
UNIVERSITY
OF TASMANIA

# The pharmaceutical and nutraceutical potential of the halophytic plant *Carpobrotus rossii*

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**BLWSc, *Sydney***

**Submitted in fulfilment of the requirements for the degree of**
**Doctor of Philosophy**
**University of Tasmania**

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## Statement of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Adam Douglas Pirie 5th June 2014

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## Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University. All animal experiments conducted in this thesis were done under the approval of the University of Tasmania's Animal Ethics Committee; approval numbers A0010751 and A0011684 respectively.

## Statement of Co-Authorship

Given that this thesis is presented as a series of papers, either published, in press or submitted, statements of co-authorship are provided for each chapter. Due to this thesis format, some repetition is inevitable.

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# Manuscripts arising from this thesis and statement of contribution

**Paper 1. "Ecophysiology of *Carpobrotus rossii* in Tasmania: Linking plant's antioxidant activity with a natural habitat**

*Chapter 2,*

*Candidate was the primary author, who in conjunction with authors1-3 contributed to experimental design and development. Authors 1,3 and 13 assisted in the conduction of experiments. Author 10 provided expertise with statistical analysis. Author 3 provided input on data interpretation and presentation. Some of the flavonoid, tannin and DPPH assay field survey data described in this paper were generated as part of author 13's Master's thesis. However, generation of the full dataset and statistical analysis was undertaken during the candidate's PhD candidature.*

**Paper 2. "Flavonoid and tannin production of Carpobrotus rossii is modulated by environmental conditions"**

*Chapter 2,*

*Candidate was the primary author, who in conjunction with authors1-3 contributed to experimental design and development. Authors 1,3 and 13 assisted in the conduction of experiments. Author 10 provided specialist expertise with statistical analysis. Author 3 provided input on data interpretation and presentation. Some of the flavonoid, tannin and DPPH assay field survey data described in this paper were generated as part of author 13's Master's thesis, however generation of the full dataset and statistical analysis was undertaken during the candidate's PhD candidature.*

**Chapter 3. Low-temperature and ultraviolet B exposure induce separate and structurally unrelated biochemical responses in *Carpobrotus rossii***

*Candidate was the primary author, who in conjunction with authors 1-3 contributed to experimental design and development and refinement. Authors 6, 8 and 15 provided specialist instrumentation and analytical skills. Authors 1,2 and 6 provided input on data interpretation. Author 3 provided input on data interpretation and presentation.*

**Chapter 4. Pirieol A from *Carpobrotus rossii*, a novel spinacetin glycoside containing apiose and HMG moieties.**

*Candidate was the primary author, who in conjunction with authors 1, 2, 14 and 7 contributed to experimental design and development and refinement. Author 14 undertook the UV spectrum analysis, hydrolysis and comparison to commercial standards, and determination of aglycone structure work described in this chapter (in conjunction with authors 1, 6 and 7), the results of which are reported in her Master's thesis. The candidate propagated the high purity plant and undertook preliminary purification of the extract used to determine the structure of the entire compound. Author 8 undertook the final large-scale fractionation step to produce a high-purity sample for NMR analysis. Authors 7,6 and 1 provided specialist instrumentation, analytical and data interpretation skills.*

**Chapter 5. Hypolipidaemic effect of crude extract from *Carpobrotus rossii* (pigface) in healthy rats**

*Candidate was the primary author, who in conjunction with authors 1, 2 and 5 contributed to experimental design and development. Authors 5 and 12 assisted in the conduction of experiments. Authors 6, 9 and 11 provided specialist analytical expertise. Authors 5, 9, 11 and 12 provided input on data interpretation and presentation.*

**Chapter 6. Flavonoids from Carpobrotus rossii improve glucose clearance in insulin resistant animals**

*Candidate was the primary author, who in conjunction with authors 1, 2, 4 and 5 contributed to experimental design and development. Author 4 assisted in the conduction of experiments. Authors 9 provided specialist analytical and statistical expertise.*

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A selection of *C. rossii* photographs taken by the candidate during the course of their PhD studies

## PhD overview

"Metabolic syndrome" refers to the triumvirate of obesity-related, cardiovascular diseases such as hyperlipidaemia, type 2 diabetes, atherosclerosis and hypertension. The worldwide prevalence of these diseases have increased to such an extent that they are now the leading cause of human morbidity and mortality. Metabolic syndrome is characterised by elevated levels of plasma lipids, hyperglycaemia, compromised insulin signalling, excessive production of reactive oxygen species (ROS) and a vasculature that is in a persistently inflamed state. Because of the increasing prevalence of these diseases, considerable research effort has gone into understanding the disease processes and developing appropriate therapies. Two metabolic syndrome targets which have been identified and for which therapeutics have been successfully developed are hyperlipidaemia and hyperglycaemia.

A common target of the lipid-lowering therapies is the HMG-CoA reductase enzyme which catalyses the rate limiting step in the cholesterol synthesis pathway namely the conversion of 3-hydroxy-3-methylglutaric acid coenzyme-A (HMG-CoA) to mevalonate. Statins are the primary class of drugs with this HMG-CoA inhibiting ability. Polyphenolic compounds produced by plants have also been shown to have hypolipidaemic activity by inhibiting HMG-CoA as well as other enzymes involved in the processes of lipid manufacture and delivery to cells. Polyphenolic compounds have also been shown to improve the glucose status of diseased subjects by improving vascular health, improving insulin signalling and glucose uptake into muscle. Of these plant-derived polyphenolic compounds, members of the flavonoid sub-family been shown to be particularly successful in treating both hyperlipidaemia and hyperglycaemia.

*Carpobrotus rossii* (CR) is a succulent halophyte native to Australia and commonly found growing along the coastal margins of southern Australia. The plant has a history of use by both the indigenous aboriginal population and early Tasmanian settlers. CR was reportedly consumed as a food, to treat gastrointestinal upsets, and applied topically for the treatment of bites and scratches. Preliminary investigations conducted at the University of Tasmania have shown that crude extracts from its leaves inhibit platelet aggregation, inflammatory cytokine release (interleukin-1-beta, tumour necrosis factor-alpha) and lipid oxidation *in vitro* (Geraghty et al., 2011). This activity is believed to be due to the flavonoid compounds that

the plant produces in its leaves. Several of these flavonoids have a known HMG-CoA inhibitor 3-hydroxy-3methylglutaric acid (HMG) present as a substituent (Jager, 2009).The presence of this moiety, in conjunction with the known hypoglycaemic and hypolipidaemic activities of other flavonoids, mean that the consumption of CR flavonoids could potentially improve endothelial health, cardiovascular function and health via a combination of effects related to both their flavonoid and statin properties.

*In planta*, the primary function of flavonoids appears to be as antioxidants, and their production has been shown to be induced under a suite of conditions which cause the plant to experience oxidative stress. The ROS generation and signalling process *in planta* are complex, and the effect of environmental conditions on a plant's redox status, and hence flavonoid production, is likely to vary between species. The effects of environment on flavonoid product has not been previously investigated for CR. The flavonoid structures described in chapter 4 are extremely complex, and based on informal discussions with an organic chemist familiar with similar compounds, not easily amenable to synthesis. As such, the ability to produce sufficient material and improve the efficiency of their production e.g. increasing biomass or increasing flavonoid concentration by the modification of environmental parameters is a key component of overall CR pharmaceutical and nutraceutical investigations.

This thesis has involved using techniques relevant to the disciplines of pharmacology, organic chemistry and plant physiology. The primary aims were to investigate the pharmaceutical and nutraceutical potential of the flavonoids derived from CR leaves. To do this, several basic questions were addressed, namely:

1. **What effect do environmental conditions have on metabolite production,**
2. **What is the structure of the CR flavonoids,**
3. **Is the consumption of CR leaf derived extracts safe, and,**
4. **Do the CR leaf flavonoids possess pharmacological activity in metabolic syndrome, specifically an improvement in either glycaemic or lipid profile.**

A suite of novel findings which pave the way for further study of this plant are the result of this research. The body of the thesis is presented as a series of articles for publication, of which three are published at the time of thesis submission.

The published articles are as follows:

PIRIE, A. D., DAVIES, N. W., AHUJA, K. D. K., ADAMS, M. J., SHING, C. M., NARKOWICZ, C., JACOBSON, G. A. & GERAGHTY, D. P. 2014. A crude extract from *Carpobrotus rossii* (pigface) lowers cholesterol in healthy rats. *Food and Chemical Toxicology,* 66**,** 134-139.

PIRIE, A., PARSONS, D., RENGGLI, J., NARKOWICZ, C., JACOBSON, G. A. & SHABALA, S. 2013. Modulation of flavonoid and tannin production of *Carpobrotus rossii* by environmental conditions. *Environmental and Experimental Botany,* 87**,** 19-31.

PIRIE, A., SHABALA, S., PARSONS, D., NARKOWICZ, C., JACOBSON, G. & RENGGLI, J. 2011. Ecophysiology of *Carpobrotus rossii* in Tasmania: Linking plant's antioxidant activity with a natural habitat. *Ecological Questions,* 14**,** 91-93.

In addition to these published manuscripts several other articles are currently undergoing the peer-review process.

Due to the multidisciplinary nature of this work, the literature review is quite detailed covering cardiovascular physiology the associated disease processes, plant physiology and organic chemistry, as appropriate. This has been done to ensure that all examiners have sufficient grounding to understand the work described in this thesis when it is outside their area of expertise.

## Plant Physiology (Chapters 2 and 3)

The survey investigating flavonoid production by wild CR (77 plants from 16 sites around the coastline of Tasmania) showed that conditions known to induce stress *in planta* were associated with altered flavonoid production. Under conditions of sub- and supra-optimal salinity (<50mM, >100mM NaCl), biomass production was reduced, whilst flavonoid

production and total antioxidant activity increased. When exposed to conditions that induce light stress (<10°C and moderate to high light), flavonoid production also increased. However, unlike reports from the literature on other plant species, the increase in flavonoid production was not due to an increase in the prevalence of a flavonoid with a dihydroxyl substituted B-ring. Instead, an increase in the concentration of other structurally-related, minor-flavonoids also based on the spinacetin aglycone, was observed. Ultraviolet B exposure led to a reduction in flavonoid concentration, but induced production of the structurally-unrelated betalain compounds, betanin/isobetanin and/or gomphrenin and its structural isomer (compounds which only vary in the site of glycolysation). Neither the exposure to low-temperatures or ultraviolet B imparted a detectable effect on gross antioxidant activity.

## Organic chemistry (Chapter 4)

During the field survey, a plant which produced almost exclusively the main flavonoid found in all CR plants was identified. This plant was cultivated and used for further investigation of the CR flavonoid group. Extensive NMR and mass spectrometry work was undertaken and the structure of the main flavonoid determined. This compound comprised the spinacetin aglycone, as well as glucose, apiose and 3-hydroxy-3-methylglutaric acid (HMG) substituents. The structures of two related, minor-flavonoids were also elucidated.

## Pharmacology (Chapters 5 and 6)

A preliminary series of experiments indicated that ingestion of crude CR leaf extract lowered non-HDL cholesterol levels and increased the concentration of HMG in the kidneys of healthy Hooded-Wistar male rats. *In vitro* vascular responses to noradrenaline, acetylcholine and sodium nitroprusside, blood pressure, haematological parameters, and blood glucose were unchanged following CR ingestion.

Based on these findings, and the reported pharmacological activity of compounds related to the CR flavonoids, such as quercetin and epigallocatechin-3-gallate, further investigations of the CR flavonoids ability to treat aspects of metabolic syndrome were undertaken. These studies showed that CR flavonoids are able to improve glucose clearance in a model of

insulin resistance (male C57/BL6 mice on a high fat diet) and, also, appear to have lipid lowering activity (male Sprague-Dawley rats on a high fat, high cholesterol diet). These effects were not observed in the groups supplemented with the crude CR leaf extract.

The findings from this research indicate that CR flavonoids are a source of novel therapeutic compounds which, in animal models, exhibit beneficial activity against more than one aspect of metabolic syndrome. Further research to confirm these activities, their mechanisms of action and potential structure activity relationships of CR flavonoids is warranted.

## 1.1 The Vascular System

### *1.1.1 Cardiovascular system*

The cardiovascular system is a complex network of tissues comprising arteries, arterioles, capillaries, venules and veins that have evolved to deliver the nutrients required for cell growth (e.g. oxygen, glucose, lipids, potassium, calcium) and remove metabolic waste products for disposal and elimination from the body. In addition, the circulatory system is used to transport immune cells around the body allowing it to be constantly monitored for signs of infection (Campbell et al., 2008).

#### *1.1.1.1 Vessel Anatomy*

To fulfil this transportation role the vascular system is composed of specialised cells which line the vessel lumen and actively transport compounds into or out of the surrounding tissue as required as well as other tissues which provide strength and vessel reinforcement. As the vessel's role changes from one of bulk fluid delivery to that of nutrient exchange individual vessel diameters reduce and the number of vessels increases, resulting in an increased total cross-sectional area and allowing mean blood velocity to drop from ~4.8cm/sec to ~0.1cm/sec (Campbell et al., 2008); (Figure 1.1).

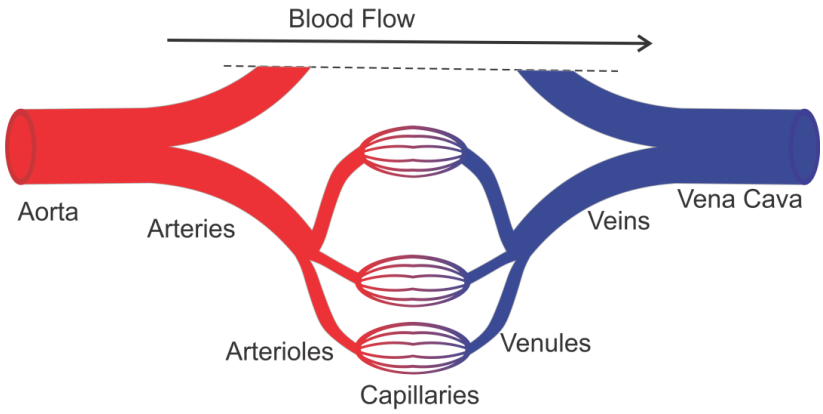



**Figure 2.1.** Vessels of the vascular system. Total vessel cross-sectional area increases as vessel diameters and blood velocity reduces and reaches the capillaries for nutrient exchange (adapted from (Campbell et al., 2008)).

In areas of high pressure and flow such as arteries and arterioles, the vasculature is comprised of elastic and collagen fibres overlaying a layer of smooth-muscle cells and an arterial intima which is then overlain by endothelial cells (Figure 1.2a). The presence of these elastic and smooth muscle cell layers allow the vessel to adjust vascular tone and absorb the pressure wave that occurs with each heartbeat. As blood gets closer to where it is needed, it moves into smaller delivery vessels arteries and arterioles until it reaches the terminal arterioles immediately upstream of the capillary beds. These terminal arterioles are characterised by the presence of precapillary sphincters which allow the controlled delivery of blood to capillaries (Campbell et al., 2008). Nervous stimulation (e.g. exercise) or hormonal signalling (e.g. insulin) results in the sphincters relaxing, the capillaries becoming recruited, perfused and able to deliver nutrients to active muscle cells (Clark et al., 2006, Rattigan et al., 2006, Rattigan et al., 2005, Clark et al., 2003, Vincent et al., 2006). Prior to recruitment by either insulin or exercise, most of these sphincters are closed and flow to the majority of muscle cells is bypassed (Clark et al., 2000). A thorough discussion of the evidence surrounding insulin and exercise mediated capillary recruitment is undertaken in Clark (2008).

The capillaries themselves are areas of both low flow volume and pressure. They are comprised solely of endothelial cells and where nutrient exchange occurs in the body (Figure 1.2b). The venules, located immediately downstream of the capillary bed, and veins complete the circulatory system, and are responsible for the low-pressure return of blood to the heart for re-oxygenation. This low flow environment means that veins have a larger diameter than their arterial counterparts and also contain valves that combine with the surrounding muscle tissue to prevent low pressure blood from back feeding or pooling in the vessels (Figure 1.2c) (Campbell et al., 2008).

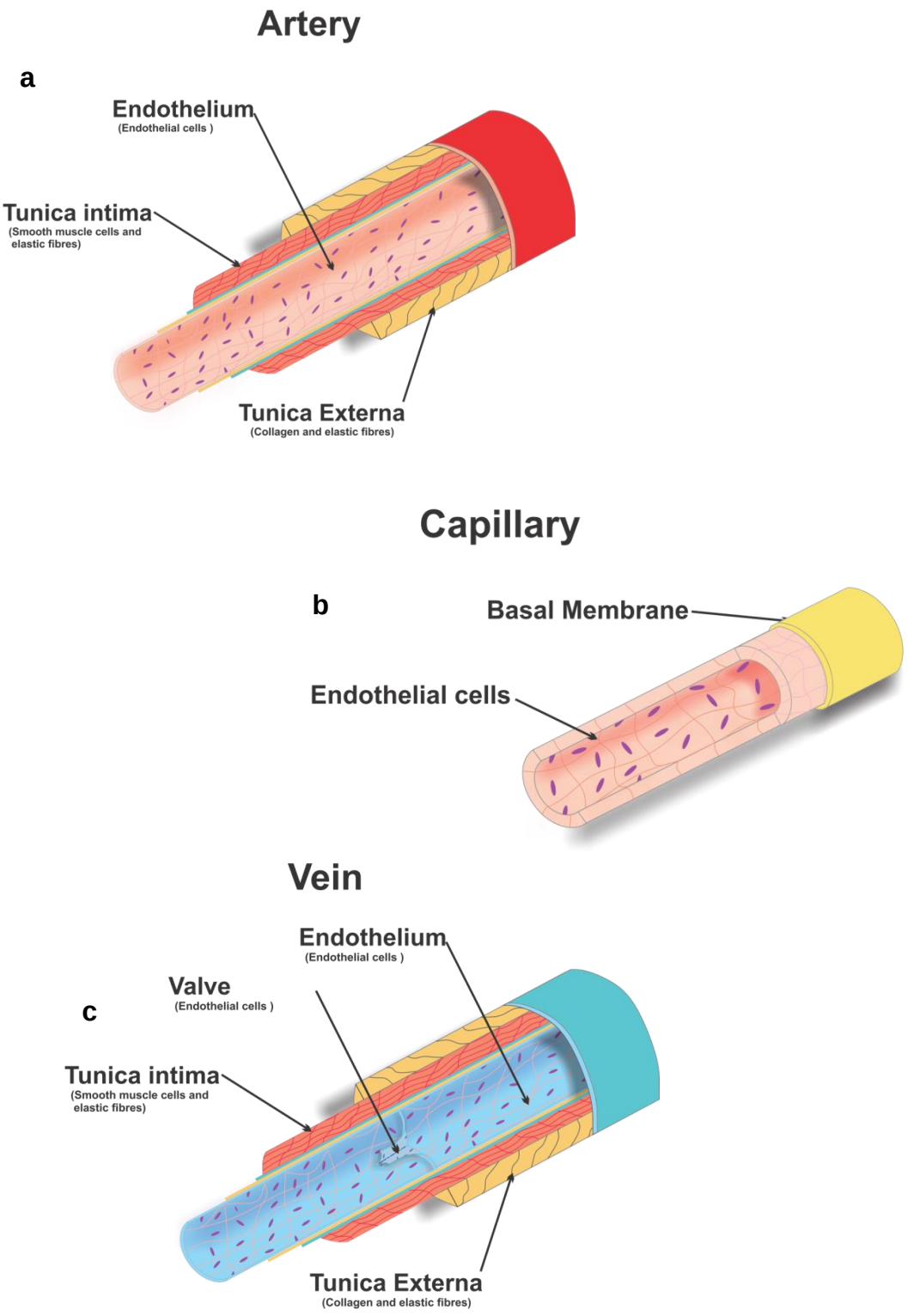



**Figure 2.2.** The variation in physiology of different blood vessel types (a) Artery, (b) Capillary and (c) Vein (Adapted from (A.D.A.M, 2012))

#### *1.1.1.1.1 The Endothelium*

Endothelial cells line the entire vasculature and the endothelium comprise one of the largest tissues in the body (Sudano et al., 2006). This 'organ' is critically important due to its involvement in signalling to regulate vascular tone and ensuring optimal blood and oxygen supply to other body tissues (Bakker et al., 2009). As the metabolic needs of the body organs change, the endothelium produces a suite of compounds such as the free radical nitric oxide (NO), prostacyclin and endothelial derived hyperpolarising factor that dilate, or compounds such as angiotensin II, endothelin-1 and prostaglandin $H_2$ which constrict the blood vessels (Osto et al., 2007). These changes in vessel state allow blood to be redirected from areas of low need (i.e. low metabolic activity) to those with higher needs. In addition to regulating vascular tone and blood supply, the endothelial cells also respond and interact with stimuli from the blood stream and circulating immune cells (Galley and Webster, 2004).

One of the most important compounds involved in signalling is NO. It is produced by the nitric oxide synthase enzymes (NOS) (Stangl et al., 2007, Yamada et al., 2000) and released into the lumen and surrounding tissue where it signals the relaxation of arterial muscle (Vita, 2005). Endothelial nitric oxide synthase (eNOS) is one of three isoforms of NOS, the others being neuronal (nNOS) and inducible (iNOS) (Domenico, 2004). In the presence of the tetrahydrobiopterin co-factor (Figure 1.3a), these enzymes convert L-arginine to L-citrulline, during which a molecule of NO is produced (Figure 1.3b) (Stoll et al., 2010).

Despite producing the same compound the roles of iNOS, e and n NOS are vastly different. e and nNOS constitutively produce low levels of nitric oxide and maintain the body in an anti-inflammatory, quiescent state. iNOS, however, is used by macrophages as an immune response to produce high levels of NO. This reacts with superoxide ($O_2^{\cdot-}$) to form peroxynitrite ($ONOO^-$) and kill pathogens (Domenico, 2004).

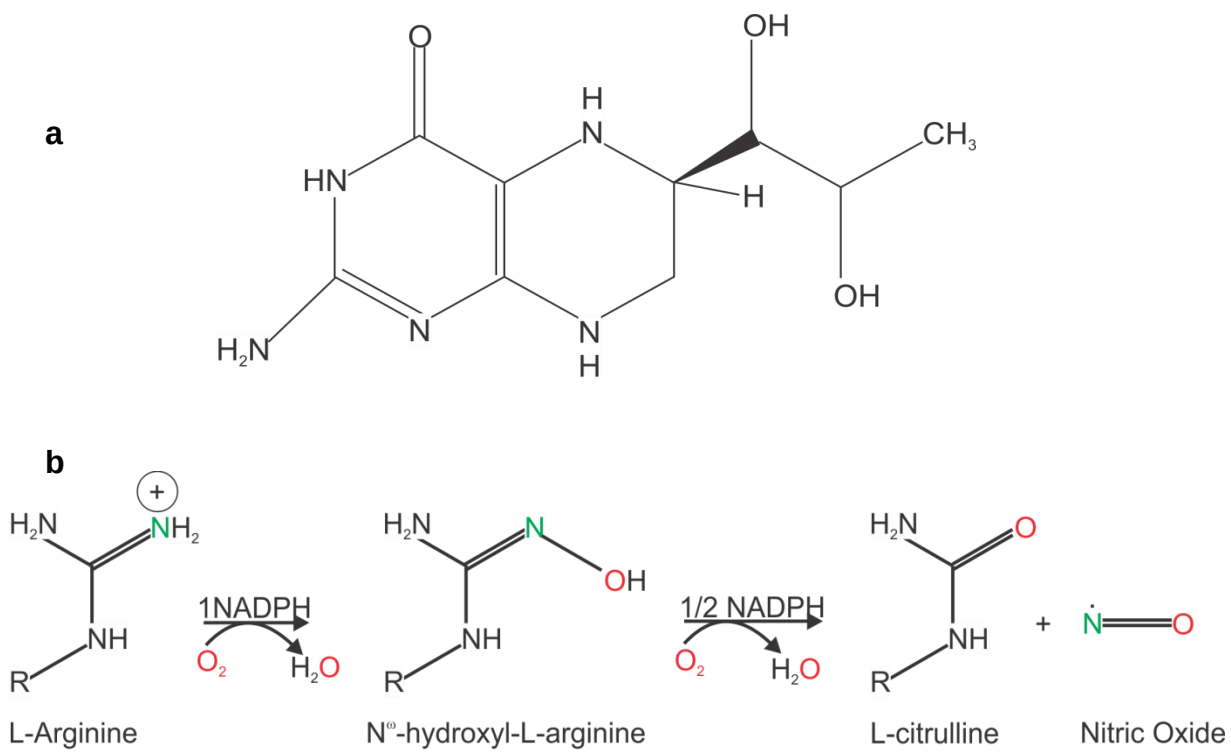



**Figure 2.3.** a) Tetrahydrobiopterin and b) NOS mediated generation of Nitric Oxide (adapted from (Stoll et al., 2010))

In contrast to the cytotoxic effects of high NO levels produced by iNOS, the low concentration of NO, produced by eNOS and nNOS has multiple beneficial effects as listed below and illustrated in Figure 1.4:

- Initiating the haemoxygenase-1 (HO-1) enzyme to start degrading haem into carbon monoxide, bilirubin and iron, thus removing pro-oxidant haem and releasing bilirubin which is shown to inhibit lipoprotein oxidation (Heeba et al., 2009),
- Maintaining vasodilatation (Liao and Laufs, 2005),
- Reducing vessel permeability (Osto et al., 2007),
- Suppression of platelet clotting (Yemisci et al., 2008),
- Suppression of smooth muscle cell proliferation (Davignon and Ganz, 2004),
- Reduced leukocyte/endothelial interaction (Martin et al., 2007), and
- General anti-inflammatory activity (Osto et al., 2007).

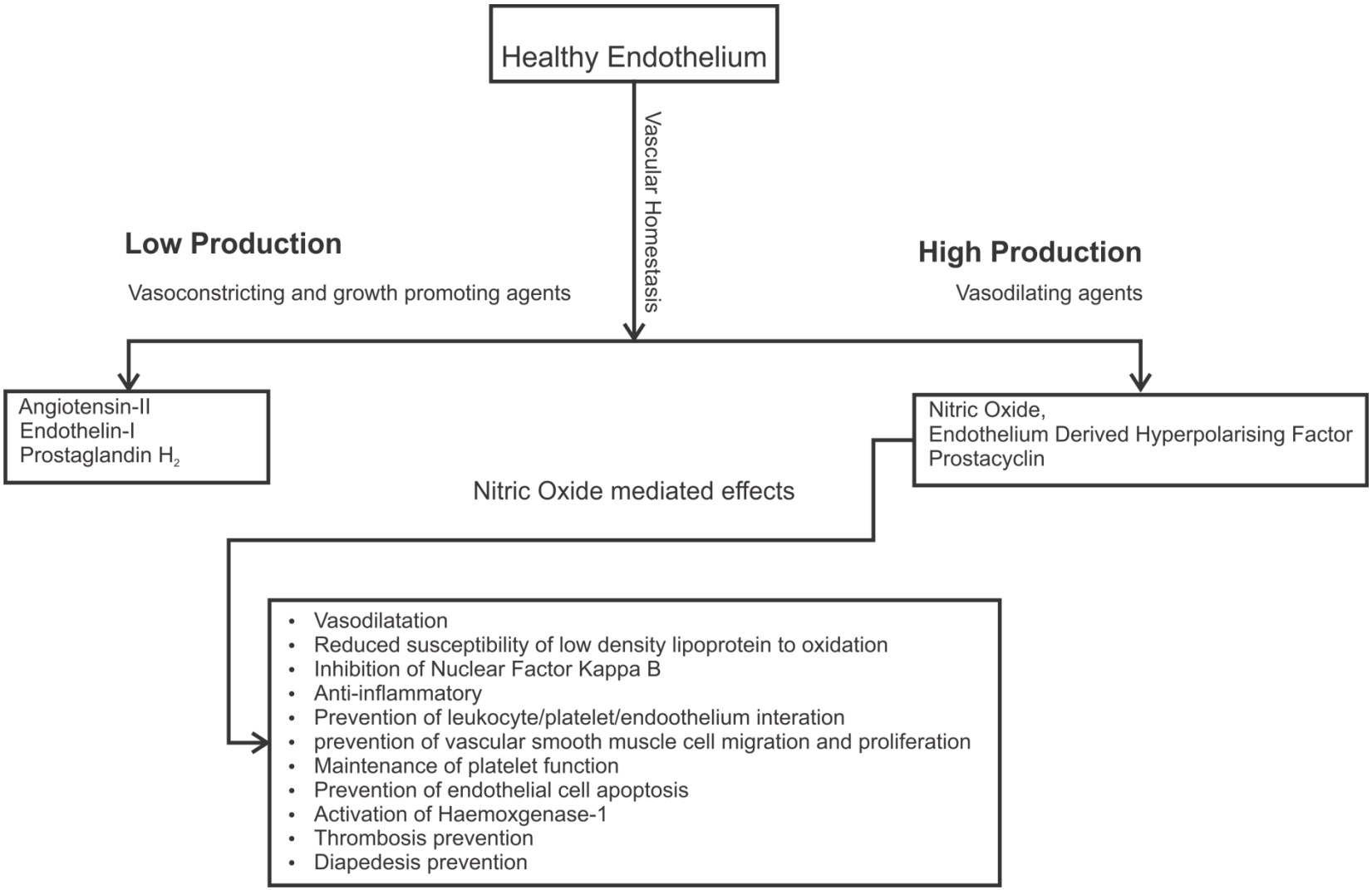



**Figure 2.4.** Characteristics of the functioning healthy endothelium.

### *1.1.2 Glucose delivery and uptake*

As well as being involved in the maintenance of general vascular health, NO also plays crucial roles in mediating glucose uptake both post-prandially and during exercise (Vincent et al., 2006). As reviewed in Clerk et al., (2004) and Vincent et al., (2003), insulin signals NO production post meal and enables the recruitment of capillaries via relaxation of precapillary sphincters leading to an increase in the number of capillary beds that are perfused (Vincent et al., 2004). Insulin also relaxes the larger resistance vessels, allowing a greater volume of blood flow to flow through them. This increased delivery of blood in turn improves the delivery of insulin to the myocytes, triggering further signalling, glucose transporter 4 (GLUT4) translocation, and the uptake of glucose into muscle cells; resulting in an increased rate of glucose removal from the bloodstream (Balon and Nadler, 1997); (Figure 1.5). The uptake of glucose by skeletal muscle is stimulated by both exercise and insulin signalling, and skeletal muscle is responsible for ~75% of postprandial glucose uptake (DeFronzo, 1988). As such, the recruitment of the macro and microvasculature, their associated glucose transport mechanisms and delivering glucose to muscle cells is essential for maintaining physiologically appropriate blood glucose levels.

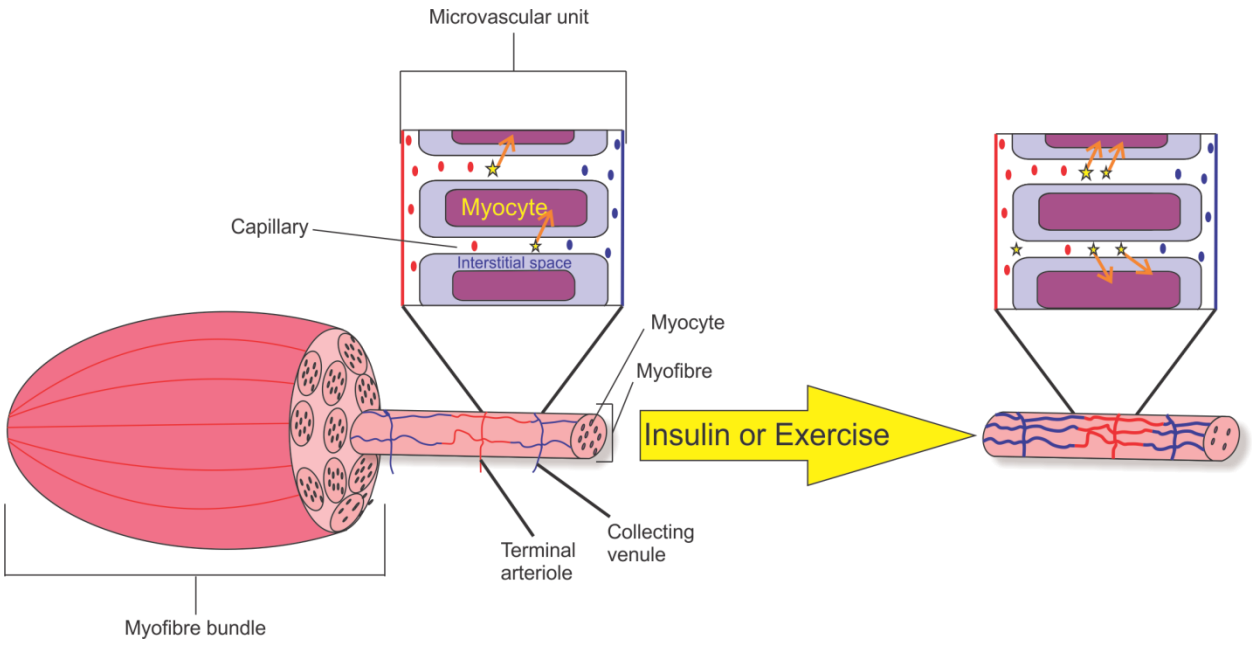



**Figure 2.5.** Insulin and exercise result in increased capillary recruitment and glucose (☆) uptake in skeletal muscle tissue (adapted from (Barrett et al., 2009)).

### *1.1.2.1 Insulin signalling*

Postprandial uptake of blood glucose is regulated by the release of insulin into the bloodstream which signals the $Ca^{2+}$ independent phosphorylation of eNOS at Serine 1177. Insulin binds to insulin receptors (IR) located on the endothelium. This leads to phosphorylation of insulin receptor substrates (IRS) and, via a signalling pathway involving tyrosine kinase, phosphatidylinositol 3-kinase (PI3K) and protein kinase B (AKT), phosphorylation of eNOS; resulting in increased activity and an elevation of vascular NO levels (Montagnani et al., 2001, Montagnani et al., 2002b). At the same time, insulin also activates a second signalling pathway involving mitogen-activated protein kinase (MAPK) that results in the synthesis of the vasoconstrictor endothelin-1 and the production of compounds such as Ras and Rho which can prenylate and affect signalling protein function (Figure 1.6); (Kim et al., 2006, Muniyappa et al., 2007, Montagnani et al., 2002a). The synthesis of both vasorelaxing and vasoconstricting agents ensures the maintenance of vascular tone during insulin release.

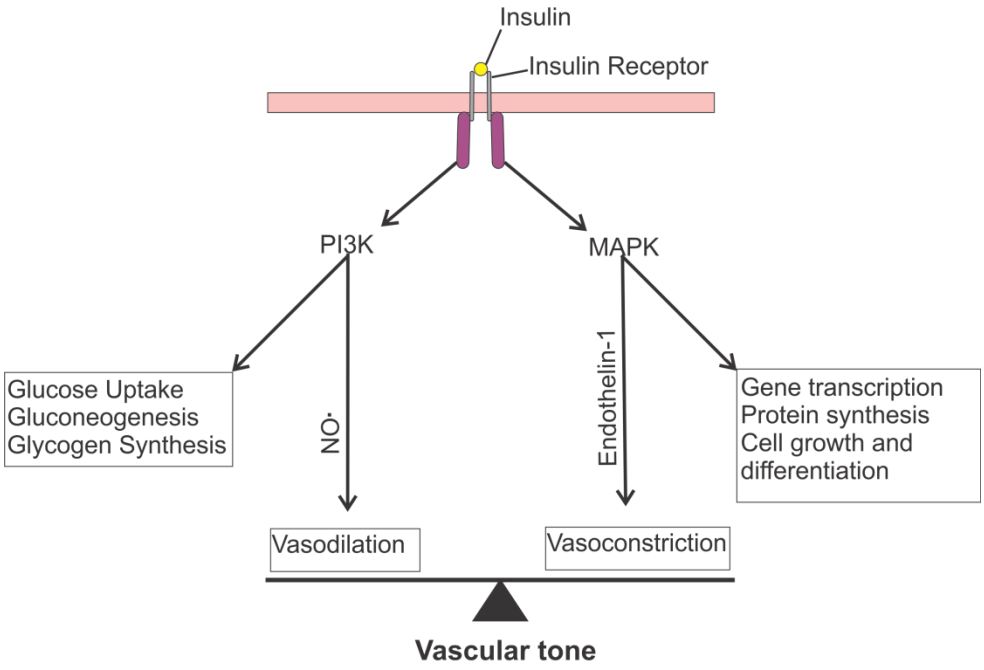



**Figure 2.6.** Cellular activities mediated by insulin signalling (adapted from (Adapted from (Kim et al., 2006) and (Muniyappa et al., 2007))

In addition to binding to endothelial IRs, insulin is also transported into the underlying interstitial space where it stimulates further NO production by nNOSμ, which is the NOS present in muscle cells (McConell et al., 2012). Unlike the rapid interaction of insulin with the endothelial receptors which occurs within 5-10 minutes of insulin release (Vincent et al., 2004), the time between insulin release and nNOSμ activation is delayed as the molecule must be actively transported across the endothelium and into the interstitial space (Vincent et al., 2002, Chiu et al., 2008). This process is most likely signalled by pathways involving PI3K as well as MAPK (Wang et al., 2008) and a major component of the transportation is mediated by caveolin-1/IRS associated caveolae. IRS/caveolin immunohistochemical staining and co-immunoprecipitation experiments (Wang et al., 2006) as well as other experiments reviewed in Vincent et al., (2005) and Barrett et al (2009) support this interpretation.

Unlike insulin mediated signalling of glucose uptake, much of the signalling cascade that occurs during exercise is yet to be unravelled (Merry and McConell, 2009). What has been demonstrated comprehensively is that exercise induces capillary recruitment (Clark et al., 2000, Rattigan et al., 2006, Rattigan et al., 2005). This process is independent of NO (Inyard et al., 2007) but some of the glucose transport itself is still mediated via NO related mechanisms (Ross et al., 2007b). Of particular interest to diabetes researchers is the fact that type 2 diabetic subjects with impaired insulin-mediated glucose uptake show no impairment of glucose uptake during exercise or muscle contraction (Inyard et al., 2009, St-Pierre et al., 2012). As such, the identification of the exercise-mediated signalling pathway and the restoration of insulin-stimulated signalling pathways both pose promising target for the treatment of diabetes mellitis.

### *1.1.3 Inflammation and the Disease process*

Various insults can cause damage to endothelial cells. These insults include infection (Deanfield et al., 2007), reactive oxygen species (ROS) and sustained high blood glucose, lipid or lipoprotein levels (Landmesser et al., 2004). Upon becoming damaged, cellular NO production is reduced, thus allowing an increase in the activity of pro-inflammatory transcription factor nuclear factor-kappa B (NF-κB) (Osto et al., 2007). This results in signalling molecules such as interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-α) being released (Tribolo et al., 2008). Adhesion molecules such as intracellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), P-selectin and E-

selectin also become expressed on the endothelial cell's surface (Landmesser et al., 2004, Desideri and Ferri, 2005, Passacquale et al., 2008).

The release of signalling molecules into the blood stream attracts macrophages and leukocytes to the damaged cell. Their role is to scavenge the toxic material and pathogens from the vascular system. This response is a normal phenomenon and is how the body targets foreign bodies and fights infection (Martin et al., 2007). NF-κB activation simultaneously activates the NF-κB inhibitor inhibitory factor kappa B alpha (IF-κBα) gene, resulting in a negative feedback loop to inflammatory response. This combined with the suppression of NF-κB by NO ensures that NF-κB induction is transient and that the activated cells return to a quiescent state (Desideri and Ferri, 2005).

#### *1.1.3.1 Causes of endothelial dysfunction*

Sustained injury to the cells, however, exhausts the endogenous anti-inflammatory system, causes endothelial cell apoptosis (Osto et al., 2007), persistent endothelial damage and formation of a dysfunctional endothelium characterised by the excessive production of ROS (Deanfield et al., 2007, Aquilano et al., 2008). This transformation involves the body moving from a homeostatic state, where cellular enzymatic and non-enzymatic antioxidants (including superoxide dismutase (SOD), catalase (CAT), glutathione (GTH) and ascorbate) are able to maintain optimum ROS levels, to one characterised by excessive ROS production, overwhelmed endogenous antioxidant systems and the redox balance tipped towards generalised oxidation (Osto et al., 2007). Consistently elevated ROS production poses a significant risk to cellular health. These species have the ability to cause lipid peroxidation which can cause an increasing in the leakage of cellular phospholipid membrane, induce the cross linking of proteins which inhibit protein function and cause damage to DNA and RNA (requiring repair to avoid mutations that lead to either apoptosis or cancer) (Kong and Lin, 2010).

This sustained increase in ROS production changes the endothelium from behaving in an anti-inflammatory manner to one of pro-inflammatory or "host defence response" (Deanfield et al., 2007). Endothelial dysfunction is known to be an independent predictor of cardiovascular disease (Landmesser et al., 2004) and multiple degenerative diseases including stroke, atherosclerosis, renal failure and diabetes are also believed to involve a

dysfunctioning endothelium as an initiating factor (Davignon and Ganz, 2004, Desideri and Ferri, 2005, Kim et al., 2006, Clark, 2008, Cersosimo and DeFronzo, 2006).

As illustrated in Figure 1.7, once the endogenous antioxidant system is overwhelmed and consistent oxidative stress is experienced by the endothelium, multiple changes occur:

- Angiotensin II (AT-II) production is increased, in turn activating nicotin amide dinucleotide phosphate (NAD(P)H) oxidase which further increases ROS production (Wassmann et al., 2001).
- NF-κB activity is increased (Desideri and Ferri, 2005, Martin et al., 2007, Osto et al., 2007).
- NO combines with the superoxide anion ($O_2^-$) to form peroxynitrite ($ONOO^-$), a highly cytotoxic compound adversely affecting cellular and vascular health (Heeba et al., 2007)
- $ONOO^-$ oxidises tetrahydrobiopterin (THB), the crucial co-factor in NO synthesis, further reducing NO levels and amplifying the oxidative stress/reduced NO bioavailability cycle (Desideri and Ferri, 2005).
- eNOS becomes uncoupled and starts producing $O_2^-$ as well as NO, resulting in further $ONOO^-$ production (Grassi et al., 2009).
- Xanthine dehydrogenase is irreversibly converted to the ROS producing enzyme xanthine oxidase (Li and Shah, 2004).

