition to extracting solvent gave higher TFC than non-acid addition. Moreover, flavonoids in blueberries are mainly anthocyanins which are more stable in acidic environment (Cabrita et al., 2000). Freeze drying treatment also resulted in higher TFCs in both fruits and pomace extracts similar to the effect on TPCs. The effect can be explained by the formation of ice crystal during freezing prior to lyophilisation that helped release anthocyanins and flavonols located in vacuoles (Haard & Chism, 1996).

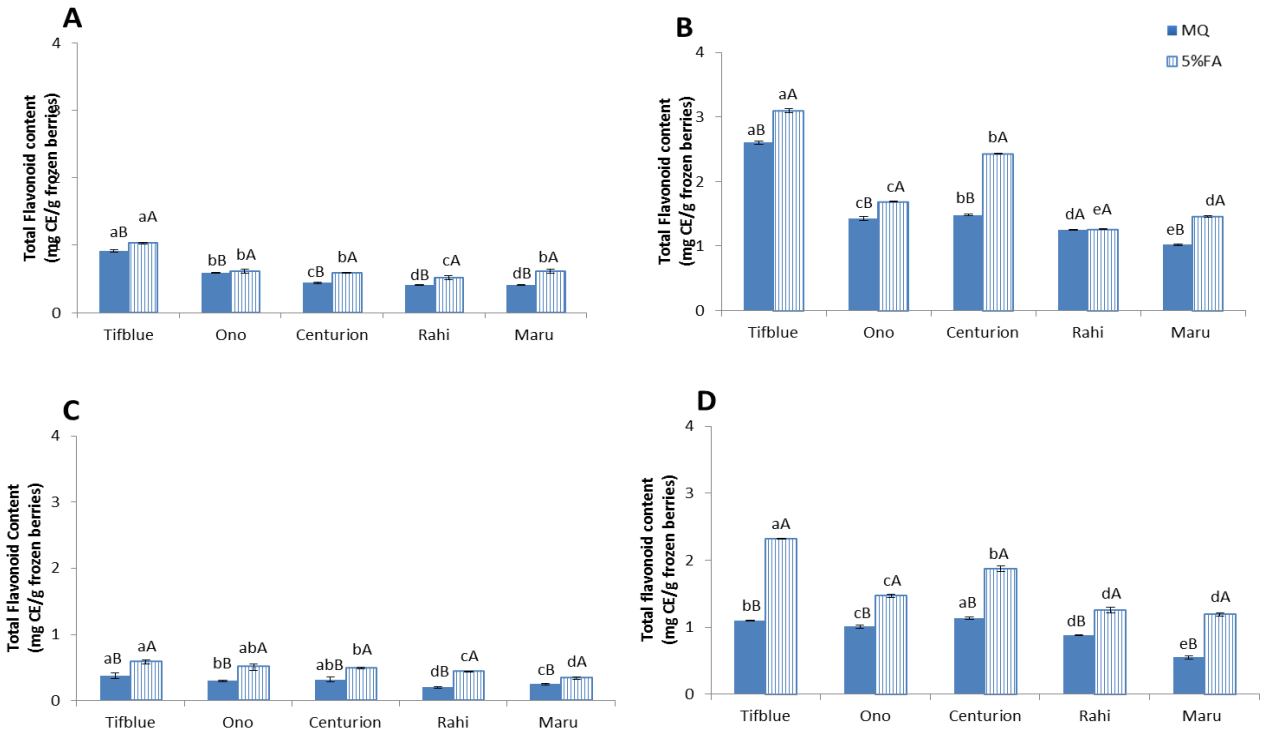


Figure 3-3 Total flavonoid concentration (TFC) of A) frozen berries, B) freeze-dried berries, C) frozen pomace and D) freeze-dried pomace of five rabbiteye blueberry cultivars extracted by MilliQ water or water plus 5% formic acid. TFC are expressed in mg catechins (CE)/g frozen berries. Data expressed as means ± SEM. Different lower-case letters indicate significant differences (*P≤ 0.05*) between blueberry cultivars within each solvent type. Different capital letters indicate significant differences (*p ≤ 0.05*) between solvents for each cultivar.

Anthocyanins

The total anthocyanin concentration of five rabbiteye blueberry cultivars extracted with Milli Q water (MQ) or 5% aqueous formic acid (5%FA) are shown in figure 3-4. Acidification of solvent increased anthocyanin extraction in all samples. Frozen berries of 'Tifblue' exhibited the highest total anthocyanin concentration (0.61 mg/g FB) when extracted with MQ while 'Rahi' exhibited the lowest total anthocyanin concentration (0.2 mg/g FB). Several studies reported that anthocyanins are mainly found in skin of berries (Burdulis et al., 2009). Total anthocyanin concentration in blueberry pomace extracted with MQ ranged from 0.08 mg to 0.17 mg /g pomace weight where the highest anthocyanin concentration was found in 'Tifblue' and the lowest was 'Maru'. Total anthocyanins concentration of blueberry pomace extracted with 5%FA ranged from 0.23 - 0.33 mg/g pomace weight where 'Ono' was the highest followed by 'Centurion', 'Rahi', 'Maru' and 'Tifblue'. The effect of 5%FA was similar in both fruit and pomace extracts. The result showed that acidification of solvent increased anthocyanin yields by 25 to 60%.

Total anthocyanins from freeze dried blueberry fruits extracted by MQ ranged from 0.9 - 1.2 mg/g FB. 'Tifblue' had the highest total anthocyanins while the lowest was 'Ono'. The freeze dried samples extracted with 5%FA showed approximately 50-80% higher yield than MQ extracts. Total anthocyanins of the freeze dried 5%FA extracts were the highest in 'Tifblue' (4.99 mg/g FB) and the lowest was 'Rahi' (2.40 mg/g FB). Freeze dried pomace also contained significant levels of anthocyanins. Total anthocyanins of the freeze dried pomace extracted with MQ were the highest in 'Centurion' (0.54 mg/g FB) and the lowest was 'Ono'. Acidification of solvent also increased total anthocyanins yields in freeze dried blueberry pomace. Total anthocyanins of 5%FA freeze dried blueberry pomace extracts ranged from 1.64 – 2.17 mg/g FB.

Total anthocyanins from our study were within the range previously reported (Hamamatsu et al., 2004; Scalzo et al., 2008; Wang et al., 2011). Thirty-six rabbiteye blueberries grown in USA were evaluated for their anthocyanin composition. Their total anthocyanins ranged from 0.9 – 3 mg/ g fruit weight (Wang et al., 2011). Another rabbiteye blueberry study reported that total anthocyanin concentration in 'Tifblue' was 1.3 mg/ g fruit weight (Hamamatsu et al., 2004).

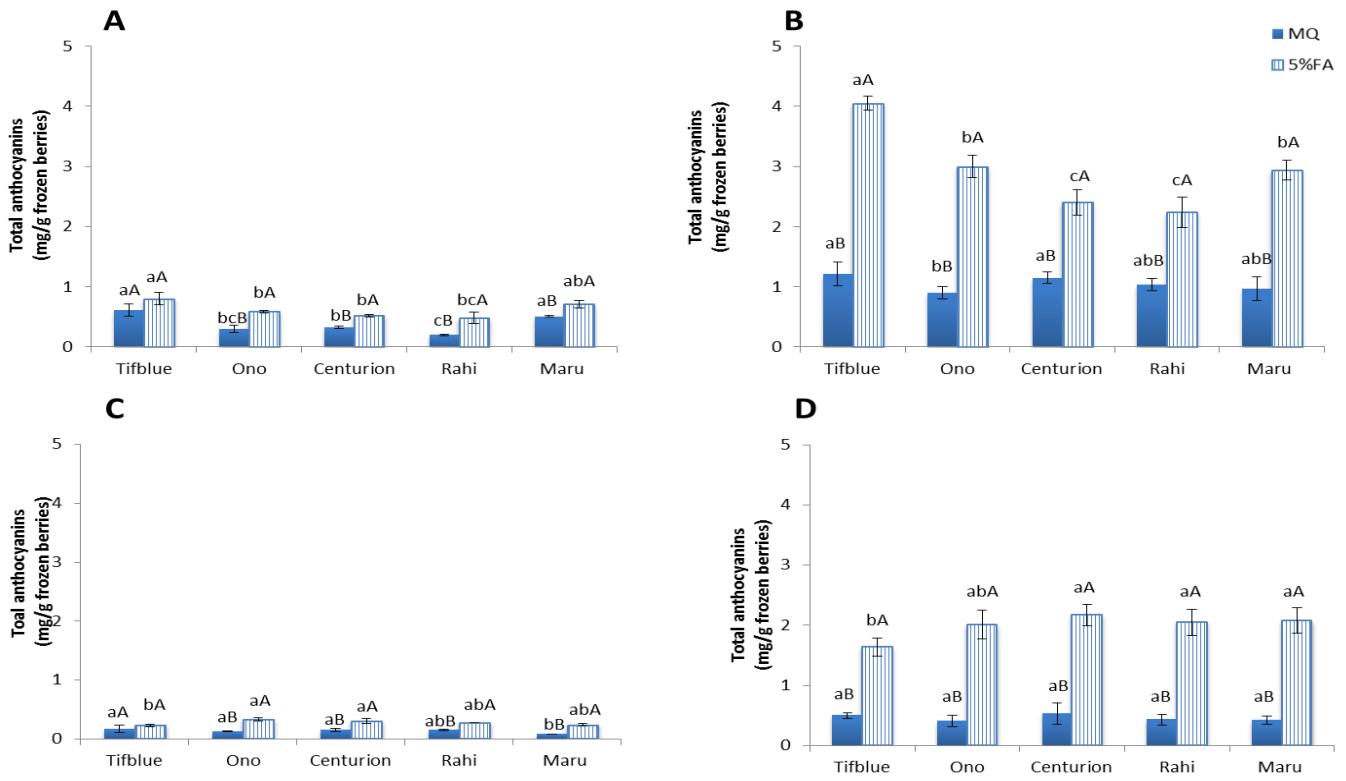


Figure 3-4 Total anthocyanin concentrations of A) frozen berries, B) freeze-dried berries, C) frozen pomace and D) freeze-dried pomace of five rabbiteye blueberry cultivars extracted by MilliQ water or water plus 5% formic acid. Total anthocyanins are expressed as mg/g frozen berries. Data expressed as means ± SEM. Different lower-case letters indicate significant differences (*P≤ 0.05*) between blueberry cultivars within each solvent type. Different capital letters indicate significant differences (*P≤ 0.05*) between solvents for each cultivar.

New Zealand grown rabbiteye blueberries extracted with acidified aqueous methanol contained total anthocyanin concentrations ranging from 0.57 – 4.2 mg/ g fruit weight (Scalzo et al., 2008). The previous study of rabbiteye blueberries grown in New Zealand used acidified methanol as an extracting solvent. Therefore total anthocyanins of blueberry fruit samples extracted with 5%FA from our study were comparable with the previous finding (0.48 – 0.80 mg/g FB). Acidification of solvent has the biggest effect on total anthocyanin yield where the concentrations of 5%FA extracts were 25 – 60% higher than their MQ extract counterparts. Solvent acidification showed that anthocyanins were more stable (Nicoué et al., 2007). Moreover, acidified solvent systems also benefit anthocyanin stability in extracting medium since anthocyanins are presented in the flavylium cation form at low pH which is more stable than the quinonodial pseudobase and chalcone forms (He & Giusti, 2010). Because anthocyanins are more stable in acidic environment than neutral conditions, therefore total anthocyanins were higher when extracted with acidified solvent than MQ.

Anthocyanins are found abundantly in the skin of blueberries. Therefore, blueberry pomace left from the first extraction still contained anthocyanins. Frozen blueberry pomace in our study extracted with MQ contained lower total anthocyanins compared to previous reports (Bener et al., 2013; Gil, 2013). In spite of using 5%FA as extracting solvent, total anthocyanins from frozen pomace were lower than previous studies. This might result from a single extraction carried out in our study, which might not be enough to complete anthocyanin extraction. Moreover, the pomace used in our study was centrifuged sediment from blueberry juice preparation. It is also clear “water extract” of pomace with or without formic acid is far less aggressive than a less polar solvent such as ethanol, methanol or hexane. The differences in extracting solvent and method of the blueberry pomace preparation might lead to TPC variation from other studies. Previous studies reported total anthocyanins from blueberry pomace ranged from 1.2 – 4.36 mg/g fruit weight (Bener et al., 2013; Gil, 2013). Moreover, pomace derived from different processes such as juice processing, wine making and vinegar yield different level of anthocyanins (Su & Silva, 2006). Therefore, the anthocyanin concentrations from different studies are difficult to compare.

There is no recommendation on the dose of daily intake of anthocyanins however, an average anthocyanin intake in U.S. adults was 12.5 mg/day which was lower than the average anthocyanin intake of people from European countries such as Spain (18.4 mg/day), Netherlands (19.8 mg/day), and Italy (44.1 mg/day) (Pojer at al., 2013). According

to our study, 1 cup (140 g) of rabbiteye blueberry provided 54 mg of anthocyanins. Twenty five grams of freeze dried blueberry pomace (approximately 3 tablespoons) would provide the same amount of anthocyanins. Anthocyanins mainly present in blueberry skin attach to fibre therefore they are difficult to extract using water. However, they might be released in the colon when fibre is fermented. Therefore, the freeze dried pomace might be another food source for additional anthocyanins.

Effect of freeze-drying on total anthocyanins seemed to be similar to the effect on TPC and TFC. Levels of total anthocyanins were higher in both freeze dried fruits and pomace compared to their non-freeze dried counterparts. Anthocyanins locate mainly in the skin of the fruits. Not only the physical deconstruction of the plant cells caused by ice crystal during the freezing period prior to lyophilisation resulted in high anthocyanins yield in freeze dried samples (Sablani et al., 2011), but also dehydration may have inactivated degradative enzyme action. Moreover, the grinding process ensured easier polyphenol release.

Anthocyanin peaks from the blueberry extracts were detected at 520 nm using HPLC coupled with PDA detection analysis (figure 3-5). The elution order of the five anthocyanidins was delphinidin, cyanidin, petunidin, peonidin and malvidin, as in previous studies (Hamamatsu et al., 2004; Li et al., 2013). Cyanidin 3-glucoside was used as a standard in our study. Specifically there were 13 to 15 individual anthocyanins detected in each chromatogram in this study: 1) Delphinidin 3-galactoside 2) Delphinidin 3-glucoside 3) Cyanidin 3-galactoside 4) Delphinidin 3-arabinoside 5) Cyanidin 3-glucoside 6) Petunidin 3-galactoside 7) Cyanidin 3-arabinoside 8) Petunidin 3-glucoside 9) Peonidin 3-galactoside 10) Petunidin 3-arabinoside 11) Peonidin 3-glucoside 12) Malvidin 3-galactoside 13) Peonidin 3-arabinoside 14) Malvidin 3-glucoside and 15) Malvidin 3-arabinoside.

There is not much published information for anthocyanin composition in New Zealand grown blueberries. All anthocyanidins except peonidin were reported in rabbiteye blueberries grown in New Zealand and a total of 12 galactosides, glucosides and arabinosides of the aglycones malvidin, delphinidin, cyanidin and petunidin were detected (Scalzo et al., 2008). Acylated anthocyanins were also found and contributed to approximately 5% of total anthocyanins. Hamamatsu (2004) reported 15 anthocyanins peaks also found in Japan grown ‘Tifblue’ blueberry extract. Li (2013) also reported that five anthocyanins were found in rabbiteye blueberries grown in Nanjing however, there were only 9 individual anthocyanins (Li et al., 2013). Another study only found four

anthocyanidins (delphinidin, cyanidin, petunidin and malvidin) and 11 glycosylated anthocyanins were found in 36 rabbiteye blueberries grown in USA (Wang et al., 2012b).

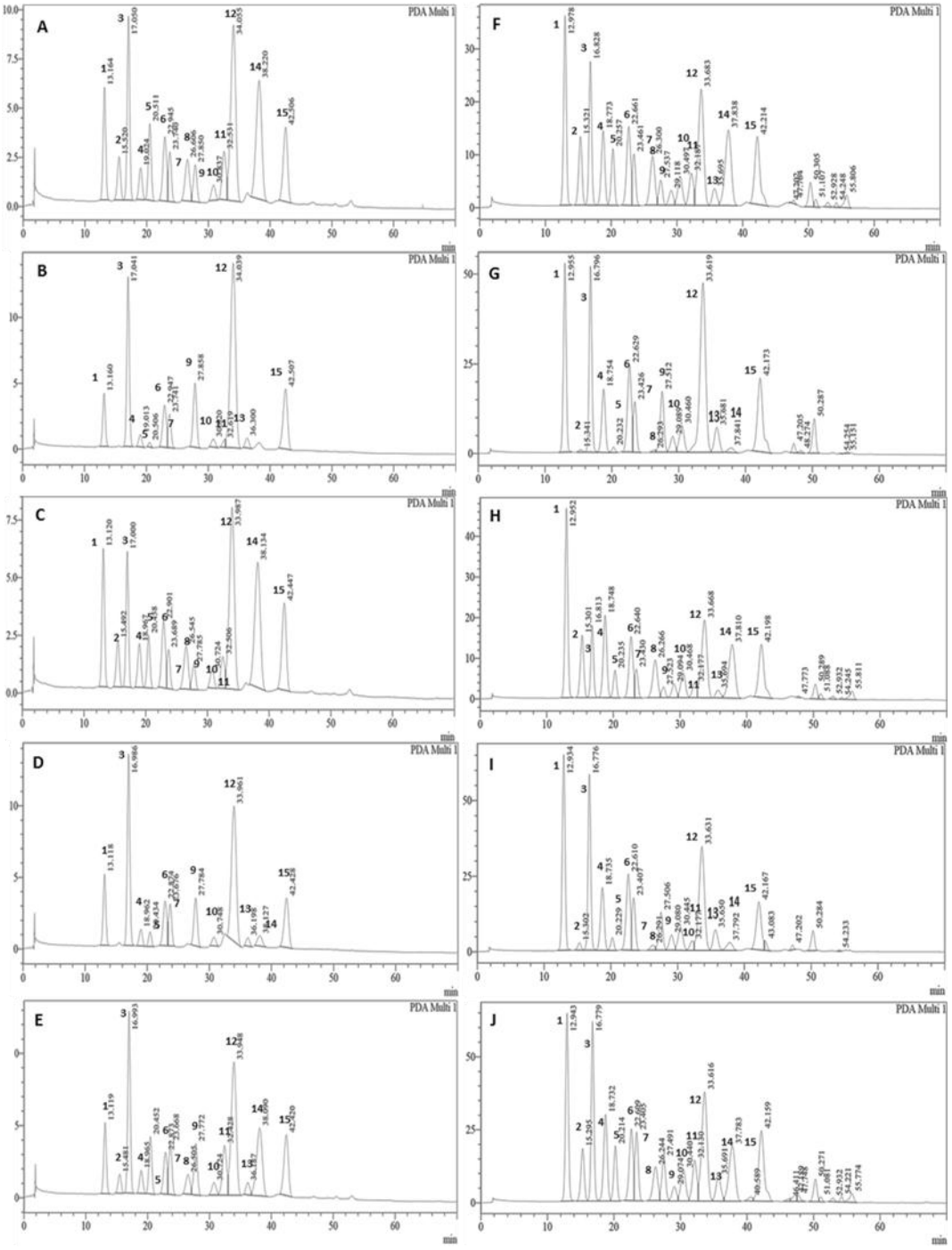


Figure 3-5 Representative anthocyanin chromatograms of five rabbiteye blueberry cultivars at 520 nm; A) 'Centurion' B) 'Maru' C) 'Rahi' D) 'Ono' and E) 'Tifblue'. A to E are MQ extracts. F) 'Centurion' G) 'Maru' H) 'Rahi' I) 'Ono' and J) 'Tifblue'. F to J are 5%FA extracts.

The same blueberry cultivars showed different anthocyanin composition in different studies. The percentage distribution of 'Tifblue' extracted with 5%FA in our study ranked from malvidin (30.1%) > delphinidin (20.4) > cyanidin (19.1%) > petunidin (13%) > peonidin (12.4%) > acylated anthocyanins (5%). 'Tifblue' extracted with acidified methanol had the anthocyanin composition ranked from malvidin (51%) > cyanidin (18%) > peonidin (14.5%) > petunidin (10.2%) > delphinidin (6.4%) (Hamamatsu et al., 2004). When acidified acetone was used as a solvent for extraction, the percentage anthocyanins of 'Tifblue' was malvidin (51.7%) > petunidin (25.1%) > cyanidin (17.7%) > delphinidin (11.1%) (Wang et al., 2012b). Even though the anthocyanin composition varied widely among blueberry cultivars, according to the data, the solvent used for extraction could also affect anthocyanin composition of the same blueberry cultivar. This finding might result from incomplete extraction using MQ as a solvent even though formic acid was added to aid anthocyanin extraction and stability. Anthocyanins have higher solubility in methanol (Ju & Howard, 2003). Collectively, variation of anthocyanin composition can be caused by genetic, geographic and experimental variation.

There are few studies on anthocyanin composition in blueberry pomace (Bener et al., 2013: Li et al., 2013). In our study, blueberry pomace contained 13 to 15 individual anthocyanins similar to the fruit extracts. An average percentage composition of blueberry pomace extracted with acidified water ranked from malvidin (29.2%) > cyanidin (20.6%) > delphinidin (18.1%) > petunidin (14.9%) > peonidin (13%) > acylated anthocyanin (4.2%). Li and colleagues (2013) found only four individual anthocyanins (delphinidin-3-galactoside, cyanidin-3-galactoside, peonidin-3-galactoside and malvidin-3-arabinoside) in rabbiteye blueberry pomace derived from wine making process while the fruit extract contained nine individual anthocyanins. Another study showed that blueberry pomace derived from juice making process contain nine individual anthocyanins. The percentage of anthocyanin composition was different from our study where malvidin contributed 57.6% of total anthocyanins followed by cyanidin (21.5%), petunidin (10.7%), delphinidin (5.4%) and peonidin (4.9%) (Gil, 2013).

Acylated anthocyanins were found in blueberry samples extracted with 5%FA but not present when MQ was used as an extracting solvent (table 3-1 to table 3-4). Acylated anthocyanin concentrations in frozen berries ranged from 23.3 - 44.5 μg/g frozen berries and from 12 – 20.3 μg/g frozen berries in freeze-dried berries. Frozen blueberry pomace also contained acylated anthocyanins which ranged from 8 – 12.2 μg/g frozen berries

however freeze-dried pomace had higher acylated anthocyanin which ranged from 13 – 18.2 µg/g frozen berries. Our finding was in agreement with acylated anthocyanin concentrations in rabbiteye blueberries, grown in New Zealand extracted with ethanol, water and formic acid mixture, which ranged from 1 – 206 µg/g frozen berries. An average acylated anthocyanin percentage in the study was 2.4% of total anthocyanin concentrations (Scalzo et al., 2008). Mostly rabbiteye blueberries in our study contained less than 5% of acylated anthocyanins except in 'Ono' frozen berries which contained 7.9% of total anthocyanins. Moreover, acylated anthocyanins were only present in 5%FA extracts. There might be several explanations for un-detected acylated anthocyanins in MQ extracts. Acylated anthocyanins might be present in small amounts in rabbiteye blueberries. Therefore, without sensitive detection methods, they are unable to be detected. Another possibility was acylation decreases water solubility of anthocyanins therefore the acylated anthocyanins were not found in the MQ extract. Acylated anthocyanins found in this study were mostly less than 5% of total anthocyanins except in frozen fruits of 'Ono' and 'Maru'.

Galactoside derivative is the first glycoside derivative detected by HPLC followed by glucoside derivative and arabinoside derivative. The major sugar moiety attached to anthocyanidin in rabbiteye blueberry extracts was galactoside. The patterns of sugar constitution in different cultivars were varied. 'Maru' and 'Ono' contained high level of galactoside (60%) followed by arabinoside (30%) and glucoside (10%), while 'Centurion', 'Rahi' and 'Tifblue' contained 40 to 45% of galactoside, 30 – 35% of glucoside and 20- 30% of arabinoside. Blueberry pomace extracts also showed similar pattern of sugar composition as the fruit extracts. Freeze-drying and acidification of solvent did not alter sugar composition. Sugar composition of 'Tifblue' in our study and Hamamatsu's study contained approximately 40% galactoside and 30% of glucoside and arabinoside. A similar trend was found in 'Centurion' extract in our study and Wang's study. The sugar composition was approximately 44% galactoside, 34% glucoside and 22% arabinoside. Even though different solvents were used in other studies (acidified methanol and acidified acetone), the sugar composition was unchanged. A pattern of sugar composition in blueberry pomace extract was similar to the fruit extracts where it varied by cultivars. In our study, galactoside was a major sugar moiety found in blueberry pomace and it accounted for 50% of total sugar moieties. Glucoside accounted for 27% and arabinoside contributed to 22% of total sugar moieties. Sugar composition of blueberry pomace derived from juice making process showed similar pattern where galactoside was the highest (40%) followed by glucoside (35%) and arabinoside (25%) (Gil, 2013).

Table 3-1: Individual anthocyanin components of the five cultivars of rabbiteye blueberry fruits extracted with MilliQ water or 5% aqueous formic acid

Peak no.	Compound	Frozen fruits (MQ)										Frozen fruits (5%FA)									
		'Centurion'		'Maru'		'Rahi'		'Ono'		'Tifblue'		'Centurion'		'Maru'		'Rahi'		'Ono'		'Tifblue'	
		µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%
1	**Delphinidin-3-galactoside**	12	3.7	40	7.8	9	4.6	11	3.7	35	5.7	48	8.9	110	14.5	68	13.5	69	12.2	82	9.7
2	**Delphinidin-3-glucoside**	10	3.1	9	1.8	9	4.6	0	0	18	3	28	5.2	55	7.3	12	2.4	11	1.9	37	4.4
3	**Cyanidin-3-galactoside**	25	7.7	49	9.6	14	7.2	36	12.1	58	9.5	43	8	48	6.3	64	12.7	0	0	76	9
4	**Delphinidin-3-arabinoside**	10	3.1	20	3.9	0	0	10	3.4	21	3.4	27	5	65	8.6	33	6.6	36	6.4	53	6.3
5	**Cyanidin-3-glucoside**	17	5.3	11	2.2	11	5.7	11	3.7	29	4.8	25	4.7	30	4	14	2.8	12	2.1	38	4.5
6	**Petunidin-3-galactoside**	13	4	33	6.5	9	4.6	13	4.4	28	4.6	34	6.3	52	6.9	38	7.6	45	8	45	5.3
7	**Cyanidin-3-arabinoside**	15	4.7	22	4.3	10	5.2	17	5.7	31	5.1	23	4.3	30	7.9	31	6.2	32	5.6	47	5.6
8	**Petunidin-3-glucoside**	13	4	13	2.5	9	4.6	10	3.4	22	3.6	29	5.4	47	6.2	12	2.4	11	1.9	34	4
9	**Peonidin-3-galactoside**	14	4.3	32	6.3	11	5.7	20	6.7	29	4.8	19	3.5	18	2.4	26	5.2	36	6.4	36	4.3
10	**Petunidin-3-arabinoside**	10	3.1	15	2.9	0	0	11	3.7	18	3	18	3.4	32	4.2	19	3.8	24	4.2	31	3.7
11	**Peonidin-3-glucoside**	16	5	0	0	12	6.2	12	4	31	5.1	22	4.1	24	3.2	12	2.4	30	5.3	38	4.5
12	**Malvidin-3-galactoside**	66	20.4	176	34.5	38	19.6	76	25.5	122	20	86	16	95	12.6	77	15.3	126	22.3	107	12.7
13	**Peonidin-3-arabinoside**	11	3.4	10	2	0	0	12	4	16	2.6	0	0	22	2.9	21	4.2	13	2.3	30	3.6
14	**Malvidin-3-glucoside**	57	17.7	21	4.1	37	19.1	24	8	88	14.4	69	12.9	14	1.8	14	2.8	11	1.9	76	9
15	**Malvidin-3-arabinoside**	34	10.5	59	11.6	25	12.9	35	11.7	63	10.4	42	7.8	72	9.5	39	7.8	65	11.5	72	8.5
Total non-acylated anthocyanin		**323**	**100**	**510**	**100**	**194**	**100**	**298**	**100**	**609**	**100**	**513**	**95.7**	**714**	**94.4**	**480**	**95.6**	**521**	**92.1**	**802**	**95**
Total acylated anthocyanin												**23.3**	**4.3**	**42.7**	**5.6**	**22.2**	**4.4**	**44.5**	**7.9**	**42.6**	**5**

Data express as mean of three replicate samples; Peak numbers refer to HPLC chromatogram in figure 3-5

Table 3-2: Individual anthocyanin components of the five cultivars of freeze-dried rabbiteye blueberry fruits extracted with MilliQ water or 5% aqueous formic acid

Peak no.	Compound	Freeze-dried fruits (MQ)										Freeze-dried fruits (5%FA)									
		'Centurion'		'Maru'		'Rahi'		'Ono'		'Tifblue'		'Centurion'		'Maru'		'Rahi'		'Ono'		'Tifblue'	
		µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%
1	Delphinidin-3-galactoside	87	7.6	65	6.7	87	8.4	76	8.5	76	6.3	280	11.1	391	12.7	351	15	471	15.2	461	10.8
2	Delphinidin-3-glucoside	65	5.7	0	0	54	5.2	0	0	54	4.4	140	5.5	40	1.3	160	6.8	60	1.9	181	5.6
3	Cyanidin-3-galactoside	108	9.4	130	13.4	87	8.4	130	14.5	130	10.7	211	8.4	371	12	150	6.4	411	13.3	431	14
4	Delphinidin-3-arabinoside	54	4.7	43	4.5	54	5.2	54	6	54	4.4	160	6.3	191	6.3	211	9	221	7.2	291	9.4
5	Cyanidin-3-glucoside	76	6.6	43	4.5	54	5.2	43	4.8	76	6.3	120	4.8	50	1.7	30	1.3	70	2.3	181	5.7
6	Petunidin-3-galactoside	76	6.6	76	7.8	65	6.3	76	8.4	65	5.4	180	7.1	271	8.8	90	3.8	280	9.1	271	8.8
7	Cyanidin-3-arabinoside	65	5.7	64	6.6	54	5.2	65	7.3	65	5.4	110	4.4	140	4.5	90	3.8	171	5.5	221	7.2
8	Petunidin-3-glucoside	65	5.7	0	0	65	6.3	0	0	54	4.4	140	5.5	40	1.3	140	6	50	1.5	170	5.5
9	Peonidin-3-galactoside	54	4.7	87	9	54	5.2	76	8.4	76	6.3	80	3.2	191	6.2	60	2.6	150	4.8	170	5.5
10	Petunidin-3-arabinoside	43	3.7	43	4.5	43	4.1	43	4.8	54	4.5	100	4	120	3.9	100	4.3	120	3.9	160	5.2
11	Peonidin-3-glucoside	65	5.7	43	4.5	54	5.2	0	0	76	6.3	100	4	0	0	70	3	70	2.3	181	5.7
12	Malvidin-3-galactoside	173	15	238	24.5	151	14.5	162	18	173	14.3	321	12.7	672	21.8	291	12.4	481	15.6	521	16.9
13	Peonidin-3-arabinoside	0	0	43	4	0	0	43	4.8	54	4.4	10	0.4	50	1.6	60	2.6	70	2.3	140	4.5
14	Malvidin-3-glucoside	130	11.3	0	0	129	12.4	43	4.8	108	8.9	251	10	110	3.6	231	9.9	110	3.6	321	10.4
15	Malvidin-3-arabinoside	87	7.6	97	10	87	8.4	87	9.7	97	8	201	7.9	301	9.8	201	8.6	231	7.5	351	11.4
Total non-acylated anthocyanin		**1148**	**100**	**972**	**100**	**1038**	**100**	**898**	**100**	**1212**	**100**	**2404**	**95.3**	**2938**	**95.5**	**2235**	**95.5**	**2966**	**96**	**4051**	**95.2**
Total acylated anthocyanin												**12**	**4.7**	**14**	**4.6**	**10.5**	**4.3**	**12**	**3.7**	**20.3**	**4.8**

Data express as mean of three replicate samples

Table 3-3: Individual anthocyanin components of the five cultivars of rabbiteye blueberry pomace extracted with MilliQ water or 5% aqueous formic acid

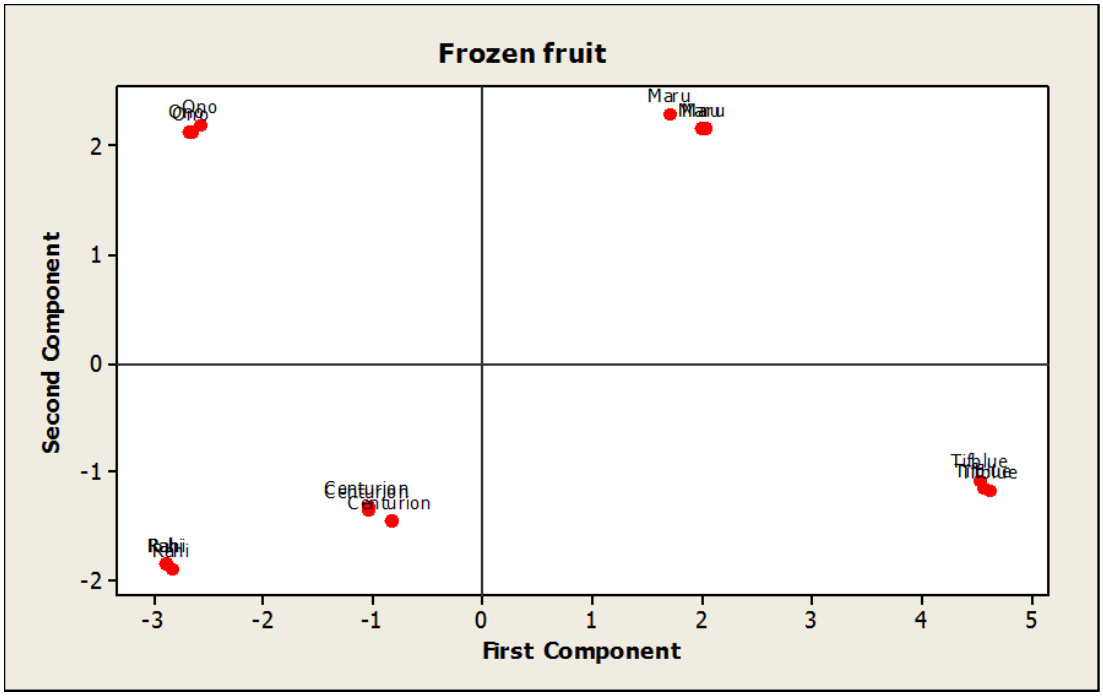
Peak no.	Compound	Frozen Pomace (MQ)										Frozen Pomace (5%FA)									
		'Centurion'		'Maru'		'Rahi'		'Ono'		'Tifblue'		'Centurion'		'Maru'		'Rahi'		'Ono'		'Tifblue'	
		µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%
1	**Delphinidin-3-galactoside**	11	7.2	6	6.8	13	8.4	12	9	12	7	40	12.3	31	12.8	42	14.8	61	18.3	28	11.5
2	**Delphinidin-3-glucoside**	7	4.6	0	0	8	5.2	4	3	7	4.1	21	6.5	4	1.7	21	7.4	6	1.8	12	4.9
3	**Cyanidin-3-galactoside**	14	9.2	11	12.5	10	6.5	16	12	18	10.5	25	7.7	30	12.4	16	5.6	43	12.9	26	10.7
4	**Delphinidin-3-arabinoside**	6	3.9	4	4.5	7	4.6	6	4.5	7	4.1	20	6.2	16	6.6	24	8.5	26	7.8	17	7
5	**Cyanidin-3-glucoside**	9	5.9	4	4.5	7	4.6	5	3.8	10	5.8	14	4.3	5	2.1	10	3.5	7	2.1	11	4.5
6	**Petunidin-3-galactoside**	10	6.5	7	8	10	6.5	10	7.5	9	5.3	23	7.1	21	8.7	21	7.4	32	9.6	15	6.2
7	**Cyanidin-3-arabinoside**	6	3.9	6	6.8	6	3.9	7	5.3	9	5.3	13	4	12	5	9	3.2	17	5.1	14	5.8
8	**Petunidin-3-glucoside**	8	5.2	0	0	9	5.8	4	3	8	4.7	20	6.2	14	5.8	17	6	6	1.8	11	4.5
9	**Peonidin-3-galactoside**	7	4.6	8	9.1	6	3.9	9	6.8	9	5.3	9	2.8	5	2.1	6	2.1	15	4.5	10	4.1
10	**Petunidin-3-arabinoside**	5	3.3	4	4.6	6	3.9	5	3.7	6	3.5	12	3.7	10	4.2	12	4.2	13	3.9	10	4.1
11	**Peonidin-3-glucoside**	8	5.2	4	4.6	7	4.5	5	3.7	12	7	11	3.4	4	1.6	8	2.8	6	1.8	11	4.5
12	**Malvidin-3-galactoside**	26	17	21	23.9	26	16.9	28	21.1	25	14.6	41	12.6	47	19.5	34	12	51	15.3	26	10.7
13	**Peonidin-3-arabinoside**	4	2.6	0	0	4	2.6	5	3.8	6	3.5	7	2.2	8	3.3	6	2.1	8	2.4	8	3.3
14	**Malvidin-3-glucoside**	21	13.7	4	4.5	22	14.3	6	4.5	20	11.7	32	9.8	5	2.1	27	9.5	10	3	17	7
15	**Malvidin-3-arabinoside**	11	7.2	9	10.2	13	8.4	11	8.3	13	7.6	25	7.7	22	9.1	24	8.4	24	7.2	17	7
Total non-acylated anthocyanin		**153**	**100**	**88**	**100**	**154**	**100**	**133**	**100**	**171**	**100**	**313**	**96.5**	**234**	**97**	**277**	**97.5**	**325**	**97.6**	**233**	**95.8**
Total acylated anthocyanin												**12.2**	**3.5**	**7.3**	**3**	**7.3**	**2.5**	**8**	**2.4**	**9.8**	**4.2**

Data express as mean of three replicate samples

Table 3-4: Individual anthocyanin components of the five cultivars of freeze-dried rabbiteye blueberry pomace extracted with MilliQ water or 5% aqueous formic acid

Centurion	Compound	Freeze-dried Pomace (MQ)										Freeze-dried Pomace (5%FA)									
		'Centurion'		'Maru'		'Rahi'		'Ono'		'Tifblue'		'Centurion'		'Maru'		'Rahi'		'Ono'		'Tifblue'	
		µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%	µg/g	%
1	**Delphinidin-3-galactoside**	34	6.5	29	7	29	6.7	29	7.3	29	5.9	226	9.9	231	10.6	264	12.3	275	13.2	148	8.6
2	**Delphinidin-3-glucoside**	24	4.6	0	0	24	5.6	0	0	20	3.9	110	4.8	22	1	121	5.6	33	1.6	66	3.4
3	**Cyanidin-3-galactoside**	49	9.3	54	12.8	34	7.9	49	12	49	9.8	198	8.7	270	12.4	138	6.4	297	14.2	176	10.2
4	**Delphinidin-3-arabinoside**	24	4.6	20	4.6	24	5.6	20	4.8	20	3.9	132	5.8	115	5.3	165	7.7	132	6.3	104	6.1
5	**Cyanidin-3-glucoside**	34	6.5	20	4.6	24	5.6	20	4.8	30	5.9	105	4.6	28	1.3	77	3.6	49.5	2.4	77	4.5
6	**Petunidin-3-galactoside**	34	6.5	29	7	29	6.8	30	7.3	29	5.9	165	7.3	192	8.8	160	7.4	192	9.2	110	6.4
7	**Cyanidin-3-arabinoside**	29	5.6	24	5.8	24	5.6	29	7.3	29	5.9	99	4.4	104	4.8	77	3.6	121	5.8	99	5.8
8	**Petunidin-3-glucoside**	29	5.6	0	0	24	5.6	24	6	24	4.9	127	5.6	22	1	121	5.6	38.5	1.8	72	4.2
9	**Peonidin-3-galactoside**	29	5.6	39	9.3	20	4.5	24	6	34	6.9	61	2.7	143	6.6	44	2.1	104	5	77	4.5
10	**Petunidin-3-arabinoside**	20	3.7	20	4.7	20	4.5	20	4.8	20	3.9	82	3.6	88	4	88	4.1	83	4	72	4.2
11	**Peonidin-3-glucoside**	29	5.5	20	4.7	24	5.6	39	9.6	34	6.9	82	3.6	0	0	60	2.8	44	2.1	82	4.8
12	**Malvidin-3-galactoside**	83	15.7	103	24.4	64	14.6	60	14.5	69	13.7	302	13.3	522	24	275	12.8	341	16.3	214	12.5
13	**Peonidin-3-arabinoside**	5	0.9	20	4.6	0	0	5	1.2	20	3.9	55	2.4	33	1.5	44	2	55	2.6	61	3.5
14	**Malvidin-3-glucoside**	64	12	0	0	54	12.4	24	6	54	10.8	226	9.9	82	3.8	220	10.3	77	3.7	138	8
15	**Malvidin-3-arabinoside**	39	7.4	44	10.5	39	9	34	8.4	39	7.8	198	8.7	226	10.4	192	9	165	7.9	148	8.6
Total non-acylated anthocyanin		**526**	**100**	**422**	**100**	**433**	**100**	**407**	**100**	**500**	**100**	**2168**	**95.6**	**2078**	**95.5**	**2046**	**95.6**	**2007**	**96.2**	**1644**	**95.8**
Total acylated anthocyanin												**100**	**4.4**	**98**	**4.5**	**95**	**4.4**	**80**	**3.8**	**72**	**4.2**

Data express as mean of three replicate samples



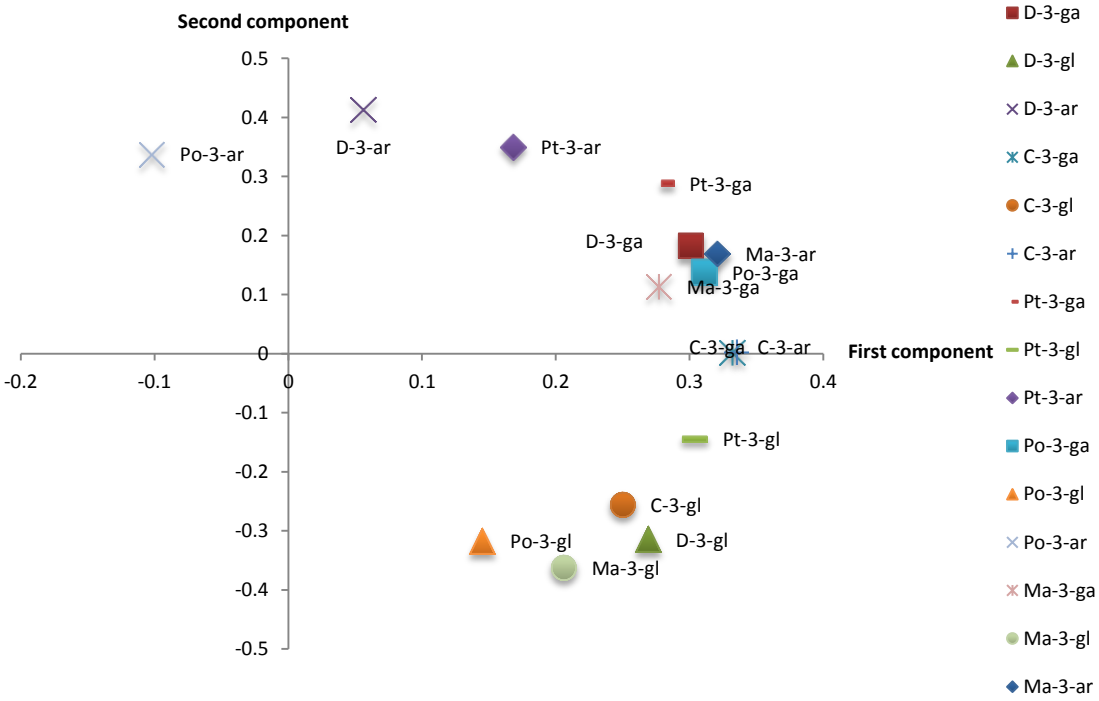


Figure 3-6 A) Principal component analysis of individual anthocyanins from five rabbiteye blueberry cultivars, B) Score plot for principal components 1 and 2 showing the contribution of each anthocyanin to the observed PCA pattern.

