Grape Sourced Bioactives: A Potential New Treatment Strategy for Intestinal Mucositis and Colon Cancer

BY

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Abbreviations

°C Celsius Degrees
AIF Apoptosis Inducing Factor
ANOVA Analysis of Variance
bwt Bodyweight
CD90 Cumulative dose at 90 min
cm Centimetre
CO₂ Carbon Dioxide
DMACA p-Dimethylaminocinnamaldehyde
DMEM Dulbecco’s Modified Eagle’s Minimum Essential Medium
DMSO Dimethylsulfoxide
DP Degree of Polymerization
DPBS Dulbecco’s Phosphate Buffer Saline
DSS Dextran Sulphate Sodium
EGCG Epigallocatechin Gallate
FC Folin-Ciocalteau
FCS Fetal Calf Serum
FRAP Ferric Reducing Antioxidant Power
g Gram
GA Gallic Acid
g/kg Gram per Kilogram
g/L Grams per Litre
GPC Gel Permeation Chromatography
GSE Grape Seed Extract
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% Inhibitory Concentration</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 Beta</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IRMS</td>
<td>Isotope Ratio Mass Spectrometry</td>
</tr>
<tr>
<td>JI</td>
<td>Junction of Jejunum and Ileum</td>
</tr>
<tr>
<td>kg</td>
<td>Kilograms</td>
</tr>
<tr>
<td>KJ</td>
<td>Kilojoules</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lethal Dose 50%</td>
</tr>
<tr>
<td>mDP</td>
<td>Mean Degree of Polymerization</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mg/kg</td>
<td>Milligram per Kilogram</td>
</tr>
<tr>
<td>mg/L</td>
<td>Milligram per Litre</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor-kappa B</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ODS</td>
<td>Overall Histological Disease Severity Score</td>
</tr>
<tr>
<td>PA</td>
<td>Proanthocyanidin</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-ADP-Ribose Polymerase</td>
</tr>
<tr>
<td>pH</td>
<td>Measurement of Acidity</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphoinositide 3-Kinase</td>
</tr>
<tr>
<td>QTY</td>
<td>Quantity</td>
</tr>
<tr>
<td>r²</td>
<td>Coefficient of Determination</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse Phase High Liquid Pressure Chromatography</td>
</tr>
<tr>
<td>SBT</td>
<td>Sucrose Breath Test</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>TNBS</td>
<td>2,4,6-Trinitrobenzene Sulfonic Acid</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor-Alpha</td>
</tr>
<tr>
<td>TPTZ</td>
<td>Tri[2-pyridyl]-s-triazine</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>wt</td>
<td>Weight</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per Volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per Volume</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µg/mL</td>
<td>Microgram per Millilitre</td>
</tr>
<tr>
<td>µL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
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Declaration

I, Ker Yeaw Cheah, certify that this work contains no material which has been accepted for the award of any other degree of diploma in any university of other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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I also give permission for the digital version of my thesis to be made available on the web, via the University digital research repository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Signed: ........................................ Date: ........................................

Ker Yeaw Cheah (Amy)
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Conference presentations arising from this thesis

Abstract:

KY Cheah, GS Howarth and SEP Bastian. Grape seed extract: a potential adjunct to chemotherapy?

1. *Australian Society for Medical Research, SA Scientific Meeting, Adelaide, June 2011.*

2. *Australian Gastroenterology Week (AGW), Brisbane, September 2011, Journal of Gastroenterology and Hepatology 2011: 26(Suppl 4).*

KY Cheah, GS Howarth and SEP Bastian. Grape seed extract improves parameters of small intestinal mucositis in a dose responsive manner in rats.

1. *Australian Society for Medical Research, SA Scientific Meeting, Adelaide, June 2010.*

2. *Postgraduate Symposium, School of Agriculture, Food and Wine, The University of Adelaide, September 2010.*


Abstract

Mucositis is a serious condition involving inflammation and ulceration to the lining of gastrointestinal tract that results from cancer chemotherapy. Grape seeds represent a rich source of proanthocyanidins (PAs) which have been reported to be strong antioxidant and chemopreventative agents. This thesis will examine in detail the potential for grape seed PAs to act as novel therapeutic adjunct in cancer treatment.

Previously, Cheah et al. (2009) demonstrated that grape seed extract (GSE; 400mg/kg) improved the parameters of intestinal damage in rats with experimentally-induced mucositis. However, its optimal dose and dose responsiveness remained undetermined. In the current study, the effects of increasing GSE doses (400, 600 and 1000mg/kg) on the severity of intestinal mucositis were investigated in a rat model. GSE at higher doses (600 and 1000mg/kg) were more effective than a lower dose (400mg/kg) at ameliorating intestinal injury induced by the chemotherapy agent, 5-Fluorouracil (5-FU) in the proximal small intestine. In addition, no deleterious effects of GSE at these doses were apparent in healthy animals.

The promising effects of GSE in a model of mucositis, and its anti-cancer activity, provided the impetus to further investigate its potential impact on the effectiveness of chemotherapy against colon cancer cells. It was decided to characterise the link between the chemical structures of the main polyphenolic compounds within GSE (PAs), and polyphenolic compounds (epigallocatechin, gallic acid (GA), resveratrol and catechin), which have all been reported to display growth inhibition on cancer cells. At a lower dose, GSE (25µg/mL) and GA (10µg/mL) significantly enhanced the capacity for 5-FU to reduce Caco-2 cell proliferation by 20-26%. Treatment with polyphenols alone, (with the exception of
Abstract

catechin) at higher doses, exerted more potent growth inhibitory activities compared to 5-FU alone.

Due to the protective effects of GSE against mucositis and the positive findings that GSE and GA reduced the viability of colon cancer cells, it was important to identify the bioactive compounds in GSE responsible for these effects. Six different PA fractions, with increasing mean degree of polymerization (mDP), were isolated from Cabernet Sauvignon grape seeds. GSE, which contained a mixture of oligomers and polymers of PAs, was included as a positive control. This study reported that smaller grape seeds PAs (mDP 2-6) were more effective chemotherapeutic agents than 5-FU alone against colon cancer and exerted greater cytotoxic activity on Caco-2 cells than the crude GSE.

As the health promoting properties of grape seeds are attributed to their PA content and GSE used in parts of this study were derived from multiple grape varieties, it was important to determine the PA profiles of grape seeds derived from different varieties and from different provenance. This study reported the difference in polyphenolic content, not only between grape varietals, but also between geographical regions. Despite Chardonnay and Tannat seeds from Nuriootpa had the highest total polyphenol and flavan-3-ol content, they were the most effective antioxidant agents. In conclusion, grape seeds are a rich source of PA (especially mDP 2-6) and may represent promising therapeutic adjuncts to conventional chemotherapy, which ameliorates mucositis.
Chapter 1 Grape seed proanthocyanidins and intestinal health: Bioavailability, mechanism of action and safety dosage
1.0 Abstract

Grape seeds have been receiving increased interest in nutritional and oncological research as a result of their potential to act as chemopreventive agents. Grape seeds are a rich source of proanthocyanidins (PAs). Certain PAs have been revealed to remain intact in the intestinal mucosa without being absorbed across the intestine. Grape seed extracts (GSE) rich in PAs therefore have the potential to be effective at promoting intestinal health. There is compelling evidence that GSEs are cytotoxic to intestinal cancer cells, yet exert anti-inflammatory activities in animal models of intestinal mucositis and ulcerative colitis. This review summarizes and highlights the latest status of the chemopreventive potential and efficacy of GSEs in the gut. Current toxicity studies demonstrate that GSEs are well tolerated and safe for consumption. Understanding the mode of action of GSE will strengthen the applicability of grape seed sourced extracts as novel adjuncts to conventional chemotherapy.
1.1 Introduction

Polyphenols are compounds commonly found in fruits, vegetables, seeds, nuts, and beverages, such as tea, coffee and wine. Polyphenols have documented beneficial effects on human health in a number of clinical settings [4]. For example, red wine reduced the mortality rate of cardiovascular disease [5], green tea has been documented to increase plasma anti-oxidant capacity in smoking patients [6], and cocoa promoted vascular health via modulation of inflammatory cytokine (IL-2, IL-4 and IL-1β) production [7]. The beneficial effects of these compounds have been attributed to the polyphenol content, including proanthocyanidins (PA)s [8]. PAs are a class of polyphenolic compounds that consist of oligomers or polymers of flavan-3-ol units [9].

PAs are deposited in the grape berry, skin, stalk and seeds, however, grape seeds contain the highest levels [10]. Grape seed extracts (GSEs) are receiving increased interest from scientists and consumers due to their purported health benefits for a variety of disorders [1, 11]. PAs have been reported to be detected in blood, liver, eye, gut and even the brain after consumption [12-14]. However, the absorption and bioavailability of PA in the gut is limited and poorly understood due to their highly polymerized structure (ranging from 2-12 subunits).

Grape seed PAs also show potential as preventative or therapeutic strategies against gastrointestinal disease. GSEs prevented ulcer formation in rat stomach [15], were cytotoxic to colon cancer cells [16], decreased severity of intestinal injury in a rat model of mucositis [17] and partially ameliorated colonic injury in a rat model of ulcerative colitis [18].

Previous studies suggested that absorption and metabolism of PAs is limited in the gut lumen, and PAs are attached to the intestinal epithelial wall, thereby increasing contact time.
with gut enterocytes, allowing them to exert their biological properties [19]. This review discusses the bioavailability of grape seed PAs in the gastrointestinal system and summarizes the latest findings on the chemopreventive potential and efficacy of GSE in the gut.
1.2 Grape seed sourced proanthocyanidins

Grapes are rich in polyphenols, although these vary in their distribution inside the grapes with less than 10% in the pulp, 60 - 70% deposited in seeds, and 20 - 35% in the skin [20]. Polyphenolic compounds can be categorized into flavanoid and non-flavanoid groups. However, this discussion will be limited to the flavanoid form only, of which PAs are a member. Flavanoids encompass flavanones, flavan-3-ols, flavonols, flavones and anthocyanidins (Figure 1.1) [1]. The major flavanoids in grape seeds are flavan-3-ol derivatives. Flavan-3-ol units have the typical C₆-C₃-C₆ flavanoid skeleton made up of 2 aromatic rings with both rings linked by a 3 carbon and oxygen containing a pyran ring [9]. Flavan-3-ol derivatives in grape seeds include monomers (catechins, epicatechin, epicatechin-3-O-gallate) and PA oligomers and polymers (also known as condensed tannins) [9, 21]. Catechin and epicatechin possess similar structures, although they exist as steric isomers. PAs are oligomers and polymers of flavan-3-ols which range from dimers to larger subunits (more than 10) and they are formed via linking the monomers through C₄-C₈ or C₄-C₆ positions (Figure 1.2) [2]. Grape seeds comprise a wide range of PAs (ranging from monomers to decamers) in variety and quantity compared to other food sources such as apples, chocolates and tea [10].

1.3 Isolation and identification of PA

Recent studies suggest that PA health benefits are greatly dependent upon their degree of polymerization (DP) [11, 22] and galloylation [23, 24]. Oligomeric PAs have been reported to be more effective radical scavengers compared to the monomers, catechin and epicatechin; reportedly due to the presence of large numbers of hydroxyl groups on the oligomers [9]. In addition, Lizarrage et al. [23] demonstrated that PAs with the highest percentage of galloylation and mean DP were more effective than PAs with a lower
percentage of galloylation and lower mean DP at inducing apoptosis in Caco-2 colonic cells. Thus, understanding DP and galloylation of PA is crucial in determining its biological activity in vivo.

Several chromatographic approaches have been developed to separate PAs based on their DP. One of the methods is cleavage of PA subunits via acid catalysis in the presence of a nucleophile [25], also known as phloroglucinolysis. This analysis is performed by reverse-phase high-performance liquid chromatography (HPLC) and provides mean DP, average molecular mass and percentage of galloylation of intact PA. Another method utilizes gel permeation chromatography (GPC), whereby the mass distribution of PAs is provided [26]. With these technologies, detailed PA profiles can be obtained easily from any food sources [10].

1.4 Bioavailability of GSE PAs

Absorption, metabolism and degradation of PAs contribute to PA bioavailability [27]. The bioavailability of these compounds is generally governed by their chemical structures and DP. Upon ingestion of PAs, these compounds react with proline-rich proteins in the mouth and the oral mucosa to elicit astringent responses [28] leaving the remainder to reach the intestinal barrier. Certain forms of PAs (DP < 3) will cross the intestinal barrier and be transported to the liver via the vasculature, where they may reach all tissues within hours following ingestion as reported in radio-labelled experiments with live rats [2].

The current available data on the absorption and metabolism of larger PAs in the gut lumen are limited [11, 29, 30]. Due to their large molecular weight, only certain smaller molecular weight compounds are absorbed during transit in the gut, predominantly leaving the larger
forms poorly absorbed in the small intestine. Deprez et al. [31] tested the absorption of
cadio-labelled PAs by Caco-2 human colonic cell lines grown on filter membranes. These
investigators reported that lower molecular weight PA oligomers (average degree of
polymerization ≤ 3) could be absorbed intact across a model of the gastrointestinal tract,
while the absorption of larger oligomers (average degree of polymerization = 7) was 10
times lower, suggesting that polymers are not likely to be readily absorbed in vivo. This
study was later supported by other studies in which monomers and dimers were detected in
the plasma of rats [29, 32] and humans [33] following ingestion of PA rich foods. However,
the polymers were still present in the small intestine up to 12 h after ingestion in rats [29].
These polymers are metabolized by colonic microflora into lower molecular-weight
metabolites, such as phenolic acids and further reabsorbed through the colonic barrier [2,
31]. Polymers have also been shown to be degraded into lower molecular-weight phenolic
acids by human colonic intestinal microflora following 48 hours of anaerobic conditions
[34]. This study was later confirmed by other studies in which metabolites were detected in
human urine following 24 hours of consumption of PA rich foods (Figure 1.3) [3].

1.5 Biological activities of GSE in the intestine

The reported health benefits of PAs in the gut could be explained by direct effects of the
PAs in their parent form or the effects of metabolites formed in the colon [3, 35]. Due to
their limited absorption through the gut barrier, the highest concentration of PA is found in
the gut lumen [11, 29, 36]. Some PAs could form a protective layer on the gut mucosa to
protect against oxidative and carcinogen-induced damage or they may be degraded into
smaller metabolites and reabsorbed across the colonic wall to exert anti-oxidant, anti-
inflammation or anti-carcinogenic biological activities in the colon [35].
1.5.1 GSE and colorectal cancer

Several studies suggest that consumption of grape seeds could provide beneficial chemopreventive effects [37]. For example, GSE has been shown to inhibit breast cancer cell proliferation and tumour growth [38], and to induce apoptosis in lung [39], prostate [40], and gastric cancer cell lines [41]. However in particular, GSE has demonstrated chemopreventive effects against colon cancer in *in vitro* settings (Table 1.1).

Colorectal cancer is the third most common cause of cancer-related mortality in Western countries and, despite improvements in the management of colon cancer patients, there has been little change in survival rates [42, 43]. GSE has been reported to reduce cell proliferation in colon cancer cell lines (HT-29, LoVo, Caco-2) in a dose- and time-dependent manner [44, 45] and also to reduce tumour volume in athymic nude mice [46]. GSE has further been demonstrated to selectively exert cytotoxic effects towards colorectal cancer cells (Caco-2) while maintaining the growth of normal colon cell lines (NCM-460) [47].

GSE treatment induced growth inhibition of cancer cells via the induction of G1 phase cell cycle arrest, eventually leading to induction of caspase-dependent activation of apoptosis, through increased caspase 3 activity and enhanced cleavage of poly-ADP-ribose polymerase (PARP) [44, 47]. This mechanism was later supported in a study by Hsu *et al.* [16] in which GSE inhibited growth of colorectal cancer cells associated with apoptosis involving the loss of mitochondrial membrane potential and caspase-3 activation. However, a recent study by Dinicola *et al.* [45] suggested the apoptosis pathway is not driven by caspase activity only, but also through enhancement of apoptosis inducing factor (AIF) [45]. The current findings
suggest that GSE could be a potential anti-cancer agent against colorectal cancer, although further human trials are required to determine its safety and appropriate dosage.

1.5.2 GSE and prevention of intestinal mucositis

High dose chemotherapy often results in serious ulceration and inflammation of the small intestine in cancer patients (known as intestinal mucositis) [48]. Intestinal mucositis is characterized by pain, nausea and diarrhoea with a resultant of cell cycle arrest and loss of gut barrier function. These factors may necessitate treatment reduction or withdrawal.

Studies of intestinal mucositis in rat models have indicated that oral ingestion of GSE is effective at preventing and treating intestinal inflammation [17, 49, 50]. Intestinal mucositis was induced in these studies by intraperitoneal injection of 5-Fluorouracil (5-FU) or intramuscular injection of Methotrexate (MTX). Rats were administered GSE by oro-gastric gavage before, during and after the induction of disease. They were euthanized, and indices of disease assessed from the intestinal tissues. GSE reduced intestinal myeloperoxidase activity (a marker of neutrophil infiltration) and maintained intestinal morphology (villus height) [17]. Gulgun et al. [50] showed that GSE reduced jejunal damage and intestinal tissue malonaldehyde levels and increased superoxide dismutase and glutathione peroxidase levels (cellular antioxidant enzymes) compared to MTX treated controls.

Therefore, the possible mechanisms by which GSE protects the intestine could involve reduction of oxidative stress, via radical scavenging through increased glutathione synthase and superoxide dismutase activity, thereby decreasing malonaldehyde. GSE further modulates inflammatory responses by reducing the activation of acute inflammatory cells such as the neutrophil. Although GSE is effective in treating specific elements of intestinal
injury arising from chemotherapy, further studies are required to investigate GSE for
efficacy in different temporal administration regimens, to determine the optimal time-points
and time-frames for administration of GSE.

1.5.3 GSE and prevention of colonic injury

Inflammatory bowel disease (IBD) is the collective term for a group of idiopathic diseases
which affect the gastrointestinal tract and include ulcerative colitis and Crohn’s disease.
Ulcerative colitis presents as episodic inflammation and ulceration of the large bowel
beginning distally in the rectum and progressing towards the proximal colon \[51\]. Patients
with ulcerative colitis have recently been shown to exhibit high expression levels of NF-κB,
a transcription factor for pro-inflammatory cytokines, together with high levels of reactive
oxygen species (ROS) \[52\].