As a result of these vascular changes, the PI3K mediated insulin signalling pathway is also interrupted, causing less NO to be produced and a subsequent reduction in NO mediated effects on vascular tissue (Endres et al., 2004, Heeba et al., 2009). At the same time, insulin production is increased to compensate for the lower PI3K sensitivity (Kim et al., 2006). A consequence of this increased insulin production is an increasingly stimulated MAPK pathway, producing endothelin-1(Osto et al., 2007), VCAM-1, E-Selectin (Montagnani et al., 2002a) and other atherogenic agents (Kim et al., 2006), as well as decreased expression of IRS2 (Kubota et al., 2011). This causes yet another feedback loop of decreasing vascular health as acute levels of endothelin-1 has been shown to inhibit insulin signalled capillary recruitment and glucose clearance *in vivo* (Ross et al., 2007a).

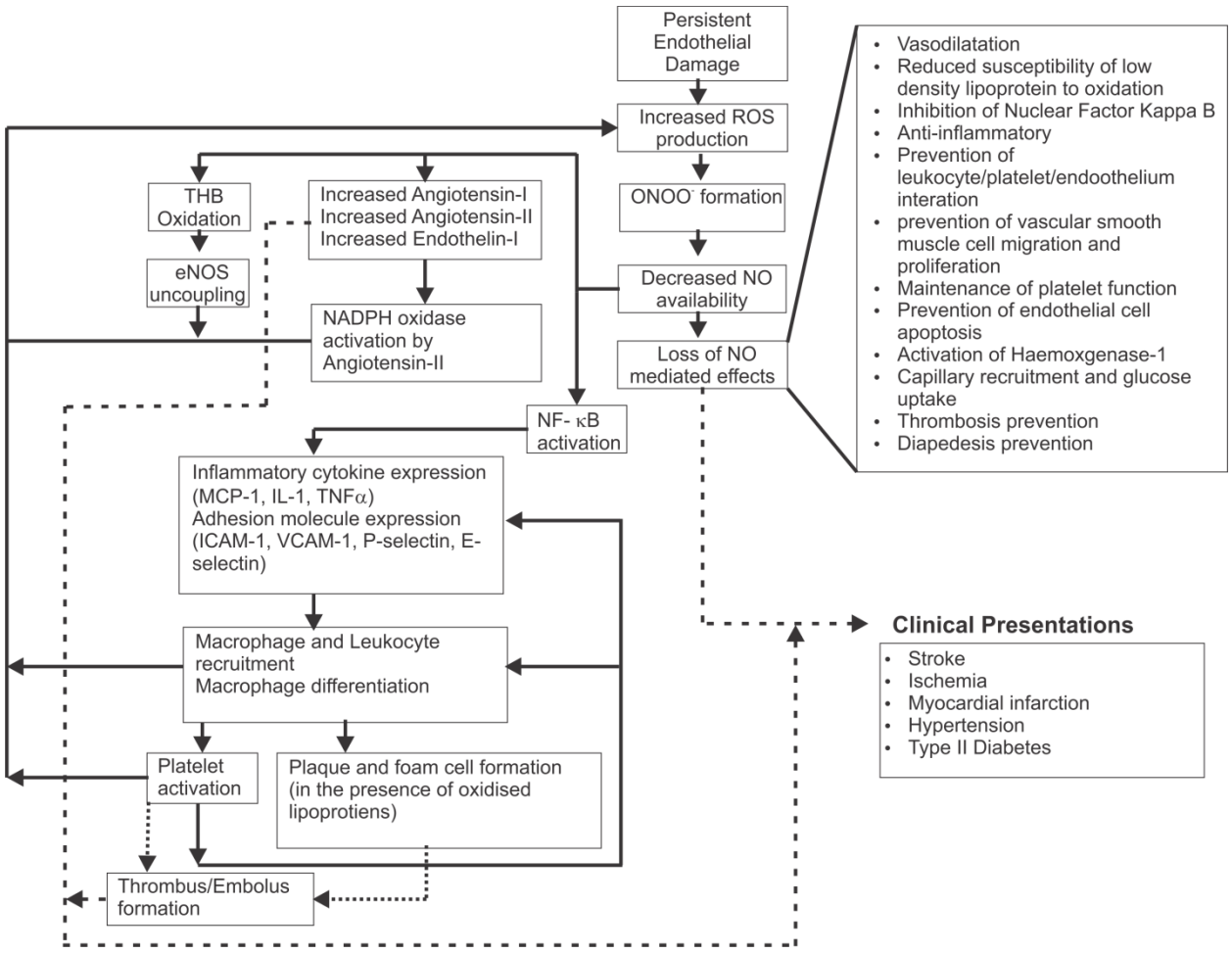



**Figure 2.7.** The feedback loop of persistent endothelial damage. Reactive Oxygen Species (ROS) generation and endothelial dysfunction. $ONOO^-$ = peroxynitrite, NF-κB = Nuclear factor Kappa B, NO = Nitric Oxide, THB = Tetrahydrobiopterin, MCP-1 = Monocyte Chemoattractant Protein-1, IL-6 = Interleukin-6, TNFα = Tumour Necrosis Factor Alpha, ICAM-1 = Intracellular Adhesion Molecule-1, VCAM-1 = Vascular Cell Adhesion Molecule-1.

#### *1.1.3.2 Platelets*

As well as leukocytes, macrophages and endothelial cells, circulating platelets are also involved in the inflammatory process. These can become activated by circulating cytokines, release inflammatory cytokines themselves, interact with the endothelium and leukocytes and exhibit inflammatory surface proteins (Weyrich et al., 2003, Weyrich et al., 2007). Once activated, platelets can further amplify the inflammatory response by activating resting endothelial cells (Varughese et al., 2006, Grassi et al., 2009) as well as interact with other platelets and macrophages forming aggregates or clots (Weyrich et al., 2007). These can then circulate and cause ischemic events when they lodge and block vascular tissue.

#### *1.1.3.3 Lipoproteins*

One source of injury comes from oxidised low density lipoproteins (oxLDL) contacting the vascular wall (Palinski et al., 1989, Sevanian and Hodis, 1997). These lipoproteins are used to transport cholesterol and other lipids around the body where free fatty-acids and triglycerides are used to power metabolic processes or stored for later use, and cholesterol is used in cellular membranes and for the synthesis of bile acids, steroid hormones and vitamin D (Liao and Laufs, 2005, Chen et al., 2008).

Cholesterol is essential for cellular function due to its role in regulating membrane fluidity, present in membrane sphinoglipid rafts where a suite of signalling molecules are located, involvement in embryonic development and cell differentiation, and as a substrate for steroid production (Oram and Vaughan, 2006). However, because cholesterol and lipids are insoluble in blood, the body manufactures lipoproteins, namely chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and intermediate density lipoproteins (IDL)) to deliver cholesterol and lipids to cells. High density lipoproteins (HDL) remove lipids from the circulation and deliver them, via reverse cholesterol transport (RCT) to the liver for excretion into the digestive tract (Figure 1.8); (Shaul and Mineo, 2004, Varughese et al., 2006). A high lipid diet, genetic predisposition or oxidative stress conditions result in the excessive production of low density "delivery" lipoproteins, endothelial damage and the uptake by macrophages to form lipid laden "foam" cells (Miller et al., 2010b). At present,

debate continues over which delivery lipoprotein(s) are the primary initiator of atherosclerosis (Tomkin, 2010).

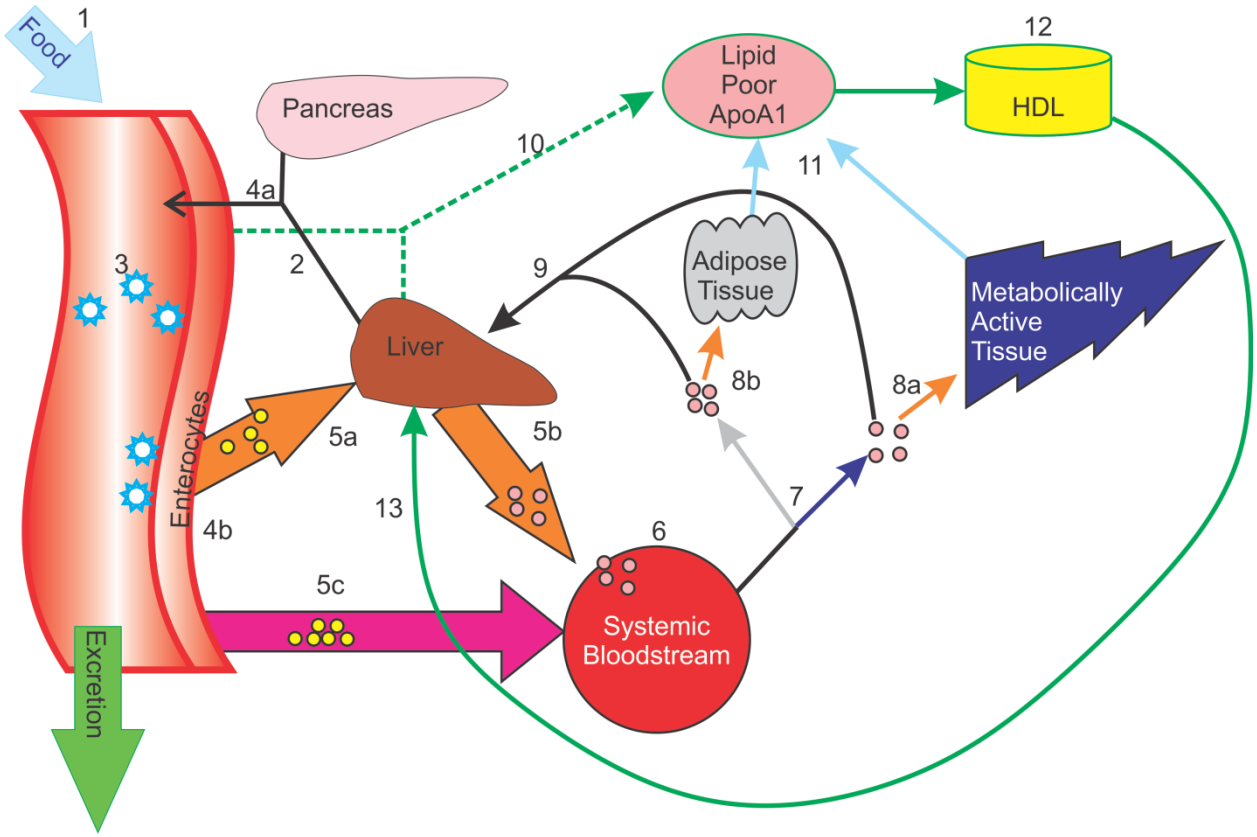



**Figure 2.8.** The digestion, absorption and cycling of cholesterol and lipids through the vasculature and bloodstream. 1 Food is consumed and reaches the gastrointestinal tract. 2 Phospholipids and bile acids in the hepatic bile emulsify food lipids to form micelles (3). Hepatic bile also delivers high levels of unesterfied cholesterol to the micelle. 4a Lipases secreted by the pancreas modify dietary lipids in the micelle so they can be absorbed by gut epithelial cells (4b). 5a Chylomicrons are taken up from the intestine and delivered to the liver which then manufactures them into VLDL. 5b the VLDL is metabolised intravascularly to LDL which then moves into the systemic bloodstream (6 and/or 5c). 6 Chylomicrons are delivered from the lymphatic system directly into the blood stream. 7 A metabolic branch point (dependent upon capillary recruitment status) is reached where the lipid and cholesterol rich particles deliver their contents to either metabolically active tissue (muscle) for combustion (8a) or to adipose tissue for storage (8 b), whilst cholesterol is delivered for use in membranes and the synthesis of vitamins and hormones. 9 Remnant lipoprotein particles move back into circulation and return to the liver where they are recycled. 10 Lipid poor ApoA1 lipoprotein particles are synthesised and excreted by both the liver and gastrointestinal tract. 11 Cholesterol (and lipids) efflux from cell into the lipid poor particles causing them to mature into HDL particles (12) which circulate back to the liver where their contents are released into the gastrointestinal tract (13) and the lipid poor ApoA1 particles recirculated again. Adapted from (Williams, 2008, Francis and Pierce, 2011) and (Miller et al., 2010b).

#### *1.1.3.4 Hyperlipidaemia*

Under conditions of hyperlipidaemia, eNOS becomes increasingly associated with caveolin-1 (Zhu et al., 2003), leading to eNOS inhibition (Ju et al., 1997, García-Cardeña et al., 1997) and decreased NO bioavailability (Desideri and Ferri, 2005, Liao and Laufs, 2005, Deanfield et al., 2007). This drop in NO bioavailability increases endothelial cell permeability, and allows the migration of LDL into the sub-endothelial space (Osto et al., 2007) where it becomes trapped and undergoes oxidative modification (Sudano et al., 2006, Varughese et al., 2006, Manduteanu and Simionescu, 2012). The oxidised LDL (oxLDL) damages endothelial cells causing a pro-inflammatory response which results in the recruitment of macrophages and leukocytes. Additionally, the presence of oxLDL leads to depletion of caveolae cholesterol and eNOS being translocated away from the plasma membrane where it is active, to internal membrane compartments (Blair et al., 1999). Elevated lipid levels also rapidly inhibit the ability of insulin to recruit capillaries (Liu et al., 2009), which in turn impairs the ability of insulin to reach the muscle interstitium (Kolka et al., 2010, Chiu et al., 2009). This process appears to be partly mediated by down-regulation of proteins such as IRS2 which are involved the insulin signalling process (Kubota et al., 2011).

The fall in bioavailable NO associated with eNOS inhibition and impaired insulin signalling allows increased NF-κB activity, an amplified inflammatory response compared to a normally functioning endothelium and the activation/attraction of circulating immune cells such as monocytes (Manduteanu and Simionescu, 2012). Once attached, the monocytes migrate into the sub-endothelial space in response to stimulation by monocyte chemoattractant protein-1 (MCP-1), macrophage colony stimulating factor (M-CSF) and interleukin-6 (IL-6). Once in the sub-endothelial space they develop into macrophages, take up oxidised LDL and become the lipid-laden foam cells (Desideri and Ferri, 2005) that form the core of the plaque ((Passacquale et al., 2008), and references therein). The foam cells continue secreting inflammatory cytokines which advance the atherosclerotic process via leukocyte recruitment, macrophage replication and increased LDL binding. This continued recruitment of new leukocytes into the shoulders of the plaque cause it to grow and expand (Desideri and Ferri, 2005). At the same time, the ongoing inflammatory response in the sub-

endothelial space stimulates vascular smooth muscle cells (VSMC) to migrate and merge into the inflamed area causing arterial wall thickening (Passacquale et al., 2008).

As these plaques develop, they can move to a secondary stage whereupon the macrophages, smooth muscle cells and platelets release metalloproteases (MMP) which degrade the elastin and collagen of the plaque. These enzymes leave the plaque weak and susceptible to rupture resulting in thrombi and emboli as the plaque releases from the vessel wall and blocks vascular tissue leading to ischemic damage (Desideri and Ferri, 2005, Vita, 2005, Osto et al., 2007).

#### *1.1.3.5 Hyperglycaemia*

Consistently raised levels of glucose also affect cellular processes by irreversibly binding to proteins and forming advanced glycation end products (AGE) (Kim et al., 2011). Such modifications impair the function of multiple proteins, decreasing both their solubility and ability to be proteolysed, which leads to their accumulation in body tissue (Busch et al., 2010). In addition, AGE formation also results in increased vascular stiffness and permeability (Meerwaldt et al., 2008). These modified proteins are recognised by multiple receptors including the receptors for advanced glycation end products (RAGE) and the macrophage scavenger receptors ScR-II and CD-36. Interactions between these receptors, for example, between AGE and RAGE, activate downstream inflammatory pathways which result in NADPH oxidase activation and generation of ROS (Busch et al., 2010).

High blood glucose levels also reduce the bioavailability of NO by increasing endothelial mitochondria ROS production (leading to peroxynitrite production) and affecting eNOS/μnNOS functionality (Li and Shah, 2004, Sasaki et al., 2008). Additionally, the elevated levels of TNFa, and ROS experienced during hyperglycaemia both inhibit insulin uptake, and the PI3K signalling pathways, whilst other insulin mediated effects such as activation of the MAPK and production of endothelin-1 remain active, leading to vasoconstriction and impaired vascular function (Montagnani et al., 2002a, Kim et al., 2006, Wang et al., 2008).

As well as causing a reduction in bioavailable NO, elevated glucose also impairs lipoprotein function, causing both an increased residence time of LDL and chylomicron particles in the

vasculature, and a loss of HDL functionality (Vergès, 2009). It is this combination of factors (lowered NO, increased LDL or chylomicron residence time and reduced reverse cholesterol transport) that explains why people with diabetes and pre-diabetes show a clearly increased (~5×) susceptibility to, and progression of atherosclerosis when compared to similar non-diabetic patients (Nigro et al., 2006).

It is clear from the preceding over-view of cardiovascular system function and disease processes that hyperlipidaemia and hyperglycaemia share many of the same damage, signalling response and process mechanisms. In many respects, hyperlipidaemia/atherosclerosis and hyperglycaemia/type 2 diabetes represent different facets of the same disease process and as such have become commonly known as "metabolic syndrome". Recent studies regularly show that treatments for one component of the disease process can have beneficial effects on other components (Schmidt-Lucke et al., 2010, Zhou and Liao, 2010, Antoniades et al., 2011).

### *1.1.4 Treatment*

Due to cardiovascular disease and diabetes being the leading causes of human morbidity and mortality worldwide (WHO, 2008), considerable research has focused on lowering the incidence of these diseases and improving endothelial cell function. This research effort has resulted in several therapeutic strategies aimed at lowering the concentration of LDL; some such as ezetimide reduce intestinal lipid absorption, whilst others, such as statins reduce the *in situ* production of cholesterol. These therapies result in a lower concentration of LDL, this in turn causes increased hepatic expression of the LDL receptor, LDL having a shorter vascular residence time, and less opportunity for oxidation of LDL and chylomicron particles (Williams, 2008).

Another focus of research has been on improving cholesterol efflux out of cells and back to the liver for excretion, i.e., reverse cholesterol transport. Recent studies have targeted the role of the HDL transport protein, whose increased concentration has been shown to improve oxidative and inflammatory conditions in the vasculature, primarily due to an LDL lowering effect (Sudano et al., 2006); also leading to atherosclerotic plaque regression and the reestablishment of endothelial function (Francis and Pierce, 2011).

The lowering of blood glucose concentration by inhibiting gluconeogenesis has also been investigated with compounds such as metformin developed into diabetic therapies (Kim et al., 2008). By reducing blood glucose levels, these compounds also lower the rate of AGE formation and the associated loss of lipoprotein functionality; which in turn creates the opportunity for re-establishment of normal endothelial function.

Additionally, increasing vascular NO production has also been proposed, due to its well documented plethora of beneficial activity and direct effect on microvasculature dilatation (Higashi et al., 2009). This in turn allows improved uptake of glucose from circulation into muscle or adipose tissue, and supplementation with several plant derived polyphenolic compounds have shown activity in this regard (Grassi et al., 2009).

As each perturbation to vascular function is associated with oxidative stress, efforts have been made to reduce disease incidence through supplementation with antioxidant such as Vitamin E and Vitamin A. Generally, these efforts have met with little to no success in reducing atherosclerotic burden (Saremi and Arora, 2010, Heinz et al., 2010, Morris and Carson, 2003). However, agents which directly lower LDL cholesterol, lipids, blood glucose or improve NO bioavailability have been shown to also improve the vasculature's redox status (Shishehbor et al., 2003).

An additional benefit of therapies such as statins or supplementation with plant derived polyphenolics is that they are able to cause plaque regression, signal the production of endothelial progenitor cells (EPC) and reverse previous damage by stimulating the repair of the previously damaged endothelium (Davignon, 2004, Besler et al., 2010, Besler et al., 2008). The ability of statins and flavonoids to interact and affect each of these therapeutic targets are summarised in table 1.1 and the precise interactions are discussed in further detail in sections 4.6.1-4.6.5.

**Table 2.1.** A comparison of the cardiovascular protective effects provided by statin and flavonoid compounds

| Activity | Statin | Flavonoid |
|---|---|---|
| Lipid lowering | | |
| - Inhibition of HMG-CoA reductase | ✓ | ✓ |
| - Inhibition of acyl CoA:Cholesterol transferase | | ✓ |
| - Increased HDL:LDL | ✓ | ✓ |
| Glucose homeostasis | | |
| - Improved uptake into muscle tissue | | ✓ |
| - Reduced uptake from the digestive tract | | ✓ |
| - Restore insulin signalling pathway and improve insulin sensitivity | | ✓ |
| Reduced ROS production | | |
| - Inhibiting oxidase enzyme activity | ✓ | ✓ |
| - Increasing antioxidant enzyme activity | ✓ | ✓ |
| - Protection of lipoproteins from oxidation | ✓ | ✓ |
| - Chelation of transition metals and ROS scavenging | | ✓ |
| Increased NO production | | |
| - Increased eNOS activity | ✓ | ✓ |
| - Increased eNOS expression | ✓ | ✓ |
| - Improved eNOS coupling | ✓ | |
| - Decreased eNOS degradation | ✓ | ✓ |
| - Decreased production/expression/activity of eNOS inhibitors | ✓ | ✓ |
| Reversal of endothelial damage | | |
| - Increased endothelial progenitor cell recruitment and mobilisation | ✓ | ✓ |
| - Reversal of atherogenic plaque | ✓ | ✓ |
| Modification of other inflammatory processes | | |
| - Decreased production of adhesion molecules | ✓ | ✓ |
| - Inhibition of TNFα production | ✓ | ✓ |
| - Reduced prenylation of compounds involved in inflammatory signalling | ✓ | ✓ |
| - Reduced expression of inflammatory cytokines | ✓ | ✓ |
| - Reduced macrophage uptake of oxLDL | ✓ | ✓ |

#### *1.1.4.1 Lowering of low-density lipoprotein*

There is a strong relationship between hyperlipidaemia, oxLDL levels and the incidence of vascular disease (Spieker and Lüscher, 2005). There is also a large body of research supporting the model of endothelial dysfunction, lipid accumulation and oxidative modification being the initiating steps of atherosclerosis (Balestrieri et al., 2003, Deanfield et al., 2007). Because of this, researchers have placed considerable effort into developing strategies to lower cholesterol and LDL levels.

One target of this research has been the rate limiting step of cholesterol synthesis, the conversion of 3-hydroxy-3methylglutaryl coenzyme-A (HMG-CoA) to mevalonate which is achieved *in vivo* by the HMG-CoA reductase enzyme (Varughese et al., 2006, Paraskevas et al., 2007). Compounds that possess HMG or a HMG-like moiety can outcompete HMG-CoA for the enzyme's active site (Figure 1.9) and several of these compounds have been developed into the statin drug class (Martinez-Gonzalez and Badimon, 2007). These drugs have been highly successful in lowering patient cholesterol levels as well as improving endothelial function and general health outcomes. This is particularly evident in patients placed on statin therapy after a myocardial infarction where statin therapy both increases survival and reduces the readmission rate of the treatment group (Sankaranarayanan et al., 2010).

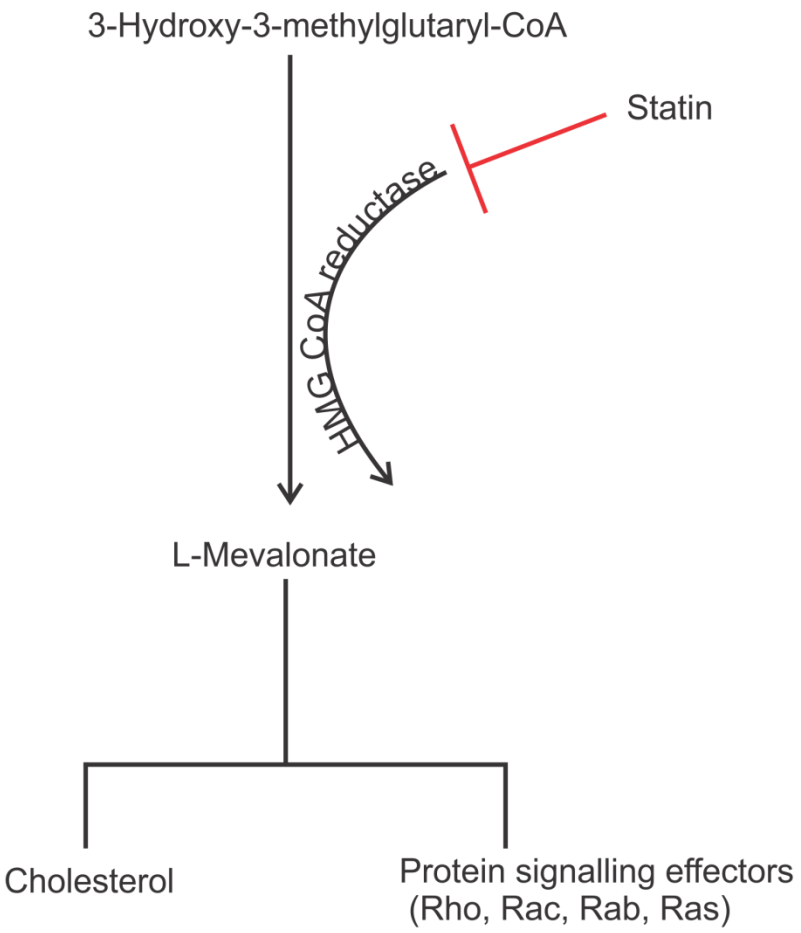



**Figure 2.9.** The cholesterol synthesis pathway, products and the HMG Coa reductase inhibition by statins. Adapted from (McTaggart, 2006) and (Zhou and Liao, 2010).

Multiple benefits are achieved by inhibiting the HMG-CoA step of cholesterol synthesis; both cholesterol manufacture and excretion is reduced, as is the production of LDL and chylomicrons. In response, hepatocyte expression of LDL receptors is upregulated improving LDL clearance from the bloodstream (Martinez-Gonzalez and Badimon, 2007). Additionally, the action of isoprenoids, which are manufactured from downstream products of HMG-CoA reductase activity and responsible for activating inflammatory proteins is simultaneously inhibited (McTaggart, 2006). Lowering the blood or plasma concentration of cholesterol and non-HDL lipoproteins, as well as increasing LDL- receptor expression raises the HDL:LDL ratio (Halcox and Deanfield, 2004). This also improves patient prognosis as raised HDL levels are associated with improved vascular function, as well as being linked to regression of

atherogenic plaques and reversal of endothelial damage (Spieker and Lüscher, 2005, Besler et al., 2010, Besler et al., 2008).

Interestingly, statins produce a greater improvement in vascular health than other lipid lowering therapies such as blocking intestinal cholesterol absorption and these effects are clearly mediated by properties outside of their lipid lowering activity, i.e., pleiotropic effects (Schmidt-Lucke et al., 2010, Carneado et al., 2002).

In addition to direct HMC-CoA reductase inhibition by statins, population based studies have highlighted the importance of consuming high levels of plant derived polyphenolic compounds to maintain good vascular health (Ross and Kasum, 2002, Manach et al., 2005). Within this group of compounds, the level of flavonoid consumption has been documented to be inversely correlated with cardiovascular risk (Peluso, 2006). Flavonoids have been shown to have direct lipid lowering effects by inhibiting both HMG-CoA reductase and acyl CoA:Cholesterol transferase (Bok et al., 1999), and increasing adiponectin levels (Shabrova et al., 2011). Adiponectin is responsible for stimulating NO production in vascular endothelial cells (Chen et al., 2003), increasing IRS2 expression and insulin sensitivity (Awazawa et al., 2011).

#### *1.1.4.2 Lowering blood glucose*

Meta-analyses have shown statin therapies to have no beneficial effect on lowering blood glucose, and in fact, statin therapy can actually increase the risk of developing diabetes (Preiss and Sattar, 2011). Additionally, exercise improves insulin sensitivity but can exacerbate statin-related myopathy in statin-treated patients (Meador and Huey, 2010). Despite these risks, in most situations, the benefits associated with decreased risk of other cardiovascular events outweighs the risk associated with diabetes (Preiss and Sattar, 2011).

Flavonoids on the other hand have shown both *in vitro* and *in vivo* glucose lowering activity, with a reduction in the production of AGEs responsible for protein binding and enzyme inhibition (Urios et al., 2007, Kim et al., 2011). Flavonoids are able to stimulate the uptake of glucose into peripheral tissue, regulate enzymes of carbohydrate metabolism and improve the patient's glycaemic profile via insulin associated pathways, as reviewed in Cazarolli et al., (2008). More significantly though, is the fact that they have been shown to increase the

activity of GLUT4 and glucose uptake into skeletal muscle tissue (Ueda et al., 2008), competitively inhibit the uptake of glucose from the digestive tract into the bloodstream by inhibiting GLUT2 and sodium-dependant glucose transporter 1 (Cermak et al., 2004, Skopec et al., 2010, Kobayashi et al., 2000, Kwon et al., 2007). They are also able to activate the PI3K pathway independently of insulin and increase the expression of GLUT4 in muscle tissue (Chi et al., 2007, Su et al., 2006).

#### *1.1.4.3 Reducing Oxidative Stress*

The production of $ONOO^-$ during conditions of oxidative stress leads to the oxidation of tetrahydrobiopterin (THB), a critical co-factor for eNOS coupling leading to a cycle of NO + ROS → $ONOO^-$ and reduction in bioavailable NO (Landmesser et al., 2004, Desideri and Ferri, 2005). Statins are able to reduce stress on the vascular system by both upregulating the activity of antioxidant enzymes such as superoxide dismutase (SOD) and downregulating the activity of the ROS producing enzyme nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase (Carneado et al., 2002, Wassmann et al., 2001).

Flavonoids also reduce vascular oxidative stress. They are able to act as scavengers of most types of oxidising species (radicals, singlet oxygen) and chelate with metal ions (Gomes et al., 2008), and in fact this was one of the earliest functions predicted for flavonoids *in vivo* (Bors et al., 1997). When the concentration of flavonoids *in vivo* is taken into consideration though, the relevance of flavonoids *in vitro* radical scavenging abilities become questionable and are an issue still under debate (Halliwell, 2008). However, evidence does suggest that flavonoid antioxidant activity is more related to effects on oxidase and antioxidant enzymes than simply ROS scavenging. Flavonoid supplementation has been reported to inhibit the activity of (NAD(P)H) oxidase and xanthine oxidase, and also increases in the activity of endogenous antioxidant enzymes such as SOD and glutathione S-transferase (GST) (Ross and Kasum, 2002, Middleton Jr et al., 2000, Sanchez et al., 2006, Gomes et al., 2008, Akhlaghi and Bandy, 2009).

#### *1.1.4.4 Increasing NO production*

Apart from the reduction in oxidative species producing an improved NO/$ONOO^-$ balance, another mechanism by which both flavonoid and statins enable an improvement in vascular health is through directly increasing NO production of platelet and endothelial cell eNOS (Endres et al., 2004, Halcox and Deanfield, 2004, Yemisci et al., 2008).

This production occurs via several mechanisms including protecting eNOS from degradation, stabilising the eNOS mRNA, increasing eNOS phosphorylation via the PI3/AKT pathway (Zhou and Liao, 2010, Chi et al., 2007, Stangl et al., 2007), and changing intracellular [$Ca^{2+}$] (Stoclet et al., 2004). Members from both groups of compounds have also been reported to hamper the production of the eNOS inhibiting Rho and caveolin-1 proteins (Endres and Laufs, 2006, Liao and Laufs, 2005, Braam and Verhaar, 2007).

Further to this, statins are able to reverse eNOS uncoupling by increasing the concentration of THB via a GTPcyclohydrolase-1 (the rate limiting enzyme of THB synthesis) mediated effect (Heeba et al., 2007, Wenzel et al., 2008).

#### *1.1.4.5 Alteration of signal transduction and receptors*

Both groups of compounds also moderate the body's inflammatory response via direct action on receptors, adhesion molecules and cytokine release. Some of these effects include reduced synthesis of the vasoconstrictor endothelin-1 (ET-1) reviewed in Stoclet et al., (2004), and Halcox and Deanfield (2004). Additionally, they are able to reduce thrombin production and the expression of tissue factor (TF), and plasmin activator type-1 (PAI-1) (Osto et al., 2007). Flavonoids and statins both have also been reported to inhibit TNFα production and down regulate the expression of VCAM-1 and E-selectin (Deanfield et al., 2007). They decrease sICAM-1, CD4 T cell expression, reduce CRP, IL-6, IL-8, MCP-1, thrombin/antithrombin complex (Varughese et al., 2006) as well as suppress the expression of macrophage CD36 receptors (Kawai et al., 2008). Additionally, both groups of compounds improve the body's ability to repair vascular damage by increasing the production, mobilisation and recruitment of endothelial progenitor cells to sites of vascular damage via NO dependant mechanisms (Davignon and Ganz, 2004, Martinez-Gonzalez and Badimon, 2007, Endres et al., 2004, Schmidt-Lucke et al., 2010, Besler et al., 2008).

Due to their ability to affect NO bioavailability, as well as impact on multiple signal pathways, statin treatment and flavonoid consumption have both been shown to be effective in protecting the body from other diseases of inflammation and degeneration including stroke (Endres et al., 1998, Laufs et al., 2000, Yamada et al., 2000, Amin-Hanjani et al., 2001, Endres et al., 2004, Endres and Laufs, 2006, Yemisci et al., 2008) and Alzheimer's disease (Spencer, 2009b, Spencer, 2010, Spencer, 2009a, Spencer, 2008, Spencer et al., 2008, Vauzour et al., 2008).

Nevertheless, at this stage it is primarily statins which have been developed into pharmaceutical therapeutic products, whilst the investigation of the pharmacological effects of most flavonoid compounds is still generally at an earlier stage. Despite this, the data being generated in relation to flavonoid compounds is quite encouraging and shows great potential in relation to both lipid lowering and improvement of glucose tolerance as well as general effects on endothelial and cardiovascular health in general. As with all pharmacological agents though, care must be taken with their use as statins have been shown to increase the incidence of patients experiencing myalgia, myopathy or rhabdomyolysis (Toth et al., 2008, Sathasivam, 2012, Harper and Jacobson, 2007), whilst certain flavonoids possess cytotoxic properties and can be pro-oxidant at high concentrations (Li et al., 2008).

## 1.2 Potential for C. rossii as a nutraceutical product

Preliminary investigations conducted by researchers at the University of Tasmania have shown that a crude extract from the native Australian plant *Carpobrotus rossii* (CR) possesses *in vitro* pharmacological activity including inhibition of platelet aggregation, inflammatory cytokine release (interleukin-1-beta, tumour necrosis factor-alpha) and lipid oxidation (Geraghty et al., 2011). This activity is believed to be due to the flavonoid compounds that this plant produces in its leaves (Figure 1.10).

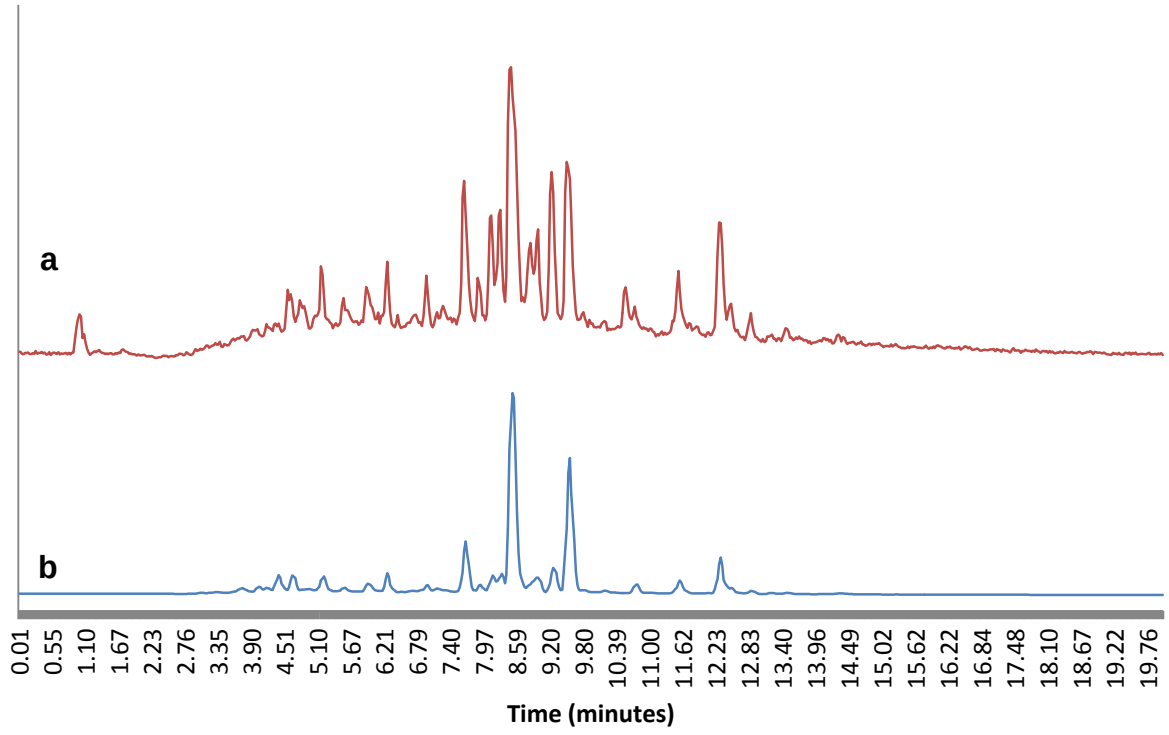



**Figure 2.10.** a) UPLC-MS/MS chromatogram of all m/z 600-1200 ions produced by CR leaf extract, b) UPLC-DAD 350nm chromatogram produced by CR leaf extract. Absorbance peaks at 350nm indicate the presence of flavonoid compounds

These flavonoid compounds have been shown to contain an HMG moiety (Jager, 2009) which is known to inhibit HMG-CoA reductase. This means that consumption of CR flavonoids could potentially improve endothelial health and cardiovascular function via a combination of effects related to both their flavonoid and possible HMG-CoA reductase inhibiting structure ("statin" activity). Fruit and leaves from this plant have been consumed for thousands of years by the Australian aboriginal people who also used juice from the leaves as a treatment for itches, insect bites and gastro-intestinal complaints (Plomley et al., 1966, Cane et al., 1979, Watson, 2007).

#### *1.2.1.1 The plant*

CR can be found along the coastal dunes of southern Australia (Winter et al., 1981, Australia, Blake, 1969, Clarke, 1994) growing as a large, spreading mat with long lateral branches searching for new locations to colonise (Blake, 1969, Venning, 1984). Its leaves are succulent and are one of several adaptions that have evolved to address the impediments to growth encountered at these highly exposed beach sites. These impediments are due to the stress induced from salt spray, wind shear, sand blast, high temperatures, excessive light and periodic overwash by seawater (Clarke, 1994, Garcia-Mora et al., 1999, Lane et al., 2008). Additionally, sand is a highly mobile and permeable substrate which can causes moisture and nutritional stress that further hinders growth (Valverde et al., 1997).

To survive these hostile conditions, foredune plants such as CR have adaptations which allow physiological functioning and growth to continue even when unfavourable conditions are present. These modifications include becoming succulent (increasing the surface to volume ratio of leaves), the storage of toxic ions in leaf tissue away from metabolically/photosynthetically active tissue and an ability to tolerate and thrive under saline conditions, i.e. becoming halophytes.

Succulence and the storage of ions in plant tissue lowers their water potential and allows enhanced uptake of moisture from the soil matrix to occur (Flowers and Yeo, 1986). Succulence also creates a larger *in planta* storage of moisture to guard against potential water stress (Winter et al., 1981) and provides a location for the safe storage of toxic ions such as $Na^+$ and $Cl^-$ away from the sensitive photosynthetic machinery (Shabala, 2012). The

thickened cuticles which have developed also protect against both moisture loss (Rabas and Martin, 2003) and abrasion damage from air-borne soil particles (Garcia-Mora et al., 1999). Additionally, halophytic plants, that is plants that require the presence of salt to achieve optimum growth, have developed membrane barriers and transport proteins to actively exclude, transport and compartmentalise ions within cell (Parida and Das, 2005, Flowers and Colmer, 2008); as well as an ability to access and directly uptake ocean water during times of low rainfall and moisture stress (Greaver and Sternberg, 2007, Greaver and Sternberg, 2006). To enable the efficient harvesting of the scarce supplies of nutrients and water present in sand, and provide strong anchor points in the highly mobile sand substrate a well-developed rhizome and root system has also evolved (Costa et al., 1996, Garcia-Mora et al., 1999, Traveset et al., 2008).

Like animals cells though, plants are also susceptible to the actions of ROS caused by unfavourable environmental conditions and thus, they have also evolved a well-developed antioxidant system to combat the effects of excessive ROS.

## 1.3 Plant Metabolism

### *1.3.1 ROS generation and function in planta*

The generation of ROS *in planta* is a complex process, owing to their heavy involvement in, and amplification of, many of the signalling pathways within the plant (Suzuki et al., 2011, Torres and Dangl, 2005). Plants use ROS as signalling molecules to control processes ranging from root growth and development (Foreman et al., 2003), through to the signalling of environmental stresses, wounding, injury and pathogen attack (Demidchik and Maathuis, 2007, Mittler et al., 2011). ROS are able to activate a host of cellular machinery including the $K^+$ and $Ca^{2+}$ ion channels to achieve further signal amplification and/or an appropriate cellular response (Demidchik, 2010, Cuin and Shabala, 2007). In addition to signalling these type of interactions, ROS signals also alter the transcription of photosystem-I (PS-I) or photosystem-II (PS-II) light centres to allow for adaption to incoming light quality and change gene expression to prevent over reduction of the photosynthetic ETC (Foyer et al., 2012). These changes to PS-I and PS-II expression revolve around changes in the oxidation state of plastoquinone (Allen et al., 2000).

As illustrated in Figure 1.11, photosynthetic cells generate ROS from a diversity of sources including organelles such as peroxisomes, the photosynthetic and the mitochondrial electron transport chains. In addition, specialised oxidase enzymes are responsible for ROS production in the apoplasm and NADPH oxidases are the main source of ROS associated with "oxidative burst" signalling (Miller et al., 2010a, Mur et al., 2005, Gill and Tuteja, 2010). *In planta* however, the chloroplast is the primary site of ROS production due to the loss of electrons from the photosynthetic electron transport chain (ETC) (Spínola et al., 2008).