The primary objective of this thesis is to evaluate the effect of blueberry extracts on DMBA-induced mammary tumorigenesis in an animal model. MQ rabbiteye blueberry extracts will be supplied in drinking liquid form without concentration or purification. Therefore principal component analysis (PCA) from five rabbiteye blueberry MQ extracts was chosen as a representative of blueberry extracts in this study. Information from 15 individual anthocyanins (table 3-1) was used to perform PCA for all five cultivars. A score plot of the first two components (figure 3-6A) revealed the differences among cultivars. The five blueberry cultivars fell into two groups with regard to the first principal component. 'Centurion', 'Rahi' and 'Ono' were on the left hand side of PC1 axis while 'Maru' and 'Tifblue' presented in the right hand side of the axis. The proportion of the effect of the first component on five rabbiteye blueberry cultivars was 0.581. The second component (PC2) also impacted distribution of blueberry cultivars (proportion = 0.231) where 'Ono' and 'Maru' were above PC2 axis and 'Centurion', 'Rahi' and 'Tifblue' were below the PC2 axis.

In order to determine major anthocyanin components that drive PC1 and PC2, the loading plot was then generated (figure 3-6B). Data from the loading plot and table 3-1 revealed that the first principal component was delphinidin. 'Maru' and 'Tifblue' were rich in delphinidin (more than 12% in the extracts). 'Ono', 'Rahi' and 'Centurion' were presented on the left hand side of the PC1 axis and contained less than 10% of delphinidin in the extracts. The second contributor was glucose molecule as presented in the loading plot. Anthocyanins glycosylated with glucose molecule were found below the PC2 axis. 'Tifblue', 'Centurion' and 'Rahi' contained at least 30% of anthocyanin glucosides while 'Maru' and 'Ono' contained less than 20% anthocyanin glucosides.

In this study, we have found that acidification of solvents affected the pattern of anthocyanins. Generally, malvidin was the highest contributor of total anthocyanin concentration. At least 30% of malvidin was found in blueberries used in this study. According to the presented data, the average percentage distribution changed with solvent acidification. Malvidin and cyanidin were the major contributors of total anthocyanin concentration when the samples were extracted with MQ while delphinidin, petunidin and peonidin contributed in a similar level. The percentage distribution of each anthocyanin changed in different degree when 5%FA was used as an extraction solvent. When the samples were extracted with 5%FA, malvidin and delphinidin were the major contributors of total anthocyanin concentration. Delphinidin percentage increased from 10 – 15% in MQ extracts to 20 – 25% in 5%FA. The percentage distribution of cyanidin also went up

approximately 20% in 5%FA. The percentage distribution of malvidin, petunidin and peonidin dropped approximately 17%, 9% and 28%, respectively.

Various organic acids have been used to enhance extraction efficiency of solvents. Kalt et al. (2008) found that the acid added to the solvents was not only enhancing anthocyanin extraction but also stabilizing anthocyanins in the extracts. Moreover, Michiels et al. (2012) showed that a small concentration of acetic acid in an extraction solvent had an influence on TPC and antioxidant activity of the plant extracts. Organic acids such as formic acid might have the ability to break the plant cell walls which lead to higher yield compared to non-acidified solvents.

Chlorogenic acid concentration

Chlorogenic acid is another polyphenol found abundantly in blueberries. Overall, the trend of chlorogenic acid concentration was similar among four types of sample. 'Tifblue' exhibited the highest chlorogenic acid concentration followed by 'Ono' and 'Maru' while 'Centurion' and 'Rahi' were the lowest (figure 3-7). Chlorogenic acid was found in much higher concentrations in frozen berries (184 -482 μg/g FB) than in the frozen pomace (3.8 – 24.8 μg/g frozen pomace) when extracted with MQ. Freeze-dried berries had significantly lower chlorogenic acid concentrations (72 – 162 μg/g FB) than their untreated counterparts. However, freeze-drying did not have much effect on blueberry pomace. Frozen pomace extracts and FD-pomace extracts displayed similar chlorogenic acid concentration ranges. Acidification of solvent also had an effect on chlorogenic acid concentration. Samples extracted with 5%FA gave higher yields in comparison with samples extracted with MQ. In frozen berries extracted with 5%FA, the chlorogenic acid concentration ranged from 233 – 634 μg/g FB. The chlorogenic acid concentration yield increased approximately 20% on average when acidified water was used. The effect of acidification seemed to be greater when applied in freeze-dried samples. There was approximately 40% increment in the chlorogenic acid concentration yield when the freeze-dried samples were extracted with 5%FA in comparison with their MQ extracts counterparts.

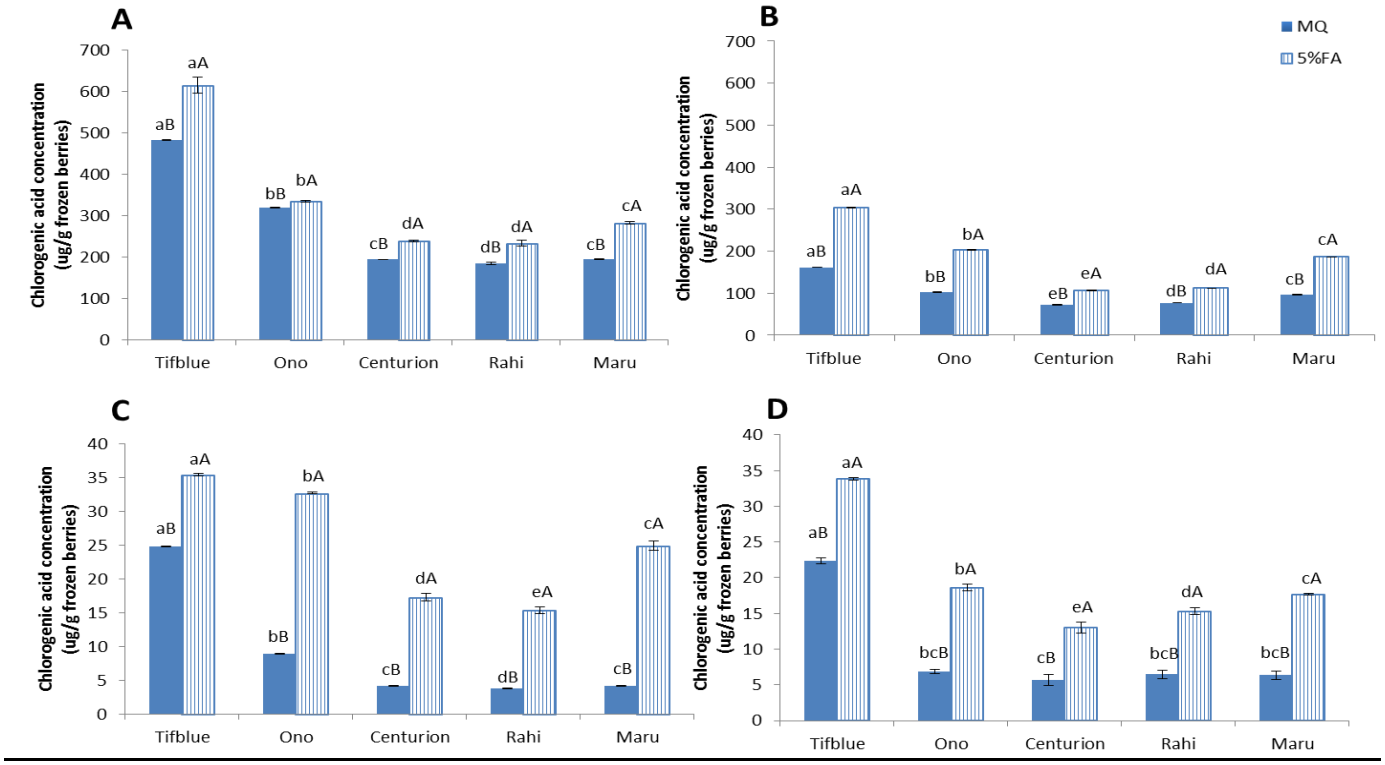


Figure 3-7 Chlorogenic acid concentrations of A) frozen berries, B) freeze-dried berries, C) frozen pomace and D) freeze-dried pomace of five rabbiteye blueberry cultivars extracted by MilliQ water or water plus 5% formic acid. Chlorogenic acid concentrations are expressed as mg/g frozen berries. Data expressed as means ± SEM. Different lower-case letters indicate significant differences (*P≤ 0.05*) between blueberry cultivars within each solvent type. Different capital letters indicate significant differences (*P ≤ 0.05*) between solvents for each cultivar.

The range of chlorogenic acid concentration in rabbiteye blueberries was 84.5 – 686 µg/g FB (Wang et al., 2012b). Blueberry pomace contained chlorogenic acid at 12.3 µg/g dried pomace when extracted with hydrochloric acid acidified distilled water (Bener et al., 2013). The chlorogenic acid concentrations in our study were within the range of what has been previously reported. Chlorogenic acid was found to be a major colourless phenolic in blueberries (Gao & Mazza, 1994). It is mainly found in flesh of blueberries (Birt, 2011). In pomace, lower concentration of chlorogenic acid was observed because pomace mainly consists of skin and seed. Chlorogenic acid is a water-soluble compound, however acidification of extracting solvent by formic acid might cause plant cell rupture yielding higher chlorogenic acid concentration compared to normal water extraction.

Conclusion:

Our study showed that rabbiteye blueberries grown in New Zealand contain biological compounds such as phenolic and flavonoid compounds within the range of other studies. 'Tifblue' contained the highest TPC, TFC, anthocyanins and chlorogenic acid. Solvent acidification resulted in higher yield in both TPC and TFC. Lyophilisation also showed higher TPC and TFC than the non-freeze dried samples. Anthocyanins in rabbiteye blueberries consisted of glycosylated delphinidin, cyanidin, petunidin, peonidin and malvidin with glucose, galactose or arabinose sugar molecule. Percentage distribution of anthocyanins varied among cultivars however malvidin and cyanidin were the major anthocyanidins found in rabbiteye blueberries in our study. Acidification of solvent with formic acid affected extraction yield and percentage distribution of anthocyanins. Instead of cyanidin, delphinidin became one of the major anthocyanins found in samples extracted with 5%FA. Moreover, when 5%FA was used as a solvent, acylated anthocyanins were present in HPLC chromatograms. Similar to TPC and TFC, total anthocyanins increased when the blueberry samples were freeze dried prior to extraction. 5%FA increased chlorogenic acid concentration in all tested samples compared to MQ extracts, however freeze drying treatment resulted in a decrease of chlorogenic acid concentration compared to their non-freeze dried counterparts.

CHAPTER 4

In vitro antioxidant, prebiotic, antimicrobial and *in ovo* anti-angiogenic activities of rabbiteye blueberry extracts

Abstract:

Blueberries exhibit several biological activities which may affect mammary tumorigenesis. Antioxidant, prebiotic, antimicrobial and anti-angiogenic activities of blueberry extracts were studied.

Water extract of frozen 'Tifblue' berries showed the highest antioxidant activities (FRAP: 6.09 mg $FeSO_4$/g frozen berries and ORAC: 11.73 µmol Trolox equivalent (TE)/g frozen berries) than other tested cultivars ('Centurion', 'Maru', 'Rahi' and 'Ono'). Part of fruit, drying treatment and extracting solvents showed significant effects on antioxidant activities of blueberry extracts. Water pomace extracts showed lower antioxidant activities than whole berry extracts. Lyophilisation slightly increased antioxidant activity of water extracts from whole berries and pomace and using 5% aqueous formic acid led to a significant increase in antioxidant activity. Blueberry extracts showed prebiotic properties for *Lactobacillus rhamnosus* and *L. acidophilus*. Bacteria population differed only at the end of the study (120 h), by which time *L. rhamnosus* and *L. acidophilus* population incubated with blueberry MQ extracts was significantly higher than their controls by 0.6 and 0.9 log CFU/mL, respectively. Blueberry extracts in this study did not show antimicrobial activity against three pathogenic bacteria (*Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus*). Blueberry MQ extracts applied on chicken chorioallantoic membrane (CAM) assay showed an anti-angiogenic property. 'Maru', 'Centurion' and 'Tifblue' fruit extracts exhibited more than 50% inhibition of blood vessel density in comparison with control samples. The pomace extracts also exhibited anti-angiogenic activity but lower than the berry extracts. Total phenolic concentration and chlorogenic acid concentration from the previous chapter strongly correlated with antioxidant activity

Material from this chapter is included in papers: a) Vuthijumnonk, J., Molan, A. L., & Heyes, J. A., (2013). Effect of freeze-drying and extraction solvents on the total phenolic contents, total flavonoids and antioxidant activity of different Rabbiteye blueberry genotypes grown in New Zealand , *IOSR Journal of Pharmacy and Biological Sciences (IORS-JPBS), 8*(1), 42-48.
b) Vuthijumnonk, J., Heyes, J. A., & Molan, A. L. (2016). Total anthocyanins, chlorogenic acid concentration, antioxidant and *in ovo* anti-angiogenic activities of rabbiteye blueberries, *International Food Research Journal, 23*(2), 515-520.

while anthocyanin content also showed positive correlation with antioxidant activity but the correlation was less strong. Interestingly, total anthocyanins showed a stronger relationship with anti-angiogenic activity than total phenolic concentration and chlorogenic acid concentration.

In conclusion, blueberries contain various phytochemicals that may be responsible for different biological activities, which contribute to health benefits of blueberries. Collectively, the prophylactic and therapeutic properties of blueberry consumption on breast cancer might result from the synergistic effect of phytochemicals and biological properties in blueberry fruits.

Introduction:

Health benefits of blueberries have been widely studied (Szajdek & Borowska, 2008; Zafra-Stone et al., 2007; Howell, 2009; Cardona et al., 2013). Major polyphenols found in blueberries are anthocyanins. Blueberries are among the fruit with the highest antioxidant concentration as measured by the oxygen radical absorbance capacity (ORAC) assay (Prior, 1998). Oxidative stress, an imbalance of free radical production and detoxification within cells, has been found to correlate with several diseases including breast cancers (Reuter et al., 2010). Antioxidants play a major role in interception of oxidative stress caused by reactive oxygen species (ROS). Therefore, ingested blueberry antioxidants might reduce breast cancer promotion or progression either directly (e.g. in the gut lumen) or indirectly (by triggering antioxidant response systems in the body) (Stoner et al., 2008).

The antioxidant capacity of blueberries was determined by generally used methods which are ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC). By using FRAP assay, the extract capacity in donating an electron which results in a reduction of Fe (III) iron to Fe (II) iron, is measured and presented as FRAP value based on concentration of standard used (eg mg $FeSO_4$ equivalent, µmol Trolox equivalent). The FRAP value correlates positively with antioxidant activity of a tested sample. ORAC assay measures a protective effect of a tested sample against peroxyl radicals generated by the free radical generator (2,2'-azobis(2-amidino-propane): AAPH). The higher ORAC value corresponds to the higher antioxidant activity of a tested sample.

Antioxidant activity of rabbiteye blueberries reported in published studies ranged from 4.6 to 55.7 µmol Trolox equivalent (TE)/g berries as measured by ORAC assay (Connor

et al., 2002; You et al., 2011; Li et al., 2013). Antioxidant activities of blueberries are affected by geographic and growing conditions. It is, therefore, necessary to evaluate antioxidant activity of the NZ grown rabbiteye blueberries used in this study. Moreover, a by-product of blueberry extraction, blueberry pomace, is of interest since anthocyanins are mainly found in the skin of the fruit. A study showed that blueberry extract from blueberry pomace reduced lipid oxidation, as measured by the level of thiobarbituric acid in microencapsulated Pollock liver oil (Li et al., 2014). Rabbiteye blueberries grown in China and their pomace were studied for their antioxidant activities (Li et al., 2013). The authors reported that the blueberry pomace extracts showed higher ORAC values than the blueberry fruit extracts based on the dried weight of the samples. Therefore, it is necessary that blueberry pomace in our study should be evaluated for its antioxidant activity as well as water extracts of whole berries.

In this chapter, the same blueberry extracts obtained from the same extraction methods described in chapter 3 were used for antioxidant activity assays.

In addition to the antioxidant activity of blueberries that might have an important role in prevention of breast cancer, intestinal microorganisms or gut microflora also play an important role in their host's health (Guarner & Malagelada, 2003). Breast cancer patients hosted different intestinal bacterial flora composition from their age-matched control healthy subjects (Minelli et al., 1990). *In vitro* studies of prebiotic and antimicrobial activities of blueberries have been carried out. In New Zealand, an extract of commercial blueberries enhanced the growth of *Lactobacillus acidophilus* cultured from commercial yogurt (Hap, 2010). *Lactobacillus* spp. and *Bifidobacterium* spp. population increased after incubation with blueberry extracts compared to the control culture without blueberry extracts (Molan et al., 2009). Blueberries do not only benefit host health by improving the growth of beneficial bacteria, they possess antimicrobial effect on some pathogenic bacteria as well. The growth of *Salmonella enteritidis*, a foodborne pathogenic bacterium, was inhibited by a phenolic-rich extract from blueberries (Park et al., 2011). Even though crude extract of lowbush blueberries was not effective against *E. coli* O157:H7, *Listeria monocytogenes*, or *Salmonella typhimurium*, the anthocyanin and proanthocyanidin rich fraction demonstrated more pronounced antimicrobial activity (Lacombe et al., 2012). Blueberries exhibited both prebiotic and antimicrobial activities which might be a target for modulation of breast cancer incidence *in vivo*. However, the effects might vary depending

on types of blueberries and experimental conditions. Therefore *in vitro* screening for both prebiotic and antimicrobial activities is necessary.

Tumour growth and cancer progression rely on a process known as angiogenesis (Fan et al., 2006). Angiogenesis is the process of new blood vessel formation. The process occurs during embryogenesis, menstruation and wound healing in normal conditions (Risau, 1997). However, excessive angiogenesis might facilitate progression of some pathogenic conditions including cancers (Fan et al., 2006). Tumours start off as small clusters of cells that grow uncontrollably however without oxygen and nutrient supply the tumours cannot grow. Furthermore, the new blood vessels that feed the growing tumours also provide a channel for tumour metastases (Weis & Cheresh, 2011). Therefore inhibition of new blood vessel formation may serve as another promising strategy for tumour chemoprevention or therapy (Ferrara & Kerbel, 2005). Several studies have shown that blueberries possess anti-angiogenic activity (Roy et al., 2002; Atalay et al., 2003; Bagchi et al., 2004; Ozgurtas et al., 2008). Blueberries exhibited anti-angiogenesis *in vitro* by suppressing vascular endothelial growth factor (VEGF) expression in human keratinocyte cells treated with H_2O_2 (Roy et al., 2002). Another assay that is widely used for screening of anti-angiogenic substances is chicken chorioallantoic membrane (CAM) assay. The assay is carried out by incubation of fertilized hen's eggs for five to seven days. Then the eggshell is opened and a known concentration of the test substance is applied on CAM. The results can be observed between 48 to 72 hours after administration of the test substance. Bilberry extract was applied on CAM and the diameter of the blood vessel underneath the extracts did not grow as large as the blood vessels in a control area after 48 hours of incubation (Ozgurtas et al., 2008).

Biological activities of blueberries have been attributed to their variety of phytochemicals. A relationship between total phenolic concentration and antioxidant activities was strong and positive (R = 0.99) (Connor et al., 2002). Even though anthocyanins possess antioxidant activity their correlation with antioxidant values was less than total phenolic concentration (Ehlenfeldt & Prior, 2001). Anti-angiogenic activity of berry fruits was studied and revealed that high antioxidant activity did not necessarily contribute to high anti-angiogenic activity (Roy et al., 2002). The authors reported that anti-angiogenic activity of the berries was a result of particular flavonoids rather than all polyphenols.

As reported in the previous chapter, we measured total phenolic concentration (TPC), total flavonoid concentration, total anthocyanins and their composition and chlorogenic acid concentration. It was found that acidification of solvent by 5% formic acid aided polyphenol extraction. In this chapter antioxidant activity of five NZ rabbiteye blueberries and their pomace extracts will be examined using FRAP and ORAC assay. Prebiotic effect of the blueberry extracts will be studied in beneficial bacteria (*L. acidophilus* and *L. rhamnosus*). Agar well diffusion method will be used for evaluation of antimicrobial activity against three pathogenic bacteria: *E. coli* (NCTC 12900)*, S.typhimurium* (ATCC 14028)*,* and *S. aureus*. Screening of anti-angiogenic activity of the blueberry extracts will be studied using CAM assay. The relationship between blueberry phytochemical concentrations from chapter 3 (total phenolic concentration, total anthocyanins and chlorogenic acid concentration), and their biological activities (antioxidant and anti-angiogenic activities) will also be examined. This study needs to be carried out in order to find the most suitable blueberry cultivars for *in vivo* study.

Materials and methods:

Raw materials

Ripe berries of five rabbiteye blueberry (*Vaccinium ashei*) cultivars ('Centurion', 'Maru', 'Rahi', 'Ono' and 'Tifblue') were purchased from Beaches Blueberries Ltd, Hamilton, New Zealand. All samples were grown under the same environmental conditions. They were all harvested at commercial maturity and stored at -20°C until used.

Blueberry extraction was performed according to the process in chapter 3. The same blueberry extracts were used for antioxidant activity determination. The extracts used for prebiotic, antimicrobial and anti-angiogenic activities were sterilised using Phenex 0.22 µm PVDF syringe filter (Phenomenex, USA), with aliquots delivered into sterilised Eppendorf tubes and kept at -20°C until used.

Antioxidant capacity

Ferric Reducing Antioxidant Power (FRAP)

Ferric reducing antioxidant power assay is a widely used method for determination of antioxidant activity of tested samples (Benzie & Strain, 1996). The philosophy of this assay is based on the ability of the antioxidant compounds to reduce the ferric-tripyridyltriazine complex to its ferrous form. In this study, the procedure described by

Benzie and Strain (1996) with some modifications (Molan et al., 2009a) was followed. Briefly, the FRAP reagent was prepared by mixing 300 mM sodium acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM ferric chloride (10:1:1). Aliquots of 8.5 µl of blueberry samples were mixed with FRAP reagent (275 µL) in a 96-well plate. The plate was incubated at 37°C in the dark for 30 minutes and then the absorbance of the mixture was measured at 595 nm using a microplate reader (ELX 808 Ultra microplate reader, BIO-TEK instrument) with KC4 software. The range of $FeSO_4$ concentration from 0 to 1000 mg was used to create a standard curve. The FRAP values were expressed as mg $FeSO_4$ equivalent /g frozen berries.

The FRAP values reported in this study are the mean FRAP value of three replicate experiments. One sample was placed in three wells (i.e. technical triplicates) in each replicate experiment. The FRAP assay was carried out three times. Different extract of the same blueberry cultivar was used in each experiment.

Oxygen Radical Absorption Capacity (ORAC)

This assay is a widely used method for measuring antioxidant activity. The assay measures the decay of a fluorescent probe by free radicals which affect the fluorescent intensity in the tested mixture (Ou et al., 2001). The damage to the probe is delayed in the presence of antioxidant compounds.

The ORAC assay was conducted as described by Ou et al., 2001 with minor modifications. 75 mM Phosphate buffer (PPB) was prepared and then stock fluorescein (FL) (2.3 µg/mL) was prepared in PPB. The FL working solution was prepared by mixing 1 part of the stock solution with 49 parts of PPB. The blueberry samples (25 µL) were mixed with the working FL solution (150 µL) in 96-well plates. Each plate was incubated at 37°C for 15 minutes and then freshly prepared AAPH (2,2' azobis(2-methylpropionamidine) dihydrochloride 25 µL) was added to the mixture to initiate the reaction. After AAPH addition, the plate was immediately placed into a VICTOR^{TM} X3 Multilable Plate Reader (PerkinElmer, USA). The signal was recorded every minute for 90 minutes. The wavelengths were set at 485 and 535 nm for excitation and emission, respectively. Each sample was measured in triplicate wells. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) ranged from 0 to 100 µM was used to create a standard curve. The ORAC values were expressed as µM Trolox equivalent (TE)/g frozen berries.

The ORAC values reported in this study are the mean ORAC values of three replicates. The ORAC assay was carried out three times. Different extracts of the same blueberry cultivar were used in each experiment.

Prebiotic and antimicrobial activities of rabbiteye blueberry extracts

Bacterial strains and growth conditions

Pure cultures of *L.rhamnosus* ATCC 299 and *L. acidophilus* NZRM 3494 were used in prebiotic study. Both strains were grown in Man-Rogosa-Sharpe (MRS) broth at 37 °C. Pathogenic pure culture, *E. coli* (NCTC 12900)*, S. typhimurium* (ATCC 14028) and *S. aureus,* were used for antimicrobial activity study. *E. coli* and *S.typhimurium,* gram-negative pathogenic bacteria, were grown in tryptic soy broth (TSB) at 37 °C while brain heart infusion media were used for gram-positive pathogenic bacteria, *S. aureus*.

Turbidity standard curve of *Lactobacillus spp.*

Turbidity/optical density measurements are an indirect but very rapid and useful method of measuring microbial growth. However, in order to relate a direct cell count to a turbidity value, a standard curve has to be established first (Harrigan, 1998). The measurements must first be correlated initially with the numbers of colony forming units (CFU) established on the surface of the agar plates. In order to achieve that, we determined the turbidity of different concentrations of each *Lactobacillus* species in MRS medium and then we utilized the standard plate count to determine the number of viable bacteria per milliliter sample. The CFU of each concentration was plotted against its OD_{600} to create the turbidity standard curve (Appendix I). Accordingly, the number of viable bacteria was read directly from the standard curve, without doing the standard plate count, which is a time consuming method.

Prebiotic property of blueberry extracts

Extracts in sterile distilled water and 5% aqueous formic acid from frozen berries, freeze-dried berries, pomace and freeze-dried pomace were used in this study. Fresh MRS broth containing 25% (v/v) of blueberry extract was prepared under aseptic conditions in sterilised tubes and then the bacterial inoculum was added. Brix value of 25% (v/v) of both MQ and 5%FA frozen berry and freeze-dried berry extracts was 6 while the Brix value of 25% (v/v) of both MQ and 5%FA frozen pomace and freeze-dried pomace extracts was 1. The pH values of all MQ and 5%FA extracts was 4.0 and 2.3, respectively.

In another set of tubes, sterile distilled water and 5% aqueous formic acid were added instead of blueberry extracts, to serve as negative controls. The tubes were incubated at 37 °C with 5% CO_2. The bacterial samples were collected at 8 hours, 24 hours, 2 days, and 5 days to determine the numbers of bacteria. The collected samples were measured for optical density at 600 nm and the log CFU/ml was calculated from the turbidity standard curve.

Screening of antimicrobial activity of blueberry extracts

All blueberry extracts were used to investigate their antimicrobial activity on three pathogenic bacteria: *E. coli* (NCTC 12900)*, S. typhimurium* (ATCC 14028)*,* and *S. aureus*. Antimicrobial properties of blueberry extracts were assessed by an agar well diffusion method. Tryptic soy agar was used for gram negative bacteria while brain heart infusion agar was used for gram positive bacteria. The sterilised agar was poured into a sterilised petri dish (90mm diameter) and left in a sterilised chamber until it solidified. The bacterial inoculum (10^6 CFU/mL, 100 µL) was added to the solidified agar and spread onto the agar surface. The plate was left in the chamber until dried then 5 holes were cut per 1 plate using a sterilised cork borer (5 mm) and agar plugs were removed. Different types of blueberry extracts (75 µL) were loaded into each hole. Brix value of both MQ and 5%FA frozen berry and freeze-dried berry extracts was 6 while the Brix value of both MQ and 5%FA frozen pomace and freeze-dried pomace extracts was 1. The pH values of all MQ and 5%FA extracts was 4.0 and 2.3, respectively.

Gentamycin (0.1 mg/mL) was used as a positive control. Sterilised MQ water and sterilised 5% formic acid were used as negative controls. After blueberry extracts, positive and negative control addition, the plates were covered and left in the sterilised chamber for 2 hours to allow diffusion. Then the plates were incubated overnight at 37°C with 5% CO_2. The diameter of the zone of inhibition was measured using a ruler. The study for each bacterium was done in triplicate and the mean diameter of the inhibition zone was reported in the study.

Evaluation of anti-angiogenic properties of blueberry extracts

Anti-angiogenesis activity of blueberry extracts was measured using the Chick Chorioallantoic Membrane (CAM) assay (Ozgurtas et al., 2008). Fertilised hen's eggs were purchased from Golden Coast Commercial, New Zealand. The eggs were incubated in an incubator (R. COM Digital incubator, 20-PRO, United Kingdom) for 5 days at 37°C with fully

humidified atmosphere. On embryonic day 5, the eggs were arranged vertically before cutting the egg shell. The egg shell was cut open using sterilised scissors at the wide end, above the air sac, and the inner membrane was left intact. The blueberry extracts (30 µL) were applied directly on the membrane and then the opening was sealed with parafilm. The eggs were incubated for two more days using the same incubator. On day 7, the membrane was removed and the blood vessels were photographically monitored using Olympus FE-190 digital camera (Olympus, Japan). A 0.25 cm^2 area of CAM midway between the embryo and the shell was selected for blood vessel counting. MQ water and 5% aqueous formic acid were used as control samples.

Gridline intersection method was used in this experiment to quantitatively measure blood vessel density (figure 4-1). The numbers of blood vessels intersected with vertical and horizontal gridlines were counted, and then the vascular density (the number of grid blocks intersected times the linear size of the grid blocks) was calculated (Knoll et al., 1999).

Percentage inhibition of blood vessel formation by blueberry extracts was calculated as follows:

$$\text{Percentage inhibition} = \frac{(\text{Blood vessel density}_{\text{MQ control}} - \text{Blood vessel density}_{\text{BB}}) \times 100}{\text{Blood vessel density}_{\text{MQ control}}}$$

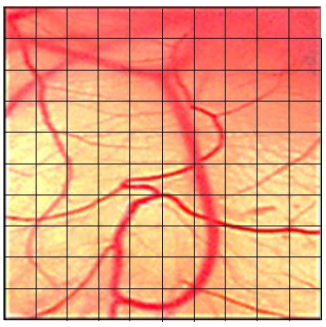


Figure 4-1 Chick chorioallantoic membrane of a control sample overlaid by the rectangular grid.

Statistical analysis

All data were tested for normal distribution using Anderson-Darling normality test. *L. acidophilus* and *L. rhamnosus* populations data were not normally distributed ($P \leq 0.05$

using Anderson-Darling normality test) therefore the data were transformed using Log. Antioxidant activities, anti-angiogenic property and the transformed bacteria population data were analysed for mean comparisons using one-way analysis of variance (one-way ANOVA) to assess significant treatment effects, and then Tukey's test was used as a posthoc comparison. The differences were considered statistically significant at $P \leq 0.05$. Determination of the correlation between two variables was performed using Pearson's correlation coefficient, R. All statistical analysis was performed on Minitab software (version 15; Minitab Inc., Pennsylvania, USA).

Results and discussion:

Antioxidant capacity

Several conditions were studied for their effect on antioxidant activity of blueberries. Antioxidant activities as measured by FRAP and ORAC assay of blueberry extracted in different conditions are summarized in figures 4-2 and 4-3, respectively. Frozen berries of 'Tifblue' showed the highest FRAP value (6.09 mg $FeSO_4$/g frozen berries) and ORAC value (11.73 µmol Trolox equivalent (TE)/g frozen berries) among all tested cultivars when water was used as solvent. The antioxidant activities of frozen pomace water extracts (FRAP; 0.44 - 0.62 mg $FeSO_4$/g frozen berries, ORAC; 0.93-1.2 µmol TE/g frozen berries) were approximately 20% of the activity measured in whole berry extracts. Freeze drying slightly increased antioxidant activities in whole berry extracts however freeze dried pomace extracted with water showed a significant increase ($P \leq 0.01$) in antioxidant activity (FRAP; 7.10 – 8.44 mg $FeSO_4$/g frozen berries, ORAC; 5.33 – 9.52 µmol TE/g frozen berries) compared to frozen pomace. The pronounced effect of freeze drying observed in blueberry pomace might result from anthocyanins, mainly located in skin of the fruits, being released by ice crystal formation during freezing before lyophilisation (Keinanen & Julkunen-Titto, 1996). Moreover, the grinding process may also facilitate the polyphenolic extraction leading to higher antioxidant activity in freeze dried samples.