In recent years, rodent models of acute and chronic colitis have been developed by addition
of dextran sulphate sodium (DSS) to the drinking water \[53\], intracolonic injection of 2,4,6-
trinitrobenzene sulfonic acid \[54\] or dinitrobenzene sulphate \[55\]. However, to date only a
few experimental colitis studies have been conducted with GSE treatment. GSE has
demonstrated an improved rate of recovery in an experimental model of acute ulcerative
colitis \[54\]. Rats were intragastrically administered GSE (100, 200, and 400 mg/kg) for 7
days after colonic inflammation was induced by intracolonic injection of 2,4,6-
trinitrobenzene sulfonic acid (TNBS) dissolved in 50% ethanol \[54\]. GSE treatment
improved the recovery of pathologic changes in the colon, as demonstrated by increased
bodyweight and a reduction of myeloperoxidase activity and malonaldehyde levels in both
colonic tissue and rat serum in GSE + TNBS groups. GSE exerted anti-inflammatory effects
in this model of TNBS-induced colitis via the increase of anti-inflammatory cytokines (IL-2
and IL-4) and a decrease in pro-inflammatory cytokines such as IL-1β in the colonic tissues, thereby, inhibiting inflammatory cell infiltration and anti-oxidative damage.

GSE also improved certain parameters of inflammation in the DSS-colitis model [18]. Rats received GSE treatment (400 mg/kg) daily and ulcerative colitis was induced by substituting drinking water with 2% DSS for 5 days. GSE treatment significantly decreased ileal villus height back toward normal values and reduced proximal colon disease severity compared to DSS treated controls. The effects of GSE on induced colitis were more pronounced in the distal ileum, becoming less effective in the distal colon. The decline of GSE activity in the intestine could be due to degradation of the bioactive components in GSE by the endogenous microflora. Administration of GSE in greater quantities might therefore extend to protection of the distal colon.

1.5.4 GSE and protection against gastric ulceration

GSE (200 mg/kg) has been demonstrated to exert anti-ulcer activity in the stomach of rats [15]. Two different fractions of GSE were prepared based on their average molecular weight and with high levels of flavanols. These conferred superior protection against stomach injury compared to GSE with low levels of flavanols. Saito et al. [15] also showed that lower molecular weight flavan-3-ol (monomers, dimers and trimers) failed to protect against stomach injury induced by 60% ethanol containing 150 mM hydrochloride. However, larger molecular weight flavan-3-ol oligomers (tetramer, pentamer and hexamer) were shown to exert anti-ulcer activities in vivo. This study concluded that different degrees of polymerization of PAs may have exerted differential effects on the gastric mucosa, whereby larger-sized PAs were able to form a physical barrier by binding proteins on the surface of the rat stomach and thus, prevent penetrating ulcer formation in the gastric mucosa.
1.5.5 GSE metabolites and promotion of colonic health

The degree of polymerization (DP) of PAs plays a major role in their fate in the body [11]. PAs are degraded and conjugated into methylated, sulfonated and glucuronidated forms during transition through the gut [2], and therefore the active compounds may be the native PAs found in foods and/or their metabolites. Larger forms of PAs are poorly absorbed in the small intestine [2], and degraded by the colonic microflora into lower molecular weight aromatic acid metabolites (Figure 1.4) which are subsequently absorbed through the colonic barrier [2, 34]. Phenylacetic, phenylpropionic, and benzoic acids were detected in urine and fecal samples of humans and rats [2, 3, 56] following consumption of PA-rich foods. These PA metabolites, bearing a free phenolic group, can act as potent radical scavengers. To date, however, there have been limited studies of GSE metabolite activity in the gut.

PA metabolites are reported to inhibit colon cancer cell proliferation [57]. 3,4-dihydroxyphenylacetic acid at 50 µmol/L significantly reduced colon cancer cell (HCT 116) proliferation to 75% of control values but did not affect cell viability of normal intestinal cells (IEC-6) [57]. These investigators proposed that 3,4-dihydroxyphenylacetic acid selectively inhibited nuclear factor-κB activation in carcinoma cells, but not in normal cells. This study, for the first time, showed that PA metabolites could be responsible for promoting gut health, encouraging further studies to apply PA metabolites in in vitro studies in order to subsequently translate the effects to the in vivo setting.

1.6 Safe dose and toxicity

Paradoxically, PA rich foods have been reported to be both beneficial and detrimental to human health due to their ability to differentially interact with proteins (enzymes, toxins,
hormones) [1, 58]. For example, a reduction of bodyweight in mice has been reported after consumption of tannin rich food [11].

Acute oral and dermal toxicity, and primary dermal and eye irritation studies have been conducted with grape seed PAs [59]. The acute LD$_{50}$ of grape seed PAs was found to be greater than 5000 mg/kg when administered orally to rats via gastric intubation [60]. This study reported no observed toxicity effects in rats for a period of 14 days. Yamakoshi et al. [61] reported that administration of grape seed PAs to rats at an acute dose of 4000 mg/kg for 14 days and a sub-chronic dose of 2 g/kg for 90 days did not display any toxic effects. This was further supported by a study in which rats were fed up to 2 g/kg bodyweight of GSE for 90 days, showing no reduction in bodyweight or toxicological effects [62]. Furthermore, studies conducted by Cheah et al. [17] revealed that ingestion of GSE (400 mg/kg) for 9 days did not affect well-being in healthy animals. Importantly, GSE (at doses up to 100 µg/mL) did not affect the cell viability of normal colon epithelial cells but significantly reduced proliferation of cancer cells in a dose- and time-dependent manner [47], suggesting potential applicability in the adjunctive treatment of bowel cancer.

1.7 Conclusions

Research studies to date reveal GSE sourced PAs to be safe and novel therapeutic agents which could be used potentially to improve intestinal health. Moreover, grape seed PAs could represent promising new anti-cancer agents as a result of their anti-cellular proliferation and anti-tumour formation properties in cancer cell lines. Grape seed PAs are also reported to be potential new chemotherapy adjunctive agents due to their capacity to combat intestinal mucositis. However, grape seed PAs, and particularly GSE metabolites, should be studied in greater detail before further development as agents to aid in the
treatment of gastrointestinal diseases. Identification and purification of the bioactive components of GSE PAs would be essential to optimize formulation of these treatments for medical or nutritional purposes, as there is currently a lack of information of the influence of chemical structure and sizes of PAs in regards to protective effects in the gut. Furthermore, mechanistic and clinical studies are required to unravel the mechanism of action of GSE.
Table 1.1 Current published studies on GSE in the context of colon cancer treatment

<table>
<thead>
<tr>
<th>Model</th>
<th>GSE dose</th>
<th>In vitro effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caco-2</td>
<td>400 mg/L</td>
<td>GSE reduced cancer cell proliferation</td>
<td>[63]</td>
</tr>
<tr>
<td>HT-29</td>
<td>25-100 µg/mL</td>
<td>GSE results in dose- and time-dependent inhibition of cell growth leads to cell apoptosis</td>
<td>[46]</td>
</tr>
<tr>
<td>Athymic nude mice</td>
<td>200 mg/kg</td>
<td>GSE reduced tumour volume in a time dependent manner</td>
<td>[46]</td>
</tr>
<tr>
<td>Caco-2</td>
<td>10-100 µg/mL</td>
<td>GSE did not affect the growth of non-cancerous cells but decreased cancer cell viability</td>
<td>[47]</td>
</tr>
<tr>
<td>Lovo, HT-29, SW480</td>
<td>25-100 µg/mL</td>
<td>GSE inhibited cancer cell growth via induction of cell cycle arrest</td>
<td>[44]</td>
</tr>
<tr>
<td>Lovo, HT-29, SW480</td>
<td>10-50 µg/mL</td>
<td>GSE induced cancer cell apoptosis via loss of mitochondrial membrane and activation of caspase 3</td>
<td>[16]</td>
</tr>
<tr>
<td>Caco-2</td>
<td>25-100 µg/mL</td>
<td>GSE induced cell apoptosis via the induction of early apoptosis-inducing factor and enhancement of caspase activity</td>
<td>[45]</td>
</tr>
</tbody>
</table>
Figure 1.1  Molecular structures of flavanoid subgroups which include flavones (e.g. apigenin and chrysin), flavonol (e.g. quercetin and myricetin), flavanone (e.g. butin and sterubin), flavan-3-ol (e.g. catechin and epicatechin), flavanonol (e.g. taxifolin and aromadedrin) and anthocyanidins (e.g. cyanidin and malvidin). Figure adapted from [1].
Figure 1.2 Molecular structures of flavan-3-ol: monomers (Catechin), dimers and trimers where PA oligomers are linked either between C4-C6 linkage or the C4-C8 linkage. Figure adapted from [2].
Figure 1.3  Molecular structures of aromatic acid metabolites detected in human urine after consumption of PA rich foods. diHPP, 3,4-dihydroxyphenylpropionic acid; mHPP, m-dihydroxyphenylpropionic acid; FE, Ferulic acid; diHPAs, 3,4-dihydroxyphenylacetic acid; mHPAs, m-hydroxyphenylacetic acid; PAc, phenylacetic acid; VA, vanillic acid; mHBA, m-hydroxybenzoic acid; pHBA, p-hydroxybenzoic acid; pHHA, p-hydroxyhippuric acid; HA, Hippuric acid. Figure adapted from [3].
Figure 1.4 Metabolic pathway of catechin degradation by colonic microflora into lower molecular weight aromatic acid. Figure adapted from [2].
1.8 Aims and Hypotheses

1.8.1 Aims

The current PhD project addressed four aims:

1. To determine the optimal and effective doses of GSE required to prevent intestinal damage induced by chemotherapeutic anti-metabolite drugs in rats.
2. To investigate if GSE and other polyphenols would affect chemotherapy effectiveness on colon cancer cells.
3. To identify the most bioactive fractions of grape seed PAs responsible for protection of the intestine.
4. To compare the PA profile of grape seed derived from different varieties of wine grapes.

1.8.2 Hypotheses

The hypotheses of this study were that,

1. GSE will dose responsively reduce intestinal damage in a rat model of mucositis.
2. GSE will not affect the capacity of chemotherapy, 5-FU in inducing growth inhibition on Caco-2 cells.
3. Smaller PAs with (mDP2-6) are more effective in exerting cytotoxic effects on Caco-2 cells than larger polymers.
4. Grape seeds sourced from different varietal will have different PA profiles.
Chapter 2 Grape seed extract dose-responsively decreased acute inflammation and improved mucosal morphology in a rat model of mucositis
2.0 Abstract

Background: Mucositis is a serious disorder of the gastrointestinal tract that results from cancer chemotherapy. Previously, grape seed extract (GSE) at a single dose (400 mg/kg) partially reduced certain indicators of intestinal damage in a rat model of mucositis; however its optimal dose and dose-responsiveness have not been determined. Objectives: We investigated the effects of increasing GSE doses on the severity of intestinal mucositis in a rat model. Methods: Female Dark Agouti rats (100 - 140 g) were oro-gastrically gavaged with GSE (400 mg/kg, 600 mg/kg and 1000 mg/kg) or water (day 3 - 11) and were injected intraperitoneally with 5-FU (150 mg/kg) or saline (control) on day 9 to induce mucositis. Daily metabolic data were collected and rats were sacrificed on day 12. Intestinal tissues were collected for histological (disease severity score, villus height and crypt depth) and myeloperoxidase analyses. Statistical analyses were performed by one-way ANOVA. Results: Compared with 5-FU controls, GSE significantly, and dose-responsively, decreased the histological damage score ($P < 0.05$) in the jejunum. GSE (1000 mg/kg) increased jejunal crypt depth by 25% ($P < 0.05$) in 5-FU treated rats compared to 5-FU controls, and attenuated the 5-FU-induced reduction of mucosal thickness (31%, $P < 0.05$). GSE (600 mg/kg) administered to 5-FU injected rats significantly decreased MPO activity by 55% ($P < 0.01$) compared to 5-FU treated controls. Conclusions: GSE was dose-responsively effective at ameliorating intestinal injury induced by the chemotherapy agent, 5-FU, with effects most pronounced in the proximal jejunum. In addition, no deleterious effects of GSE were apparent in normal animals, indicating its safety for human administration. GSE may represent a new therapeutic option to decrease the symptoms of intestinal mucositis.
2.1 Introduction

Mucositis is a serious, debilitating consequence of cancer therapy, which significantly reduces quality of life in cancer patients [48]. Mucositis is a painful condition associated with inflammation and ulceration of the gastrointestinal tract; most commonly affecting the mucosa of the mouth (oral mucositis) and small intestine (intestinal mucositis). Intestinal mucositis is characterized by reduced enterocyte proliferation and increased apoptotic rate of crypt cells, resulting in malabsorption and disrupted barrier function [64, 65]. Symptoms of mucositis include intense pain, diarrhoea, nausea, vomiting and anorexia. Often there is an increased risk of bacterial infection with associated mortality and morbidity [48]. Sometimes, gastrointestinal toxicity may lead to a reduction, or even termination, of the chemotherapy regimen [66]. Currently, there is no effective treatment for intestinal mucositis. However, a number of alternative therapies have been developed including a whey-derived growth factor extract [67], keratinocyte growth factor [68], velafermin (fibroblast growth factor-20) [66] and, more recently, emu oil [69]. However, these treatments are still under experimental or clinical investigation, and are not yet available to cancer patients.

Grape seed extract (GSE) is consumed widely as a dietary supplement on the basis of its potent anti-oxidant [70], anti-inflammatory [71] and purported, anti-cancer [45] activities. Proanthocyanidins are believed to be the key bioactive constituents in GSE [10, 11], which are also widely found in other food sources such as tea, apples and red wine [10]. Proanthocyanidins are a class of polyphenolic compounds that are composed of flavan-3-ol subunits (oligomers and polymers) [72]. Several studies have reported that the absorption and bioavailability of proanthocyanidins in the gut is dependent upon their chemical structure and degree of polymerization [35]. Proanthocyanidins (degree of polymerization =
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7 or higher) are retained in the intestinal tract, thereby increasing contact time with gut enterocytes to promote intestinal health [11]. A number of studies have described the biological properties of GSE-sourced proanthocyanidins in the gut. For example, GSE has been reported to reduce colonic inflammation in a rat model of ulcerative colitis [54] and also to be toxic to colon cancer cells [16].

Previously, GSE reduced intestinal damage in in vitro and in vivo models of intestinal mucositis [73]. However, the optimal dose required to achieve maximal therapeutic benefit, dose-responsiveness and safety of this effect remained undefined. Accordingly, the current study has investigated high doses of GSE for their potential to safely reduce the severity of intestinal mucositis in a rat model.
2.2 Materials and methods

2.2.1 Grape seed extract (GSE) preparation

Grape seed extract was kindly donated by Tarac Technologies (GrapEX seed tannin; South Australia, Australia) and stored in an air-tight, light-resistant pack until being dissolved in distilled water prior to use. The GSE was derived from condensed tannin made from Australian white wine marc (residual skins and seeds from winemaking). The nutrition and chemical profile of the proanthocyanidin content in GSE is listed in Table 2.1.

2.2.2 Animal studies

Female Dark Agouti rats (100 - 140 g, n = 64) were housed in individual metabolic cages (Tecniplast, Exton, PA, USA) in a temperature-controlled room (22°C) with a light-dark cycle of 12 h. Rats were given *ad libitum* access to water and food (18% casein-based diet) [74] in the Animal Care Facility of the Children, Youth and Women’s Health Service, North Adelaide, South Australia. This study followed the Australian Code of Practice for the Care and Use of Animals for Scientific purposes and was approved by both the Animal Care and Ethics Committees of the Children, Youth and Women’s Health Service and University of Adelaide.

Rats were randomly allocated to 8 groups (n = 8) : Water + Saline injection; GSE 400 mg/kg+ Saline injection; GSE 600 mg/kg + Saline injection; GSE 1000 mg/kg+ Saline injection; Water + 5-FU injection; GSE 400 mg/kg + 5-FU injection; GSE 600 mg/kg + 5-FU injection; and GSE 1000 mg/kg + 5-FU injection (150 mg/kg; MaynePharma Pty Ltd, Mulgrave, Vic, Australia). Rats acclimatized in cages from day 0 - 2 and were gavaged (1 mL) with GSE dissolved in water (400 mg/kg, 600 mg/kg or 1000 mg/kg) or water from day 3 - 11. At day 9, all rats were intraperitoneally injected with either 5-Fluorouracil or saline.
Daily measurements of body weight, food and water intake, and urine and faecal output were recorded. Rats were sacrificed by CO$_2$ asphyxiation followed by cervical dislocation on day 12. All visceral organs were weighed and discarded. The lengths and weights of the gastrointestinal organs (duodenum, small intestine and colon) were recorded. Representative samples (2 cm) of gastrointestinal organs were collected and fixed in 10% buffered formalin for histological analyses. Further 4 cm samples were snap frozen in liquid nitrogen and stored at -80°C for biochemical analysis.

### 2.2.3 $^{13}$C-sucrose breath test (SBT)

The SBT is an indirect measure of intestinal sucrase activity and was performed according to the method described by Tooley et al. [75]. SBTs were conducted by placing the rats in a 600 mL sealed Perspex container for 2 min to collect expired breath. The breath was collected by a 20 mL syringe which was attached to a two-way tap fitted to the chambers. The collected breath was later evacuated to a 10 mL labelled glass tube as baseline breath samples (T0). The container was then opened to re-equilibrate to room air. Next, rats were oro-gastrically gavaged with 1 mL of sucrose solution containing $^{13}$C (250 mg/kg) and immediately placed back in the container. 5 min later, the container lids were shut for a period of 2 min to collect the expired breath. The breath samples were collected at 15 min intervals for the next 120 min. Breath samples were analyzed for $^{13}$CO$_2$ content by isotope ratio mass spectrometry (IRMS) equipped with a V410 data collection system (Europa Scientific, ABCA 20/20, Crewe, United Kingdom). The data were expressed as percentage cumulative dose at 90 min (%CD90) by calculating the change in breath $^{13}$CO$_2$ levels from baseline for each time point of breath collection throughout the period of interval sampling. SBT determinations were performed at day 3 (before GSE treatment), day 9 (before 5-FU injection) and day 12 (before kill).
2.2.4 Myeloperoxidase (MPO) assay

Small intestinal tissue samples (4 cm) of jejunum, junction of jejunum and ileum (JI) and ileum were thawed on ice and homogenized with 1.5 mL of phosphate buffer (10 mM, pH 6.1) for 60 seconds until the solution was homogenous. The homogenates were kept frozen at -80°C until required.

MPO is an enzyme present in the intracellular granules of neutrophils, acting as an acute inflammation marker. The level of MPO in the small intestine was determined by a slight modification of the assay described by Krawisz et al. [76]. Tissue homogenates were thawed on ice and centrifuged at 13000 g for 13 min. The supernatant was discarded and cell pellets were resuspended in hexadecyltrimethyl ammonium bromide (HTAB; 0.5%, pH 6.0). The samples were vortexed for 2 min and further centrifuged at 13000 g for 3 min. Supernatants were reacted with o-dianisidine reagent (280 mg/mL o-dianisidine and 0.0005% hydrogen peroxide in phosphate buffer; Sigma Chemical Co. Ltd, St Louis, MO) whereby MPO oxidizes the hydrogen peroxide to form oxygen radicals which react with o-dianisidine to form a coloured compound. The absorbance measured at 450 nm at 1 min intervals for a period of 15 min using a microplate reader (Sunrise Microplate Reader, Tecan Austria GmbH, Grodig, Austria). MPO activity was expressed as Units MPO activity per gram of tissue.