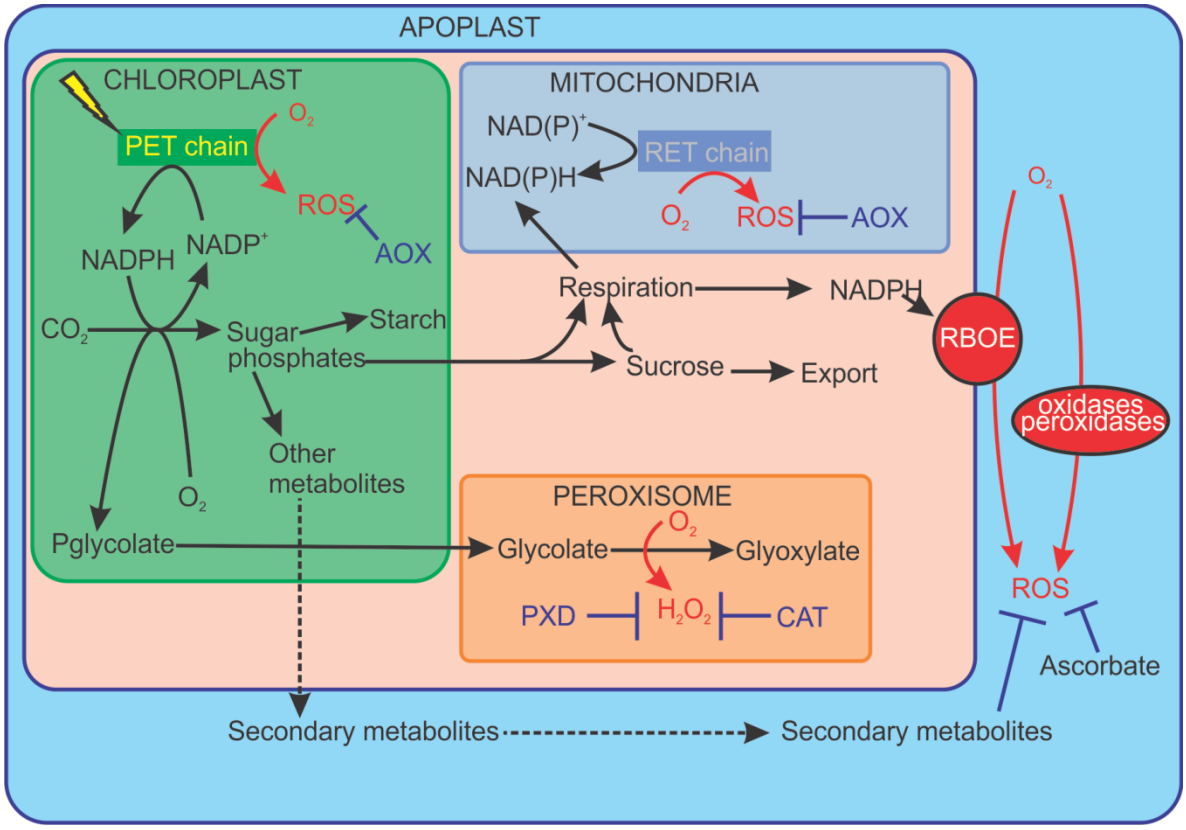



**Figure 2.11.** Location of the major sites of ROS production in photosynthetic cells. AOX, antioxidant system; CAT, catalase; PET, photosynthetic electron transport; PXD, peroxidase; RBOE, respiratory burst oxidase enzymes; RET respiratory electron transport. Adapted from (Foyer and Noctor, 2009).

Photosynthetic organisms such as bacteria, algae and plants possess specialised organelles (chloroplasts) which allow the harvesting of light energy to drive the generation of redox and proton gradients. These gradients are in turn used to power the production of NADPH and ATP which are needed for cellular growth and metabolism. In plants, the chloroplast houses two light harvesting systems, namely photosystem II and photosystem I. These two systems are linked together by an electron transport chain (ETC) and split water to provide electrons (e-) and enable the reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$) to NADPH. This system also generates a proton gradient across the thylakoid membrane for ATP generation via ATP synthase (Figure 1.12); (Campbell et al., 2008).

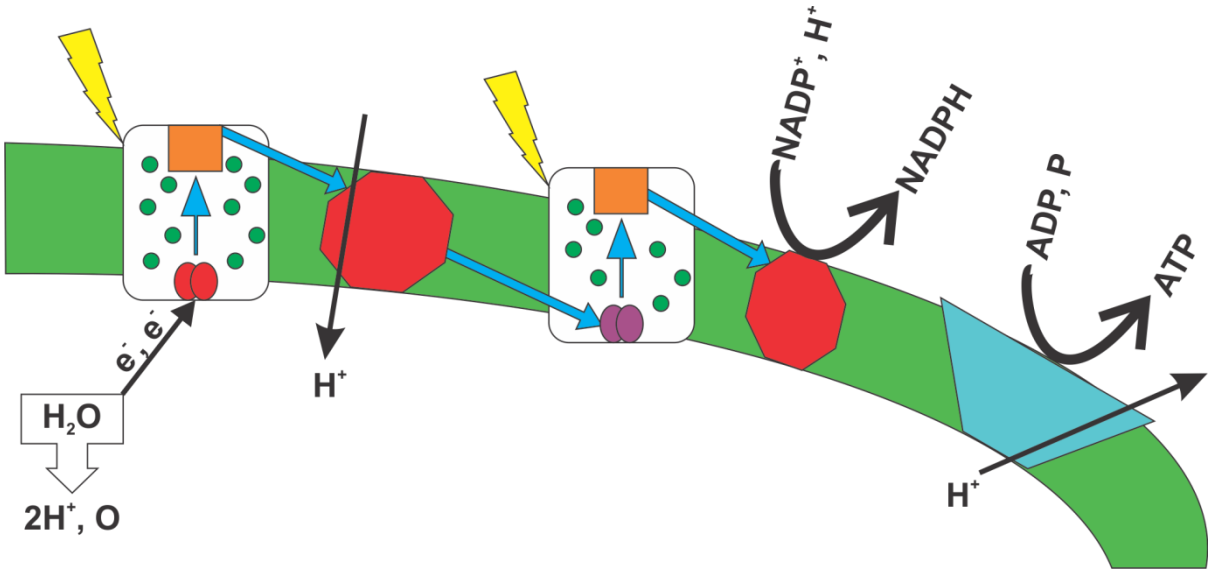



**Figure 2.12.** An overview of the photosynthetic process (adapted from (Campbell et al., 2008).

In addition to supplying electrons for the reduction of $NADP^+$, the ferredoxin protein also supplies electrons to power the reduction of ammonia via nitrite reductase, sulphur via sulphur reductase, thioredoxin via ferredoxin-thioredoxin reductase and oxygen via the Mehler reaction (Figure 1.13); (Oelze et al., 2008).

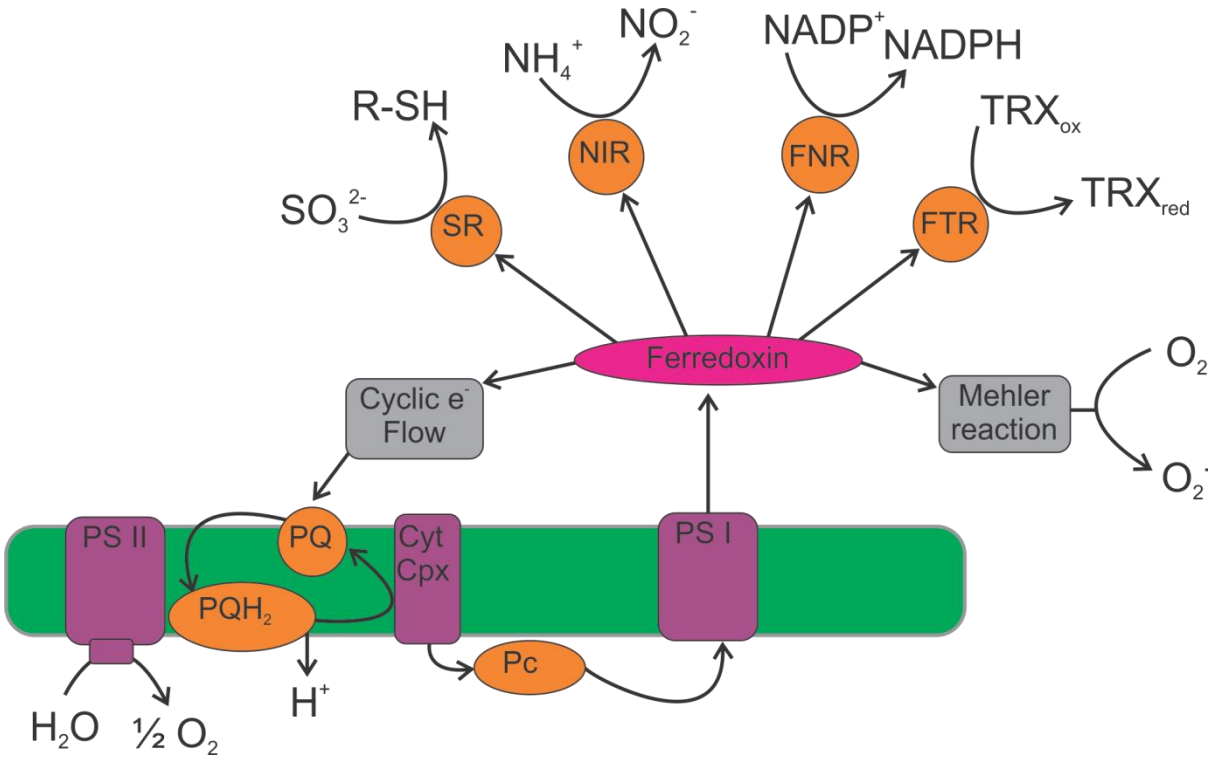



**Figure 2.13.** Potential electron acceptors of photosynthesis. Sulfite reductase (SR), ferredoxin-nitrite reductase (NIR), ferredoxin $NADP^+$ reductase (FNR), ferredoxin-thioredoxin reductase (FTR) (adapted from (Oelze et al., 2008)).

The photosynthetic process begins with the interception of photons by the light harvesting compounds chlorophyll a, chlorophyll b and carotenoids, and ends with the reduction of $NADP^+$ and the generation of ATP. This newly generated NADPH and ATP move to the stroma where it can be used to power the conversion of $CO_2$ into carbohydrates such as glucose and starch during the Calvin cycle. These metabolites are then transported throughout the plant to provide substrate for growth and power respiration in cell mitochondria (Campbell et al., 2008).

The light harvesting process of photosynthesis isn't perfect though and there is at least 15 orders of magnitude difference in the reaction rates of light harvesting compared to downstream metabolic processes (Huner et al., 1998). This results in a number of electrons being lost from the transport chain, producing superoxide ($O_2^{\cdot-}$) and other ROS.

Many unfavourable environmental conditions lead to an increase in ROS production *in planta*. Primarily, this increased production is due to these conditions hindering the metabolic processes which consume ATP and NADPH to produce ADP and $NADP^+$, respectively. Subsequently, an imbalance between electrons captured and the amount of substrate ($NADP^+$) available for reduction occurs. Put simply, the rate of $CO_2$ uptake to produce glucose/starch and the downstream metabolic processes which consume this product are slower than photon/electron capture speed. This results in the ETC being held in a reduced state and becoming more likely to lose an electron to $O_2$, forming superoxide ($O_2^{\cdot-}$) as part of the Mehler reaction from PSI (Figure 1.13); (Takahashi and Murata, 2008). Additionally an over-reduced transport chain results leads to a higher chance of chlorophyll reaching the highly unstable triplet state ($_3Chl^*$) and dismutating its energy from PSII to form singlet oxygen (Foyer et al., 2012). Potential ROS and reactive nitrogen species (RNS) generated *in planta* are given as Table 1.2, whilst a schematic of ROS generating mechanisms are shown in Figure 1.14.

**Table 2.2.** Reactive Oxygen and Nitrogen Species (adapted from (Foyer and Noctor, 2009, Møller et al., 2007)).

| Name | Symbol | Relative Reactivity | Primary Source in planta | Reacts with |
|---|---|---|---|---|
| Singlet Oxygen | $^1O_2$ | Extreme | Excited chlorophyll | Poly-unsaturated fatty acids, DNA and proteins. |
| Superoxide | $O_2^{\cdot-}$ | Average | Electron transport chain | Iron-sulphur centres of proteins |
| Hydrogen Peroxide | $H_2O_2$ | Low | Reduction/dismutation of superoxide | Proteins |
| Hydroxyl Radical | $OH^{\cdot}$ | Extreme | Reduction/cleavage of $H_2O_2$ | Poly-unsaturated fatty acids, DNA, carbohydrates and proteins |
| Peroxynitrite | $ONOO^-$ | | Reaction of NO and $O_2^{\cdot-}$ | Poly-unsaturated fatty acids, DNA and proteins |

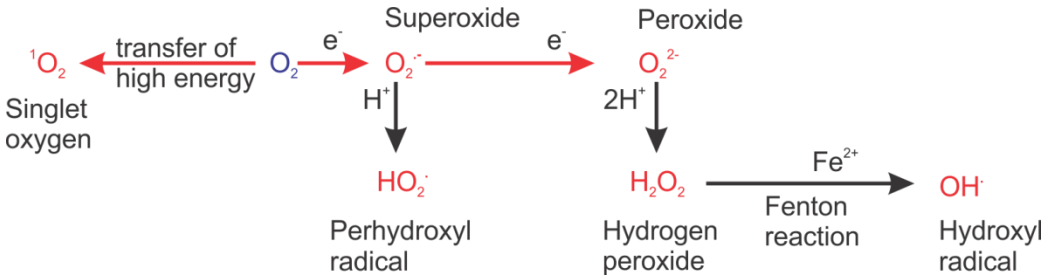



**Figure 2.14.** Schematic of ROS generation mechanisms (adapted from (Gill and Tuteja, 2010)).

As well as the effect of environmental conditions on the consumption of NADPH and ATP, interactions with the environment directly increase the production of ROS from oxidase enzymes, the peroxisome and mitochondria. A prime example of ROS generated from these sources is the commonly observed oxidative burst and hypersensitive response which occurs as part of the pathogen signalling. During this process a combination of oxidase enzymes as well as ROS from the mitochondrial ETC are involved in signalling the PCD process (Mur et al., 2005, Reape and McCabe, 2010). Respiratory burst oxidases are also involved in processes including pollen tip growth, root hair formation, stomatal closing, seed ripening and mechanosensing (Suzuki et al., 2011).

### *1.3.2 ROS induced damage*

Despite their critical role in signalling, the reactive nature of these ROS and RNS means that, as in animals, their production must be tightly regulated to avoid the deleterious effects associated with their over production (Figure 1.15).

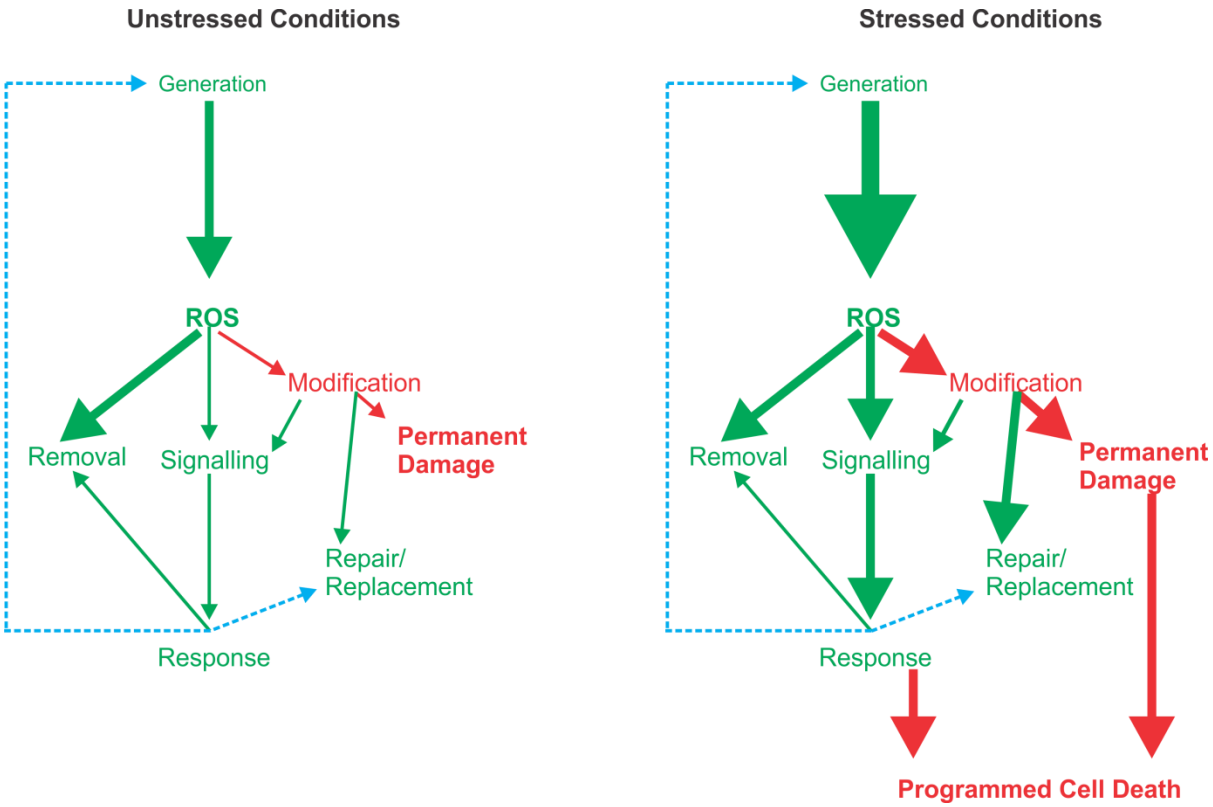



**Figure 2.15.** The interaction of ROS generation/signalling and damage under unstressed and stressed conditions. Arrow width indicative of relative effect of ROS production under each condition (adapted from (Møller et al., 2007)).

Mechanisms of damage caused by ROS include the peroxidation of phospholipid membranes which cause decreased membrane fluidity and increased membrane permeability, thus affecting the ability of cells to generate and maintain biochemical gradients; the oxidation of proteins, altering their charge, shape, function, and hence their catalytic activity (Kourie, 1998, Møller et al., 2007); and damage to sugars, DNA and RNA (Tuteja et al., 2001, Møller et al., 2007). Because ROS also activate membrane ion channels ($Ca^{+}$, $K^{+}$), there is the potential for them to causing a potentially uncontrolled ion flux, loss of intracellular $K^{+}$, programmed cell death (PCD) and the death of the whole plant if their production isn't kept under control (Demidchik et al., 2010, Casolo et al., 2005, Demidchik et al., 2007, Shabala, 2009). These processes are illustrated in Figure 1.16.

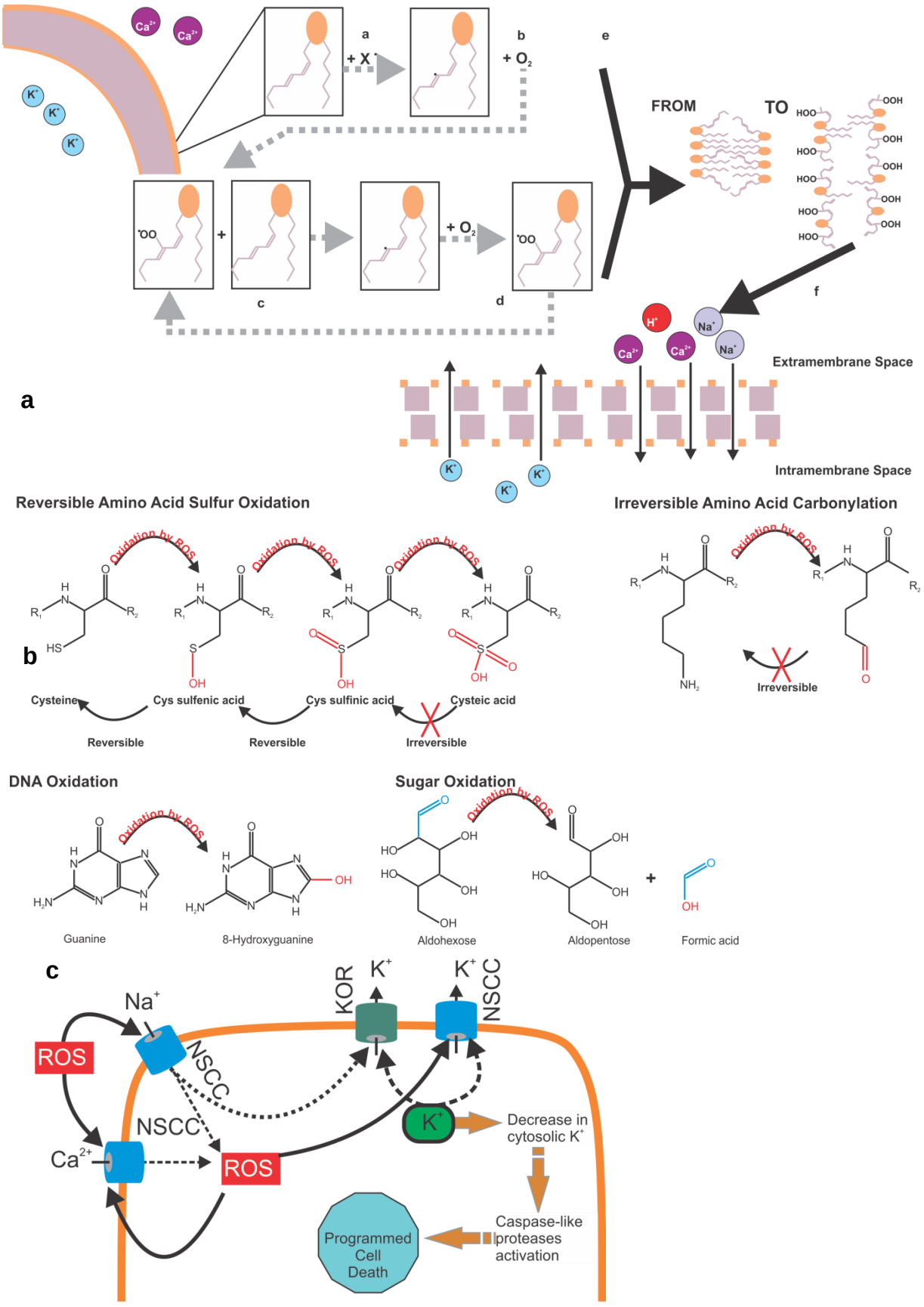



**Figure 2.16.** Mechanisms of ROS cellular damage (a) lipid peroxidation, (b) amino acid, DNA and sugar oxidation, and (c) activation of membrane ion channels. (b) and (c) Adapted from (Møller et al., 2007, Shabala, 2009) respectively.

In addition to PCD induction, damage to cellular constituents and machinery by reactive species impacts cellular function in several other ways. Firstly, they are immediately lost from involvement in metabolic processes, and secondly there is the longer term energetic cost of repairing and replacing the damaged components (Table 1.3); (Møller et al., 2007).

**Table 2.3.** Approximate cost of replacing damaged cellular components (adapted from (Møller et al., 2007)).

| Component | Size | ATP equivalents to replace |
|---|---|---|
| Single amino acid molecule | 100-200 Da | 10 |
| Single polyunsaturated fatty acid (PUFA) | 250-300 Da | 4 × 10 |
| Single phospholipid molecule containing two PUFAs | 700-800 Da | $10^2$ |
| Single average protein molecule | 50000 Da | $2 \times 10^3$ |
| Importation of single average protein molecule across a membrane | 50000 Da | $10^4$ |
| Single mitochondrion | 1 pg protein | $2 \times 10^{10}$ - $10^{11}$ |

Carbon fixation by the Calvin cycle also virtually ceases during $H_2O_2$ scavenging as electrons from the ETC reduce antioxidant compounds instead of $NADP^+$ (Wise, 1995). The repair of D1 protein located in PSII and degraded under normal photosynthetic conditions also becomes impaired, resulting in photoinhibition (Takahashi and Murata, 2008).

Unfavourable environmental conditions often expose plants to a combination of stress sources. For example, whilst experiencing drought a plant experiences moisture limitation, light and temperature stress. This means the plant has to deal with a combination of low moisture by closing stomata, which in turn causes low $CO_2$ exchange; high light levels providing an excess of high energy electrons, as well as potential UV damage; and temperatures outside of a physiologically optimum range, meaning that metabolic processes are impaired and that use of NADPH and ATP is hindered.

#### *1.3.2.1 Low Temperature, high light conditions and exposure to ultra-violet light*

In many ways, low temperature and high light can be considered different manifestations of the same problem, namely an excess of electrons captured by the light harvesting complex compared to the rate of substrate use (Huner et al., 1998).

At temperatures lower than approximately 10°C, the Calvin-cycle enzymes become impaired due to simple thermodynamic effects. This in turn causes a reduction in the supply of $NADP^+$ available for reduction and ADP and phosphate ions for phosphorylation. If this occurs in the presence of moderate light which continues to be captured and moved into the ETC (causing a high PSII excitation pressure), the ETC becomes held in an over-reduced state which increases the opportunity for ROS formation (Wise, 1995). Light which is absorbed by the manganese component of PSII's oxygen evolving complex can then create primary photo damage, whilst the interaction of excess light with light harvesting complexes inhibit the PSII repair process through ROS production (Takahashi and Badger, 2011).

Additionally, low temperatures reduce membrane fluidity as unsaturated membrane lipids start to solidify and to counteract this cold tolerant species possess a higher proportion of unsaturated lipids than their cold sensitive counterparts (Uemura and Steponkus, 1994). Low temperatures also cause plants to experience dehydration from and imbalance in water lost from the leaves by transpiration and the reduced hydraulic conductivity of the roots (Aroca et al., 2012).

Alongside the excess of electrons captured vs. rate of metabolic use, light possesses a specific threat to photosynthesis in the form of ultraviolet radiation. Despite only comprising a small proportion of the solar spectrum it is able to cause direct damage to proteins, DNA and membranes due to its high energy levels (Ibdah et al., 2002). The PSII complex is a major target of UV damage, PSII is directly damaged by UV and it is estimated that approximately one third of damage to PSII is due to ultraviolet radiation (Takahashi and Murata, 2008, Jansen et al., 1996b, Jansen et al., 1996a). As well as direct effects, ultraviolet radiation induces the production of ROS through both signalling pathways and the direct homolytic fission of $H_2O_2$ to twin hydroxyl radicals (Halliwell, 2006, Takahashi and Badger, 2011). These ROS then inhibit the replacement of the PSII D1 protein, leading to photoinhibition (Takahashi and Badger, 2011). Ultraviolet radiation also inhibits the activity of ribulose bisphosphate carboxylase (Rubisco) leading to inhibition of the uptake of $CO_2$ and use of NADPH and ATP during its conversion to glucose during the Calvin cycle (Jansen et al., 1998).

#### *1.3.2.2 High Sodium and low potassium*

The presence of excessive salts in the soil profile cause problems for plants wishing to grow there. One problem is the lower osmotic potential that must be generated to draw moisture out of the surrounding salty soil and into the plant where it is used to maintain turgor and cellular processes in the cytosol. This leads to the plant experiencing moisture stress, stomatal closure, reduced $CO_2$ uptake and increased ROS production (Chaves et al., 2009).

The second, larger problem that salinity poses is its effect on cellular metabolic processes due to sodium toxicity. The influx of sodium into a plant is primarily through its root hairs and is driven by two factors 1) diffusion down a concentration gradient and 2) the attraction of positive cations to the electronegativity of the cellular cytosol (Amtmann and Beilby, 2010, Tester and Davenport, 2003). The reason for sodium's toxicity lies in the similarity sodium possesses to potassium, i.e. a positive monovalent ion with small ionic size (Marschner, 2012). Potassium is involved in over 50 different enzymatic reactions; under conditions of low-potassium enzymatic reactions cannot be catalysed leading to increased ROS production. Sodium is also recognised by and able to compete for the $K^+$ binding sites. However, sodium is unable to catalyse any reactions and thus the enzyme is taken out of metabolic production (Marschner, 2012), a dearth of electron acceptors ($NADP^+$) occurs and an increased loss of electrons via the Mehler reaction to generate superoxide and other ROS occurs.

The influx of sodium to the cytosol impacts the sodium to potassium ratio in two ways. Firstly, an increase in sodium ions directly affects the $K^+$:$Na^+$ ratio, and secondly the influx of positive sodium ions causes membrane depolarisation forcing the cell to efflux potassium through outward rectifying channels (KOR) in order to maintain cellular electronegativity (Shabala et al., 2005, Shabala et al., 2006). This loss of $K^+$ not only results in a loss of metabolic processes reliant on $K^+$ and increased ROS production, but can also catalyse programmed cell death as $K^+$ is no longer able to inhibit proteases activity (Shabala, 2009). For these reasons, plants place considerable effort in maintaining a minimum (~100mM) potassium concentration and a high potassium to sodium ratio in the cytosol (>10:1); (Apse and Blumwald, 2007). This dual-strategy ensures potassium is ability to outcompete sodium at the catalytic sites (Shabala and Pottosin, 2010).

### *1.3.3 Antioxidants to combat ROS*

As outlined previously, it is critically important for plants to maintain ROS concentrations at physiologically relevant levels and avoid their deleterious effects. To do this, plants employ an array of chemical and enzymatic antioxidant compounds to modify and convert reactive species into safe forms (Table 1.4).

**Table 2.4.** Plant antioxidant systems (adapted from (Agati et al., 2007, Rafat Husain et al., 1987, Furuno et al., 2002, Procházková and Wilhelmová, 2007)).

| **Antioxidant** | **Dismutation reaction** |
|---|---|
| Enzymatic | |
| Superoxide dismutase (SOD) | $2\ O_2^{\cdot -} + 2\ H^+ \rightarrow O_2 + H_2O_2$ |
| Ascorbate peroxidase (APX) | $H_2O_2 + 2$ Asc $\rightarrow 2\ H_2O + 2$ MDHA |
| Monodehydroascorbate reductase (MDHAR) | $2$ MDHA$^{\cdot}$ + NADPH + $H^+ \rightarrow 2$ Asc + $NADP^+$ |
| Dehydroascorbate reductase (DHAR) | DHA + 2 GSH → Asc + GSSG |
| Glutathione reductase (GR) | GSSG + NADPH + $H^+ \rightarrow 2$ GSH + $NADP^+$ |
| Catalase (CAT) | $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$ |
| Glutathione peroxidase (GPX) | $H_2O_2 + 2$ GSH $\rightarrow 2\ H_2O +$ GSSG |
| Chemical | |
| Glutathione (GSH) | $H_2O_2 + 2$ GSH $\rightarrow 2\ H_2O +$ GSSG |
| | GSH + OH$^{\cdot} \rightarrow H_2O +$ GS$^{\cdot}$ |
| Ascorbate (Asc) | Asc + $H_2O_2 \rightarrow 2\ H_2O +$ DHA |
| α-tocopherol (α-toc) | α-toc + ROO$^{\cdot} \rightarrow$ α-toc$^{\cdot}$ + ROOH |
| Carotenoids e.g. β-carotene (β-car) | β-car + ROO$^{\cdot} \rightarrow$ β-car$^{\cdot}$ + ROOH |
| | β-car + $^3$Chl$^*$ → β-car$^*$ + $^1$Chl |
| Flavonoids (Flav) | Flav + $^3$Chl$^*$ → Flav$^*$ + $^1$Chl |
| | Flav ROO$^{\cdot} \rightarrow$ Flav$^{\cdot}$ + ROOH |
| | Flav + OH$^{\cdot} \rightarrow$ Flav$^{\cdot}$ + $H_2O$ |
| | Flav + $O_2^{\cdot -} \rightarrow$ Flav$^{\cdot}$ + $H_2O_2$ |

APX= Ascorbate Peroxidase, Asc = Ascorbate, CAT = Catalase, $^1$Chl = Chlorophyll, $^3$Chl$^*$ = triplet state chlorophyll, DHA = Dehydroascorbate, DHAR = Dehydroascorbate reductase, Flav = Flavonoid, GPX = Glutathione Peroxidase, GR = Glutathione Reductase, GSH = Reduced Glutathione, GSSG = oxidised glutathione, MDHA$^{\cdot}$ = Monodehydroascorbate reductase radical, MDHAR = Monodehydroascorbate reductase, SOD = Superoxide dismutase, β-car = β-carotene, α-toc = α-tocopherol. ROO$^{\cdot}$ = peroxyl radical, OH$^{\cdot}$ = hydroxyl radical, $O_2^{\cdot -}$ = Superoxide

Enzymatic antioxidants convert reactive species such as $O_2^{\cdot -}$ to less harmful hydrogen peroxide ($H_2O_2$) and then to water, whilst many chemical antioxidants acts by donating an electron to the reactive species and becoming a stable radical, or chelate metallic ions to prevent them participating in Fenton reactions and related mechanisms (Figure 1.17); (Valko et al., 2005).

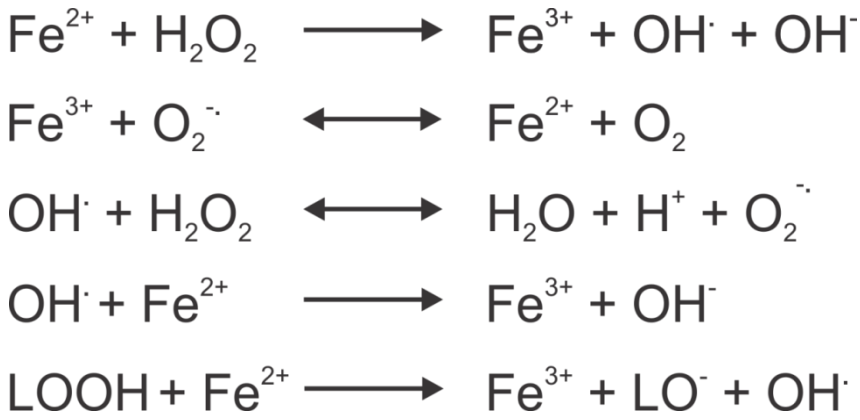
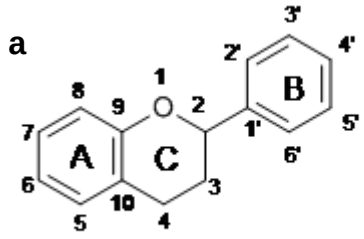



**Figure 2.17.** Potential ROS generation mechanisms of transition metals (adapted from (Valko et al., 2005)).

In addition, chemical antioxidants help the enzymatic systems deal with the burden of ROS and have been shown to accumulate under long term stress conditions where the enzymatic antioxidant system becomes slowly overwhelmed as reviewed in Fini et al., (2011). Environmental conditions known to perturb metabolism such as salinity, light stress, and poor nutrition have shown an increase in the concentration and activity of both enzymatic and non-enzymatic antioxidants (Agati and Tattini, 2010, Potters et al., 2009, Hernandez et al., 2004).

### *1.3.3.1 Flavonoid structure*

One group of non-enzymatic antioxidant compounds that plants produce are the flavonoids. These are a large family of polyphenols characterised by two aromatic (C6) rings linked together by a three carbon chain as illustrated in Figure 1.18a. Variations in the site and degree of aglycone oxidation break the flavonoids into their various sub-class or family (anthocyanin, aurone, chalcone, coumarin, dihydrochalcone, dihydroflavonol, flavanone, flavone, flavonol, flavan-3,4-diol, flavan-3-ol, isoflavone) as shown in Figure 1.18b (Crozier et al., 2009).

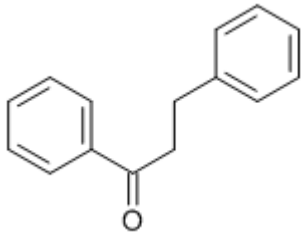



Anthocyanin

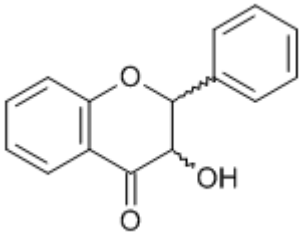
Aurone

Chalcone

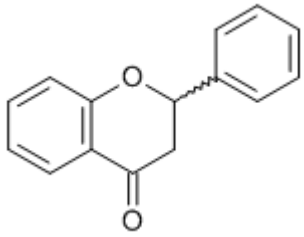
Coumarin



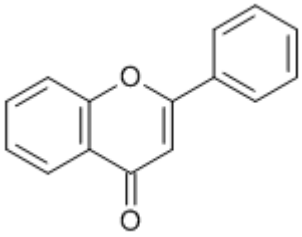

Dihydrochalcone



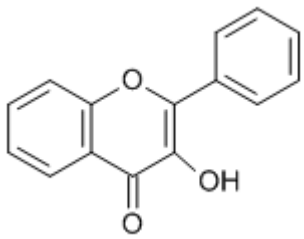

Dihydroflavonol



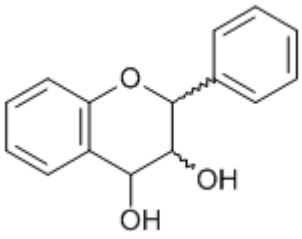

Flavanone



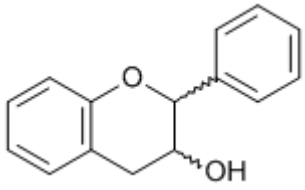

Flavone



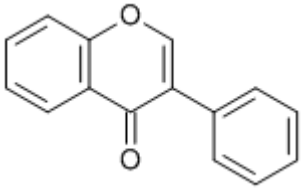

Flavonol,




Flavan-3,4-diol,




Flavan-3-ol




Isoflavone

**Figure 2.18.** (a) The primary flavonoid skeleton, (b) flavonoid families.

As well as oxidation of the aglycone, substitution of the hydrogen ions present on the basic aglycone commonly by hydroxyls or less commonly by methoxy groups ($OCH_3$) can also occur (Valant-Vetschera et al., 2006). This variation in substituent location and number determine whether the compound is for example, the flavonol quercetin, isorhamnetin or nobiletin (Figure 1.19).

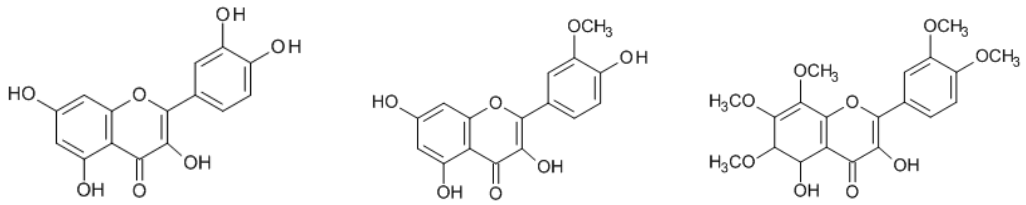



**Figure 2.19.** Variation in substituents to yield various flavonol aglycones.

*In planta* though, flavonoids are most commonly found as glycosides. Here, the presence of aglycone hydroxyl groups and attached sugars improve compound hydrophilicity, whilst other substituents such as methyl and isopentyl groups make the compound more hydrophobic. As such, the combination of individual substituents can have a large effect on whether the compound partitions in the lipid or water phase. Flavonols, the family which CR flavonoids belong, can conjugate at the 5, 7 3', 4' and 5' carbons (Crozier et al., 2009). However, apart from OH and $OCH_3$ groups most flavonol substituents attach via the 3 hydroxyl to become 3-O-glycosides (Williams and Markham, 2006).

### *1.3.3.2 Role of flavonoids in Planta*

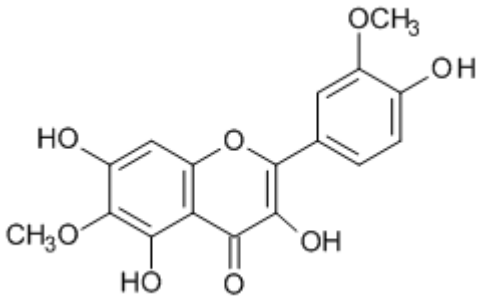
Originally, flavonoids and more specifically flavonols were proposed to protect plants from UV-B light damage by acting as a sunscreen. This was because production was noted to increase in response to UV exposure (Burchard et al., 2000) However, it has since been shown that the flavonoid precursors, hydroxycinnamate acids and their derivatives, have better UV absorption than the flavonoids (Tattini et al., 2004). Further to this, recent evidence

has shown that flavonoid production is induced by a number of environmental factors including salinity, drought, and excess light (Agati et al., 2011a, Hernandez et al., 2004). All the above factors cause stress to the plant and result in ROS production. Hence, it appears that flavonoids are not being employed as sunscreens, but rather as antioxidant compounds. Agati et al., (2007) was able to show that flavonoids are co-located with chloroplasts and proposed that in this location their purpose is to scavenge superoxide.

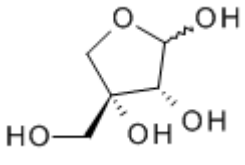
## 1.4 *C. rossii* novel flavonoid - pharmaceutical potential

As discussed earlier in section 4.6 of this review, flavonoids, and inhibitors of HMG-CoA reductase have significant health benefits associated with their consumption. These benefits relate to a plethora of diseases including atherosclerosis and diabetes, as well as stroke, cancer, Parkinson's and Alzheimer's disease (Liao and Laufs, 2005, Manach et al., 2005, Erdman et al., 2007, Aquilano et al., 2008, Grassi et al., 2009, Paraskevas et al., 2007). Some of the strongest evidence for benefit to the disease process is in the treatment of lipid disorders and hyperglycaemia due to type 2 diabetes. These activities are related to the flavonoid and statins ability to both inhibit cholesterol synthesis, as well as restore insulin signalling, endothelial health and vascular function.

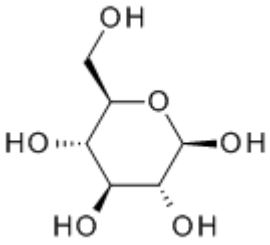
Pharmacologically, it is the flavonoids produced by CR which are of interest. This is because investigations recently conducted at the University of Tasmania's School of Pharmacy have shown the presence of unusual and novel flavonoid compounds in its leaves. These flavonoids include the uncommon aglycone spinacetin, with several substituents including glucose, the uncommon sugar apiose, ferulic acid, as well as the known HMG-CoA reductase inhibitor, HMG, attached in various combinations (Jager, 2009, Renggli, 2010); (Figure 1.20).