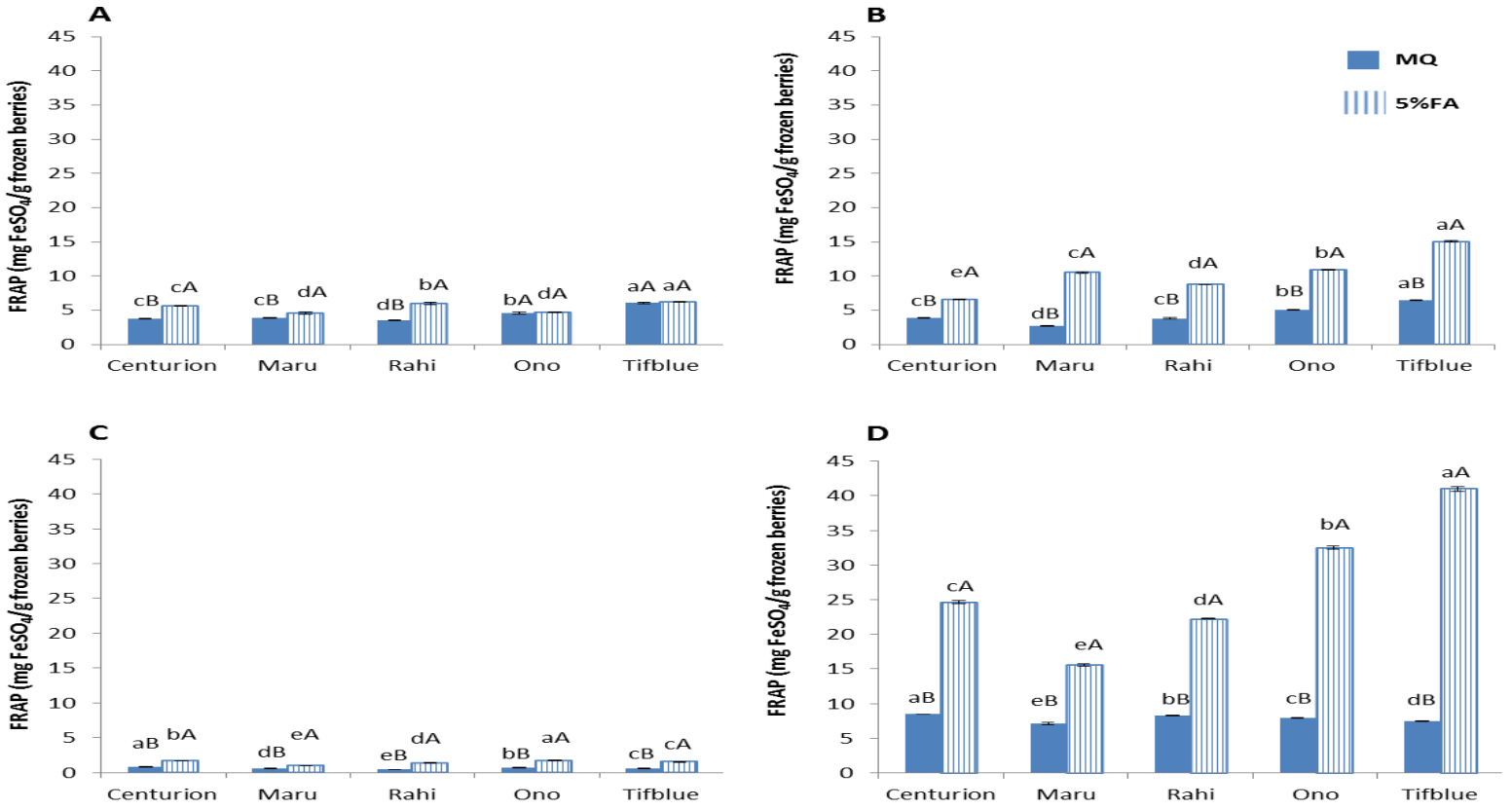


Figure 4-2 Ferric reducing antioxidant power **(**FRAP; mg $FeSO_4$/g frozen berries) of A) frozen berries, B) freeze-dried berries, C) frozen pomace and D) freeze-dried pomace of five rabbiteye blueberry cultivars extracted by MilliQ water or water plus 5% formic acid. Data expressed as means ± SEM. Different lower-case letters indicate significant differences (*P≤ 0.05*) between blueberry cultivars within each solvent type. Different capital letters indicate significant differences (*P ≤ 0.05*) between solvents for each cultivar. MQ water and 5%FA were used as blanks as required.

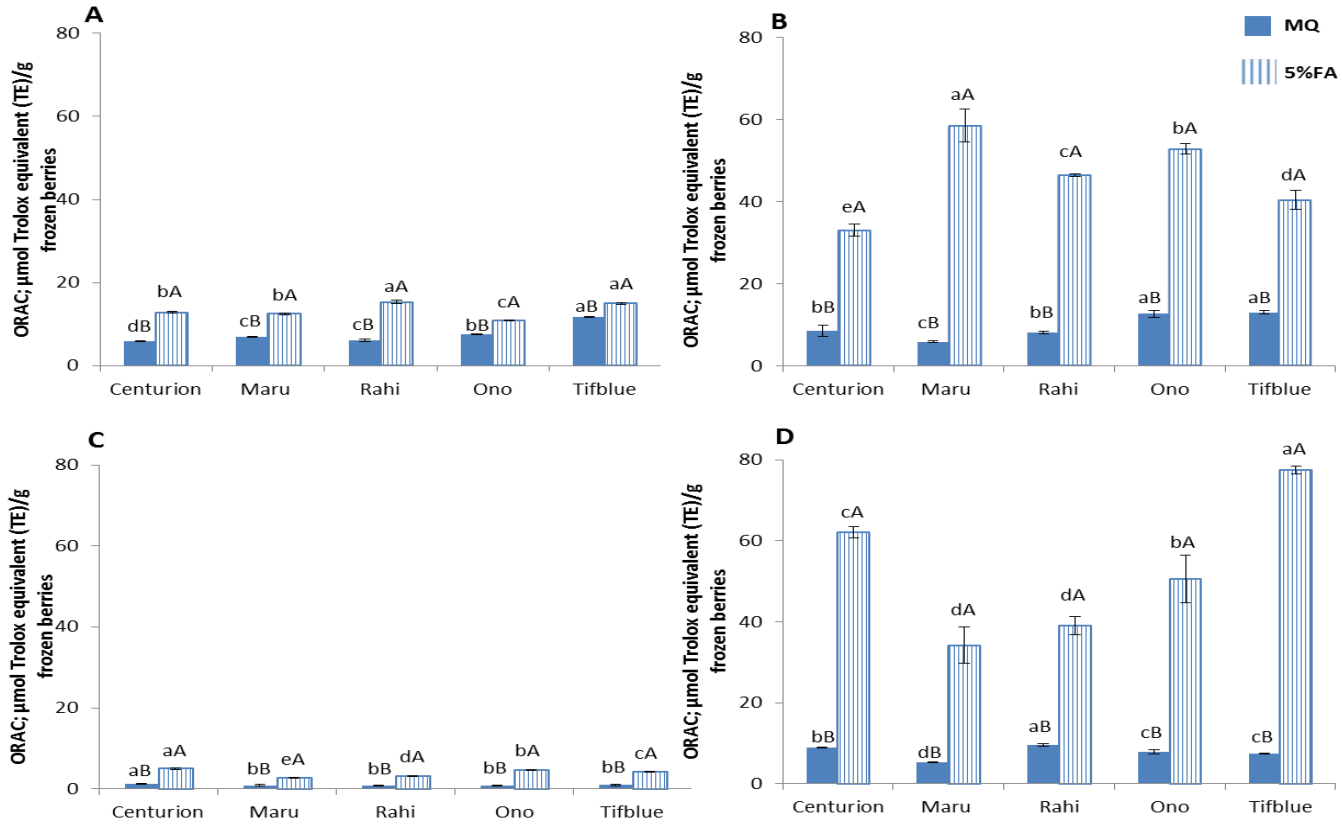


Figure 4-3 Oxygen radical absorption capacity (ORAC; µmol Trolox equivalent/g frozen berries) of A) frozen berries, B) freeze-dried berries, C) frozen pomace and D) freeze-dried pomace of five rabbiteye blueberry cultivars extracted by MilliQ water or water plus 5% formic acid. Data expressed as means ± SEM. Different lower-case letters indicate significant differences ($P \leq 0.05$) between blueberry cultivars within each solvent type. Different capital letters indicate significant differences ($P \leq 0.05$) between solvents for each cultivar. MQ water and 5%FA were used as blanks as required.

Antioxidant activities of frozen blueberries extracted with 5%FA (FRAP: 4.58 - 6.25 mg $FeSO_4$/g frozen berries, ORAC: 10.87 – 15.33) µmol TE/g frozen berries) were higher than the antioxidant values from the MQ extracts. A similar trend was also found with frozen pomace extracted with 5%FA, which had higher FRAP and ORAC values (FRAP: 0.99 – 1.64 mg $FeSO_4$/g frozen berries, ORAC: 3.20 - 5.07 µmol TE/g frozen berries) than their MQ extract counterparts. Interestingly, both freeze dried berries and pomace exhibited higher antioxidant activities than their frozen counterparts. The effect of freeze drying on antioxidant activity of blueberries is discussed further below.

Blueberries have long been studied for their antioxidant activity. They are among the fruit that possess the highest antioxidant activity per gram fresh weight (Prior, 1998). Antioxidant activity of blueberries varied amongst cultivars, growing conditions and experimental conditions. Our results were in agreement with the previous studies of rabbiteye blueberries. Reported FRAP values of freeze dried rabbiteye blueberry fruit extracted with acidified 70% methanol were 4.76 mg $FeSO_4$/ g berries while freeze dried blueberry pomace extract showed higher antioxidant activity at 5.33 mg $FeSO_4$/ g berries. ORAC values also showed a similar trend with the FRAP values. The ORAC value of the pomace extracts was higher than the fruit extract at 9.54 and 4.56 µmol TE/g berries, respectively. Methanol was used in the study which gave a very complete extraction of phenolic antioxidant. The study also indicates that phenolic antioxidants were more concentrated in blueberry pomace than the whole berries (Li et al., 2013).

The effect of freeze drying on blueberry phytochemicals and antioxidant activities has also been studied. Freeze drying was shown to increase total antioxidant activity in ‘Reka’ blueberries by 36% compared to the non-freeze dried counterparts (Sablani et al., 2011). Freeze drying caused a reduction of ascorbic acid in blueberries; however, total phenolic concentration and antioxidant activity of freeze dried blueberries increased (Reyes et al., 2011). Freeze drying might be able to preserve heat sensitive polyphenols which may be lost during heat drying treatment and facilitate polyphenols extraction as mention in chapter 3. The higher polyphenols released from the plant cells then led to higher antioxidant activities as expected. Relationship between polyphenols and antioxidant activity is discussed further in this chapter.

Acidification of extracting solvent showed a significant effect on antioxidant activities of blueberry extracts. The pH of crude blueberry MQ extracts and crude blueberry 5%FA extracts in the current study were approximately 4.0 and 2.3, respectively (data not

shown). This finding is supported by reports elsewhere. Blueberry extracted with low pH solvent (pH 1) showed greater antioxidant activity as measured by ORAC assay than the extracts of blueberry derived from higher pH (pH 4 and 7) (Kalt et al., 2000). The presence of weak organic acids such as formic acid, acetic acid, citric acid or tartaric acid in extracting solvents led to denaturation of plant cell which then facilitated anthocyanin or polyphenolic extraction (Dai & Mumper, 2010). Moreover, an acidified solvent system also benefits anthocyanin stability in the extracting medium since anthocyanins are present in the flavylium cation form at low pH, which is more stable than the quinonodial pseudobase and chalcone forms (He & Giusti, 2010). By increasing solvent extractability and stabilization of anthocyanins, blueberries extracted with acidified water showed higher antioxidant activities than antioxidant activities of blueberry sample extracted solely with MQ.

Prebiotic properties of blueberry extracts

The growth and bacteria population of *L. rhamnosus* was measured at four time points during the total period of the study (120 hours) (figure 4-4A). In the MQ water extracts, there was no significant difference in the bacteria population between all experimental groups both with and without blueberry after 24 hours of incubation. The bacteria reached the maximum growth at 48 hours with no significant difference in bacteria population among experimental groups. A significant difference in the bacteria population was observed only after 120 hours of incubation (5 days). On the fifth day, the numbers of bacteria in the water control incubation dropped significantly ($P \leq 0.05$) relative to the numbers of bacteria on the second day of incubation. In contrast, the numbers of bacteria in incubations containing water blueberry extracts were slightly lower on the fifth day of incubation in comparison with the number of bacteria on the second day of incubation. The decrease of bacteria population in the cultures incubated with the blueberry extracts was not significant relative to the bacterial population at 48 hours of incubation. The *L. rhamnosus* population of blueberry MQ extract treatment was approximately 13 log CFU/mL after 5 days incubation. It was 0.6 log CFU higher than the control group. The difference between control incubations and those containing blueberry extracts samples was significant ($P \leq 0.05$) on the fifth day of incubation. No significant difference was found between blueberry cultivars, freeze drying treatments (frozen/freeze dried) and different fruit parts (whole berries/pomace). Statistical analysis showed that the

only variable that affected the *L. rhamnosus* population was the solvent used for blueberry extraction.

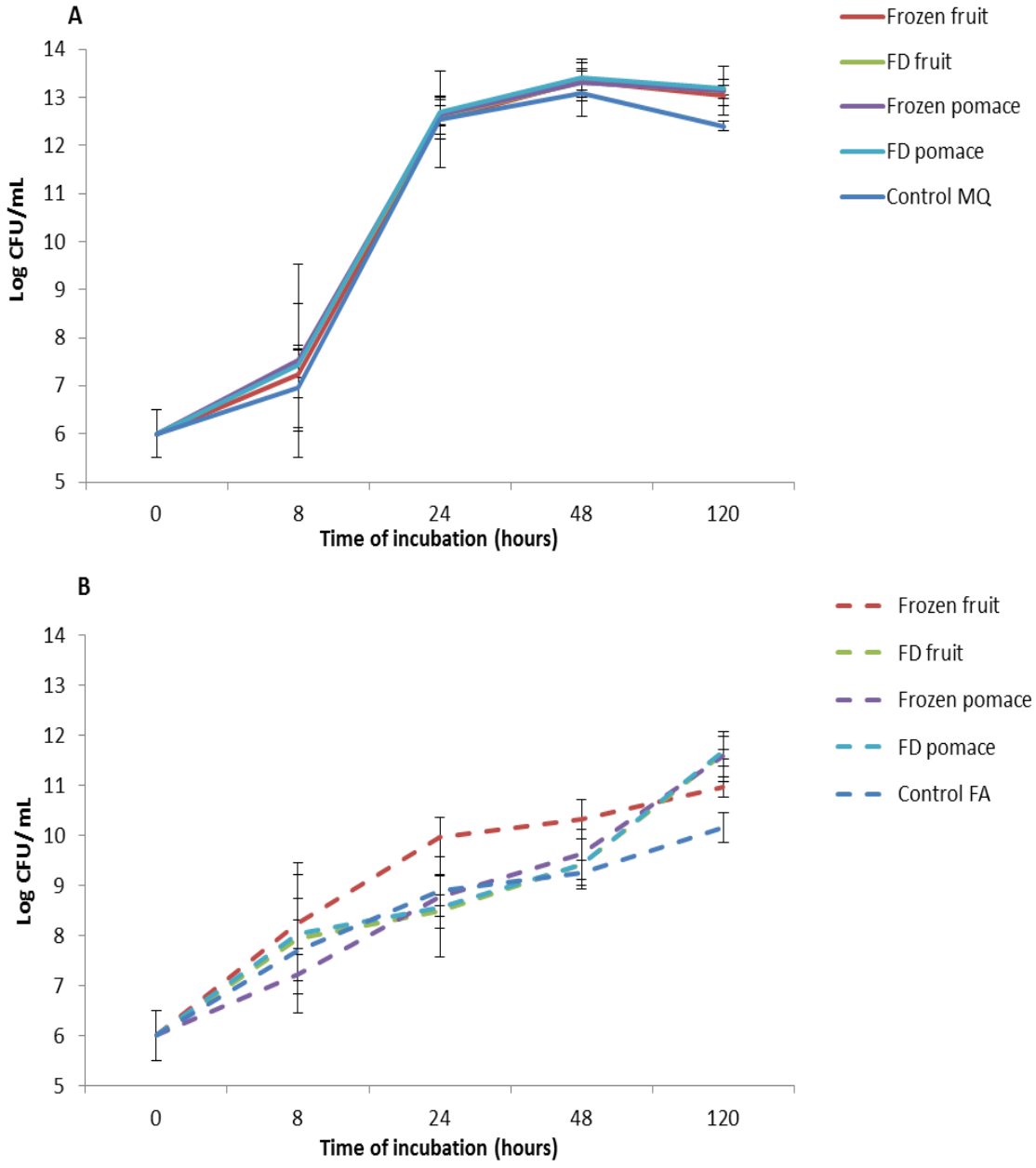


Figure 4-4 Enumeration [Log10 colony forming units (CFU)/mL] of *L. rhamnosus.* The bacteria were incubated at 37°C in MRS medium containing extracts from blueberry fruits or pomace extracted with (A) Milli Q water and (B) 5% aqueous formic acid.

In the 5%FA extracts, *L. rhamnosus* incubated with blueberry extract exhibited a different growth pattern from the bacteria incubated with blueberry MQ extracts (figure 4-4B). The population increased slowly and was approximately 1.5 log CFU/mL lower than the population of bacteria culture incubated with blueberry MQ extract. The bacterial population of bacteria incubated with blueberry 5%FA extract continued to increase by the end of the study. In comparison with the control (5%FA without blueberry extract), the bacterial population also peaked at 10.2 log CFU/mL at day 5 of incubation. The difference between the control and the blueberry treatments, when 5%FA was used as a solvent, was significant ($P \leq 0.05$).

Regarding the effects of blueberry extracts on the growth of *L. acidophilus*, the results showed that extracts of all five cultivars showed similar trends where the bacterial numbers reached the maximum at 24 hours and then slightly dropped (figure 4-5). No significant differences were observed in the numbers of *L. acidophilus* between incubations containing water extracts from different blueberry cultivars at any incubation period (figure 4-5A). The bacteria population peaked at 13.4 log CFU/mL and no significant difference was found between MQ blueberry extract incubations and control incubation. The bacterial population of the culture with blueberry 5%FA extracts slightly decreased to 12.6 log CFU/mL on the fifth day of incubation (figure 4-5B). The population of the 5%FA control incubation on day 5 was significantly lower ($P \leq 0.05$) relative to the highest bacterial population (figure 4-4B). The bacteria population in the control incubation dropped to 11.5 log CFU/mL which was significantly lower ($P \leq 0.05$) than the bacteria population with blueberry MQ extract treatments measured on the last day of the experiment.

Similar to *L. rhamnosus*, the growth pattern of *L. acidophilus* was affected by 5% formic acid blueberry extract (figure 4-5). The bacteria population was not significantly different from the bacteria population in blueberry MQ extract treatments during the first 8 hours of incubation. However, the bacterium grew slowly and was 2.7 log CFU/mL lower than bacteria treated with blueberry MQ extract. The bacteria population in the water extract incubations started to decline after 24 hours, but the bacteria population of 5% formic acid incubations continued to rise. By the end of the study, the bacteria population in a control of 5% aqueous formic acid extract (10.4 log CFU/mL) dropped significantly ($P \leq 0.05$) relative to the highest bacterial population (11 log CFU/mL) found while the population of bacteria with blueberry extracts increased.

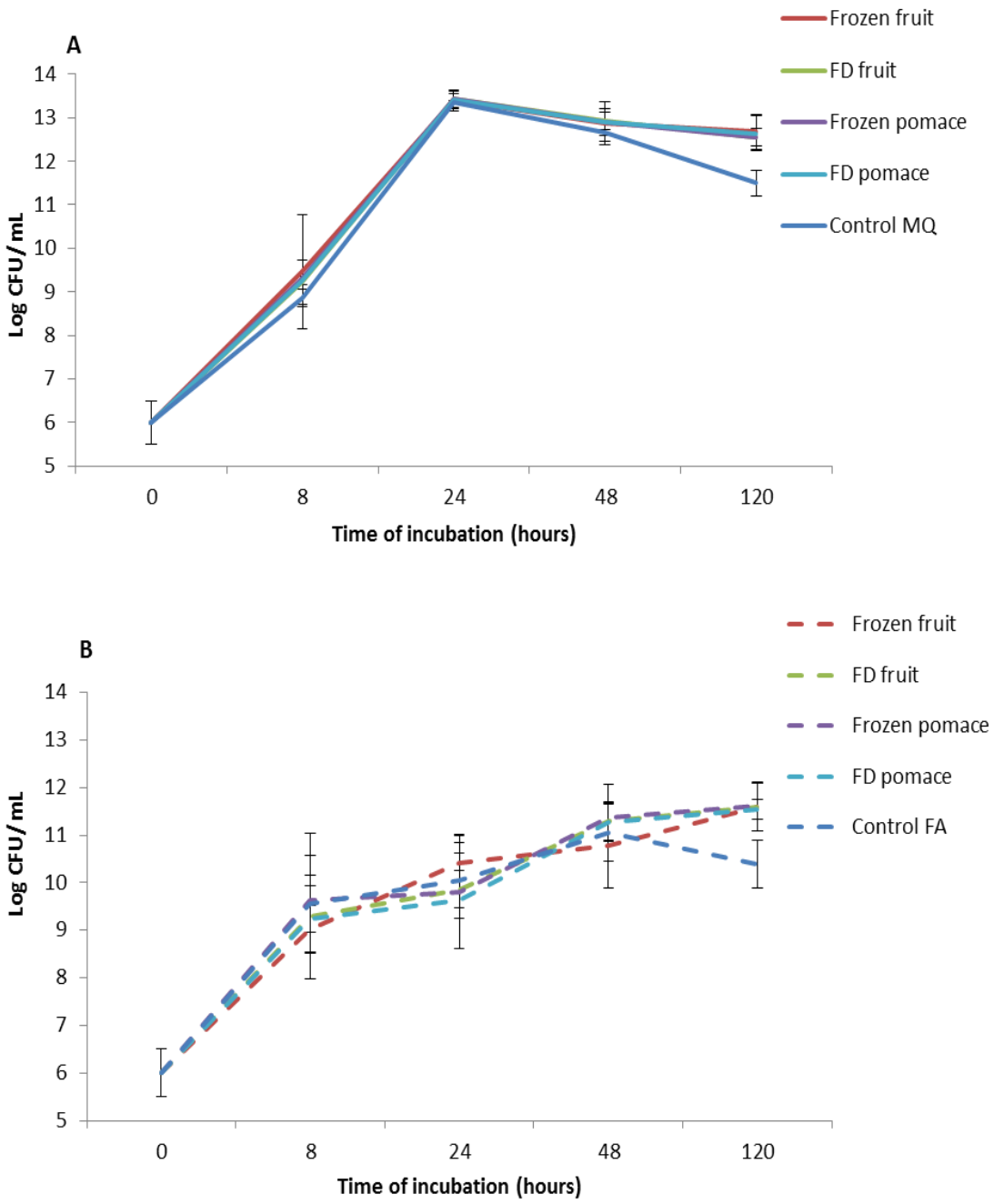


Figure 4-5 Enumeration [Log10 colony forming units (CFU)/mL] of *L. acidophilus.* The bacteria were incubated at 37°C in MRS medium containing extracts from blueberry fruits or pomace extracted with (A) Milli Q water and (B) 5% aqueous formic acid.

The major effect of 5%FA on *L. rhamnosus* and *L. acidophilus* was significantly slowing the rate of growth compared to water alone. The major effect of both MQ and 5%FA blueberry extracts on *L. rhamnosus* and *L. acidophilus* was delaying the normal significant drop of bacteria population observed after five days in culture.

Similar findings have been reported elsewhere. Numbers of *L. rhamnosus* and *Bifidobacterium breve* in incubations containing blueberry extracts from the berries of 'Centurion' and 'Maru' cultivars were significantly higher than the bacterial populations of the control incubations (Molan 2009b). Moreover, the author also reported that the *in vivo* study showed that the numbers of lactobacilli and bifidobacteria in rat colons increased after administration of extracts from both blueberry cultivars to rats daily for 6 days compared to control rats gavaged with water. The growth of *L. acidophilus* isolated from commercial yoghurt was stimulated by crude blueberry extract. The lowest concentration of blueberry extract that exhibited the prebiotic effect was 0.12 g/L where the strongest stimulating effect was found at 10 g/L concentration (Hap, 2010). Growth of several probiotic organisms including *L. rhamnosus* was stimulated by aqueous blueberry extract which was attributed to sugar and small proteins that are commonly present in blueberry aqueous extracts (Sutherland et al., 2009).

The relationship between probiotic microflora and phenolic compounds is a two-way relationship. Bacteria benefit from typical compounds like sugars that serve as an energy source. Some phenolic compounds are activated by bacterial enzymes and exhibit their biological activities (Selma et al., 2009). *L. acidophilus* incubated with a low concentration of fructose (0.5% w/w) showed higher viable counts than bacteria incubated in normal media without fructose added (Saxena et al., 1994). The presence of glucose and fructose in MRS media with simulated gastric juice addition enhanced *Lactobacillus* spp. survival compared to the bacterial population incubated in the media without glucose or fructose (Corcoran et al., 2005). The mechanism of glucose in enhancing survival of *Lactobacillus* spp., suggesting that glucose metabolism generated ATP which enables proton exclusion from the cells by F_0F_1-ATPase enzyme. The proton exclusion process then enhanced survival of *Lactobacillus* spp. in low pH condition. The active antioxidant compounds might be able to reduce harmful effects generated by cell metabolism and provide a more suitable environment for bacteria survival (Molan et al., 2009b). Moreover, anthocyanins which are responsible for antioxidant activity in blueberries might also have a positive effect on probiotic growth stimulation. Two anthocyanins, cyanidin 3-monoglucoside and delphinidin

3-monoglucoside, boosted *L. acidophilus* growth (Pratt et al., 1960) which might result from enhanced β-glucosidase enzyme activity from the bacteria catalysing anthocyanin conversion into their active form that possesses antioxidant activity (Ávila et al., 2009).

In the present study, the positive prebiotic effect observed might be attributed to both sugar and blueberry polyphenols. In order to further investigate the effect of blueberry polyphenols, control media with addition of sugar found in blueberries must be included. The prebiotic effects of blueberry fruit and blueberry pomace were not significantly different. However, fiber and xylooligosaccharides found in blueberry skin have been shown to possess prebiotic activity (Hsu et al., 2004; Patel & Goyal, 2012). By using blueberry pomace extract in the present study *in vitro*, the *in vivo* prebiotic effect of dietary fiber in blueberry pomace was neglected. In the next chapter, prebiotic effect of blueberry extracts and blueberry pomace will be investigated *in vivo* using an animal model of breast cancer. In order to thoroughly investigate the prebiotic effect of the blueberry pomace, dried blueberry pomace will be mixed with standard rat chow instead of using blueberry pomace extracted with MQ.

Antimicrobial activity of rabbiteye blueberry extracts

In this study, both blueberry fruit and pomace extracted by either MQ or 5%FA did not exhibit antimicrobial activities against all three pathogenic bacteria. There was no clear zone presented in all wells with the blueberry MQ extracts. Even though clear zones representing bactericidal effect were present in agar wells with blueberry 5%FA extracts, the clear zone sizes were not significantly different from 5%FA control (Appendix II).

Sutherland and colleagues demonstrated that concentrated aqueous blueberry extracts at either neutral (pH 7) or low pH (pH 2) exhibited antimicrobial effect against *E. coli* O157:H7 and *E. coli* LF82 (Sutherland et al., 2009). Moreover, several studies demonstrated that phenolic acid-rich extract, anthocyanin-rich extract or sugar-free extract from blueberry exhibited antimicrobial activities. Phenolic extract from blueberries was studied for its antimicrobial activity against foodborne pathogenic bacteria *Salmonella enteritidis*. Blueberry phenolic water extract effectively reduced the growth of the bacteria by approximately 3 log CFU/mL (Park et al., 2011). Anthocyanin and proanthocyanidin fractions from lowbush blueberry extract demonstrated stronger antimicrobial activity against *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium* than crude blueberry extracts (Lacombe et al., 2012). Blueberry phenolic extracts effectively inhibited the growth of *B. cereus* and *S. enterica* sv. *typhimurium* but only showed small changes that

were not statistically significant effect against *S. aureus*, *S. epidermidis* and *E. coli*. Moreover, blueberry phenolic extract caused lipopolysaccharide (LPS) released in *S.* sv. *typhimurium* and *S.* sv. *infantis* which indicated outer membrane disintegration. Phenolic compounds and weak organic acids present in blueberry were found to be responsible for the outer membrane disintegration in gram-negative bacteria (Nohynek et al., 2006). Inhibition of bacterial extracellular enzyme, inhibition of oxidative stress phosphorylation and deprivation of substrate necessary for bacterial growth have been proposed as possible mechanisms of tannin antimicrobial activity (Scalbert, 1991). Other flavonoids beside anthocyanins such as kaempferol and quercetin demonstrated antimicrobial effect against both gram-positive and gram-negative pathogenic bacteria (Hendra et al., 2011). The plausible mechanisms of flavonoids in pathogenic inhibition included inhibition of nucleic acid synthesis, cytoplasmic membrane function and energy metabolism (Cushnie & Lamb, 2005).

The blueberry phenolic extracts in the studies cited above went through purification, fractionation and concentration processes; therefore, higher concentrations of bioactive compounds could be achieved. According to the literature, the concentrations of the compounds used in the studies were extremely high and did not match physiological concentrations achievable from diet supplementation. Our study aims to investigate the synergistic effect of blueberry extracts at physiological concentrations achievable in the human diet; therefore, fractionation or purification of the extracts is not the main focus. The blueberry extracts from our study failed to demonstrate antimicrobial activity *in vitro*, which likely resulted from insufficient concentration of the bioactive compounds.

Anti-angiogenic property of blueberry extracts

A series of experiments was conducted to evaluate the anti-angiogenic effects of extracts from whole berries or pomace from five rabbiteye blueberry cultivars using the CAM assay. Neovascularization from the existing blood vessels of each tested CAM was compared with control CAM.

In the first experiment, embryonic death was found in fertilised eggs treated with blueberry 5%FA extracts and 5%FA control and there was no blood vessel development on the CAM (figure 4-6). Embryonic death was presumably caused by the highly acidic extract (pH 2.2). At day five, eggs treated with MQ extracts showed good development of blood vessel network on CAM (figure 4-6). Thus, even though the blueberry 5%FA extracts

showed higher TPC values, total anthocyanins and antioxidant activities, the extracts were not suitable for use directly in CAM assay. The blueberry 5%FA extracts might be purified to obtain polyphenolic compounds; however, acid residue present after purification process might still cause harmful effects to embryos in the CAM assay. Therefore, only the crude blueberry MQ extracts were used in this study.

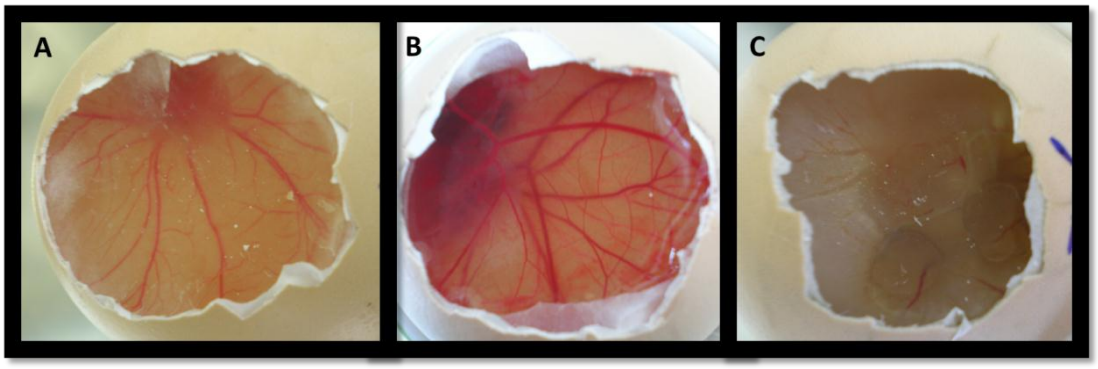


Figure 4-6 Macroscopic monitor of embryos in anti-angiogenic activity using CAM assay. A) Five day old fertilised egg B) Seven day old fertilised egg treated with MQ C) Seven day old fertilised egg treated with 5%FA.

Although there was no harmful effect of blueberry extracts on the major blood vessels on treated CAMs visible macroscopically, the generation of new micro blood vessels was affected (figure 4-7). The blueberry extracts from both frozen fruit and pomace inhibited the formation of new capillaries from the existing blood vessels. As shown in figure 4-7, a network of blood vessels is formed in a control CAM (A) while on CAM treated with blueberry fruit and pomace extracts (B and C) even though small capillaries elongated, most of them failed to interconnect. Elongation is a part of sprouting angiogenesis where new capillaries sprout and elongate in a similar pattern as plant rooting. Another angiogenesis type is called intussusceptive angiogenesis where the original blood vessel diverges and new blood vessels grow into the center. Both types of angiogenesis are very important for blood network formation in all tissues and organs (Risau, 1997).

The qualitative effect of edible bilberry extract on neovascularization using CAM assay has been reported. The study showed that the blood vessels beneath the extract were thinner and less branched (Ozgurtas et al., 2008).

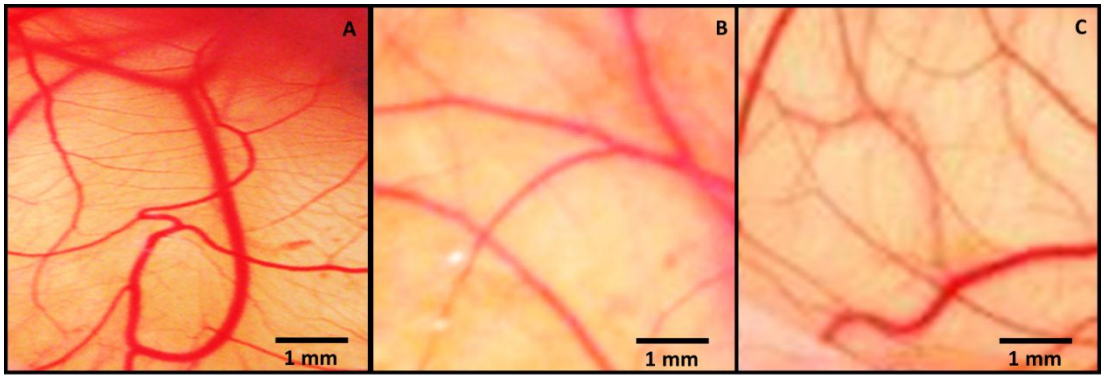


Figure 4-7 Representative photomicrographs for qualitative observation of anti-angiogenic properties of A) Control B) Frozen blueberry fruit extract C) Frozen pomace extract (30 µL taken from 100 g berry fruit in 100 mL MQ water) on CAM.

Interestingly, the effects of blueberry extracts in our study were not localized. There were no thinner blood vessels observed underneath the area that the blueberry extract was placed however there was less new blood vessel formation and less branching of the blood vessels on CAM treated with the blueberry extracts compared with the control CAMs treated with MQ water. Delphinidin, anthocyanidin found in blueberries, also showed anti-angiogenesis of the developing embryo. Delphinidin exhibited anti-angiogenic activity on CAM in a dose-dependent manner (Favot et al., 2003). Sugar present in blueberry extract might also play a part in anti-angiogenesis in CAM assay. A study for determination of short-term and long-term effects of optical clearing agent on blood vessels using laser speckle contrast imaging to monitor the changes of blood vessels in CAM. The researchers reported that 40% glucose solution showed a slow inhibitory effect on blood flow and blood vessels formation. Within 48 hours after the application of glucose on CAM, most of the vessels were blocked and new blood vessel formation was not found (Zhu et al., 2008). In our study, the MQ blueberry extracts contained approximately 4% glucose which was lower than the concentration used in Zhu's study. Therefore, anti-angiogenic property of the MQ blueberry extracts from our study might be a synergistic effect of blueberry polyphenols and glucose.

Therefore, it is difficult to conclude that anti-angiogenic activity of blueberries is primarily a result of polyphenols or anthocyanins. Further investigation using purified concentrated polyphenols from blueberries coupled with sugar solutions as a control are required.

In our study, the anti-angiogenic effects of blueberry extracts were also determined quantitatively using 10 X 10 grids. Blood vessel density of CAM treated with blueberry extracts were compared with blood vessel density of a control CAM. Percentage inhibitions of blueberry extracts were calculated as shown in figure 4-8. According to statistical analysis, 'Maru', 'Centurion' and 'Tifblue' extracts were in the same group that exhibited a high percentage inhibition of angiogenesis while 'Ono' and 'Rahi' showed significantly lower percentage inhibition ($P \leq 0.05$). Percentage inhibition from blueberry pomace extracts ranged from 15 to 38 %.

Mechanisms of anti-angiogenesis by blueberry extracts in CAM assay are not clear. In general, *in vitro* studies have used vascular endothelial growth factor (VEGF) as a biomarker for angiogenesis. Human keratinocyte HaCaT cells, were pretreated with berry extracts then VEGF expression was induced in the pretreated cells using either TNFα or H_2O_2 (Roy et al., 200. The cells pretreated with blueberry extract showed significant reduction in VEGF-expression by 70% and 75% induced by TNFα and H_2O_2, respectively. Grape seed extract was also studied. It exhibited high antioxidant activity as measured by ORAC assay but failed to demonstrated anti-angiogenesis. This finding led to a conclusion that anti-angiogenesis did not depend on antioxidant activity of the extracts; however, it might depend on some particular compounds in the extracts (Roy et al., 2002).

Similar research has also demonstrated anti-angiogenic activity of pure flavonoid compounds, which were ferulic acid, rutin, and catechin, compared to α-tocopherol (high antioxidant activity) using H_2O_2-induced VEGF expression in the HaCaT cell model. All flavonoids significantly reduced VEGF expression while α-tocopherol did not cause changes in VEGF expression compared to the H_2O_2 induced cells (Bagchi et al., 2004). Interestingly, procyanidin-rich extract from grape seed exhibited anti-angiogenic activity by binding with VEGF or VEGFR and then resulted in deactivation of a downstream signaling pathway. The extract inhibited the kinase activity of purified VEGF receptor 2 and the signaling pathway through VEGF receptor/mitogen-activated protein kinase (MAPK). Chicken aortic ring assay was used for as an organ culture model. They found that the rings cultivated in media containing the extracts showed less sprouting than the rings cultured in normal media. Effects of the grape seed extracts in both *in vitro* and organ culture model were dose-dependent. The researchers confirmed that procyanidin was responsible for anti-angiogenic activity of the grape seed extract by using procyanidin-free extract both *in vitro* and organ culture models. The results showed that without procyanidin, VEGFR2 kinase

activity was high and the sprouting in chicken aortic ring assay was similar to a vehicle control. They then concluded that procyanidin was the compound in grape seed extract that was responsible for their anti-angiogenic activity (Wen et al., 2008). Procyanidin is also found in blueberries (Kasim et al., in press). The researchers also studied mode of action of delphinidin using cell migration assay and cell proliferation assay. Human umbilical vein endothelial cell (HUVEC) culture was stimulated with VEGF after incubation with delphinidin. The results showed that HUVEC migration and proliferation was reduced in a concentration-dependent manner. Delphinidin showed anti-angiogenic activity by inhibition of cell migration and cell proliferation in VEGF-induced HUVECs. It also caused cell cycle arrest at G0/G1 phase that was regulated by $p27^{kip1}$. Level of $p27^{kip1}$ was not affected with delphinidin treatment in normal conditions. However, when the cells were treated with VEGF, $p27^{kip1}$ was up regulated. The increase of $p27^{kip1}$ molecules led to inhibition of cdk2/cyclin A and cdk4/cyclin D that subsequently caused cell cycle arrest (Favot et al., 2003).