2.2.5 Histological analysis

Gut tissue samples (2 cm) were embedded in paraffin wax and 4μm sections were stained with haemotoxylin and eosin. The overall histological disease severity score (ODS) of intestinal sections was rated semi-quantitatively (0 - 3) based on 11 independent histological criteria according to a protocol described by Howarth et al. [77] (Table 2.2). Saline control
rat intestinal tissue was used as a baseline reference to grade each of the 11 parameter criteria. The ODS was expressed as a median score for each criteria.

Villus heights and crypt depths (40 villi and 40 crypts per section) were determined in the small intestinal sections including jejunum, junction of jejunum and ileum (JI) and ileum as described in Howarth et al. [77]. The combined measurement of villus heights and crypt depths provided an approximation of total mucosal thickness in each small intestinal specimen. All microscope-based analyses were performed in a blinded fashion using a light microscope (Nikon, ProgRes®CS, Tokyo, Japan) and image ProPlus software version 5.1 (Media Cybernetics, Silver Spring MD, USA).

2.2.6 Statistical analyses

Statistical analyses were conducted using PASW 18 (SPSS, Inc., Chicago, IL, USA). All parametric data including bodyweight, daily metabolic data, SBT, MPO and villus height and crypt depth were compared using analysis of variance (ANOVA) with a Tukey’s post-hoc test. The overall disease severity score (ODS) was compared by a Kruskal-Wallis test with a Mann Whitney U-test to identify significance between groups. Data were considered significant at \( P < 0.05 \).
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2.3 Results

2.3.1 Daily metabolic parameters and bodyweight

Oral administration of GSE (400, 600 or 1000 mg/kg) between days 3 and 9 did not significantly affect body weight, food or water intake and urine or faecal output compared to rats receiving water (Table 2.3A). 5-FU injection significantly increased water intake and urine output and reduced body weight, food intake and faecal output compared to rats receiving saline injection between days 10 and 12 (Table 2.3B). GSE 600 and 1000 mg/kg significantly returned faecal output ($P < 0.01$) in 5-FU treated rats, back towards the values for normal saline-injected control rats.

2.3.2 Visceral organs

5-FU injection significantly reduced thymus weight by 51% ($P < 0.001$) and spleen weight by 20% ($P < 0.01$) compared to saline-injected rats. GSE did not prevent any of the 5-FU-induced changes in thymus and spleen weight. None of the GSE doses impacted on visceral organ weights (Table 2.4) and gastrointestinal organ weights and lengths (Table 2.5) in healthy animals. However, GSE 1000 mg/kg significantly increased stomach weight by 13% ($P < 0.05$) compared to normal controls.

2.3.3 Sucrose breath test (SBT)

5-FU injection significantly ($P < 0.001$) decreased the SBT (%CD90) by 70% compared to water + saline treated rats, indirectly, indicating 5-FU injection had disrupted brush border sucrase activity (Figure 2.1). There were no significant differences in %CD90 among any of the 5-FU treated rats receiving GSE compared to 5-FU treated-control rats. In addition, no significant differences in %CD90 were observed between GSE and water treatment,
implying none of the GSE doses had affected brush border sucrase activity in healthy rats (Figure 2.1).

2.3.4 Myeloperoxidase activity (MPO)

Following 5-FU injection, there was a significant ($P < 0.001$) increase in MPO activity in the proximal jejunum, junction of jejunum and ileum (JI) and ileum of water + 5-FU treated rats (1092%, 357% and 297% respectively) compared to water + saline treatment (Figure 2.2). GSE 600 mg/kg significantly ($P < 0.01$) reduced MPO activity in 5-FU treated rats by 55% compared to water + 5-FU treated rats in the JI (Figure 2.2B). No significant difference in MPO activity was detected between GSE and water treatment in healthy rats (Figure 2.2), indicating that GSE did not affect MPO activity in healthy animals.

2.3.5 Overall disease severity scores

Administration of 5-FU significantly increased disease severity score in the proximal jejunum when assessed by the semi-quantitative histological severity score (Figure 2.3). 5-FU controls attained the highest damage score (median score = 30) and were significantly greater than water + saline treated rats (median score = 1, $P < 0.01$). GSE treatment significantly reduced disease severity score in 5-FU treated rats in a dose-responsive manner (GSE 400 = 21 (15.5-25.5), $P < 0.01$; GSE 600 = 15.75 (9-24), $P < 0.01$; and GSE 1000 = 11.75 (7-19), $P < 0.01$) compared to 5-FU controls. No significant difference in disease severity score was observed between GSE and water treated rats receiving saline injection (Figure 2.3), indicating that GSE did not induce intestinal damage in healthy animals.
2.3.6 Villus height and crypt depth

5-FU injection resulted in shortening of the villi in the jejunum (38%, $P < 0.001$), JI (39%, $P < 0.001$) and ileum (29%, $P < 0.01$) compared to water controls (Figure 2.4). 5-FU also reduced crypt depth in the jejunum (40%, $P < 0.001$), JI (24%, $P < 0.05$) and ileum (18%, $P < 0.05$). In the jejunum, GSE treatments tended to dose RESPONSIVELY improve villus height and crypt depth, although only GSE at a dose of 1000 mg/kg significantly ($P < 0.05$) increased crypt depth compared to 5-FU controls (Figure 2.4A). Importantly, none of the GSE treatments impacted negatively on villus height and crypt depth in healthy animals (Figure 2.4). 5-FU injection significantly reduced mucosal thickness in the jejunum (39%, $P < 0.001$), JI (39%, $P < 0.001$) and ileum (19%, $P < 0.01$) compared to water controls (Figure 2.5). GSE tended to dose RESPONSIVELY increase mucosal thickness in the jejunum, although only GSE 1000 mg/kg significantly increased mucosal thickness in 5-FU treated rats (31%, $P < 0.05$) compared to water + 5-FU treatment (Figure 2.5A).
2.4 Discussion

Overall, the present study represents the first report of GSE dose-responsively reducing severity indicators of mucositis. Our findings indicated that higher doses of GSE (600 and 1000 mg/kg) were more effective at reducing the severity indicators of intestinal mucositis in rats. The GSE-induced effects were largely dose-dependent and were more evident in the proximal jejunum compared to the distal small intestine.

Injection of 5-FU impacts on the small intestine to a greater extent than the large intestine presumably due to the greater cell turnover rate in the more proximal regions of the gut [65]. Consistent with previous studies, the 5-FU mucositis model resulted in severe intestinal injury 72 h after the induction of mucositis. This damage was characterized by a reduction of intestinal brush border enzyme activities [78], increased neutrophil infiltration [79], increased disease severity score [80] and decreased mucosal thickness [17]. Moreover, blunting of the villi (intestinal structures responsible for nutrient absorption) and disorganization of crypts (location of stem cells) were the primary events associated with severe mucositis [73].

Proanthocyanidin rich foods (tea, chocolate and grape seed) have been shown to be protective against chronic diseases such as cardiovascular disease and breast cancer [81] and gastrointestinal disorders which include ulcerative colitis and intestinal mucositis [17, 50, 54]. Indeed, anti-inflammatory, anti-infectious, anti-carcinogenic and anti-viral properties have been attributed to proanthocyanidins. The bioavailability of proanthocyanidins in the gut system has been well documented in other studies [27]. Their unique polymerized structures inhibits absorption of proanthocyanidins across the small intestine, as they adhere to the gut mucosa [82]. Tsang et al. [29] detected larger forms of proanthocyanidins in the
small intestine of rats up to 12 h after ingestion. Thus, an accumulation of relatively high proanthocyanidin concentrations can occur in the gut lumen to protect the intestinal barrier. In the current study, higher doses of GSE (1000 mg/kg) were effective at maintaining crypt depth and mucosal thickness in the jejunal region with most end-point approaching healthy control levels. Furthermore, the current study also showed improvement of fecal output (less severe diarrhoea) in chemotherapy-treated rats receiving higher doses of GSE (600 mg/kg and 1000 mg/kg), suggesting reduced disruption of the mucosal lining of the small intestine.

Although GSE in the current study was more effective in the jejunum, the site of major intestinal injury, bioactivity was decreased in the distal small intestine. This may have been due to reduced bioavailability in the distal regions of the small intestine. Future studies could examine protection of GSE, possibly by microencapsulation, or via suppository application, to better target GSE and improve its bioavailability in the more distal regions of the bowel.

In consumers, interest in GSE has been primarily due to its high antioxidant content. GSE is reported to be a more potent radical scavenger than other known anti-oxidants such as vitamin C and E [83]. In the present study, the partial reduction in acute inflammation, as indicated by the decrease of MPO activity, and reduction in lymphocyte infiltration as recorded by the disease severity score analysis, could strengthen the potential role of GSE as a potent anti-oxidant and anti-inflammatory agent. A number of studies have described GSE as an anti-inflammatory agent. For example, GSE has been reported to reduce the expression of pro-inflammatory cytokines (TNF-α and IL-6) in mesenteric lymph nodes [84], rat plasma [85] and carrageenan-induced paw oedema in rats [86]. The reduction of these activities may represent a consequence of GSE and its ability to prevent NF-κB activation and
subsequently reduce the activation of nitric oxide and pro-inflammatory cytokines. Thus, inhibition of NF-κB activation may have been a possible mechanism by which GSE reduced mucosal injury and hence mucositis severity, in the current study.

The current study provides important information on the safety of GSE usage. Oral administration of GSE (400 mg/kg, 600 mg/kg and 1000 mg/kg) for nine days did not induce any deleterious side-effects in healthy animals. GSE did not impact negatively on daily metabolic parameters, nor induced any side-effects in the small intestine. Moreover, the sucrose breath test indicated that GSE did not affect small intestinal brush border enzyme activity. These data are in agreement with other studies [62] in which rats consuming up to 2 g/kg of GSE showed no abnormal metabolic findings or toxicological effects. In the current study, oral administration of GSE (1000 mg/kg) for nine days significantly increased stomach weight in healthy rats. Although these findings were not reported in other studies [61], it could be possibly due to differences in rat strains. Thus, histological analyses of rat stomach should be conducted in future. Indeed, promising findings with high doses of GSE in the proximal small intestine suggested applications for GSE in other small intestinal disorders including infectious enteropathy, radiation, enteritis and possibly celiac disease.

The effects of GSE on mucositis could lead to further investigations of GSE and its potential impact on the effectiveness of chemotherapy against cancer cells, as it is currently unknown if GSE in combination with chemotherapy increases or decreases the effectiveness of chemotherapy. The combined effects of GSE and chemotherapy on neoplasia (in vitro and in vivo) should be addressed in further studies. In addition, the present study was conducted using female Dark Agouti rats which can be manipulated to develop breast cancer [87].
These studies would facilitate further investigations into GSE and its potential to modify tumour growth.

In conclusion, the present investigation provides the first evidence for GSE to partially reduce severity indicators of intestinal mucositis in a dose-responsive manner. In addition, oral administration of GSE at doses up to 1000 mg/kg, for a period of 9 days, did not result in any deleterious side-effects in healthy animals. These findings suggest that dietary GSE could act as a promising approach for combating intestinal mucositis.

Although the current study revealed that GSE only minimally improved parameters of intestinal mucositis (disease severity score), future studies could examine higher doses of GSE or alternatively more purified proanthocyanidin compounds to determine whether these would be more effective. Additionally, other biomarkers such as inflammatory cytokines in tissue and blood could be measured to provide new endpoints to support the dose-related reduction in disease severity score.
Table 2.1  Nutrition and chemical profile of GSE.

<table>
<thead>
<tr>
<th>Nutrition profile¹</th>
<th>Qty per 100g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (KJ)</td>
<td>1480</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>4.5</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>0.2</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>79.1</td>
</tr>
<tr>
<td>sugars</td>
<td>0.2</td>
</tr>
<tr>
<td>Sodium (g)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemical profile²</th>
</tr>
</thead>
<tbody>
<tr>
<td>mDP³</td>
</tr>
<tr>
<td>Mass conversion⁴ (%)</td>
</tr>
<tr>
<td>Galloylation (%)</td>
</tr>
<tr>
<td>Molecular mass⁵ (g/mol)</td>
</tr>
<tr>
<td>Polyphenol⁶ (%)</td>
</tr>
</tbody>
</table>

¹The nutrition profile of GSE is represented in quantity/100 g.

²All the chemical profile data were obtained from phloroglucinolysis.

³Mean degree of polymerization (mDP) based on subunit composition identified from phloroglucinolysis.

⁴Mass conversion (MC) was calculated based on the recovery of proanthocyanidin detected by phloroglucinolysis.

⁵Estimated molecular mass based on subunit composition from phloroglucinolysis.

⁶Total polyphenols was measured by Folin-Ciocalteau method [17].
Table 2.2 The histological scoring criteria for assessment of the mucosa, submucosa and muscularis externa. The scoring comprised of 11 criteria where each criteria were scored between 0 (normal) to 3 (damaged) to give a maximum damage score of 33 for each intestinal region.

| Mucosa                                      | villus fusion and stunting / villus:crypt ratio |
|                                            | enterocyte disruption                           |
|                                            | reduction in goblet cell numbers                |
|                                            | reduction in mitotic figures                    |
|                                            | crypt disruption                                |
|                                            | crypt cell disruption                           |
|                                            | crypt abscess formation                         |
|                                            | lymphatic and polymorphonucleocyte infiltration |
|                                            | capillary and lymphatic dilatation              |
| Submucosa                                  | thickening/oedema                               |
| Muscularis externa                         | thickening                                     |
Table 2.3  Effects of increasing doses of GSE (mg/kg) on cumulative body weight change, food and water intake, urine and faecal output in saline-injected rats over day 3-9 (A) and in 5-FU injected rats from day 10-12 (B).

<table>
<thead>
<tr>
<th></th>
<th>Day 3-9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
</tr>
<tr>
<td></td>
<td>(n = 16)</td>
</tr>
<tr>
<td>Body Weight Change (g)</td>
<td>12.0 ± 0.9</td>
</tr>
<tr>
<td>Water Intake (mL)</td>
<td>174.2 ± 7.9</td>
</tr>
<tr>
<td>Food Intake (g)</td>
<td>66.2 ± 0.8</td>
</tr>
<tr>
<td>Urine Output (mL)</td>
<td>117.8 ± 6.7</td>
</tr>
<tr>
<td>Faecal Output (g)</td>
<td>8.2 ± 0.1</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM.
B

<table>
<thead>
<tr>
<th></th>
<th>Water + Saline (n = 8)</th>
<th>Water + 5-FU (n = 8)</th>
<th>Day 10-12 GSE 400 + 5-FU (n = 8)</th>
<th>GSE 600 + 5-FU (n = 8)</th>
<th>GSE 1000 + 5-FU (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight Change (g)</strong></td>
<td>-4.3 ± 1.1</td>
<td>-8.9 ± 0.8*</td>
<td>-9.8 ± 0.5**</td>
<td>-9.4 ± 0.9**</td>
<td>-9.8 ± 0.1**</td>
</tr>
<tr>
<td><strong>Water Intake (mL)</strong></td>
<td>73.3 ± 2.0</td>
<td>109.4 ± 10.8*</td>
<td>100.0 ± 5.3</td>
<td>89.8 ± 12.2</td>
<td>113.4 ± 10.7*</td>
</tr>
<tr>
<td><strong>Food Intake (g)</strong></td>
<td>28.7 ± 0.8</td>
<td>13.8 ± 0.9***</td>
<td>13.8 ± 0.8***</td>
<td>12.7 ± 1.4***</td>
<td>13.3 ± 1.0***</td>
</tr>
<tr>
<td><strong>Urine Output (mL)</strong></td>
<td>56.3 ± 2.6</td>
<td>85.8 ± 6.4*</td>
<td>87.3 ± 4.5*</td>
<td>80.0 ± 7.0*</td>
<td>89.8 ± 11.0*</td>
</tr>
<tr>
<td><strong>Faecal Output (g)</strong></td>
<td>3.6 ± 0.2</td>
<td>2.3 ± 0.3**</td>
<td>2.8 ± 0.2</td>
<td>3.5 ± 0.3##</td>
<td>3.3 ± 0.1#</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM. Statistical significance compared to water + saline, where * indicates $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. # $P < 0.05$ and ## $P < 0.01$ compared to Water + 5-FU
Table 2.4  Effect of increasing doses of GSE on organ weights of female Dark Agouti rats 72 h after 5-FU or saline injection

<table>
<thead>
<tr>
<th></th>
<th>Water + Saline</th>
<th>GSE 400 + saline</th>
<th>GSE 600 + Saline</th>
<th>GSE 1000 + Saline</th>
<th>Water + 5-FU</th>
<th>GSE 400 + 5-FU</th>
<th>GSE 600 + 5-FU</th>
<th>GSE 1000 + 5-FU</th>
<th>(n = 8)</th>
<th>(n = 8)</th>
<th>(n = 8)</th>
<th>(n = 8)</th>
<th>(n = 8)</th>
<th>(n = 8)</th>
<th>(n = 8)</th>
<th>(n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>397 ± 6</td>
<td>393 ± 6</td>
<td>406 ± 8</td>
<td>402 ± 8</td>
<td>405 ± 7</td>
<td>414 ± 16</td>
<td>408 ± 6</td>
<td>403 ± 6</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Lung</td>
<td>611 ± 8</td>
<td>593 ± 10</td>
<td>625 ± 18</td>
<td>657 ± 38</td>
<td>721 ± 49</td>
<td>711 ± 40</td>
<td>663 ± 47</td>
<td>580 ± 58</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Liver</td>
<td>3111 ± 57</td>
<td>3008 ± 43</td>
<td>3072 ± 63</td>
<td>3114 ± 45</td>
<td>3370 ± 144</td>
<td>3334 ± 85</td>
<td>3353 ± 73</td>
<td>3311 ± 41</td>
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<td></td>
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</tr>
<tr>
<td>Kidneys</td>
<td>832 ± 26</td>
<td>816 ± 15</td>
<td>815 ± 18</td>
<td>857 ± 13</td>
<td>874 ± 20</td>
<td>896 ± 21</td>
<td>880 ± 13</td>
<td>891 ± 19</td>
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</tr>
<tr>
<td>Thymus</td>
<td>181 ± 18</td>
<td>148 ± 17</td>
<td>178 ± 13</td>
<td>183 ± 14</td>
<td>88 ± 18***</td>
<td>86 ± 7***</td>
<td>91 ± 15***</td>
<td>69 ± 8***</td>
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<tr>
<td>Spleen</td>
<td>198 ± 5</td>
<td>205 ± 5</td>
<td>193 ± 8</td>
<td>205 ± 4</td>
<td>159 ± 6**</td>
<td>155 ± 5**</td>
<td>150 ± 13***</td>
<td>153 ± 3***</td>
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<td></td>
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</tr>
<tr>
<td>Stomach</td>
<td>600 ± 10</td>
<td>630 ± 10</td>
<td>640 ± 10</td>
<td>680 ± 10*</td>
<td>590 ± 30</td>
<td>650 ± 10</td>
<td>650 ± 20</td>
<td>680 ± 20*</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Caecum</td>
<td>370 ± 10</td>
<td>360 ± 10</td>
<td>410 ± 40</td>
<td>400 ± 10</td>
<td>470 ± 40</td>
<td>490 ± 50</td>
<td>440 ± 30</td>
<td>440 ± 30</td>
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</tr>
</tbody>
</table>

Organ weights are expressed as (wt g/kg bwt) %. Data are expressed as means ± SEM. Statistical significance compared to Water + Saline, * indicates $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.  