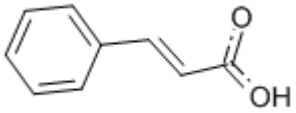

Spinacetin



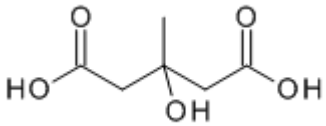

Apiose




Glucose




Ferulic Acid




3-Hydroxy-3-methylglutaric Acid

**Figure 2.20.** Substituents of the *C. rossii* flavonoid

Therefore, consumption of CR derived flavonoids may produce health benefits due to properties related to both their flavonoid and statin structure. This, combined with a history of the plant's fruit and leaves being used as a traditional food and medicine make the CR leaf extracts pharmacologically interesting and worthy of investigation.

## 1.5 PhD Aims

The aims of this thesis were to:

**1.** Determine the level of variation in metabolite production exhibited by wild grown plants in order to a) identify environmental factors influencing the level of metabolite production, and b) identify plant cultivars that possess characteristics worthy of further investigation (high metabolite producers, plant(s) with an unusual metabolic profile).

**2.** Determine the effects specific environmental factors have on CR metabolite production.

**3.** Elucidate the structure of the CR flavonoid metabolites.

**4.** Investigate whether CR leaf extract, and specifically the CR leaf flavonoids possess pharmacological activity.

# 2 Flavonoid and tannin production of Carpobrotus rossii is modulated by environmental conditions

## 2.1 Abstract

Dietary supplementation with plant-derived polyphenolic compounds such as the flavonoids epigallocatechin-3-gallate (EGCG), quercetin and resveratrol can result in a beneficial effect on degenerative disease processes through both radical scavenging and activation of cellular ion channels. Preliminary investigations have shown that extracts from the halophyte species *Carpobrotus rossii* (CR) have high in vitro antioxidant and in vivo low-density-lipoprotein-lowering activities. In this study, we investigated the environmental conditions responsible for inducing flavonoid production in CR in an attempt to maximise production of these compounds. Both field surveys and controlled glasshouse experiments were conducted. Flavonoid production appears to be related to conditions known to cause oxidative stress in plants such as exposure to excessive light, reduced water availability, and low soil potassium levels. Flavonoid production was minimal under salinity levels optimal for CR growth (around 50 mM NaCl) and increased dramatically at sub- and supra-optimal salinities. Flavonoids were clearly concentrated in metabolically active palisade mesophyll tissue rather than the spongy parenchyma. Non-optimal (outside 50 to 100 mM range) NaCl levels significantly increased flavonoid concentration on a per leaf basis. However, the reduction in biomass production at sub and supra optimal salinities diminished absolute flavonoid production per plant. As a result, saline conditions favouring optimal plant growth appear to be most suitable for maximising production of flavonoids in CR.

Parts of this Chapter have been published as the following journal articles

PIRIE, A., PARSONS, D., RENGGLI, J., NARKOWICZ, C., JACOBSON, G. A. & SHABALA, S. 2013. Modulation of flavonoid and tannin production of *Carpobrotus rossii* by environmental conditions. *Environmental and Experimental Botany,* 87**,** 19-31.

PIRIE, A., SHABALA, S., PARSONS, D., NARKOWICZ, C., JACOBSON, G. & RENGGLI, J. 2011. Ecophysiology of *Carpobrotus rossii* in Tasmania: Linking plant's antioxidant activity with a natural habitat. *Ecological Questions,* 14**,** 91-93.

## 2.2 Introduction

Diseases including cancer, diabetes and cardiovascular disease are the leading causes of death worldwide (WHO, 2008). There is considerable interest in disease aetiology and compounds that provide protection against the development of these diseases. Oxidative stress and generation of reactive oxygen (ROS) and nitrogen (RNS) species have been implicated in many disease processes due to their potential to create RNA damage, induce lipid peroxidation, modify phospholipid and lipoproteins to immune stimulating forms as well as alter membrane properties and cellular signalling (Kong and Lin, 2010, Münzel et al., 2010). Each of these processes can alter cellular function and impact on organism health. Additionally, body metabolites such as excessive plasma levels of low-density lipoproteins (LDL) and glucose have been associated with poor diabetic and cardiovascular disease prognosis (Boullier et al., 2006, Gautam and Banerjee, 2011).

Reactive species such as hydrogen peroxide and nitric oxide are also involved in signalling within normal cells and can play an important role in the body's natural defence mechanisms. Thus, the potential of ROS and RNS to direct damage, interfere and modulate cellular pathways during disease processes (Li and Shah, 2004) and how these processes can be ameliorated by antioxidants is of much interest.

An extract from the succulent halophyte *Carpobrotus rossii* has been shown to have both potent *in vitro* antioxidant activity, as well as *in vivo* LDL lowering activity (Geraghty, DP, personal communication, Geraghty et al., 2011). These properties make the extract of interest to both cardiovascular and general degenerative disease prevention research (diabetes, cardiovascular, cancer, Alzheimer's). Current evidence suggests that these activities are due to the presence of novel flavonoid and tannin compounds (unpublished data).

Like animals, plants produce ROS (such as superoxide and $H_2O_2$) for use as secondary messengers (Noctor and Foyer, 1998, Scandalios, 1993). Traditionally these compounds were considered to be toxic by-products of aerobic metabolism. However it has become apparent that plants actively produce ROS as signalling molecules to control numerous physiological processes including defence response and cell death, cross tolerance, gravitropism, stomatal aperture, cell expansion and polar growth, hormone action, as well as leaf and flower development (reviewed in Shabala and Munns (2012)). The signalling role of ROS appears to be highly tissue-specific, for example, occurs only in actively growing root cells, but not mature ones (Demidchik et al., 2007).

Under stress conditions, however, ROS production in plants may increase to the point where physiological damage can occur. This includes a range of biochemical alterations such as lipid peroxidation in cellular membranes, DNA damage, protein denaturation, carbohydrate oxidation, pigment breakdown and an impairment of enzymatic activity (Blokhina et al., 2003, Noctor and Foyer, 1998, Scandalios, 1993), as well as direct regulation of cation ($Ca^{2+}$, $K^+$) permeable ion channels at the cellular membranes (Demidchik and Maathuis, 2007, Demidchik et al., 2003, Demidchik et al., 2007, Demidchik et al., 2010, Foreman et al., 2003, Zepeda-Jazo et al., 2011). The latter has direct impact on intracellular $K^+$ homeostasis and, ultimately, the cell fate by controlling activities of numerous proteases and endonucleases involved in programmed cell death in plant cells (Demidchik et al., 2010, Shabala, 2009). Thus, plants face the extremely challenging dilemma of having to delicately balance ROS required for signalling with scavenging to avoid their deleterious effects (Miller et al., 2008).

ROS in plants production can be initiated by a suite of stresses such as inadequate nutrition (N, P, K), the presence of toxic ions ($Na^+$, $Al^{3+}$, $Cu^{2+}$, $Se^{2-}$, $Cd^{2+}$), excessive levels of solar radiation, exposure to ultraviolet light or moisture stress (Ahmad et al., 2008). The main lines of defence that protect cells against oxidative injury are various antioxidant components that include a number of enzymes and low molecular weight compounds capable of quenching ROS without themselves undergoing conversion to a destructive radical (Scandalios, 1993). Both enzymatic and non-enzymatic components contribute to detoxification of ROS species (Mittler et al., 2004).

Among the latter group are flavonoids, a group of compounds highly conserved across plant families (Pollastri and Tattini, 2011) and originally proposed to serve as a UV protective "sunscreen" for the plant (Burchard et al., 2000). This view of their role has changed though, with reporting of their increased production under non-light-induced conditions of oxidative stress such as salinity (Melgar et al., 2009), drought (Sangtarash et al., 2009) or temperature extremes (Bilger et al., 2007), in addition to grazing and pathogen attack as reviewed by Treutter (2005). It is proposed, therefore, that being co-located with photosynthetic machinery, flavonoids are responsible for scavenging both singlet oxygen and superoxide at their source, thereby protecting the chloroplast and surrounding tissue from oxidative damage (Agati et al., 2007).

Tannins are another group of non-enzymatic antioxidant compounds also accumulated during times of oxidative stress such as drought and low temperature (Tharayil et al., 2011). They have also been shown to play a role in the scavenging of ROS as well as protection of photosynthetic machinery (Gould et al., 2002).

Plant tannins, such as those found in CR are of less interest pharmacologically due to their use by plants as anti-herbivory compounds, demonstrated ability to bind and decrease protein digestion (Barbehenn and Peter Constabel, 2011) as well as data showing that their excessive consumption can be deleterious to human health as is the case with many other plant phenolics (Galati and O'Brien, 2004). In addition to this, preliminary mass spectrometry (MS) and nuclear magnetic resonance (NMR) data indicate that structurally the flavonoids of CR are of most interest pharmacologically due to their proposed novel structure.

As reactive species generation increases under adverse conditions it is expected that exposure to challenging environmental situations will increase the production of anti-oxidant compounds in CR**.** This study aimed to identify the major environmental conditions responsible for inducing CR flavonoid production and allow optimisation of the conditions needed to maximise production of these pharmaceutically interesting compounds. Two major questions were asked: (1) which environmental factor(s) will have the largest effect on flavonoid and tannin production in CR, and (2) how can this knowledge be used to optimise their production under controlled conditions?

## 2.3 Material and Methods

### *2.3.1 Sampling methods and location*

A permit to allow plant material collection was obtained from the Tasmanian state government. Coastal reserves around the state were then surveyed for the presence of CR by A. Pirie, C. Narkowicz and J. Renggli. When plant material was found in suitable quantities (i.e. multiple plants greater than 1 metre in diameter), soil (0-15, 15-30, 30-45 cm) and plant samples were taken. The GPS coordinates, site description and amount of shelter plants had (High = 3, Medium = 2, Low = 1) was recorded. Chlorophyll fluorescence characteristics were measured non-invasively on each plant.

### *2.3.2 Chlorophyll fluorescence*

Chlorophyll fluorescence characteristics (Fo, Fm, and Fv/Fm) were measured in the field using an Opti-Sciences OS-30P chlorophyll fluorometer (Opti-Sciences, New Hampshire, USA). Measurements were conducted on 15 minute dark-adapted samples by using leaf clips equipped with light shutters. For each plant, three laterals were selected. Leaf clips were placed on the first mature leaf at the growing tip and the next three mature leaves. On occasions when new laterals were growing close to the tip the next large main-branch leaf was chosen. During preliminary investigations some plants were found to have extremely small leaves and the leaf clip aperture was reduced to 4 mm by gluing black cardboard "washers" onto the leaf clips. This ensured a proper fit on the leaf and prevented erroneous readings from either excess light exciting the leaf chlorophyll or fluorometer radiation bypassing the leaf surface.

### 2.3.3 Soil Sampling

For each plant or group of plants (if less than 10 m apart), 500 g of soil was collected from 0-15, 15-30, and 30-45 cm depth. This was taken back to the laboratory, air dried, passed through a 2 mm screen (Endecotts, London, England) and then stored prior to analysis. Sub-samples were weighed into 50 ml centrifuge tubes, mixed 1:5 (w/v) with distilled water and shaken. $Na^+$ and $K^+$ levels were determined via flame photometry (PFP7, Jenway Pty Ltd, Essex, England), $Cl^-$ with an ion specific electrode (ISE) (TPS Ltd, Brisbane, Australia), pH using a pH probe (EZ DO PL 600, M.R.C Ltd, Holon, Israel) and electrical conductivity (EC) using an electrical conductivity meter (labCHEM-C, TPS Ltd, Brisbane, Australia).

### 2.3.4 Stomata density

The epidermis from two leaves per sample were removed, placed on a microscope slide and stomatal counts were conducted using a Leica DM 500 microscope (Leica Microsystems, Germany). Five locations per leaf were counted with density defined as the number of stomata within the 0.52 mm diameter field of view.

### 2.3.5 Leaf sap nutrient analysis and osmolarity

Leaf sap nutrient analysis and osmolarity were conducted as described by Cuin et al., (2010). In brief, freeze-thawed leaf samples were crushed to extract the leaf sap. The supernatant then transferred to a second tube, frozen and stored until thawing for analysis. Leaf osmolarity was assessed using a VAPRO vapour pressure osmometer 5520 (Wescor Inc, Logan, Utah, USA). Sap samples were then diluted 100 times and measured for $Na^+$ and $K^+$ concentration using flame photometry (as before).

### 2.3.6 Flavonoid concentration by HPLC

Leaves were randomly selected, washed under deionised water, dried and ground with a mortar and pestle. The ground plant material was then placed in an Eppendorf tube and centrifuged at 15000 g for 5 minutes. Following this the supernatant was transferred to a HPLC sample vial for analysis.

HPLC analysis was conducted using a Varian Pro-Star 230 solvent delivery system coupled with a Varian 410 auto sampler and Varian 335 diode array detector (DAD); operation of these modules was achieved using Varian Star software (all HPLC modules and software supplied by Varian Inc, Melbourne, Australia). The column was an Apollo 5 µm x 250 mm C-18 (Grace, Deerfield, USA) fitted with a guard column (Phenomenex, Security Guard®, Torrance, USA). Eluent profiles used were those optimised for CR by Renggli (2010) and summarised in Table 2.1. Injection volume was 15 µl with the spectrum between 200 nm and 450 nm scanned and wavelengths of 254 nm and 350 nm monitored.

**Table 2.1.** HPLC conditions for flavonoid and tannin detection. Solvent A was 2% acetic acid in $H_2O$; Solvent B was 2% acetic acid in methanol. Dual wavelength (254 nm, 350 nm) monitoring and a C18 analytical column were used. A five minute equilibration time occurred between runs.

| Time (min) | Flow rate (mL\minute) | % solvent A | % solvent B |
|---|---|---|---|
| initial | 0.8 | 60 | 40 |
| 5.00 | 0.8 | 45 | 55 |
| 5.01 | 0.6 | 45 | 55 |
| 20.00 | 0.6 | 45 | 55 |
| 25.00 | 0.6 | 45 | 55 |
| 25.01 | 0.8 | 45 | 55 |
| 30.00 | 0.8 | 0 | 100 |

For each sample, area under the curve (AUC) integration was performed for the 350 nm wavelengths. Peaks showing simultaneous 254 nm and 350 nm absorbance with a retention time of less than 9 minutes were assigned as tannins (or flavonoids of non-pharmaceutical interest), whilst peaks from 9 minutes onwards were deemed to be flavonoids. This assignment was based on previous mass spectroscopy results.

### *2.3.7 Antioxidant activity assessment*

Antioxidant activity was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay reviewed by MacDonald-Wicks et al., (2006). The method was slightly modified to enable analysis by a microplate reader and absorbance was measured at 490 nm instead of a wavelength between 515 nm and 528 nm. Briefly, DPPH (Sigma-Aldrich, St Louis, United States of America) was diluted in methanol until a 250 µl aliquot placed in a flat bottomed 96 well microplate (Greiner Bio-one, Monroe, United States of America) gave an absorbance at 490nm of 1.50 ± 0.05 units. A 12 µl aliquot of gallic acid (either 100, 50,

25, 12.5, 6.25, 3.125 μg/ml) or leaf sap (undiluted, diluted 1:2, 1:4, 1:8, 1:16, 1:32) was added to sequential microplate wells, followed by 250 μl of DPPH solution. The plate was covered with foil and stored in the dark for 30 min. After this time the absorbance at 490 nm was measured on a microplate reader (Model 680, Bio Rad Laboratories Pty Ltd, Sydney, Australia) and extract antioxidant activity converted to gallic acid equivalents.

### *2.3.8 Glasshouse experiment*

Concentration-dependent responses to salinity were studied at the University of Tasmania's glasshouse complex (Hobart, Tasmania, Australia). The average day/night temperatures were 23/17$^{o}$C; relative air humidity 55 to 70%; natural day length of approximately 15 hours. Cuttings from donor clones were taken and 5 cuttings were planted in each 14 × 200 mm pot. These were then grown under ambient conditions for 2 weeks until they had established and then treatments were applied. Treatments consisted of distilled water with NaCl added to make concentrations of 0, 50, 100, 200, 300, 400 and 500 mM respectively, with two pots used per treatment. All plants were monitored and watered to saturation every 2-3 days until the completion of the trial at 21 days.

After 21 days, soil was washed out of the pots and plants were individually assessed for root, stem and leaf length; fresh weight; and the number of new leaves produced. The two newest mature leaves from each plant were taken for more in depth analysis including leaf weight, length, width, and height before the photosynthetically active outer leaf was separated from its vacuolated inner core. These segregated sections were frozen and then analysed for osmolarity, antioxidant activity (DPPH), flavonoid content (HPLC), $Na^{+}$ and $K^{+}$ content (as described earlier). The remaining plant was separated into roots and stems then oven dried (60 $^{o}$C, 48hrs) prior to assessment of dry matter production.

### *2.3.9 Statistical Analysis*

Multiple regressions were performed in Minitab 15 (Minitab Inc, Sydney, Australia) using site modelled climatic variables obtained from the SILO website (QCCE, 2011) and as individual soil test and plant parameter results.

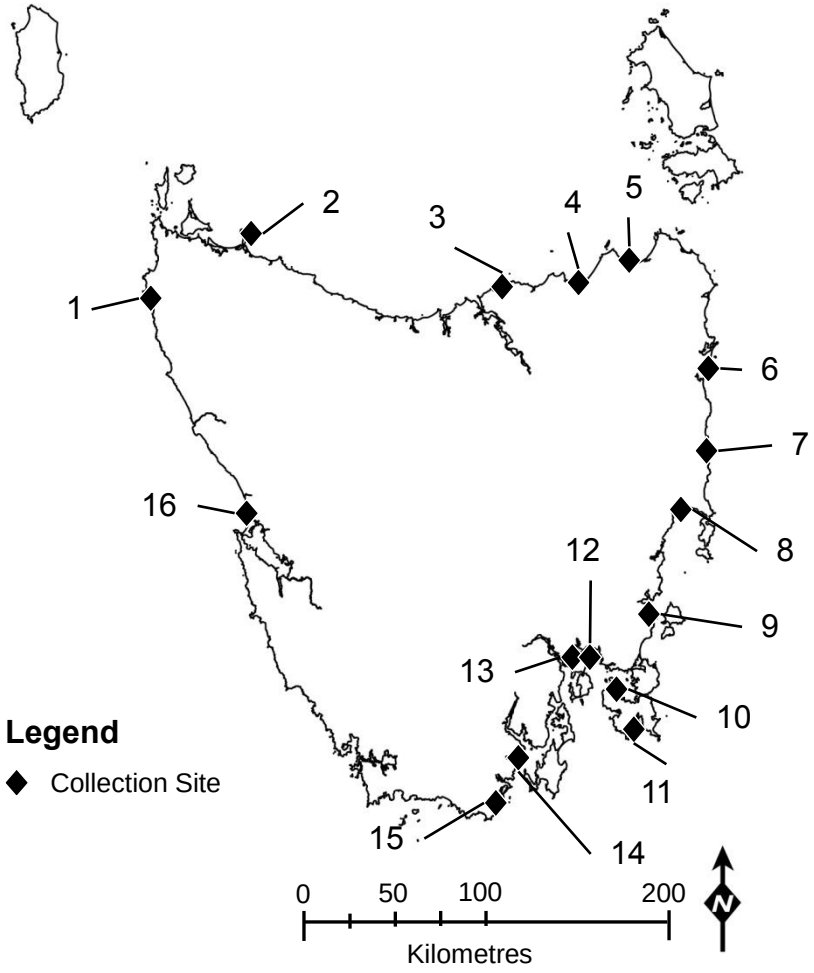
The means for each plant were subjected to a hierarchical cluster analysis in Minitab 15, using the variables flavonoid and tannin concentration (centroid linking method for measuring Euclidean distance, after transforming using Z-scores to standardise across variables measured in different units), to generate a dendrogram illustrating relationships between plants. Differences between the treatment groups in glasshouse trail were assessed using the statistical program JMP8 (SAS Institute Inc., North Carolina, United States of America) using an ANOVA analysis. Differences between treatments and the control (50mM NaCl) were assessed using Dunnett's method.

## 2.4 Results

### *2.4.1 Field Survey*

In total, 77 samples from a total of 16 locations were collected along the coastal line of Tasmania (Figure 2.1, Appendix Chapter 2, Appendix Table 2.1).

Soil samples showed a high degree of variability for all parameters measured. The 0-15 cm depth pH values ranged from 4.3 to 8.1, EC from 10 to 2760 μS/cm, soil $K^+$ from 0.06 to 0.70 mM, and soil $Na^+$ from 0.13 to 23.6 mM. Further investigation of the soil results showed that the high EC, $K^+$ and $Na^+$ values were from the Stanley (STAN 1-5) and Southport 1 (SP 1) sites which were composed of a clay/colluvium soil rather than the podzol/beach sand present elsewhere. Because of this, all Stanley and the Southport 1 results were excluded from further statistical analysis. The exclusion of these samples resulted in 0-15 cm depth soil variables of the remaining dataset ranging from pH 4.3 to 8.1, EC from 10 to 237 μS/cm; soil $K^+$ from 0.06 to 0.22 mM and soil $Na^+$ from 0.13 to 2.46 Mm (Appendix Chapter 2, Appendix tables 2.2 and 2.3).




| Number | Location | Sample Code |
|---|---|---|
| 1 | Arthur River | AR 1-5 |
| 2 | Stanley Nut | STAN 1-5 |
| 3 | Beechford | BFD 1-5 |
| 4 | Bridport | BPT 1-2 |
| 5 | Tomahawk | THK 1-5 |
| 6 | St Helens | STH 1-5 |
| 7 | Bicheno | BIC 1-5 |
| 8 | Dolphin Sands | DS 1-5 |
| 9 | West Shelly Beach | WSB 1-5 |
| 10 | Sloping Main Beach | SLO 1-5 |
| 11 | Safety Cove Beach | SCB 1-4 |
| 12 | Seven Mile Beach | 7MB 1-5 |
| 13 | Southport | SP1-5 |
| 14 | Belrieve Beach | BEL 1-8 |
| 15 | Recerche Bay | RB 1-5 |
| 16 | Strahan Ocean Beach | SOB 1-5 |

**Figure 2.1.** Field survey locations, showing sites of soil and plant collection.

The plant material showed huge variation in the leaf concentration of both flavonoid and tannin metabolites (Figure 2.2). Flavonoid production of the highest producing plant (BFD 5) was 27 times that of the lowest producing one (WSB 1) and a similar scenario was seen in tannin production where there was a difference of 22 times between the highest (SP 5) and lowest (BEL 1) producers. Large differences were found both between sites and also between specimens collected from the same site. As an example of intra-site variability, the highest flavonoid producing plant at Beechford (BFD 5) outperformed the lowest producing one (BFD 4) approximately five fold. Thus, either genetic variability, or subtle variation in growing conditions within each site, plays a key role in antioxidant production.

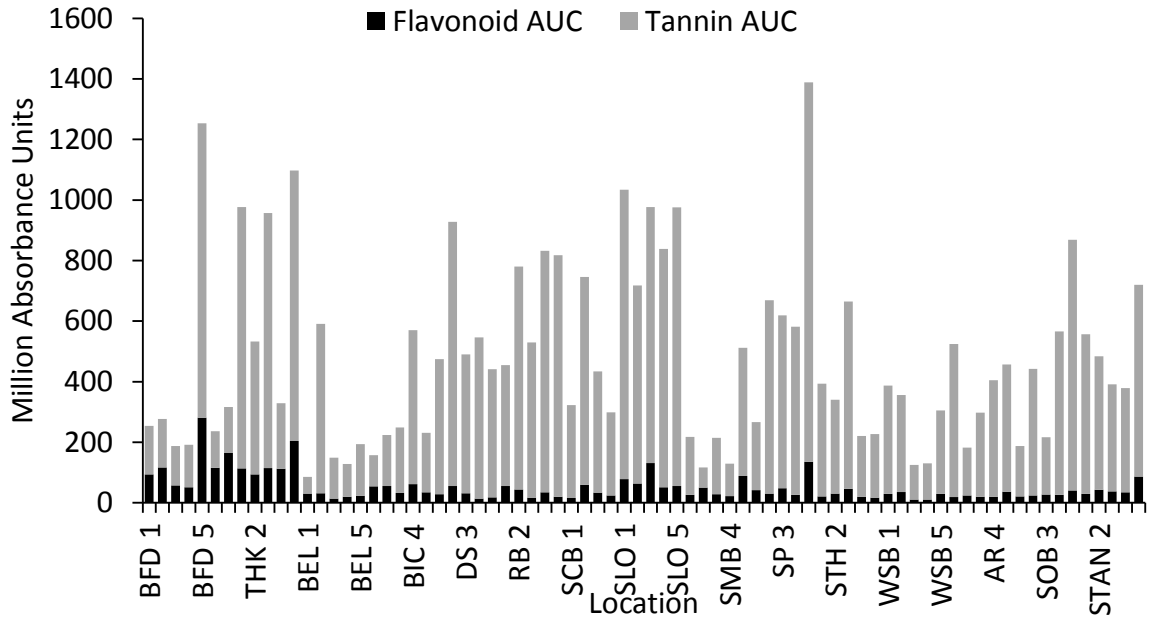



**Figure 2.2.** Individual plant flavonoid and tannin production highlighting the intra and inter-site variation in metabolite production between plants. AUC – Area under the curve absorbance units calculated from 350nm chromatogram.

In order to unravel the parameters underpinning differences in antioxidant production between the plants, both cluster analysis and multiple linear regression modelling was used. Cluster analysis of the field-collected plants revealed there to be a small group of high antioxidant producing plants, and a larger general population of plants with lower tannin and flavonoid production; these have been defined as Cluster 1 and Cluster 2, accordingly (Figure 2.3).

Comparison of means using Student's t-test showed there to be significant differences between the two clusters for a range of environmental, soil and plant physiological characteristics (Figure 2.4a-f). Environmentally, higher plant flavonoid and tannin production was associated with lower soil EC (Figure 2.4b) and lower soil $Na^+$ and $K^+$ content (Figure 2.4c). Plants with higher antioxidant activity had higher stomata density (Figure 2.4d) and leaf sap osmolality (Figure 2.4e) and showed a reduced number of efficiently operating photosystem II centres (Fm value; Figure 2.4f). Higher producing plants were also more exposed than the general population (lower shelter index; Figure 2.4a).

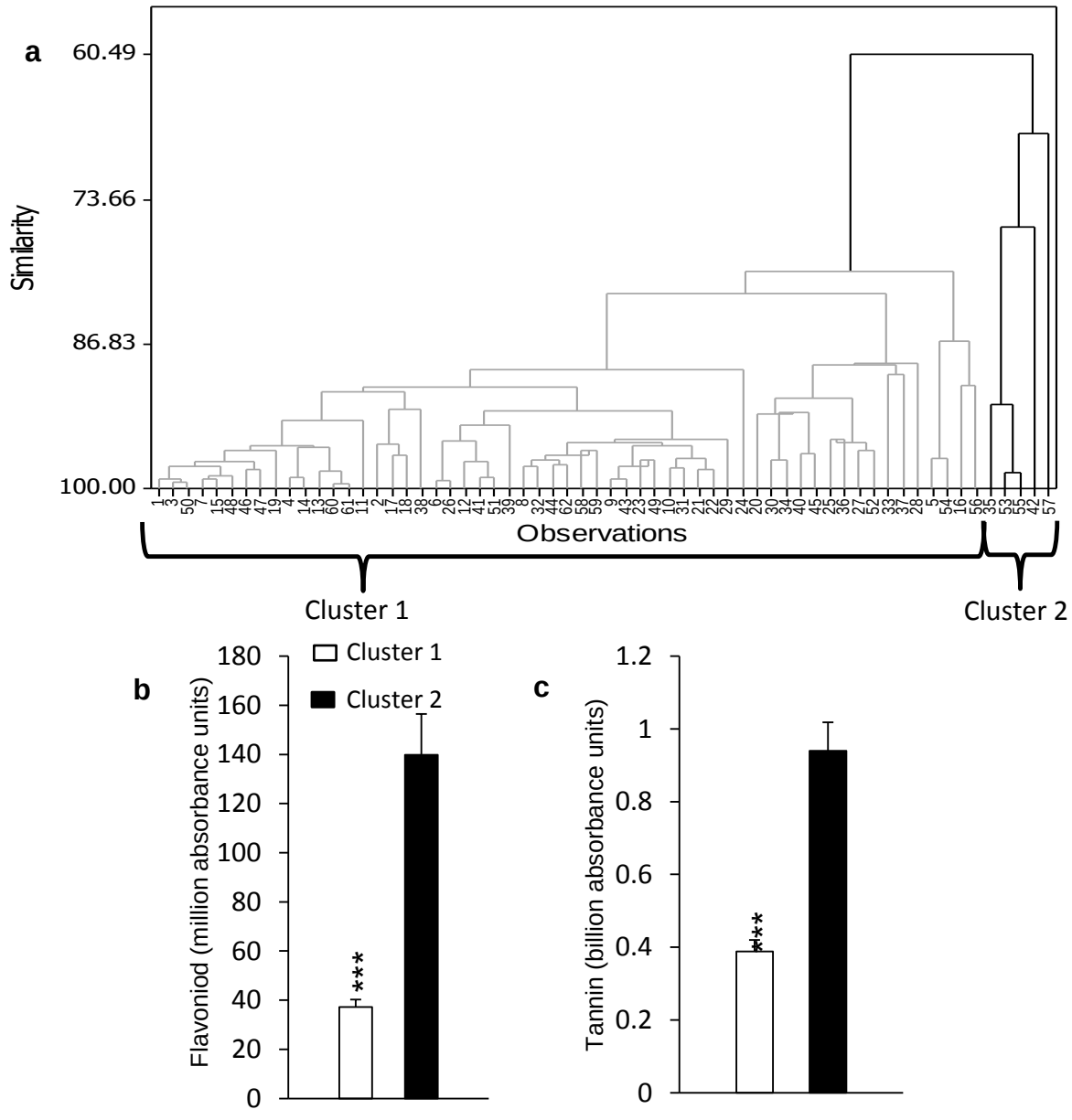



**Figure 2.3.** (a) Dendogram of cluster analysis showing a small group of high flavonoid and tannin producing plants with a larger population of low producing plants. Means of clusters 1 and 2 for (b) flavonoid concentration, (c) tannin concentration, Data expressed as Mean ± SEM, ***= P<0.001 Cluster 1 n = 72, Cluster 2 n = 5.

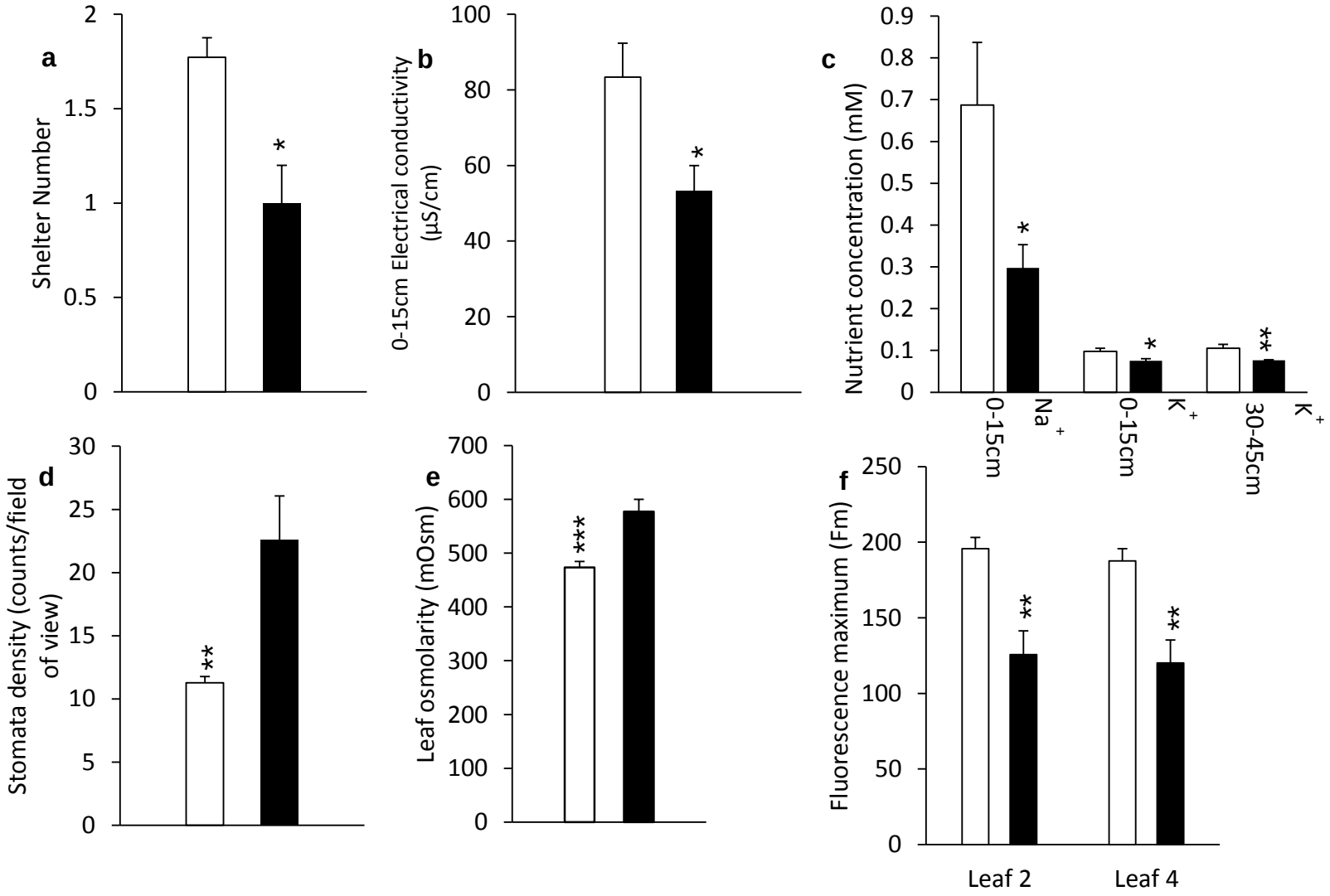



**Figure 2.4.** Means of clusters 1 (□) and 2 (■ ) for (a) shelter index, (b) soil electrical conductivity, (c) soil Na and K concentration, (d) stomatal count, (e) osmolarity, and (f) leaf fluorescence characteristic. Data expressed as Mean ± SEM *, **, *** indicate statistically significant response when compared to 50 mM NaCl ($p<0.05$, 0.01, 0.001 respectively).

Single factor regression models were able to effectively predict flavonoid, tannin and overall antioxidant activity (gallic acid equivalents) of the leaf sap with adjusted $R^2$ of 36.4, 19.3 and 29.0, respectively (Appendix Chapter 2, Appendix Table 2.4). When variables were combined into multiple linear regression models they were able to predict tannin, flavonoid and total antioxidant production with much improved accuracy. The best prediction models (highest accuracy without over-fitting as determined by Mallow's Cp analysis) for flavonoids and tannins contained 4 factors. A frequency analysis was performed on the 10 best 4-factor prediction models, which showed that flavonoid production was best predicted by stomata density and efficiency of PSII (100% and 70% occurrence in models) and was also highly affected by soil potassium content (80% occurrence) (Table 2.2). A very similar observation was made for tannins, with both stomata counts and soil K being present in all the best regression models (Table 2.2); however, in contrast to the flavonoid models, daily evaporation (Evd) was also present in every model analysed (Table 2.2).

### *2.4.2 Biomass and flavonoid production under controlled conditions*

The negative correlation between polyphenolic antioxidant production (tannin and flavonoid) and soil Na content (Figure 2.4c) was unexpected and worth further investigation. As a result, a series of glasshouse experiments was undertaken where plants were grown using water containing NaCl from 0 to 500 mM (seawater level). As evident from Figure 2.5, CR exhibits a classical halophytic concentration-dependency response, with optimal growth observed at around 50 mM NaCl, and growth inhibition at both sub-optimal (< 50 mM) and above-optimal (> 100 mM) NaCl concentrations. This increase in biomass production at optimal salinity was driven by two separate factors. Firstly, the plant produced more leaves at this concentration; and secondly, these leaves were larger (longer and more succulent) than under non-optimal conditions leading to heavier more numerous leaves at the 50 mM concentration (Figure 2.6).

**Table 2.2.** Frequency (%) of prediction variable occurrence in the 10 best 4-factor prediction models for flavonoid, tannin and antioxidant production in *C. rossii* leaves.

| Variable Related To | Prediction Variable | Flavonoid | Tannin | Gallic Acid Equivalents |
|---|---|---|---|---|
| Plant | S | 100 | 100 | 20 |
| Plant | $F_{v/Fm}$ | 70 | 10 | 40 |
| Plant | Fm | 30 | 10 | 20 |
| Plant | O | 10 | 10 | 70 |
| Plant | Nap | 20 | 10 | 10 |
| Plant | Kp | 10 | 10 | - |
| Soil | Ks | 80 | 100 | 100 |
| Soil | Nas | - | 10 | - |
| Environment | Evd | 30 | 100 | 100 |
| Environment | $T_6$ | - | 10 | - |
| Environment | $Ev_6$ | - | 10 | - |
| Environment | Ra | 10 | - | 10 |
| Environment | $Ra_6$ | - | 10 | - |
| Environment | Wf | 30 | - | 10 |
| Environment | $W_3$ | 10 | 10 | 20 |

Ec = soil electrical conductivity (15-30cm), Evd= onsite daily evaporation, $Ev_6$= average evaporation occurring at the collection site in the 6 months preceding plant collection, Fm = maximum fluorescence yield, $F_{v/Fm}$= maximum quantum efficiency of Photosystem II , Kp = $K^+$ concentration of leaf sap, Ks= soil $K^+$ concentration (15-30cm), Nap = $Na^+$ concentration of leaf sap, Nas= soil $Na^+$ concentration (15-30cm), O = osmolarity of tissue sap, Ra = cumulative radiation in the fortnight prior to plant collection, $Ra_6$ = cumulative radiation occurring at the collection site in the 6 months preceding plant collection, S= Stomata density,$T_6$ = average daily maximum temperature experienced in 6 months preceding plant collection, Wf = cumulative rainfall in the fortnight prior to plant collection, $W_3$= cumulative rainfall in the 3 months prior to plant collection.

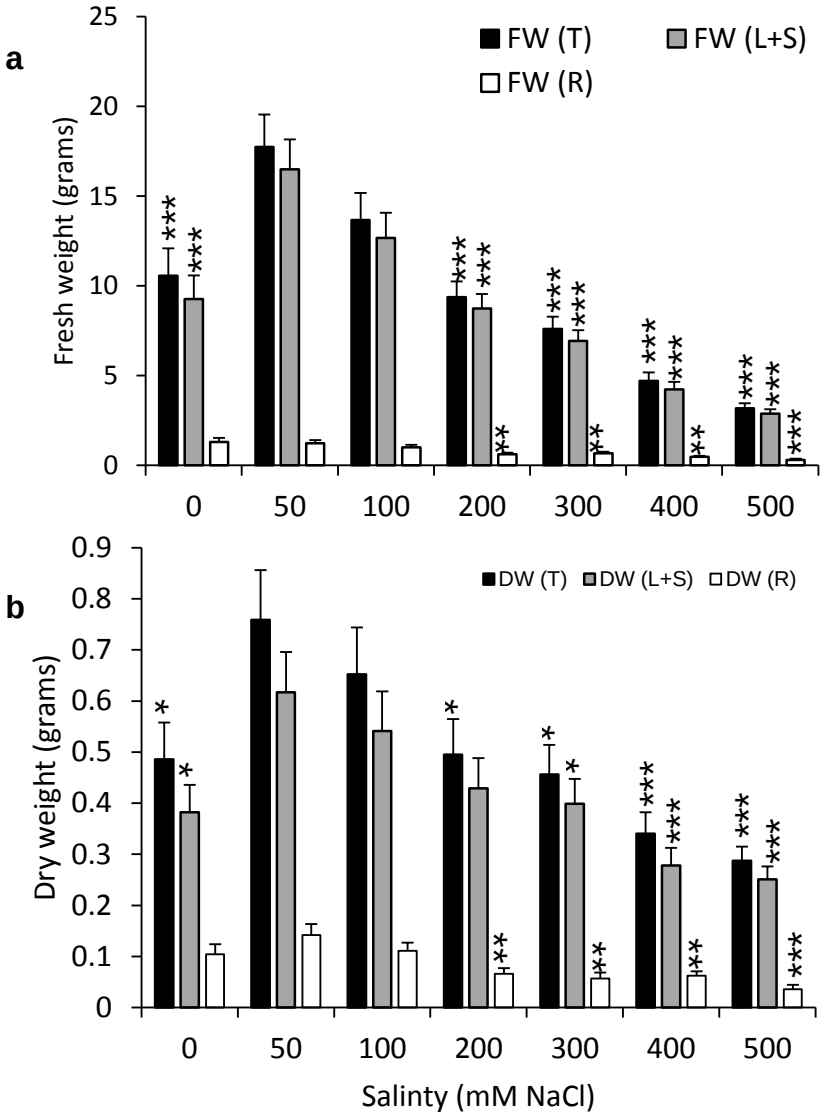



**Figure 2.5.** Effect of salt (NaCl) treatment on plant biomass production. (a) - fresh weigh (FW) of the entire plant (total, T), leaf and stem (L+S), and plant root (R). (b) - dry weight (DW) of the entire plant (total, T), leaf and stem (L+S), and plant root (R). Mean ± SE (n = 9 to 10). *, **, *** indicate statistically significant response when compared to 50 mM NaCl ($p<0.05$, 0.01, 0.001 respectively).