As reported previously in chapter 3, delphinidin was the second most abundant anthocyanin found in blueberry extracts. 'Maru', 'Tifblue' and 'Centurion' contained 10 – 14 percent of delphinidin while 'Rahi' and 'Ono' had less than 10% delphinidin in total anthocyanin composition. This might explain the different level of anti-angiogenesis in each cultivar. Other polyphenols found in blueberries, such as ellagic acid, also showed modulation of downstream signaling pathway of MAPK and PI3K/Akt and direct inhibition of VEGFR2 tyrosine kinase activity. The researchers also found an anti-angiogenic property of ellagic acid attributed to anti-proliferation, anti-migration and inhibition of tube formation (Wang et al., 2012c). Hypoxia-inducible factor-1 (HIF-1) activates transcription in several genes related to tumour angiogenesis. Chlorogenic acid decreased HIF-1α expression, which subsequently reduced VEGF expression in A549 lung cancer cells (Park et al., 2015).

Collectively, anti-angiogenic activity of blueberries might result from various polyphenols in the fruits, specifically flavonoids. This study is the first report on screening of anti-angiogenic activity of rabbiteye blueberries using CAM assay. The mechanisms responsible for anti-angiogenesis by polyphenols or flavonoids from blueberry need to be further examined using biomarkers related to angiogenesis such as VEGF.

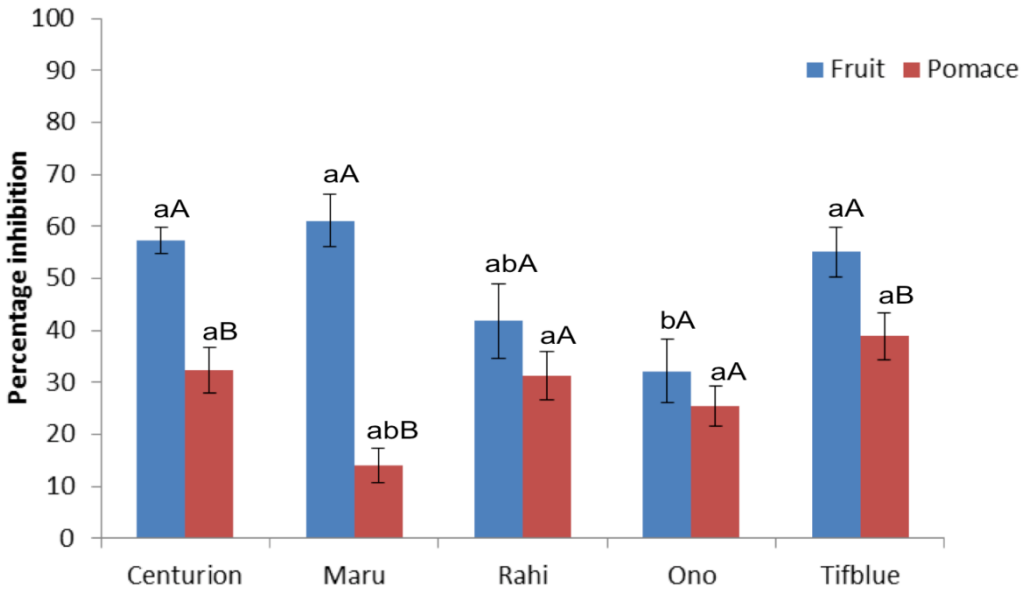


Figure 4-8 Percentage inhibition of new blood vessel formation in a chick chorioallantoic membrane assay by five rabbiteye blueberry cultivars. Data expressed as mean ± SEM of five replicate samples of two independent experiments. Columns denoted with the same small letter are not significantly (*P*> 0.05) different among cultivars. Columns denoted with the same capital letter are not significantly (*P*> 0.05) different between solvents.

Correlation between blueberry phytochemicals and their biological properties

Biological properties of blueberries are attributed to various phytochemicals in the fruits. The correlations between biological properties of blueberries and their phytochemicals are summarised in table 4-1. In this study, the strong correlation between total phenolic concentration and antioxidant activities was established with R values that equalled 0.921 and 0.952 in TPC/FRAP and TPC/ORAC, respectively. Chlorogenic acid also showed strong correlation with antioxidant activities. Total anthocyanin revealed lower but still positive correlation with antioxidant activities with R values of 0.824 and 0.869 in ACY/FRAP and ACY/ORAC, respectively. Percentage inhibition of angiogenesis in CAM assay correlated well with total anthocyanin and total phenolic concentration. Chlorogenic acid concentration exhibited lower correlation with percentage inhibition of angiogenesis with R value of 0.614.

A relationship between blueberry phytochemicals and their biological activities was reported in several studies. Linear relationships between ORAC and anthocyanin or total phenolic concentration were 0.77 and 0.92, respectively (Prior et al., 1998). They also mentioned that health benefits of anthocyanins might not relate to their antioxidant

activity. *Lonicera japonica* (Flos Lonicerae) showed high antioxidant activity as measured by FRAP assay. Their antioxidant activity was mainly attributed to chlorogenic acid (Wu, 2007). The chemical structure of polyphenolic compounds could alter their antioxidant activity (Rice-Evans et al., 1996; Heim et al., 2002; Pereira et al., 2009). The effect of structure modulation in anthocyanins was well documented. Cyanidin (with five hydroxyl groups) showed higher ORAC value than other anthocyanidins (pelargonidin, malvidin and peonidin) which contain 4 hydroxyl groups (Wang et al., 1997). This research group also demonstrated an effect of glycosylation in anthocyanidins on their antioxidant activities. Glycosylation at 3-OH position on C ring might elevate, retain or decrease antioxidant activity of cyanidin depending on the type of sugar molecule. Cyanidin glycosylated with glucose showed higher ORAC value while galactose substitution lowered antioxidant activity compared to aglycone molecule (Wang et al., 1997).

In our study, 15 anthocyanins present in blueberry extracts were in glycosylated form. According to the modulation of anthocyanins antioxidant activity by hydroxylation, methylation and glycosylation, antioxidant activities of anthocyanins showed lower correlation than chlorogenic acid which was determined in the study in its free form.

Table 4-1 Correlation coefficient (*R* values) between blueberry phytochemicals [total phenolic concentration, total anthocyanin and chlorogenic acid concentration] and their biological properties (antioxidant activities (FRAP and ORAC values) and percentage inhibition of angiogenic activity).

	Total phenolic concentration (TPC)	**Total anthocyanin (ACY)**	**Chlorogenic acid concentration (CGA)**
FRAP	0.921	0.824	0.974
	P< 0.001	*P* = 0.003	*P*< 0.001
ORAC	0.952	0.869	0.984
	P< 0.001	*P* = 0.001	*P*< 0.001
% inhibition of angiogenic activity	0.791	0.796	0.614
	P = 0.006	*P* = 0.021	*P* = 0.059

Conclusion:

Variation of antioxidant activity of blueberry extracts depended on cultivars, the part of fruit, drying treatment and solvents used for extraction. In our study, 'Tifblue' showed the highest antioxidant activity when frozen fruits were extracted with water. Frozen berry pomace showed slightly lower antioxidant activity than berries extracted with water. Acidification of solvent by formic acid enhanced antioxidant activity of blueberry extract which might relate to higher phenolic compounds extracted from the fruit matrix. The blueberries extracted with 5% aqueous formic acid showed slightly higher antioxidant activity when used to extract frozen berries and pomace. Interestingly, lyophilisation coupled with acidification of solvent exhibited the highest antioxidant activity.

Blueberry extracts exhibited prebiotic effect on tested *Lactobacillus* spp. (*L. rhamnosus* and *L. acidophilus*). Bacteria populations in culture media with blueberry extract addition were significantly higher than bacteria populations in a control group (no blueberry extract) as measured at the end of the experiment (5 days after incubation). There were no significant differences in bacteria population and growth pattern of bacteria incubated with blueberry extracts from the same solvent. However, the growth pattern and bacteria population did show significant differences when 5% aqueous formic acid was used for blueberry extract preparation. Blueberry extracts in this study did not demonstrate antimicrobial activity against three pathogenic bacteria (*E. coli*, *Salmonella typhimurium* and *Staphylococcus aureus*). This might result from lower polyphenol contents in the extracts than previous studies which used purification and concentration techniques prior to the screening of antimicrobial activity. Anti-angiogenic activity of blueberry extracts was also observed in the study. New blood vessel formation was inhibited by frozen berries and pomace water extracts without harmful effect on major blood vessels and embryos. Extracts from blueberry fruit showed approximately 30 – 60% inhibition compared to a control sample while pomace water extracts were less effective.

Antioxidant activity of blueberry extracts correlated well with total phenolic concentration and chlorogenic acid concentration. Anthocyanin concentration also showed positive correlation with antioxidant activity but it was not as strong as total phenolic concentration and chlorogenic acid concentration. Anti-angiogenic activity showed a stronger positive correlation with anthocyanin concentration than chlorogenic acid concentration. It can be concluded that phytochemical compounds in blueberry extracts

may be responsible for different biological activities of the fruits. Blueberries exhibit health benefits which may result from different phytochemical components of the fruits.

Even though the blueberry extracts prepared with 5% aqueous formic acid showed higher total phenolic concentration, anthocyanin content chlorogenic acid concentration, and antioxidant activity, the extracts were not suitable to apply *in vivo* because they slowed the growth of *Lactobacillus* spp. and caused embryocidal effects in CAM assay. Therefore, blueberry fruit water extracts will be used to supplement drinking water and dried pomace will be supplemented to animal feed to examine the effect of blueberry extracts on chemically-induced mammary carcinogenesis in the *in vivo* studies for this project.

CHAPTER 5

Impact of blueberry extracts and blueberry pomace on 7,12-dimethylbenz[a]anthracene-induced breast carcinogenesis in rat model

Abstract:

This study was designed to examine blueberries for their chemopreventive effect on mammary tumorigenesis both *in vitro* and *in vivo*. Rabbiteye blueberry extract exhibited high antioxidant, prebiotic and anti-angiogenic activity reported in the previous chapter. Therefore in this chapter, we examined the hypothesis that blueberry extracts and blueberry-pomace supplemented diets would be able to decrease tumour incidence in DMBA-induced mammary tumorigenesis *in vivo*.

One hundred virgin female Sprague-Dawley rats (*Rattus norvegicus*) were divided into five groups after a one week acclimation period. Animals in the negative and positive control groups received normal rat chow and normal drinking water while rats in 'Centurion' and 'Maru' groups were fed with normal rat chow but received blueberry extracts in drinking water form (1:3, w/w). Animals in the Pomace group were fed a 5% Highbush blueberry pomace supplemented diet and normal drinking water. Animals in all experimental groups were gavaged with 50 mg DMBA/kg body weight in corn oil except animals in a negative control group were gavaged with 1 mL corn oil (vehicle control). The DMBA and corn oil were given once at the beginning of the study. The experiment was terminated 13 weeks after DMBA-administration. No tumours were found in the vehicle control group while 15 out of 20 animals were found with tumours in the positive control group. Animals that received blueberry treatments (either juice or pomace) showed lower tumour incidence. The 'Maru'-fed group had the lowest tumour incidence among all blueberry-treated groups with only 4 out of 20 animals found with tumours. However, the 'Maru' extract did not delay tumour onset, whereas the 'Centurion' and Pomace groups

extended the first tumour onset by 15 and 24 days in comparison with the positive control group. A total of 35 tumours were found in the positive control group and total tumour volume was 79,975 mm^3. There was approximately 70-80% reduction in total tumour volume in animals that received any blueberry treatment. Two animals from the positive group were found bearing 6 tumours while the highest tumour multiplicity in blueberry treated animals was 4. However, only rats in the Pomace group showed significantly lower tumour multiplicity than the positive control group when affected rats were considered.

From this experiment, we concluded that the major effect of blueberry extracts and a blueberry pomace-supplemented diet was reducing tumour incidence. Only animals that received the pomace-supplemented diet had significantly delayed tumour onset ($P \leq 0.05$). Total tumour number significantly reduced in animals with blueberry intervention ($\chi^2 = 22.1$, $P < 0.001$) Blueberry treatments showed small changes that were not statistically significant on tumour volume and tumour progression. When affected animals were considered, we found that only animals that received the blueberry-pomace diet showed a significant reduction in tumour multiplicity ($P \leq 0.05$).

Introduction:

Female breast cancer incidence is the highest cancer incidence and it is the second most common cause of cancer death in New Zealand (Ministry of Health, 2013). Approximately 80 million NZD are spent each year in regard to breast cancer diagnosis and treatments. Therefore research for breast cancer prevention is required.

In general, carcinogenesis is a complex process including three major stages which are initiation, promotion and progression. Phytochemicals with chemopreventive properties may be able to interfere with any of these carcinogenesis stages (Tan et al., 2011). Blueberries contain phytochemicals which are beneficial to human health. Blueberries and their phytochemicals have been reported to affect breast cancer both *in vitro* and *in vivo* (Faria et al., 2010; Adams et al., 2011; Ravoori et al., 2012). A 2.5% blueberry powder-supplemented diet was found to reduce tumour incidence by 69% in estrogen-induced mammary tumour *in vivo* (Aiyer & Gupta, 2010). Resveratrol, a phytochemical found in blueberries, effectively delayed tumour onset for 19 days in DMBA-induced mammary tumorigenesis (Whitsett et al., 2006).

A by-product from juice- or wine-making industries, known as pomace, consists of seed pulp and skin. Anthocyanins present in blueberries are found mainly in the skin

therefore blueberry pomace is a good source of anthocyanins. Moreover, dietary fiber contributes to approximately 40% of blueberry pomace dry weight (Bränning et al., 2009). Dietary fiber benefits on the reduction of breast cancer risk are well documented. A study in 20 premenopausal women showed that serum estrone and estradiol decreased after 2 months consumption of high fiber/low fat diet which may indicate a role for dietary fiber in breast cancer prevention (Bagga et al., 1995). To our knowledge, there is no study of the effect of blueberry pomace on breast cancer.

Even though the antioxidant activity of blueberry phytochemical is widely claimed to be the bioactivity responsible for cancer prevention, other biological properties such as anti-proliferation, induction of detoxification and antioxidant enzymes, induction of apoptosis, and anti-angiogenesis may also be anti-cancer properties attributable to blueberries (Whitsett et al., 2006; Gordillo et al., 2009; Samad et al., 2014).

7,12-Dimethylbenz[a]anthracene-induced mammary tumorigenesis in Sprague-Dawley (SD) rats is one of the most widely used animal models for studying breast cancer. The DMBA administration period has an important influence on tumour incidence. The most susceptible period for neoplastic formation in the mammary gland of female SD rats is when they are approximately 6 to 8 weeks old (Russo & Russo, 1994). 7,12-Dimethylbenz[a]anthracene-induced mammary tumorigenesis results in estrogen receptor-positive (ER+) breast cancer, which accounts for 85% of breast cancers found in humans. Even though the histopathology of DMBA-induced mammary tumorigenesis in the rat model has some structural differences in comparison with human breast carcinomas, morphologic similarities have been established (Irmgard et al., 2002).

More than 200 epidemiological studies have suggested that fruit and vegetable consumption provides significant protective effects on several diseases including breast cancer (Block et al., 1992). However, this was not confirmed in more recent analyses eg EPIC (European Prospective Investigation into Cancer and Nutrition) study (Bradbury et al., 2014). Quercetin and resveratrol, phytochemicals found in blueberries, were studied for their synergistic effect and it was found that quercetin inhibited resveratrol glucuronidation which might result in more bioavailability of resveratrol when consumed together (Aggarwal et al., 2004). Cancer chemoprevention research often focuses on bioactive food components. Commonly, the component is purified and used in *in vitro* studies. However, even if the purified component exhibits a chemopreventive effect *in vitro*, it is sometime less effective when the component is applied *in vivo*, (Aiyer & Gupta, 2010). Therefore in

this study, we used blueberry crude extract with MilliQ water without purification and given in a drinking water to avoid stress from gavaging in an *in vivo* model.

Two blueberry cultivars, 'Centurion' and 'Maru', were chosen for the *in vivo* study. Even though both cultivars exhibited similar biological activities, 'Centurion' was rich in glucosidated anthocyanins while galactosylation was dominant in 'Maru'. Commercially available highbush blueberry pomace was supplemented to animal's diet as another form of blueberry intake.

To determine the effect of blueberries in DMBA-induced mammary tumorigenesis in rat model, 100 animals were divided into five groups where each group received different blueberry treatments. We hypothesized that blueberry as a nutritional intervention treatment would affect tumour incidence, tumour latency and tumour multiplicity (number of tumours in each animal).

Materials and methods:

Animals and protocols

One hundred Sprague-Dawley, 6-weeks old female rats were purchased from the Massey University Small Animal Production Unit (SAPU). Rats were acclimated at SAPU and fed with standard diet for one week prior to DMBA gavaging. They were housed in pairs in clean shoebox cages in an environmentally controlled room with 12 h dark/light cycle, 22 ± 2°C and 45 to 50% humidity. The animals had free access to feed and to water or blueberry extracts throughout the experimental period. All experimental protocols were approved by Animal Ethics Committee, Massey University (Approval number 13/11).

Chemicals

All reagents were purchased from Sigma-Aldrich Pty. Ltd., Australia. All the reagents used were of analytical grade except xylene which was histopathological grade. Blueberry extracts were prepared with deionized water. All standard rat chow materials were provided by the Massey University Feed Mill, Palmerston North. Ultrapure Milli-Q (MQ) water obtained from a Milli-Q system (Millipore, Milford, MA) was used for all chemical preparations. Spectrophotometric measurements were performed by ELX 808 Ultra Microplate reader (KC4 version 3.01 software Bio-Tek. Instrument Inc, USA). Centrifugations were performed on a Beckman Coulter (Allegra™ 64R) high-speed

centrifuge or Eppendorf Minispin microcentrifuge in blueberry extract preparation and DNA extraction, respectively.

Preparation of blueberry extracts

Two cultivars of frozen rabbiteye blueberry fruits (*Vaccinium ashei*) 'Centurion' and 'Maru' were purchased from Beaches Blueberries Ltd, Hamilton, New Zealand. Freshly-thawed blueberries were mixed with deionized water (1:3, w/w) using a stick blender (Sunbeam, SM6200). The extract was centrifuged at 10,000*g* for 5 minutes. The supernatant was collected and kept at -20°C until used.

The blueberry extracts or deionized water was supplied in clean polyethylene water bottles to rats *ad libitum*. The blueberry extract or deionized water (for negative control, positive control and Pomace groups) was the sole source of drinking fluid throughout the 13 week experimental period. The extracts were changed every day and fluid intake was measured. The water bottles for negative control, positive control and pomace fed groups were cleaned once a week using an automatic cleaning facility at Small Animal Unit Production (SAPU, Massey University, Palmerston North, New Zealand). However, the water bottles for the blueberry extracts were cleaned thoroughly every day to prevent spoilage from bacteria.

Standard diet and blueberry pomace diet

Rat chow was given in pellet form. It was formulated to meet the requirements for normal rat growth based on the AIN-93G recommendation (table 5-1). Corn oil was used in this rat chow formula in order to avoid any effect of phytoestrogens from soybean oil.

Highbush blueberry pomace was provided by Mamaku blueberry orchard, Hamilton, New Zealand. Pomace came from commercial juicing residue from highbush blueberries (mix cultivars). The pomace was oven-dried (40°C, 48 hours), ground using coffee grinder (Breville, CG2B) and kept at -20°C until used. The powdered pomace was supplemented (5% w/w) on top of the standard rat chow formula. Both standard diet and blueberry pomace diet were prepared in bulk at the Massey University Feed Mill, Palmerston North.

Experimental design

After a one-week acclimation period, 7-week old rats were weighed and randomly assigned into five experimental groups (n = 20 per group): Negative control (gavaged with

corn oil and given standard rat chow and deionized water), Positive control (gavaged with 50 mg DMBA in corn oil/kg body weight, given standard rat chow and deionized water), 'Centurion' (gavaged with 50 mg DMBA in corn oil/kg body weight, given standard rat chow and 'Centurion' blueberry extract), 'Maru' (gavaged with 50 mg DMBA in corn oil/kg body weight, given standard rat chow and 'Maru' blueberry extract), Pomace (gavaged with 50 mg DMBA in corn oil/kg body weight, given blueberry pomace feed and deionized water) (Figure 5-1). DMBA was given to animals only once at the beginning of the study.

The blueberry extract and blueberry pomace feed began 2 hours after DMBA administration. The experimental period was 13 weeks after DMBA administration. This is the only study where blueberries were introduced immediately after administration of carcinogen.

Table 5-1 Standard animal diet and 5% pomace supplemented diet formulation

Ingredient	**Control diet (g/kg)**	**Blueberry supplemented diet (g/kg)**
Wheat	420	400
Barley	300	285
Skim milk powder	90	85
Broll	50	47.5
Lucern	50	47.5
Corn oil	39	37.5
MBM/LO	46	42.5
Micronutrient		
Smethi	1	1
(Commercial synthetic methionine)		
Vitamin/Mineral Premix	4	4
Blueberry pomace powder		50

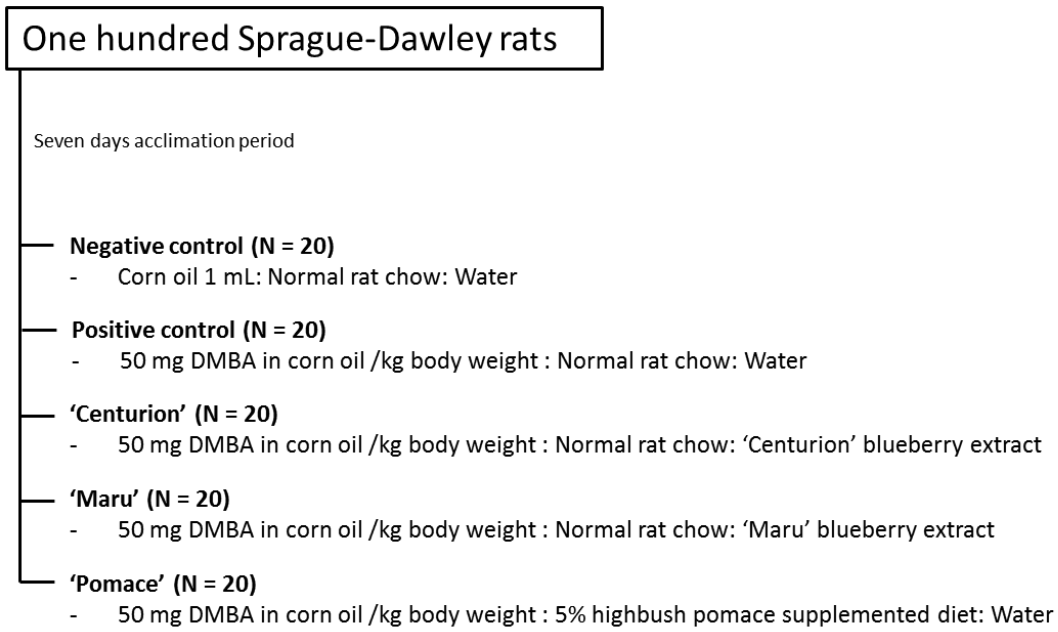


Figure 5-1 Animal experimental design

Rats were weighed weekly to monitor effects of DMBA and blueberry extracts on rat health. Feed intake and deionized water intake were recorded weekly while blueberry extract intake was measured daily. All these measurements were done between 9 and 11 am. The difference in weight of standard or pomace pellet rat chow before and after each feeding period was calculated for rat daily feed intake. The fluid intakes were determined by the weight difference of water bottle containing deionized water or blueberry extracts.

Animal health was checked daily using an animal daily health score record (Appendix III) specifically developed for the experiment (Workman et al., 1989). Animals were palpated once a week starting from week 5 after DMBA administration in order to check for tumour incidence. Any rats with tumours were palpated every second day. Tumour size was measured by digital vernier callipers (Insize, Germany).

At week 13 after DMBA administration, rats were sacrificed by using anaesthesia followed by intracardiac puncture and exsanguination. Four animals from each group were selected at random and a total of twenty animals were euthanased and dissected each day through five days period. There was no significant change in biological markers in animals' blood serum during the period of euthanasation. All animals were anaesthetized with an intraperitoneal injection (25G x 5/8" needle) of 0.12 mL/100 g body weight of the injectable anaesthetic mixture. The anaesthetic mixture contains APC (Acepromazine) (2

mg/mL), Ketamine (100 mg/mL), Xylazine (10%) and sterile water at the ratio 2:5:1:2, respectively. Animals were anaesthetized one at a time by experienced animal technicians. An animal was placed on a towel on a working bench and then covered with a towel. The towel covered the entire body except the tail. The first technician held the tail of the animal by placing the thumb under the tail base and the hand was placed on top of the tail and covered the lower body. This holding provided gentle but firm restraint to the animal. Then the hind part of the animal was gently lifted exposing the animal's abdomen without injuring the spine. The second animal technician performed intraperitoneal injection lateral to the midline on the right side of the animal to avoid the caecum. The animal was returned to the cage. The animals were anaesthetized within 3 – 10 minutes. For terminal blood collection, the animal was monitored closely and once anaesthetised, terminal heart puncture was performed. The animal was in dorsal recumbency. It was checked for response before cardiac puncture by firmly pinching its footpad. Provided the animal did not respond to the stimulus, a needle was inserted slowly under the sternum and blood was withdrawn gently to avoid haemolysis.

Animal dissection and sample collection

Blood from cardiac puncture was collected in a pre-labelled BD Vacutainer® red-top tubes (Ref No: 367895). The blood was left at room temperature for 30 min then centrifuged. The blood serum was aliquotted into pre-labelled Eppendorf tubes (1.5 mL). The serum was kept at -80°C until used.

The liver was dissected out, weighed and cut into small pieces. Some were fixed in 10% buffered formalin and some were snap frozen in liquid nitrogen. A post-mortem assessment of all internal organs was performed. If abnormal tissues were found, the tissue was dissected out and fixed in 10% buffered formalin for further investigation.

Rat mammary tumours were dissected out. The position of the tumour found in each animal was recorded in the diagram shown in figure 5-2. Tumours were measure using digital vernier calliper. Tumour volume was calculated from the calliper measurement using the formula from a previous study on rat mammary tumours [Volume = (Width x Depth x Length)/2] (Faustino-Rocha et al., 2013). One half of the tumour was fixed in 10% buffered formalin for histopathological exam and another half was immediately frozen in liquid nitrogen. The right side of the 4th pair mammary gland was fixed in 10% buffered formalin. The left side of the 4th pair mammary gland was dissected out and fixed in Carnoy's fixative

(ethanol: chloroform: glacial acetic acid (6:3:1)) for evaluation of whole mount mammary gland (Dunphy, 2008). Rat caecum (caecal content and mucosal lining) was collected for q-PCR analysis of commensal and pathogenic bacteria. The caecum contents were extruded manually into a pre-labelled sterilised Eppendorf tube (1.5 mL). Mucosal lining was scraped from the inner surface of the caecum using an ethanol-sterilised spatula into a pre-labelled sterilised Eppendorf tube (1.5 mL). Samples were frozen on dry ice before transfer to -80°C freezer.

Results of food and water intake, body weight, tumour incidence, tumour latency, tumour multiplicity, tumour volume and tumour progression are presented here in chapter 5. Results of the histopathological exam, whole mount mammary gland, analysis of intestinal microflora, level of oxidative stress and circulatory estrogen in blood serum are presented in chapter 6.

Statistical analysis

All normally distributed data were analysed for mean comparisons using one-way analysis of variance (one-way ANOVA) to assess significant treatment effects, and then Tukey's test was used as a posthoc comparison. The differences were considered statistically significant at $P \leq 0.05$. A Chi-square goodness of fit was used to analyse tumour incidence and tumour number and the differences were considered statistically significant at $P \leq 0.05$. A Kruskal-Wallis test was used for non-normalised data, tumour volume, for comparison as an alternative to one-way ANOVA. The differences were considered statistically significant at $P \leq 0.05$. All statistical tests were analysed using Minitab software (version 15; Minitab Inc., Pennsylvania, USA).

Animal ID:

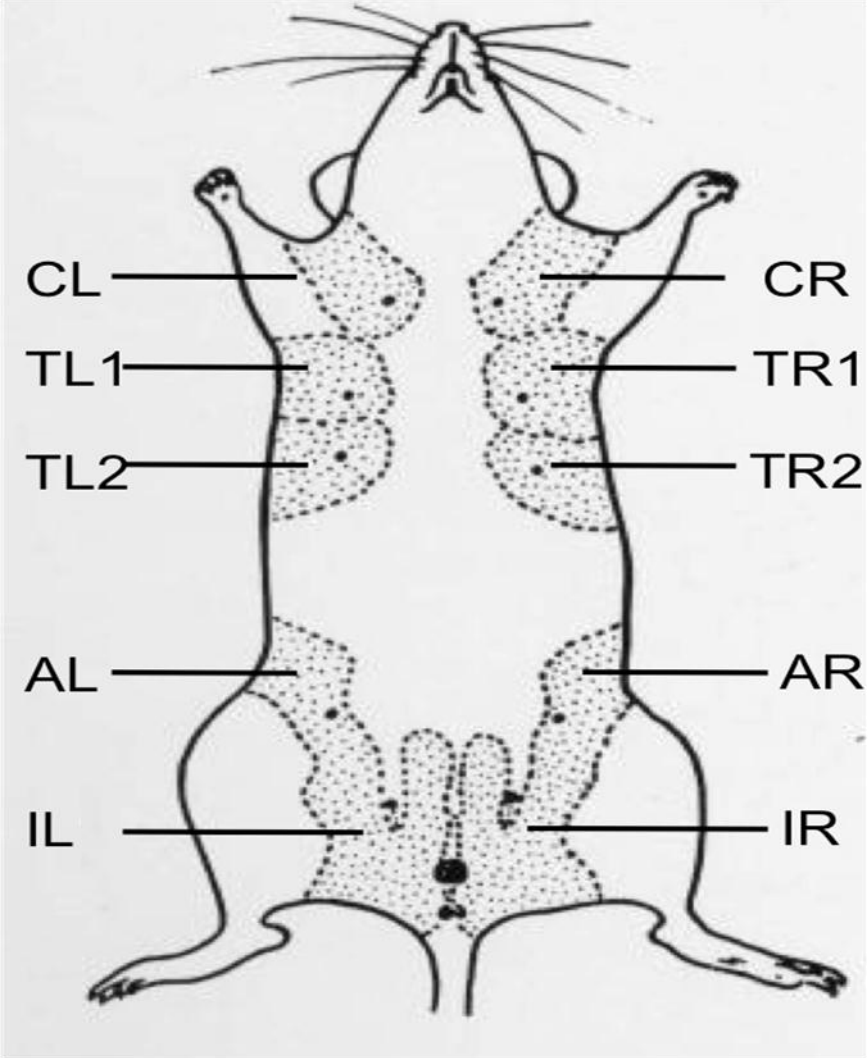


Figure 5-2 Scheme reporting the localization of mammary glands for mammary tumours record. (Source: modified from Murphy, 1966). Mammary gland left cervical = CL, Mammary gland right cervical = CR, Mammary gland left thoracic = TL, Mammary gland right thoracic = TR, Mammary gland left abdominal = AL, Mammary gland right abdominal = AR (the right side 4th pair mammary gland for a mammary gland whole mount), Mammary gland left inguinal = IL, Mammary gland right inguinal = IR.

Results and discussion:

Body weight, liver weight and diet consumption

All animals remained healthy and survived until the end of the experiment except one. Six weeks after DMBA administration, the DMBA-treated rat from 'Maru' group received scores of 4, 4, 3 and 3 in spontaneous activity, appearance, body weight and food intake, respectively. The animal was euthanased. A necropsy result showed that the rat had lymphoma and was excluded from the experiment (Appendix IV).

As it was possible that the blueberry extract, blueberry pomace supplemented diet and/or DMBA treatment might affect animal health and induce weight loss, animal body weights were measured and recorded once a week. Average animal weight for each experimental group is shown in table 5-2. Animals in the 'Maru' group had the lowest average starting weight (animals age 7 weeks) (205.2 g); however, this was not statistically different to the other experimental groups. At the end of the study (animals age 20 weeks), animals in the negative control group had the highest body weight (366 ± 30 g) among all the experimental groups followed by Pomace, 'Centurion', positive control, and 'Maru' at the lowest (345 ± 23 g). The variation in animal weight was not statistically significant. A growth chart of female SD rats from the Charles river's website ("Sprague-Dawley® Rat | Charles River," n.d.) shows that at seven weeks of age the rat weighed approximately 170 g which was lower than the body weight of animals used in our experiment at the same age. The animals gained weight rapidly during the first five weeks of the study (age 7 to 12 weeks old) with an average of 20 g per week. According to information provided by the company, average weight gain of the animals at the same age is approximately 8 g per week. There is no information on rat weight after 13 weeks of age provided by the company. In our study, animals gained approximately 6 g per week at age 13 weeks old until the end of the experiment (20 weeks old). An adult body weight of female SD rats is between 250-300 g however, in our study animal weight ranged from 311 to 426 g at the end of the experiment (animals age 20 weeks). The higher weight gain and body weight of the animals in our experiment might result from different rat chow or variations in biological subjects, as each breeding colony tends to become inbred and develop slight variations in physiology.

Diet consumption in each group varied significantly. In the first three weeks of the study, the average diet intake was not stable, likely due to change of diet and handler.

From week four onward, the trend was more stable compared to the beginning of the study (figure 5-3). The animals consumed slightly less diet in week seven which may result from slowing of their growth as they become adult. Disease progression might be another reason explaining reduction of diet consumption in week seven onward. The first tumour appeared as a sign of disease progression at seven weeks after DMBA administration. As the disease progressed the animals required more attention and handling which might result in unstable diet consumption observed at the end of the study. The highest mean consumption was found in the Pomace group followed by the negative control. Animals in the positive control and animals that received 'Centurion' extract showed a similar level of diet consumption. Animals that received 'Maru' extract consumed less feed than the negative control, positive control and 'Centurion' groups but it was not statistically significant.

However, the average feed intake in 'Maru' was significantly lower than the Pomace groups ($P< 0.014$). In our study, animals that received 'Centurion' and 'Maru' extract showed lower dietary intake by 4.5% and 9% respectively compared to the negative control. In a previous research, 'Centurion' and 'Maru' extracts have been shown to exhibit appetite-suppressing effects in male SD-rats. Animals that received blueberry extracts showed lower diet intake by 6.2% ('Centurion') and 8.6% ('Maru') (Molan et al., 2008).

Even though animals in the Pomace group, consumed significantly more feed than other groups, their final weights were slightly but not significantly lower than the negative control (figure 5-3). The dietary fiber in blueberry pomace may slow body weight gain by reducing fat absorption.

Average water consumption varied among each experimental group (figure 5-4). The highest was found in 'Centurion' group with approximately 28 g per rat per day. In other reports assessing chemopreventive or therapeutic effects of plant extracts, the extracts were usually gavaged instead of being supplied in drinking water form because the water intake is not a very reliable measurement as a lot of variability might occur such as water dripping from animals contact. However, in this experiment blueberry extracts were given in drinking water for ethical reasons, in order to avoid stress from gavaging. The water consumption of animals in our study was within a normal range of female SD rats at the age of study (Laaksonen et al., 2013).

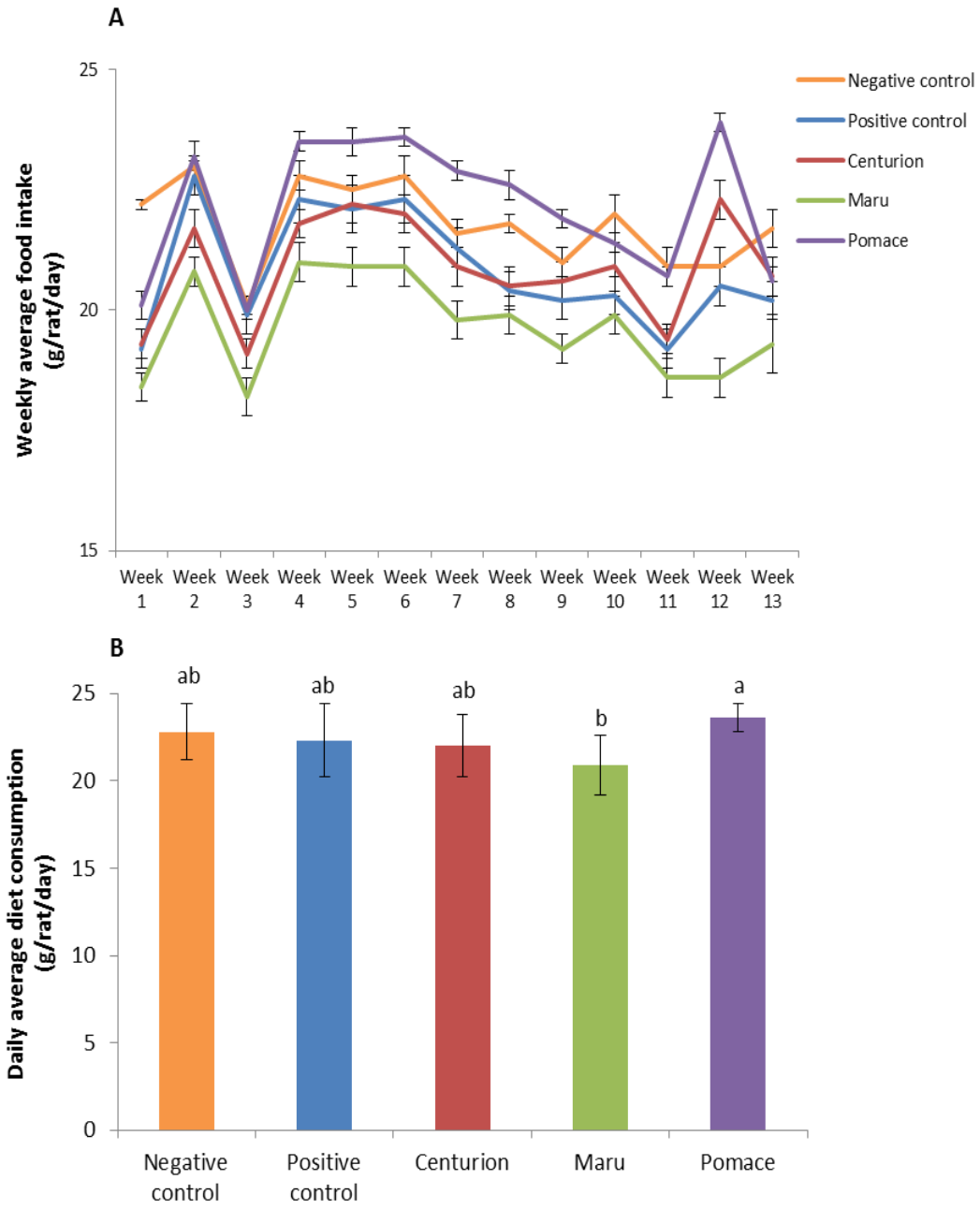


Figure 5-3 Data of average diet consumption (A) Weekly mean diet consumption for total study period (13 weeks) (B) Daily mean diet consumption at week 6. Data are shown as mean ± SEM. Different letters denote statistically significant differences as assessed by one way ANOVA with posthoc Tukey's test ($P \leq 0.05$). (N = 20 rats in all groups except 'Maru' N = 19).