40
Table 2.5  Effects of increasing GSE doses on gastrointestinal organ weights and lengths of female Dark Agouti rats 72 h after 5-FU or saline injection.

<table>
<thead>
<tr>
<th></th>
<th>Water + Saline (n = 8)</th>
<th>GSE 400 + Saline (n = 8)</th>
<th>GSE 600 + Saline (n = 8)</th>
<th>GSE 1000 + Saline (n = 8)</th>
<th>Water + 5-FU (n = 8)</th>
<th>GSE 400 + 5-FU (n = 8)</th>
<th>GSE 600 + 5-FU (n = 8)</th>
<th>GSE 1000 + 5-FU (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duodenum</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g/kg)</td>
<td>0.20 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.22 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>5.06 ± 0.28</td>
<td>5.4 ± 0.3</td>
<td>5.2 ± 0.2</td>
<td>5.6 ± 0.1</td>
<td>5.3 ± 0.1</td>
<td>5.2 ± 0.2</td>
<td>5.2 ± 0.2</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td><strong>Jejunum + Ileum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g/kg)</td>
<td>1.99 ± 0.05</td>
<td>2.13 ± 0.05</td>
<td>2.04 ± 0.05</td>
<td>2.28 ± 0.05</td>
<td>1.93 ± 0.03</td>
<td>1.93 ± 0.05</td>
<td>1.98 ± 0.07</td>
<td>2.08 ± 0.04</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>71.0 ± 1.4</td>
<td>72.5 ± 1.1</td>
<td>70.4 ± 1.6</td>
<td>73.8 ± 1.9</td>
<td>68.5 ± 1.1</td>
<td>68.4 ± 0.9</td>
<td>70.9 ± 1.2</td>
<td>68.6 ± 0.6</td>
</tr>
<tr>
<td><strong>Colon</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g/kg)</td>
<td>0.53 ± 0.02</td>
<td>0.53 ± 0.02</td>
<td>0.52 ± 0.02</td>
<td>0.56 ± 0.02</td>
<td>0.64 ± 0.03</td>
<td>0.55 ± 0.02</td>
<td>0.63 ± 0.06</td>
<td>0.60 ± 0.04</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>11.8 ± 0.4</td>
<td>11.3 ± 0.4</td>
<td>10.9 ± 0.5</td>
<td>12.1 ± 0.5</td>
<td>10.2 ± 0.4</td>
<td>10.5 ± 0.4</td>
<td>11.4 ± 0.4</td>
<td>11.5 ± 0.2</td>
</tr>
</tbody>
</table>

Gastrointestinal organ weights are expressed in (wt g/kg bwt)% and lengths are expressed in (cm). Data are expressed as means ± SEM.
Figure 2.1  Effects of GSE on small intestinal sucrase activity assessed by the sucrose breath test on Day 12 (72 h after 5-FU or saline injection). Data expressed as mean (%CD90) ± SEM. *** indicates $P < 0.001$ compared to Water + Saline.
Figure 2.2  Effects of GSE on Myeloperoxidase (MPO) activity in the jejunum (A), JI (B) and ileum (C) 72 h after either saline or 5-FU injection. Data are expressed as mean (MPO units/g tissue) ± SEM. * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$ compared to rats receiving water and saline injection. ## indicates $P < 0.01$ compared to rats receiving water and 5-FU injection.
Figure 2.3  Effects of GSE on histological severity scores in the jejunum 72 h after either saline or 5-FU injection. The box plots represent the range of disease severity score and the horizontal lines represent the median disease severity score. ** indicates $P < 0.01$ compared to Water + Saline. ## indicates $P < 0.01$ compared to Water + 5-FU.
Figure 2.4  Effects of GSE on villus height and crypt depth in the jejunum (A), JI (B) and Ileum (C) 72 h after either saline or 5-FU injection. Data are expressed as mean (µm) ± SEM. * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$ compare to Water + Saline. # indicates $P < 0.05$ compared to Water + 5-FU.
Figure 2.5 Effects of GSE on mucosal thickness in the jejunum (A), JI (B) and Ileum (C) 72 h after either saline or 5-FU injection. Data are expressed as mean (µm) ± SEM. * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$. # indicates $P < 0.05$ compared to Water + 5-FU.
Chapter 3 Potential chemopreventative effects of dietary polyphenols on colorectal cancer cells
3.0 Abstract

**Background:** Grape seed extract (GSE), epigallocatechin gallate (EGCG), gallic acid (GA), resveratrol and catechin have been reported to exert anti-neoplastic activity in transformed cell lines in addition to protective effects against oxidative stress *in vivo*. However, it is currently unknown if polyphenols in combination with chemotherapy increase or decrease anti-neoplastic effectiveness. We investigated the combined effects of GSE, EGCG, GA, resveratrol or catechin on the viability of Caco-2 cells following 5-FU chemotherapy.

**Methods:** The antioxidant capacity of each polyphenol was determined by the ferric reducing antioxidant power (FRAP) assay. Caco-2 cell viability was examined in response to polyphenols tested, both alone or in combination with 5-FU, for either 24 or 48 h by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT) assay. **Results:** GA (26.9 mM/g) and EGCG (12.4 mM/g) attained significantly (*P* < 0.05) higher antioxidant activity compared to other polyphenols. With the exception of catechin, all polyphenols dose-dependently (*P* < 0.05) reduced the proliferation rate of Caco-2 cells. When GSE (25 µg/mL) was added in combination with 5-FU, the growth inhibitory effects of 5-FU were significantly enhanced by 26% (*P* < 0.05; combined treatment vs. both agents alone) at 24 h. GA (10 µg/mL) significantly enhanced the ability of 5-FU to induce growth inhibition by 20% (*P* < 0.05; combined treatment vs. both agents alone) at 48 h. Treatment with polyphenols alone, with the exception of catechin, at the higher doses (25-100 µg/mL) exerted more potent growth inhibitory activity (*P* < 0.05) compared to 5-FU chemotherapy.

**Conclusion:** Dietary polyphenols are potent chemopreventative agents which could represent a promising approach to combat intestinal mucositis while retaining or enhancing anti-neoplastic activity.
3.1 Introduction

Colorectal cancer is the second most deadly, and fourth most frequently diagnosed, form of cancer in the United States [88]. Currently, treatments for colorectal cancer include surgical resection of the bowel and chemotherapy, primarily with the anti-metabolite, 5-FU [89]. However, 5-FU chemotherapy often results in severe gastrointestinal toxicity and the development of mucositis that may limit the dose of chemotherapy and reduce cure rates and quality of life [65]. Current available treatment approaches for mucositis include amifostine, cryotherapy and palifermin [90]. Conversely, these agents are not fully effective as they do not target pathogenesis of the condition [48]. Thus, there is a need to seek new treatment approaches to enhance chemotherapeutic action without affecting patient well-being. To date, an optimal combination of agents which could enhance chemotherapeutic cytotoxicity against cancer cells, with minimal effect on normal cells, has not been developed.

GSE has demonstrated partial amelioration of intestinal damage in a model of chemotherapy-induced mucositis (chapter 2) and has reduced gastrointestinal cell toxicity following chemotherapy treatment in normal IEC-6 intestinal cells [17]. We would like to investigate the effects of GSE in modulating colonic neoplasia in combination with chemotherapy.

GSE represents a crude mixture of polyphenolic compounds and therefore it is necessary to test other commercial purified polyphenolic compounds which are reported to hold potential as chemopreventative agents [4, 91-94]. Indeed, there is a growing interest in purified polyphenolic compounds with a protective role against colorectal cancer [95, 96], however there is limited study performed on their structure-functionality relationship in terms of their bioactivity on colon cancer cell.
Resveratrol is mainly found in grape skin (50-10 µg/mL) and red wines (1.5-3 mg/L) [97]. It has been reported to exert growth inhibitory and cell cycle arrest on human colorectal cell lines [98] whereas the chemopreventive properties of green tea against colon cancer have been attributed primarily to its flavan-3-ol constituent [99]. Moreover, green tea catechin and epigallocatechin gallate (EGCG), have demonstrated anti-neoplastic activity on colon cancer cell lines such as HT-29, T84, Caco-2 and SW837 [100-102]. Gallic acid (GA; 3,4,5-trihydroxybenzoic acid) can be found in tea, grape seeds and red and white wine [103]. Recent studies suggest that GA inhibits cell proliferation in Caco-2 cells [104]. GSE contains high levels of flavan-3-ol subunits, proanthocyanidins, which similarly have been reported to reduce cell proliferation in the HT-29 and LoVo colon cancer cell lines [47] and to reduce tumour volume in athymic nude mice [46].

Due to the reported anti-neoplastic activities of polyphenolic compounds, we investigated the effects of GSE, EGCG, GA, resveratrol or catechin on the viability of Caco-2 colorectal cancer cells with 5-FU chemotherapy.
3.2 Materials and methods

3.2.1 Instrumentation

An Agilent model 1100 HPLC (Agilent Technologies Australia Pty Ltd., Melbourne, Australia) with Chemstation software was used for chromatographic analyses.

3.2.2 Material

Purified polyphenols: epigallocatechin gallate (EGCG), gallic acid (GA), resveratrol and catechin were purchased from Sigma-Aldrich (St. Louis, MO). Phloroglucinol, ascorbic acid, tri[2-pyridyl]-s-triazine (TPTZ), ferrous chloride, dimethylsulfoxide (DMSO), (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). 5-FU was purchased from DBL® Mayne Pharma (Victoria, Australia). All the cell culture solutions were purchased from Invitrogen (Victoria, Australia).

3.2.3 Grape seed extract (GSE)

A powdered GSE was kindly donated by Tarac Technologies (GrapEX seed tannin; South Australia, Australia) and stored in an air-tight, light-resistant pack. The nutrition and chemical profile of the proanthocyanidin content in GSE is listed in Table 2.1(Chapter 2).

3.2.4 Phloroglucinolysis

The proanthocyanidin profile of GSE was characterised by phloroglucinolysis. Phloroglucinolysis determined the subunit composition, mean degree of polymerization (mDP) and galloylation of proanthocyanidins. Phloroglucinolysis was performed according to a previously described method [106]. GSE was dissolved in methanol (10 mg/mL, v/v) and 25 µL of GSE was added to an equal volume of phloroglucinol solution (0.2 N HCL in
methanol, 100 g/L of phloroglucinol and 20 g/L of ascorbic acid). The phloroglucinolysis reaction was carried out at 50°C for 25 min and analysed by RP-HPLC using (-)-epicatechin as quantitative standard [106].

3.2.5 Ferric reducing antioxidant power (FRAP) assay

The antioxidant activity of GSE, EGCG, GA, resveratrol and catechin was measured by FRAP assay [107] with slight modifications. FRAP reagent (300 mM acetate buffer of pH 3.6; 10 mM TPTZ (2,4,5-tri[2-pyridyl]-s-triazine) in 40 mM HCL; 20 mM ferrous chloride; in 10:1:1 ratio) was prepared and kept in the dark at 37°C prior to analysis. All the polyphenolic compounds were dissolved in DMSO (0.1 mg/mL) and 15 µL samples were added in triplicate to non-sterile 96-well plates (Grenier Bio-one, Victoria, Australia). 150 µL of FRAP reagent was added and the plate was read at 593 nm after 4 min (Multiskan® Spectrum, Therma Electron Corporation, Vantaa, Finland) using Skanit software 2.2. Ferrous sulphate solutions were prepared (0.1 - 1 mM) and used to generate a calibration curve. FRAP values of test compounds were expressed as mM Fe(II)/g of sample.

3.2.6 Cell culture

The human colon cancer cell line, Caco-2 was obtained from the American Type Culture Collection (ATCC, Manassas, USA). Caco-2 cells were maintained at 37°C in a humidified incubator with 5% CO₂ – 95% air, and 90% relative humidity in Dulbecco’s Modified Eagle’s Minimum Essential Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and 1% antibiotics (penicillin, gentamicin and streptomycin) (v/v). The cells were grown in 75 cm² vented tissue culture flasks, culture medium was changed twice a week and cells were passaged when they were 80-90% confluent.
3.2.7 Cell proliferation activity

The proliferation activity of Caco-2 cells was determined by MTT assay according to a previously described method by Huynh-Delerme et al. [108]. Cells (5 x 10^3 cells/well) were seeded on 96-well tissue culture plates for 48hrs to allow attachment. In all experiments, the concentration of DMSO in control and treated samples was less than 0.025%. All the polyphenols were prepared in DMSO and diluted with DMEM and further filter-sterilized through a 0.22 µM filter (Millipore, South Australia, Australia). After 48 h, culture medium was replaced with serum free media containing polyphenols at different concentrations (µg/mL) and 5-FU (µM) and further incubated for either 24 or 48 h. MTT solution was prepared in Dulbecco’s Phosphate Buffered Saline (1 mg/mL) and sterile-filtered to remove any biological contaminants. Next, 50 µL of MTT solution was added to each well and further incubated at 37°C for 4 h. Medium was replaced with 100 µL of DMSO to extract the formazan product. Plates were placed on a shaking incubator for 15 min and read by spectrometer at 570 nm. Data were expressed as number of viable cells as a percentage of control cells treated with serum free medium only.

3.2.8 Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using XLSTAT version 2011.4.02 (Addinsoft SARL, France). All the data was analysed using one-way ANOVA, with a Tukey’s post hoc test. P <0.05 was considered statistically significant.
3.3 Results

3.3.1 Proanthocyanidin profile of GSE

The chemical profile of GSE is illustrated in Table 3.1. Phloroglucinolysis characterized the proanthocyanidin subunit composition as a proportion of either terminal or extension subunits. GSE contained 23.8% of measurable proanthocyanidins and the average mean degree of polymerization was 5.9.

3.3.2 Antioxidant levels of polyphenols

Ascorbic acid, a well characterized antioxidant, was included as a positive control to determine the antioxidant activity of polyphenols. Despite the similar chemical structure of polyphenols studied (Figure 3.1), GA had the highest antioxidant activity (26.9 mM/g) followed by EGCG (12.4 mM/g), catechin (6.7 mM/g), GSE (4.9 mM/g) and resveratrol (4.2 mM/g) (Figure 3.2). Compared to ascorbic acid (9.3 mM/g), GA attained the highest level of antioxidant capacity (2.9 - fold increase, \( P < 0.05 \)) followed by EGCG (1.3-fold increase, \( P < 0.05 \)).

3.3.3 Effects of 5-FU on proliferative activity of Caco-2 cells

The dose responses of 5-FU (0 - 100,000 \( \mu \)M) on Caco-2 cells for either 24 or 48 h are illustrated in Figure 3.3. The proliferative activity of Caco-2 cells was inhibited by 5-FU in a time- and dose-dependent manner. At 24 h, 5-FU at 100 \( \mu \)M reduced proliferative activity in Caco-2 cells to 88% of control values and a further reduction of cell proliferation was observed to 70% of control values at 48hrs. The proliferative activity of Caco-2 cells was reduced to 73% (24 h) and 48% (48 h) by 5-FU (10 mM) compared to controls (Figure 3.3).
3.3.4  Effects of GSE and 5-FU on Caco-2 cell proliferative activity

In order to establish the cytotoxicity of GSE on Caco-2 cells, GSE (10-100 µg/mL) was applied to cells for either 24 or 48 h (Figure 3.4). GSE treatment inhibited cell proliferation in a dose- and time-dependent manner. GSE treatments significantly ($P < 0.05$) reduced cell proliferation ($IC_{50} = 50.24$ µg/mL) at 24 h and became more toxic to the Caco-2 cells at 48 h ($IC_{50} = 37.84$ µg/mL). When the cells were exposed to the combination of GSE (10-100 µg/mL) and 5-FU (100 µM), greater numbers of dead cells were evident compared to cells exposed to 5-FU alone (Figure 3.4). At 24 h, 5-FU significantly reduced cell viability to 84% ($P < 0.05$) of control values. Interestingly, when Caco-2 cells were exposed to the combination of GSE and 5-FU, the growth inhibitory effects of 5-FU were significantly enhanced by 26% (GSE = 25 µg/mL; $P < 0.05$; combined treatment vs. both agents alone) at 24 h. GSE at higher doses (50-100 µg/mL) exerted greater growth inhibition compared to 5-FU alone. At 24 h, GSE induced significant growth inhibitory effects on Caco-2 cells (GSE 50 = 33% and GSE 100 = 27%; $P < 0.05$) compared to 5-FU control (84% of control value) (Figure 3.4A). In addition, GSE significantly ($P < 0.05$) decreased the proliferative activity of Caco-2 cells (GSE 50 = 31% and GSE 100 = 29%; $P < 0.05$) compared to 5-FU control (64% of control value) at 48 h (Figure 3.4B).

3.3.5  Combination of other polyphenols with 5-FU and effects on Caco-2 proliferation

In order to investigate possible synergistic or inhibitory effects of polyphenols on 5-FU-induced cytotoxicity, polyphenols (EGCG, GA, resveratrol and catechin) and 5-FU were cultured on Caco-2 cells. At 48 h, all polyphenols dose-responsively reduced proliferation with the exception of catechin (Figure 3.5) which enhanced proliferation at the highest dose (100 µg/mL). None of the polyphenols interfered with the chemotherapeutic properties for
5-FU to kill Caco-2 cells. GA (10 µg/mL) significantly enhanced the ability for 5-FU to induce growth inhibition by 20% ($P < 0.05$; combined treatment vs. both agent alone) compared to 5-FU alone (74% of control value) (Figure 3.5B). With the exception of catechin, all the polyphenols demonstrated greater growth inhibition compared to 5-FU alone. Compared to 5-FU control, EGCG (100 µg/mL) significantly ($P < 0.05$) reduced cell viability to 32%, whereas GA and resveratrol (25-100 µg/mL) exerted growth inhibition (GA: 3-57%; resveratrol: 23-57%). Catechin was included in the study as a negative control. Catechin (10-50 µg/mL) did not affect Caco-2 cell proliferation and did not show any effects upon combination with 5-FU (Figure 3.5D). The data for all polyphenols at 24 h were similar to 48 h data (data for 24 h not shown).
3.4 Discussion

Colon cancer has one of the greatest mortalities in western countries. Current available treatment is incomplete due to chemotherapeutic insufficiency and development of intestinal mucositis [90]. Natural products, in particular polyphenolic compounds, may represent a potential source of chemoprevention for colon cancer [95, 109]. The purpose of the current study was to evaluate whether the combined effects of 5-FU with representative polyphenolic compounds in the human diet, would enhance or inhibit the impact of 5-FU chemotherapy on the viability of colon cancer cells. The selected polyphenols (GSE, EGCG, GA, resveratrol) have been reported to induce growth inhibition on colon cancer in vitro [110], but their effectiveness in combination with chemotherapy, 5-FU remains unknown.