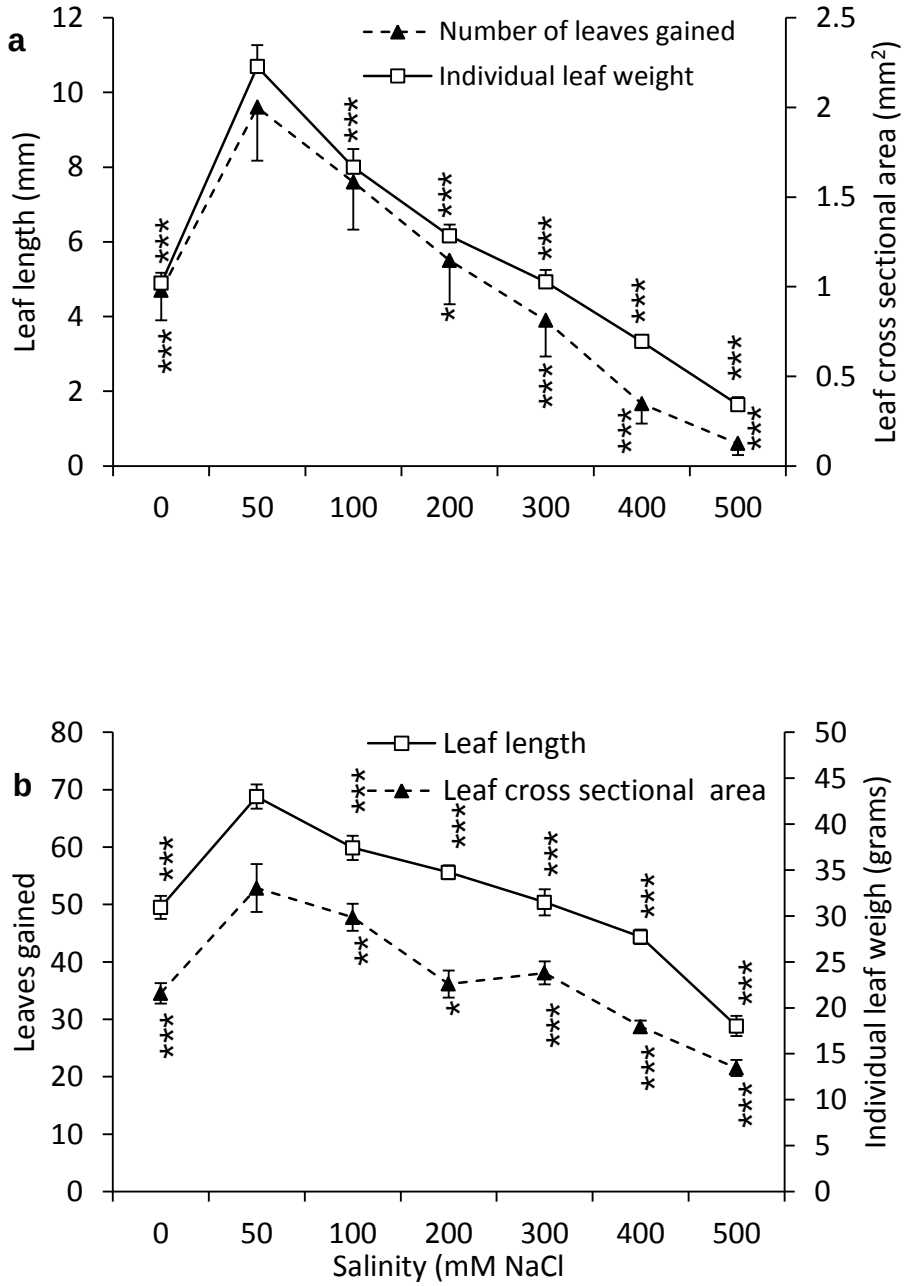



**Figure 2.6.** (a) - Effect of NaCl concentration on number of leaves gained (n = 9 to 10) and individual leaf weight (size) (n = 18 to 20). (b) - leaf length and cross-sectional area as affected by NaCl concentration. Mean ± SE (n = 18 to 20). *, **, *** indicate statistically significant response when compared to 50 mM NaCl ($p<0.05$, 0.01, 0.001 respectively).

Almost all whole-plant and individual leaf parameters measured were affected by the concentration of NaCl and showed significant correlation to both the salt treatment and to each other (Appendix Chapter 2, Appendix Tables 2.5 and 2.6).

Flavonoid production correlated inversely with individual leaf biomass (LW *vs* $F_{P,}$ Appendix Chapter 2, Appendix Table 2.6), with CR grown under non-optimal conditions of NaCl (ie. above or below ~50 mM NaCl) showing significant increases in both flavonoid production and total antioxidant activity (Figure 2.7a, 2.7b). Although a similar trend was observed in both palisade and spongy parenchyma tissues (Figure 2.7c), the amount of flavonoid and overall antioxidant status (as measured by gallic acid equivalents) produced in palisade mesophyll (a photosynthesising green tissue on a periphery of CR leaf) tissue was several orders of magnitude higher compared with spongy parenchyma (a highly vacuolated transparent white "flesh" tissue making the major bulk of the leaf; Figure 2.7d, Appendix Chapter 2, Appendix Figure 2.2).

Despite the fact that non-optimal saline conditions increased the flavonoid concentration of each individual leaf, the depression in biomass associated with these growing conditions i.e. fewer leaves produced and a reduction in their size meant that maximum flavonoid production per plant occurred under the optimal saline conditions of 50 mM NaCl (Figure 2.8a to 2.8c).

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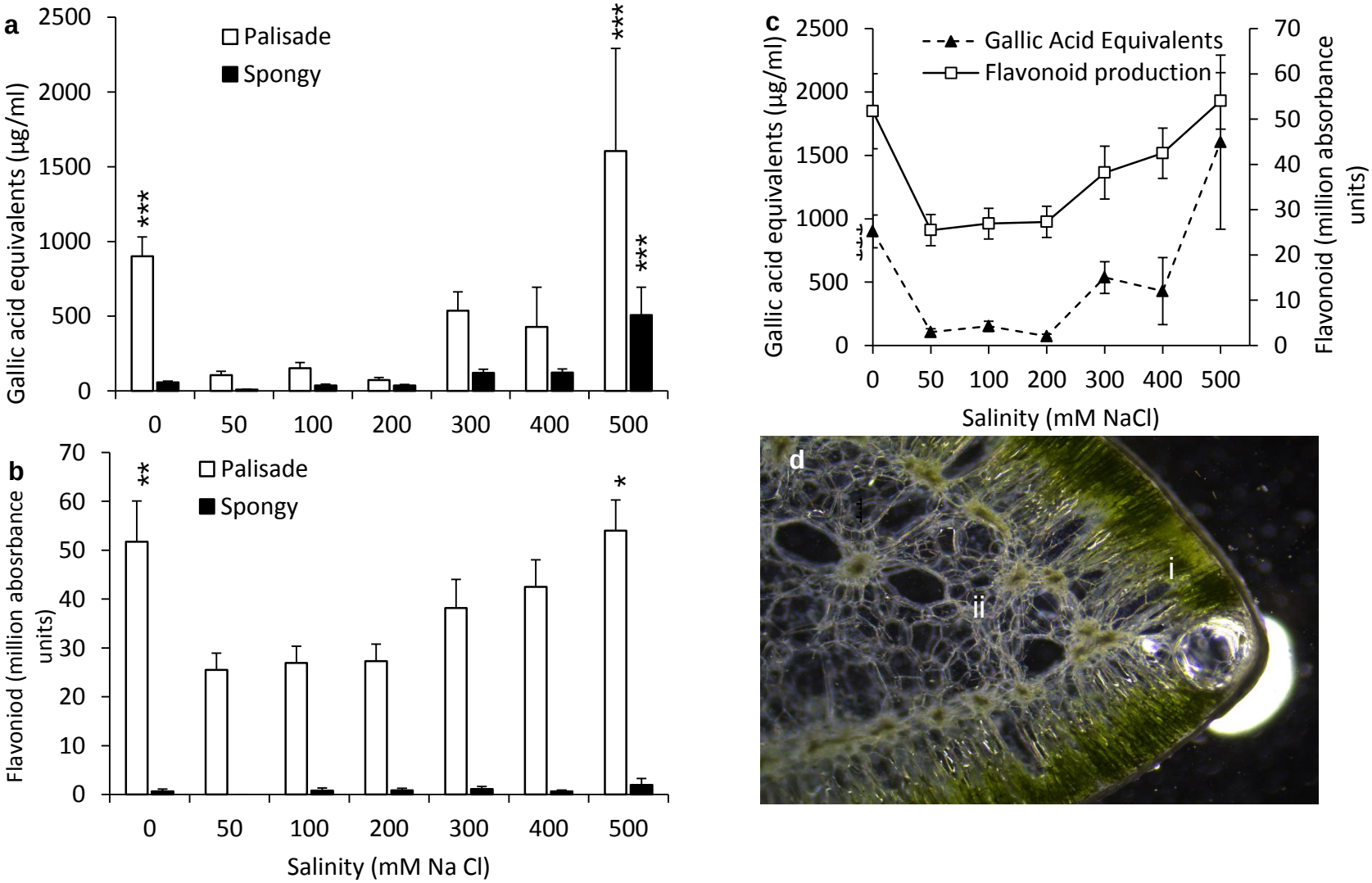


**Figure 2.7.** (a) Effect of NaCl concentration on the antioxidant activity of palisade and spongy leaf tissue. (b) Effect of NaCl concentration on the flavonoid production of palisade and sponge leaf tissue. (c) Concentration-dependency of NaCl effects on palisade antioxidant activity and flavonoid production, (d) Cross section of CR leaf showing the clear segregation between (i) palisade mesophyll and (ii) spongy parenchyma. Mean ± SE (n = 8 to 20). *, **, *** indicate statistically significant response when compared to 50 mM NaCl ($p<0.05$, 0.01, 0.001 respectively).

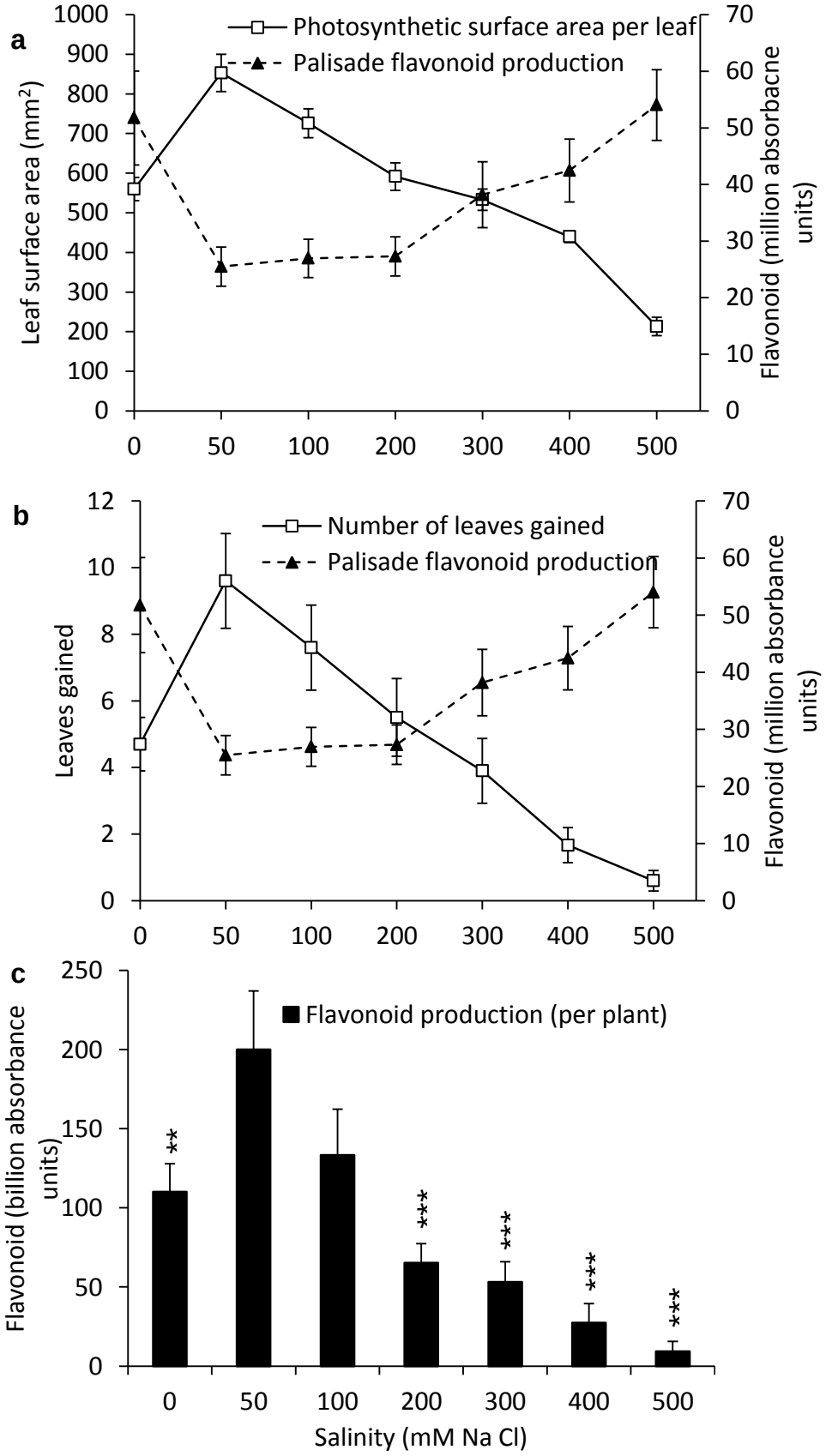


**Figure 2.8.** Determination of optimal flavonoid production conditions. (a) individual leaf photosynthetic area vs palisade flavonoid concentration. (b) Leaves gained vs individual leaf flavonoid production (calculated as flavonoid concentration x photosynthetic leaf area). (c) Total flavonoid production per plant (number of leaves gained x individual leaf flavonoid production). Mean ± SE (n = 9 to 10). *, **, *** indicate statistically significant response when compared to 50 mM NaCl ($p<0.05$, 0.01, 0.001 respectively).

### *2.4.3 Leaf ionic relations under saline conditions*

Plants were able to increase osmolarity to all parts of the leaf upon exposure to saline conditions of up to 400 mM NaCl (Figure 2.9a). Plotting the measured palisade and spongy sap osmolarity against osmolarity produced by the inorganic ions $Na^+$, $K^+$, $Cl^-$ (Figure 2.9b, 2.9c) revealed that the mechanism used to achieve this adjustment varied between the tissue types and magnitude of osmotic potential needed.

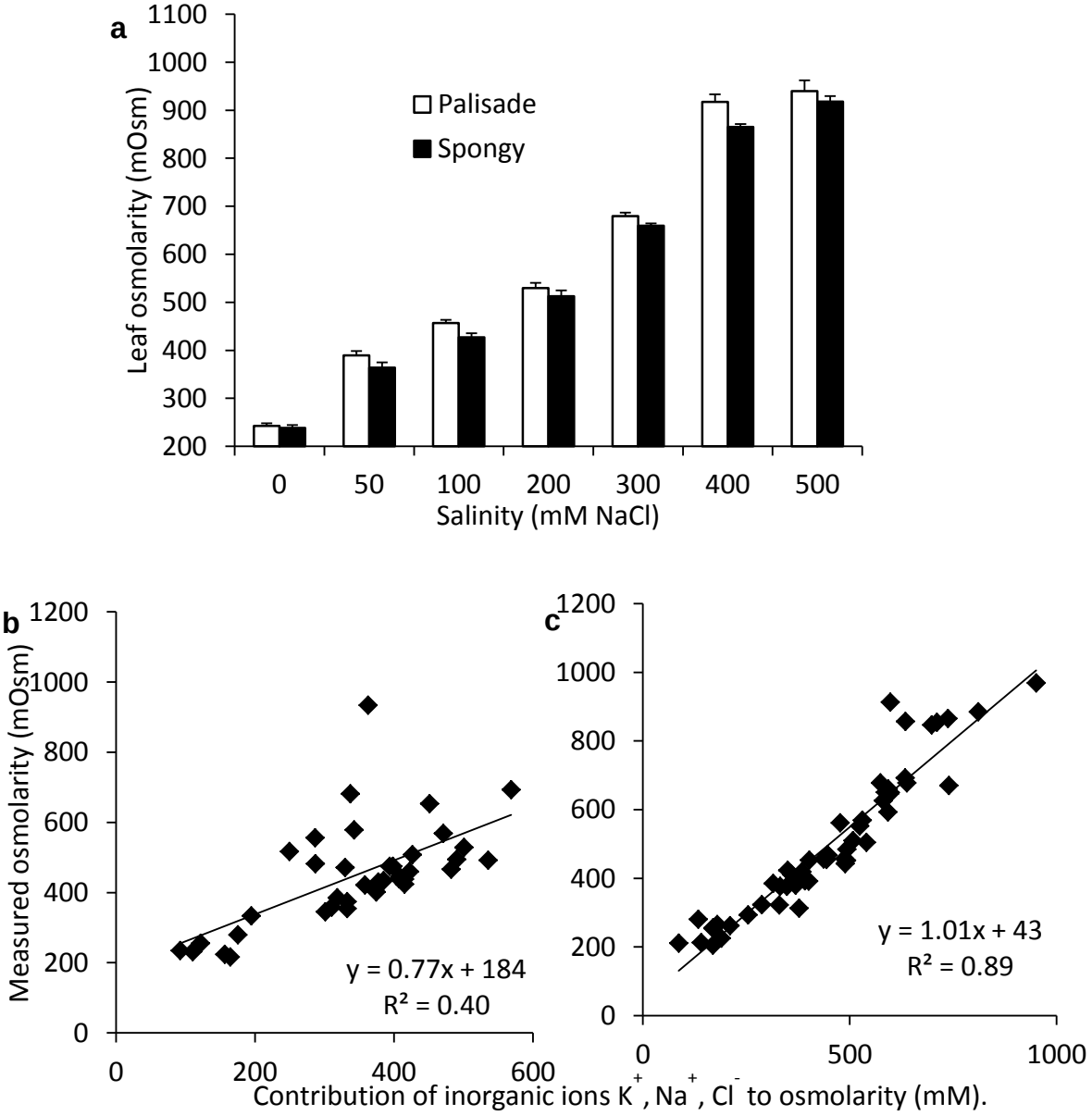


**Figure 2.9.** Osmotic and Ionic response to NaCl concentration. (a) sap osmolarity of spongy parenchyma and palisade mesophyll. Inorganic ion (Na+, K+ Cl-) generated osmolarity versus total sap osmolarity of (b) palisade mesophyll and (c) spongy parenchyma.

As shown in Table 2.3, at concentrations above 0 mM NaCl (i.e. once $Na^+$ and $Cl^-$ ions were supplied), the spongy parenchyma primarily adjusted its osmolarity with a combination of the three inorganic ions $Na^+$, $K^+$ and $Cl^-$. This pattern was not seen in the palisade tissue where $Na^+$, $K^+$ and $Cl^-$ ions contributed to just over 50 percent of the osmotic adjustment at 0 mM, suggesting that organic osmolytes may play a considerable role in adjustment for this tissue. Interestingly, the proportion of organic osmolytes (or non $K^+$, $Na^+$, $Cl^-$ ions) decreased under optimum growing conditions (50-100 mM), but increased again for plants growing at higher NaCl concentrations (Table 2.2), mirroring the flavonoid and antioxidant production trend seen in the palisade tissue's response to salinity concentration (Figure 2.7a-c).

**Table 2.3.** Relative contribution of inorganic osmolytes ($K^+$, $Na^+$, $Cl^-$) towards overall osmotic adjustment in parenchyma and mesophyll leaf tissue of C. rossii at various salinity treatments. Leaf $Na^+$ and $K^+$ content were measured in direct experiments, and leaf $Cl^-$ was assumed to be equal to $Na^+$.

| Sap source | External NaCl (mM) | Measured Osmolarity (mOsm) | Contribution of inorganic ions ($Na^+$, $K^+$, $Cl^-$) (mM) | inorganic ion % contribution to osmolarity | Calculated contribution of organic osmolytes (mM) | Organic ion % contribution to osmolarity |
|---|---|---|---|---|---|---|
| **Parenchyma** | 0 | 238 | 161 | 68 | 77 | 32 |
| | 50 | 364 | 328 | 90 | 36 | 10 |
| | 100 | 427 | 409 | 96 | 19 | 4 |
| | 200 | 513 | 513 | 100 | 0 | 0 |
| | 300 | 659 | 616 | 93 | 43 | 7 |
| **Mesophyll** | 0 | 243 | 137 | 56 | 106 | 46 |
| | 50 | 389 | 337 | 87 | 52 | 13 |
| | 100 | 459 | 417 | 90 | 42 | 10 |
| | 200 | 530 | 376 | 71 | 154 | 29 |
| | 300 | 682 | 453 | 66 | 229 | 34 |

The above differences in osmotic adjustment strategies between photosynthetically active and non-active leaf tissues were also reflected in differential accumulation of $Na^+$ and $K^+$ (Figure 2.10). While $Na^+$ accumulation in spongy parenchyma showed almost a linear relationship with external NaCl concentrations (Figure 2.10a), the amount of accumulated $Na^+$ plateaued in palisade mesophyll (Figure 2.10b) and was about the same (around 200 mM) in the broad range of external NaCl concentrations (between 100 and 500 mM). Tissue $K^+$ content declined with increasing NaCl levels in palisade mesophyll (Figure 2.10d), while in spongy parenchyma, $K^+$ levels showed no discernible trend (Figure 2.10c).

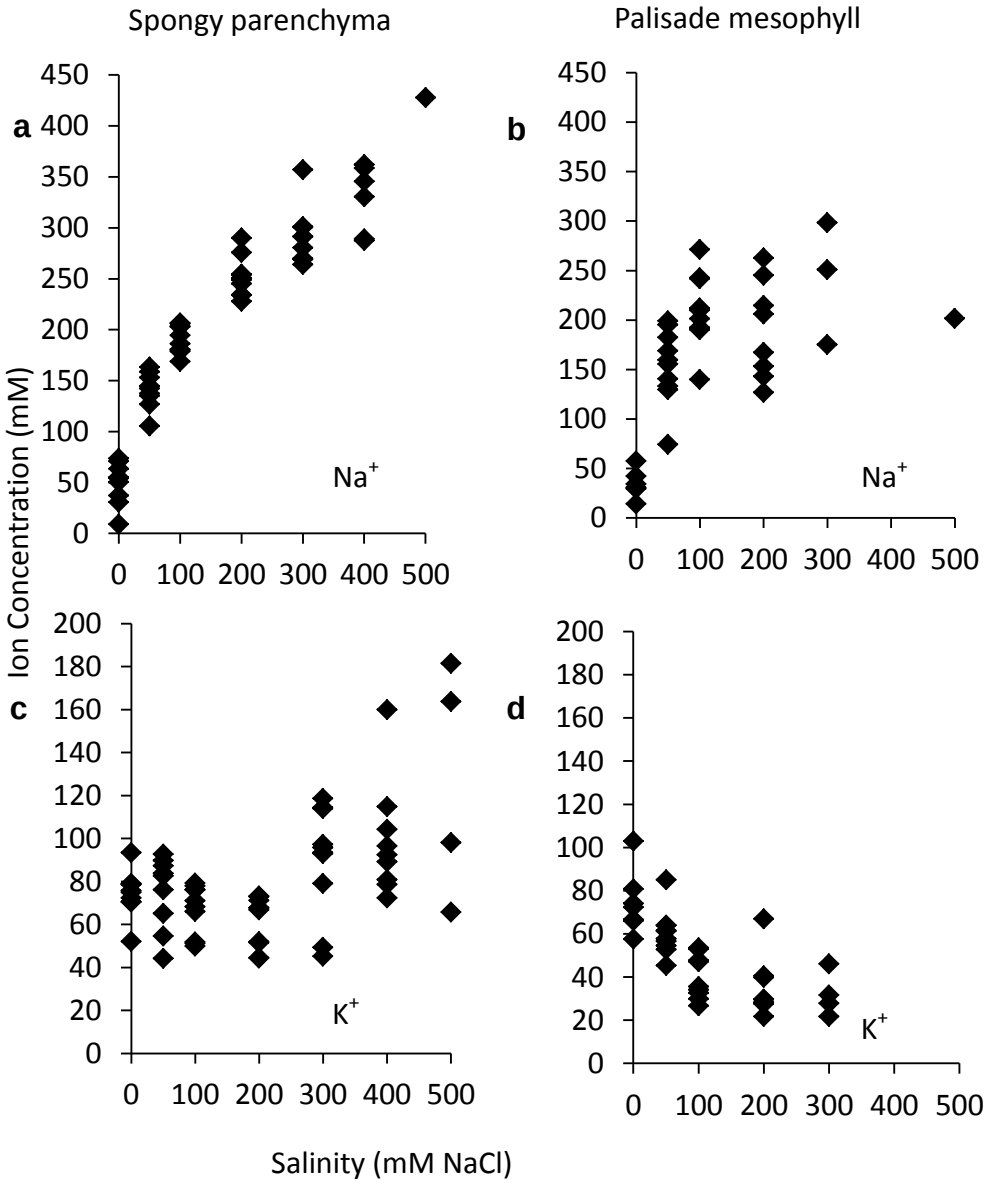


**Figure 2.10.** Specific ion response of leaf sap to NaCl treatment (a) palisade $Na^+$, (b) palisade $K^+$, (c) spongy $Na^+$, (d) spongy $K^+$.

## 2.5 Discussion

It is now generally accepted that phenolic compounds such as flavonoids and tannins are produced by plants in response to and as part of a strategy to minimise cellular damage from oxidative stress (Agati and Tattini, 2010, Pollastri and Tattini, 2011). Of critical importance to plants is the need to protect their photosynthetic machinery as this is both the site where light is converted to chemical energy (powering growth and metabolic activities) and a major site of ROS production in plant.

The importance of photosynthetically active tissues and their involvement in ROS production is illustrated when one compares the difference in flavonoid and total antioxidant (gallic acid equivalents) production between the spongy parenchyma and the photosynthetically active palisade of the various salt-treated plants. The preferential concentration of flavonoids in the photosynthetic tissue as seen during this experiment has also been documented by several other researchers (Agati et al., 2007, Melgar et al., 2009). Agati et al (2007) were able to show flavonoid co-location with chloroplasts and proposed that their role was to directly scavenge singlet oxygen due to its short diffusion time and high reactivity. Other researchers have highlighted their ability to scavenge hydrogen peroxide, superoxide and other reactive species (Rafat Husain et al., 1987, Tattini et al., 2004).

Our hypothesis that non-optimal environmental conditions cause elevated ROS levels, which the plant needs to counteract through increased antioxidant (flavonoid, tannin) production, is supported by both our observations in the field and under controlled conditions. Higher producing plants were more exposed to sunlight (e.g. lower shelter index) than lower producing plants, under natural conditions. Given the geographical location of field sites, namely coastal foredunes, and the high levels of UV radiation in Australia, this higher exposure may lead to increased ROS production. Two mechanisms may contribute to this process. First, increased irradiance and photon interception may exceed the photochemical competence of PSII, raise superoxide production and lead to photoinhibition (Foyer and Noctor, 2000). Evidence of this is the decreased Fm (the number of open reaction centres of PSII) at higher producing cluster 2 sites. Secondly, UV-B component *per se* can destroy chlorophyll as well as carotenoids, in addition to degrading photosystem II and rubisco function (Zhang and Zhao, 2008). Other factors shown to be significantly different between

the high and low producing clusters (soil electrical conductivity, nutrient levels and stomatal density) can also be explained in the context of higher ROS and hence higher antioxidant (flavonoid, tannin) production.

Tannin and flavonoid production was positively correlated with higher stomatal density and daily evaporation (Appendix Chapter 2, Appendix Table 2.4), suggesting that ROS production (and, hence a need for antioxidant protection) was causally related to water stress. Plants respond to a lack of moisture by closing their stomata, which impairs the ability of photosynthetic machinery to fully utilise light that was absorbed by photosynthetic pigments and leads to increased ROS (superoxide radical) production in chloroplasts (Flexas and Medrano, 2002b, Flexas and Medrano, 2002a, Miller et al., 2010a). In addition, the lack of moisture leads to an increased rate of photorespiration. All these processes exacerbate the effects of increased exposure to irradiance (including UV) and photosystem impairment. The water stress experienced by the higher-producing cluster is a function of both being exposed to higher evaporative conditions (a lower shelter index) and having increased moisture loss via uncontrolled stomatal conductance i.e. a higher stomatal count providing more points to lose water from. The role individual plant physiology plays in ROS and hence subsequent antioxidant (flavonoid, tannin) production is further highlighted by the frequency analysis which showed 100% of tannin and flavonoid regression models included this factor.

In addition to the influence of climatic variables on ROS production, soil nutrient availability also plays an important role. This is evidenced by both the frequency with which soil $K^+$ appears in the regression models and the differences in soil $K^+$ and $Na^+$ levels between the high (cluster 2) and low (cluster 1) producing clusters. Potassium is an essential nutrient for cellular metabolism since it is involved in the activation of over 50 enzymes (Marschner, 2012) and as such $K^+$ deficiencies are likely to perturb metabolism, resulting in the increased production of ROS (Cakmak, 2005). The increased ROS concentration has been shown to induce $K^+$ loss via outward rectifying potassium channels (KOR) (Cuin and Shabala, 2007) and if not addressed results in programmed cell death (Shabala, 2009). Therefore it is not surprising to see the plant produce antioxidants in an attempt to scavenge excessive ROS produced under $K^+$ deficient conditions.

The importance of potassium is highlighted when comparing the response of the

metabolically active palisade mesophyll tissue with the heavily vacuolated (storage organ) spongy parenchyma under controlled conditions. In the palisade cells, a clear reduction in potassium concentration occurs in response to increased salinity. High NaCl levels in the leaf apoplast have been reported to cause a massive $K^+$ leak from photosynthetically active mesophyll tissues (Shabala et al., 2007, Shabala et al., 2005, Shabala, 2000). This process is mediated by depolarization-activated outward rectifying $K^+$ channels (Shabala et al., 2006) and results in severe depletion of the cytosolic $K^+$ pool. In addition, $K^+$ leak from the cytosol may occur via ROS-activated $K^+$ permeable channels, both selective (Cuin and Shabala, 2007) and non-selective (Demidchik and Maathuis, 2007, Demidchik et al., 2003). Thus, an increased ability to scavenge the excess ROS produced under saline conditions may be essential to control cytosolic $K^+$ homeostasis and prevent PCD resulting from activation of caspase-like endonucleases and proteases (Reape and McCabe, 2010, Vartapetian et al., 2011) under low $K^+$ conditions (Demidchik et al., 2010).

Of special interest was the negative correlation between amount of soil $Na^+$ and flavonoid and tannin production in plants under field conditions. Normally, one would expect higher soil salinity to increase ROS production through perturbed metabolism and membrane depolarisation. However, CR is an obligate halophyte and as such has a physiological need for a minimum soil sodium concentration. Many halophytes use inorganic ions (such as $Na^+$ and $Cl^-$) stored in vacuoles to generate lower osmotic potential to draw water out of the soil and into the plant for growth processes (Flowers and Colmer, 2008). Because of this, the absence of $Na^+$ in the growth media can be considered as a stress for CR species. This explains the inverse relationship between soil $Na^+$ content and antioxidant activity under field conditions (Figure 2.4c) where soil salinity levels were not high enough (e.g. <50 mM NaCl) to provide optimal plant growth at any site and the lower $Na^+$ concentration at cluster two sites simply increased stress levels. Whether the increased stress is due to reduced carbon fixations, a fall in turgor, changes in cell wall elasticity or futile cycling of ions remains unclear (Flowers and Colmer, 2008). However, the corresponding increase in antioxidant activity at suboptimal salinities indicate that it is not merely a physical (turgor) effect, but mediated by ROS signalling interplay and metabolic perturbation is clearly occurring.

Once the optimum level of sodium is exceeded, the plants start to encounter its' toxic effects

including membrane depolarisation, potassium efflux, and interference with cellular enzymatic processes which can potentially lead to programmed cell death (Shabala, 2009, Shabala and Cuin, 2007). So, although insufficient and excess salt are physiologically different problems, both result in the plant experiencing stress, producing ROS and upregulating antioxidant (including flavonoid) production.

Highlighting the complex role ROS play in the plant signalling pathway is the fact that it was not a single factor alone that best predicted flavonoid/tannin production, but rather a complex interplay between several stress-causing variables. This interplay has been documented on many occasions where it has been shown that exposure to one stress agent affords protection against a second e.g.salinity to UV-B stress (Cakirlar et al., 2008, Melgar et al., 2009) and is referred as cross-tolerance (Bowler and Fluhr, 2000) due to the commonality of downstream targets in the signalling process. As such, when there are multiple stress agents present, a complex nonlinear effect is to be expected.

In addition to their ROS scavenging activity, flavonoids inhibit the transportation of auxins through tissue. Whether the flavonoid inhibition of auxin transport (Brown et al., 2001) or ROS induced ion fluxes (Demidchik and Maathuis, 2007) were responsible for the outcome was not determined, however the role of flavonoids as signalling molecules is widely known (recently reviewed in Pollastri and Tattini (2011)) and the effect on biomass production is most likely the result of a complex interplay between all of these factors.

## 2.6 Conclusion

Our results show that flavonoid and tannin production by CR is enhanced under conditions favouring ROS production (e.g. higher irradiance; low potassium and water availability) and, as such, may be causally related to oxidative stress. This conclusion is further supported by the fact that these secondary metabolites clearly accumulated in the metabolically active palisade mesophyll tissue of CR rather than the spongy parenchyma. Both sub- and supra-optimal concentrations of NaCl also resulted in a dramatic increase in antioxidant concentration.

Non-optimal NaCl levels significantly increase per leaf flavonoid concentration. However, the concomitant decrease in plant growth and biomass production caused stressed plants to

produce less flavonoids on a per plant basis. As a result, saline conditions favouring optimal plant growth appear to be most suitable for maximising production of flavonoids in CR. Future experiments should reveal whether the relationship reported here for NaCl holds for other stress-causing agents (UV-B, $K^+$ nutrition levels, light stress).

## 2.7 Acknowledgements

The authors would like to thank the assistance of Phil Andrews from the University of Tasmania for preparation of plant material for glasshouse salinity dosing trials. Adam Pirie is the recipient of an Australian Postgraduate Award from the Australian Government.

This work was supported by the Australian Research Council grants (DP1094663 and LP1020456) to Sergey Shabala.

# 3 Low-temperature and ultraviolet B exposure induce separate and structurally unrelated biochemical responses in *Carpobrotus rossii*

## 3.1 Abstract

Liquid chromatography, tandem mass-spectrometry and ultraviolet detection techniques were used to investigate the effects of low-temperature and ultraviolet B (UVB) exposure on the production of flavonoids, antioxidants, and other metabolites in the facultative halophyte *Carpobrotus rossii* (CR). Under low-temperature conditions there was an increase in gross flavonoid production and a change in flavonoid profile due to the increased concentration of less-substituted flavonoids. However, contrary to many reports where flavonoid production increases under stress conditions, the aglycone of these CR-produced flavonoids did not possess a dihydroxyl B-ring substitution. The concentration of minor flavonoids did not increase equally and the accumulation of flavonoids without a 3-hydroxy-3-methylglutaric acid substituent was favoured. It appears that the accumulation of lesser-substituted flavonoids is an important part in the response of CR to low-temperature conditions. UVB exposure resulted in lower concentrations of flavonoids and induced the production of structurally unrelated betalain pigments. It appears that a permanent positive charge and visible pigmentation are the key attributes needed by plants to respond to UVB exposure. Despite the considerable changes in metabolite profile, no difference in antioxidant activity was detected for either low-temperature or UVB exposed plants.

## 3.2 Introduction

*In planta*, the production of flavonoids is induced under conditions that cause the plant to experience stress (Agati et al., 2011a, Hernandez et al., 2004). These stresses all result in either a mismatch between the use of NADPH/ATP in the Calvin cycle and the capture of electrons by light harvesting antennae, leading to an over-reduced electron transport chain (ETC) (Takahashi and Murata, 2008); or direct damage to the photosynthetic machinery itself, such as the degradation of the D1 and D2 proteins of photosystem II (PSII) by exposure to ultra-violet B (UVB) radiation (Jansen et al., 1998). Inhibition of Calvin-cycle enzymes leads to an over-reduced ETC and the production of reactive oxygen species (ROS) via the Mehler reaction, whilst UVB exposure and damage leads to the generation of singlet oxygen, inhibited repair of the damaged PSII proteins and impaired Rubisco activity.

ROS species produced under these conditions are able to interfere with cellular processes through a suite of mechanisms including lipid peroxidation, activation of ion channels ($K^+$, $Ca^{2+}$) and alteration of protein function, in addition to causing damage to cellular DNA and RNA (Møller et al., 2007). Elevated ROS production can cause enough damage to make a cell unviable, resulting in programmed cell death and (if occurring on a large enough scale) death of the whole organism (Demidchik et al., 2010, Shabala, 2009, Shabala et al., 2007). To ensure that ROS concentrations are maintained at levels physiologically appropriate for cellular signalling, plants employ a suite of enzymatic and non-enzymatic antioxidants to ensure that these ROS are safely disposed of.

Current research indicates that the protective scavenging of ROS damage is one of the main roles for flavonoids *in planta*. Evidence suggests that flavonoids both chelate with transition metals to prevent Fenton reactions and form stable radicals after interacting with ROS such as the hydroxyl and superoxide radicals, however, their exact function(s) *in planta* are yet to be resolved (Hernandez et al., 2009). Agati et al (2007) have shown that flavonoids are preferentially located in the chloroplast, where they can directly scavenge ROS from the photosynthetic machinery and ETC before ROS can react with other cellular constituents and cause damage.

Data presented in chapter 2 on *Carpobrotus rossii* (CR) (Schwantes, 1928) lend support to the importance of flavonoids in protecting photosynthetic tissue. Our analysis has shown a strong preference for the accumulation of flavonoids in photosynthetically active palisade tissue versus the heavily-vacuolated spongy parenchyma which comprises the inner core of CR leaves and where virtually no flavonoids were detected. The relationship between flavonoid production and exposure to oxidative stress is further strengthened when one looks at the reports of other researchers who have found flavonoid production to be induced by a suite of conditions that include excess light, low temperatures, ultra-violet radiation and drought. Our results with CR also support this interpretation. As described in chapter 2, we have observed that when CR (a facultative halophyte) was grown at non-optimal salinity (<50-100mM> NaCl), there was an increase in flavonoid concentration, and total antioxidant activity, with reduced biomass production.

Previous work conducted in our laboratory has allowed the optimisation of techniques for CR flavonoid analyses and the determination of various substituents (spinacetin, glucose, apiose, ferulic acid, and 3-hydroxy-3-methylglutaric acid (HMG)); which in specific combinations comprise the majority of flavonoids in CR (Jager, 2009, Renggli, 2010). The objective of this study was to determine the effect two environmental stresses (low-temperature and UVB exposure) reported to alter flavonoid production in other species had on CR flavonoid production.

## 3.3 Experimental

### *3.3.1 Materials and reagents*

DPPH was purchased from Sigma-Aldrich (St Louis, USA), temperature recording was performed with Madgetech Temperature Retriever ® data loggers (Warner, USA), model T20.M ultra-violet B tubes were obtained from Vilber-Lourmat (Eberhardzell, GER), all other materials were of analytical grade or higher. Plant material propagated for these experiments was taken from verified CR plants collected during the work described in chapter 2.

### *3.3.2 Low Temperature experiments*

#### *3.3.2.1 Plant propagation*

Thirty cuttings were taken from a single donor plant and potted into 200 mm pots holding 5 cuttings per pot. Three pots (n= 15) were grown under control (glasshouse) and three pots under low-temperature conditions (an adjacent open sided glasshouse) for 30 days at which time they were harvested. Plants were watered to excess with 50 mM NaCl every 3 days to ensure optimum growing salinity (Chapter 2) Ambient air temperature was recorded every 5 minutes using two data loggers per treatment placed at random locations around the pots. The number of leaves present at the trial's start was also recorded.

#### *3.3.2.2 Sample collection*

On day 30 the plants were removed from their pots and washed with tap water to free the roots of potting mix, and separated into leaf plus stem, and roots. These were then weighed and measured for overall length (from base of stem to furthest leaf tip). The number of leaves at experiment end point, the distance between the nodes of fully mature leaves and root length were also recorded. The two newest fully-mature leaves were removed from the plant, weighed and measured individually for length and width. These leaves were then peeled and separated into palisade and parenchyma as described in chapter 2. Following this, palisade samples were analysed by HPLC-UV detection and UPLC-MS/MS to separate, identify and allow assessment of changes in the individual CR flavonoids. These samples were then assessed for their overall antioxidant activity with DPPH as described in chapter 2 with the only modification being that absorbance was measured at 515 nm with a Multiskan™ GO microplate spectrometer (Thermo Fischer Scientific, Waltham, USA). Samples were then diluted 1:100 before $Na^+$ and $K^+$ levels analysis was conducted using ion chromatography (IC). HPLC-UV, UPLC-MS/MS and IC conditions are given as supplementary material 3.8.1-3.8.3. The remaining plant material (roots, leaves and stem) was taken and oven dried for 72 hours at 40 °C to allow assessment of dry weight.

### 3.3.3 Ultraviolet B radiation exposure

#### 3.3.3.1 Plant propagation and growing conditions- Ultra-violet B exposure

A further thirty cuttings were taken from the same donor plant and potted into a 200 mm pots as per the temperature trial. Three pots (n=15 plants) were then subjected to UVB radiation, whilst three pots (n=15 plants) were used as the control treatment. Plants were grown at 24°C on a 12 hour light/dark cycle. UVB exposures consisted of 15 minutes UVB exposure followed by 15 minutes "recovery" starting 5 hours into the light cycle to generate a cumulative UVB exposure of 1.5 hours on day 1 and 45 minutes exposure on days 2 and 3. Spectra and spectroradiometric data were recorded at pot height for both control and UVB treated on and off conditions (Appendix Chapter 3, Appendix figure 3.1).

#### 3.3.3.2 Sample collection

The two newest fully mature leaves were removed, separated into palisade and spongy parenchyma tissue and analysed as per the low-temperature experiments.

### 3.3.4 Statistical analysis

Data were recorded and analysed in Prism 6 (GraphPad Software, La Jolla, USA) using Student's t test, results were considered significant when $P<0.05$. Levels of significance on figures are indicated as *=$P<0.05$, **=$P<0.01$, ***=$P<0.001$.

## 3.4 Results

### 3.4.1 Temperature Experiments

#### 3.4.1.1 Temperatures and growing conditions

The low-temperature study was conducted in Hobart, Tasmania (Latitude 43° S, Longitude 147° E) during August (the last month of winter). Plants grown in the open-sided glass house experienced significantly lower temperatures than control plants grown in an adjacent glasshouse ($P< 0.001$, Figure 3.1a). Overnight temperatures experienced by the low-temperature treated plants frequently dropped below 5°C and 16 percent of daytime temperatures (recordings between 7am and 5pm) were under 10°C. At no stage did the control plants experience temperatures lower than 10°C (Figure 3.1b). The maximum daily

temperature experienced by the control and low-temperature treatments was similar on many days of the trial. Exposure to this environment made it certain that the low-temperature plants would have experienced sustained and consistent periods of light stress during the experimental period.

