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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 \textit{Lactobacillus. rhamnosus} and \textit{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 (\textit{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 (\textit{Lactobacillus} spp., \textit{Bifidobacterium} spp. and \textit{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.
Acknowledgements

One of the greatest creations in nature is the human body. How the human body functions and responds to its environment is something I am passionate about and was the primary reason I decided to pursue a PhD in this area. A PhD study is a great journey where you meet many new people, learn new concepts, have to step out of your comfort zone and finally reach your destination. I would not have been able to reach my destination without the support from a number of people I have met along the way.

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<tbody>
<tr>
<td>16α-OHE1</td>
<td>16α-hydroxyestrone</td>
</tr>
<tr>
<td>2-MeOE2</td>
<td>2-methoxyestradiol</td>
</tr>
<tr>
<td>2-OHE2</td>
<td>Hydroxyestradiol</td>
</tr>
<tr>
<td>4-OHE2</td>
<td>4-hydroxyestradiol</td>
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<tr>
<td>5%FA</td>
<td>5% aqueous formic acid</td>
</tr>
<tr>
<td>AAPH</td>
<td>2,2’ azobis(2-methylpropionamidine)dihydrochloride</td>
</tr>
<tr>
<td>AB</td>
<td>Alveolar bud</td>
</tr>
<tr>
<td>ACY</td>
<td>Total anthocyanins</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AV</td>
<td>Alveoli</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CAM</td>
<td>Chicken chorioallantoic membrane</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CE</td>
<td>Catechin equivalent</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CGA</td>
<td>Chlorogenic acid concentration</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>C&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>Cytochrome P450 1A1</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>Cytochrome P450 1B1</td>
</tr>
<tr>
<td>DAGDL</td>
<td>2,5-di-O-acetyl-D-glucaro-1,4:6,3-dilactone</td>
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<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
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<td>DMBA</td>
<td>7,12-dimethylbenz[a]anthracene</td>
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<tr>
<td>E1</td>
<td>Estrone</td>
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<td>E2</td>
<td>17β-estradiol</td>
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<tr>
<td>E3</td>
<td>Estriol</td>
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<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FB</td>
<td>Frozen berries</td>
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<tr>
<td>FeCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Ferric chloride</td>
</tr>
<tr>
<td>FeSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Iron (II) sulfate</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FL</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>FOS</td>
<td>Fructooligosaccharides</td>
</tr>
<tr>
<td>FRAP</td>
<td>Ferric reducing antioxidant power</td>
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</tbody>
</table>
GAE  Gallic acid equivalent
GPx  Glutathione peroxidase
H&E  Heamatoxylin and eosin
H₂O₂ 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
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>TAC</td>
<td>Total antioxidant capacity</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TE</td>
<td>Trolox equivalent</td>
</tr>
<tr>
<td>TEB</td>
<td>Terminal end bud</td>
</tr>
<tr>
<td>TFC</td>
<td>Total flavonoid concentration</td>
</tr>
<tr>
<td>TPC</td>
<td>Total phenolic concentration</td>
</tr>
<tr>
<td>TPTZ</td>
<td>2,4,6-tripyridyl-s-triazine</td>
</tr>
<tr>
<td>Trolox</td>
<td>6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factors</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XREs</td>
<td>Xenobiotic response elements</td>
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