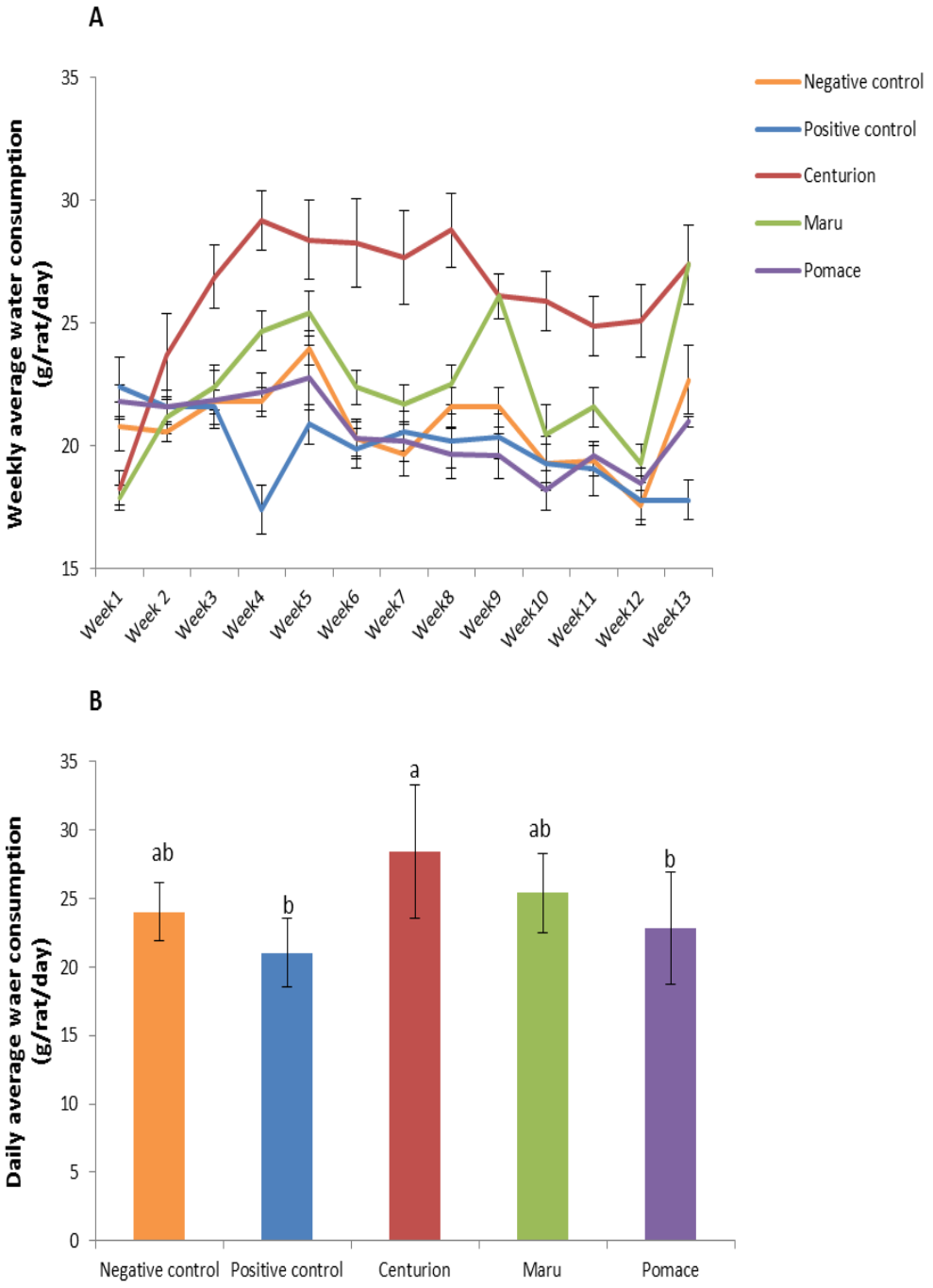


Figure 5-4 Data of average water consumption (A) Weekly mean water consumption for total study period (13 weeks) (B) Daily mean water consumption at week 6. Data are shown as mean ± SEM. Different letters denote statistically significant differences as assessed by one way ANOVA with posthoc Tukey's test ($P \leq 0.05$). (N = 20 rats in all groups except 'Maru' N = 19).

In the positive control group, animals gained less weight and had lower average weight at the end of the study compared to non-treated animals, which correlated with disease progression. Interestingly, animals in 'Centurion' and 'Maru' groups, which exhibited better health score than the positive control groups, also showed less weight gain and lower body weight at the end of the study which might be an effect of blueberry consumption.

Effects of blueberry juice and whole blueberry powder on obesity has been studied elsewhere and revealed that animals that received blueberry juice consumed less food than animals that received whole blueberry powder fed a similar diet, which may result from higher sugar available in blueberry juice compared to whole blueberry powder (Prior et al., 2010).The effect of blueberries on satiety has also been studied in humans; obese subjects reported that they felt more satiated after blueberry supplemented meal consumption, compared to normal BMI subjects who consumed the blueberry supplemented diet (Magrane, 2009). Therefore, in our study, disease progression might not be the only cause of body weight variation; blueberry extracts and blueberry pomace might also play a part.

Table 5-2 Mean body weight at week 0 and week 13 of the study, mean liver weight and liver/ body weight ratio. Data are shown as mean ± SEM. Superscript letters denote statistically significant differences as assessed by one way ANOVA with posthoc Tukey's test ($P \leq 0.05$).

Group	Body weight (g)		Liver weight (g)	Liver/ Body weight ratio (g liver/kg bodyweight)
	Week 0	Week 13		
Negative control	206.2 ± 2.1	366.6 ± 6.7^{a}	11.3 ± 0.3^{ab}	31.0 ± 0.6
Positive control	206.2 ± 2.1	348.6 ± 8.1^{b}	11.1 ± 0.4^{ab}	31.8 ± 0.7
'Centurion'	206.3 ± 2.4	361.3 ± 5.3^{a}	11.6 ± 0.4^{a}	32.1 ± 0.8
'Maru'	205.2 ± 2.5	345.3 ± 5.3^{b}	10.4 ± 0.3^{b}	30.0 ± 0.6
Pomace	206.3 ± 2.1	363.9 ± 4^{a}	11.3 ± 0.2^{ab}	31.1 ± 0.5

The liver is a major organ in the detoxification process and thus is susceptible to damage from chemical carcinogens like DMBA. In this study, there was no significant different between average liver weight in vehicle control group and DMBA-treated groups. The only significant difference was found between animals that received 'Centurion' extract and 'Maru' extract where their average liver weights were 11.6 g and 10.4 g, respectively (table 5-2). Liver-to-body weight ratio of all experimental groups ranged from 30 to 32.1 g liver/kg body weight where the lowest was 'Maru' and the highest was 'Centurion'. The differences between the liver-to-body weight ratios were not significant. A previous study found that liver weight of DMBA-treated animals increased in comparison with the vehicle-control groups, which led to an increase in liver weight/body weight ratio (Deshpande et al., 1998).

Effect of blueberry extracts and blueberry pomace on tumour indices

No tumours were found in the negative control group, which confirmed our expectation that all tumours present in other experimental groups were induced by DMBA. Fifteen animals in the positive control group developed mammary tumours, which accounted for 75% of total animals in the group. Blueberry extracts and blueberry pomace-supplemented diet reduced the number of rats bearing tumours (table 5-4). The data collected from the study revealed that more than 50% of animals in blueberry treated groups combine did not develop tumours by the end of the study. However, a Chi-square analysis showed that there was almost statistically significant different of number of animals with tumours in DMBA-treated groups (P = 0.06). The percentages of animals in each test group bearing tumours during the study period are shown in figure 5-5. The expected values derived from the Chi-square analysis were the sum of the observed values from each category divided by the number of groups; for example, the expected values of animals with tumours was 8.75 which was the sum of number of animals bearing tumours (15 + 7 + 4 + 9) divided by number of groups (4).

This finding was similar to a study of Aiyer and Gupta. They studied preventive effects of blueberry, black raspberry and ellagic acid. At the end of the experiment, positive control animals were found with 100% tumour incidence while animals that had received 2.5% blueberry-supplemented diet had 69% tumour incidence and animals fed with 400 ppm ellagic acid or 2.5% black raspberry had 80% tumour incidence (Aiyer & Gupta, 2010).

The first palpable tumours appeared 47 days after DMBA induction in the positive control and 'Maru' groups. The first tumour found in 'Centurion' and Pomace group appeared 62 and 71 days after DMBA administration, respectively. This suggests that 'Centurion' and Pomace supplemented diet increased tumour latency by 15 days and 24 days, respectively. However, a more reliable statistic is the mean palpable tumour latency as shown in table 5-3. There was no significant different between the positive control and both blueberry extract groups ('Centurion' and 'Maru') but the mean tumour latency of palpable tumours was significantly delayed by six days in animals that received blueberry pomace supplemented diet compared to the positive control. A volume of the first tumour found at day 47 in the positive group exceeded 2 mm^3 which may indicate mis-palpation earlier in the study (figure 5-6). According to the size of the tumour, it is possible that the tumour might have appeared approximately 7-10 days before it was palpated.

It is important to emphasise that average tumour latency data presented here were derived from palpable tumours only. Therefore, it cannot be definitively concluded that blueberry pomace altered tumour latency of all tumours in the experiment since small tumours were difficult to detect by palpation.

Table 5-3 Mean palpable tumour latency of animals that received DMBA treatment. Data are shown as mean ± SEM. Superscript letters denote statistically significant differences as assessed by one way ANOVA with posthoc Tukey's test.

Group	Tumour latency (days)
Positive control (N = 20)	83 ± 2^{b}
'Centurion' (N = 20)	81 ± 4^{b}
'Maru' (N = 19)	72 ± 4^{b}
Pomace (N = 20)	89 ± 3^{a}

Sprague-Dawley rats, fed 2% quercetin-supplemented diet followed by DMBA administration, showed later tumour onset by approximately one week (Verma et al., 1988). Resveratrol, a polyphenolic compound found in blueberries, was found to delay tumour onset by 19 days compared to animals fed a normal diet in a study of a chemopreventive effect of resveratrol on DMBA-induced mammary tumorigenesis (Whitsett et al., 2006). In a study on ER+ breast cancer, female ACI rats that received 5% blueberry powder diet showed 24 days delay in tumour onset in comparison with the control diet group (Ravoori et al., 2012). Blueberry-supplemented diet increased tumour latency by 8 days in estrogen-induced mammary carcinogenesis study; however, the result was not statistically significant in comparison with animals that received control diet (Aiyer, 2007).

The total number of mammary tumours collected in this study was 72 tumours with 60% in the thoracic region and 40% in the abdominal region. The areas of tumour found in rats were similar to the study of Dias who found 67.9% tumour in cervical and thoracic regions and 32.1% in the abdominal region (Dias et al., 2000). The difference between mammary tumorigenesis susceptibility of mammary glands in different areas can be explained by the structure of the mammary glands. Terminal end buds are the most susceptible structure to chemically-induced carcinogenesis because of their high proliferative rate of mammary epithelia, and higher numbers of terminal end buds are found in the thoracic and cervical region compared to the abdominal and inguinal region (Russo & Russo, 1994).

At the end of the experiment, a total of 35 tumours were found in the 15 tumour-bearing rats in the positive control group (table 5-4). Two animals with palpable tumours were found in Pomace group; therefore, 5% blueberry pomace supplemented diet seemed to be the most effective treatment for decreasing tumour incidence. However, after dissection, animals from Pomace group were found to contain a total of 12 tumours. Most of the tumours found in the Pomace group were impalpable due to their small size. The total tumour number in rats that received 'Centurion' extracts was 15. Only 10 tumours from 4 rats were found in the 'Maru' group. It seemed that 'Maru' extract effectively inhibited tumour initiation. The effect of blueberry treatment on reduction of tumour numbers was confirmed using a Chi-square analysis. There was a statistical significant difference between DMBA-treated animals ($P \leq 0.05$) (table 5-4).

Tumour multiplicity was also decreased in blueberry-treated groups. Two animals from the positive group were found bearing 6 tumours at the end of the experiment while the highest tumour number found in a single animal in blueberry-treated groups was 4. However, when only affected animals were considered, tumour multiplicity in the positive control group was not significantly different from 'Centurion' and 'Maru' groups. However, rats that received the blueberry pomace diet had significantly lower tumour multiplicity in comparison with positive control. Tumour indices data are shown in table 5-4.

Amount of blueberry intake from all blueberry treated animals did not show correlation with any tumour indices (data not shown).

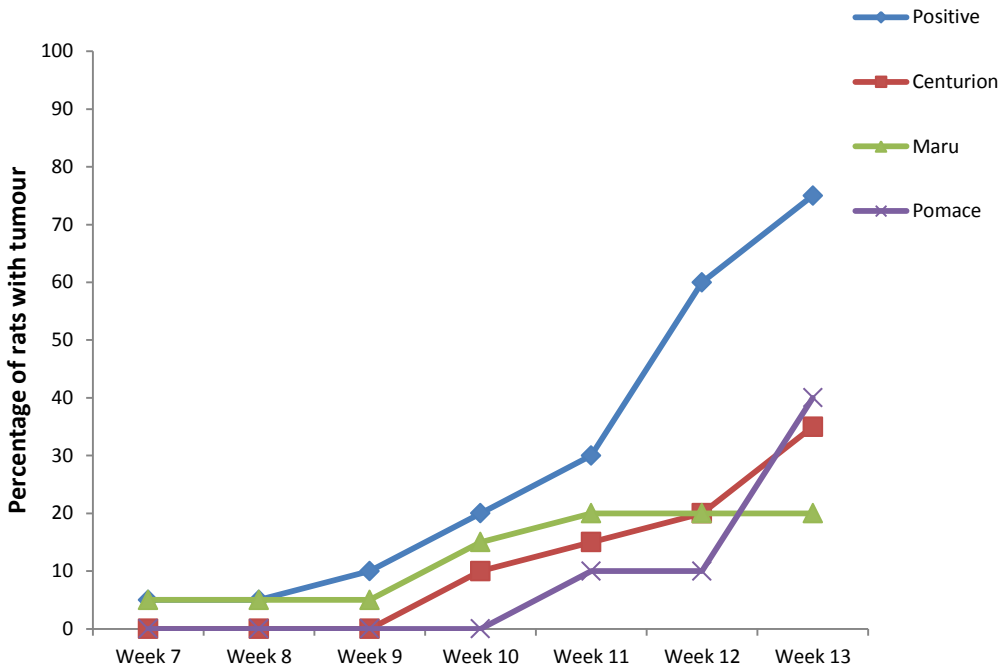


Figure 5-5 Percentage of palpable tumour incidence in all DMBA-treated groups started from week 7 to week 12 after DMBA administration. The tumour incidence data of week 13 was post mortem. During week 7 to week 12, tumour incidence was calculated on the basis of palpation of the mammary tumours (but tumours in blueberry treated groups were smaller therefore they were less palpable).

Table 5-4 Comparison of post mortem tumour indices between experimental groups. Tumor multiplicity data are shown as mean ± SEM. Superscript letters denote statistically significant differences as assessed by one way ANOVA with posthoc Tukey's test ($P \leq 0.05$).

Group	Animals with tumour		Total number of tumours in all rats		Tumour multiplicity (tumours per tumour-bearing rat)
	Observed	Expected	Observed	Expected	
Negative control (N = 20)	0	0	0	0	NA
Positive control (N = 20)	15	8.75	35	18	2.3 ± 0.4^{a}
'Centurion' (N = 20)	7	8.75	15	18	2.1 ± 0.5^{a}
'Maru' (N = 19)	4	8.75	10	18	2.5 ± 0.9^{a}
Pomace (N = 20)	9	8.75	12	18	1.3 ± 0.2^{b}
χ^2	7.4		22.1		
P value	0.06		0.00		

NB: Chi-square analysis was analysed using data from DMBA-treated groups only.

Similar results were reported in several published studies, either with whole blueberries or blueberry polyphenols. A chemopreventive study of rats fed with diet supplemented with resveratrol (0.1% w/w) showed approximately 50% reduction in tumour multiplicity in comparison with animals that received a control diet (Whitsett et al., 2006). Dietary quercetin also decreased tumour multiplicity in rats with DMBA-induced mammary tumours by 50% (Verma et al., 1988). Five percent whole blueberry powder significantly lowered tumour multiplicity in ACI rats with estrogen-induced mammary tumour (Ravoori et al., 2012).

In this study, only information regarding tumour indices was collected. Possible mechanisms regarding anti-cancer properties of blueberries and blueberry pomace was not included. However, several potential mechanisms related to the anti-cancer properties of blueberries and their phytochemicals have already been identified: for example, antioxidant, prevention of DMBA-DNA adduct formation and antagonistic effect on aryl hydrocarbon receptor (AhR).

Antioxidant activity of blueberries might have an important role during tumour initiation especially in DMBA-induced mammary tumorigenesis model or others where high numbers of free radicals are generated. In this study, DMBA, a polycyclic aromatic hydrocarbon (PAHs), was used as a carcinogen. It is not a direct action carcinogen but its metabolites, especially those arising from hydroxylation of the 7-methyl group, are key compounds for the carcinogenicity and mutagenicity of DMBA. After hydroxylation at 7-methyl group resulting in 7-hydroxy DMBA formation, the compound forms an unstable derivative resulting in a highly reactive carbonium ion. The carbonium ion initiates cellular chain reactions, which lead to initiation of cancer (Flesher & Sydnor, 1971).

In another study, the process of mammary tumorigenesis by DMBA initiation was elucidated by *ex vivo* studies. A whole mammary gland, in organ culture study, fully absorbed DMBA within 12 hours after DMBA treatment. DMBA was slowly released from the mammary fat pad and caused DMBA-DNA adducts in mammary epithelial cells. DMBA-DNA adduct formation peaked at 27 hours after DMBA treatment. After the initiation period, DNA repair was found when the mammary gland was incubated in DMBA-free medium (Das et al., 1989). However, different results were found in an *in vivo* study, in which DMBA-DNA adduct formation occurred longer than 120 hours after DMBA administration (Lenoir et al., 2005). According to the former studies, DMBA metabolites might be scavenged by blueberry antioxidants when blueberry treatment is introduced within the initiation stage. Antioxidant activity of blueberry extract and pomace, therefore, can affect mammary tumour initiation. Moreover, blueberry polyphenols might alter antioxidant status of serum by altering antioxidant enzymes or molecules such as catalase, glutathione and superoxide dismutase (Elks et al., 2011).

Prevention of DMBA-DNA adduct formation is a possible mechanism in inhibition of tumour initiation. Apart from their antioxidant activity, flavonoids and other polyphenolic compounds found in blueberries may prevent DMBA-DNA adduct formation. Plant phenolic compounds including chlorogenic acid and resveratrol were studied for their effect on DMBA-DNA adduct formation. Chlorogenic acid was found to be the least effective in preventing DMBA-DNA adduct formation among all tested phenolic compounds (Ignatowicz et al., 2003). However, another *in vitro* assay using mouse epidermis model showed that phenolic acids such as chlorogenic acid interacted with DMBA metabolites while resveratrol was able to decrease DMBA-DNA adduct (Szaefer et al., 2004). The study showed a synergistic effect of blueberry polyphenols compared to using a single concentrated

compound. The prevention of DMBA-DNA adduct formation has also been demonstrated for purple grape juice: consumption prior to DMBA administration inhibited formation of DMBA-DNA adducts. Purple grape juice contains a similar flavonoid profile to blueberries (Jung et al., 2006).

Another possible mechanism is modulation of aryl hydrocarbon receptor (AhR) by blueberries and their polyphenols. The effect involves a separate pathway for decreasing DNA-DMBA adduct formation and inhibiting tumour initiation. AhR is up-regulated in mammary gland by exposure to polycyclic aromatic hydrocarbon (PAH) compounds such as DMBA (Currier et al., 2005). 7,12-Dimethylbenz[a]anthracene is not a carcinogen; it needs to undergo cellular metabolism and transform into an active carcinogen. AhR plays an important role in transformation of DMBA into its active form. Generally, AhR is in the cytoplasm but when DMBA is present, AhR binds with DMBA and is translocated into the nucleus. In the nucleus, the AhR/DMBA complex interacts with xenobiotic response elements (XREs) resulting in transcription of cytochrome P450 genes. Enzymes in cytochrome P450 family such as CYP1A1 and CYP1B1 are involved in phase I drugs or xenobiotic metabolism. DMBA is metabolised by CYP1A1 or CYP1B1 into a potent carcinogen. Therefore, AhR and phase I enzymes are promising targets for mammary tumour chemoprevention.

In an ER+ breast cancer cell line (MCF-7), kaempferol bound with AhR but did not result in CYP1A1 transcription (Ciolino et al., 1999). AhR pathway closely relates with phase I enzymes in xenobiotic metabolism. In normal mammary glands, AhR expression was detected at a modest level, however, AhR expression was high in DMBA-induced mammary tumorigenesis. The researchers also found that 6 hours after DMBA administration, AhR-regulated CYP1A1 and CYP1B1 mRNA up-regulation was found in breast tissue. Therefore, AhR and CYP1B1 are possible biomarkers in DMBA-induced mammary tumorigenesis (Trombino et al., 2000). Quercetin, kaempferol, myricertin and luteolin were found to act antagonistically to an AhR-responsive breast cancer cell line (MCF7) (Zhang et al., 2003). Resveratrol also acted as an antagonist of AhR. It bound with AhR and allowed AhR nuclear translocation but did not induce CYP1A1 expression (Casper et al., 1999).

Even though blueberry antioxidant properties are generally believed to account for their health benefits, in breast cancer studies, several distinct molecular pathways appear to be involved. Blueberries and their phytochemicals appear able to modulate these

molecular pathways which effectively lower or inhibit mammary tumour incidence (Aiyer et al.,2012).

Blueberry treatment in our study started two hours after DMBA administration. Antioxidant activity, antagonism to AhR and/or prevention of DMBA-DNA adduct formation may be responsible for this chemopreventive effect of blueberries in our study where numbers of tumour-bearing rats were reduced by at least 40% compared to the positive group (table 5-4). Moreover, numbers of total tumour decreased more than 50% in blueberry treated groups compared to the positive control. Effect of blueberries on tumour latency was less significant since latency was not increased in 'Maru' or 'Centurion' group and only the tumour multiplicity of Pomace was found to be significantly different from the positive group. Tumour size and tumour progression will be investigated further in this chapter.

Table 5-5 Total number of tumours in test group, total tumour volume and volume/tumour. Data was assessed for statistical significant different by Kruskal-Wallis test.

Group	Total number of tumours	Total tumour volume (mm^3)	Volume/tumour (mm^3)	
			Mean± SEM	Median
Negative control	0	NA	NA	NA
Positive control	35	79,975	2285 ± 709	727.3
'Centurion'	15	12,134	809 ± 212	470.7
'Maru'	10	17,858	1,623 ± 286	1465.8
Pomace	12	9,896	899 ± 277	536.2

The largest tumour volume found in positive control was 16,600 mm^3 and the total tumour volume was 79,975 mm^3 (table 5-5). The lowest total tumour volume, found in Pomace group, was 9896 mm^3. Blueberry treatments effectively reduced total tumour volume by 70 – 80 % when compared with no-blueberry treatment counterparts. Mean volume/tumour in the positive group was 2285 ± 709 mm^3.

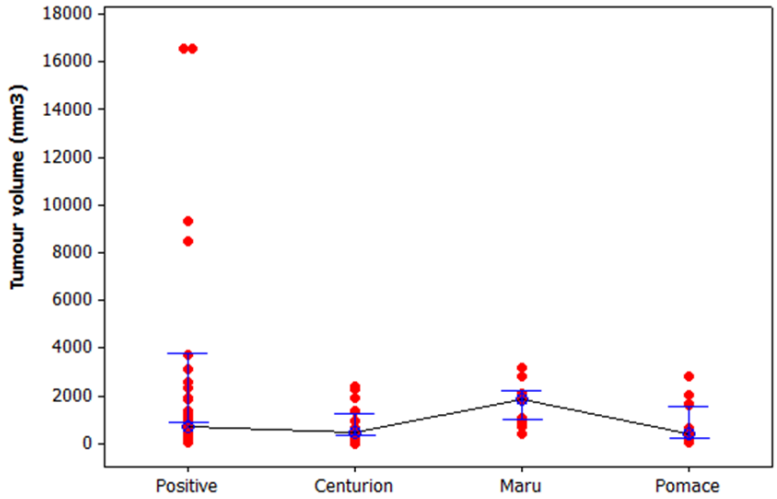


Figure 5-6 Individual value plot of tumour volume found in all DMBA-treated groups.

It initially appeared that blueberry treatment successfully reduced mean tumour size especially in the 'Centurion' and Pomace groups. However, the data of tumour volume were tested for normal distribution using Minitab software. It was found that the data were not normally distributed; therefore it could not be concluded that blueberry treatment effectively reduced tumour volume. There were two tumours in the positive control that grew bigger than 2 mm^3 which may cause negatively skewed data (figure 5-6). Therefore these two tumours were excluded from the calculation and the remaining data was re-tested for normal distribution. Even though the two largest tumour volumes were excluded, overall tumour volume was not normally distributed. Therefore, Kruskal-Wallis test for nonparametric was used as an alternative to one-way ANOVA where median of the data is used for comparison instead of mean. Both mean and median of the tumour volumes from each group are shown in table 5-5. By using Kruskal-Wallis test, the analysis showed that there was no significant different between the positive control and blueberry-treated groups ($P = 0.247$).

Several studies demonstrated an effect of blueberry consumption on reduction of tumour volume (Aiyer et al., 2008; Aiyer & Gupta, 2010; Ravoori et al., 2012; Jeyabalan et al., 2014). Animals that received ellagic acid or 2.5% blueberry supplemented diet showed 75% and 40% reduction in tumour volume respectively compared to animals fed control diet; however, the reduction of tumour volume in animals fed 2.5% blueberry supplemented diet was not statistically significant (Aiyer et al., 2008). The researchers concluded that ellagic acid may be responsible for mammary tumour chemoprevention by

some berry species. Since blueberries are low in ellagic acid, they exhibited only a modest mammary tumour chemopreventive effect that might result from anthocyanins and other polyphenols present in blueberries such as procyanidin b2 and chlorogenic acid. A similar result was found in a study from the same laboratory where 1% and 2.5% blueberry supplemented diet showed reduction of tumour volume but it was not statistically significant (Aiyer & Gupta, 2010). Diet supplemented with 5% blueberry powder significantly reduced tumour volume in E2-induced mammary tumorigenesis compared to animals fed control diet (Ravoori et al., 2012). Moreover, another study using E2-induced mammary tumorigenesis showed that 5% blueberry supplemented diet significantly reduced tumour volume regardless of time of administration. The blueberry diet was given prior to E2 induction or after the first palpable tumour was observed, and exhibited a similar effect on reduction of tumour volume in both of these treatments, compared to animals fed control diet (Jeyabalan et al., 2014).

According to previous studies, an effect of blueberries on tumour volume reduction seems to be dose-dependent. Identification of the optimum concentration in the human diet is required. Mechanisms by which blueberries and their polyphenols reduced tumour size were not explored in this study; however, an anti-proliferative effect of blueberries might be responsible for tumour size reduction. Abnormal cell division or uncontrollable cell proliferation is a hallmark of cancer development. A study found that expression of oncogenic molecules such as *c-myc, cyclin D1* and hyperphospho-related retinoblastoma (Rb) protein were elevated in the DMBA-induced mouse mammary model. These molecules affected cell proliferation in several cancers including breast cancer (Currier et al., 2005). Blueberry polyphenols, or resveratrol (1 mg/g diet), given to animals at pupping for 50 days resulted in lower cell proliferative index than in animals without resveratrol treatment (Whitsett et al., 2006). The study showed that the polyphenol reduced mammary cell proliferation in healthy animals. Moreover, blueberries and their polyphenols have also been studied extensively for their anti-proliferative effect in mammary tumorigenesis. Synthetic resveratrol showed anti-proliferative effect in 3 types of breast cancer cell lines, MCF-7, MCF-10F and MDA-MB-231, which have differing estrogen receptor status. The researchers concluded that the anti-proliferative effect of resveratrol occurred regardless of the cancer estrogen status (Mgbonyebi et al., 1998). Interestingly, at low concentration (<4 μM), resveratrol enhanced cell proliferation while it inhibited cell proliferation at higher concentration (>44 μM) in ER+ breast cancer cell line (Nakagawa et al., 2001). Another study on a hormone-dependent breast cancer cell line (T47D) showed that phenolic acids

such as caffeic acid and syringic acid had anti-proliferative effects (Kampa et al., 2004). A synergistic effect of anthocyanins found in blueberries, delphinidin, cyanidin, malvidin, petunidin and peonidin, on anti-proliferation of breast cancer cell line was documented (Ravoori et al., 2012). The study showed that IC_{50} of individual anthocyanins ranged from 50-120 µM while the anthocyanin combination required only 10 µM to achieve a similar result. *In vivo* anti-proliferative effect was also investigated. Animals receiving a blueberry-supplemented diet showed 37.4% lower proliferative index than animals on a control diet as measured by immunohistochemical staining for proliferating cell nuclear antigen (PCNA) in E2-induced mammary tumorigenesis.

Collectively, biological activities of blueberries such as antioxidant activity, anti-proliferative effect, modulation of AhR expression, and prevention of DMBA-DNA adduct formation may play an important role in anti-cancer properties of blueberries.

Tumour progression

Progression of all palpable tumours was recorded and plotted as shown in figure 5-7. Animals in positive group had the highest number of palpable tumours followed by animals in 'Maru', 'Centurion' and Pomace as shown earlier in figure 5-5.

The palpation data indicated that tumours generally grew at a slow rate until their size reached approximately 2 mm^3 then accelerated. In our study, there were only two tumours that reached this size category. The P7-1 tumour was larger than 2 mm^3 when it was first felt which indicated that it might have appeared at least one week before but was missed. The P7-2 tumour took approximately 30 days for the tumour to reach 2 mm^3 in volume after its first palpation.

However, there were two tumours found on the same day (47 days after DMBA administration), one in a positive control animal (P7-1) and one in a 'Maru'-fed animal (M14-1). The volume of the tumour in M14-1 was six times lower than P7-1 when the tumours were found. The P7-1 tumour grew to become the biggest tumour found in this experiment while the M14-1 tumour grew slowly and was 8-fold smaller than P7-1 at the end of the experiment. The progress of these tumours is shown in figure 5-8. It was obvious that the size of this tumour increased more rapidly in rats without blueberry treatment than tumours in animals receiving blueberry treatment.

In the previous chapter, blueberry extracts were shown to possess anti-angiogenic activity. The activity might explain slower tumour progression found in this *in vivo* study. Even though the modes of action were not investigated in our study, blueberries have shown *in vivo* anti-angiogenic activity in some type of cancers. Mice receiving blueberry treatment showed anti-angiogenic activity in a vascular tumour model, hemangioendothelioma, compared to non-treated mice (Gordillo et al., 2009).

According to our statistical analysis, it is difficult to conclude whether blueberry treatment had an effect on reduction of tumour volume and slower tumour progression. Further study regarding inhibitory effect of blueberries during tumour progression is required such as down-regulation of VEGF expression, which relates to tumour angiogenesis.

Conclusion:

In this study, DMBA-induced mammary tumorigenesis was found to be consistent with other studies. DMBA successfully induced mammary tumours in SD rats. We found that tumour incidence was affected by blueberry treatment in both extract and diet-supplemented form. The major effect of blueberry treatments was the reduction of tumour number. However, only animals fed blueberry pomace supplemented diet showed a significant reduction in tumour multiplicity and delaying of palpable tumour latency. Both 'Centurion' and 'Maru' extracts showed similar results. No tumours in blueberry treated groups reached over 2 mm3 and one animal from 'Maru' that developed a palpable tumour on the same day as an animal in the positive control group showed slower tumour progression. However, the deduction that blueberry feeding reduced tumour progression requires further study for validation, due to low sample size. The tumour size (volume/tumour) and tumour progression also appeared to be reduced by blueberry treatments but these results were not statistically significant. Antioxidant activity, apoptotic induction, and anti-proliferation might be mechanisms underlying these phenomena. Delaying tumour progression might result from anti-angiogenic property of blueberries. The findings support the hypothesis that blueberry consumption as a drink or as pomace in the diet helped in reducing tumour incidence of DMBA-induced mammary carcinogenesis in rats *in vivo*.

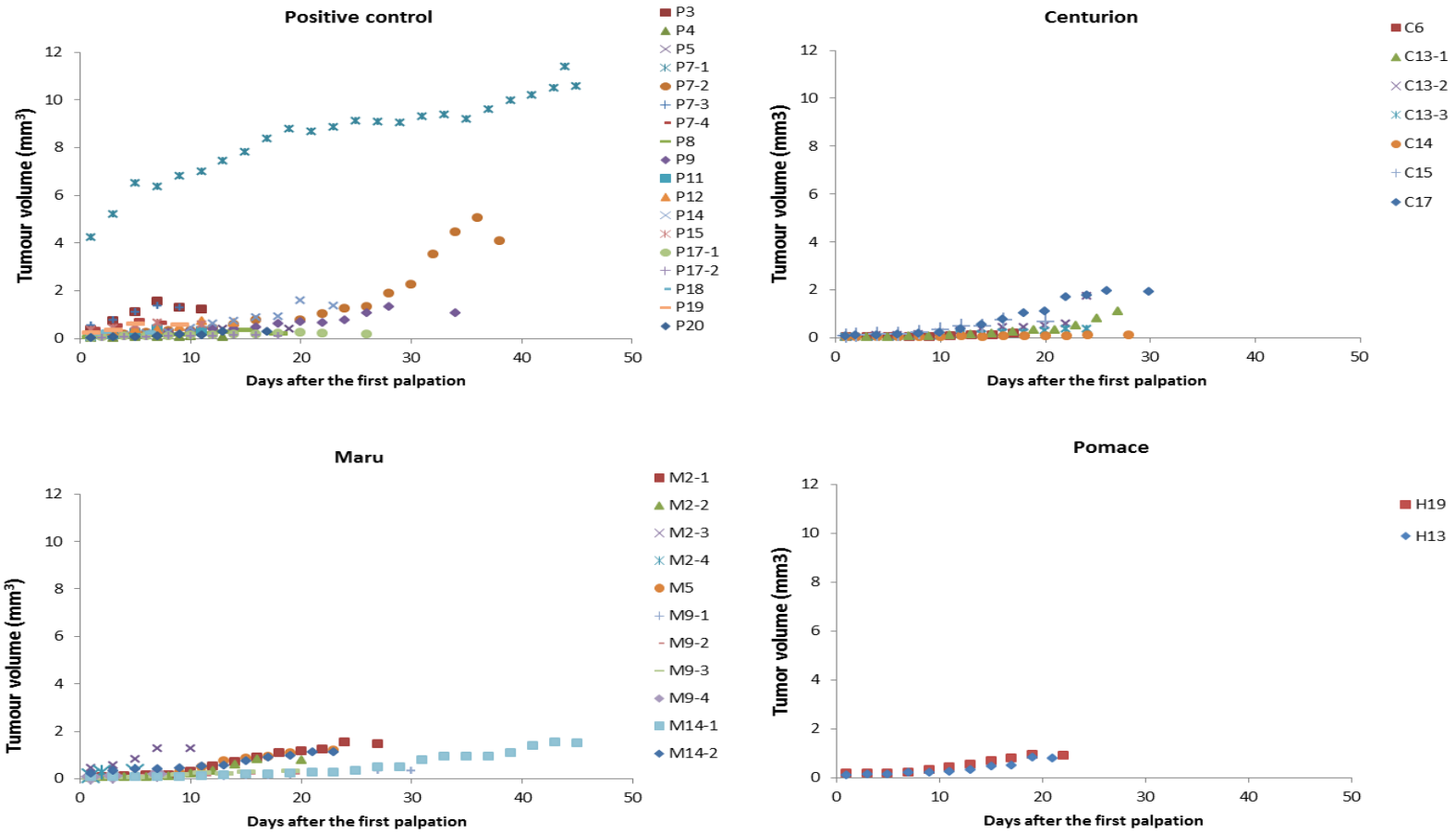
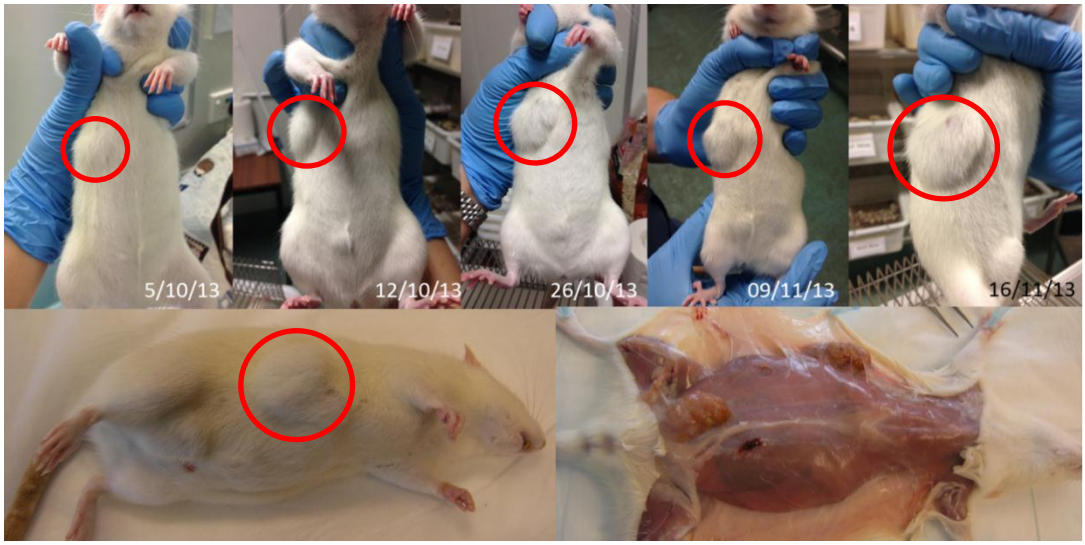


Figure 5-7 Progression of all individual palpable tumours from DMBA treated groups. Volume of each palpable tumour was plotted against the day it was found. Volumes of all palpable tumours were collected until the end of the experiment.