#### *3.4.1.2 Flavonoid production*

Exposure to low temperatures significantly increased overall flavonoid production by CR ($P<0.01$, Figure 3.2a). The concentration of the main flavonoid (Molecular Weight (MW) 784) did not significantly increase under low-temperature conditions ($P=0.246$). An increased concentration of the structurally-related minor flavonoids (MW 640, 652, 816, 960) was recorded (all $P<0.001$, Figure 3.2b, Table 3.1). Not only was there an increase in the minor CR flavonoids, but a preferential accumulation of flavonoids lacking the ester linked HMG (MW 640, 816) was observed (all $P<0.001$) (Figure 3.2c).

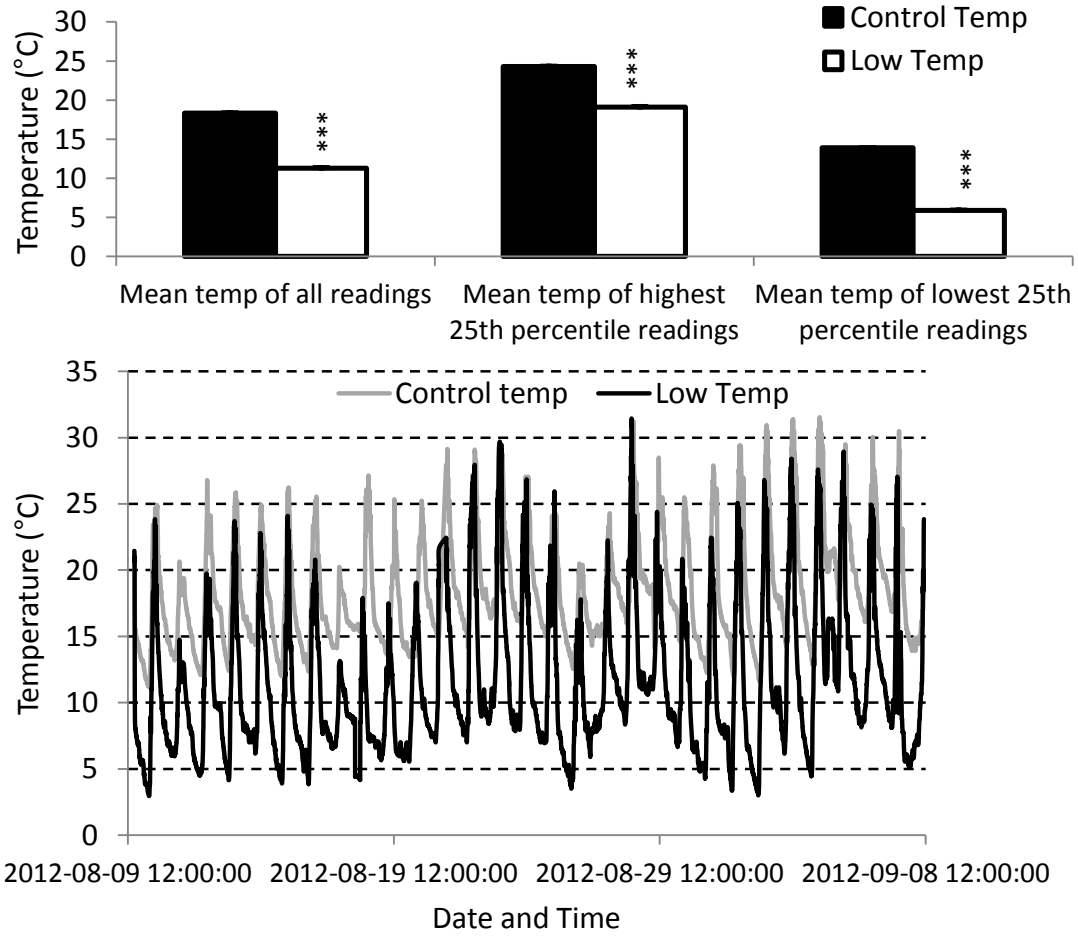


**Figure 3.1.** (a) Statistical summary of control and low-temperature treatments (Mean ± SEM), (b) temperature logs of control and low-temperature treatments.

**Table 3.1.** Substituents of the *C. rossii* flavonoids and the effect of low–temperature on their concentration *in planta*.

| | **Mass contribution** | **Flavonoid MW 640** | **Flavonoid MW 652** | **Flavonoid MW 784** | **Flavonoid MW 816** | **Flavonoid MW 960** |
|---|---|---|---|---|---|---|
| spinacetin | 346 | 1 | 1 | 1 | 1 | 1 |
| glucose | 162 | 1 | 1 | 1 | 1 | 1 |
| apiose | 132 | 1 | 0 | 1 | 1 | 1 |
| HMG | 144 | 0 | 1 | 1 | 0 | 1 |
| ferulic acid | 176 | 0 | 0 | 0 | 1 | 1 |
| Flavonoid MW | | 640 | 652 | 784 | 816 | 960 |
| Increase in concentration compared with control plants (%) | | 500 | 100 | - | 3960 | 130 |

### *3.4.1.3 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay performance*

The DPPH assay for both low-temperature and UVB experiments demonstrated adequate performance over the range 0.84-108 µg/ml gallic acid equivalents from the 11 different batch analyses. Precision (%RSD) was 0.9% and 3.5% at the 0.84 µg/ml and 108 µg/ml levels respectively; accuracy ((observed- expected)/expected) at the 27 µg/ml level was between -2.7% and +1.1%; and linearity estimated by correlation coefficient $r^2$ was greater than 0.994 on all batch calibration curves.

### *3.4.1.4 Antioxidant Activity*

Despite the significant (P<0.01) increase in flavonoid production under low temperatures, analysis of overall cellular antioxidant activity by DPPH was not able to detect a difference between the control and low-temperature groups (P=0.767) (Figure 3.2d).

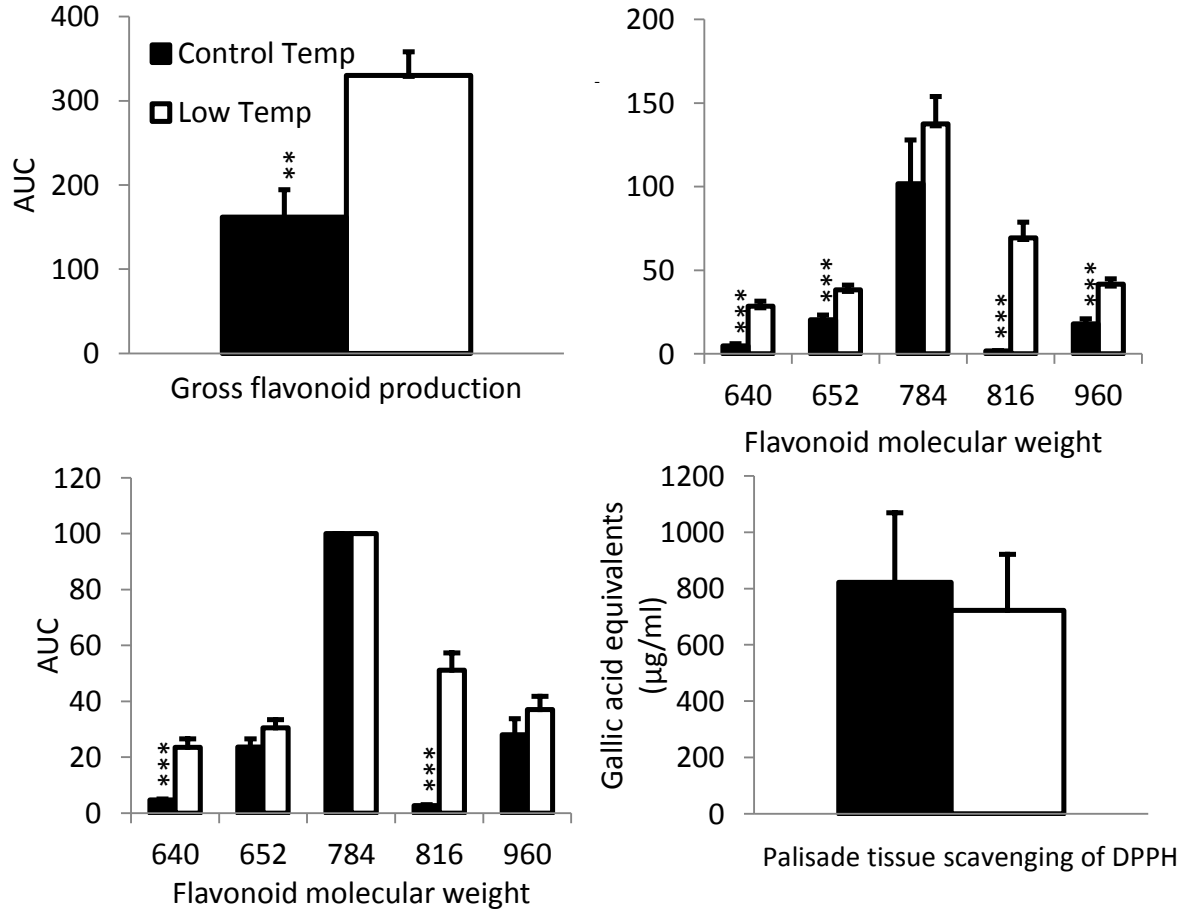


**Figure 3.2.** Analysis of low and control temperature CR leaf palisade tissue. (a) Gross (Mean ± SEM n=8, 10), (b) individual (Mean ± SEM n=8, 10), and (c) standardised to flavonoid MW 784 (Mean ± SEM n=8, 10), flavonoid production as assessed by HPLC-DAD analysis at 350 nm, expressed as chromatographic area under the curve (AUC) chromatogram, (d) antioxidant activity as assessed by DPPH (Mean ± SEM n=22, 20). **=P<0.01, ***=P<0.001.

### *3.4.1.5 Biomass production*

Plants grown under lower temperature conditions produced more biomass than the controls. This increased biomass was due to longer, wider and heavier leaves than those of the controls (P=0.0483, P<0.001 and P<0.001, respectively). Root growth was also increased under low-temperature conditions (P<0.001) (Table 3.2). The same differences were observed in dry matter production.

**Table 3.2.** Biomass production of control and low-temperature grown plants (n = 15, # n=30)

| | Control | Low Temp | P value |
|---|---|---|---|
| Leaves gained | 8.6 | 9.0 | 0.822 |
| Leaf width (mm)# | 5.6 | 7.2 | < 0.001 |
| Leaf length (mm)# | 58.0 | 66.8 | 0.048 |
| Leaf Weight (g)# | 1.2 | 2.2 | < 0.001 |
| Leaf and stem length (mm) | 128.9 | 119.9 | 0.518 |
| Fresh leaf and stem weight (g) | 9.15 | 12.58 | 0.036 |
| Internode length (mm) | 32.9 | 29.2 | 0.289 |
| Root length (mm) | 241.0 | 255.4 | 0.539 |
| Fresh root weight (g) | 0.42 | 1.34 | < 0.001 |
| Dry leaf and stem weight (g) | 0.42 | 0.63 | 0.012 |
| Dry root weight (g) | 0.20 | 0.40 | 0.012 |

### *3.4.1.6 Nutrient levels*

Low-temperature grown plants showed an increase in the sodium concentration of palisade tissue (P<0.001, Figure 3.3). There was no difference in the potassium levels of low-temperature plants' spongy parenchyma, but levels did appear to be trending downwards (P=0.120).

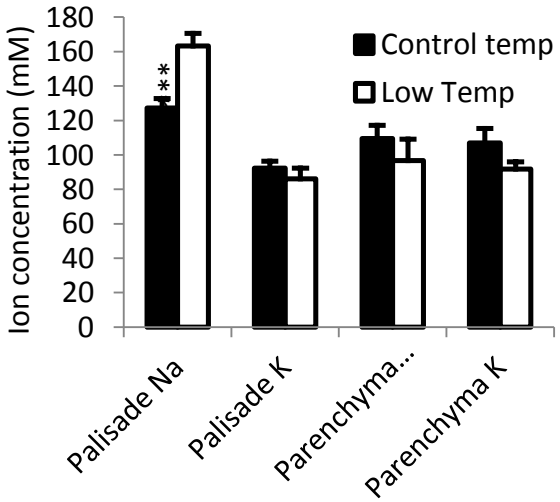


**Figure 3.3.** Na+ and K+ levels of low and control temperature mesophyll and palisade tissue (mean ± SEM n= 10). ***=P<0.001.

### *3.4.2 Ultra-violet B exposure*

#### *3.4.2.1 Flavonoid and betalain production*

UVB exposure reduced total flavonoid content ($P<0.001$, Figures. 3.4a, 3.4b). However, unlike the low-temperature treatment, no preferential accumulation or change in the proportion of specific flavonoids was observed (Figure 3.4c).

The exposure of CR to UVB induced the production of pigment compounds ($P<0.001$, Figure 3.4b). UPLC-MS/MS analysis determined the pigments to be betalain compounds; either betanin and isobetanin or gomphrenin and its structural isomer, compounds which only vary in the site of glycolysation (Mabry, 1980). These pigments were not detected in any of the controls or in low-temperature grown plants.

#### *3.4.2.2 DPPH Antioxidant Activity*

Despite the significant reduction in flavonoid concentration and induction of betalain production after exposure to UVB, no change in DPPH assessed antioxidant activity was detected ($P=0.160$, Figure 3.4d).

#### *3.4.2.3 Effect of UVB on Biomass*

As this experiment was of such a short duration (3 days), no formal measurements of biomass were made. However, it was observed that in the leaves collected for individual analysis, those from UVB exposed plants were noticeably less turgid than their control counterparts.

#### *3.4.2.4 Nutrient levels*

Plants exposed to UVB radiation showed an increased concentration of sodium in the palisade tissue ($P<0.001$) and the spongy parenchyma showed an increased concentration of both sodium ($P<0.001$) and potassium ($P<0.05$, Figure 3.5).

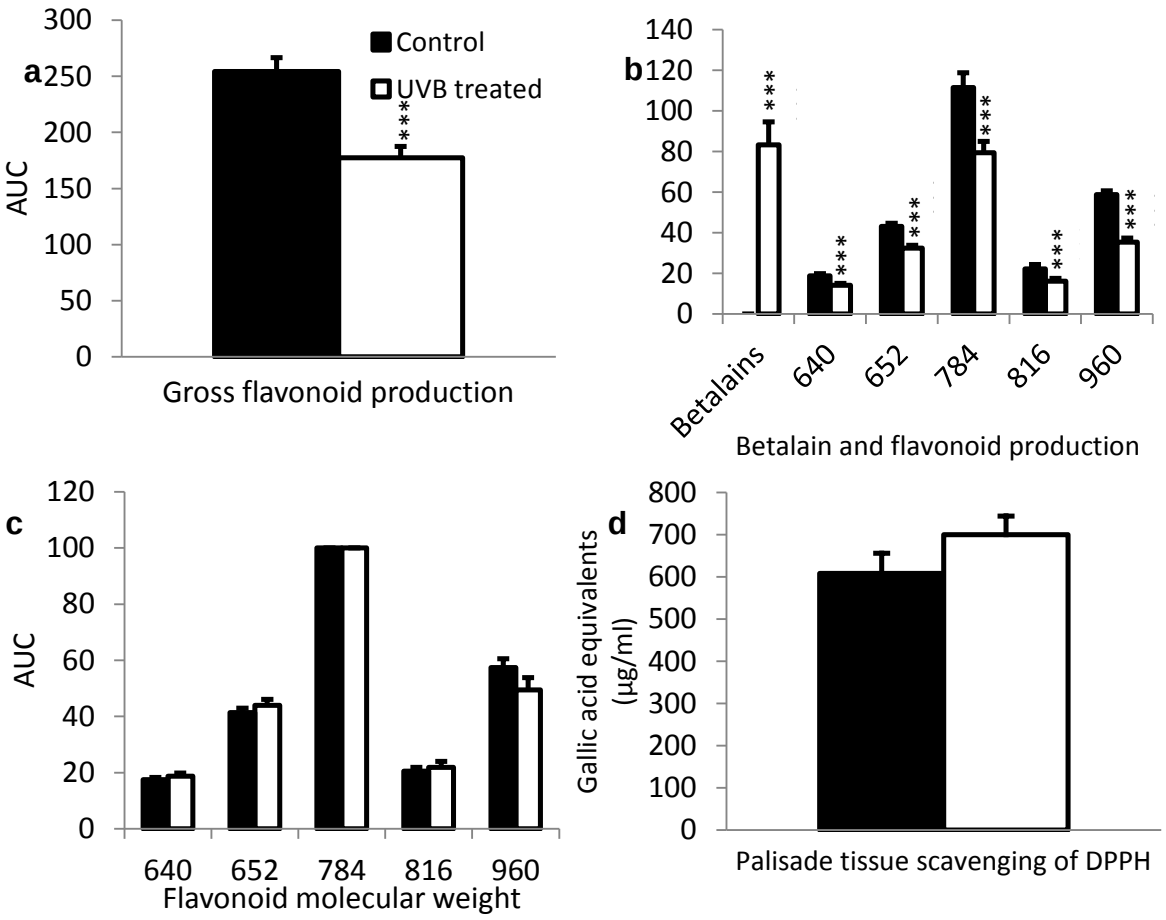


**Figure 3.4.** Analysis of UVB exposed and control CR leaf palisade tissue. (a) Gross (Mean ± SEM n= 30), (b) individual (Mean ± SEM n= 30), and (c) standardised to flavonoid MW 784 (Mean ± SEM n=30), flavonoid and betalain production as assessed by HPLC-DAD analysis at 350 nm (flavonoid), 535 nm (betalains), expressed as chromatographic area under the curve (AUC) (Mean ± SEM n= 10), (d) antioxidant activity as assessed by DPPH (Mean ± SEM, n= 30) ***=P<0.001.

## 3.5 Discussion

### *3.5.1 Mechanisms and specificity of ROS generation*

Many studies have reported an increased concentration of total flavonoids upon imposition of the stresses used in our experiments (Treutter, 2005), but few have investigated changes in the metabolite profile. There are limited reports of changes in the production of flavonoids having different parent aglycones (Agati et al., 2011b, Agati et al., 2007), but we are not aware of any reports showing the environmentally induced effects on a flavonoid series with the same parent aglycone. Because the flavonoids produced by CR are predominantly based

on the spinacetin aglycone, it was possible, in the present study, for such an investigation to occur. Additionally, because UVB exposure induced CR to produce structurally unrelated (and mutually exclusive (Mabry, 2001)) betalain compounds rather than that of another flavonoid subfamily (the anthocyanins) we were able to gain a greater understanding of the biochemical features important in UVB stress response and mitigation in the cell.

Considerable differences exist in the mechanisms and species of ROS produced by the two stresses used in our experiment. These differences resulted in CR employing a ROS-specific biochemical strategy to counter their production and mitigate their damage. Exposure to moderate (or higher) light levels and temperatures below 10 degrees Celsius leads to the thermodynamic inhibition of Calvin-cycle enzymes, an over-reduced electron transport chain and generation of superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), and the hydroxyl radical ($OH^\cdot$) (Wise, 1995). Safe disposal of $O_2^-$, $H_2O_2$, and $OH^\cdot$ is reliant on the donation of an electron (or electrons) by a compound able to form a stable radical intermediate. On the other hand UVB exposure causes direct damage to the D1 and D2 proteins of photosystem II (Jansen et al., 1998). This damage stops PSII from being able to accept the high energy electrons from the light harvesting antennae, leading to increased formation of triplet-state chlorophyll and singlet oxygen. Scavenging of these species relies on non-photochemical quenching (NPQ), which involves quenching to the ground state and then dissipating the energy to the environment (Foyer et al., 2012). Comparison of our results with those of others, where the increased production of flavonoid pigment compounds has been reported, allows the identification of the core characteristics needed to effectively achieve this outcome, namely visible pigmentation and a permanent positive charge (Figure 3.5). Presumably it is these features that are critical for the efficient acceptance of surplus excitation energy and subsequent NPQ.

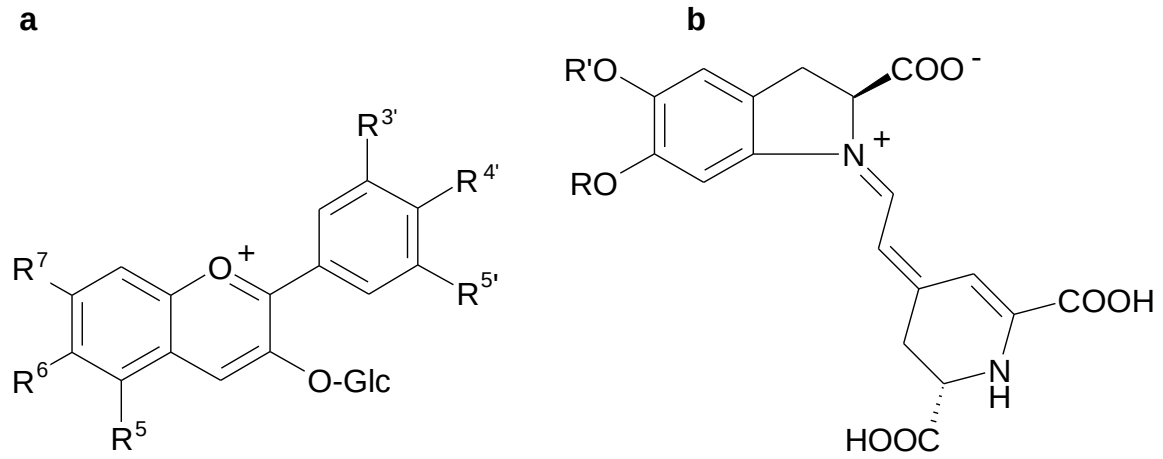


**Figure 3.5.** Anthocyanin (a) and the Betalain aglycone (b) both possess a permanent positive charge as well as visible pigmentation. Flavonoid positions $R^{5-7}$, $R^{3'-5'}$ may be substituted by H, OH or $OCH_3$. Betalain substitution are R'= glucose, R = H for betanin, whilst gomphrenin substitution are R'= H, R= glucose (Mabry, 1980).

UVB exposure caused the concentration of non-pigment flavonoids to decrease, despite an increase in both betalains and flavonoids being reported in the related species *Mesembryanthemum crystallinum* when exposed to high light and increased UV irradiance for 5 days (Ibdah et al., 2002). A decline in non-pigment flavonoids has been reported in *Arabidopsis thaliana*, in which flavonoid concentrations declined after 14 days of UVB exposure and during the recovery period. However, *A. thaliana* flavonoids have also been reported to increase during longer periods of UV exposure (Comont et al., 2012 and references therein). Whether a recovery in flavonoid production would have occurred in CR is uncertain.

Variations in the flavonoid response between this and other experiments may be due to differences in the species or in the spectra and intensity of radiation used. Some experiments excluded ambient background UVB (Agati et al., 2007, Pollastrini et al., 2011), whilst our experiment used an artificial UVB source.

It has been reported that flavonoids with a dihydroxyl-substituted B-ring are the most important flavonoids for ROS-scavenging due to their ability to act as peroxidase substrates, complex with transition metal ions and act as reductants by donating either electrons or hydrogen atoms (Agati et al., 2012, Agati et al., 2011b, Agati et al., 2007). As the spinacetin aglycone contains only one hydroxyl group on its B ring (Figure 3.6) it would appear that this is not a critical requirement for ROS scavenging in CR.

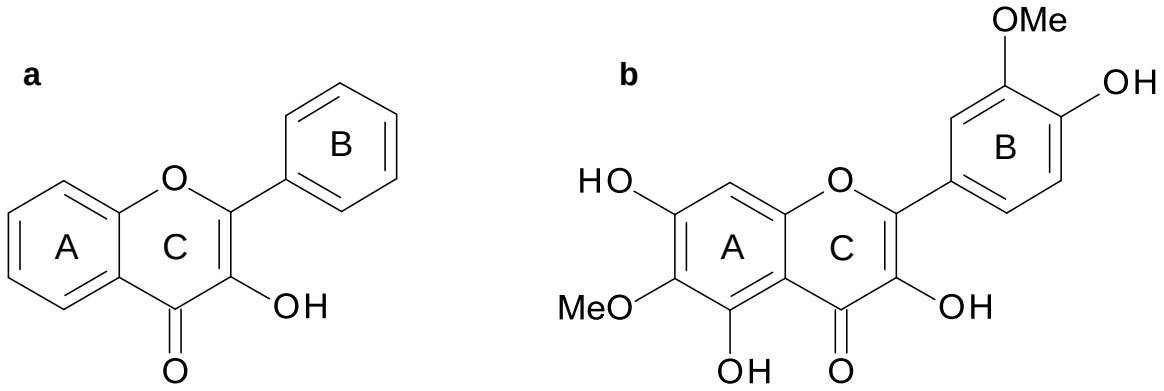


**Figure 3.6.** (a) The generalised naming structure of flavonoid aglycones (Adapted from Crozier et al. (2009)), and b) spinacetin.

Our analysis showed that low-temperature conditions, which produce ROS ($O_2^-$,$H_2O_2$, and $OH^\cdot$) generated a 100% higher gross flavonoid content compared to control CR leaves**.** Investigation of the differences in individual flavonoids showed that the concentrations of 4 of the 5 main flavonoid constituents were greater in the low-temperature treated plants compared with controls (Figure 3.2b).

The difference in concentration between control and low-temperature plants was not consistent and varied between the five flavonoids, an effect not seen after UVB exposure. The greatest increase observed was for the MW 816 flavonoid (3960%), followed by the MW 640 flavonoid, the MW 960 flavonoid and the MW 652 flavonoid (500%, 130%, 100%, respectively). The MW 784 flavonoid showed no significant difference. By far the greatest changes observed were in the concentrations of the flavonoids with MWs 816 and 640. Of the main CR flavonoids, these are the only two that do not contain an HMG substituent. There are several possible explanations for these observations, namely differences in the rate of

individual flavonoid biosynthesis and/or degradation in the presence of low-temperature associated ROS.

If increased ROS production induces the selective hydrolysis of HMG from CR flavonoids a flavonoid pool enriched with flavonoids lacking HMG such as MW 816 and 640 would result. However, it would also be reasonable to expect hydrolysis of the MW 652 flavonoid to occur and yield measurable quantities of a MW 508 flavonoid (652 – 144). This MW 508 flavonoid was not detected.

Therefore, it is more likely that the low-temperature treatment upregulated flavonoid biosynthesis, but the induction signal was not expressed equally for each individual flavonoid or enzyme involved in the flavonoid synthesis process. The greater representation of flavonoids lacking HMG may be due to relatively lower up-regulation of enzyme(s) responsible for either HMG biosynthesis or flavonoid-HMG acylation.

The greatest increase in flavonoid concentration observed was in the MW 816 flavonoid, which is substituted with glucose, apiose and ferulic acid. Presumably the MW 640 flavonoid (substituted with glucose and apiose) is a precursor of MW 816. This would indicate that, in contrast with HMG, under low-temperature stress there is a higher bioavailability of, and greatly increased acylation with, ferulic acid. Whether flavonoids acylated with ferulic acid confer greater protection to the low-temperature-stressed plant than flavonoids substituted with HMG is a question that requires further experimentation to answer.

### *3.5.2 Nutritional levels, sodium and potassium, biomass*

Unlike our previously reported results, where flavonoid concentration was inversely related to leaf size, and biomass production (Chapter 2), low-temperature exposed plants experienced both an increase in flavonoid concentration and leaf, stem, and root weight. In addition to inhibiting Calvin cycle enzymes, low-temperature causes roots to exhibit reduced hydraulic conductivity and under conditions of medium to high light an imbalance between moisture taken up through the root system and that lost through evapotranspiration occurs (Aroca et al., 2012). Being a succulent halophyte with access to free soil sodium, CR is able to counteract this perceived moisture limitation by increasing the sodium content of palisade tissue and lowering its osmotic potential. A consequence of this strategy is increasingly

succulent leaves (which allow a greater volume of water to be transpired before moisture stress is experienced) and hence heavier biomass weights for low-temperature grown plants. A second strategy to combat this perceived moisture stress is to increase the ability to harvest moisture by enhancing root growth, as was observed during our experiments. We believe this increase in biomass reflects a short term stress response rather than a preference for low-temperature conditions, as we have observed that CR cuttings grow considerably faster under glasshouse conditions than when they are struck and grown at lower temperatures (unpublished observation). Based on this we expect that if the experiment had continued for an extended period, the increases in biomass due to leaf size (and moisture storage) would have been negated by the increased number of leaves produced by control-temperature plants.

ROS ($O_2^-$, $H_2O_2$, and $OH^-$) induce the efflux of potassium from plant cells (Cuin and Shabala, 2007, Velarde-Buendía et al., 2012, Zepeda-Jazo et al., 2011) yet the concentration in low-temperature palisade tissue was virtually identical to that of control plants. Despite not being significant (P=0.120), the trend of reduced potassium in low-temperature spongy parenchyma would suggest that CR may be using this tissue body as a virtual super-vacuole from which to draw potassium and maintain the metabolic processes of photosynthetic tissue. The large volume of the spongy parenchyma compared to the photosynthetically active palisade means that a relatively small reduction in the concentration of parenchyma potassium would lead to a substantial increase in the palisade potassium concentration.

UVB exposure also resulted in significant changes in the sodium and potassium concentration of leaf tissue. Like exposure to low-temperature, exposure to UVB also increased sodium in the palisade tissue without affecting the potassium concentration. UVB exposure has been shown to stimulate both stomatal opening or closing depending on the metabolic state of the guard cell, and that once this stimulation occurs the leaf is unable to re-adjust stomatal aperture in response to relevant environmental stimuli such as changes in light, temperature or leaf moisture conditions (Jansen and Van Den Noort, 2000). If UVB exposure caused CR stomata to be locked into their open state, then the increased sodium and potassium concentrations of the vacuolated parenchyma tissue likely reflects moisture loss due to uncontrolled evapotranspiration. If this is the case, the increased sodium and undetectable change in potassium of the palisade tissue actually reflects an efflux of potassium that is

being masked by the loss of moisture volume. A loss of moisture would also explain the observed reduction in turgidity, and the increased concentration of both sodium and potassium in the spongy parenchyma of UVB exposed plants.

### *3.5.3 Antioxidant activity*

Despite the use of treatments known to induce oxidative stress and the production of compounds (flavonoids, betalains) with documented antioxidant activity, DPPH assessed antioxidant activity was unable to detect the difference between either control group and their respective treatment. Several explanations exist for this; firstly, the antioxidant system of plants is composed of a complex mix of enzymatic and low-molecular-weight compounds. These have been shown to react independently of each other depending upon the type, intensity, and duration of ROS-inducing event (Gill and Tuteja, 2010, Vanacker et al., 1998). As such, it is quite feasible that net antioxidant activity of the control and treatment groups had not changed, but that various sub-groups of antioxidants adjusted in a compensatory manner to maintain the same gross antioxidant status; we have found that an analogous process involving glutathione peroxidase occurs in mammalian systems (Jacobson et al., 2007). Adding further complexity to this scenario is the fact that the redox status of individual antioxidants changes depending on whether the plant is in the induction, compensation or acclimatised stage of stress response (Polkowska-Kowalczyk et al., 2007). In addition, there is considerable ongoing discussion as to the relevance and most physiologically appropriate way to measure ROS production *in planta* and *in vivo* (Halliwell, 2009, MacDonald-Wicks et al., 2006).

## 3.6 Conclusion

Our data show that the metabolite profile of CR stress response is stress specific, with the induction of betalain pigment compounds upon UVB exposure and flavonoid compounds upon exposure to low temperatures. The results indicate that it is the presence of a permanent positive charge and visible pigmentation which is most important in providing protection against UVB damage. In response to low-temperature conditions, differential enzyme activation plays a major role in determining the changes in CR flavonoid profile with the preferential accumulation of less-substituted flavonoids, rather than the more often reported production of flavonoids with a dihydroxyl-substituted B-ring being observed. The accumulation of less-substituted minor flavonoids is likely to be an important process in the stress response of other species and is worthy of further investigation.

### *3.6.1 Acknowledgements*

The authors would like to thank Mr Philip Andrews for propagating the plant material.

Adam Pirie is the recipient of an Australian Postgraduate Award funded by the Australian Government. This project was supported by the ARC Discovery and Linkage projects to Sergey Shabala.

# 4 Pirieol A from Carpobrotus rossii, a novel spinacetin glycoside containing apiose and HMG moieties.

## 4.1 Abstract

*Carpobrotus rossii* (CR) is a plant native to Australia where it is found growing along its southern coastal areas. The plant has a history of traditional use by both indigenous Australians and early European settlers. Despite this history of use and the recent reports of *in vitro* pharmacological activity, no detailed structural analysis of the compounds contained have been published. This paper reports the elucidation of three CR flavonoids, primarily the predominant flavonoid compound produced by CR, as well as two related minor flavonoid compounds. We have shown these flavonoids to be comprised of a common spinacetin aglycone, and common glucose substituents. The presence/absence of apiose and 3-hydroxy-3-methylglutaric acid (HMG) moieties determines whether the flavonoid is the predominant flavonoid (all substituents present) or one of the minor flavonoids (apiose or HMG missing, respectively). The predominant flavonoid's IUPAC name was determined to be 5-[[4-[3,4-dihydroxy-4-(hydroxymethyl)tetrahydrofuran-2-yl]oxy-3,5-dihydroxy-6-[2-(4-hydroxy-3-methyl-phenyl)-5,6,7-trimethyl-4-oxo-chromen-3-yl]oxy-tetrahydropyran-2-yl]methoxy]-3-hydroxy-3-methyl-5-oxo-pentanoic acid.

**NB:** Chapter 4 has been written in a format common to many organic chemistry journals, for example J Nat. Prod. In such journals there is often a combined results and discussion section when the determination of new structures (but no bioactivity) has occurred.

## 4.2 Introduction

The succulent halophyte *Carpobrotus rossii* (CR) (Schwantes, 1928) is a member of the Aizoaceae family (Blake, 1969) and commonly found growing along the coastline of southern Australia (Australia). The stresses associated with surviving in these locations, namely exposure to salinity, high light, poor nutrition, extremes of temperature, and aridity (Garcia-Mora et al., 1999) induce the plant to produce high levels of polyphenolic compounds which function as antioxidants in the photosynthetic machinery of the leaves (Chapter 2). Both indigenous Australians and early settlers consumed CR leaf material as a food, and also used it to treat a variety of ailments including burns, stings, and gastrointestinal disorders (Plomley et al., 1966, Watson, 2007).

Despite this history of use, no reports detailing the structure of compounds produced by CR has been published. Recent pharmacological investigations have shown that the extract is flavonoid rich, has strong antioxidant activity, and inhibits the release of inflammatory cytokines (IL-10, TNF-α, MCP-1) *in vitro* (Geraghty et al., 2011). Additionally, preliminary data indicate that consumption of crude CR extract may have beneficial effects on cardiovascular disease risk by lowering the concentration of lipid levels in *vivo* (Geraghty, DP, personal communication).

UPLC-UV and -MS/MS analysis of CR leaf extracts from plant samples collected during a previous field survey (Chapter 2) showed that the leaves contained a suite of flavonoid compounds (Figure 4.1a, 4.1b). Tandem MS/MS analysis revealed that many of these compounds formed common daughter ions, indicating the presence of common underlying structures (Figure 4.1c, 4.1d).

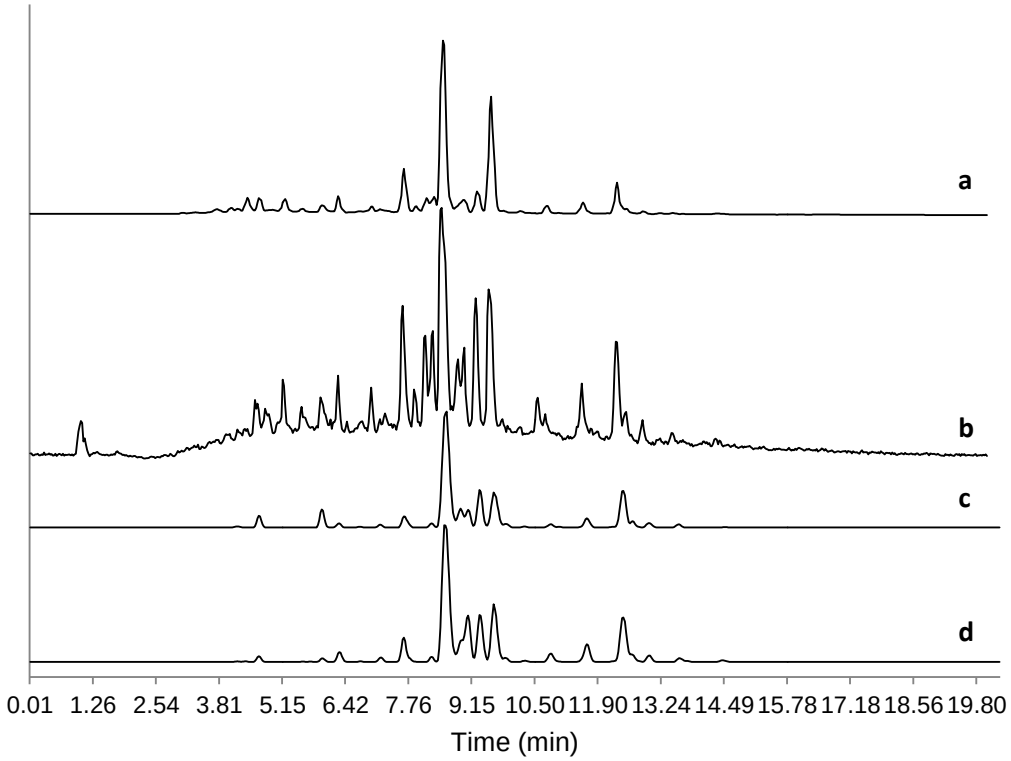


**Figure 4.1.** UPLC chromatogram of CR leaf extract (a) UV-DAD 350 nm chromatogram, absorbance peaks indicate the presence of flavonoids,(b) MS [M-H] total ion chromatogram of all compounds m/z 400-1600 Da, (c) MS/MS generation of m/z345 Da product ions, (d) MS/MS generation of m/z 344 Da product ions.

The flavonoid profiles of surveyed plants showed considerable intra- and inter-site variation (data not presented). However, a dominant/co-dominant peak with a molecular weight (MW) of 784 was observed in all samples. This peak generated daughter ions common to many of the other flavonoids observed in the CR extract and so was selected as an appropriate candidate for detailed structural investigation.

## 4.3 Material and methods

### *4.3.1 General experimental procedures*

UV spectra were measured using a Cary 100 (Varian Inc., Palo Alto, USA) spectrophotometer, NMR data were collected using a Bruker Avance 800 MHz spectrometer (Bruker Corp., Billerica, USA) and referenced to $d^6$-DMSO. HRESIMS was recorded using an Thermo Electron LCQ Orbitrap mass spectrometer (Thermo Fischer, Waltham, USA)

whilst UPLC-MS/MS and UV data was generated using a Waters Acquity UPLC system coupled to a H-series PDA detector, located in series with a Xevo triple quadrupole mass spectrometer (Waters Corp. Milford, USA). Fractionation into a single compound was conducted using a Waters 2695 Chromatographic station coupled to a Waters 2487 dual wavelength photometric detector operating at 254 and 350 nm as well as a Waters 120 differential refractometer (Waters Corp. Milford, USA)**.** UPLC-MS/MS, Orbitrap MS and chromatographic conditions for single compound fractionation are detailed in Appendix Chapter 4.

Plant material (~200g) collected during an earlier field trip (Chapter 2) was propagated and grown out to produce sufficient plant material for structural elucidation. UPLC-MS/MS comparison of the collected plants metabolite profile to those of authenticated specimens (HO 529461, HO 529462) held at the Tasmanian Herbarium (Hobart, Australia) ensured that the plants collected were indeed CR and not other closely related species (data not shown).

Plant leaves were removed, washed under tap water to remove surface contaminants and dried using paper towel. Plant material was macerated in a stainless steel blender and filtered sequentially through commercially bought cheese cloth and Whatman #1 filter paper to remove solids. The filtered homogenate was loaded onto C18 silica gel, eluted with 95% aqueous methanol and dried under reduced atmosphere at 42$^{o}$C (Heidolph Instruments, Schwabach, GER). This dried material was then made up as a 200 mg/ml solution in 10% methanol, 90% $H_2O$ for aglycone and substituent structure determination.

An aliquot of the solution was hydrolysed using a 1:1:4 mixture of extract:32% hydrochloric acid:methanol heated to 85$^{o}$C for 1 hour. The sample was then loaded onto a C18 reverse phase cartridge (strata-X ®, Phenomenex, Sydney, Australia), washed with 90% aqueous methanol and the aglycone eluted with 100% methanol. This eluent was then dried under reduced vaccum (as before) and re-dissolved in methanol to run through an 500 mm × 20 mm ID column of Sephadex LH20 gel (Sigma-Aldrich, St Louis, USA). Fractions determined via HPLC-UV to contain the aglycone were combined and dried down once again.

The high purity aglycone was then redissolved in methanol for UV spectrum analysis as per Mabry et al. (1970). The remaining non-hydrolysed extract was also run through Sephadex

LH-20 gel. Fractions exhibiting large co-absorbing peaks at 254 and 350 nm during HPLC-UV analysis were deemed to be flavonoid-rich, combined together and dried. A portion of this dried fraction was made into a 100 μg/ml methanol solution and analysed via LC Orbitrap MS/MS analysis, masses recorded as [M+Na] adducts with the remainder retained for NMR analysis.

Separate hydrolyses of the flavonoid-rich fractions were conducted to allow the identification of substituent compounds via mass spectrometry and comparison to commercial standards. Comparison to commercial sugars was done using LC-MS/MS following hydrolysis in acetic acid (1 ml sample: 5 ml 20% acetic acid, 100$^{o}$C, 8 hours). Comparison to authentic 3-hydroxy-3-methylglutaric acid (HMG) was done following hydrolysis (as with aglycone preparation), methylation according to Presser and Hüfner (2004) with GC-MS retention times and mass spectra compared.

The remainder of the dried, flavonoid-rich sample was reconstituted in water prior to fractionation into a single compound for NMR analysis. The collected peak was stored under refrigerated conditions, adjusted to pH 7 using ammonia hydroxide, freeze dried and stored until being re-dissolved in d$^{6}$-DMSO for NMR structural analysis ($^{1}$H-1D, $^{13}$C-1D, COSY, TOCSY (120 ms), NOESY (500 ms), $^{1}$H-$^{13}$C HSQC, $^{1}$H-$^{13}$C HMBC, HSQC-TOCSY (120 ms)).

