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

A thesis presented in partial fulfilment of
the requirements for the degree of

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JANYAWAT VUTHIJUMNONK

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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

16 α -OHE1	16 α -hydroxyestrone
2-MeOE2	2-methoxyestradiol
2-OHE2	Hydroxyestradiol
4-OHE2	4-hydroxyestradiol
5%FA	5% aqueous formic acid
AAPH	2,2' azobis(2-methylpropionamide) 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-1,4:6,3-dilactone
DCIS	Ductal carcinoma in situ
DMBA	7,12-dimethylbenz[a]anthracene
E1	Estrone
E2	17 β -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 ₂ O 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
Na ₂ CO ₃	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