The bioactive compounds in GSE are known as proanthoacyanidins, which are made up of flavan-3-ol monomers, catechin [9].

5-FU is an anti-metabolite chemotherapeutic drug commonly used against breast, oesophageal and colorectal cancer [90]. However, its toxicity is a common issue for many cancer patients, with gastrointestinal toxicity and myelo-suppression being the most commonly observed side-effects [79]. Due to this, the dose of 5-FU administered to cancer patients is restricted. Recently, various strategies have been developed to counter the development of mucositis (reduced gastrointestinal toxicity) or to enhance the chemotherapeutic activity of 5-FU. To date, a number of strategies to enhance 5-FU efficacy on colon cancer have been investigated although none are clinically available. These include omega 3-fatty acid [111], chloroquine [112], violecin [113] and ginseng [114] which have been reported to effectively improve 5-FU efficiency at killing cancer cells in vitro compared to the chemotherapy agent acting independently.
A variety of phytochemical compounds have been identified with potential anti-carcinogenic properties against colon cancer [95]. Among them, polyphenols have been reported to exert a protective role [96]. Although there is much evidence to support a protective role for these polyphenolic compounds in colon cancer, little knowledge exists regarding their toxicity on cancer cells when combined with 5-FU. Green tea polyphenols such as EGCG have been reported to enhance chemotherapy-induced apoptosis in human cholangiocarcinoma cells, decrease tumour growth and to increase sensitivity to chemotherapy in xenograft nude mice [115]. Moreover, EGCG has been reported to selectively induce toxicity in colon cancer cells (HT-29) but not in human fetal colon cells [102]. GSE (400-1000 mg/kg) has been reported to dose-dependently reduce chemotherapy-induced damage in rats (Chapter 2). In addition, GSE has been reported to prevent chemotherapy-induced damage to normal intestinal cells (IEC-6) [17].

A number of investigators have reported the radical scavenging and apoptotic properties of polyphenolic compounds to be largely governed by their molecular structure: galloylation moiety and degree of polymerization (Figure 3.1) [23, 24, 101, 116]. In the current study, galloylated compounds (GSE and GA) demonstrated higher cytotoxic activity compared to a non-galloylated monomer (catechin) on Caco-2 cells. This supports previous findings of galloylated compounds exerting toxic effects in cancer cell lines compared to non-galloylated compounds [116]. In the present study, GSE, and GA acted synergistically with 5-FU to inhibit Caco-2 cell proliferation. These polyphenols may act as potent chemotherapeutic agents as they have been demonstrated to selectively exert cytotoxicity against tumour cells compared to normal cells [47, 117, 118]. It has been suggested that GSE induces growth inhibition in cancer cells via induction of cell cycle arrest which eventually leads to the induction of caspase-dependent apoptosis [45] and disruption of the
mitochondrial membrane [16]. GA (3,4,5-trihydroxybenzoic acid) has been identified as the major active constituent in GSE at inducing growth inhibition and apoptotic death of human prostate cancer cells [119]. In addition, Gifton et al. [103] reported GA anticancer properties against 1,2-dimethyl hydrazine-induced carcinogenesis in the rat colon.

The current study demonstrated that certain polyphenols applied alone at high doses (25-100 μg/mL) were superior anti-neoplastic agents compared to 5-FU alone. At higher concentrations, the polyphenols (GSE, EGCG, GA and resveratrol) alone tended to induce greater growth inhibitory effects on Caco-2 cells compared to 5-FU alone. Thus, the current data support polyphenols as promising anti-neoplastic adjuncts to cancer treatment.
Figure 3.1 Molecular structures of the dietary polyphenolic compounds examined in this study. The figure shows catechin (A), proanthocyanidin (B), EGCG (C), GA (D), and resveratrol (E).
Figure 3.2  Total antioxidant capacity of polyphenols (GSE, EGCG, GA, resveratrol, catechin and ascorbic acid) measured by FRAP assay. Data are expressed as means ± SEM of 3 independent experiments. Bar data not sharing the same letter are significantly different $P < 0.05$. 

Figure 3.3  Proliferative activity of Caco-2 cells treated with 5-FU for either 24 or 48 h, assessed by MTT assay. Data are expressed as percent of cell proliferation relative to proliferation of untreated controls. Data are presented as means ± SEM of 2-3 independent experiments.
Figure 3.4  Combination effects of GSE (µg/mL) and 5-FU (µM) on proliferation of Caco-2 cells for either 24 (A) or 48 h (B). Data are expressed as percent of cell proliferation relative to untreated controls. Data are presented as means ± SEM of 4 independent experiments. Bar data not sharing the same letter is significantly different \( P < 0.05 \).
Figure 3.5  Combination effects of 5-FU (µM) and EGCG (A), GA (B), resveratrol (C) or catechin (D) in µg/mL on proliferation of Caco-2 cells for 48 h. Data are presented as percent of cell proliferation relative to proliferation of control. Data are presented as means ± SEM of 2-3 independent experiments. Bar data not sharing the same letter is significantly different $P < 0.05$. 

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Chapter 4  Grape-derived proanthocyanidin fractions and effects on colon cancer cells
4.0 Abstract

**Introduction:** Grape seed extract (GSE), which contains proanthocyanidins (PAs), has been reported to be a potent anti-cancer agent. Previously, GSE has been reported to reduce small intestinal injury arising from administration of chemotherapy (mucositis) and also to enhance the capacity for chemotherapy to kill cancer cells. **Aim:** We sought to investigate the effects of purified PA fractions in combination with 5-FU chemotherapy on the viability of colon cancer cells. **Methods:** Six different PA fractions with increasing mean degree of polymerization (mDP) were isolated from Cabernet Sauvignon seeds at two ripeness stages: pre-veraison green (immature) and ripe (mature). The fractions were characterized by phloroglucinolysis and gel permeation chromatography (GPC). The antioxidant capacity of the fractions was determined by ferric reducing antioxidant power (FRAP) assay. Fractions were tested on Caco-2 cells, alone and in combination with 5-FU. Cell viability was determined by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT) assay. **Results:** The antioxidant capacity of all six fractions was negatively correlated with PA mDP ($r^2 = -0.81, P < 0.05$). All isolated fractions significantly reduced cell proliferation compared to the control ($P < 0.05$). However, F2 and F3 were the most active fractions (immature F2, 32%; F3, 35% and mature F2, 13% and F3, 17%; percentage of viable cells) on Caco-2 cells. When combined with 5-FU, immature fractions F1-F3 enhanced the growth inhibitory effects of 5-FU by 27%-73% ($P <0.05$; compared to 5-FU control). Mature seed PA fractions (F1-F4) significantly enhanced the toxicity of 5-FU against Caco-2 cells ($P < 0.05$; 60-83% compared to 5-FU control). Moreover, some fractions were more potent at decreasing viability in Caco-2 cells ($P < 0.05$; immature, 65-68%; mature, 83-87%) than 5-FU alone (37%). **Conclusions:** Smaller grape seed PAs (mDP of 2-6) were more effective chemotherapeutic agents, representing a promising approach to combat intestinal mucositis.
4.1 Introduction

Colorectal cancer is the second most lethal, and fourth most frequently diagnosed, form of cancer in the United States [88]. The primary treatment of colon cancer involves surgical bowel resection and chemotherapy, most often by 5-FU. Unfortunately, chemotherapy treatment does not discriminate between healthy and cancer cells, and it targets areas where cells are replaced at a high rate, such as in the mouth and gut [65]. This leads to the development of mucositis (gastrointestinal toxicity)[120]. Current mucositis treatments are largely ineffective as they target only the symptoms, but not the pathogenesis of the condition [90]. Thus, it is important to seek new alternative treatments which not only target mucositis but also enhance chemotherapeutic action without compromising the well-being of the patient.

Increasingly, grape seed extracts (GSEs) are being studied due to their reported health benefits for a variety of disorders such as cancer, cardiovascular disease and atherosclerosis [20, 121-123]. The health-beneficial effects of GSEs have been attributed to polyphenolic proanthocyanidins (PAs) [122]. PAs are made up of flavan-3-ol subunits and the polymer length of the PA is described by the mean degree of polymerization (mDP). Due to their polymerized structure, cellular absorption has been shown to be restricted to oligomers with a lower mDP, leaving the larger mDP molecules for adsorption to the gut lumen [35]. A number of studies have reported anti-proliferative effects of PAs on cancer cells, and these have indicated that PA mDP and galloylation enable their specificity of action [23, 24, 124]. This was also supported in previous chapter (Chapter 3) where galloylated compound (GSE and GA) demonstrated higher cytotoxic activity compared to a non-galloylated monomer (catechin) on Caco-2 cells.
Previously, GSE has been demonstrated to enhance the potency of 5-FU chemotherapy, in conferring toxicity against colon cancer cells (Caco-2). Moreover, GSE has been identified as a superior chemotherapy agent to 5-FU in Caco-2 cells (Chapter 3). However, the bioactive components in GSE responsible for improving colon health remain unknown. The primary aim of the current study was to investigate potentially bioactive PA fractions, differing in their mDP (or molecular mass), in combination with the 5-FU chemotherapy agent, for their effects on the viability of colon cancer cells.
4.2 Methods and Materials

4.2.1 Instrumentation

An Agilent model 1100 HPLC (Agilent Technologies Australia Pty Ltd., Melbourne, Australia) was used with Chemstation software for chromatographic analyses.

4.2.2 Grape sampling and preparation

Cabernet Sauvignon grape samples were obtained from a commercial vineyard in the Langhorne Creek growing region of South Australia in the 2009 season. Grape samples were collected at two different stages of ripeness: pre-veraison, green (immature) and ripe (mature; 25-26°Brix). Grape berries were prepared according to a previously reported method [126] in which grapes were collected and kept frozen at -20°C. While still frozen, seeds were removed from the flesh with a scalpel. The flesh residue was removed from seeds with a paper towel and seeds were re-frozen at -20°C prior to extraction.

4.2.3 Preparation and extraction of seed proanthocyanidins (PAs)

PA extraction commenced by extracting seeds (100 g) overnight for 18 h in 70% aqueous acetone (200 mL, v/v) and ascorbic acid (1 g/L). The extract was concentrated under reduced pressure at 35°C (Heidolph Laborota 4011 rotary evaporator, John Morris Scientific, Adelaide, Australia), and lyophilized to a dry powder (Dynavac FD3 freeze drier, Dynavac Pty Ltd, Sydney, Australia). Yields for each sample were; immature, 6.31 g and mature, 10.29 g, respectively. PA seed powder (5 g) was dissolved in 50 mL of aqueous methanol (60%, v/v) containing trifluoroacetic acid (TFA) (0.05%, v/v) and then applied (~18.3 mL/min) to a 300 mm x 21 mm glass column (Michel-Miller, Vineland, NJ, USA) containing Sephadex LH20 chromatography resin (Amersham, Uppsala, Sweden) to an approximate bed volume of 93 mL, and washed in 250 mL of methanol (60%, v/v)
containing TFA (0.05%, v/v) to remove low molecular weight monomers. PA was then recovered in 150 mL of aqueous acetone (70%, v/v) and the extract was concentrated in a rotary evaporator at 35°C to remove acetone. The aqueous solution was extracted with hexane to remove residual lipophilic material and recovered using a separatory funnel. The aqueous extract was freeze-dried into powder, and recovered amounts for the two extracts were; immature, 2.5 g and mature, 0.9 g, respectively. Powders were kept under nitrogen and at -20°C prior to fractionation.

For fractionation of PAs, 0.5 g of powder was dissolved in aqueous methanol (60%, v/v) containing TFA (0.05%, v/v) and applied to the same column under identical conditions. PA seed extract was fractionated according to a solvent system method described previously [106] to produce 6 fractions of increasing mDP, designated F1 to F6 (Table 4.1). The eluted fractions were concentrated under pressure at 35°C to remove organic solvents and lyophilized to dry powder. The isolated fractions were stored at -20°C prior to analysis.

Grape seed extract (GSE) was a generous gift from Tarac Technologies (GrapEX seed tannin; North Adelaide, South Australia) and was included in the study as a control. GSE (1 g) was dissolved directly in aqueous methanol (60%, v/v) containing TFA (0.05%, v/v) and fractionated according to the same procedure applied to the grape seed extracts as mentioned above [106], with the eluant from the loading solvent discarded.
4.2.4 Acid catalysis of PA in the presence of excess phloroglucinol (phloroglucinolysis)

Phloroglucinolysis was used to determine subunit composition, mDP and galloylation of PA. Phloroglucinolysis was performed according to a previously described method [106]. Briefly, seed fractions (obtained from section 4.2.3) were dissolved in methanol (10 mg/mL, v/v) and equal volumes (25 µL) of extract and phloroglucinol solution (0.2 N HCL and 100 g/L phloroglucinol and 20 g/L ascorbic acid) were prepared to give a final PA concentration of 5 g/L. The phloroglucinolysis reaction was carried out at 50°C for 25 min and analysed by RP-HPLC according to the conditions outlined in previous method using (-)-epicatechin (Sigma Aldrich, St. Louis, MO) as the quantitative standard [25].

4.2.5 Gel Permeation Chromatography (GPC)

Gel permeation chromatography was performed based on a previously described method [26]. The GPC technique characterises information on the size distribution of PA for each fraction. The GPC system consisted of 2 PLGel columns (300 mm x 7.5 mm, 5 µm, 100 and 500 Å ) connected in series and protected by a guard column containing the same material (PLGel, 50 x 7.5 mm, 5 µm) (Varian Inc., Mulgrave, Victoria, Australia). Fractionated samples were dissolved in methanol (10mg/mL) and further diluted (25 µL) with 4 volumes (100 µL) of HPLC mobile phase (N,N-dimethylformamide containing glacial acetic acid (1%, v/v), water (5%, v/v) and 0.15 M lithium chloride). The flow-rate was maintained at 1 ml/min with a column temperature of 60°C and elution was monitored at 280 nm. The maximum amount of PA injected onto the column was 40 µg. Seed PA fractions of differing molecular mass from a previous study [106] were used as standards for calibration. Calibration curves of fractionated PAs with their cumulative mass distribution were plotted and the mean molecular mass of the fractions was predicted at 50% elution.
4.2.6 Ferric reducing antioxidant power (FRAP) assay

This assay was carried out following a modified protocol [127]. Briefly, FRAP reagent was prepared (300 mM acetate buffer, pH 3.6, 10 mM 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ) solution in 40 mM HCL, 20 mM ferrous chloride; in 10:1:1 v/v) and kept in the dark at 37°C prior to analysis. Fractions were dissolved in dimethylsulfoxide (DMSO) (0.1 mg/mL) and 15 µL were added to 96-well plates. Fifteen µL of FRAP reagent was added to the wells containing the fractions and the plate was read at 593 nm after 4 min (Multiskan® Spectrum, Therma Electron Corporation, Vantaa, Finland) using Skanit software 2.2. Ferrous sulphate (0.1-1 mM) was used to construct a standard curve and FRAP values of test compounds were expressed as mM Fe(II)/g of sample.

4.2.7 Cell preparation and experimental treatment

The human colon cancer cell line, Caco-2, was obtained from the American Type Culture Collection (ATCC, Manassas, USA). Cells were maintained in Dulbecco Modified Eagle Medium (DMEM) at 37°C in a 5% CO₂ incubator. Medium was replaced twice in each week. PA fractions were dissolved in DMSO and kept at -20°C prior to analysis. Caco-2 cells were seeded at 1000 cells/well on 96-well tissue culture plates (Grenier Bio-one, Vic) and incubated at 37°C in 5% CO₂ for 48 h to allow attachment. After 48 h of incubation, the medium was replaced with (25 µg/mL) of seed fractions dissolved in DMEM (µg/mL) containing < 0.025% (v/v) of DMSO and 5-FU (µM) (DBL®, Mayne Pharma Pty. Ltd., Victoria, Australia). Cells were further incubated at 37°C, 5% CO₂ for 72 h.

4.2.8 MTT assay

The (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT) assay was performed based on a previously described method, with slight modification [108]. After 72
h exposure to the treatments (seed extracts and 5-FU), 50 µL of MTT (1mg/mL in Dulbecco’s Phosphate Buffered Saline, DPBS) was added to each well and further incubated at 37°C, 5% CO₂ for 4 h. After 4 h, the medium was aspirated and 100 µL of DMSO added to dissolve the formazan product. Plates were placed on a shaking incubator for 15 min and read at 570 nm by a UV spectrophotometer.

### 4.2.9 Statistical analysis

Each cell-based experiment was performed at least 3 times. Statistical analyses were performed using XLSTAT and PASW statistic. Statistical analysis was determined by ANOVA using the Tukey’s post-hoc test. A Pearson’s correlation test was performed to determine the relationship between cell viability, PA composition and antioxidant value. Statistical significance was considered at $P < 0.05$. 
4.3 Results

4.3.1 Characterization of PA fractions

GSE was included in the current study as a control. The PA composition of GSE is illustrated in Table 4.2. Compared to Cabernet Sauvignon seed extracts (Table 4.3), GSE had a low mass conversion (23.8% w/w) and also a lower molecular mass, as measured by phloroglucinolysis and GPC. The PA terminal subunits in GSE were mostly dominated by (-)-epicatechin-3-O-gallate. Due to the apparently low contribution of PAs in GSE, we isolated PAs from Cabernet Sauvignon seeds harvested at different stages as previous reports showed a high mass conversion in unprocessed grape seeds [126].

Comparing purified PA from both immature and mature seed samples, the immature seeds had higher mass conversion (87%) compared to mature seeds (71%) (Table 4.3). The purified PAs were further fractionated into 6 fractions of increasing mDP or molecular mass. The mass conversion yields for conversion of PA into known PA subunits was > 49% except for the highest mDP F6 (approximately 37%). The major differences between immature and mature seed fractions were that mature fractions had a higher proportion of (-)-epicatechin terminal subunits (9 - 30%) compared to the immature fractions (2 - 11%). Both mature and immature seed fractions showed a similar pattern of decreasing (-)-epicatechin terminal subunits with increasing mDP. Interestingly, the increases in polymerisation or molecular weight were mainly controlled by (-)-epicatechin-3-O-gallate extension subunits. The increase of polymerisation across the fractions was primarily driven by (-)-epicatechin-3-O-gallate-phloroglucinol extension units. The galloylation of immature fractions ranged from 15 - 23% and in mature fractions ranged from 9 - 24%. Immature F2 had the highest galloylation (35%), driven by a high proportion of (-)-epicatechin-3-O-gallate (88%).
4.3.2 Antioxidant capacity of seed fractions

The antioxidant capacity of seed fractions was measured by the FRAP assay (Figure 4.1). GSE, which contained a mixture of oligomers and polymers of PAs, was included as a positive control to determine the antioxidant activity of the seed fractions. Compared to GSE (5 mM/g), all the fractions had higher FRAP values, ranging from 5.4 - 8.8 mM/g. The antioxidant capacity of the fractions decreased in the more polymerized PA fractions. The FRAP values were negatively correlated with mDP ($r^2 = -0.81, P < 0.05$).