A.



B.

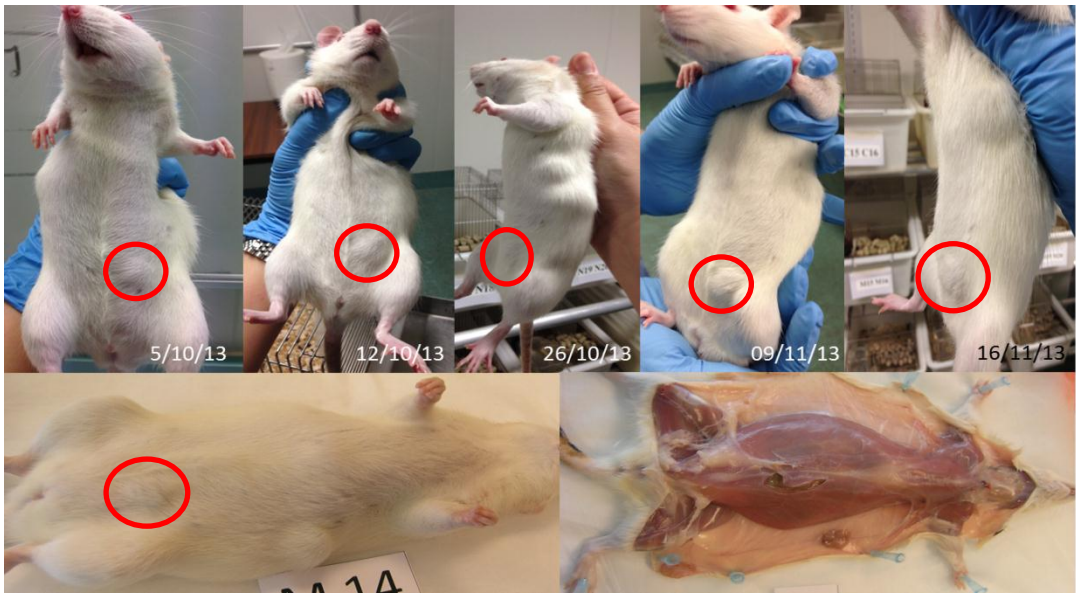


Figure 5-8 Tumour progression of (A) Positive group and (B) 'Maru' group. The tumours in red circles were initially palpable 47 days after DMBA administration.

CHAPTER 6

Impact of blueberry extracts and blueberry pomace on mammary gland morphology, oxidative stress, circulatory estrogen level and intestinal microflora of 7,12-dimethylbenz[a]anthracene-induced mammary tumorigenesis rat model

Abstract:

Blueberries may exhibit chemopreventive and therapeutic effects on breast cancer via a number of different pathways. In this chapter, the effects of selected blueberry extracts and constituents on mammary gland maturation, tumour histopathology, oxidative stress, circulating estrogen levels, gut microflora population and β-glucuronidase enzyme activity were studied in female Sprague-Dawley (SD) rats.

Blueberry consumption in either extract or pomace supplemented diet form did not significantly alter mammary gland maturation. Blueberry consumption exhibited more pronounced effects on tumour histopathology. Rats in positive control group (DMBA without blueberry treatment) were found with adenocarcinomas (malignant tumours) and only adenomas (benign tumours) were found in 'Centurion' and Pomace groups. The absence of adenocarcinomas might result from increased tumour latency. Oxidative stress status of blood *in vivo* was also increased by blueberry consumption. Blood serum antioxidant activity was measured by FRAP assay, and increased in all three blueberry treated groups. Malondialdehyde (MDA) was also measured as a biomarker for lipid peroxidation in serum and it elevated in all DMBA treated groups. However, all blueberry treatments decreased serum MDA levels. Catalase, an antioxidant enzyme, is important in the cellular defence system. The positive control group showed the lowest catalase activity among all experimental groups. Even though tumours were present in all three blueberry treatments, the level of catalase enzyme activity was similar to the healthy non-DMBA

control. Estrogen, a steroid sex hormone produced mainly from the ovaries during the premenopausal period, plays an important role in estrogen receptor positive (ER+) breast cancer. Levels of circulating estrogen were not affected by blueberry extract consumption; however, consumption of the Pomace supplemented diet significantly ($P \leq 0.05$) reduced circulating estrogen in the blood serum in comparison with animals from other experimental groups.

All bacterial populations (*Lactobacillus* spp., *Bifidobacterium* spp. and *E. coli*) were affected by DMBA. All three bacterial populations were lower in animals that received DMBA than in non-treated animals. Prebiotic and antimicrobial properties of blueberries were also investigated but no significant change was found among all DMBA-treated groups.

β-glucuronidase enzyme produced from intestinal microflora plays an important role in enterohepatic reabsorption of steroid sex hormones such as estrogen or xenobiotic compounds. Even though the bacterial population was unchanged, a reduction in β-glucuronidase activity was observed in animals receiving blueberry pomace supplemented diet. The reduction would result in enhanced excretion of xenobiotic compounds and steroid sex hormones.

This study has demonstrated that blueberry consumption decreased oxidative stress in a DMBA-induced breast cancer model in rats. However blueberry consumption, just prior to tumour initiation, did not affect mammary gland maturation. Blueberry pomace exhibited a more pronounced effect on the reduction of circulating estrogen and β-glucuronidase enzyme activity which might relate to the dietary fiber content in the pomace. Collectively, the extracts of two cultivars of New Zealand grown rabbiteye blueberries 'Centurion' and 'Maru' blueberries and dried blueberry pomace from blueberry juice industry showed positive effects on DMBA-induced mammary tumorigenesis in SD rat model. The results may be used as preliminary data for a human clinical study.

Introduction:

The impacts of blueberry extracts on breast cancer incidence and tumour indices (tumour multiplicity, tumour latency and tumour size) were demonstrated in the previous chapter (chapter 5). In this chapter, we are presenting possible pathways that might be altered by blueberry consumption and may be responsible for the chemopreventive effect found.

The mammary gland is a unique organ that is not fully functional at birth but plays an important role during the prepubertal period. The main structures of the rat mammary gland are the terminal end buds (TEBs). Steroid sex hormones such as estrogen and progesterone trigger mammary structure alteration when the animals reach puberty. During puberty, TEBs transform into alveoli (AV) and lobules (LB) which remain throughout the animal's life. Unless they are going through parity (full term pregnancy with gestation), the mammary glands are not fully developed (Hennighausen & Robinson, 2001). In chemically-induced mammary carcinogenesis in SD rats, the window of susceptibility is when the numbers of TEBs are equal to number of AV+LB at the onset of puberty. Terminal end buds are highly proliferative mammary gland structures. They are targeted by carcinogens, which stimulate cell proliferation (Russo & Russo, 1994).

Mammary gland maturation occurs during human pregnancy, which results in a higher number of lobule type 3 and fewer TEBs compared to the breast of nulliparous women. The mammary gland structures of parous women are also less susceptible to carcinogens than the mammary gland structures of nulliparous women (Russo & Russo, 2004). Therefore, mammary gland maturation by induction of mammary gland structure differentiation might serve as a chemopreventive strategy in breast cancer prevention. Blueberry polyphenol (resveratrol) consumption has been previously shown to successfully induce maturation of rat mammary glands which might indicate less susceptibility to carcinogens (Whitsett et al., 2006).

Oxidative stress is considered as an imbalance of oxidants generated via oxygen metabolism and antioxidants in the body. Cancer initiation, promotion and progression stages relate closely to oxidative stress (Reuter et al., 2010). Evidence from both animal and human trials shows increases in lipid peroxidation level and decreases in antioxidant enzyme (catalase, CAT) in breast cancer incidence compared to healthy controls (Tas et al., 2005; Sener et al., 2007; Batcioglu et al., 2012). Blueberries and their polyphenols reduced oxidative stress in various pathogenic conditions such as Alzheimer's disease (Tong-un et al., 2010), liver fibrosis (Wang et al., 2013), hypoxia-induced oxidative stress in male reproductive function (Zepeda et al., 2012) and osteoporosis (Hubert et al., 2014).

Estrogen is a steroid sex hormone that has an important role in regulation of normal mammary gland development and mammary tumorigenesis (Russo & Russo, 2004; Yager & Davidson, 2006). The type of breast cancer caused by DMBA is estrogen receptor positive (ER+) breast cancer; therefore, cancer promotion and progression are highly impacted by

exogenous and endogenous estrogen (Hakkak et al., 2007). Phytoestrogens from various plant sources exhibited a preventive effect in breast cancer (Adlercreutz, 2002). Blueberry phytochemicals, such as resveratrol, possessed both estrogenic and antiestrogenic properties under different experimental conditions (Bhat et al., 2001). Not only phytochemicals in blueberries can influence circulating estrogen level; dietary fiber was found to enhance the faecal estrogen level, which inversely correlated with the estrogen level in plasma (Rose, 1990). The intestine was found to be one of the major metabolic sources of estrogen which may result from intestinal microflora metabolism (Minelli et al., 1990).

Beside estrogen metabolism, gut microflora also effected host health via various functions such as protecting the host from pathogens, vitamin production, immune system development and fermentation of non-digestible dietary residue (Guarner & Malagelada, 2003). Even though bacterial composition in human intestine varies in each individual, the composition is generally stable in a person unless modulated by environmental factors or pathogenic conditions (Vendrame et al., 2011). Fermented milk products from *Lactobacillus* spp. and *Bifidobacterium* spp. showed antiproliferative effects on breast cancer cell lines (Biffi et al., 1997). The prebiotic effects of blueberries have been studied both *in vitro* and *in vivo* (Molan et al., 2010). An anthocyanin-rich fraction of blueberries showed antimicrobial effects against intestinal pathogenic bacteria such as *Salmonella enterica* sv. *typhimurium*, *Salmonella enterica* sv. *infantis* and *Staphylococcus aureus* (Puupponen-Pimiä et al., 2005).

A part of the detoxification system in the human body includes conjugation of xenobiotics or steroid sex hormones by sulfate or glucuronic acid. The conjugation enhances hydrophilicity of the compounds and facilitates excretion via urine or bile (Raftogianis et al., 2000). However, β-glucuronidase enzyme, present in lysosome and colon, hydrolyses glucuronyl conjugates and makes the compounds available for reabsorption. Blueberry consumption in an animal trial led to a reduction of fecal β-glucuronidase activity (Molan et al., 2010). Therefore decreasing β-glucuronidase enzyme activity may facilitate estrogen excretion which could make blueberry supplementation a promising strategy in breast cancer prevention.

In this study, the various effects of blueberry extracts and diet supplemented with 5% blueberry pomace including mammary gland maturation, oxidative stress, prebiotic and antimicrobial properties and fecal β-glucuronidase activity were studied. We hypothesised

that all blueberry treatments may improve oxidative stress status, decrease circulating estrogen concentration, enhance beneficial bacteria population and reduce *E. coli* population and decrease fecal β-glucuronidase enzyme activity.

Materials and methods:

Animals and experimental design

All samples used for experiments in chapter 6 derived from the animal study described in chapter 5. The data can therefore be interrogated using all the insights of chapter 5 about individual animals. Animal feed, experimental design and sample collection are described in chapter 5.

Whole mount mammary gland

A whole mount mammary gland preparation and evaluation was carried out (de Assis et al., 2010). The left side of 4^{th} pair mammary gland was dissected and spread on a glass slide (75 x 50 mm). The mammary tissue was left to adhere to the glass slide for 30 minutes then the slide was put in Carnoy's fixative (ethanol: chloroform: glacial acetic acid (6:3:1)). The tissue was fixed for at least 2 days then washed with 70% ethanol for 1 hour followed by distilled water for 30 minutes. The slide was then stained in Carmine alum stain (1 g carmine and 2.5 g aluminium potassium sulfate in 1 L MilliQ water) for 2 days or until stained through. After staining, the slide was dehydrated in series of ethanol washes (70%, 95% and absolute ethanol) each for 1 hour. Then the mammary gland was defatted in xylene for 7 days or until the mammary gland became clear.

Histopathological examination of rat mammary tumours

After the mammary tumours were fixed in 10% formalin, the tumours were embedded in paraffin blocks, sectioned and stained with haematoxylin and eosin (H&E staining). Tumour grading was done by an experienced veterinary pathologist, Assoc Prof John Munday (BVSc, PhD, DipACVP), from Institute of Veterinary, Animals and Biomedical Sciences, Massey University. Three morphologic features according to Scarff-Bloom-Richardson scheme were used for tumour grading in this study: tubule formation, nuclear pleomorphism and number of mitoses. The extent of solid area in tumour was used to categorise tubule formation. Tubule formation grade 1 had less than 25% of solid area, grade 2 had more than 25% but less than 75% solid area, and grade 3 was applied to tumours with more than 75% solid area. The number of mitoses per 10 HPFs (high power

field) was used to categorise the tumours. Tumour group 1 had fewer than 10 mitoses/10 HPF, group 2 showed 10 to 20 mitoses/10 HPF and group 3 had more than 20 mitoses/10 HPF. Size and shape of nuclei, chromatin pattern and the presence of nucleoli were used to grade (Bloom & Richardson, 1957).

Caecal content and mucosal lining DNA extraction and quantification

Caecal contents and caecal mucosa lining were collected and weighed. The DNA from aliquots of caecal contents or caecal mucosal lining (150 mg) was extracted using ISOLATE fecal DNA kit (Bioline, NSW, Australia) following manufacturer's instructions. Final DNA concentration was determined using a spectrophotometer (Nanophotometer 2000 IMPLEN, Munchen, Germany). The DNA samples were stored at -20°C until analysed.

Bacterial culture and DNA extraction for quantitative PCR

Stock cultures of *L. rhamnosus*, *B. breve* and *E. coli* kept at -80°C were revived in tryptic soy broth (TSB) for *E. coli* and Man-Rogosa-Sharpe (MRS) broth for *L. rhamnosus* and *B. breve*. The cultures were incubated at 37°C for 24 hours. The cultures (100 µL) were ten-fold serially diluted to achieve the final concentration at 10^{-10} and the bacterial cultures were kept at 4°C for further use. Three concentrations of diluted bacteria cultures (10^{-8}, 10^{-9}, 10^{-10}) were plated on MacConkey agar plates for *E. coli* and MRS agar for *L. rhamnosus* and *B. breve*. Bacterial culture plating was done in triplicate. The plates were incubated at 37°C overnight. The bacterial colonies were counted and calculated for number of bacteria in the culture media.

For bacterial DNA extraction, the bacterial cultures kept at 4°C were serially diluted (10-fold dilution) in their culture media with the final volume of 5 mL in 15 mL centrifuge tube. The tubes were centrifuged at 10,000 *g* for 20 minutes to collect bacterial pellet. The pellet was washed in phosphate buffered saline (PBS), mixed thoroughly and centrifuged at 10,000 *g* for 10 minutes. The PBS was discarded. The washed bacterial pellet was extracted using QIAamp DNA mini kit (QIAGEN, USA) following manufacturer's instructions. Final DNA concentration was determined using a spectrophotometer (Nanophotometer 2000 IMPLEN, Munchen, Germany) and stored at -20°C until analysed.

Quantitative PCR (qPCR)

Bacterial levels in fecal samples were evaluated by a quantitative real-time polymerase chain reaction (qPCR) using the LightCycler™ 480 (Roche Diagnostics Ltd.,

Indianapolis, USA). SYBR Green I Mastermix (Roche Ltd.), which contains *Taq* polymerase, reaction buffer, a deoxynucleotide triphosphate mixture, SYBR Green I dye and hot start antibody and three primers sets (Geneworks, Adelaide, Australia) of *Lactobacillus*, *Bifidobacterium* spp. and *E. coli* as follows: Lab-0159-F (5'-GGA AAC AGR TGC TAA TAC CG-3'), UnivL-0515-R (5'-ATC GTA TTA CCG CGG CTG CTG GCA-3'), Bif1-F (5'-TCG CGT CYG GTG TGA AAG-3'), Bif2-R (5'-CCA CAT CCA GCR TCC AC-3'), IEC-UP (5'-CAA TTT TCG TGT CCC CTT-3') and IEC-DNr (5'-GTT AAT GAT AGT GTG TCG-3') respectively, were used in this study (Collier et al., 2003; Khan et al., 2007; Rinttila et al., 2004).

Total of 20 µL qPCR reaction contained 10 µL SYBR Green I Mastermix, 1 µL of each primer and a total volume of 8 µL of template DNA diluted with nuclease-free water. The PCR cycle consisted of three steps which were denaturation, amplification and melting-curve determination. The cycle started with an initial denaturation and anti-*Taq* DNA polymerase antibody-inactivation step at 95°C for 5 min followed by an amplification step (45 cycles of 15 s at 95°C, 20 s at 63°Cand 10 s at 72°C) and a melting-curve determination step where temperature increased from 65°C to 95°C with 30 s hold. SYBR green fluorescence signal was measured at the end of each amplification step. The first detection of a significant increase of the signal generated by the PCR reaction was defined as a threshold cycle (C_T).

The range of 10-fold serial dilution of each bacterial sample was used to establish a standard curve. A linear relation of the bacterial concentration against C_T value was used to quantify the number of specific bacteria in unknown samples using the LightCycler™ software based on the C_T value detected in each DNA sample.

β-glucuronidase enzyme activity

Sample preparation

Rat caecal contents (100mg) were suspended in cold 0.1 M potassium phosphate buffer (1 mL) then vortexed for 3 minutes. The mixture was centrifuged at 13,400 *g* for 2 minutes to remove debris. The supernatant was transferred into new microcentrifuge tubes then allowed to freeze (-70°C)/thaw (37°C) three times. After freeze/thaw cycles the supernatant was filtered through 0.22 µm syringe filter. The filtered supernatant was used for determination of β-D-glucuronidase enzyme activity.

Determination of β-D-glucuronidase enzyme activity

Colorimetric method was used for determination of β-D-glucuronidase enzyme activity in caecal samples as described by De Preter et al. (2008) with some modifications. The supernatant (10 µL) was mixed with 40 µL reaction mixture (0.02 M potassium phosphate buffer, 0.1 mM EDTA, 0.05 phenolphthalein β-glucuronide (substrate)) in a 96-well plate. The plate was incubated at 37°C for 60 min, and then 0.2 M glycine buffer (250 µL) was added to stop the reaction. The amount of phenolphthalein was determined by measuring the UV absorption at 550 nm using microplate reader (ELX 808 Ultra microplate reader, BIO-TEK instrument) with KC4 software. The supernatant mixed with the reaction mixture without substrate was used as a control. Phenolphthalein (concentration 0-1 mg/mL) was used to establish a calibration curve.

Blood serum antioxidant

Ferric reducing antioxidant power assay is a widely used method for determination of antioxidant activity of tested samples (Benzie & Strain, 1996). This assay is based on the ability of the antioxidant compounds to reduce the ferric-tripyridyltriazine complex to its ferrous form. In this study, the procedure described by Benzie and Strain (1996) with some modifications (Molan et al., 2009) was followed. Briefly, the FRAP reagent was prepared by mixing 300 mM sodium acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM ferric chloride (10:1:1). Aliquots of 8.5 µL of animals' blood serum were mixed with FRAP reagent (275 µL) in a 96-well plate. The plate was incubated at 37°C in the dark for 30 minutes and then the absorbance of the mixture was measured at 595 nm using microplate reader (ELX 808 Ultra microplate reader, BIO-TEK instrument) with KC4 software. The FRAP values were expressed as mM $FeSO_4$.

Blood serum catalase activity

Catalase activity in animal blood serum was measured using Cayman catalase assay kit (item no. 707002). The assay was performed following the manufacturer's instruction. Briefly, 20 µL of serum sample was added in to the well then 100 µL diluted as+say buffer and 30 µL methanol were added. Twenty µL of 35 mM hydrogen peroxide was added to the well to start the reaction. The plate was then covered and incubated on a shaker for 20 minutes at room temperature. After the incubation, potassium hydroxide (10 M) 30 µL was added to terminate the reaction. Catalase Purpald (Chromogen: 30 µL) was added to the well, then the plate was incubated on a shaker at room temperature for 10 minutes for color development. Finally, catalase potassium periodate (10 µL) was added and the plate

was incubated for five minutes. After incubation, absorbance was read at 540 nm using a microplate reader (SPECTROStar Nano, BMG Labtech) with Spectrostar nano software.

Lipid peroxidation in blood serum

Lipid peroxidation in animal blood serum was measured by TBARS (TCA method) using assay kit from Cayman Chemical (Item No. 700870). Malondialdehyde (MDA) is produced when lipid peroxidation occurs. This assay measured the formation of MDA and thiobarbituric acid (TBA) using a colorimetric method.

The experiment was conducted according to the method described by the the manufacturer's instruction. Briefly, samples or standards (100 µL) were placed in screw cap microcentrifuge tubes then 100 µL of the TCA assay reagent and 800 µL of the color reagent were added to each vial. The vials were heated in vigorously boiling water. After 1 hour, the reaction was stopped by placing the vials in ice bath for 10 minutes. Then, the vials were centrifuged at 1600 *g* at 4°C. The supernatant (200 µL) from each vial was carefully pipetted from the vials and placed in 96-wells plate in triplicate. The absorption was read at 540 nm within 30 min after completing the reaction using a microplate reader (SPECTROStar Nano, BMG Labtech) with Spectrostar nano software. Malondialdehyde from 0.0625 to 5 µM was used to establish a standard curve; MQ water was used as a blank.

Level of estradiol in blood serum

Circulating estrogen in experimental rats was measured with Estradiol EIA Kit from Cayman Chemical (Item No. 582251). This enzyme immunoassay (EIA) is a competitive assay between ability of estradiol and estradiol-acetylcholineesterase (tracer) to bind to estradiol antiserum.

The study was performed according to the instructions from the company. In this assay each plate contained 2 blank wells, 2 non-specific binding (NSB) wells, 2 maximum binding (B0) wells and 8 concentrations of estradiol standard. Briefly, samples or standards (50 µL) were added to the wells and EIA buffer was added into B0 (50 µL) and NSB wells (100 µL). Then the Tracer (50 µL) was added to all wells except the blank wells. Lastly, estradiol antiserum was added to all wells except NSB and blank wells. The plate was covered with a plastic film and incubated for 1 hour on orbital shaker. After 1 hour, contents from all wells were emptied and rinsed with wash buffer 5 times. Then Ellman's reagent was added to each well for color development. The plate was covered again with

plastic film and placed on a covered orbital shaker to allow color development in the dark. Each blood serum sample was analysed in triplicate. Estradiol standard curve was established using estradiol from 6.6 to 4,000 pg/mL.

The plate was suitable for reading absorption when the absorbance of B0 well was between 0.3-1.0 after blank subtraction. If the absorbance from B0 well was higher than 1.5, the color development step was repeated. The wavelength reading at 410 nm was performed using a microplate reader (SPECTROStar Nano, BMG Labtech) with Spectrostar nano software.

The concentration of estradiol in animal blood serum was calculated using computer software available from the Cayman chemical website (www.caymanchem.com/analysis/eia).

Statistical analysis

All normally distributed data (which included numbers of each mammary gland structure from mammary gland whole mount, blood serum antioxidant, levels of MDA, catalase enzyme activity, level of 17β-estradiol in animal blood serum and β-glucuronidase enzyme activity in animal caecal content were analysed for mean comparisons using one-way analysis of variance (one-way ANOVA) to assess significant treatment effects, and then Tukey's test was used as a posthoc comparison. The differences were considered statistically significant at $P \leq 0.05$. Number of affected animals and number of tumours in DMBA-treated groups were analysed for statistical difference using Chi-square analysis. Determination of the correlation between two variables was performed using Pearson's correlation coefficient, R. Data of gut bacteria population were not normally distributed therefore nonparametric Kruskal-Wallis test was used for pairwise comparison. The differences were considered statistically significant when $P \leq 0.05$. All statistical analysis was carried out using Minitab software (version 15; Minitab Inc., Pennsylvania, USA)

Results and discussion:

Mammary gland whole mount

The fourth left side mammary glands of all animals were used in a mammary gland whole mount preparation. A structure called terminal end bud (TEB), found in immature mammary gland, is highly proliferative and highly susceptible for tumour initiation (Whitsett et al., 2006). Total number of mammary gland structures including TEBs, alveoli

buds (ABs) and lobules (LB) were counted. The structures located in zone c (the area of the gland distal to the nipple in 21 days old female SD rats, figure 6-1 (A)) were counted, (Moral et al., 2004). Figure 6-1 (B) shows a large highly developed mammary gland from a 140-day old rat from our study. Therefore, five 1 mm^2 fields were randomly selected and all mammary gland structures were counted in these fields. Average number of each mammary structure (TEBs, ABs, and LBs) in 1 mm^2 was calculated and shown in figure 6-2.

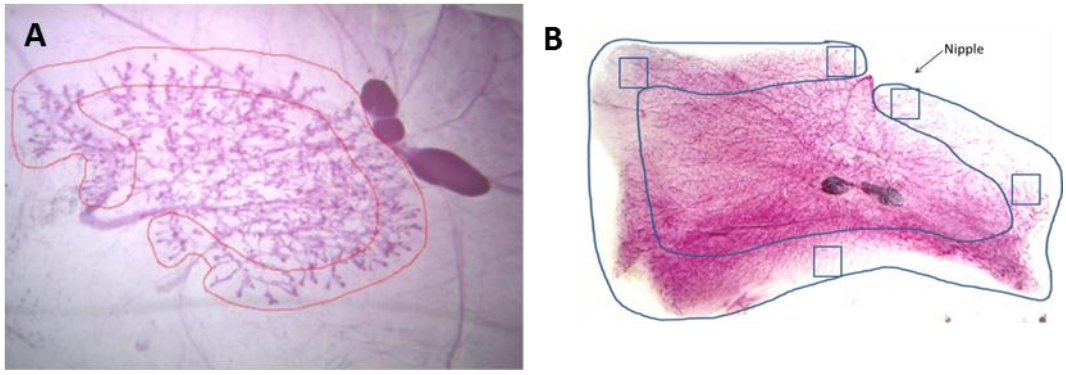


Figure 6-1 Representative whole mount mammary gland of rat showing "zone C" area. Mammary gland from (A) 21-days old rat (Modified from Moral et al., 2004) (B) 140-days old rats mammary gland structures (TEBs, ABs, LBs) in zone C were counted.

There was no significant difference found in the numbers of TEBs between each experimental group. However, the number of ABs was modified by DMBA treatment but not by blueberry treatments. The negative control group showed the lowest number of ABs while the numbers of ABs from all DMBA treated animals were not significantly different. LB structure appeared higher in the positive control group however the difference was not significant. The average number of TEBs present in our experiment was in agreement with the study of Russo and Russo (1994). They identified the window of susceptibility for mammary tumorigenesis in mature virgin female Sprague-Dawley rats by measuring the numbers of TEBs, ABs and LBs at different ages. Terminal end buds are a dominant structure of immature mammary gland. During prepuberty, TEBs differentiate into ABs and LBs. When the animals reach puberty, the number of TEBs is similar to the summation of ABs and LBs type 1 structure. This period is the most susceptible period for chemically induced mammary tumorigenesis which is around 40-50 days of rat age. They also demonstrated that as the animals age the number of their TEBs decrease while the sum of ABs and LBs increased. Mammary gland of 140 days old animal consists of approximately 2

TEBs and 20 Abs +LBs per mm^2 (Russo & Russo, 1994). The average number of ABs + LBs in our study was approximately 8 which was lower than expected. The variation might be caused by lack of experience on whole mount mammary gland technique or the random fields selected for analysis.

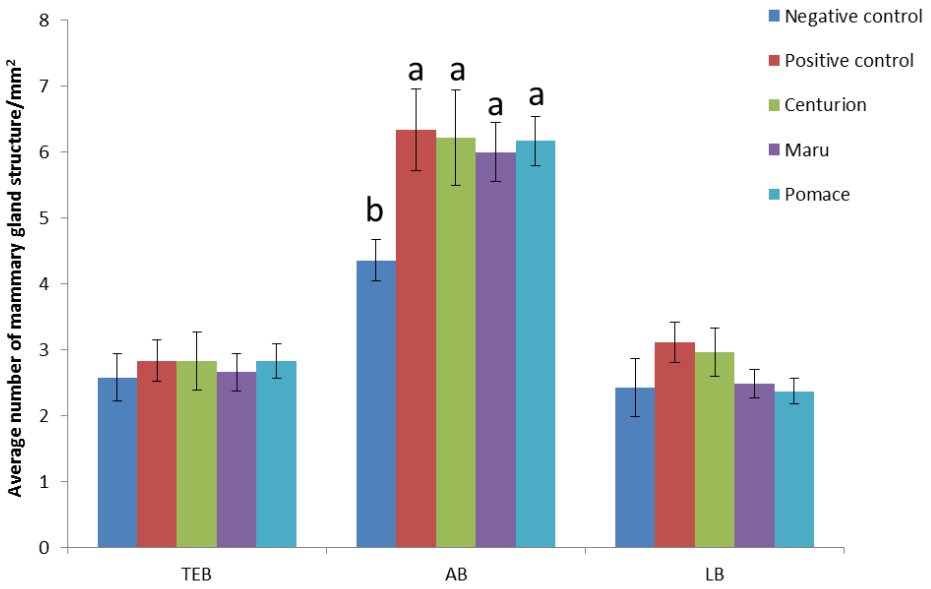


Figure 6-2 Average number of mammary gland structures (Terminal end bud (TEB), (Alveolar bud) AB and (Louble) LB) per mm^2 counted under light microscope for 140 day old rats. The data shown in the graph was mean ± SEM of each mammary gland structure. The differences were analysed using Tukey's HSD. Bars not sharing the same letter are statistically different at $P \leq 0.05$.

Terminal end buds are the most susceptible structure when exposed to carcinogens such as estrogen or DMBA (de Assis et al., 2010). Therefore, mammary glands that have differentiated to mature glands would be less susceptible for mammary tumour initiation. A protection from mammary gland maturation is similar to the protective effect resulting from full-term pregnancy. Several studies have found that blueberry or blueberry polyphenol administration prior to carcinogenesis may affect mammary gland structures resulting in less susceptibility to tumour initiation. Resveratrol given to female SD rats from birth resulted in a higher number of lobule type I plus lobule type II than in animals that received control diet. The result implies that mammary glands of animals receiving resveratrol supplementation from birth were less susceptible to DMBA-induced carcinogenesis, which may have contributed to the reduction in tumours found in these animals (Whitsett et al., 2006). A study of mammary tumorigenesis using transgenic mice

found that Wnt1-Tg offspring of mice receiving blueberry treatment had fewer TEBs than the Wnt1-Tg offspring of animals without blueberry treatment, and this lower number of TEBs indicated lower risk of developing breast cancer (Rahal et al., 2013). According to the previous studies, timing of blueberry or blueberry phytochemical exposure was important in modification of mammary gland remodeling. Blueberry treatment in utero or from birth resulted in less susceptible mammary glands; while if there was an anti-tumour effect resulting from blueberry treatment after mammary gland remodeling, it must imply protective effects via a different pathway. Our data confirm that blueberry addition to 49 days old rats did not remodel mammary tissue.

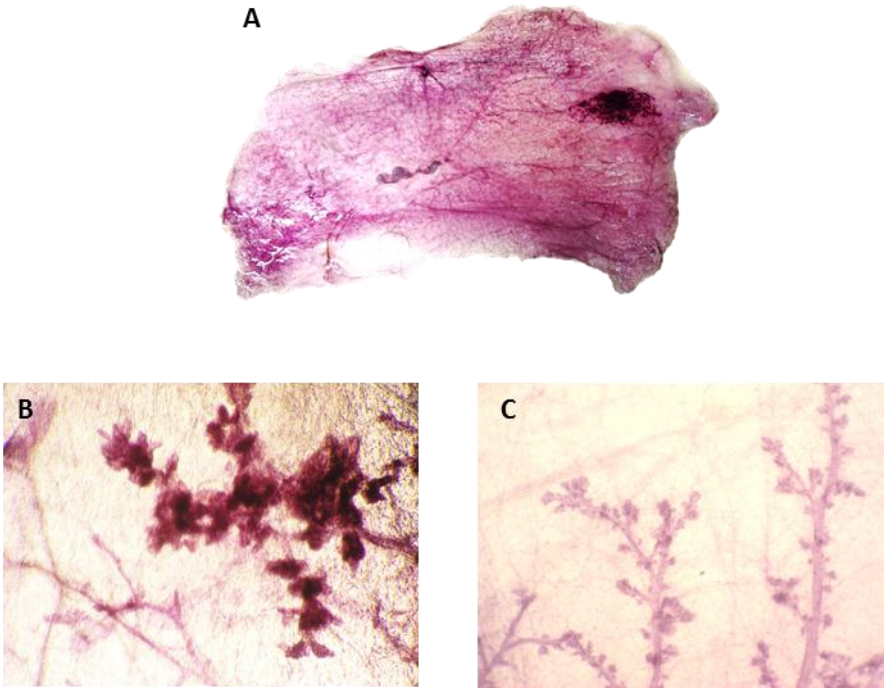


Figure 6-3 Representative images of mammary gland abnormality A) Whole mount B) Abnormal mammary lesion, microscopic structure C) Normal mammary gland, microscopic structure (100X magnification).

A representative mammary gland from H-12 rat (DMBA treated and received blueberry pomace supplemented diet) with a mammary gross lesion is shown in figure 6-3. The lesion is seen in figure 6-3A as an abnormal growth of mammary gland on the right side of the mammary gland compared to the normal mammary gland presented in figure 6-1B. When the lesion was observed under a light microscope (figure 6-3B), the mammary gland structures did not differentiate into alveolar buds or ductules as presented in figure 6-3C (from N-8 rat: no DMBA treatment).

The mammary glands of all animals from the experiment were examined. Animals with mammary gland abnormalities were found (table 6-1). It was reported in the previous chapter that 15 animals in a positive group were found bearing tumours. After whole mount mammary glands were observed, another 3 animals were found with mammary gland abnormalities. Therefore, a total of 18 animals in the positive group were affected which accounts for 90% of total animals in the group. Total affected rats with blueberry treatment were 10, 9 and 13 in 'Centurion', 'Maru' and Pomace, respectively (table 6-1).

Table 6-1 Number of tumour incidence and mammary gland abnormality

Group	Animals with tumour		Animal without tumour but with mammary gland abnormalities		Total affected animals	
	Observed	Expected	Observed	Expected	Observed	Expected
Negative control	0	0	0	0	0	0
Positive control (n = 20)	15	8.75	3	4	18	12.5
'Centurion' (n = 20)	7	8.75	3	4	10	12.5
'Maru' (n = 19)	4	8.75	5	4	9	12.5
Pomace (n = 20)	9	8.75	5	4	13	12.5
χ^2	7.4		1		3.92	
P value	0.06		0.801		0.270	

NB: Chi-square analysis was analysed using data from DMBA-treated groups only.

In the previous chapter (chapter 5), we demonstrated that the number of animals with tumours were not significantly different between animals that received blueberry treatments and animals without blueberry treatment. Observations on mammary gland abnormalities and Chi-square statistical analysis also confirmed that even though blueberry treatment showed a reduction in the number of animals with tumours, the effect of the treatments was not statistically significant.

The DMBA induced mammary tumour model exhibited higher number of tumours in thoracic area (67%) (Dias et al., 2000). Therefore, using only one mammary gland from an abdominal area might not be suitable for representing tumour incidence. For this reason, all tumours present in every animal were collected and quantified. However, it is common to use the 4th pair left or right side of an experimental subject for whole mount mammary gland analysis (de Assis et al., 2010; Rowlands et al., 2002; Whitsett et al., 2006). Mammary gland whole mounts cannot be done on mammary glands that have tumours present. In our study, in the few animals with tumours on the fourth pair left side, the fourth pair right side was substituted.

Tumour mass histology

At the end of the experiment, all tumour samples were processed and submitted for histopathology examination. According to the histopathological data, tumours were categorised into 3 different groups which are adenoma (benign), low-grade adenocarcinoma, and adenocarcinoma (malignant). Microscopic images of mammary gland and mammary tumours are presented in figure 6-4. It is important to mention that each animal has different levels of susceptibility. While all animals received the same DMBA treatment, some developed multiple tumours while others never showed signs of neoplasia.

A total of 35 tumours were found in the positive control group. More than 80% of tumours found in positive group were adenocarcinomas with less than 20% categorised as low-grade adenocarcinoma. Blueberry treatment reduced tumour incidence and multiplicity as mentioned previously in chapter 5. Percentage of adenocarcinoma in rats that received 'Centurion' and 'Maru' extract was approximately 60% of total tumours found in each group. Interestingly, 20% of tumours found in rats that received 'Centurion' extract were adenomas (benign tumour). Animals that received Pomace supplemented diet had the lowest tumour multiplicity and the lowest tumour volume. Percentage of histopathological results of tumours found in this group was 30%, 40% and 30% in adenoma, low-grade adenocarcinoma and adenocarcinoma, respectively (figure 6-5).