## 4.4 Results and Discussion

High resolution orbitrap electrospray ionisation MS (HRESIMS) data of the predominant peak showed a $[M+Na]^{+}$ parent ion of 807.1957 Da, corresponding to a compound with a molecular formula of $C_{34}H_{40}O_{21}Na$. Product ions were consistent with substitution by a hexose sugar (162.0521 Da, $C_6H_{10}O_5$), a pentose sugar (132.0419 Da, $C_5H_8O_4$) and an acyl substituent (144.0419 Da, $C_6H_8O_4$), and an aglycone (346.0700 Da, $C_{17}H_{14}O_8$). Hydrolysed flavonoid products were compared with authentic standards of sugars and 3-hydroxy-3-methylglutaric acid (HMG). Retention times and mass spectra confirmed the presence of D-glucose (LCMS) and HMG (GCMS) in the flavonoid hydrolysate. The common pentose sugars xylose, arabinose, ribose and rhamnose were absent, suggesting the presence of an alternative pentose substituent.

The flavonoid aglycone was isolated from other hydrolysis products by the C18 reverse phase cartridge. In brief, HRESIMS of the aglycone ion $[M+Na]^+$ yielded a mass of 369.0577 Da ($C_{17}H_{14}O_8Na$), consistent with the orbitrap MS data generated from the unhydrolysed flavonoid sample. Analysis of the aglycone UV spectrum showed absorbance maxima at 256.00 nm (Band II) and 368.50 nm (Band I) indicating a flavonol (Mabry et al., 1970). Further spectrum analysis indicated the presence of hydroxyl groups at the 3, 7, 4'and 5 positions (Mabry et al., 1970) (Table 4.1).

**Table 4.1. UV Spectrum analysis of the *C. rossii* flavonoid aglycone.**

| Reagent added | Band I λ max | Band II λ max | OH position indicated |
|---|---|---|---|
| None | 368.50 | 256.00 | |
| $NaOCH_3$ | 421.50 | 276.00 | |
| λ shift | 53.00 | 20.00 | 4' |
| $NaOCH_3$ | - | - | |
| + 10 minutes | | | |
| λ shift | Spectrum decomposed | Spectrum decomposed | 3, 4' |
| $CH_3COONa$ | 383.00 | 269.00 | |
| λ shift | 14.50 | 13.00 | 7 |
| $AlCl_3$ | 431.50 | 268.50 | |
| λ shift | 63.00 | 12.50 | |
| $AlCl_3$ + HCl | 430.00 | 268.00 | |
| λ shift | 61.5 | 12.00 | 3, 5 |

NMR analysis showed the presence of protons at positions 8, 2', 5' and 6', with methoxy groups at 6 and 3' (Table 4.2). These assignments indicate that the aglycone was the previously described (but relatively uncommon) flavonol, 3,5,7-trihydroxy-2-(4-hydroxy-3-methoxyphenyl)-6-methoxy-4H-chromen-4-one, commonly known as spinacetin. This aglycone has been found in *Artemisia copa*, *Brickellia chlorolepsis*, some species of chenopodiaceae and *Spinacia oleracea* (spinach) (Gorzalczany et al., 2013, Ulubelen et al., 1980, Sanderson et al., 1988, Aritomi et al., 1985).

Apart from a shift of approximately 10 ppm downfield for C2, indicative of attachment via a 3-O linkage (Agrawal, 1989), the carbon NMR spectrum of **1** (Table 4.2) contained signals consistent with those for the spinacetin aglycone. Attachment via a 3-O linkage was confirmed by an HMBC coupling between the glucose anomeric proton H1" and C3. The anomeric proton coupling constant (7.8 ppm) was indicative of a *β*-glucose conformation (Agrawal, 1992).

Evidence of HMG attachment via an ester linkage to glucose C6" was seen with the H6" and H4""protons both coupling to C1"". Connection via an ester linkage to the C6 of glucose is the most common site of HMG attachment; and is the favoured site for attachment amongst isomeric hexose compounds (Wang et al., 2012).

The assignment of the signal as a 4-bond HMBC coupling (H4"" to C1"") rather than a 2-bond HMBC coupling (H2"" to C1"") was based on the presence of broadened $CH_2$ peaks (H2"") in the $^{1}H$ spectrum (1.59, 1.49 ppm, Appendix Chapter 4, Appendix figure 4.1). Steric hindrance is known to lead to the broadening of proton peaks, and in **1** presumably occurs due to steric hindrance of free rotation of the H2"" protons. These protons also showed no resonance in the multiplicity edited HSQC when the default C-H coupling frequency of 146Hz was used (Appendix Chapter 4, Appendix figure 4.2), but altering this to 160 Hz showed them unambiguously.

The presence of apiose was confirmed by interpreting the data from several NMR experiments. Firstly, the presence of two methylene groups was observed in the molecule. Naturally occurring apiosides are the only branched plant cell wall monosaccharide (Mølhøj et al., 2003), and are found exclusively in the $_{D-}$ form (Su et al., 2003). One methylene group

exhibited a large difference in the proton shift values (3.26, 2.91 ppm) indicating their location on the furanose ring. HMBC experiments showed these protons coupled to the anomeric carbon (C1'''), a second carbon (C2''') and the other methylene carbon (C5'''). The second $CH_2$ group protons (H5''') only showed 3-bond coupling with C4''' and C2''', whilst the anomeric proton (H1''') exhibited strong 3-bond coupling to the quaternary C3''' as well as coupling with C4'''. A weaker 2-bond coupling from the H5''' protons to C3''' was also observed (Appendix Chapter 4, Appendix figure 4.3). The shift value of the anomeric carbon (108.8 ppm) is consistent with that of apiose in the $\beta$ conformation (Snyder and Serianni, 1987). Therefore the sugar was determined to be $_{D}$-apiose in $\beta$ conformation.

A NOESY experiment showed coupling between the anomeric apiose proton (H1''') and proton H3'', giving the site of attachment via a 1'''$\rightarrow$ 3'' ester linkage (Table 4.2). In addition, the NOESY data showed coupling between H2' and H2'' as well as between H2' and the protons of the methoxy group at C3'. From these data, the structure of **1** (Pirieol A, Figure 4.2) was determined. Proton and $^{13}C$ spectra for **1** are included in Appendix Chapter 4.

LCMS/MS analysis of the CR extract revealed the presence of two other compounds related to **1**. The first was found to have a MW of 652 and to generate ions consistent with a spinacetin aglycone and the loss of HMG and glucose substituents, whilst the other had a mass of 652 and generated ions consistent with a spinacetin aglycone and the loss of apiose and glucose substituents. Structures **2** and **3** were proposed for these compounds (Figure 4.3).

**Table 4.2.** NMR Spectroscopic Data Structure 1 aglycone (800 MHz, d6-DMSO)

| Position | 13C (δC), type | 1H (δH) | COSY | TOCSY | NOESY | HMBC | HSQC-TOCSY |
|---|---|---|---|---|---|---|---|
| 2 | 156.0, C | | | | | H2', H6' | |
| 3 | 132.4, C | | | | | H1'' | |
| 4 | 177.3, C | | | | | | |
| 5 | 152.3b, C | | | | | | |
| 6 | 131.5, C | | | | | H8, 6OCH3 | |
| 7 | 151.7b, C | | | | | H8 | |
| 8 | 94.1, CH | 5.99 s | | | | H8 | |
| 9 | 158.6, C | | | | | | |
| 10 | 104.0, C | | | | | H8 | |
| 1' | 121.2, C | | | | | H5' | |
| 2' | 113.3, CH | 7.40 d (2.2) | | H5', H6' | H2'', 3'OCH3 | H6' | |
| 3' | 147.0, C | | | | | H5', 3' OCH3, 2bond H2' | |
| 4' | 149.4, C | | | | | H2',H 6', 2 bond H5' | |
| 5' | 115.2, CH | 6.35 d (8.4) | H6' | H2' | | | |
| 6' | 121.93, CH | 6.99 dd (8.4, 2.1) | H5' | H2' | | | |
| 6OCH3 | 59.9, CH3 | 3.20 s | | | | | |
| 3'OCH3 | 55.8, CH3 | 3.33 s | | | H2' | | |

[a] assignments based on directly obtained experimental data and comparison with published values, [b] assignments are interchangeable

**Table 4.3**. NMR Spectroscopic Data Structure 1 Substituents (800 MHz, d6-DMSO)

| | $^{13}C$ ($\delta_C$) | $^{1}H$ ($\delta_H$) | COSY | TOCSY | NOESY | HMBC | HSQC-TOCSY |
|---|---|---|---|---|---|---|---|
| 1'' | 99.0, CH | 5.04 d (7.8) | H2'' | H3'' | | | H5'', H2'' |
| 2'' | 74.3, CH | 3.25 m | H1'' | | H2' | | H1'', H3'' |
| 3'' | 73.4, CH | 3.07 m | | H1'' | H1''' | | H2'', H4'' |
| 4'' | 68.5, CH | 3.10 m | | | | | |
| 5'' | 72.6, CH | 3.10 m | | | | | H6'', H1'' |
| 6'' | 62.8, $CH_2$ | 3.47, 3.48 dd (11.4, 7.5) | | | | | |
| 1''' | 108.8, CH | 4.75 s | | | H3'' | H3''', H4''' | |
| 2''' | 76.0, CH | 3.25 m | | | | H5''', H4''' | |
| 3''' | 79.3, C | | | | | H1''', 2 bond H4''', H5''' | |
| 4''' | 74.1, $CH_2$ | 3.26 m, 2.91 d (9.1) | | | | H1''', H4''', H5''', | |
| 5''' | 64.6, $CH_2$ | 2.94 d (11.2), 2.84 d (11.2) | | | | | |
| 1'''' | 170.2, C | | | | | 6'', 2'''' | |
| 2'''' | 68.8, $CH_2$ | 1.60 d (14.8), 1.48 d (15.0) | | | | H4'''' 2 bond | |
| 3'''' | 74.1, C | | | | | | |
| 4'''' | 46.0, $CH_2$ | 1.77 d, 1.66 d (13.5) | | | | H2'''' 2 bond | |
| 5'''' | 174.4, C | | | | | H4'''' 2 bond | |
| 6'''' | 27.3, $CH_3$ | 0.38 s | | | | | |

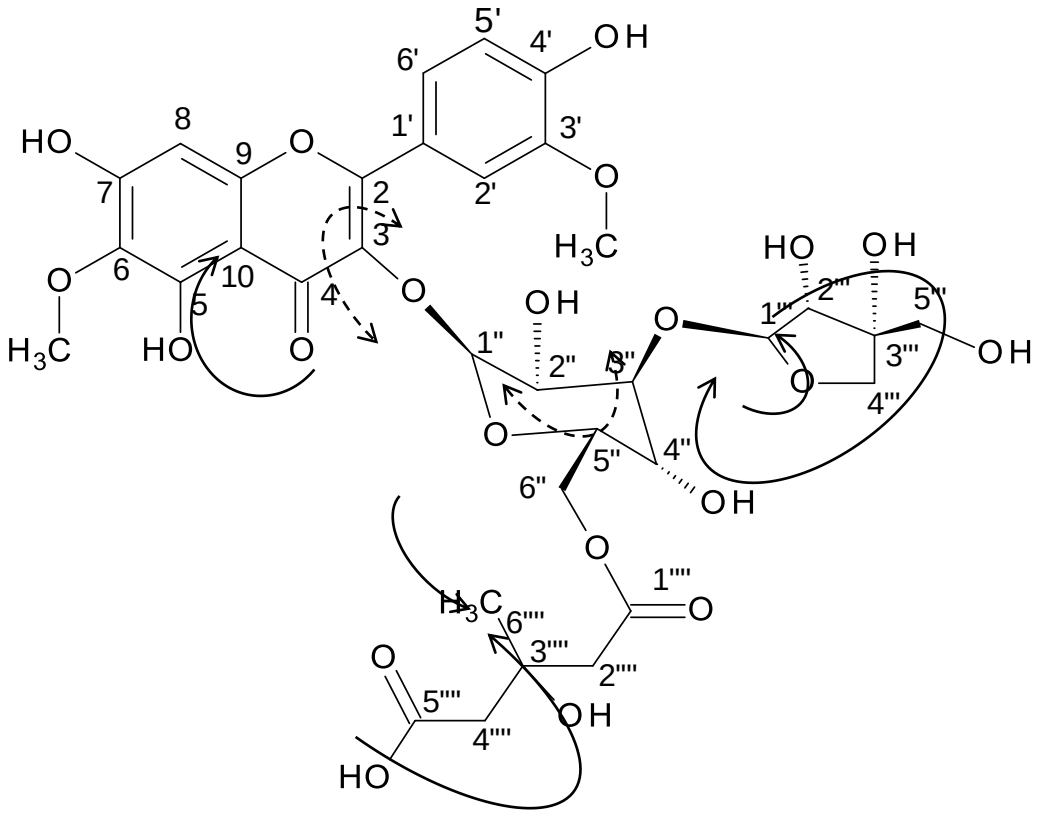


**Figure 4.2.** Pirieol A, Key HMBC ( ) and NOESY (- - -) correlations.

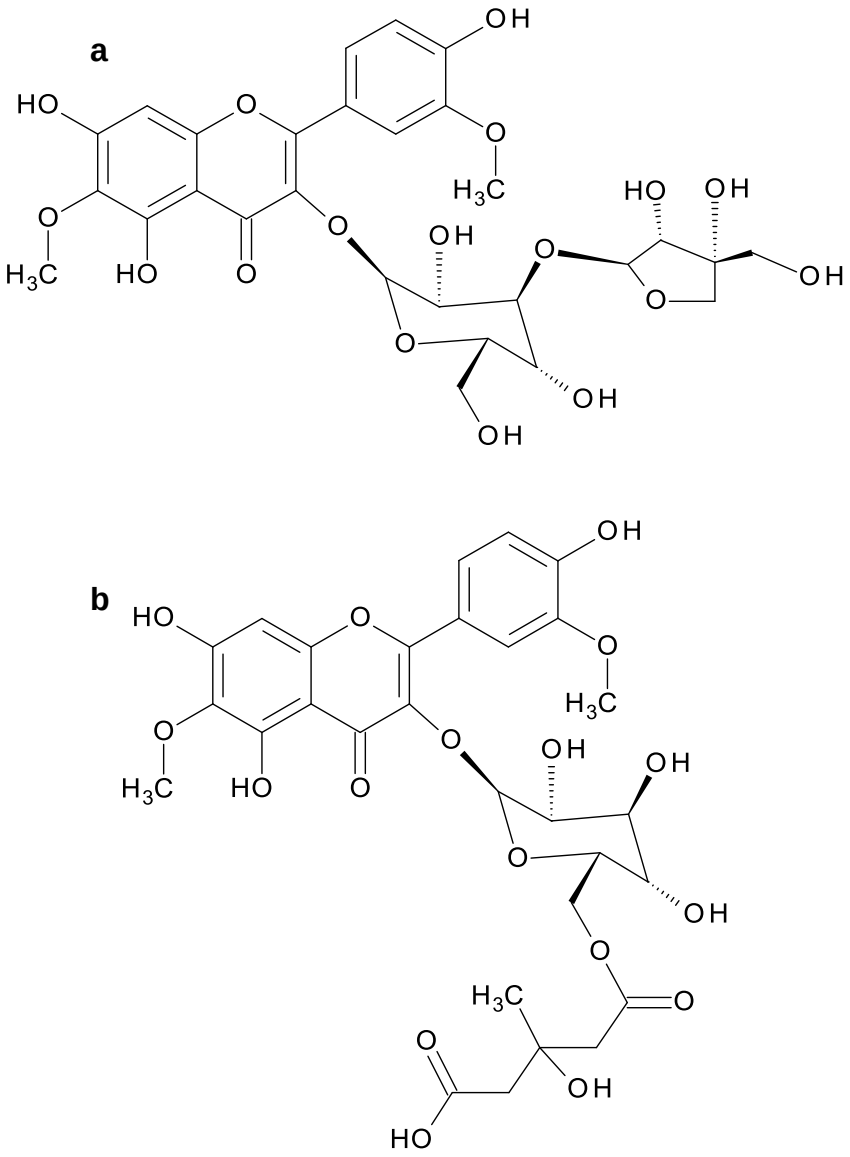


**Figure 4.3.** Proposed structures related to compound 1. (a) Compound 2, missing the HMG substituent and (b) Compound 3, missing the apiose substituent.

## 4.5 Acknowledgements

We would like to express our appreciation to Dr Ann Kwan (University of Sydney) for running the NMR experiments. Adam Pirie is the recipient of an Australian Postgraduate Award from the Australian Government.

# 5 Hypolipidaemic effect of crude extract from *Carpobrotus rossii* (pigface) in healthy rats

## 5.1 Abstract

*Carpobrotus rossii* (CR) was used by the Aboriginal population and early European settlers both as a food and therapeutic agent. Based on the presence of flavonoids in CR and results from our previous *in vitro* investigations, this study aimed to determine whether consumption of CR crude leaf extract: (a) affected lipoprotein profile, resting glucose, systolic blood pressure and vascular function, and (b) produced toxic effects (haematological measures, organ weight) in healthy rats. Male Hooded-Wistar rats (~230 g) were supplemented for 4 weeks with CR extract in their drinking water (35 mg/kg body weight daily). CR extract produced a significant decrease (18%, p=0.033) in atherogenic lipoproteins (but not high density lipoprotein). CR supplemented animals showed no signs of haematological toxicity and body and organ weight, daily fluid and food consumption and *in vitro* vascular responsiveness were similar for both groups. CR also increased (40%, p=0.049) the renal concentration of 3-hydroxy-3-methylglutaric acid (HMG), consistent with HMG-containing CR flavonoids being bioavailable, and therefore possessing the potential to interfere with cholesterol synthesis pathways. CR extract appears to be safe to ingest and may reduce cardiovascular risk.

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## 5.2

## Introduction

Carpobrotus species are large trailing succulent perennials which grow on the Pacific coast of the Americas, South Africa, and Australia. The fruit of *C.edulis* is eaten in South Africa, and *C.rossii* ((Haw.) Schwantes) has an extensive history of use as a food source by the Aboriginal population of Tasmania, Australia, in addition to anecdotal use for medicinal purposes (Plomley et al., 1966, Watson, 2007). The loss of native languages shortly after European settlement combined with the lack of indigenous written language(s) means that first-hand documented reports of CR's use (such as Plomley et al. (1966)) are exceedingly rare. Thus, information on the plant's historical use must largely be gleaned from other anecdotal sources.

We recently reported that extracts from this plant are pharmacologically active *in vitro* (Geraghty et al., 2011). The crude CR extract, inhibited platelet aggregation, inflammatory cytokine release (interleukin-1-beta, tumour necrosis factor-alpha) and lipid oxidation, effects if replicated *in vivo* reduce the risk of cardiovascular disease. Additionally, the plant has now been shown to produce several flavonoids that contain 3-hydroxy-3-methylglutaric acid (HMG) moieties (Jager, 2009), and as such may have the potential to interfere with cholesterol synthesis pathways via HMG-Coenzyme A (HMG-CoA) reductase inhibition, potentially acting as statins. HMG disrupts two key steps of cholesterol biosynthesis; it inhibits the conversion of acetoacetate to HMG-CoA and the reduction of HMG-CoA to mevalonic acid by HMG-CoA reductase (Moorjani and Lupien, 1977).

Polyphenolic plant compounds (especially flavonoids) have been shown to offer protection against atherosclerosis and metabolic disorder (hyperlipidemia, hyperglycaemia, hypercholesterolemia) related processes (Hooper et al., 2008). These compounds provide protection via a range of mechanisms including lowering the concentration of plasma non-HDL cholesterol, reducing serum lipid oxidation, lowering vascular resistance (Stoclet et al., 2004) and altering cellular inflammatory signalling pathways (Gomes et al., 2008). Benito et al., (2002) reported the vasorelaxant properties of several flavonoid compounds (quercetin, catechin and red wine polyphenols) and determined that this activity was due to an increase in $NO^{\cdot}$ bioavailability. As well as improving the response to vasodilators and reducing blood pressure, polyphenolic extracts inhibit absorption of glucose through the digestive tract

(Kobayashi et al., 2000) and lower fasting glucose levels (Chi et al., 2007). Polyphenolic compounds such as epigallocatechin and resveratrol derivatives have also been shown to improve muscle glucose uptake (Ueda et al., 2008).

Despite the benefits generally associated with their consumption, some flavonoids exhibit cytotoxicity or function as pro-oxidants at high concentrations (Li et al., 2008). Therefore, the potential toxicity of these compounds must also be assessed when determining whether their consumption produces health benefits.

Based on the presence of flavonoids in CR, this study aimed to determine whether consumption of CR crude leaf extract; (a) affected lipoprotein profile, resting glucose, systolic blood pressure and vascular function, and (b) produced toxic effects (haematological measures and organ weight) in healthy rats.

## 5.3 Materials and Methods

### *5.3.1 Materials and Reagents*

3-Hydroxy-3-methylglutaric acid (HMG), deuterated 3-hydroxy-3-methylglutaric acid (dHMG), acetylcholine (ACh), sodium nitroprusside (SNP) and noradrenaline (NA) were purchased from Sigma-Aldrich (MO, USA). Sodium pentobarbitone was purchased from Boehringer Ingelheim (NSW, AUS). All other standard reagents and materials were of analytical grade.

### *5.3.2 Preparation and flavonoid content of C. rossii extract.*

CR leaf homogenate was prepared as previously described (Geraghty et al., 2011). After filtration, the aqueous juice was filtered and loaded onto C18 silica gel, washed with water to remove salts, eluted with methanol:water (95:5) and dried under reduced pressure at 42°C using a rotary evaporator (Heidolph Instruments, BY, GER**).** Sample integrity was validated by comparing high performance liquid chromatography ultraviolet detection traces of the extract with samples from authenticated voucher specimens (HO 529461, HO 529462) held at the Tasmanian Herbarium (TAS, AUS). Once dry, the plant extract was reconstituted in water at 100 mg/ml. CR extract was found to contain a suite of flavonols; the main flavonols consisted of the spinacetin aglycone, LC-MS/MS data indicative of multiple acylated

flavonoid glycosides and consistent with the presence of an HMG substituent was generated (Appendix Chapter 5). Crude CR extract was found to consist of approximately 33% of flavonoids by weight, confirmed by HPLC-DAD. The detailed structure of the major CR HMG-containing flavonoid has been elucidated using detailed MS and NMR studies, and is the subject of chapter 4. UPLC MS, 270 nm and 370 nm chromatograms of the crude CR extracts are included in Appendix Chapter 5, Appendix Figures 5.4, 5.5

### *5.3.3 Animal treatment*

Animal experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes - 7$^{th}$ Edition (NHMRC, 2004), approved by the University of Tasmania's Animal Ethics Committee (approval number A10751). Individually-caged male Hooded-Wistar rats (n= 8 per group, each ~ 230 g) had a 7 day 'run-in period' during which time all animals had access to tap water and standard rat chow (9% fat, 22% protein, 65.8% carbohydrate, 3.2% crude fibre, total digestible energy 13.2 MJ/Kg; Ridley Agri Products, VIC, AUS) *ad libitum.* Animals were then given tap water with CR extract at an arbitrary dose of 0.25 mg/ml (treatment) or tap water (control) for 4 weeks whilst continuing on the standard rat chow diet. Animals had their body weight, food and fluid consumption recorded daily whilst systolic blood pressure (SBP) was recorded non-invasively on days 0, 9, 18 and 27 of supplementation by tail cuff method using an NIBP Controller (ADInstruments, NSW, AUS).

### *5.3.4 Blood and tissue collection*

Food was removed from animal cages the night prior to surgery (day 27). On the morning of surgery, animals were individually warmed under a heat lamp, injected with 60 mg/kg sodium pentobarbitone, had their common carotid artery cannulated and were exsanguinated into lithium heparin and ethylenediaminetetraacetic acid (EDTA) tubes. EDTA collected blood was used for full blood analysis using a Sysmex 1000i (Sysmex Corporation, Kobe, Japan), whilst the lithium heparin blood was transferred to Eppendorf ® tubes, centrifuged for 15 minutes at 850 g, divided into 100 µl aliquots and stored at -80$^{\circ}$C for subsequent analysis.

The urinary bladder, testes, kidneys, heart, lungs, liver and brain were immediately removed from each animal *post mortem,* individually weighed and stored at -80°C. Differences between haematological measures, and body and tissue weights of the two groups were used as surrogate markers of toxicity.

### *5.3.5 In Vitro vascular responsiveness*

Aortic tissue was prepared as described by Lexis et al., (2006). Briefly, the aortic arch and descending aorta were removed and immediately placed in modified Krebs-Henseleit solution (mM: NaCl 136.9, KCl 5.4, $MgCl_2$ 1.05, $NaH_2PO_4$ 0.42, $NaHCO_3$ 22.6, $CaCl_2$ 1.8, glucose 5.5, ascorbic acid 0.28 and $Na_2EDTA$ 0.05), cleared of adventitial tissue, sectioned into rings (3 x 4mm) and mounted in 5ml Radnoti organ baths (Grass Technology Incorporated, CA, USA). Leftover tissue was weighed, snap frozen and stored at -80°C. Vessel contractility was measured with Grass FT03 strain gauges (Grass Technology Incorporated, CA, USA) and recorded with Chart 4 software (ADInstruments, NSW, AUS). Cumulative concentration-response curves to noradrenaline (NA) were constructed. Endothelium dependant (sodium nitropruside, SNP) and independent (acetylcholine, ACh) relaxation was measured in aortic segments previously contracted with NA.

### *5.3.6 Lipid, cholesterol, glucose analysis*

Plasma glucose, triglycerides, cholesterol and high density lipoprotein (HDL) were measured by spectrophotometric enzymatic methods (Konelab 20XT, Thermo Fisher Scientific, VA, USA) using commercially available kits (Thermo Fisher Scientific, VA, USA), according to the manufacturer's instructions. It has been reported that the formula Friedewald et al., (1972) developed for estimating cholesterol (total cholesterol - (HDL-C + triglycerides/5)), has the potential to either over or underestimate LDL-C levels (Sanchez-Muniz and Bastida, 2008, Martin et al., 2013). All cholesterol not associated with HDL was classified as atherogenic cholesterol using the following formula; (total cholesterol – HDL cholesterol).

### *5.3.7 Determination of kidney HMG levels*

Kidneys were roughly macerated on ice using a surgical scalpel blade and then homogenised with 130 µl water per 100 mg kidney using a tissue grinder (Wheaton Scientific, NJ, USA). An aliquot of the tissue homogenate (575 µl) containing 250 mg of kidney tissue was transferred to an Eppendorf® tube, spiked with dHMG (225.6 ng/g tissue) and vortex mixed for 10 seconds. Glacial acetic acid (20 µl) was then added to inhibit protein binding and facilitate protein precipitation, the sample vortex mixed and centrifuged at 15000 g for 10 minutes. The supernatant was transferred to a high performance liquid chromatography sample vial with an Omix® C18 pipette tip (Varian, CA, USA) to remove lipids and subjected to ultra-performance liquid chromatography tandem mass spectrometric (UPLC-MS/MS) analysis (Aquity UPLC-Xevo triple quadrupole MS, Waters Corporation, MA, USA). Full LC and MS conditions are given as Appendix Chapter 5.

The ratio of deuterated to non-deuterated peak area was used to calculate the renal concentration of free HMG. This calculation was based on a standard curve ($R^2$= 0.9999, RSD=0.46%, accuracy=0.08%, n=6 at 600ng/g) generated using 'blank' kidney homogenates spiked with known HMG concentrations and prepared under identical conditions to the experimental samples. Accuracy (deviation from true value) of the HMG assay was determined as ((observed-expected/expected)/number of samples) * 100. Hence the lower the number the better the assay's performance

### *5.3.8 Statistical analysis*

SBP, *in vitro* aortic responses to NA, SNP and ACh were analysed using 2-way ANOVA, whilst all other parameters were analysed using an unpaired Student's t-test in Prism 6 (GraphPad Software, CA, USA). Outliers were detected using the ROUT method, Q set at 1 % in Prism 6. Results were considered statistically significant when $p<0.05$ and are presented as mean ± SEM.

## 5.4 Results

Animals consumed on average 35 ± 0.52 mg/kg body weight of CR extract per day (mg/kgBW/day). There were no differences in systolic blood pressure (P=0.280), post supplementation body weight (P=0.106), food intake (day 27, P=0.308) or fluid consumption (day 27, P=0.106) between the CR supplemented and control groups (Figure 5.1).

There were no differences in the weight of any weighed internal organs or blood parameters between the two groups (Table 5.1).

**Table 5.1.** Organ weights of control (water) and *C. rossii* supplemented (CR) animals (mean ± SEM).

| Organ weight (mg/100g body weight) | Water (n= 8) | CR (n= 8) | P value |
|---|---|---|---|
| Bladder | 26 ± 2 | 29 ± 3 | 0.436 |
| Testes | 913 ± 27 | 883 ± 26 | 0.453 |
| Kidneys | 780 ± 17 | 750 ± 24 | 0.334 |
| Liver | 4420 ± 62 | 4521 ± 125 | 0.482 |
| Heart | 275 ± 4 | 276 ± 6 | 0.930 |
| Spleen | 186 ± 6 | 182 ± 8 | 0.733 |
| Lungs | 367 ± 9 | 336 ± 26 | 0.283 |

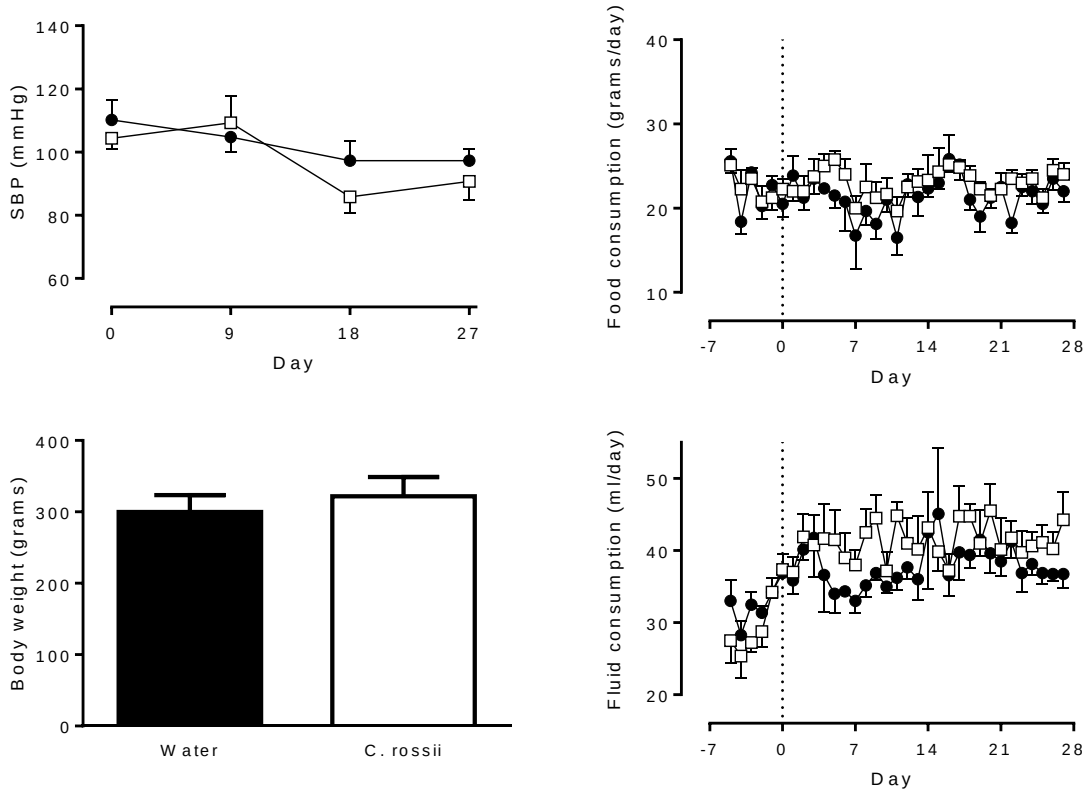


**Figure 5.1.** Control (water) and *C. rossii* extract supplemented animals showed no significant difference (P<0.05) in (a) systolic blood pressure (SBP), (b) food consumption, (c) post-supplementation body weight or (d) fluid Consumption. (Mean ± SEM, n= 8 per group).

Table 5.2 shows total cholesterol was lower in the CR-supplemented rats compared with controls (~14% reduction, P=0.027), but other blood chemistry parameters including HDL and triglycerides were similar. Non-HDL cholesterol concentrations were also lower (~18%, P=0.033) in CR treated animals.

**Table 5.2.** Full blood analysis and blood biochemistry of control (water) and *C. rossii* supplemented (CR) animals (mean ± SEM).

| | Water (n= 8) | CR (n= 6) | P value |
|---|---|---|---|
| RBC ($10^{12}$/L) | 8.72 ± 0.33 | 8.48 ± 0.10 | 0.540 |
| HGB (g/L) | 153 ± 6 | 150 ± 2 | 0.712 |
| HCT % | 0.44 ± 0.12 | 0.44 ± 0.01 | 0.810 |
| MCV (fL) | 50.9 ± 0.58 | 51.8 ± 0.65 | 0.346 |
| MCH (pg) | 17.6 ± 0.14 | 17.7 ± 0.14 | 0.387 |
| MCHC (g/L) | 300 ± 43 | 343 ± 3 | 0.419 |
| Platelets ($10^9$/L) | 995 ± 27 | 967 ± 46 | 0.604 |
| WBC ($10^9$/L) | 4.49 ± 0.44 | 4.46 ± 0.54 | 0.974 |
| Neutrophils ($10^9$/L) | 0.77 ± 0.23 | 0.59 ± 0.25 | 0.616 |
| Lymphocytes ($10^9$/L) | 3.14 ± 0.38 | 2.57 ± 0.67 | 0.447 |
| Monocytes ($10^9$/L) | 0.28 ± 0.10 | 0.29 ± 0.08 | 0.904 |
| Eosinophils ($10^9$/L) | 0.07 ± 0.02 | 0.09 ± 0.02 | 0.467 |
| Basophils ($10^9$/L) | 0.12 ± 0.12 | 0.16 ± 0.10 | 0.804 |
| Cholesterol (mg/ml) | 1.94 ± 0.09 | 1.67 ± 0.07* | 0.027 |
| HDL(mg/ml) | 0.66 ± 0.04 | 0.62 ± 0.04 | 0.540 |
| Triglycerides (mg/ml) | 0.85 ± 0.24 | 0.97 ± 0.18 | 0.701 |
| Glucose (mmol/L) | 11.2 ± 1.6 | 11.7 ± 1.3 | 0.819 |
| Atherogenic cholesterol (mg/ml) | 1.29 ± 0.05 | 1.05 ± 0.08 * | 0.033 |

The effects of NA, SNP and ACh on aortic rings from control and CR-treated rats are presented in Table 5.3 and Figure 5.2. Concentration-response curves for NA, SNP and ACh were similar for both groups of rats and no statistically significant differences in $EC_{50}$ values were obtained.

**Table 5.3**. $EC_{50}$'s and 95% confidence intervals (CI) for noradrenaline (NA)-, sodium nitroprusside (SNP)- and acetylcholine (ACh)-induced *in vitro* vascular (aortic) responses from control (water) and *C. rossii* (CR) treated animals.

| **Agonist** | **Water** | **CR Extract** |
|---|---|---|
| | **$EC_{50}$ (nM, 95% CI)** | **$EC_{50}$ (nM, 95% CI)** |
| NA | 45 (36 to 55) | 48 (39 to 59 ) |
| SNP | 35 (22 to 54) | 52 (28 to 98) |
| ACh | 116 (51 to 265) | 213 (146 to 312) |

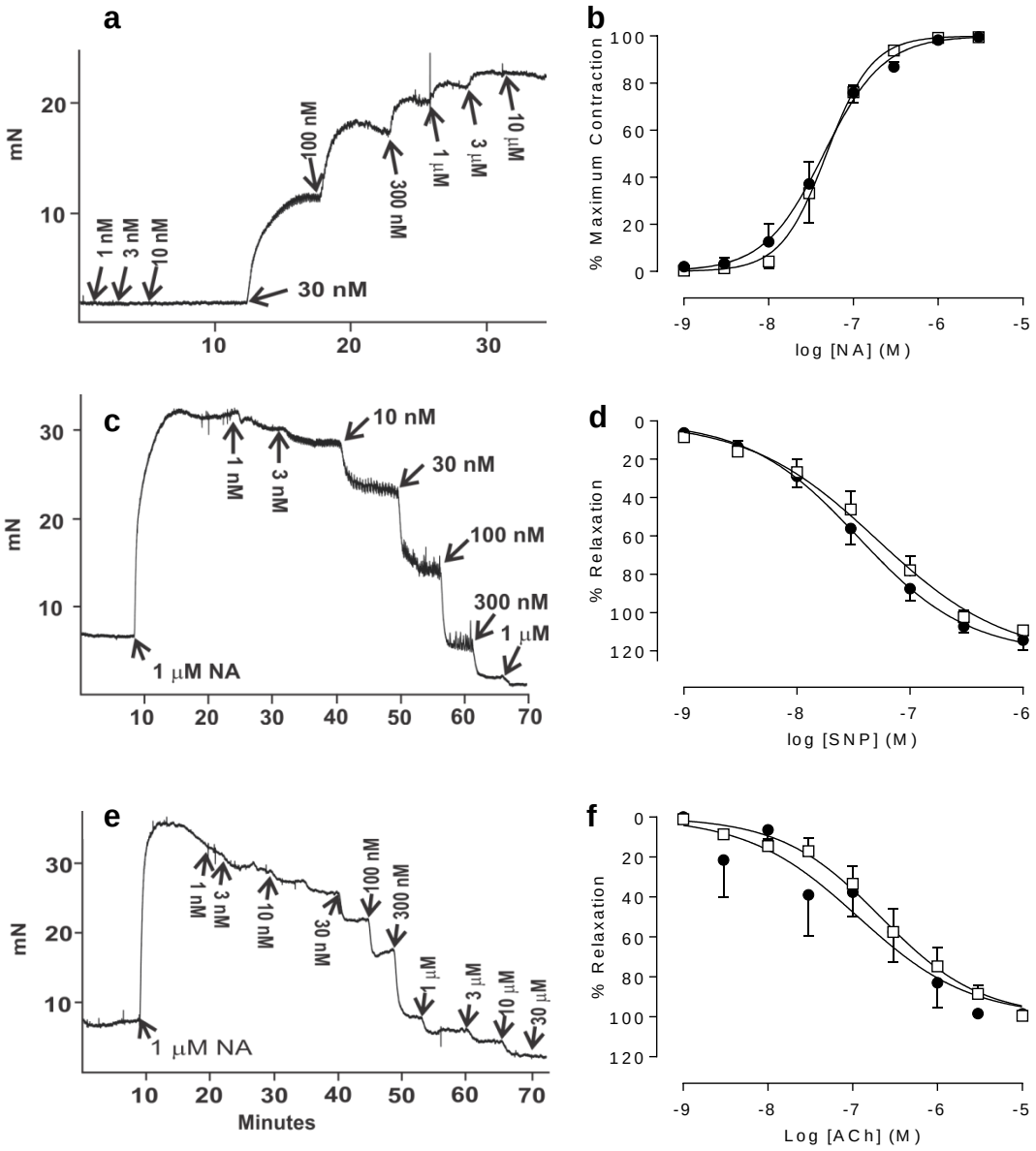


**Figure 5.2.** Representative plots and Mean ± SEM vascular response of control ●(water) and *C. rossii* □ supplemented animals to (a, b) noradrenalin (NA) (n= 7, 5); (c, d) sodium nitroprusside (SNP) (n= 7, 6) and (e, f) acetylcholine (ACh) (n= 4, 6).

LC-MS/MS analysis of renal tissue showed that free HMG levels were higher (~40%, P= 0.049) in CR supplemented animals compared with controls (Figure 5.3), consistent with the presence of a flavonoid HMG substituent as suggested by LC-MS/MS analysis of the CR extract.

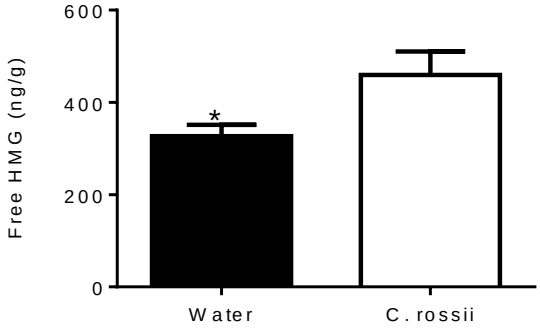


**Figure 5.3.** Level of free HMG in the kidney tissue of control (water) and *C. rossii* supplemented rats. Concentration is given as ng/g of kidney tissue (Mean ± SEM, n= 6, 7 per group).

## 5.5 Discussion

Comparison of body and organ weights, and haematology did not show any indications of toxicity at 35 mg/kgBW/day over the duration of treatment. Previous toxicology studies conducted with flavonoid compounds have produced a spectrum of results ranging from gossypol supplemented rabbits (20 mg/kgBW/day) which lost body weight, developed muscle paralysis and breathing difficulties (Saksena et al., 1981) through to grape seed extract doses of up to approximately 1600 mg/kgBW/day (male) and 1900 mg/kgBW/day (female) in rats which resulted in no mortalities and only a minor reduction in serum iron levels recorded after treatment for 90 days (Wren et al., 2002). Morita et al., (2009) reported levels of safety in rats for the green tea catechins of 1200 mg/kgBW/day (male) and 400 mg/kgBW/day (female) as this was the point where reduced body weight gain was observed. In the present study, there was no change in food consumption of CR supplemented rats, unlike the increased food consumption previously reported in grape seed extract flavonoid supplemented animals (Wren et al. (2002). The extensive haematological data was almost identical for controls and CR treated rats, suggesting no gross detrimental effects on either erythrocyte or immune system function.

Pharmacologically, the most interesting findings from this study were the lowering of both total and non-HDL cholesterol in CR treated rats as well as an increase in kidney HMG levels. This is despite all animals being on a normal (non pro-atherogenic) diet. Debate continues as to whether it is LDL-C alone, the chylomicron fraction, or all cholesterol not associated with HDL that is responsible for inducing atherosclerosis (Tomkin, 2010). As such, for this study we have taken a conservative view and treated all cholesterol not associated with HDL as atherogenic. Kidney tissue was specifically targeted for HMG analysis as it is the primary site of HMG accumulation and storage *in vivo* (Savoie and Lupien, 1975). The increased HMG in the kidney after CR may have been due to glycoside hydrolysis of the HMG-containing flavonoids during digestion (Walle, 2004), or possibly by indirect effects on HMG-CoA reductase activity.