4.3.3 Effect of seed fractions on Caco-2 cell proliferation

The cytotoxic effects of seed fractions on Caco-2 cells were determined by the MTT assay (Figure 4.2). Caco-2 cells were exposed to seed fractions for 72 h and the data were expressed as IC$_{50}$, defined as the dose of each compound that inhibited cell proliferation to 50%. All the fractions tested showed a degree of toxicity as indicated by decreased absorbance values. The IC$_{50}$ value for immature seed fractions ranged from 17.7 - 70.2 µg/mL (Figure 4.2A). The number of viable cells was positively correlated with the mDP of immature seed fractions ($r^2 = 0.48, P < 0.05$) i.e., the fractions with highest mDP (F5 and F6) were less toxic to Caco-2 cells. Similar trends in terms of cytotoxic effect were also observed for the application of mature seed fractions (Figure 4.2B), where the number of viable cells was similarly correlated with the mDP of the fractions ($r^2 = 0.50, P < 0.05$). Mature seed fractions exhibited stronger cytotoxicity compared to immature seed fractions (Figure 4.2A and 4.2B).

4.3.4 The synergistic effect of isolated seed fractions and 5-FU on Caco-2 proliferation

The combination effect of seed fractions and 5-FU on Caco-2 cell viability was investigated (Figure 4.3). For immature seed fractions, the proliferation activity of Caco-2 cells was
significantly inhibited by all the seed fractions tested (Figure 4.3A). Compared to the GSE control (67%), F2 and F3 of immature seed extracts were more toxic to Caco-2 cells (F2, 32% and F3, 35% of control value, \( P < 0.05 \)). However, when seed fractions were present with 5-FU (100uM), the growth inhibitory effects of 5-FU were significantly enhanced (\( P < 0.05 \)) (Figure 4.3A). 5-FU significantly reduced cell proliferation to 62% of control values (\( P < 0.05 \)). F1, F2 and F3 significantly enhanced the growth inhibitory activity of 5-FU (27%, 73% and 56% respectively compared to 5-FU control; \( P < 0.05 \)). F2 could therefore be considered a more potent chemotherapy agent than unfractionated commercially available GSE, which only enhanced the growth inhibitory of 5-FU by 55% (\( P < 0.05 \); compared to 5-FU control). Moreover, we also found that immature F2 (32%) and F3 (35%) were more potent chemotherapeutic agents than 5-FU (62% of control value; \( P < 0.05 \)).

Mature seed fractions behaved in a similar manner to immature seed fractions (Figure 4.3B). Mature seed fractions significantly reduced cell proliferation (\( P < 0.05 \)). F2 and F3 were more cytotoxic to Caco-2 cells (13% and 17% respectively, \( P < 0.05 \)) than GSE (47%) (Figure 4.3B). When cells were exposed to seed fractions and 5-FU, some seed fractions enhanced the capacity of 5-FU to reduce cell proliferation. GSE significantly enhanced the growth inhibition of 5-FU by 49%. However, F1-F4 significantly enhanced the growth inhibitory effect of 5-FU (62%, 83%, 80% and 60% respectively compared to 5-FU control; \( P < 0.05 \)). Moreover, the F2 (13%), F3 (17%) and F4 (50%) fractions were more potent than 5-FU alone (65% of control value; \( P < 0.05 \)) (Figure 4.3B).
4.4 Discussion

Grape seed PAs have been reported to exert health-promoting properties, particularly in the gut [44, 45, 47]. In addition to its anti-cancer efficacy, GSE has also been demonstrated to reduce gastrointestinal toxicity following chemotherapy treatment in healthy animals and in normal intestinal cells [17]. When combined with 5-FU, GSE not only enhanced the growth inhibition of 5-FU on Caco-2 cells but also tended to be a more potent chemotherapeutic agent than 5-FU [125]. Although there is much evidence to support the chemotherapeutic properties of GSE in colon cancer [44, 45, 47], identification of the bioactive components responsible remains undefined.

In the current study, PA fractions isolated from commercially available GSE showed different mDPs and molecular sizes. However, these fractions had very low conversion yields (< 37%), indicating that less than 40% of the fractions were characterised using the selected analysis techniques, which was unacceptable for this study. Thus, it was decided to isolate PA fractions from fresh grapes collected at different ripeness levels: immature (pre-veraison) and mature (ripe). This decision was based on the observation that immature seeds have been reported to have a higher conversion yield than mature seeds [26], and this was substantiated by the current study.

The cytotoxic effects of PA fractions on cancer cells have been reported previously [23, 124, 128]. However, to determine whether these effects occurred through extracellular signalling or following uptake of PAs was not attempted. The present study showed that the most active fractions affecting the viability of Caco-2 cells contained smaller oligomers: F2 and F3. The more potent cytotoxic effects observed in Cabernet Sauvignon seed fractions compared to GSE may have reflected a lower PA yield in GSE (~24%) compared to PA
isolated from fresh grapes (~60%). GSE was included as a positive control in the cell model also consistent with previous work in which GSE was identified as a potential anti-cancer and chemotherapy adjunct for colon cancer cells [125].

Absorption of PA across cell membranes was greatly dependent upon their mDPs [31, 129]. Only certain lower mDP PAs are absorbed during transit in the gut, leaving the larger mDP PAs (mDP > 7) deposited in the gut lumen. This results supports the current data whereby F2 and F3 (mDP 2-6) are potentially absorbed across the cell membranes, exerting a more potent intracellular effect within the cell than fractions with a higher mDP, which exert a surface effect only. However, F2 and F3 are more bioactive than F1, possibly due to their higher percentage of galloylation and proportion of epicatechin-3-O-gallate as terminal subunits, compared to F1. This result is in agreement with other studies in which PA fractions with higher galloylation were more cytotoxic to cancer cells than PA fractions with lower galloylation [24, 116]. However, the actual mechanism of PAs on cell growth remains unknown. Absorbed PA may play a vital role in interfering with cell signalling pathways. PAs have been reported to be inhibitors of androgen receptors in prostate cancer cells [130] and epidermal growth factor receptors on colon cancer cells [131]. In addition, PAs are known to attenuate the PI3-kinase (serine/threonine protein kinase) pathway [47] which could lead to induction of cell cycle arrest at the G1 phase [44] and activation of the apoptosis-inducing pathway [45].

Previous findings demonstrated that cytotoxic effects of PAs are dependent on their mDP [23, 24]. Nevertheless, the current study showed that fractions with higher mDP (7 - 16) failed to exert cytotoxic effects on colon cancer cells. However, the current study used a different solvent system to isolate PA fractions [26] compared to other studies [24, 132].
This solvent system manages to isolate PA fractions with a wider range of molecular mass (619-6063 g/mol) and mDP (2 - 19) compared to other methods (molecular mass of 552 - 1232 g/mol, mDP =1 - 4); implying that the previous results only measured limited PA fractions (< 1200 g/mol) for their biological activities.

Previous studies have demonstrated that the cytotoxic effects of PAs are governed by their antioxidant activities [23, 133, 134]. The antioxidant capacity of PAs is greatly dependent upon their degree of polymerization and galloylation [24, 135]. The reduction of metal ions, as measured by the FRAP assay, is believed to be positively correlated with the number of hydroxyl groups present in the molecules, and that the points of attachment to transition metal ions in the flavonoid molecules are at the o-catechol group of ring B [136]. However, this was not the case in the current study. PA fractions with higher mDP were weaker antioxidants, which is in agreement with previous studies [134, 137]. Fractions with larger subunits tend to self-aggregate and cause stereochemical hindrance [134, 138], thereby exposing fewer hydroxyl groups for radical scavenging activities.

When seed extract fractions were combined with 5-FU, they not only acted synergistically with 5-FU in killing Caco-2 cells, but also surpassed 5-FU as an anti-cancer agent. The current study also revealed that mature seed fractions were superior to immature seed fractions as chemotherapeutic agents against colon cancer in vitro. The chemical profile of the seed fractions showed that these effects could be driven by their higher proportion of epicatechin as terminal subunits and (-)-epicatechin-3-O-gallate as extension subunits compared to the immature seed extract. Another possible explanation might be that the distribution within each fraction has a higher proportion of low molecular weight material in the mature seeds than immature seeds.
In conclusion, further studies are warranted to determine the molecular mechanism of F2 and F3 on cellular pathways associated with colonic neoplasia. Mature seed PA extracts not only have potential uses in human health, but may also value-add to the wine industry. Our data provides compelling evidence that isolated PA seed fractions can enhance the impact of chemotherapy on cancer cells, and secondly, that F2 and F3 of grape seed extract could be a promising adjunctive approach to combat intestinal mucositis.
Table 4.1 Solvent systems used to fractionate proanthocyanidins from grape seeds.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Solvent system</th>
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<tr>
<td>1</td>
<td>75% v/v methanol</td>
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<tr>
<td>2</td>
<td>90% v/v methanol</td>
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<tr>
<td>3</td>
<td>10% v/v acetone; 80% v/v methanol</td>
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<td>20% v/v acetone; 65% v/v methanol</td>
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<tr>
<td>5</td>
<td>30% v/v acetone; 40% v/v methanol</td>
</tr>
<tr>
<td>6</td>
<td>60% v/v acetone</td>
</tr>
</tbody>
</table>
Table 4.2  The chemical profiles of GSE and its isolated fractions characterized by phloroglucinolysis and GPC.

<table>
<thead>
<tr>
<th>Proanthocyanidin Fraction</th>
<th>MC&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>mDP&lt;sup&gt;b&lt;/sup&gt; (%)</th>
<th>galloylation (subunit)</th>
<th>MM&lt;sup&gt;c&lt;/sup&gt; (GPC 50%)</th>
<th>MM&lt;sup&gt;d&lt;/sup&gt; (%)</th>
<th>Mass&lt;sup&gt;e&lt;/sup&gt; (%)</th>
<th>Terminal subunits&lt;sup&gt;f&lt;/sup&gt;</th>
<th>Extension subunits&lt;sup&gt;f&lt;/sup&gt;</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C</td>
<td>E</td>
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<tr>
<td>GSE Control&lt;sup&gt;g&lt;/sup&gt;</td>
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<td>19</td>
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<td>22.4</td>
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<sup>a</sup>Mass conversion based on % recovery of proanthocyanidin by phloroglucinolysis based on the gravimetric mass.  
<sup>b</sup>Mean degree of polymerization in epicatechin units.  
<sup>c</sup>Molecular mass as determined by phloroglucinolysis.  
<sup>d</sup>Molecular mass as determined by GPC at 50% proanthocyanidin elution.  
<sup>e</sup>Percent composition of proanthocyanidin fractions by gravimetric recovery.  
<sup>f</sup>Percent composition of subunits (in moles) with the following subunit abbreviations: (-P), phloroglucinol adduct of extension subunit; C, (+)-catechin; EC, (-)-epicatechin; ECG, (-)-epicatechin-3-O-gallate.  
<sup>g</sup>Crude grape seed extract of which 55% w/w eluted in F0 and 45% w/w was recovered as proanthocyanidin for further fractionation.
Table 4.3  The chemical profile of immature and mature fractions characterized by phloroglucinolysis and GPC.

<table>
<thead>
<tr>
<th>Proanthocyanidin Fraction</th>
<th>MC(^a) (%)</th>
<th>mDP(^b) (%)</th>
<th>Galloylation (subunit)</th>
<th>MM(^c) (GPC 50%)</th>
<th>Mass(^d) (%)</th>
<th>Terminal subunits(^f)</th>
<th>Extension subunits(^f)</th>
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<td></td>
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<td></td>
<td></td>
<td>C</td>
<td>E</td>
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<td>Immature(^g)</td>
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<td>7400</td>
<td>8.7</td>
<td>22.3</td>
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\(^a\)Mass conversion based on % recovery of proanthocyanidin by phloroglucinolysis based on the gravimetric mass. \(^b\)Mean degree of polymerization in epicatechin units. \(^c\)Molecular mass as determined by phloroglucinolysis. \(^d\)Molecular mass as determined by GPC at 50% proanthocyanidin elution. \(^e\)Percent composition of proanthocyanidin fractions by gravimetric recovery. \(^f\)Percent composition of subunits (in moles) with the following subunit abbreviations: (-P), phloroglucinol adduct of extension subunit; C, (+)-catechin; EC, (-)-epicatechin; ECG, (-)-epicatechin-3-O-gallate. \(^g\)Purified proanthocyanidin from immature (preveraison) and mature (ripe) Cabernet Sauvignon seeds prior to fractionation.
Figure 4.1  Antioxidant activity of the fractions from immature (A) and mature (B) seed extracts measured by FRAP assay. Results are expressed as mean ± SEM of 3 independent experiments. Different letters indicate significant difference at $P < 0.05$. 
Figure 4.2  Proliferation activity of Caco-2 cells after exposure to fractions from immature (A) and mature (B) seed extracts for 72 h. Data are expressed as IC$_{50}$ or dose (µg/mL) inhibiting cell viability to 50% (mean ± SEM) of 3 independent experiments. Different letters indicate significant difference at $P < 0.05$. 
Figure 4.3  Combination effects of immature (A) and mature (B) fractions (25 µg/mL) and 5-FU on Caco-2 cells for 72 h. Data are presented as percent of cell proliferation relative to proliferation of control. Data are expressed as mean ± SEM of 3 independent experiments. Different letters indicate significant difference at $P < 0.05$. 
Chapter 5  
Comparison of grape seed polyphenolic content and antioxidant activity in five different varieties of wine grapes
5.0 Abstract

**Introduction:** Grape seeds are the by-products of winemaking and grape juice processing. Grape seeds are a rich source of proanthocyanidins (PAs) which have become of interest to nutritionists due to their antioxidant and chemopreventative activities in the cancer setting. We hypothesized that grape seeds derived from different wine grape varieties may have differing PA composition and therefore affect their antioxidant capacities. **Methods:** Grape seeds were sourced from Australian commercial winegrapes (Vitis vinifera, L. cvs.): Merlot, Chardonnay, Viognier, Tannat (Adelaide Hills), Tannat (Nuriootpa) and Shiraz. The polyphenolic composition of the seed extracts was determined by Folin-Ciocalteau, p-(dimethylamino)-cinnamaldehyde (DMACA) and phloroglucinolysis assay. Antioxidant activity was determined by the Ferric Reducing Antioxidant Power (FRAP) assay. **Results:** Chardonnay and Tannat (Nuriootpa) grape seeds had the highest polyphenolic and flavan-3-ol content ($P < 0.05$) compared to other seed extracts. These extracts also attained the highest antioxidant activity compared to other grape seed sources (2.78-2.43 mM/g; $P < 0.05$). Tannat seeds sourced from Nuriootpa had a higher polyphenolic content (321 mg CAT/g; $P < 0.05$) compared to Tannat seeds sourced from Adelaide Hills (225 mg CAT/g seed). Principal component analysis (PCA) revealed the antioxidant activity of seed extracts not only applies to total phenolic and flavan-3-ol content, but also on the galloylation and mDP value of PA. **Conclusion:** There was a difference in polyphenolic content, not only between grape varietal, but also between geographical regions. Australian wine grapes differentially represent important sources of antioxidant species that could be effective in combating health disorders characterised by oxidative stress.
5.1 Introduction

Proanthocyanidins (PAs), also known as condensed tannins, are commonly found in fruits and vegetables as well as leaves and barks [139]. PAs are not only responsible for plant protection against pathogens and herbivores [139], but also play an important role in winemaking and contribute to wine quality and mouth-feel, such as colour, astringency and bitterness [140, 141]. Recently, PA rich foods (lychee, cocoa and red wine) have received attention for their plethora of beneficial health effects [21]. PAs have demonstrated anti-carcinogenic activity in various cell lines, including lung, colon and prostate cancer [37, 39, 142]. A recent in vitro study reported PAs exhibited anti-viral activity against coxsackie and herpes simplex virus on HeLa and Vero cell lines [107].

PAs are made up of multiple extensions of the flavan-3-ol subunits, (+)-catechin and (-)-epicatechin and (-)-epicatechin-3-0-gallate [1, 143]. These compounds are also present in grapes, Vitis vinifera, and mainly deposited in grape seeds [122]. Grape seed PAs exert powerful antioxidant properties and other beneficial biological activities [59, 123]. Grape seed extract (GSE) has been reported to induce apoptosis in human prostate carcinoma cells [144], to protect cardiac cells from apoptosis via induction of endogeneous antioxidant enzymes [94] and to decrease severity of intestinal injury in a rat model of intestinal mucositis [17]. GSE has also been identified as a potent radical scavenger compared to other antioxidants such as Vitamin C [83].

The health benefits of wines are attributed to their PA content [5]. PA compounds are transferred from grapes (skins, seeds and stems) to the wine during the winemaking process (crushing, maceration and fermentation) [145]. However, large quantities of wine by-products (grape skins and seeds) are produced by the winemaking and grape juice industries.
worldwide. At present, there is a growing interest in the exploitation of these waste products. The recovery of antioxidant polyphenolic compounds as a winery waste product could represent a means to minimize the waste and maintain the environmental equilibrium.

Numerous studies have reported on the varying PA composition in grape seeds from different wine grape varieties using high performance liquid chromatography (HPLC) and Folin-Ciocalteau assay [145-151]. For example, Tannat red wines have been reported to have higher polyphenolic content compared to Merlot wine and offer great health benefits in promoting cardiovascular health [5, 146]. A number of studies have also reported the higher polyphenolic content in Chardonnay grapes compared to other varieties [152, 153]. For example, Chardonnay grape seeds were reported to have higher polyphenolic content than Merlot grape seeds [152]. Landrault et al. [153] reported higher antioxidant activity in Chardonnay wine compared to other dry white wine (Viognier and Sauvignon Blanc).

However, a detailed analysis of the varietal differences in the complete PA profiles, determined by phloroglucinolysis has not been undertaken. Furthermore, the relationships between grape seed antioxidant capacity and PA profile parameters are also not understood. If we can identify grape varieties with consistently higher PA composition, these could be targeted to produce a more enriched starting material for further purification as nutritional supplements. Thus, the objectives of this study were therefore to compare the influence of both variety and grape seed provenance on PA composition and antioxidant capacities of grape seeds sourced from different varieties and geographical regions: Nuriootpa and Adelaide Hills, South Australia, Australia. The aims of this study were to 1) define in more detail, the phenolic composition of grape seeds derived from wine grapes commonly used in global winemaking and reported to have differing phenolic content, 2) undertake a
preliminary examination of whether these profiles differed between grape varietals, 3) examine whether the profile was influenced by region and 4) whether the resultant anti-oxidative capacity of each grape varietal seed extract differed. This information is essential to confirm the potential of the use of grape seed PAs in health disorders characterised by oxidative stress and continue to uncover the compounds within these extracts that afford these health benefits.
5.2 Methods and materials

5.2.1 Instrumentation

An Agilent model 1100 HPLC (High Pressure Liquid Chromatography; Agilent Technologies Australia Pty. Ltd., Melbourne, Australia) was used with Chemstation software for chromatographic analyses.