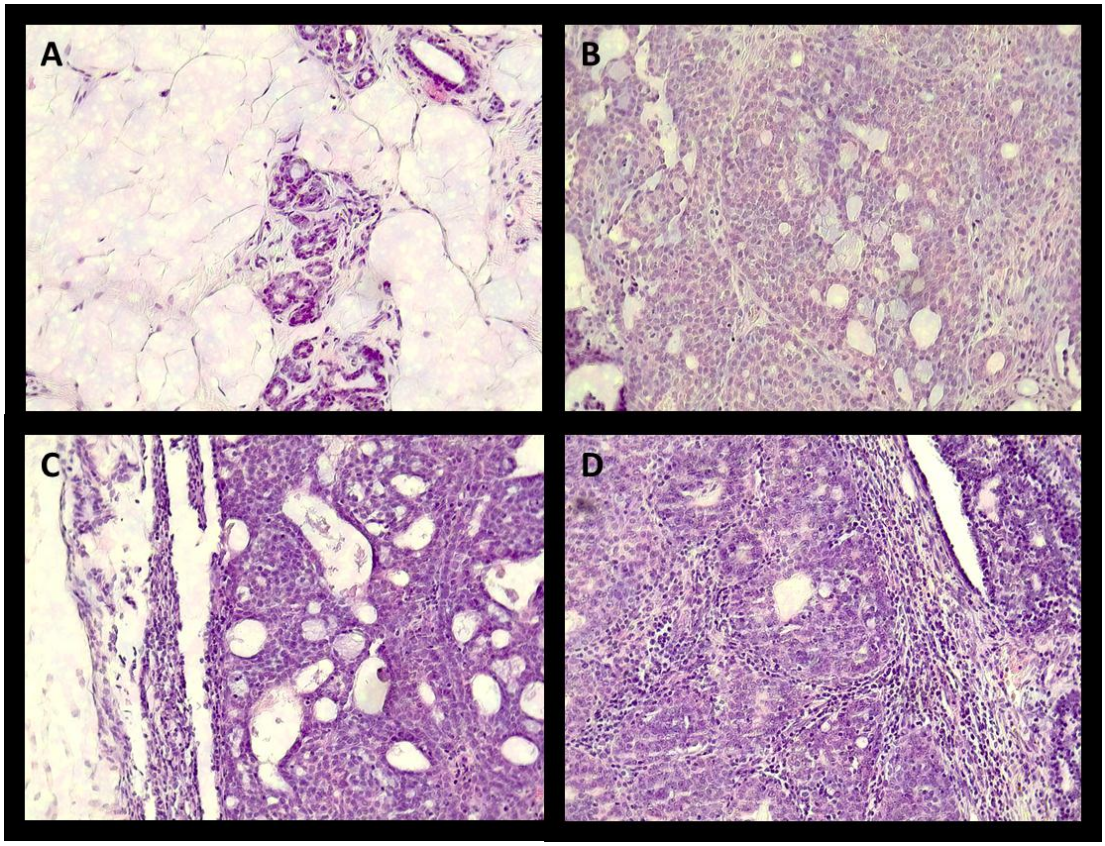


Figure 6-4 Representative microscopic image (200X magnification) of A) Normal mammary gland structure B) Adenoma C) Low grade adenocarcinoma D) Malignant adenocarcinoma

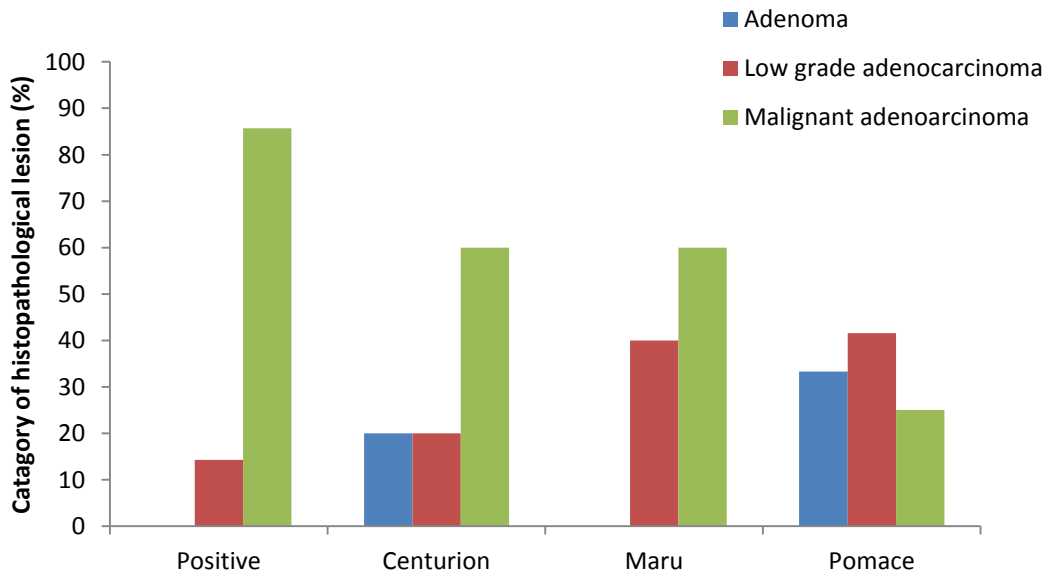


Figure 6-5 Effect of blueberry extracts ('Centurion' and 'Maru') and diet supplemented with 5% pomace on distribution of the malignancy of tumour based on the histopathological report.

Table 6-2 Histopathological categories of tumours found in DMBA treated groups. Statistical significant different was evaluated using Chi-square analysis. The differences were significantly difference when $P \leq 0.05$.

Group	Adenoma		Low grade adenocarcinoma		Adenocarcinoma	
	Observed	Expected (Chi-Square contribution)	Observed	Expected (Chi-Square contribution)	Observed	Expected (Chi-Square contribution)
Positive control (n = 20)	0	3.40 (3.043)	5	8.26 (1.289)	30	23.33 (1.905)
'Centurion' (n = 20)	3	1.46 (1.630)	3	3.54 (0.083)	9	10.00 (0.100)
'Maru' (n = 19)	0	0.97 (0.972)	4	2.36 (1.138)	6	6.67 (0.067)
Pomace (n = 20)	4	1.17 (6.881)	5	2.83 (1.657)	3	8.00 (3.125)

$\chi^2 = 22.249$, DF = 6, $P < 0.01$
Warning: 1 cell with expected counts less than 1 (Chi-square approximation probably invalid). 7 cells with expected counts less than 5.

In a study of DMBA-induced mammary tumours in Wistar rats, the percentage of benign and malignant tumours was 38.5 and 61.5, respectively; 85% of benign tumours in this study were diagnosed microscopically (Dias et al., 1999). Animals in Pomace group had the highest percentage of benign tumours which may result from the delay of tumour onset. The major effect of blueberry treatment in our study is delaying the progression of tumour from abnormalities caused by DMBA to malignant tumours (Yokota, 2000). Even though there was no significant difference between number of adenoma and low grade adenocarcinoma in all DMBA-treated groups with or without blueberry treatments, the number of tumours that progressed to carcinoma was significantly different (table 6-2). However, the statistical analysis suggested that the low expected count might affect validity of Chi-square approximation. Yate's correction could be used for correction of Chi-square where the expected values are under 5. However, some statisticians recommended that Yate's correction is too strict (Hitchcock, 2009). Therefore, in this study we did not use the Yate's correction in addition to the Chi-square analysis. Thirty tumours out of 35 tumours from the positive control group were adenocarcinomas, which approximates 85% of all tumours. Percentages of adenocarcinoma in animals that received blueberry extracts were 60% of all tumours and 25% in animals that received the pomace supplemented diet. A similar finding was reported for a 10% grape and blueberry juice mixture delayed tumour

latency, and malignant tumours found in the juice group were lower by 4% in comparison with animals without treatment (Wang et al., 2013).

Effect of blueberry extracts and blueberry pomace supplemented diet on oxidative stress

Modulation of oxidative stress by fruit and vegetable consumption is believed to play an important role in inhibition of initiation of tumorigenesis (Stoner et al., 2008). All three blueberry treatments exhibited a positive effect on antioxidant activity measured in animal blood serum by FRAP assay. Average level of antioxidants in serum of animals from the positive group was the lowest (but not significantly different from the negative control group) among 5 experimental groups (figure 6-6). Pomace-fed rats showed the highest FRAP value in serum among three blueberry treated groups. Therefore, blueberry intake could elevate antioxidant activity in animal blood serum.

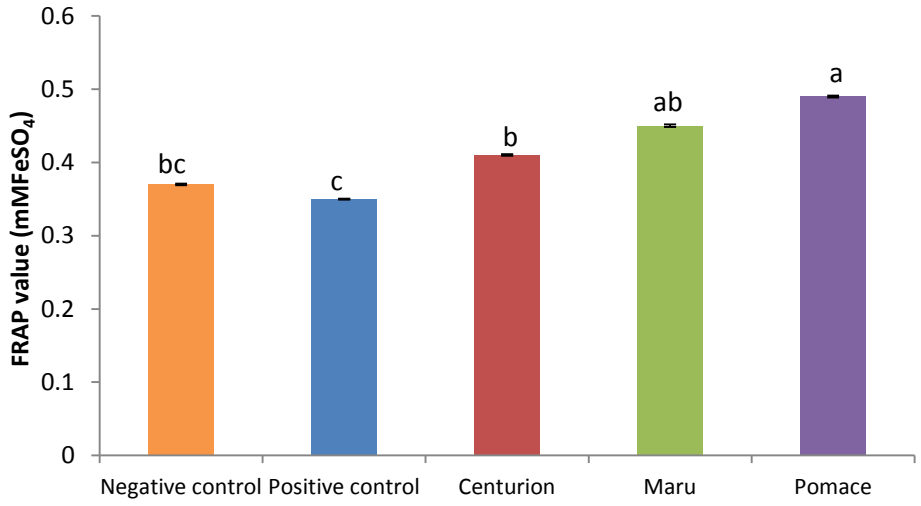


Figure 6-6 Effect of blueberry extracts ('Centurion' and 'Maru') and diet supplemented with 5% pomace on blood serum antioxidant. The data are expressed as mean±SEM. The differences were analysed using posthoc Tukey's test. Bars not sharing the same letter are statistically different at $P \leq 0.05$.

7,12-Dimethylbenz(a)anthracene-induced mammary carcinogenesis in rats led to a significant decrease in both enzymic and non-enzymic antioxidant in their blood serum in comparison with control animals (Anbuselvam et al., 2007). A similar trend was also found in human subjects with breast cancer. Total antioxidant capacity (TAC) was lower in subjects with breast cancer compared to the healthy control (Sener et al., 2007). However, a conflicting finding was reported that breast cancer patients exhibited higher blood serum antioxidant than their age matched control as measure by FRAP assay. The authors

indicated that increasing FRAP value may be linked to systemic inflammatory reaction found in breast cancer patients (Stolarek et al., 2010).

Blueberry consumption elevated blood serum antioxidants in both animals and human subjects. A study on chronically stressed rats found that rats that received 5% blueberry water under normal conditions led to rats with a higher antioxidant activity in their blood serum compared to the control group (Lee et al., 2012). A study of rat satiety modulation by blueberry extracts in female SD-rats showed that antioxidant status in animal blood serum increased after 6 days of blueberry extract gavaging (Molan et al., 2008). Freeze-dried blueberry powder consumed with high-fat meal showed anthocyanin absorption and increase of blood serum antioxidant measured by ORAC assay. The researchers also found a positive correlation between total anthocyanins and antioxidant capacity in blood serum (Mazza et al., 2002).

Oxygen metabolism yields reactive oxygen species (ROS) or free radicals. An imbalance of free radicals within cells leads to oxidative stress and can cause damage to proteins, lipids and DNA (Reuter et al., 2010). Malondialdehyde (MDA) is produced when cells are injured. It has been used as a marker for lipid peroxidation. In this experiment, MDA concentration determined by TBARS was the highest in animals that received DMBA without any blueberry treatments. The level of MDA was significantly lower in negative control group ($P \leq 0.05$). Levels of MDA in all three blueberry treated groups were also significantly lower than the positive group (figure 6-7).

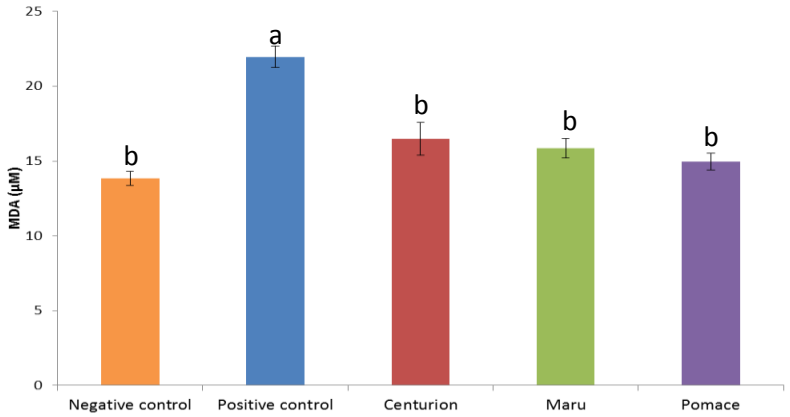


Figure 6-7 Effect of blueberry extracts ('Centurion' and 'Maru') and diet supplemented with 5% pomace on malondialdehyde concentration in animal blood serum. The data are expressed as mean±SEM. The differences were analysed using posthoc Tukey's test. Bars not sharing the same letter are statistically different at $P \leq 0.05$.

Another study of DMBA-induced breast cancer in a rat model found that the level of lipid peroxidation measured by TBARS assay was significantly higher in blood serum of animals receiving DMBA treatment. Feeding with quercetin (a flavonol compound found in fruits and vegetables including blueberries) as well as DMBA significantly reduced the products of lipid peroxidation compared to controls treated with DMBA (Reddy et al., 2012). Another group of researchers showed a similar result where lipid peroxidation level was increased by DMBA. They demonstrated an up-regulation of MDA level in animals treated with DMBA in comparison with animals receiving vehicle control (Vennila et al., 2010).

Cell membrane consists of phospholipid and protein molecules. These biomolecules are targets of reactive oxygen species. Several studies showed that DMBA-induced mammary carcinogenesis also resulted in up-regulation of MDA levels in blood serum, mammary gland homogenate, liver and kidney (Perumal et al., 2005; Pattanayak & Mazumder, 2011). As mention previously, DMBA is metabolized into its active form (7-hydroxy DMBA) which is a potent reactive oxygen species that can cause DNA mutation as well as lipid peroxidation (Flesher & Sydnor, 1971).

Blueberries, high in antioxidant activity, have been studied for their ability to reduce lipid peroxidation. An *in vivo* study found that healthy animals that received 10% freeze-dried whole blueberry supplemented diet had similar levels of lipid peroxidation to non-blueberry-treated healthy animals (Dulebohn et al., 2008). Consumption of 50 g freeze-dried blueberry beverage showed MDA and hydroxynonenal (a lipid peroxidation marker) reduction in obese male and female human study participants after 8 weeks of blueberry beverage consumption (Basu et al., 2010). In agreement with other studies, blueberry treatment may not reduce the level of lipid peroxidation in the normal condition but in pathogenic conditions such as breast cancer the blueberry treatments are able to normalize lipid peroxidation level. To our knowledge, our study is the first study to report an effect of blueberries on modulation of MDA levels in DMBA-induced mammary tumorigenesis.

Catalase is an antioxidant enzyme involved in cellular defence against free radicals. It catalyses the decomposition of hydrogen peroxide (H_2O_2), a harmful by-product from oxygen metabolism, into less harmful molecules, oxygen and water (Khan et al., 2010). Catalase activity in blood serum is modified when free radicals and antioxidant enzyme imbalance occurs within cells. Our study found that catalase activity of animals in the positive group was lower by 26.5% in comparison with the negative group (figure 6-8). This

might result from high amounts of reactive oxygen species generated in animals bearing tumours. Blueberry extracts, 'Centurion' and 'Maru', were able to increase catalase activity in animal blood serum to a similar level seen in non-DMBA treated animals. Rats receiving 5% blueberry pomace-supplemented diets appeared to have the highest catalase activity in their blood serum (although this activity was not statistically different from 'Maru' treated rats or the negative control) (figure 6-8).

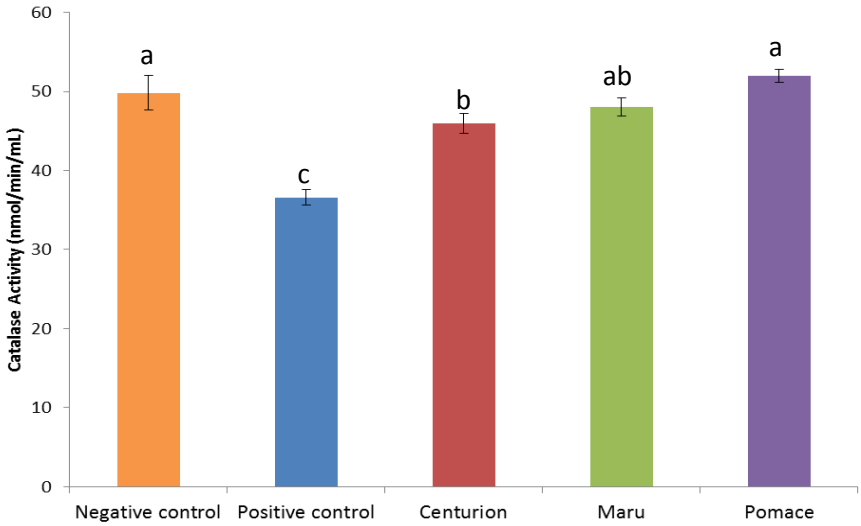


Figure 6-8 Effect of blueberry extracts ('Centurion' and 'Maru') and diet supplemented with 5% pomace on catalase activity in animal blood serum. The data are expressed as mean±SEM. The differences were analysed using posthoc Tukey's test. Bars not sharing the same letter are statistically different at $P \leq 0.05$.

An effect of oxidative stress on reduction of catalase level in human studies has been well documented. When tissue homogenates of 40 breast cancer patients were analysed, in comparison with tissue homogenates of 10 patients with fibroadenoma, for oxidative stress using MDA, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) as markers, up-regulation of SOD and GPx was found, which might be a result of higher production of reactive oxygen species. However, cancerous tissue had low level of CAT and led to lower H_2O_2 detoxification. An accumulation of H_2O_2 therefore resulted in lipid peroxidation as seen from MDA up-regulation (Tas et al., 2005). An oxidative stress marker in blood serum from breast cancer patients could be used as an indication of breast cancer incidence since the level of CAT was lower by 65% in breast cancer patients compared to their age-matched controls (Ray et al., 2000).

The effect of blueberry extract in amelioration of catalase activity *in vitro* has been demonstrated. Catalase activity increased by 44% in N2a neuroblastoma cell line treated with bio-transformed blueberry juice (Vuong et al., 2010). A recent study showed that blueberry extracts made with water or ethanol possessed moderate catalase activity compared to bovine liver catalase used as positive control. From the result, it could be assumed that blueberry extracts, with catalase activity, were able to metabolize H_2O_2 into a non-dangerous form, which led to lower oxidative stress in a controlled environment (Samad et al., 2014). Even though, blueberries possessed catalase activity *in vitro*, it is less likely for the enzyme activity to be active *in vivo* due to harsh conditions during digestion. Therefore, a study of blueberries or their polyphenols in up-regulation of catalase activity *in vivo* is required.

Quercetin increased catalase activity in female imprinting controlled region (ICR) mice treated with DMBA (Choi et al., 2012). Another breast cancer study showed that female Wistar rats treated with DMBA had lower catalase activity than a vehicle control group but the catalase activities were restored to a similar level to the negative control group, or even greater, in animals that received quercetin treatment (Reddy et al., 2012). Purple grape juice contains several flavonoids including anthocyanins similar to blueberries and when given to female SD-rats prior to DMBA treatment, successfully enhanced catalase activities compared to animals without the juice (Jung et al., 2006). However, a study of an effect of grape antioxidant dietary fiber consumption on catalase activity in healthy animals showed that the levels of catalase in both control and animals that received grape antioxidant dietary fiber supplemented diet were similar (Alía et al., 2003). It could be assumed that blueberries might not be able to increase catalase enzyme activity in normal or healthy conditions but effectively restored the catalase activity in pathogenic conditions.

For all oxidative stress variables in the current study, catalase activity was found inversely correlated with level of MDA (R = -0.541, P< 0.01) and positively correlated with blood serum antioxidant measured by FRAP (R = 0.369, P< 0.01). Collectively, blueberry extracts and blueberry pomace supplemented diet exhibited positive effects on oxidative stress *in vivo.* Therefore, alteration of tumour incidence and tumour indices might result from reduction of oxidative stress by blueberry consumption.

The delay of tumour onset in our study might have resulted from reduction of oxidative stress during tumour initiation stage. Moreover, the effect of blueberry treatments on oxidative stress might affect tumour progression from adenoma to

carcinoma. Oxygen radicals generated during tumour progression can cause genetic instability which leads to high malignant potential of the tumours (Szatrowski & Nathan, 1991).

Therefore, blueberry treatments in our study showed effective modulation of oxidative stress and subsequently resulted in lower progression to malignant tumours.

Effect of blueberry extracts and blueberry pomace-supplemented diet on level of 17β-estradiol in animals' blood serum

Estrogen plays an important role in breast cancer development. DMBA causes estrogen receptor positive (ER+) breast cancer; therefore, a higher exposure to estrogen results in a higher rate of development and progression of breast cancer (Huggins et al., 1958). In this study, levels of circulating estrogen were slightly altered in the positive control group and animals fed blueberry extracts compared to the negative control group; however it was not statistically significant. Animals that received diet supplemented with blueberry pomace exhibited significantly lower circulating estrogen than other groups ($P \leq 0.05$). The average level of estradiol in animal blood serum is presented in figure 6-9.

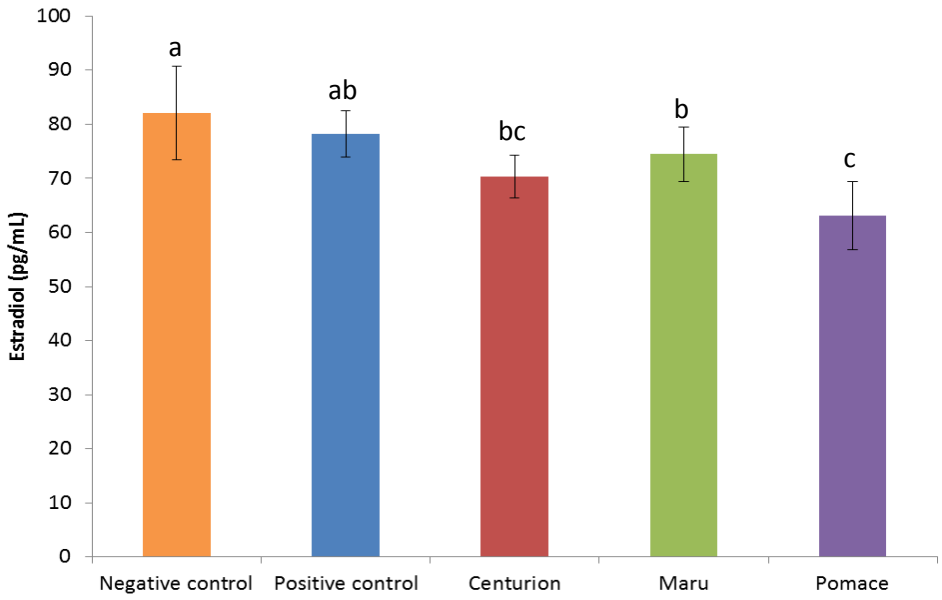


Figure 6-9 Effect of blueberry extracts ('Centurion' and 'Maru') and diet supplemented with 5% pomace on estrogen level (pg/mL) in animal blood serum. The data are expressed as mean±SEM. The differences were analysed using posthoc Tukey's test. Bars not sharing the same letter are statistically different at $P \leq 0.05$.

Up-regulation of ERα is the most critical step for initiation and progression of estrogen receptor positive breast cancer. The DMBA carcinogenesis model causes ER+ breast cancer, with higher number of estrogen receptors (ER) (Keightley & Okey, 1974). The increase of ERα in breast cancer coupled with high level of circulating estradiol leads to the higher rate of mammary gland proliferation which contributes to disease progression. Since level of estradiol in plasma is closely correlated with breast cancer risk in both pre- and postmenopausal women (Key, 1999), reduction of circulating estradiol might be another option for reduction of breast cancer incidence. 5% blueberry supplemented diet did not alter estrogen level in healthy animals; but the diet was able to lower circulating estrogen level in rat blood serum when treated with 17β-estradiol (E2). Expression of mRNA levels of CYP1A1 gene was also reduced in animals fed 5% blueberry supplemented diet. CYP1A1 plays a critical role in estrogen metabolism into the active form (4-hydroxy metabolites) (Ravoori et al., 2012). Another study on effect of blueberries on E2 metabolism reported down-regulation of CYP1A1 gene expression in early and intermediate stages of E2-induced mammary tumorigenesis which might result in lower carcinogenic metabolites of estrogen (Aiyer & Gupta, 2010). It was clear that blueberry-supplemented diet showed chemopreventive effect via regulation of estrogen metabolism.

It would be also of interest to examine numbers of estrogen receptors in tumour samples using real time PCR method in a future study in order to assess an effect of blueberries on estrogen receptors which may contribute to breast cancer chemoprevention and therapy.

Two major estrogen receptors found in mammary tissue are ERα and ERβ. ERα is increased in breast cancer tissue and results in cell proliferation, however ERβ, when activated, counteracts ERα effects (Matthews & Gustafsson, 2003). Blueberries contain various polyphenols which have different affinities to different types of ER. Quercetin, which exhibited anti-proliferation effect in mammary cancer, was further examined for its affinity to ERα and ERβ. It was found that ERβ activation was higher with quercetin than E2 by 4.5 fold but quercetin ERα activation was only 1.7 fold higher than E2 induction. The researchers concluded that anti-proliferative effect of quercetin could be attributed to ERβ responses which counteract ERα effect (van der Woude et al., 2005). Kaempferol in red wine also showed strong binding to ERβ (Zoechling et al., 2009).

Effect of blueberry extracts on reduction of circulating estrogen in our study was not statistically significant; however the effect was more pronounced in animals fed 5%

blueberry pomace supplemented diet. An effect of diet on estrogen excretion was evidenced in a human study. The researchers found that vegetarian female volunteers, who consumed 28 g fiber on average, excreted more estrogen than group that consumed only 12 g fiber per day. They also found that the level of excreted estrogen positively correlated with fecal bulk (Goldin et al., 1981). Unconjugated estrogen was found to bind effectively to several types of fiber such as lignin (Shultz & Howie, 1986) and lignin comprises approximately 14% of blueberry pomace (Håkansson et al., 2012).

Amount of blueberry intake from all blueberry treated animals did not show any correlation with tested biological markers (MDA concentration, catalase enzyme activity and estradiol concentration) in animals' blood serum.

Enterohepatic recirculation of estrogen is caused by β-glucuronidase enzyme. In the detoxification process, estrogen binds to glucuronic acid and is excreted via bile or urine. B-glucuronidase enzyme is produced by intestinal microflora. It cleaves glucuronide bond releasing estrogen to enter blood stream and results in higher circulating estrogen level (Gorbach, 1984). An effect of blueberries on β-glucuronidase enzyme and intestinal microflora was also studied and will be presented in the next section of this chapter.

Effect of blueberry extracts and blueberry pomace supplemented diet on beneficial and pathogenic bacteria in rat caecum

In general caecum weight was approximately 1% of total body weight of each animal (figure 6-10). Blueberry supplement in either extract or pomace form did not have any effect on total caecum weights of animals. Percentage dry matter of animals' caecal contents were also not significant different.

The caecum weight of animals fed blueberry pomace (30% w/w) as reported elsewhere was significantly higher than animals in a fiber-free diet control group (Bränning et al., 2009). The non-significant result on animals' caecum weight in the current study may be due to lower percentage of blueberry pomace in our experiment (5% w/w).

Prebiotic activity of blueberry extracts on *Lactobacillus acidophilus* and *L. rhamnosus in vitro* is discussed in chapter 4. In this chapter, prebiotic activity of blueberries was further investigated *in vivo*. Bacterial population in this study was calculated based on total caecum content weight of each animal. DMBA treatment slightly reduced mean population number of both beneficial and pathogenic bacteria in comparison with negative group. Blueberry

treatments did not cause significant differences in bacterial populations in any bacteria studied (figure 6-11).

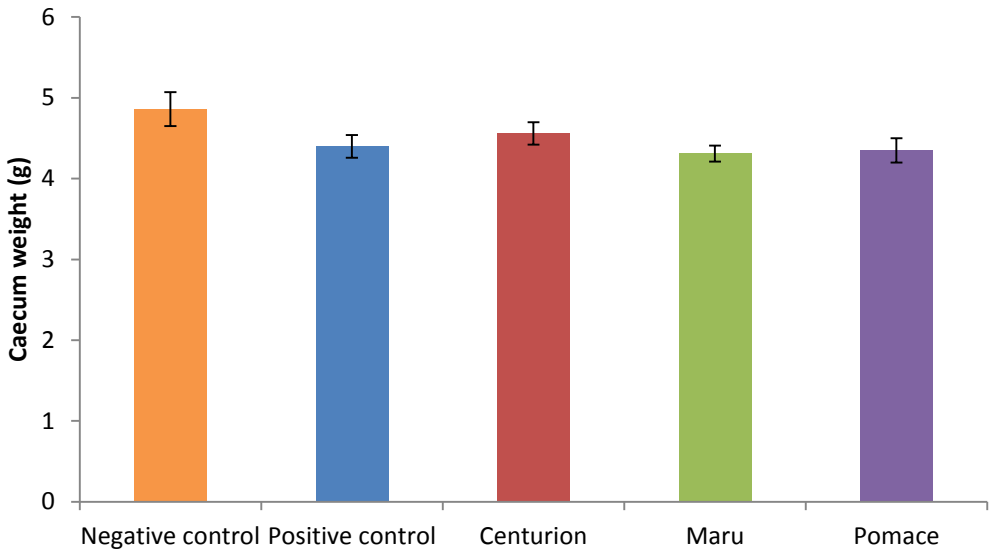


Figure 6-10 Average caecum weights of animals from all experimental groups. The data expressed as mean ± SEM. The differences were analysed using posthoc Tukey's test. Treatments were not statistically different.

Alteration of bacterial population has been observed in some pathogenic conditions. Patients with systemic inflammatory response syndrome were found with lower bacterial counts of beneficial bacteria (*Bifidobacterium* spp. and *Lactobacillus* spp.) and higher bacterial count of *Staphylococcus* and *Pseudomonas*, pathogenic bacteria, compared to healthy subjects (Shimizu et al., 2006).

A study showed that faecal samples derived from colorectal cancer (CRC) patients had lower butyric-producing bacteria, and butyric acid regulated apoptotic effects on colon tissue, than samples from healthy controls. Bacteria of genera including *Enterococcus*, *Escherichia/Shigella*, *Klebsiella*, *Streptococcus* and *Peptostreptococcus* were abundant in CRC samples (Wang et al., 2012d). Interestingly, even though bacterial populations are not always altered significantly, pathogenic conditions can still cause modulation in gut bacterial enzymes. Aerobic and anaerobic microflora profile from large bowel cancer patient faeces and healthy subjects were not significantly different, but bacterial enzymes including 7α-dehydroxylase and cholesterol dehydrogenase were up-regulated in CRC patients (Mastromarino et al., 1978).

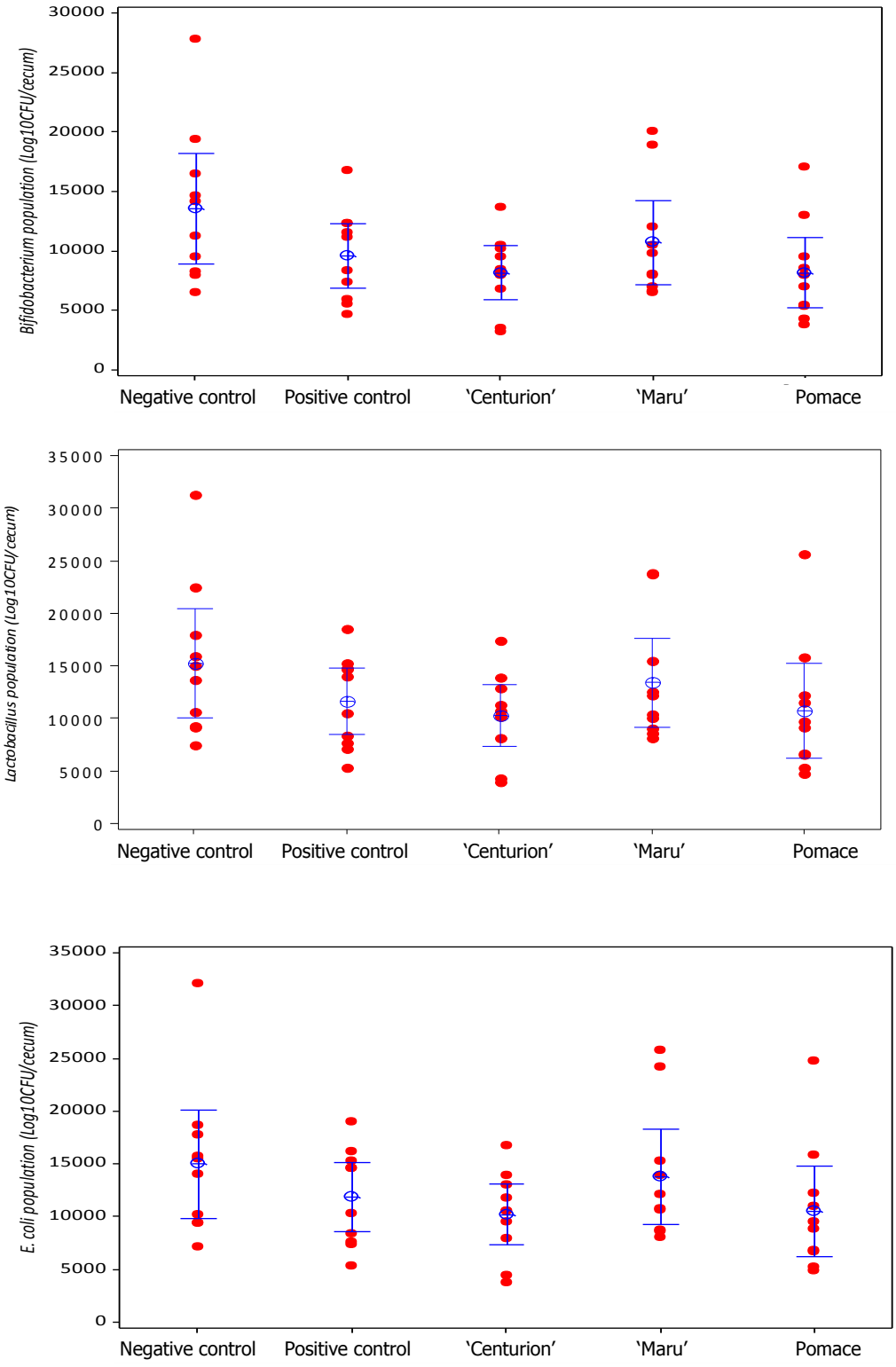


Figure 6-11 Individual value plots showing the intestinal bacterial population (A) *Lactobacillus* spp., (B) *Bifidobacterium* spp. and (C) *E. coli* affected by DMBA-treatment and different blueberry treatments. The data are expressed as 95%CI of mean.

A relationship between gut microflora and breast cancer was demonstrated in an animal study using germ-free rats. Germ-free rats fed dietary lignan exhibited higher tumour numbers per tumour bearing rat and larger tumour size compared to rats inoculated with lignan-converting bacteria using DMBA for mammary tumour induction (Mabrok et al., 2012). Their finding was consistent with the concept that gut bacteria play a critical role in breast cancer prevention by converting dietary compounds into their active form, which possess anti-cancer function. Prebiotic and antimicrobial properties of blueberries and their polyphenols are well evidenced. Prebiotic effect of blueberries was tested on *Lactobacillus* and *Bifidobacterium in vivo* using fluorescence in situ hybridization (FISH) method. Rats that received 4 mL/ kg body weight of blueberry extracts ('Centurion' and 'Maru') for 6 consecutive days increased both bacterial populations compared to rats that received distilled water (Molan & Lila, 2009). A prebiotic property of blueberries was examined in a human trial. *Bifidobacterium* and *Lactobacillus* population increased significantly after six weeks' consumption of wild blueberry powder drink while others, *Bacteroides* spp*., Prevotella* spp*., Enterococcus* spp., and *Clostridium coccoides* group were not affected by blueberry consumption (Vendrame et al., 2011). Some contrast was found in a study of bacterial composition in a mouse model of inflammatory bowel disease. Even though *E. coli* population decreased compared to mice fed standard diet, *Lactobacillus* population was also reduced while *Bifidobacterium* population was unchanged in animals fed with 10% blueberry supplemented diet (Paturi et al., 2012).

Neither prebiotic nor antimicrobial properties of blueberry extracts were found in this study. The prebiotic effect of blueberries on beneficial microflora in our study might be overwhelmed by the pathogenic condition. According to previous studies, an impact of blueberries on gut microflora is still inconclusive. It might vary by health condition of subjects or individual variation when tested *in vivo*.

Effect of blueberry extracts and blueberry pomace supplemented diet on β-glucuronidase enzyme activity from animals' caecum

β-glucuronidase is an enzyme mainly produced by bacteria such as *E. coli* in the human gut. It is responsible for hydrolysis of glucuronide bond. The level of β-glucuronidase was not significantly different between negative and positive control rats, however, rats fed blueberry extracts or a blueberry pomace- supplemented diet had significantly reduced level of β-glucuronidase. β-glucuronidase activity in rats from 'Centurion' and Pomace

group was significantly lower than positive group. The β-glucuronidase activity was slightly higher in rats that received 'Maru' extract than in other blueberry-treated groups but it was lower than the positive group (figure 6-12). Percentage dry matter of animals' caecal contents were not significantly different between rats fed normal diet and those fed blueberry pomace-supplemented diet.

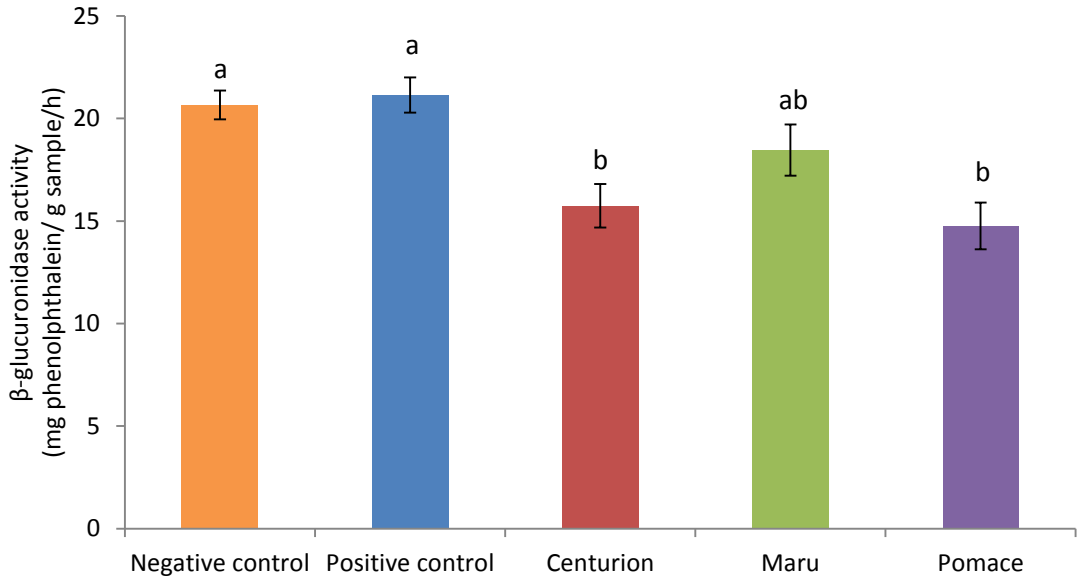


Figure 6-12 β-glucuronidase enzyme activity in caeca from rats in all experimental groups affected by DMBA-treatment (all groups except the negative control) and different blueberry treatments ('Centurion', 'Maru' and Pomace). The data are expressed as mean±SEM. The differences were analysed using posthoc Tukey's test. Bars not sharing the same letter are statistically different at $P \leq 0.05$.

Glucuronidation is a process of detoxification in animals. The process is the formation of glucuronide which transform hydrophobic compounds into more hydrophilic compounds that can be more readily released from the body via urine or faeces (Hughes & Rowland, 2000). However, with the presence of β-glucuronidase in colon, the glucuronide bond is hydrolysed. This results in enterohepatic circulation of the toxic compounds. In some pathogenic conditions including cancer β-glucuronidase enzyme activity is increased. Bacterial β-glucuronidase enzyme activity was 1.7 times higher in patients with colon cancer in comparison with their normal control (Kim & Jin, 2001). β-glucuronidase enzyme activities from blood serum of breast cancer patients were higher than their age-matched healthy controls. Similar results were found in both pre- and post-menopausal women (Thangaraju et al., 1998).