5.2.2 Chemicals

All HPLC-grade solvents, including methanol, acetone, acetonitrile, formic and hydrochloric acid were purchased from Sigma-Aldrich (St. Louis, MO). Folin-Ciocalteau’s reagent, catechin, gallic acid, sodium bicarbonate, 4-(dimethylamino)cinnamaldehyde (DMACA), ferrous sulphate, ferrous chloride, 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), phloroglucinols and ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO).

5.2.3 Grape seeds preparation and extraction of seed proanthocyanidins (PAs)

Wine grape samples of 20kg for each varietal (Tannat, Shiraz, Merlot, Viognier and Chardonnay) were obtained from commercial vineyards in the Adelaide Hills and Nuriootpa growing regions of South Australia. Grape samples were collected at commercial ripeness (25° Brix) during the 2008 growing season. Grape berries of each varietal were kept frozen at -20°C. Seeds were removed from flesh with a scalpel and rinsed with Milli-Q water and dried on a piece of paper towel. The collected seeds were re-frozen at -20°C prior to extraction.

PAs were extracted from seeds of the 6 different grape berry varieties based on a previously described method with slight modification [150]. Seeds were ground by a coffee grinder and incubated with 70% acetone (v/v, 25 mg/mL) at 37°C for 2 h in a shaking incubator. The
solution was filtered (Whatman no. 1) and was further concentrated by a rotary evaporator (BUCHI, Medos Company Pty. Ltd., Adelaide) to remove acetone. The concentrated seed extract solutions were kept in a 200 mL round bottomed flask and aliquots (1 mL) were prepared and kept at -20°C for further analyses.

5.2.4 **Quantification of polyphenolic content in grape seeds**

The total phenolic content of each seed extract was determined by Folin-Ciocalteau (FC) method as previously described by Singleton and Rossi and adapted for use in a 96-well microtitre plate [154]. Catechin and gallic acid were used as standards to generate standard curves. Briefly, 50 µL of FC solution (1/10 with Milli-Q water) was added to 10 µL of seed extract for 10mins. Sodium bicarbonate solution (40 µL, 7.5% w/v) was added to samples and incubated in the dark for 2 h before being read by a spectrophotometer (Multiskan® Thermo Electron Corporation, Vantaa, Finland) at 740 nm. The total phenolic content of seed extracts was expressed as mg catechin (CAT) or gallic acid (GAE)/g seed.

5.2.5 **Total flavan-3-ol content in grape seeds**

Total flavan-3-ol content was determined by p-(dimethylamino)-cinnamaldehyde (DMACA) described by [155] with slight modification for adaptation to a 96-well microtitre plate. Catechin was included to generate a standard curve. DMACA solution (0.1%, w/v) was prepared in acid methanol (0.75 M Sulphuric acid). Seed extracts (20 µL) were transferred to a 96-well microtitre plate in triplicate and 100 µL of DMACA solution was added to each well. Plates were read at 640 nm by a spectrophotometer after 20 min. The flavan-3-ol content of seed extracts was expressed as mg CAT/g seed.
5.2.6 *Grape seed PA compositional information by phloroglucinolysis*

Phloroglucinolysis was performed according to the method described by Bindon et al. [106]. 10 mL of each seed extract was lyophilized to dry powder and kept at -20°C prior to analysis. Seed extracts were dissolved in HPLC-grade methanol (10 mg/mL).

For phloroglucinolysis, equal volumes of seed extract and phloroglucinol solution (0.2 N HCL and 100 g/L phloroglucinol and 20 g/L ascorbic acid) were prepared to yield a final concentration of 5 g/L. The phloroglucinolysis reaction was carried out at 50°C for 25 min and analysed by RP-HPLC according to the condition using (-)-epicatechin as the quantitative standard outlined previously by Kennedy and Jones [25].

5.2.7 *Antioxidant activities of grape seeds*

The antioxidant activity of seed extracts was measured by ferric reducing antioxidant power assay (FRAP) according to a protocol described by Benzie and Szeto [127] and adapted to use in 96-well microtitre plates. FRAP reagent was prepared in a 10:1:1 v/v solution (300 mM acetate buffer pH 3.6, 10 mM TPTZ solution in 40 mM HCL and 20 mM Ferrous chloride). The reaction was started by adding 150 µL of FRAP solution to 15 µL of seed solution. Ferrous sulphate was used to construct a standard curve and FRAP values were expressed as mM Fe(II)/g of seed.

5.2.8 *Statistical analysis*

Statistical analyses were determined by GraphPad Prism 5 and PASW statistics version 18. Statistical analysis was determined by ANOVA with a Tukey’s *post-hoc* test. Pearson’s correlation test was performed to determine the correlation relationship between PA level and antioxidant value. Statistical significance was considered at $P < 0.05$. Principal
Component Analysis (PCA) of total phenolics, PA profile parameters and antioxidant capacity was undertaken using XLSTAT version 2011.4.02 (Addinsoft SARL, France).
5.3 Results

5.3.1 Polyphenol content of grape seed

The total polyphenol content of grape seeds sourced from different varieties are illustrated in Figure 5.1. The FC assay indicated the total polyphenolic content in grape seeds sourced from different varietals range from 125–321 mg catechin equivalent (CAT)/g seed or 83–192 mg gallic acid equivalent (GAE)/g seed. Chardonnay and Tannat (Nuriootpa) grape seeds had the highest total polyphenolic content (321 and 312 mg CAT/g grape seed, respectively; \( P < 0.05 \)), followed by Tannat (Adelaide Hills; 225 mg CAT/g seed), Shiraz (176 mg CAT/g seed), Merlot (162 mg CAT/g seed) and Viognier (125 mg CAT/g seed). Interestingly, the same varietal Tannat seeds sourced from different regions showed very different total polyphenol content (Nuriootpa, 321 mg CAT/g and Adelaide Hills, 225 mg CAT/g seed; \( P < 0.05 \)).

5.3.2 Total flavan-3-ol content

Flavan-3-ol composition in grape seeds was measured by the DMACA assay (Figure 5.2). Chardonnay and Tannat (Nuriootpa) grape seeds had the highest flavan-3-ol level (169 and 153 mg CAT/g seed, respectively) and were significantly different to the rest of the tested grape seeds (\( P < 0.05 \)). Tannat (Nuriootpa) grape seeds had significantly higher flavan-3-ol levels (153 mg CAT/g seed) compared to Tannat (Adelaide Hills) grape seeds (94 mg CAT/g seed). Flavan-3-ol level was highly correlated with polyphenol content measured by FC assay (\( r^2 = 0.81, P < 0.001 \)).

5.3.3 Proanthocyanidins profile of grape seeds

Although FC and DMACA assays measure the total phenolic and flavan-3-ol content in the seed extract, it fails to provide the subunit composition of the phenolic compounds. The
phloroglucinolysis HPLC method produces information on subunit composition, conversion yield and mean degree of polymerization. The PA profile of grape seeds is illustrated in Table 5.1. The overall mass conversion of recovered PAs measured by phloroglucinolysis ranged from 19.8-40.2%. Chardonnay seed tannin had the highest mDP (8.7) and galloylation (24%). The PA terminal subunits in Chardonnay seeds were mostly dominated by (-)epicatechin-3-O-gallate (74%). Tannat grape seeds sourced from two different regions showed very similar PA profiles, with mDP (6.7-7.6), galloylation (22-23%) and a higher proportion of (-)epicatechin-3-O-gallate as terminal subunits. However, Tannat (Nuriootpa) had a higher mass conversion (40%) compared to Tannat (Adelaide Hills). Merlot, Viognier and Shiraz had a lower mDP, molecular mass and galloylation status. The seed extracts had a higher proportion of catechin as terminal subunits compared to Chardonnay seed extracts.

5.3.4 Antioxidant activity of grape seeds

The antioxidant capacities or the reducing power of grape seeds was tested using the FRAP method. FRAP values from grape seeds ranged from 1.48 - 2.78 mM/g (Figure 5.3). Similar trends were also observed for antioxidant activity in grape seeds sourced from different varietals. The greatest antioxidant capacity was found in Tannat (Nuriootpa) and Chardonnay grape seeds (2.78 and 2.38 mM/g, respectively). On the contrary, the same varietal Tannat sourced from different regions showed very different FRAP values (Adelaide Hills, 1.84 mM/g, Nuriootpa, 2.78 mM/g; P < 0.05). Correlation between total phenolic content and flavan-3-ol content with antioxidant activity of grape seeds from different grape varietals was highly significant (r² = 0.77, r² = 0.86, respectively; P < 0.001). Galloylation is weakly correlated with FRAP (r² = 0.19, P < 0.05).
5.3.5 **Principal component analysis (PCA)**

A correlation matrix was generated from the mean values of replicate grape seed extract samples for total phenolics and flavan-3-ol levels, FRAP values and PA profile parameters. The first and second principal components (PC) accounted for 47.45% and 23.27% of the variance in the PCA data for the six grape seed varieties, respectively. As shown in Figure 5.4, PC1 contrasted the grape seed extracts based on mDP, galloylation, molecular mass, DMACA, FC and FRAP values plus epicatechin terminal subunits. The second PC separated the different seed extracts on the basis of mass conversion and catechin terminal subunit content. The third PC accounted for a further 14.6% of the variance in the PCA of the data for the six seed extracts and is correlated with the ECG and epicatechin extension subunits (data not shown). Chardonnay and Tannat (Nuriootpa) had greater antioxidant capacity than Viognier, Merlot, Shiraz and Tannat (Adelaide Hills) which all shared similar FRAP values. Chardonnay and Tannat (Nuriootpa) grape seed extracts tended to have higher mDP values, greater galloylation, flavan-3-ol content and total phenolics. Relative to Tannat (Nuriootpa), the Chardonnay grape seed extract contained more ECG terminal subunits, while Tannat (Nuriootpa) had a higher mass conversion compared to Chardonnay. Tannat (Adelaide Hills), Shiraz, Merlot and Viognier had more epicatechin terminal subunits relative to Tannat (Nuriootpa) and Chardonnay. Shiraz extracts contained more catechin terminal subunits. Interestingly, Tannat (Nuriootpa) had higher epicatechin extension subunits, while the Tannat extracts from Adelaide hills had higher contents of ECG extension and epicatechin terminal subunits.
5.4 Discussion

The project examined grape seeds of 5 different wine grape varietals, two white grape varieties (Chardonnay and Viognier) and 3 red grape varieties, (Tannat [from Nuriootpa and Adelaide Hills, South Australia], Merlot and Shiraz). Previous studies have shown the PAs differ within a variety depending on the ripeness levels [26, 156]. These findings were also reported in Chapter 4, where mature seeds (25° Brix) had lower mass conversion (lower measurable PA in seed extracts) compared to immature seeds (unripe) with higher mass conversion (higher measurable PA in seed extracts). We wanted to avoid ripeness factors affecting our results. As such, fruit for this study was collected from commercial vineyards and harvested at 25° Brix, a ripeness level reflecting the average total soluble solids levels of commercial wine grapes at harvest for the winemaking process.

The current findings are consistent with other studies in which different grape varietals display differing PA profiles in the grape seeds, skins or whole berries [152, 157]. For example, total polyphenol content for our grape seeds ranged from 83 (Viognier) - 192 (Tannat Nuriootpa) mg GAE/g seed. Chardonnay seeds attained the highest polyphenolic (180 mg GAE/g seeds) and flavan-3-ol (170 mg CAT/g seeds) content. This agrees with previous studies indicating Chardonnay seeds (53 mg GAE/g seeds) attained the highest polyphenolic content compared to Merlot grape seeds (39 mg GAE/g seeds) [158]. Using spectrophotometry at 280nm, Tannat red wine has been reported to have higher total phenolic content compared to other red wines such as Merlot and Cabernet Sauvignon [146, 159]. Our findings confirm this as Tannat from Nuriootpa had higher total polyphenolic content compared to both Merlot and Shiraz. Using the FC assay, Viognier wine has been reported to have lower total polyphenol content than Chardonnay wine (288 mg GAE/L versus 379 mg GAE/L, respectively) [153]. Our data agrees with earlier findings that
Chardonnay seed extracts has higher total polyphenolic content than Viognier (187 mg GAE/g seeds and 83 mg GAE/g seeds, respectively).

The polyphenolic content in our seed extracts as measured by the Folin-Ciocalteau assay (83 – 192 mg GAE/ g seed), was higher than those reported in previous studies possibly due to a different solid: liquid ratio used in other study (32 – 53 mg GAE/g seed) [158]. The current study used (1 g in 40 mL) compared to others (50 mg in 2 mL). Additionally, de-oiling seed extracts with hexane was performed to remove lipid material by Yilmaz and Toledo[158], which was not applied in our study. Thus, the presence of sugars and protein in our seed extracts may have potentially interfere with the measurement and lead to an overestimation of total phenolic content by the Folin-Ciocalteau assay [160].

PAs are made up of complex mixtures of flavan-3-ol subunits, they exist in oligomers (hydrophilic) and polymers (lipophilic), and it is difficult to isolate the specific compounds [1]. There has been a lot of activity in this extraction methodology area and as a result numerous extraction methods have been employed in order to achieve maximal extraction of PA. A search of the literature indicates a number of extraction methods differing mainly in extraction time and solvent used for the isolation of PAs from grape seeds [23, 40]. The extraction of PAs is greatly dependent upon their diffusion into the extraction solvent [41]. Extraction times varying from 30 min [40] to 24 h [29] have been used previously. The shorter extraction times aim to reduce PA degradation by oxidation, while longer extraction times are undertaken to achieve maximal extraction. Moreover, various solvents (methanol, acetone, and ethanol) have been used for the extraction of polyphenolic compounds from grapes. Monomers and oligomers are more soluble in methanol, while higher molecular weight PAs, are better extracted with acetone [29, 42]. In addition, extraction of PA
compounds is more effective in a mixture of solvents compared to a single solvent only [22, 43]. Higher polyphenol content was reported when extracted from grape seeds using a solvent mixture of acetone and water compared to acetone alone [44]. Taken together, with the aim of examining the bioactivity of our extracts, the current study used an aqueous acetone solution with a 2 h incubation time in order to achieve maximal extraction but decrease damage to PAs by oxidation.

Since both Chardonnay and Tannat (Nuriootpa) contained higher total phenolic and flavan-3-ol content compared to other seed extracts, we wanted to identify what drives the total phenolic and flavan-3-ol content in these seed extracts using phloroglucinolysis. Phloroglucinolysis enables determination and characterisation of the complete PA profile such as subunit composition, mDP, galloylation and mass conversion status [25]. The PA profiles of Chardonnay and Tannat (Nuriootpa) seed extracts showed that they have higher mDP, molecular mass and galloylation status compared to other seed extracts. Moreover, Chardonnay’s PA subunits are mainly driven by a higher proportion of epicatechin-3-O-gallate as terminal subunits, while Tannat (Nuriootpa) PA has a higher mass conversion (40%) compared to other seed extracts (< 28%). In contrast, other seed extracts (Merlot, Viognier, Tannat [Adelaide Hill] and Shiraz) have a lower mDP, galloylation and molecular mass and their PA profile are driven by higher proportion of epicatechin as terminal subunits. This indicates that total phenolic profile of seed extracts are control by mDP, galloylation, molecular weight and epicatechin-3-O-gallate as terminal subunits.

Although phloroglucinolysis characterised the PA profiles of each seed extracts, our seed extracts had very low conversion yields (< 40%), indicating that less than 40% of the seed fractions were characterised, compared to previous studies (65 - 81%) [25, 26]. The possible
explanation for this could be due to the variation of extraction method applied. The current study had a shorter extraction time (2 h) compared to other studies (18 - 24 h), indicating less PAs are extracted in the shorter time point and confirming earlier findings. Future studies are warranted using both ours and previously described extraction methods [26] and compare the effects on the resultant extracts’ bioactivity.

The present study also revealed that differences exist not only between varieties, but also within the same varietal. Tannat seed extracts sourced from different regions (Adelaide Hills and Nuriootpa) showed very different total polyphenolic and flavan-3-ol compositions and, PA profiles. For example, Tannat (Nuriootpa) had a higher polyphenolic content and mDP, compared to Tannat seeds sourced from Adelaide Hills. The major difference could be due to soil and climate differences between the two regions, important factors influencing PA levels in grape seeds. The polyphenol content in grape seeds may be affected by type of cultivar, viticultural practises and environmental condition [19, 46-48]. For example water deficit during the veraison to harvest period was reported to enhance the biosynthesis and concentration of polyphenol in grape berries [46]. Vineyards located at sites of lower altitudes and, higher temperature and humidity enhanced the biosynthesis of polyphenols in grape berries compared to vineyards located at higher altitude [156, 161]. The Adelaide Hills wine grape growing region is located at a higher altitude and dominated by a cooler climate (average temperature: 10 - 20°C) with heavier rainfall (total rainfall in February: 7.2mm) compared to Nuriootpa which is located at lower altitude dominated by warmer climate (average temperature: 13 – 27 °C) and receives less rainfall (total rainfall in February: 0.4mm). Since we found higher mDP values and levels of polyphenolics in the Tannat extracts from Nuriootpa, our study supports previous findings that the warmer climatic conditions enhance PA biosynthesis and metabolism in grape seeds [156, 161].
However, further studies are warranted to examine grape seeds sourced from different varieties, from different regions across 2 - 3 vintages to take into account seasonal differences.

Consumption of red wine has been reported to reduce the mortality rate from cardiovascular disease [162]. The beneficial effects of red wine have been attributed to its polyphenolic content and antioxidant activities [5, 163]. Chronic consumption of red wine was demonstrated to protect rats from oxidative stress in vivo [164]. Additionally, the antioxidant properties of red wine have been shown to protect against atherosclerosis in hamsters [165]. The bioactive compounds probably responsible are mainly located in the seed and skin relative to pulp of grape berries [122]. Red wine has been reported to possess higher antioxidant activity than white wines [166, 167]. This is most likely due to the presence of grape skin and seeds during red wine fermentation which is not the general case for white wine production. However, the selection of grape varietal for winemaking, including different red grape varieties for red wine production, may also influence the PA and antioxidant content of wines.

Our findings showed that Chardonnay grape seed extract was a more powerful antioxidant than three other red grape varieties and one white grape variety seed extract. This supports the findings reported by Yilmaz and Toledo [158], where Chardonnay seed powders were stronger antioxidant agents compared to Merlot seed powders measured by a different antioxidant assay (ORAC, oxygen radical absorbance capacity). Tannat red wine has been reported to improve vascular health and human longevity due to its antioxidant properties [5]. This was also substantiated in our study which showed Tannat (Nuriootpa) had similar antioxidant capacity as Chardonnay seeds. When we examined the PCA plot, some of the
PA profile parameters possibly driving the higher antioxidant activity of Chardonnay and Tannat (Nuriootpa) seed extracts could be the higher mDP values, greater galloylation, and flavan-3-ol content and total phenolics. This implies the antioxidant capacity of grape seed PA may not only apply to total phenolic and flavan-3-ol content, but also on the galloylation and mDP values of PA.