There was a risk that the DM concentration of caecal contents could explain the beta-glucuronidase difference, if extra fibre in the blueberry-fed rat diets had led to higher hydration. This was not the case. Reduction of β-glucuronidase enzyme activity might affect breast cancer initiation since DMBA undergoes detoxification process, is conjugated with glucuronic acid and is excreted via urine and faeces. DMBA might be reabsorbed with β-glucuronidase enzyme present. A study using high dose of 2,5-di-O-acetyl-D-glucaro-l,4:6,3-dilactone (DAGDL) (a β-glucuronidase enzyme inhibitor) showed that mammary tumour incidence in DMBA-fed rats that received DAGDL treatment was reduced by 70% compared to animals without DAGDL treatment (Walaszek et al., 1984). In animals that received the β-glucuronidase enzyme inhibitor, administered DMBA might go through glucuronidation and be excreted but in animals without the inhibitor, β-glucuronidase enzyme hydrolysed the glucuronide bond and allowed reabsorption of DMBA to the blood stream. The research established a link between breast cancer and β-glucuronidase enzyme. Supporting evidence was reported that β-glucuronidase enzyme activity in breast tumours was 6 times higher than activity in peritumoral tissue from the same patient (Albin et al., 1993). Circulating estrogen, which plays a crucial role in mammary tumour promotion, might be affected by reduction of β-glucuronidase enzyme activity in the same way as DMBA metabolism (Goldin et al., 1981).

A chemoprotective effect of fruit and vegetable consumption might relate to modulation of β-glucuronidase enzyme activity. The data collected for fruit and vegetable consumption in human subjects showed that dietary fiber consumption inversely correlated with β-glucuronidase enzyme activity in blood serum. However, not all types of fruit and vegetable were related to β-glucuronidase enzyme activity since a correlation could not be established between total fruit or vegetable consumption and β-glucuronidase enzyme activity. Intake of plants in Cucurbitaceae, Rosaceae and Leguminosae families such as cucumbers, apples and string beans exhibited significant negative correlation with β-glucuronidase enzyme activity (Lampe et al., 2002). Rats gavaged with blueberry extracts for 4 weeks showed reduction in β-glucuronidase activity (Molan et al., 2010). In relation to intestinal bacterial population, lactic acid bacteria consumption was able to reduce intestinal β-glucuronidase production and *E. coli* population (Han et al., 2005) which may result in reduction of β-glucuronidase activity *in vivo*. In our study, blueberry consumption failed to demonstrate antimicrobial activity on *E. coli* but the activity of β-glucuronidase enzyme was reduced in two of three treatments. Even though β-glucuronidase enzyme is

produced by *E. coli*, *Clostridium perfringens* and *Bacteroides* species may also be responsible for the production of this enzyme (Skar et al., 1988).

In the current study, a relationship between *E. coli* population and β-glucuronidase activity was unable to be established; however β-glucuronidase activity correlated with level of circulating estrogen (R = 0.455, P = 0.001)

The population of other gut bacteria might require further investigation. As presented here, the reduction of β-glucuronidase enzyme activity may contribute to the beneficial effect of blueberry consumption on mammary tumorigenesis *in vivo*.

Conclusion:

Blueberry consumption in both extract and diet-supplemented forms exhibited chemopreventive effect in DMBA-induced mammary tumorigenesis *in vivo*. Blueberries reduced oxidative stress by enhancing catalase activity and blood serum antioxidants and reducing lipid peroxidation. The level of circulating estrogen was significantly lower in rats that received 5% Pomace supplemented diet which is likely to be an effect of dietary fiber present abundantly in blueberry skins, while blueberry extracts resulted in slightly lower circulating estrogen compared to non-treated animals. Intestinal bacteria population was affected by DMBA treatment as the treatment slightly reduced average populations of both beneficial and pathogenic bacteria in comparison with negative control. However, blueberry treatments in either extracts or diet supplemented form did not affect any studied intestinal bacterial populations. 7,12-Dimethylbenz(a)anthracene alone did not have any effect on faecal β-glucuronidase enzyme because the enzyme activities in negative and positive group were similar; however the enzyme activity was reduced by all blueberry treatments.

Collectively, two New Zealand grown rabbiteye blueberry extracts and 5% pomace supplemented diet exhibited chemopreventive effects on DMBA-induced mammary tumorigenesis in virgin female Sprague-Dawley rat model. The findings from this study should be useful as preliminary data for further investigation of blueberries and their phytochemical effects on human health, specifically breast cancer. Moreover, the use of blueberry pomace as a food additive with health benefits might be of interest.

CHAPTER 7

General discussion and conclusion

This study's primary aims were to evaluate the potential of selected NZ rabbiteye blueberries and blueberry pomace, a by-product from blueberry juice processing, in the prevention or delay of chemically induced mammary tumorigenesis *in vivo*. In pursuance of these objectives, NZ rabbiteye blueberry cultivars ('Centurion', 'Maru', 'Rahi', 'Ono' and 'Tifblue') were screened for total phenolic concentration, total flavonoid concentration, individual anthocyanins and chlorogenic acid concentration *in vitro*. Furthermore, antioxidant, prebiotic and anti-angiogenic activities of all blueberry fruit and pomace extracts were evaluated. Two blueberry cultivars, 'Centurion' and 'Maru', exhibited moderate antioxidant activity and high anti-angiogenic activity but contained different compositions of anthocyanins ('Centurion' was rich in glucosides while 'Maru' was dominated by galactosides). They were both also slightly beneficial for *Lactobacillus* growth *in vitro*.

Water extracts of 'Maru' and 'Centurion' berries were compared with commercially available blueberry pomace to determine if they had any potential chemopreventive effect in the DMBA induced mammary tumorigenesis rat model and to investigate if there was any effect in modulation of oxidative stress, endogenous estrogen level, populations of intestinal bacteria and intestinal β-glucuronidase activity. The results obtained from this study will provide more information about polyphenols and the potential health benefits of NZ grown blueberries. Moreover, the study presents useful information for a recommendation of blueberry consumption by consumers, both in healthy people or those with breast cancer, and the role of blueberry extract and blueberry pomace in the modulation of breast cancer parameters.

Blueberry polyphenols and their effects on DMBA-induced mammary tumorigenesis

Blueberries are a rich source of various polyphenols such as anthocyanins, procyanidins and chlorogenic acid. Types and concentration of blueberry polyphenols have been studied previously but the types and concentration of blueberry polyphenols are affected by several factors such as genetic variation and cultivation condition (Howard et al., 2013). Apart from genetic variation and cultivation condition which affect types and concentrations of polyphenols found in blueberries, different extracting solvents used can effect complete extraction of polyphenols in blueberries (Lee & Wrolstad, 2004; You et al., 2011; Li et al., 2013).

In the present study, water was used for extraction. Even though the concentration of blueberry polyphenols extracted with water is low, the blueberry extracts were intended for use *in vivo* without contamination from organic solvent residue. In chapter 4, blueberries extracted with 5% aqueous formic acid was tested in anti-angiogenic assay *in ovo* and caused embryonic death. Moreover, synergistic effect of anthocyanidins in blueberries was studied and found that lower concentration of a mixture of five blueberry anthocyanidins was required to kill 50% of T47D breast cancer cells while it required 5-10 times higher concentration of individual anthocyanidins to provide similar 50% inhibition (Ravoori et al., 2012). Therefore, in our *in vivo* study, blueberries extracted with water were used without purification.

The study began by characterising anthocyanin composition and antioxidant activity of blueberries which allowed some limited correlations to be made with *in ovo* bioactivity of particular attributes. Once the animal trial was under way the focus of the study changed to a more fundamental analysis of observed effects of blueberries on mammary tumorigenesis. Oxidative stress plays an important role in several diseases including breast cancer. An increase in free radicals in human body system might lead to an initiation or progression of breast cancer. Blueberries have high antioxidant activity and are believed to be able to modulate oxidative stress *in vivo* (McAnulty et al., 2011). However, the major polyphenols in blueberries are anthocyanins, which have low bioavailability *in vivo* because they are poorly absorbed (McGhie & Walton, 2007). Moreover, blueberry polyphenols

chlorogenic acid and procyanidins also possess antioxidant properties but require bacterial metabolism before they are functional (Gonthier et al., 2003: Gonthier, et al., 2003).

Therefore, antioxidant activity of blueberries might not have a direct effect on modulation of oxidative stress in blueberry consumers. Instead, polyphenolic-rich diets have been shown repeatedly to alter the antioxidant status of the body indirectly by modifying catalase activity (Samad et al., 2014) and level of lipid peroxidation (Zepeda et al., 2012) in pathogenic conditions. Moreover, another blueberry polyphenol, quercetin, at high dose (20 – 100 mg/kg bodyweight) has been shown to modulate oxidative stress by normalizing lipid oxidation and rejuvenating antioxidant enzymes such as catalase and superoxide dismutase in DMBA-induced mammary tumorigenesis in rat model (Reddy et al., 2012).

Blueberry consumption has shown several possible inhibitory and preventive effects in multiple steps of DMBA-induced mammary tumorigenesis as summarized in figure 7-1.

During tumor initiation, DMBA, a pre carcinogen, is metabolized into active metabolites. The metabolites are reactive oxygen species and cause oxidative stress at the cellular level (Flesher & Sydnor, 1971). An organ study showed that DMBA is absorbed to the mammary fat pad and slowly released to mammary epithelium. The maximum level of DMBA-DNA adduct formation occurs within 24 hours after DMBA administration (Das et al., 1989). In SD rats circulating DMBA has not been found longer than approximately 120 h after administration. Tumour incidence among DMBA-treated groups were not significantly different. Tumour latency significantly increased only in the Pomace group compared to the positive control group (without blueberry treatments). In chapter 6, a post mortem mammary morphology examination showed that the number of animals affected by DMBA in each experimental group was not statistically different. Therefore, we are unable to conclude that blueberry consumption in this study affected the tumour initiation process. In order to investigate the effect of blueberry consumption on mammary tumour initiation, DMBA-DNA adduct formation would need to be measured. Formation of DMBA-DNA adduct has been studied and proved to be one of the causes of mammary tumorigenesis in animal models of breast cancer (Jung et al., 2006). A larger number of animals would be required to quantify DMBA-DNA adduct formation in mammary glands. Even though the DMBA-DNA adduct formation was not evaluated in this study, it could be done in a future study.

Oxidative stress plays an important role in tumour initiation. It also has a key role in tumour progression (Lin et al., 2003). In this study, blueberries showed antioxidant activities *in vitro* (chapter 4) and modulation of oxidative stress *in vivo* (chapter 6). Blueberry polyphenols might act directly as an antioxidant in scavenging free radical DMBA metabolites after the polyphenols have been modulated by gut microflora and absorbed. Moreover, animals that received blueberry treatment showed modulation of oxidative stress by normalizing the level of lipid oxidation and catalase enzymes to match that of the negative control group as presented in figure 7-1. Blueberry consumption has also been reported to lower MDA level in obese male and female subjects after 8 weeks consumption (Basu et al., 2010). Recently, a study also reported that quercetin inhibits lipid peroxidation in DMBA-induced skin cancer in mice (Ali & Dixit, 2015). The blueberry polyphenol, quercetin, restored catalase activity in animals that had received DMBA to a similar level of healthy controls (Reddy et al., 2012). We also found that catalase activity showed a weak but positive correlation with the blood serum antioxidant (FRAP) but was negatively correlated with MDA level.

Even though blueberries appeared to have only a small effect on the initiation stage of DMBA-induced mammary tumors, they had a significant effect on tumour progression from adenoma to carcinoma as observed by histopathological exam (chapter 6). Most tumours (> 85%) found in the positive control group were carcinomas while fewer than 50% of tumours in blueberry treated animals progressed to carcinoma stage. The proportion of carcinomas in the positive control group was significantly higher than those from blueberry treated groups.

Angiogenesis is a key process for tumor progression. We observed anti-angiogenic activity of blueberry extracts *in ovo* (chapter 4). In our *in vivo* rat study we have found benign tumors (adenomas) only in the blueberry treated groups. This finding suggested that blueberry supplementation might affect tumor progression from the benign to the malignant form. However, quantification of blood vessels around tumors or the rate of tumour growth might not effectively assess the anti-angiogenic activity of blueberries *in vivo*. Measurements of VEGF level would serve as a better biomarker for anti-angiogenic activity of blueberries (Bagchi et al., 2004). Unfortunately, the detection and quantification of VEGF in mammary tumors by immunohistochemical staining is challenging and was not attempted here; but this could make a useful study on its own in future work (Maae et al., 2011).

The major effect of blueberry treatment in our study was the prevention of tumour progression from adenoma to carcinoma. There are different genetic alterations in each step of tumour progression such as over expression of cyclin D1 and Neu (T/D) in late carcinoma which are not observed in hyperplasia and adenoma (Lin et al., 2003). Blueberries and their polyphenols might have an effect on genetic alteration which may prevent tumour progression from adenoma to carcinoma. Molecular study on gene expression modulation by blueberries would allow this to be specifically tested.

Taken together, blueberry consumption caused a reduction in oxidative stress in the DMBA-induced mammary tumorigenesis rat model, both by increasing catalase and decreasing MDA. These positive effects might contribute to a slowing or inhibition of breast cancer progression.

The type of breast cancer induced by DMBA is estrogen receptor positive (ER+) breast cancer. This type of breast cancer is dependent on estrogen for its cellular proliferation. Therefore, a reduction of circulating estrogen, either from increased estrogen excretion or decreased estrogen synthesis, would serve as a promising strategy for ER+ breast cancer treatment. Animals that received the 5% pomace supplemented diet had significantly lower levels of circulating serum estrogen than other blueberry treated groups (chapter 6). Therefore, the mechanisms by which circulating estrogen were reduced is likely due not only to their anthocyanins but may additionally result from the dietary fibre present in blueberry pomace that plays an important role in estrogen excretion (Bagga et al., 1995) and also carries anthocyanins and procyanidins further though digestive tract which benefit their bioacessibility and bioavailability (Palafox-Carlos et al., 2010).

Up-regulation of estrogen receptors in mammary tumours is found in DMBA-induced mammary tumorigenesis. It is possible to measure this up-regulation using the real-time polymerase chain reaction (qPCR) in formalin-fixed paraffin-embedded tumour samples (Al-Bader et al., 2010). We did not attempt to measure estrogen receptors in this study; however, it would be of interest to carry out these assays in future on stored or newly acquired samples.

Blueberry consumption in either extract or pomace supplemented diet form did not significantly alter the population of intestinal microflora. However, β-glucuronidase enzyme activity was lower in animals that received blueberry pomace supplemented diet. This may have been due to an antimicrobial effect of the blueberry on β-glucuronidase enzyme

producing bacteria other than *E. coli*. In addition, D-glucaric acid, a β-glucuronidase enzyme inhibitor, is found in fruits and vegetables such as cherries. Calcium-D-glucarate supplemented diet has been shown to cause a 70% inhibition of mammary tumours in DMBA-induced mammary tumorigenesis in the rat model compared to rats without calcium-D-glucarate supplemented diet (Walaszek et al., 1986). To our knowledge, there is no study on D-glucaric acid in blueberries or blueberry pomace. Therefore, it would be of interest to determine D-glucaric acid content in blueberries and to establish a correlation between the level of the acid and β-glucuronidase enzyme activity. This information may provide more knowledge on the effect of blueberries on the modulation of β-glucuronidase enzyme activity and mammary tumorigenesis.

The blueberry pomace extracts used in our *in vitro* studies (chapter 3 and 4) did not match the dried pomace used *in vivo* since the fibre in the pomace was not included. It seems that blueberry consumption in dried pomace form was more effective than blueberry extract. Fibre found in pomace might also have an important role. Anthocyanins attach to fibre in the food matrix and may therefore be more likely to reach the lower gastrointestinal tract and be metabolized into their active form (Mann, 2002). Moreover, fibre functions as a prebiotic to promote the growth of beneficial bacteria such as lactobacilli and bifidobacteria (Anderson et al., 2009) and also help with estrogen excretion (Bagga et al., 1995). Polyphenol content in 25% blueberry juice was approximately five times higher than polyphenol content in 5% pomace supplemented diet. The effect of blueberries in modulation of breast cancer were more pronounced in animals that received 5% pomace supplement diet than animals consumed blueberry juice without fibre. Taken together, not only do the polyphenols found in blueberries have an effect on mammary tumorigenesis, fibre is also found to play a part. Evidence suggests that consumption of polyphenols such as anthocyanidins or proanthocyanidins with fibre facilitate the release, bioaccessibility and bioavailability of the compounds (Palafox-Carlos et al., 2010). Therefore, it should be recommended that blueberries should be consumed in a whole fruit form or any other forms which include the fibre component.

Our study showed that blueberry consumption in either extract or pomace supplemented diet form reduced tumour incidence in the animal population that received DMBA for mammary tumour induction. We also provided evidence that blueberry consumption was active against multiple steps of breast cancer pathophysiology. However, blueberry consumption did not prevent breast cancer formation in all animals, and these

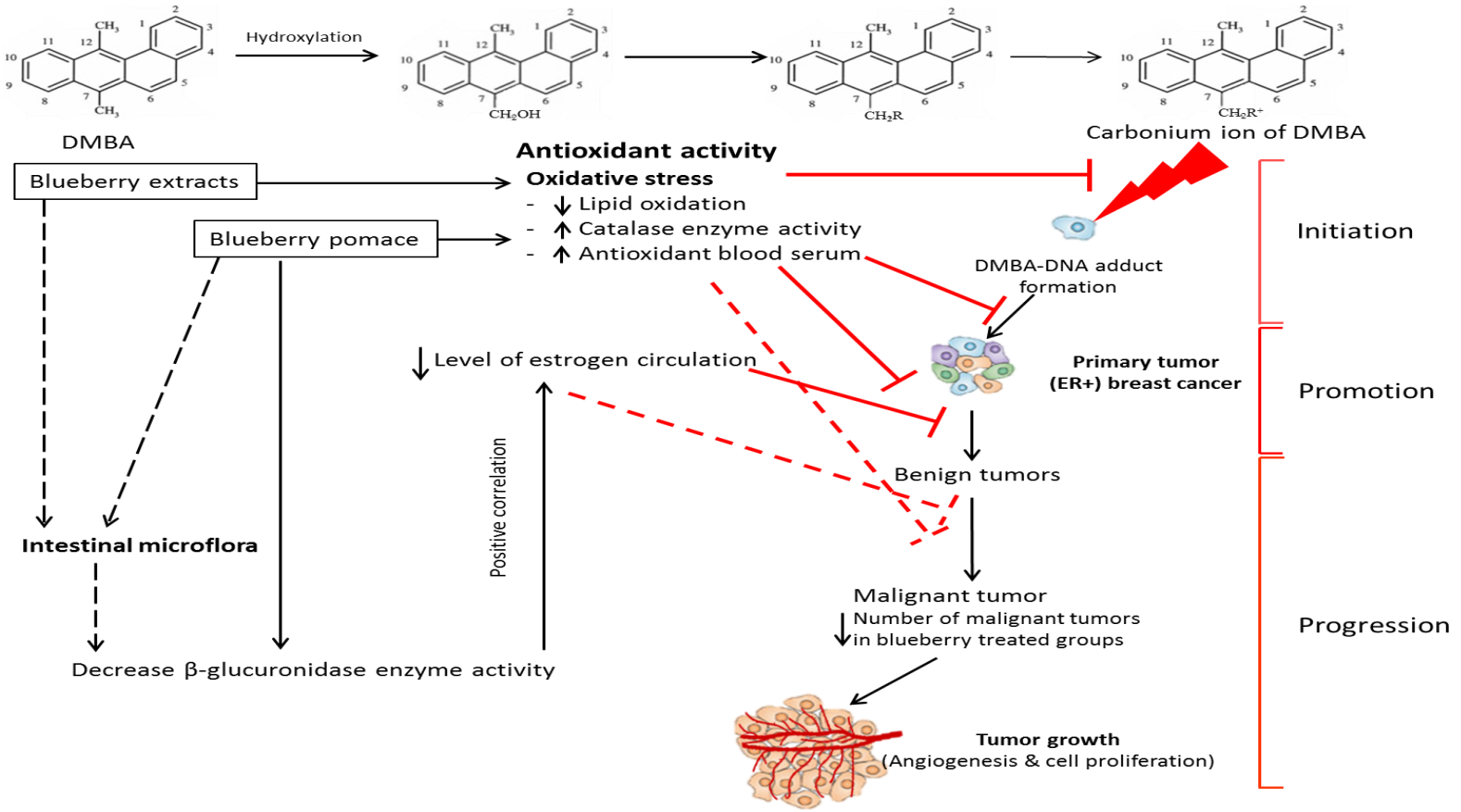


Figure 7-1 Contribution of evidence in this thesis to prevention of DMBA-induced mammary tumour initiation and promotion. ↑ = Increase, ↓ = Decrease, ⊣ = Inhibition or prevention, → = Significant effect, - - → = Insignificant effect, - - ⊣ = Possible inhibition.

findings may not extrapolate to humans. While it is possible to speculate on how these results may extrapolate to breast cancer risk in humans, it is clear that only very cautious conclusions may be drawn. It seems likely that increased blueberry consumption (as part of a healthy lifestyle, avoiding smoking and with adequate exercise) could reduce breast cancer incidence at a population level, as it may contribute to an increase in serum antioxidant status and a reduction in circulating estrogen concentration and contribute to a reduction in vascular neogenesis. Together these effects may reduce the risk of breast cancer initiation and progression. These suggestions can in no way be interpreted as suggesting that breast cancer could be managed through diet; a population-wide benefit does not mean any particular individual will not contract breast cancer on the healthiest of diets. Clinical advice is still an absolute requirement for anyone who is concerned they may have breast cancer. Clinicians may be supportive of patients who are under active medical attention being advised to include a blueberry-rich diet as part of their diet and exercise advice. Clearly much remains to be found about the molecular mechanisms of blueberry extracts and their polyphenols during each step of breast cancer in animal models and in humans.

Implication of blueberry consumption on breast cancer

In this study, blueberry consumption especially in pomace supplemented diet form affected breast cancer in DMBA induced mammary tumorigenesis in rat model. A positive effect of increased blueberry consumption is likely to be beneficial at a population level; but of course does not abolish the risk of breast cancer for each individual.

According to the results of this study, blueberry consumption should provide benefits for healthy subjects in reducing the risk of breast cancer that is a result of environmental insults such as chemical exposure, smoking or alcohol consumption. However, blueberry consumption in any forms (fruit extract, dried powder or supplement) is not recommended as a sole source of breast cancer treatment. Breast cancer patients should strictly follow conventional medical practice. A study demonstrated a synergistic effect of pterostilbene and Tamoxifen in animals with breast cancer (Mannal et al., 2010). Therefore, increased blueberry consumption, especially in dried pomace form might be beneficial for post-surgery breast cancer patients, and this could be safely tested in a clinical trial.

Moreover, we also observed that blueberry consumption in an extract form led to a decrease in food consumption which may have been a result of enhanced satiety in those

animals, leading to a lower average body weight and overall healthy condition. Therefore, studies of blueberry consumption to increase satiety and decrease appetite either in healthy or pathogenic conditions should be further investigated.

Recommendations for future research

Even though this study has provided more knowledge about the impact of blueberries on breast cancer, emerging questions from this study require further investigation. In this study, blueberries were administered two hours after DMBA administration and one of their modes of action may have been a simple chemical reduction in the efficacy of DMBA as an agent causing mammary tumours. Therefore, delaying the time of blueberry administration to 120 hours after DMBA administration might ensure that all mammary glands would have had 'maximal' contact with DMBA at this dose, shown to induce palpable breast tumours in 75% of treated animals in our study. Blueberry administration could be done after the first palpable tumour was felt in order to study a therapeutic effect of blueberries.

The type of breast cancer caused by DMBA is ER+ breast cancer. It is the most found breast cancer but it is not the most aggressive breast cancer. Apart from level of estrogen, level of oxidative stress was affected by blueberry treatments. Moreover, blueberries and their polyphenols have been found to modulate immune status (Peluso et al., 2015). Other type of hormone-independent breast cancer that result in alteration of immune system might be affected by blueberry consumption and need further studies.

The major effect of blueberry consumption found in this study was slower tumour progression. Tumour progression is a result of several genetic alterations such as over expression of VEGF, cyclin D1 and Neu (T/D). Blueberry consumption might have been able to alter gene expression in breast cancer which requires further investigation. Moreover, oxidative stress was restored back to normal level in animals that received blueberry treatments. Molecular pathways related to oxidative stress in breast cancer also require future research.

This study has shown that lower circulating estrogen level might result from higher excretion; however blueberries might act as an aromatase inhibitor which affects estrogen synthesis *in vivo*. A study of the function of blueberries as an aromatase inhibitor would

benefit breast cancer patients and people with other estrogen dependent pathogenic conditions. Therefore future studies in this area are essential.

Even though the effect of blueberry consumption on modulation of intestinal microflora was unclear, the level of bacterial β-glucuronidase decreased and correlated with circulating estrogen level. Further investigation should be carried out to completely explore intestinal bacteria population as affected by blueberry consumption in both healthy and pathogenic condition. Blueberry pomace was more effective than blueberry extract which may be attributed to the fibre or the concentration of phytochemicals in food. A study should be carried out to compare the effect of fibre alone or polyphenols alone with a combination of fibre and polyphenols on breast cancer.

The major objective of this study was to demonstrate whether there was a chemopreventive effect of NZ rabbiteye blueberries, and blueberry pomace, a by-product from blueberry juice industry, on DMBA-induced estrogen positive breast cancer.

The major findings attained indicate that the selected NZ rabbiteye blueberries contained relatively high amounts of polyphenols, specifically anthocyanins. The blueberry pomace also presented with considerable amount of polyphenols. Antioxidant activities of both blueberry fruit and pomace extracts examined *in vitro* were within the range of those previously demonstrated. The extracts showed prebiotic activity on pure cultures of *L. rhamnosus* and *L. acidophilus;* however, they failed to exhibit antimicrobial activity against *E. coli*, *S. typhimurium* and *S. aureus* as measured by agar well diffusion method. The sterilised aqueous extracts of both blueberry fruit and blueberry pomace showed anti-angiogenic activity when examined by CAM assay using fertilized hen eggs. Lastly, consumption of rabbiteye blueberry extracts or pomace-supplemented diet affected DMBA-induced estrogen positive mammary tumorigenesis in rats. Animals receiving blueberry pomace had increased tumour latency, but blueberry treatments did not significantly reduce tumour multiplicity or tumour volume. Blueberry extracts and pomace consumption increased the level of antioxidants in blood serum. The chemopreventive effect of blueberry extracts and pomace might result from increasing the antioxidant level in blood serum, up-regulating catalase enzyme activity, lowering the level of lipid oxidation and lowering the level of estrogen in blood serum. Even though blueberries did not show any direct effects on modulation of intestinal microflora, β-glucuronidase enzyme activity was affected by blueberry consumption, in particular by the blueberry pomace

supplemented diet. Interestingly, histopathological exam showed that blueberry treatment effectively prevented tumour progression from adenoma to carcinoma.

The results of this thesis will help to improve the understanding of the chemopreventive effect of rabbiteye blueberries and blueberry pomace on ER+ breast cancer. The study also exemplifies the possible application of NZ blueberries and their by-products for modulation of breast cancer for better outcomes when used with standard clinical treatments. Blueberries should be consumed regularly as whole fruit or in any forms including their fibre for reducing breast cancer risk. They may also benefit breast cancer patients as part of a healthy diet when receiving standard clinical treatment.

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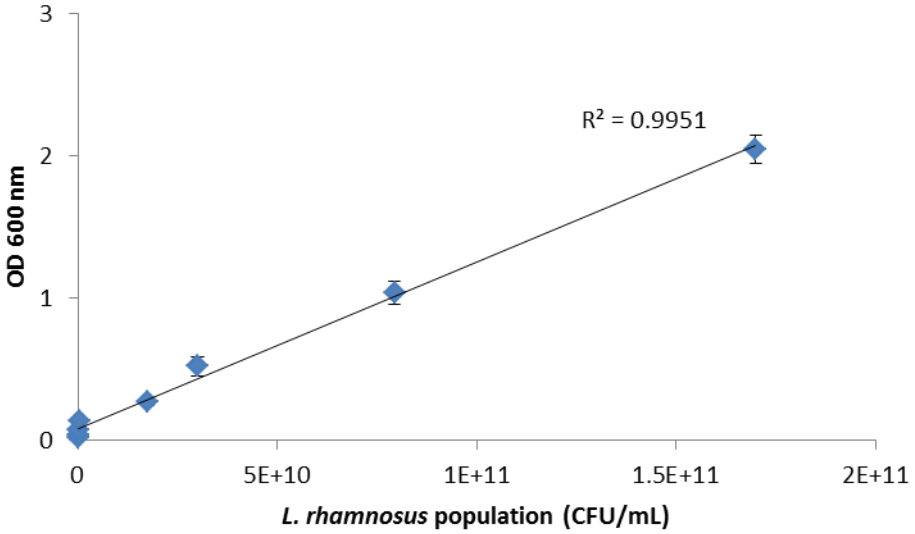
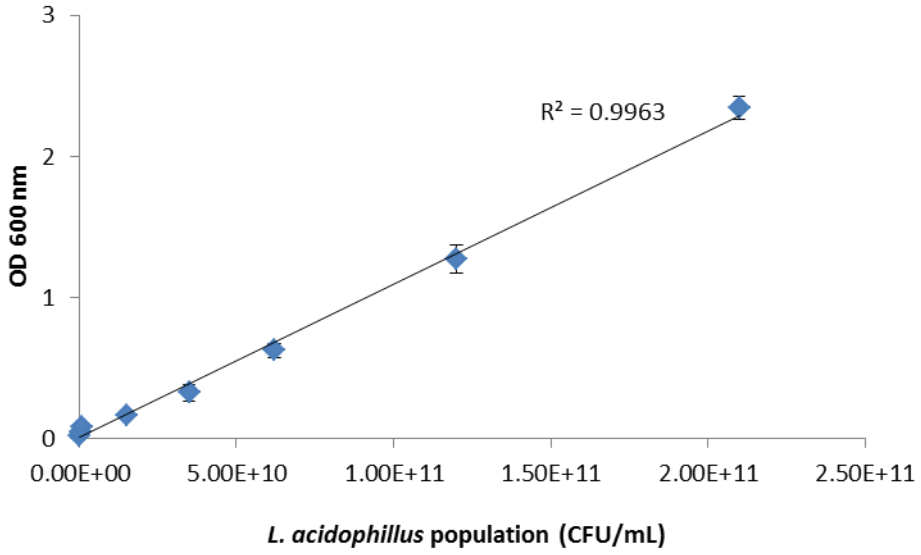
Turbidity standard curves of *Lactobacillus* spp. Data are expressed as mean±SEM of triplicate





Antimicrobial activities of crude blueberry extract against different pathogenic bacteria strains

Blueberry extracts and controls		Zone of inhibition (mm)		
		E. coli	*Salmonella typhimurium*	*Staphylococcus aureus*
Positive control (Gentamycin 0.1 mg/mL)		18.6 ± 1.2	14 ± 1	26 ±.3 1.2
Water extract control		-	-	-
5% formic acid control		15.3 ± 1.5	12.7 ± 0.6	20.0 ± 1.0
Frozen berries extracted with water	**Centurion**	-	-	-
	Maru	-	-	-
	Rahi	-	-	-
	Ono	-	-	-
	Tifblue	-	-	-
Frozen pomace extracted with water	**Centurion**	-	-	-
	Maru	-	-	-
	Rahi	-	-	-
	Ono	-	-	-
	Tifblue	-	-	-
Frozen berries extracted with 5%FA	**Centurion**	16.0 ± 2.0	13.0 ± 1.0	20.3 ± 0.6
	Maru	16.0 ± 2.0	12.7 ± 0.6	20.7 ± 1.5
	Rahi	16.0 ± 1.7	12.3 ± 1.5	20.0 ± 1.0
	Ono	16.3 ± 1.6	12.7 ± 2.1	20.3 ± 1.2
	Tifblue	16.3 ± 1.5	13.6 ± 0.6	20.7 ± 1.2
Frozen pomace extracted with 5%FA	**Centurion**	16.3 ± 2.1	12.6 ± 0.6	21.0 ± 1.0
	Maru	16 ± 1	12.3 ± 1.5	19.7 ± 0.6
	Rahi	15.7 ± 1.5	11.6 ± 1.2	19.7 ± 1.2
	Ono	15.3 ±1.2	11.3 ± 0.6	20.3 ± 1.2
	Tifblue	16.3 ± 1.5	11.7 ± 0.6	20.7 ± 1.2
Freeze dried berries extracted with water	**Centurion**	-	-	-
	Maru	-	-	-
	Rahi	-	-	-
	Ono	-	-	-
	Tifblue	-	-	-
Freeze dried pomace extracted with water	**Centurion**	-	-	-
	Maru	-	-	-
	Rahi	-	-	-
	Ono	-	-	-
	Tifblue	-	-	-
Freeze dried berries extracted with 5%FA	**Centurion**	16 1	12.3 0.6	19.7 1.2
	Maru	16.7 1.2	12.7 1.2	21 1
	Rahi	16.3 1.2	12.7 1.5	19.3 1.5
	Ono	15.3 0.6	12.3 1.2	19.3 1.2
	Tifblue	16.3 2.1	12.3 0.6	19.7 2.1
Freeze dried pomace extracted with 5%FA	**Centurion**	16 1.7	12.3 0.6	19.7 1.5
	Maru	15. 0.6	12.7 1.2	20.7 0.6
	Rahi	16 1.7	12.7 1.5	19.7 1.5
	Ono	16 2	12.7 1.2	19.6 2.1
	Tifblue	15.3 0.6	12 1	19.7 1.5

Animal daily health score record

Animal ID:		Treatment:						
Variables		Score						
Date								
Time								
Spontaneous activity								
0	Normal							
1	Minor reduction in activity							
2*	Moderate reduction in activity							
3	Prefers to sleep							
4	Marked reduction in activity, slow response to stimulation, listless. Eyes are not open much and may be sunken due to dehydration							
5	No response to stimulation							
Appearance								
0	Bright shiny eyes; smooth coat							
1	Coat staring							
2*	Piloerection							
3	Dirty tail and fur, slightly hunched posture							
4	Moderately hunched posture							
5	Fur totally sticking up and dull, hunched posture							
Porphyrin staining								
0	No staining							
1	Mild stain on either eyes/tail/nose							
2*	Mild stain on more than one region							
3	Increased stain on eyes/ tail/ nose							
4	Increased stain on more than one region and across shoulder							
5	Heavy stain							
Largest Tumor size (mm) (L x W)								
0	0-10							
1	10-20							
2	20-30							
3	30-35							
4	35-40							
5	>40							
Body weight (g)								
0	No weight loss							
1	<5% weight loss***							
2*	5.1-10% weight loss***							
3	10.1-15% weight loss***							
4	15.1-19.9% weight loss***							
5	20% weight loss***							
Food intake (g)								
0	Normal**							
1	5% less food intake							
2	5.1-10% less food intake							
3*	10.1-15% less food intake over several days							
4	15.1-19.9% less food intake over several days							
5	>20% less food intake							
Tumor Ulceration		Presence			Absence			

Animal necropsy result

10th October 2013

History

Rat M17 was reported to be 12 weeks old and had been dosed with a carcinogen 7 weeks previously. In the last few days the rat had been reported to be anorexic and had lost approximately 10% of its body weight since it was last weighed at the weekend.

Gross examination

This rat was presented dead on the 8th of October. She was judged to be in a poor state of nutrition, adequately hydrated, and in a good state of preservation. Careful examination of the skin over the ventrum of the rat did not reveal the presence of any evidence of mammary hyperplasia or neoplasia. The most significant lesion observed during gross examination of this rat was visible on examination of the abdomen. Here, the liver was observed to be markedly enlarged and occupied approximately a half of the abdominal cavity. The liver weighed 37 grams and was pale. Samples of liver did not float when placed in formalin. The intestinal tract contained little ingesta with few feces visible in the colon. The spleen appeared mildly enlarged. No other significant lesions were visible on gross examination of this rat.

Histology

Only sections of liver were examined histologically. Here, large numbers of a moderately monomorphic population of small to medium-sized lymphocytes has diffusely infiltrated the hepatic parenchyma. The neoplastic cells have resulted in disruption of normal hepatic architecture and marked separation of hepatic cords is visible within the sections. There are greater numbers of neoplastic lymphocytes within the sections than hepatocytes. This massive infiltration of the liver explains the marked increase in organ size and weight.

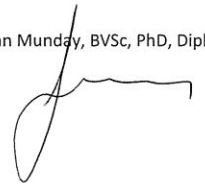
Diagnosis

Liver, lymphoma.

Comments

Lymphoma is moderately common in rats, although unusual in such a young animal. As only one rat has so far been affected, it is not possible to determine whether the development of this neoplasm was related to the carcinogens administered in this experiment. However, if additional neoplasms of this type are observed, it should be strongly suspected that the lymphoma has been predisposed to by the experimental manipulation.

John Munday, BVSc, PhD, Diplomate ACVP



List of Presentation

Vuthijumnonk, J., Molan, A. L., & Heyes, J. A. (2012). Free radical scavenging and anti-angiogenic properties of three rabbiteye blueberry extracts. (abstract) Proceedings of the Nutrition Society of New Zealand, 36, 88-88. Poster presentation at the Nutrition Society of New Zealand Conference, Auckland, New Zealand.

Vuthijumnonk, J., Heyes, J.A., Wolber, F.M., Chua, W-H., Molan, A.L. (2014). *In vitro* and *in vivo* prebiotic and antimicrobial activities of rabbiteye blueberry extracts Poster presentation at the Nutrition Society of New Zealand Conference, Queenstown, New Zealand.

Vuthijumnonk, J., Heyes, J.*A.,* Wolber, F.M., Chua, W-H., Molan, A.L. (2015) Biological activities of rabbiteye blueberries 'Maru' and their chemopreventive effect on 7,12-dimethylbenz[a]anthracene-induced mammary tumorigenesis in rats. (abstract) Oral presentation at the NZIAHS/IAPB Plants for the Future Conference, Palmerston North, New Zealand.

Vuthijumnonk, J., Heyes, J.A., Wolber, F.M., Chua, W-H., Molan, A.L. (2015*) In vitro* and *in vivo* antimicrobial activity of blueberry pomace and modulation of β-glucuronidase activity in animal model of breast cancer (abstract) Poster presentation. Winner of "the best student poster presentation" prize at at the NZIAHS/IAPB Plants for the Future Conference, Palmerston North, New Zealand.