The higher percentage of galloylation and proportion of (-)-epicatechin-3-O-gallate as terminal subunits in Chardonnay seed extracts also supports previous findings indicating the antioxidant capacity of PAs to be greatly dependent upon the presence of galloyl compounds and the number of hydroxyl groups present in the molecules [52, 53]. Both studies reported antioxidant activity increases with galloylation units. Additionally, Tannat seeds (Nuriootpa) also displayed potent antioxidant activity relative to the other varieties, which could be due to its higher mass conversion (40%) compared to the other seed extracts. The higher mass conversion indicates that the PA in Tannat seeds (Nuriootpa) would depolymerise more easily relative to the other extracts under study, thus could be more soluble and hence more active in the FRAP assay. Moreover, the higher mass conversion in Tannat (Nuriootpa) grape seeds indicated more bioactive PA present in their seeds when they are grown in a warmer climate. Future studies should be conducted on Chardonnay and Tannat grape seeds in relation to gaining a better understanding of the structure-function relationships of more bioactive PA compounds.

In conclusion, the current preliminary study demonstrated a large variation in PA profile parameters, not only between grape varietals, but also within the same varietal emanating from different grape growing regions. It has also indicated the drivers of antioxidant activity are likely to be linked to not only total phenolic content but galloylation status, mean degree
of polymerisation, and the likelihood of the larger polymers to depolymerise giving rise to PA forms that maybe more soluble and/or provide more readily accessible hydroxyl groups. Future studies should repeat these experiments on the different grape varieties to confirm the observed relative differences in antioxidant capacity and PA profile status. Previous findings have reported that dried grape seeds obtained from wine marc still retain significant amounts of PAs [54]. Thus, the recovery of polyphenolic compounds rich in antioxidants could represent a very economical source of PAs which could be used as dietary supplements or for the production of phytochemicals. This is also environmentally and economically favourable for the global wine industry as it provides a use for a substantial waste product of the wine grape industry whilst value adding to the same crop. Our data provides compelling evidence that Chardonnay and Tannat grape seeds sourced from Nuriootpa may have potential uses in human health due to their strong anti-oxidant activity.
Table 5.1 The chemical profile of seed extract PAs characterised by phloroglucinolysis and GPC

<table>
<thead>
<tr>
<th>Proanthocyanidin</th>
<th>MC(^a)</th>
<th>mDP(^b)</th>
<th>galloylation</th>
<th>MM(^c) (subunit)</th>
<th>Terminal subunits(^d)</th>
<th>Extension subunits(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(%)</td>
<td>(%)</td>
<td>(subunit)</td>
<td>C</td>
<td>E</td>
<td>ECG</td>
</tr>
<tr>
<td>Merlot</td>
<td>19.8</td>
<td>7.3</td>
<td>19</td>
<td>2338</td>
<td>20.0</td>
<td>19.9</td>
</tr>
<tr>
<td>Chardonnay</td>
<td>19.9</td>
<td>8.7</td>
<td>24</td>
<td>2852</td>
<td>15.1</td>
<td>10.6</td>
</tr>
<tr>
<td>Viognier</td>
<td>27.6</td>
<td>7.1</td>
<td>22</td>
<td>2296</td>
<td>23.8</td>
<td>13.5</td>
</tr>
<tr>
<td>Tannat (Adelaide Hill)</td>
<td>24.9</td>
<td>6.7</td>
<td>22</td>
<td>2151</td>
<td>24.5</td>
<td>22.3</td>
</tr>
<tr>
<td>Tannat (Nuriootpa)</td>
<td>40.2</td>
<td>7.6</td>
<td>23</td>
<td>2467</td>
<td>29.7</td>
<td>12.0</td>
</tr>
<tr>
<td>Shiraz</td>
<td>27.3</td>
<td>6.5</td>
<td>21</td>
<td>2088</td>
<td>22.8</td>
<td>17.1</td>
</tr>
</tbody>
</table>

\(^a\)Mass conversion based on % recovery of proanthocyanidin by phloroglucinolysis based on the gravimetric mass. \(^b\)Mean degree of polymerization in epicatechin units. \(^c\)Molecular mass as determined by phloroglucinolysis. \(^d\)Percent composition of subunits (in moles) with the following subunit abbreviations: (-P), phloroglucinol adduct of extension subunit; C, (+)-catechin; EC, (-)-epicatechin; ECG, (-)-epicatechin-3-O-gallate.
Figure 5.1 Total Folin-Ciocalteau measured of total polyphenol content of grape seeds sourced from different grape varietals, expressed in catechin (A) and gallic acid (B) equivalents. Results are expressed as mean ± SEM of 3 independent experiments. Different letters indicate significant difference at $P < 0.05$. 
Figure 5.2 DMACA measures of total flavan-3-ol levels in grape seeds from different grape varietals. Results are expressed as mean ± SEM of 3 independent experiments. Different letters indicate significant difference at $P < 0.05$. 
Figure 5.3 Total antioxidant capacity of grape seeds from different grape varietals, measured by FRAP assay. Results are expressed as mean ± SEM of 3 independent experiments. Different letters indicate significant difference at $P < 0.05$. 
Figure 5.4  Principal component analysis of the mean values of each replicate grape seed extract sample for total phenolics and flavan-3-ol levels, FRAP values and PA profile parameters. FC indicates total phenolic measured by Folin-Ciocalteau assay; DMACA indicates total flavan-3-ol measured by DMACA assay; FRAP indicates antioxidant activity; mDP indicated mean degree of polymerization; MC indicates mass conversion of PA in seed extract measured by phloroglucinolysis; -P indicates phloroglucinol adduct of extension subunits; C indicates (+)-catechin; E indicates (-)-Epicatechin and ECG indicates (-)-Epicatechin-3-O-gallate.
Chapter 6  Discussion
6.1 Discussion

Collectively, the results contained within this thesis provide significant insight into the potential for GSE to serve as a promising therapeutic adjunct to combat intestinal mucositis and also to expand options as anti-neoplastic agents against colon cancer beyond conventional chemotherapy. The results suggest that GSE treatments (1000 mg/kg) are safe for consumption by rodents and this may reflect a safety dosage for human administration. Of even greater importance, the study demonstrates that the beneficial effect of GSE is likely attributed to the PA content. Furthermore, the study concludes that the selection of grape seeds is important for the isolation of bioactive PA compounds.

**GSE dose-responsively reduced intestinal injury in a rat model of intestinal mucositis**

Initially, a dose-response study of GSE was conducted in a rat model of intestinal mucositis. The key findings from this study were the reduction of intestinal injury, as a result of chemotherapy administration, at higher doses of GSE. This study represents the first report of GSE to dose-responsively reduce the severity of intestinal mucositis. This effect was more evident in the proximal small intestine, the principal site of intestinal injury, compared to the distal small intestine. As discussed in Chapter 1, the bioavailability of GSE is greatly dependent upon the chemical structure of PA. Smaller PA oligomers are absorbed across the small intestine, whilst larger polymers are adsorbed to the mucosal surface [29]. A possible explanation for the greater effectiveness of GSE in the proximal intestine could be an accumulation of the most active GSE PA components (mDP 2-6, as discussed in Chapter 4) in the gut lumen, which target inflammatory cytokines and reactive oxygen species, and result in diminished bioactive components reaching the distal regions of the small intestine. Importantly, this chapter reveals that oral administration of GSE (400mg/kg, 600mg/kg and
1000mg/kg) for nine days did not induce any deleterious side-effects in healthy animals, suggesting safety applications for GSE in humans.

**GSE acts synergistically with 5-FU to kill colon cancer cells**

The promising findings of GSE in the treatment of experimental mucositis, combined with the research findings of others who have shown the effectiveness of PAs in cancer settings [16, 44, 45], led to our further investigation of GSE and its potential impact on the effectiveness of chemotherapy on colon cancer cells (Chapter 3). The results of this study provided evidence that GSE acts synergistically with 5-FU chemotherapy to inhibit colon cancer cell proliferation. Moreover, this study also described that GSE preparations at high doses (50-100µg/mL) were superior anti-cancer agents compared to the chemotherapy agent, 5-FU, acting independently. Findings from Chapter 2 and 3 strengthen the potential role of GSE, not only as a natural source of nutraceuticals to combat mucositis, but also as a potent anti-neoplastic agent.

GSE has been demonstrated to selectively exert cytotoxicity towards colorectal cancer cells while maintaining the growth of normal colon cell lines [47]. In addition, our preliminary study indicated some protection by GSE was afforded against 5-FU chemotherapy damage in normal intestinal cell lines (IEC-6) [17]. The selective effects of GSE could be that oxidative damage may be prevented at lower GSE concentrations, as observed in earlier studies [17] whereas at higher concentrations, GSE may have greater impact at the cellular level, including induction of apoptosis and DNA damage [168]. These studies suggest that GSE treatment may provide a potent targeted therapy for killing cancer cells, without affecting normal cells.
Gallic acid (GA) acts synergistically with 5-FU to kill colon cancer cells

Since the health-promoting properties of GSE are attributed to their polyphenolic content, we investigated other polyphenolic compounds (EGCG, GA, resveratrol and catechin) to determine if they would enhance or inhibit the effect of chemotherapy on the viability of colon cancer cells. GSE is a complex mixture containing GA, catechin, epicatechin and oligomers of catechin and epicatechin (also known as PA), some of which are esterified to GA. GA demonstrated greater cytotoxicity against colon cancer cells compared to GSE. A possible explanation was that GSE represents a crude mixture of polyphenolic compounds and less potentially bioactive compounds (24% PA) compared to GA, which is a pure extract of active ingredients. Furthermore, GA has been identified as one of the major active constituents in GSE at inducing growth inhibition and apoptotic death of human prostate cancer cells [119]. The esterification of GA with catechin or epicatechin contributes to galloylation, reported to be an important factor in inducing cancer cell death [24]. Both GA and GSE are effective in combination with 5-FU at killing cancer cells at a lower dose, whilst at higher concentrations they are more potent anti-neoplastic agents compared to 5-FU alone.

This study also revealed that the chemical structure of polyphenolic compounds is responsible for their toxic effects on colon cancer cells. Many studies have demonstrated that cytotoxicity against cancer cells is dependent on the extension subunits of flavan-3-ol, degree of polymerization and galloylation units [124, 128, 169]. GSE comprises a mixture of oligomeric and polymeric forms of flavan-3-ol subunits, which are more potent than catechin (monomers of flavan-3-ol). Conversely, presence of a galloyl residue at the 3 position on the C-ring can also enhance the cytotoxic response of polyphenolic compounds [116]. In the current study, EGCG (galloylated monomers) displayed higher cytotoxic
activity compared to catechin (non-galloylated monomers). Therefore, the presence of galloyl compounds may be an important determinant for polyphenolic compound health benefits. Nevertheless, there is much discussion about galloylation with respect to anti-neoplastic properties, and the underlying mechanism remains undefined.

**Smaller PA oligomers (mDP 2-6) with higher galloylation are more potent on colon cancer cells**

Although there is significant evidence to support the chemotherapeutic properties of GSE in colon cancer, identification of the bioactive components responsible remains undefined. As discussed earlier, the chemotherapeutic properties of GSE are reliant on mDP and galloylation of PA. In addition, the findings in Chapter 2 suggested bioactivity of GSE in the proximal small intestine. Therefore, it is necessary to identify which forms and sizes of PA from GSE are responsible for promoting intestinal health (Chapter 4).

PA fractions isolated from fresh Cabernet Sauvignon grape seeds showed differing mDP or molecular sizes. We elected to use fractions isolated from Cabernet Sauvignon seeds in preference to the fractions of commercially available GSE (applied in experiments described in Chapters 2 and 3) because the GSE had a very low level of measurable PAs in the fractions. The study showed that the most active fractions affecting the proliferation rate of Caco-2 were F2 and F3. However, whether these effects occurred through extracellular signalling or following uptake of PAs is not known. There is a possibility that the cytotoxic effects of these fractions may be due to auto-oxidation of PA and from generation of hydrogen peroxide. F2 and F3 were made up of smaller oligomers of PA (mDP 2-6) which may be absorbed across cell membranes and interfere with cell signalling pathways rather than fractions with a higher mDP (mDP 7-16), which only remained adsorbed to the gut
mucosa. Further studies are warranted to look at the intestinal absorption of PA fractions on intestinal cell lines such as measuring the transepithelial electrical resistance value. Additionally, studies looking at apoptosis activity such as caspase-3 could provide evidence of specific sizes of PAs impact on cell signalling pathway.

Previous findings demonstrated that cytotoxic effects of PAs were dependent on their mDP [23, 24]. Nevertheless, the current study showed that fractions with higher mDP 7-16 failed to exert cytotoxic effects on colon cancer cells. However, the current study used a different solvent system to isolate PA fractions [26] compared to other studies [24, 132]. This solvent system managed to isolate PA fractions with a wider range of molecular mass (619-6063 g/mol) and mDP (2-19) compared to other methods (molecular mass = 552-1232 g/mol, mDP = 1-4); implying that the previous results only measured limited PA fractions (<1200g/mol) for their biological activities.

The current study discovered that the cytotoxic activity of PA fractions is correlated with small molecular mass PAs, suggesting that small PA oligomers could be absorbed across the cell membrane and exert an impact on cancer cell viability. Conversely, this study also reported that galloylation is another factor responsible for the observed effects in colon cancer cells. F2 and F3 were more potent than F1 with respect to cancer cell cytotoxicity, possibly due to the presence of higher concentrations of galloylated compounds, as measured by phloroglucinolysis. Due to the unique structure of galloylated compounds, fractions with smaller oligomers may be able to be absorbed across the intestinal mucosa and allowing the attached galloylated compound to have direct impact on cell function such as inhibit cell proliferation and induce cell apoptosis. However, future studies should
compare purified galloylated and non-galloylated oligomers on cell signalling pathway such as apoptosis activity to confirm such effects.

**Mature seed fractions are more potent than immature seed fractions**

When PA fractions (Immature: F1-F3 and Mature F1-F4) were combined with 5-FU, similar trends were observed to the commercially available GSE described in Chapter 4. Seed fractions not only acted synergistically with 5-FU at killing Caco-2 cells, but also surpassed 5-FU as an anti-cancer agent. The current study also revealed that PA fractions isolated from mature seeds (ripe) were superior to immature seeds (unripe) as chemotherapeutic agents against colon cancer *in vitro*. The chemical profile of the mature seed fractions showed that these effects could be driven by their higher proportion of epicatechin as terminal subunits and (-)-epicatechin-3-*O*-gallate as extension subunits compared to the immature seed extract. Also, PA fractions were more potent antioxidants compared to immature seed fractions. This study supports value-adding to the wine industry, as mature grape seeds are the major by-product of winemaking.

**Tannat and Chardonnay grape seeds represented the optimal candidates for isolation of PA compounds**

The data obtained from Chapter 4 suggests that the bioactivity of grape seeds is attributed to their PA content. Therefore, the selection of grape seed varietals for isolation of bioactive PAs may be important. The final study (presented in Chapter 5) addressed the differing PA profiles, not only between grape varietals, but also between geographical regions. Tannat and Chardonnay grape seeds sourced from Nuriootpa represented the optimal candidates for isolation of PA compounds due to their higher polyphenol and flavan-3-ol content compared to other grape seeds.
Although, there were limitations in extraction methods, the current study managed to quantify the PA profiles of the grape seeds. The results in this study concur with previous findings in that polyphenolic content in grape seeds is affected by genetic factors, viticultural practises and environmental conditions. Grape seeds sourced from differing varieties within the same geographical region (Nuriootpa) displayed differing polyphenolic content. Moreover, Tannat grape seeds sourced from different locations displayed differing polyphenolic content. These findings also suggest climate, viticultural practices and soil conditions may affect polyphenolic content in grapes [145, 170]. Chardonnay and Tannat (Nuriootpa) seed extracts were more powerful antioxidants than other seed extracts tested. The drivers of antioxidant activity in these extracts are not only linked to total phenolic and flavan-3-ol content, but also on the galloylation status, mean degree of polymerisation, and the likelihood of the larger polymers to depolymerise giving rise to PA forms that maybe more soluble and/or provide more readily accessible hydroxyl groups.

The present study concludes that grape seeds represent rich source of PA are strong antioxidants and may have potential uses in human health in combating health disorders characterised by oxidative stress.
6.2 Future directions

Further research is required to strengthen GSE-sourced PAs as a potential therapeutic treatment for intestinal diseases. The important findings that GSE reduced the severity of intestinal damage in the rat model of mucositis from Chapter 2, indicates further examination of the safety endpoints regarding ingestion of GSE is required to strengthen the safety of GSE for potential future use in humans. The promising finding that higher doses of GSE are more effective in the proximal small intestine suggests protection of GSE, possibly by microencapsulation, or via suppository application, could be employed to better target GSE and improve its bioavailability in the more distal regions of the bowel. Furthermore, various sized components of GSE PA (with differing mDP) could be tested using radio-labels in vivo to assess the absorption of GSE PA in the small intestine. This further suggests applications for GSE in other small intestinal disorders including infectious enteropathy, radiation, enteritis, Crohn’s, and possibly celiac disease. Moreover, investigation into GSE and its potential to modify tumour growth in vivo is warranted. Identification of the likely mechanism of GSE PAs in reducing intestinal damage in vivo is crucial.

The cytotoxicity of selected PA fractions against colon cancer cell viability highlights the importance to examine absorption of PAs by colon cell lines. Further studies are warranted to determine the molecular mechanisms of seed fractions F2 and F3 on cellular pathways associated with colonic neoplasia such as PI3-K cell signalling pathway. Moreover, conducting a dose-response trial of F2 and F3 in intestinal mucositis or in a combined tumour and mucositis model in vivo is strongly recommended.
6.3 Conclusions

This thesis provides insight into GSE as a safe, non-pharmaceutical and novel therapeutic agent which could be used potentially to improve intestinal health. The current findings propose GSE as a promising new adjunct to combat mucositis while concomitantly providing an anti-neoplastic agent to augment conventional chemotherapy. This should broaden medical options against cancer and increase patient tolerability and adherence to chemotherapy regimens. In addition, identification and purification of bioactive PAs from GSE would provide an optimized formulation of PAs for medical or nutritional purposes. This may permit the wine industry to undertake management practices to produce extracts enriched in PAs. This thesis provides compelling evidence that PA seed fractions, F2 and F3 (mDP: 2-6) can enhance the impact of chemotherapy on cancer cells, and secondly, that these seed fractions could be a promising adjunctive approach to combat intestinal mucositis. This thesis concludes that grape seed sourced PA may be a potential new treatment strategy for intestinal mucositis and colon cancer; and that further exploration of grape seed PAs for potential uses in human health will value-add to the wine industry.
Chapter 7  References


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