HMG-containing flavonoids are not common and most studies have reported cholesterol lowering activity and other beneficial cardiovascular effects from flavonoids that do not contain HMG (Hooper et al., 2008). This activity has been shown to be due to both inhibition of acyl-CoA cholesterol acyltransferase and HMG-CoA reductase (Bok et al., 1999). Surprisingly, the effects of flavonoid-conjugated HMG on HMG-CoA reductase activity (and hence cholesterol synthesis) is poorly described in the literature. However, obese patients supplemented with extracts from *Rosa spp* (shown to contain HMG (Porter et al., 2012)) showed an improved plasma cholesterol profile (Andersson et al., 2012), and the administration of free HMG (750-3000 mg per day) decreased total cholesterol (11-13%) and LDL levels (8%) over an eight week period (Lupien et al., 1979). The lowering of cholesterol observed in the present study is potentially due to a combination of flavonoid and HMG mediated effects.

We did not observe any flavonoid mediated effects on other cardiovascular risk factors such as glycaemic control or vascular health (systolic blood pressure and vessel reactivity). Many factors could explain these finding; size of effect, dosing regimen and duration of treatment, using a crude versus purified extract (Van Dam et al., 2013), but most likely the explanation lies in the healthy animals used in the present study which contrasts with disease models used in previously published studies. A lack of improvement in the vascular function of healthy subjects has been reported previously by Baer et al., (2011) and Van Mierlo (2010) who

found that supplementing the diet of healthy adults did not improve glucose metabolism, despite these same flavonoids (catechins and resveratrol) showing high levels of activity when introduced to diseased subjects (Kang et al., 2012, Nagao et al., 2009).

One potential risk that needs to be considered regard CR extract's safety is it's potential statin-like activity. Statin compounds can induce myalgia, myopathy and rhabdomyolysis in a small percentage of patients but the exact mechanism is unknown (Toth et al., 2008). Nevertheless, CR extract possesses cholesterol lowering activity, and whilst this effect may be due to HMG-CoA inhibition or other mechanisms, it may present a safer alternative to conventional statin therapy.

## 5.6 Conclusion

CR extract improved the lipoprotein profile, but had no effects on other measured cardiovascular parameters in rats. There were no gross anatomical or haematological changes to indicate CR extract was toxic at the level and duration tested. CR increased the renal concentration of HMG consistent with CR flavonoid bioavailability, and therefore a potential for either flavonoid or HMG mediated interference with cholesterol synthesis pathways. As consumption of CR extract appears to be safe and may reduce cardiovascular risk, further work needs to be undertaken to validate the cardioprotective properties of CR extracts in disease models and determine which of the compound(s) impart this protection.

## 5.7 Acknowledgements

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# 6 Flavonoids from *Carpobrotus rossii* improve glucose clearance in insulin resistant animals

## 6.1 Abstract

We recently reported that a crude extract from the plant *Carpobrotus rossii* (CR) improves the lipid profile of rats fed a normal (standard) diet. The aims of this pilot study were to determine whether (a) CR extract(s) improved glucose tolerance under conditions of insulin resistance, (b) the previously observed improvements in lipid profile were maintained under hyperlipidaemic conditions, and (c) effects could be attributed to specific components of the CR extract, or only to the unfractionated crude extract. C57/BL6 mice on a high fat diet were used to model insulin resistance, whilst Sprague-Dawley rats were fed a high fat, high cholesterol diet to induce hyperlipidaemia. The diets of both rats and mice were supplemented with either a crude CR extract or a flavonoid-rich, fractionated CR extract in drinking water, in addition to healthy and unhealthy controls. In the hyperlipidaemic rat experiment a commercially prescribed treatment group (simvastatin) was also ran. The flavonoid rich fractionated extract significantly ($P<0.05$) lowered blood glucose of insulin resistant mice 45, 60 and 90 minutes post glucose challenge, and normalised glucose clearance to that of the normal healthy diet group. This effect was not observed in animals supplemented with crude CR extract. Neither supplementation with the CR extracts, or simvastatin improved the lipid profile in high fat fed rats. The flavonoid-rich extract of CR appears to have activity against the insulin resistance of metabolic syndrome. Further investigation of the improved glucose clearance and the potential lipid lowering activity is warranted.

## 6.2 Introduction

Obesity, type II diabetes, and the other disease states associated with metabolic syndrome (hypertension, hyperlipidaemia, stroke) continue to be the leading cause of human morbidity and mortality (WHO, 2008). Despite considerable research and interventions over many years, their prevalence continues to rise. Obesity and hyperlipidaemia impact vascular health, most notably through impaired insulin signalling (leading to inhibited glucose clearance, elevated blood glucose and progression to type II diabetes) and atherosclerosis or stroke, associated with damage to the vascular wall due to high lipid levels (Cersosimo and DeFronzo, 2006). As such, significant research effort has been expended to identify new therapies that treat one or several of the disease states associated with obesity and metabolic syndrome.

We have recently shown that a crude extract of the Australian native plant, *Carpobrotus rossii* (CR), improves the lipid profile of rats fed a normal diet by lowering the concentration of non-high-density lipoprotein (Chapter 5). Among the suite of phytochemicals that this plant produces are high concentrations of novel flavonoid compounds. Flavonoids extracted from other plants have been reported to exhibit activity against multiple facets of metabolic syndrome. They may activate the phosphatidylinositol 3-kinase (PI3K) pathway independently of insulin, leading to endothelial nitric oxide synthase (eNOS) activation and increased nitric oxide ($NO^{\cdot}$) production (Zhou and Liao, 2010). Additionally, they may increase the expression and activity of glucose transporter 4 in muscle tissue which is responsible for ~75% of glucose uptake in the post-prandial state (Ueda et al., 2008, Cao et al., 2007, DeFronzo, 1988), and inhibit the activity of oxidase enzymes which produce superoxide, in turn inhibiting peroxynitrite formation ($ONOO^{-}$) (Sanchez et al., 2006). These actions lead to an increase in $NO^{\cdot}$ bioavailability and a subsequent improvement in vascular health.

Flavonoids have also been shown to impart protection against hyperlipidaemia. Shabrova et al., (2011) reported that a flavonoid rich extract from cranberries improved both the insulin signalling and lipid profile of mice fed a high fat diet. The flavonoids, naringin, hesperidin and epigallocatechin-3-gallate (EGCG), have been reported to inhibit acyl CoA:cholesterol O-acyltransferase (ACAT) and 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA

reductase) activity (Bok et al., 1999, Cuccioloni et al., 2011). In addition to their flavonoid structure, which may enable inhibition of enzymes involved in lipid manufacture and transport such as HMG-CoA reductase and ACAT (Bok et al., 1999), several CR flavonoids possess a 3-hydroxy-3-methylglutaric acid (HMG) substituent. Free HMG is a known HMG-CoA reductase inhibitor (Lupien et al., 1973), and many commercial statins (HMG-CoA reductase inhibitors) possess a HMG-like moiety (Martinez-Gonzalez and Badimon, 2007). Conjugation of flavonoids with an HMG moiety is not common, although they have been reported in extracts from *Chamaemelum nobile* (Konig et al., 1998), namely the flavonoid chamaemeloside, *Oxytropis falcata* which is used in traditional Tibetan medicine (Wang et al., 2012), and *Rosa spp* (Porter et al., 2012).

A small trial in 32 obese patients supplemented with *Rosa canina* extract demonstrated improvement in plasma cholesterol levels (Andersson et al., 2012), whilst chamaemeloside was shown to have an *in vivo* hypoglycaemic activity comparable with free HMG (Konig et al., 1998). Thus, there exists the potential for CR flavonoids to lower blood lipids by increasing the *in vivo* concentration of HMG (as recently reported by our group in Chapter 5) or other mechanisms. Based on the activity of chamemeloside, there is also the potential that the CR compounds may have hypoglycaemic activity.

In addition to lowering cholesterol, statins have several pleiotropic effects, particularly on the vasculature. These effects appear to be related to increased $NO^{\cdot}$ availability due to improved eNOS coupling, inhibition of oxidase enzymes (less $ONOO^{-}$ production), increased eNOS phosphorylation (Antoniades et al., 2011, Martinez-Gonzalez and Badimon, 2007), and reduced eNOS translocation away from cellular membranes under high lipid conditions (Blair et al., 1999).

Based on reports centering on the hypoglycaemic and/or hypolipidaemic activity of other flavonoid compounds, and data generated by our group using CR extract in healthy rats, we expect that the CR flavonoids may have potential to treat of type II diabetes and/or hyperlipidaemia. The aims of this pilot study were to determine whether CR extract(s); (a) improved glucose tolerance in a model of insulin resistance, (b) improved the lipid profile of hyperlipidaemic animals as they have been previously observed to in healthy animals, and (c) whether any effects observed were due specifically to CR flavonoids or required consumption of the entire CR extract.

## 6.3 Materials and Methods

### *6.3.1 Materials and Reagents*

Whatman #1 filter paper (Whatman, Kent, UK), methanol (MeOH), Sephadex LH20 (Sigma-Aldrich, St Louis, USA), simvastatin (Sigma-Aldrich, St Louis, USA), trichloro(octadecylsilane) (ODS) (Sigma-Aldrich, St Louis, USA), trimethylchlorosilane (TMS) (Sigma-Aldrich, St Louis, USA), dichloromethane ($CH_2Cl_2$) (Sigma-Aldrich, St Louis, USA), and silica gel (Sigma-Aldrich, St Louis, USA) were purchased from the companies indicated. All other reagents and materials were of analytical reagent AR grade or higher.

### *6.3.2 Preparation of crude and flavonoid-rich C. rossii extracts*

Preparation of C18 was essentially as per Evans et al., (1981) with the major change being the replacement of carbon tetrachloride with $CH_2Cl_2$. In brief, 15 grams of ODS was dissolved in 600 ml of $CH_2Cl_2$, and reacted with 150 grams of silica at room temperature for 2 hours with constant stirring. The silica was then filtered with Whatman #1 filter paper, washed under vacuum three times with 100 ml of $CH_2Cl_2$ followed by two washes of 100 ml MeOH, and a further 2 × 100 ml $CH_2Cl_2$ and dried. 20 ml of TMS was then added to 600 ml $CH_2Cl_2$, and the silica added and reacted for a further 2 hours with constant stirring at room temperature. The silica was refiltered and unreacted TMS was then eluted with 2 × 250 ml $CH_2Cl_2$ before being allowed to air dry overnight.

Sufficient CR leaf material was taken from cultivated plants previously collected during the work reported in chapter 2 to produce approximately 40 litres of crude juice extract. This leaf material was homogenised using a commercial food blender, filtered through cheesecloth, refrigerated overnight at 4°C (to allow the settling of leaf chloroplasts) and then filtered through Whatman #1 filter paper. A slurry of C18 silica was prepared and added to 2L aliquots of the filtered extract to allow the adsorption of non-polar compounds. The C18 silica was then filtered out, washed with approximately 500 mL of distilled water to elute residual salt, and polar compounds. Less polar compounds were then eluted with 500 mL 95% MeOH/5% $H_2O$ and the process repeated until all of the filtered crude extract had been processed.

The 95% MeOH elution (CR crude) was then freeze dried under reduced atmosphere to powder. To ensure sample homogeneity, the powder was hand ground using a mortar and pestle and a large sub-sample (approximately 1/3) reconstituted in AR grade MeOH for further fractionation. Aliquots were applied to a Sephadex LH20 column (500 mm ×20 mm ID) and eluted with AR grade MeOH. The LH20 fractionation process was repeated until the reconstituted sample had been processed. Twenty five millilitre eluent fractions were collected, diluted 1:100 and analysed using high-performance liquid chromatography with diode array detection (HPLC-DAD) monitoring 350 nm for the elution of the main flavonoid peaks. The flavonoid rich fractions from each fractionation run were combined and dried as per the 95% MeOH/C18 elution before being hand ground once again to ensure sample homogeneity.

Samples of the LH20 (CR flavonoid rich) and 95% MeOH C18 elutions (CR crude) were weighed and redissolved in distilled water to generate solutions of identical mg/ml flavonoid concentration. Flavonoid peak areas were analysed by HPLC-DAD to determine the concentration effect that LH20 purification produced and ensure consistent flavonoid dosing across the different treatment groups. Essentially, the average flavonoid peak size was increased approximately 1.8 times by LH20 purification and hence the mg/kg dosing of animals on the flavonoid rich dosing was in turn reduced by this amount.

### *6.3.3 Animal models*

All animal experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 2013 - 8th Edition (2013) and approved by the University of Tasmania Animal Ethics Committee (approval number A0011682)

#### *6.3.3.1 Animal Housing*

Animals were housed 2 or 3 per cage. Food and water consumption was monitored for each cage, individual animal weights were recorded every 3-4 days at which time the water (or water plus extract) and food was replaced.

#### *6.3.3.2 Insulin resistance model (high fat diet C57/BL6 mice)*

C57/BL6 male mice were fed a high fat diet (HFD) (22% wt/wt, SF12-050, Specialty Feeds, Glen Forest, AUS) or a normal control diet (NDM) (9% fat wt/wt, Barastoc Stockfeed, Pakenham, AUS) for four weeks. CR (crude and flavonoid rich) was administered at a flavonoid dose equivalent to 70 mg/kg/day of crude extract. Treatment groups consisted of i) normal healthy controls (NDM), ii) insulin resistant (HFD), iii) HFD supplemented with CR crude (HFD + Crude) and iv) HFD supplemented with CR flavonoid rich (HFD + FLAV). The macronutrient composition of the diets is shown in table 6.1.

**Table 6.1.** Macronutrient dietary composition of insulin resistant (HFD) and normal (NDM) diets used in insulin resistant C57/BL6 mice.

| | HFD | NDM |
|---|---|---|
| Protein | 19.0 % | 22.0 % |
| Carbohydrate | 53.7 % | 65.8 % |
| Fat | 22.6 % | 9.0 % |
| Total Mono-unsaturated Fats | 38.9 % | 36.6 % |
| Total Poly-unsaturated Fats | 9.2 % | 42.1 % |
| Total Saturated Fats | 51.9 % | 20.4 % |
| Crude Fibre | 4.7 % | 3.2% |
| Total Digestible Energy | 19.9Mj/Kg | 13.2MJ/Kg |

At 28 days, a glucose tolerance test was performed on the mice (Andrikopoulos et al., 2008). Briefly, they were fasted for 6 hours, resting blood glucose measured from a tail snip, and the animals injected with a 30% w/v glucose solution to provide a dose of 2g glucose/kg body weight intraperitoneally. Blood glucose was then measured at 15 minute intervals for the first hour and at 30 minute intervals for the remaining hour. Measurements were performed with an Accuchek® Performa Nano blood glucose monitor (Roche Diagnostics, Castle Hill, AUS).

#### *6.3.3.3 Hyperlipidaemia model (high cholesterol diet rats)*

To mimic the alteration in lipid profile seen in humans with metabolic syndrome, male Sprague-Dawley rats weighing approximately 150 grams were placed on a hyperlipidaemic diet for 4 weeks.

Again, CR (crude and flavonoid rich) was administered at a flavonoid dose equivalent to 70 mg/kg/day of crude extract. The hyperlipidaemia (HL) inducing diet consisted of 10% fat, 2% cholesterol (SF09-090, Specialty Feeds, Glen Forest, AUS) and was supplemented with either CR crude (HL + Crude), CR flavonoid rich (HL + FLAV) or 2mg simvastatin/kg body weight (HL + SIMV). This dose of simvastatin has been shown to produce ~99% inhibition of HMG-CoA reductase in rats (Chao et al., 1991). In addition to these treatment groups, a 5% fat wt/wt healthy (NDR) diet (SF00-229, Specialty Feeds, Glen Forrest, AUS) and hyperlipidaemic (HL) control groups were included (n= 5 for all groups). The macronutrient profile of these diets is shown in table 6.2.

**Table 6.2** Macronutrient dietary composition of hyperlipidaemic (HL) and normal (NDR) diets used in hyperlipidaemic Sprague-Dawley rats.

| | NDR | HL |
|---|---|---|
| Protein | 19.4 % | 19.6 % |
| Carbohydrate | 70.7 % | 65 % |
| Fat | 4.8 % | 10.00 % |
| Total Mono-unsaturated Fats | 38.5 % | 46.1 % |
| Total Poly-unsaturated Fats | 43.8 % | 33.4 % |
| Total Saturated Fats | 17.3 % | 18.5 % |
| Cholesterol | - | 2.0 % |
| Crude Fibre | 5.1 % | 3.4 % |
| Total Digestible Energy | 14.0 MJ/Kg | 15.0 MJ/Kg |

#### *6.3.3.4 Blood collection and measurement*

Two days after the glucose clearance experiments, mice were fasted overnight and a lethal dose of sodium pentobarbitone administered. Blood was obtained by cardiac puncture using heparinised syringes, centrifuged at 13000 g for 5 minutes in micro-centrifuge tubes and the plasma frozen. The same procedure of overnight fasting, administration of a lethal dose of sodium pentobarbitone and collection of blood via cardiac puncture was used to terminate the rat experiment after 4 weeks.

Blood samples from mice and rats were analysed for serum total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides, and plasma glucose on a Konelab 20XT autoanalyser (Thermo Fisher Scientific, Scoresby, Victoria, Australia) using Thermotrace reagents (Thermo Fisher Scientific).

### *6.3.4 Statistical analysis*

Results were analysed using Prism 6 (GraphPad Software, CA, USA). Statistical comparisons employed were one and two way ANOVA followed by Fishers' post-hoc test. $P<0.05$ was considered statistically significant. Power calculations to assess the likelihood of a type II error were performed on the rat data using the mean and standard deviations of the HL and HL + SIMV groups. These calculations were undertaken using StatMate 2.0 (GraphPad Software, CA, USA).

## 6.4 Results

### *6.4.1 Insulin resistant (C57/BL6) mice*

#### *6.4.1.1 Body weight gain, food and water consumption*

No differences in weight gain, water consumption or daily energy consumption were detected between any group (Figures 6.1a-c).

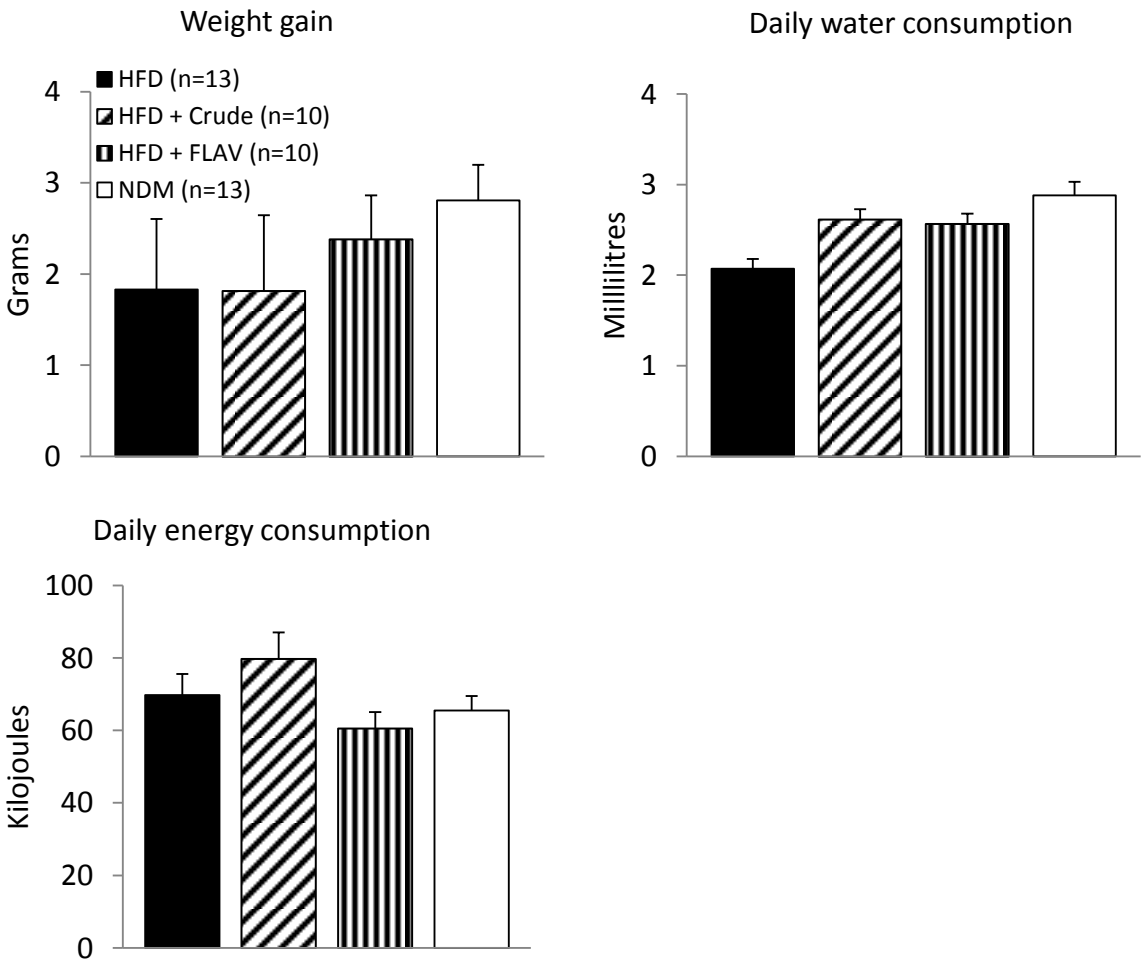


**Figure 6.1.** (a) Animal weight gain, (b) daily water consumption, and (c) daily energy consumption of C57/BL6 mice. All data expressed as mean ± SEM and analysed using one-way ANOVA followed by Fisher's LSD post-hoc test.

### *6.4.1.2 Glucose clearance*

Supplementation of drinking water with the CR flavonoid rich fraction improved glucose clearance compared to HFD. Blood glucose levels were lower in the HFD + FLAV compared to HFD mice at 45, 60 and 90 minutes after glucose administration (P=0.023, 0.047, 0.050, respectively). There was no significant difference between HFD + FLAV and NDM animals. In contrast to supplementation with the CR flavonoid rich fraction, HFD + CR crude mice showed no improvement in glucose clearance compared to the HFD animals (Figure 6.2).

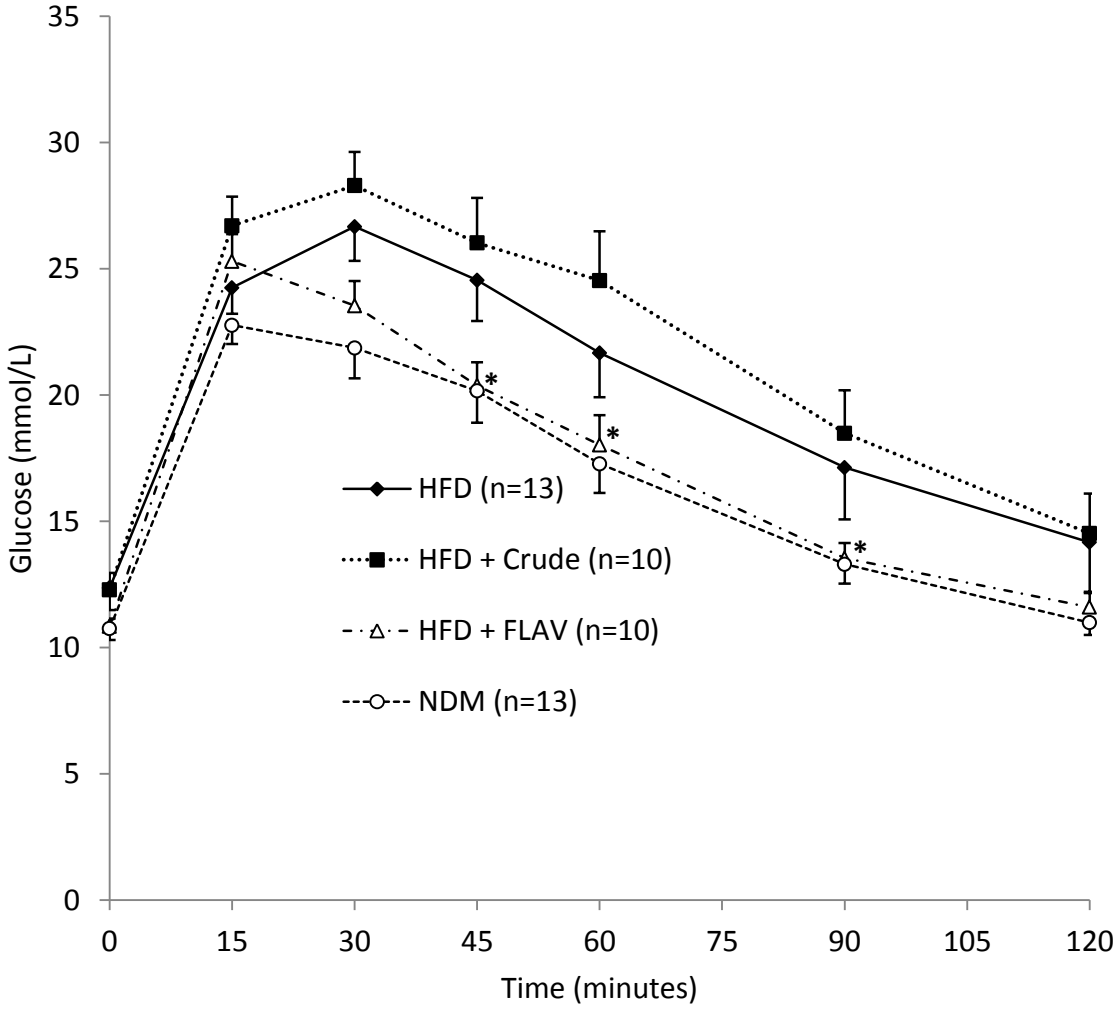


**Figure 6.2.** Plasma glucose concentration following a 2mg/kg ip bolus injection of glucose (*=P<0.05). All data are expressed as mean ± SEM and were analysed using a two-way ANOVA followed by Fisher's LSD post-hoc test.

#### *6.4.1.3 Blood lipids*

There were no differences in total cholesterol, HDL, LDL, LDL:HDL and triglycerides between any treatment group (Table 6.3).

**Table 6.3.** Blood lipids of C57/BL6 mice. Data expressed as mean ± SEM. Analysed using one-way ANOVA with Fisher's LSD post-hoc test and NDM as control. There were no statistically significant differences between treatments.

| **mmol/L** | **HFD Control (n=5)** | **HFD + CR crude (n=5)** | **HFD + CR Flavo rich (n=5)** | **NDM (n=13)** |
|---|---|---|---|---|
| Total Cholesterol | 2.61 ± 0.26 | 2.46 ± 0.26 | 2.71 ± 0.10 | 2.32 ± 0.09 |
| HDL | 2.24 ± 0.21 | 2.13 ± 0.22 | 2.33 ± 0.08 | 2.07 ± 0.07 |
| LDL | 0.27 ± 0.05 | 0.25 ± 0.06 | 0.30 ± 0.04 | 0.19 ± 0.02 |
| LDL:HDL | 0.11 ± 0.01 | 0.11 ± 0.02 | 0.13 ± 0.014 | 0.09 ± 0.01 |
| Triglycerides | 0.22 ± 0.04 | 0.20 ± 0.05 | 0.25 ± 0.12 | 0.22 ± 0.02 |

### *6.4.2 Hyperlipidaemic rats*

#### *6.4.2.1 Body weight gain, food and water consumption*

No differences in final body weight, weight gained during the experiment or daily energy consumption between treatment groups were detected (Figure 6.3a-c). However, an increase in the fluid consumption of HL + FLAV (~3.5mL) and HL + SIMV (~4.0mL) was observed (Figure 6.3 d, P=0.016, 0.006, respectively).

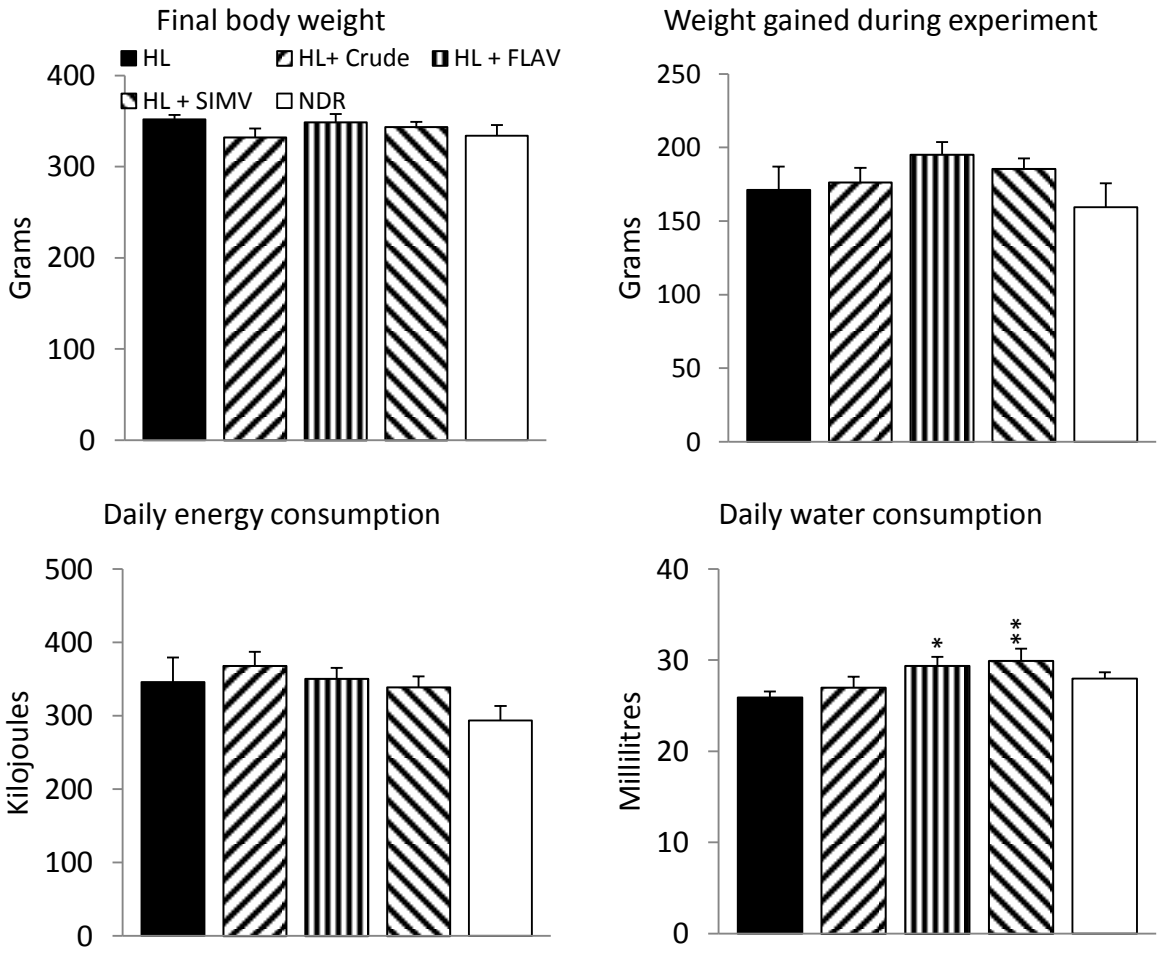


**Figure 6.3.** (a) Final body weight, (b) weight gained during experiments, (c) daily energy consumption, and (d) daily water consumption of hyperlipidaemic Sprague-Dawley rats (*=P<0.05, **=P<0.01). n= 5 for all groups, all data expressed as mean ± SEM and analysed using one-way ANOVA followed by Fischer's LSD post-hoc test.

### *6.4.2.2 Blood lipids*

Significant differences in HDL and LDL:HDL were only observed between the HL and NDR groups (Table 6.4, P<0.001 and P=0.017, respectively). LDL levels were trending lower in the NDR group (P=0.062). Supplementation of the HL diet with either CR crude, CR flavonoid rich or simvastatin failed to significantly alter blood lipids. Despite this, a trend towards lower LDL:HDL, LDL and total cholesterol was observed for both the simvastatin and CR flavonoid rich treatments.

**Table 6.4.** Blood lipids of Sprague-Dawley rats. Data expressed as mean ± 95% CI. Analysed using one-way ANOVA with Fisher's LSD post-hoc test with HL as the control. (*=P<0.05, ***=P<0.001).

| mmol/L | HL (n=5) | HL + Crude (n=5) | HL + FLAV (n=5) | HL + SIMV (n=5) | NDR (n=5) |
|---|---|---|---|---|---|
| Total Cholesterol | 2.61 ± 0.59 | 2.91 ± 1.51 | 2.02 ± 0.44 | 2.11 ± 0.53 | 2.39 ± 0.62 |
| HDL | 0.95 ± 0.16 | 1.01 ± 0.12 | 0.92 ± 0.18 | 0.97 ± 0.16 | 1.61 ± 0.37*** |
| LDL | 1.26 ± 0.56 | 1.45 ± 1.49 | 0.81 ± 0.43 | 0.82 ± 0.51 | 0.48 ± 0.18 |
| LDL:HDL | 1.39 ± 0.83 | 1.46 ± 1.46 | 0.92 ± 0.55 | 0.84 ± 0.50 | 0.30 ± 0.07* |
| Triglycerides | 0.46 ± 0.31 | 0.72 ± 0.28 | 0.45 ± 0.11 | 0.49 ± 0.26 | 0.62 ± 0.46 |

## 6.5 Discussion

### *6.5.1 C57/BL6 mice body weight gain, energy and water consumption.*

We have previously shown that male rats supplemented for 28 day with 35 mg/kg body weight per day of CR crude, administered in drinking water, showed no signs of toxicity when fed a normal diet (Chapter 5). The lack of difference in body weight at experiment termination, weight gained during the experiment, daily energy or fluid consumption indicates that CR crude and CR flavonoid rich extract supplementation in drinking water does not impact growth when given at 70 mg/kg body weight/day.

### *6.5.2 C57/BL6 Mice glucose tolerance*

Animals that were supplemented with CR crude showed no improvement in glucose clearance compared to the control group, whilst the CR flavonoid rich supplemented group did. Because the initial fractionation procedure collects all but the most polar/ionic compounds, the CR crude fraction contains a complex mix of compounds. This mixture may contain betalains, flavonoids as well as many other unidentified phytochemicals (Chapters 3, 4). Each compound may have effects on vascular function and glucose clearance ranging from improving function through to causing damage and injury to the vessel wall. The fact that under modified dietary conditions no obvious beneficial or detrimental effect was observed, suggests that in aggregate, supplementation with crude CR has a negligible effect on the glucose clearance of animals experiencing metabolic syndrome.

Flavonoid rich supplemented animals had significantly improved glucose clearance, with the restoration of glucose clearance back to that of non-insulin resistant animals (Figure 6.2). Because CR flavonoids belong to the same family as the hypoglycaemic compounds resveratrol, epigallocatechin gallate and quercetrin, but several of them also possess a HMG moiety, the observed improvements in glucose disposal may be due to effects related to their flavonoid structure, statin activity or a combination of both properties (Chapter 1). Increased glucose clearance may be due to an increased concentration of tetrahydrobiopterin and eNOS coupling (Antoniades et al., 2011, Wenzel et al., 2008), increased eNOS phosphorylation and expression (Stoclet et al., 2004), a reduction in the expression of the eNOS inhibiting protein caveolin-1, increased translocation of eNOS to the cellular membrane (where it is active) (Gelosa et al., 2007), an increase in insulin receptor substrate 2 expression, effects on GLUT4 expression and activity (Qin et al., 2010, Ueda et al., 2008), or a reduction in the production of superoxide (Sanchez et al., 2006, Antoniades et al., 2011); all of which are effects reported for either flavonoid or statin compounds. The exact mechanism(s) by which supplementation with the CR flavonoid rich extract affected glucose clearance were not investigated in the present study. Nevertheless, our data clearly demonstrate an improvement in glucose clearance, and this action is independent of any effects on lipid profile. Given the history this plant has as a traditionally used therapeutic agent, and if this improvement in glucose clearance continues to be observed, there is considerable potential that CR (or a CR derived extract) could once again be used for its medicinal properties.

### *6.5.3 C57/BL6 Mice blood lipids*

The lipoprotein profiles of rodents are considerably different to those found in humans. In addition, rodents are able to readily compensate for conditions where they consume a diet containing a high percentage of dietary fats (Sanchez-Muniz and Bastida, 2008). This is why a specific dietary regimen and animal model (2% cholesterol, 10% fat, male, Sprague-Dawley rat) was used to investigate the potential hypolipidaemic activity of the CR extracts. As such, the CR extracts inability to impart changes to the C57/BL6 mouse blood lipids may be due to the model's inappropriateness, or the effects of overnight fasting on mouse biochemical profiles (Andrikopoulos et al., 2008).

### *6.5.4 Sprague-Dawley weight gain, energy and water consumption*

Like the C57/Bl6 mouse experiment, no differences were observed in the rats' body weight at experiment termination, weight gained during the experiment or daily energy consumption. The increase in fluid consumption observed in the HL+ FLAV and HL + SIMV are relatively small, and it is our view that these differences are most likely a statistical quirk due to the averaging of measurements (fluid consumed/number of days of between measurements/number of animals in box), rather than being of actual physiological relevance. This is supported by the analysis of rats weight gain during the experiment and final body weight. Both of these parameters were individually measured and recorded, and failed to show any difference between groups. The data from this experiment are consistent with that reported in chapter 5 and indicate that supplementing drinking water with either a CR crude or CR flavonoid rich extract does not impact growth when given at 70 mg/kg body weight/day.

### *6.5.5 Sprague-Dawley at blood lipids*

Unlike our glucose clearance data, the only differences observed in the hyperlipidaemic rats were between the healthy and ‘unhealthy’ control groups. The lack of appropriate data regarding population variance and treatment effect meant that the experimental pilot data was used for power calculations post-hoc. Power analysis of our results showed that with only 5 animals in each experimental group, we had over an 80% chance of making a type II statistical error (a false negative) when comparing results between a commercially prescribed treatment for hyperlipidaemia (simvastatin) and the hyperlipidaemic controls. The mean and standard deviation of the HL + FLAV group LDL and LDL:HDL results are nearly identical to that of the HL + SIMV group. Based on the differences in mean and standard deviations generated by this pilot dataset, 30 animals per group for LDL and 40 animals per group for LDL:HDL would be needed to detect differences between HL + SIMV and HL groups (based on an alpha of 0.05 and a beta of 0.95).

It appears that in addition to the previously discussed ability to improve glucose clearance, the CR flavonoid rich extract may also have lipid lowering activity. This interpretation is based on the similar effects that CR flavonoid rich and the simvastatin treatments had on total cholesterol, LDL, and LDL:HDL levels (Table 6.4). Further investigation with adequate statistical power are needed to determine whether these effects are real or not.

## 6.6 Conclusion

The lipid lowering effects of CR crude previously reported by our group appear to be due to the novel CR flavonoid compounds. Our data indicate that supplementation with these compounds can alleviate the glucose clearance problems associated with metabolic syndrome. The CR flavonoids may also be active in treating the hypercholesterolaemia commonly seen in metabolic syndrome patients; these pilot data support further work in an suitably powered study of around 40 rats per group. Further investigation of the potential hypoglycaemic and hypolipidaemic effects of CR flavonoids with more sensitive techniques such as the euglycaemic-hyperinsulemic clamp involving sufficiently large group numbers are warranted to enable a better understanding of their ability to treat the symptoms of metabolic syndrome.

## 6.7 Acknowledgements

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## 6.8 Declaration of interests

There are no interests to declare.

## 6.9 Contributors statement

Planning of experiments was undertaken by AP, GJ, DG, CN and MK

Experiments and lipid analysis were undertaken by AP, MK and KA

Manuscript preparation was conducted by AP, DG, MK and KA

# 7 Thesis overview and direction of future work

## 7.1 Overview

The research described in Chapters 2-6 of this thesis has been broad in scope, covering the disciplines of plant physiology and biochemistry, organic chemistry, and basic pharmacology. During the course of the work, a number of novel findings were generated. The plant physiology results support the general consensus that flavonoid accumulation is enhanced under conditions that are known to induce oxidative stress *in planta*. Interestingly, and for the first time, these studies have shown that environmental conditions don't affect the accumulation of all flavonoid metabolites equally; and that not all plants respond to increased oxidative stress by producing flavonoids with a dihydroxylated B ring. Information regarding the features of chemicals involved in the non-photochemical quenching process was also determined.

From an organic chemistry perspective, the structures of three new and novel flavonoid compounds were determined. This work also provides an NMR template for determining the structure of two other related flavonoids which have an additional ferulic acid substituent (Jager, 2009, Renggli, 2010). Pharmacologically, there is now considerable evidence that the *Carpobrotus rossii* (CR) extract improves both the glucose tolerance in insulin resistant C57/BL6 mice, and lipid profile of high fat fed rats. Moreover, the dosages needed to achieve these effects are not toxic. These characteristics mean the CR flavonoids or even related synthetic derivatives show considerable promise as a potential alternative therapy in the treatment of hyperglycaemia or hyperlipidaemia associated with metabolic syndrome.

## 7.2 Thesis Limitations

Undertaking research that spans multiple disciplines rather than the narrow focused route common to many PhD's is not without challenges. The need to be 'across' so many subjects and conducting experiments in several different fields concurrently; these challenges have generated the main limitation of this thesis.

As was shown in the analysis of field survey results (Chapter 2), other conditions known to induce oxidative stress, namely potassium and moisture levels, also appear to affect flavonoid accumulation in CR. Long-term, single factor experiments to investigate both of these conditions were initiated. However, approximately halfway through the three month study, a fungal/viral infection was noted in the plants. As infections are known to induce ROS production (Mur et al., 2005, Mittler, 2002) and hence were likely to alter CR leaf flavonoid levels, these studies had to be abandoned and could not be completed with existing time constraints. The desire to select plants for their metabolic profile for use in the later pharmacological and organic chemistry investigations also meant that plant experiments were conducted with a limited number of CR cultivars. As such, there is a possibility that the results reported do not reflect the response of the majority of the CR population. However, it must be noted that all CR plants sampled during Chapter 2 experiments produced chromatograms consistent with flavonoids based on the spinacetin aglycone. Investigations into the long term effect of UVB or different exposure regimes, and investigation of enzymatic processes to determine whether it is increased accumulation, biosynthesis or a combination of these factors which lead to the increased leaf-tissue flavonoid concentration are all possible avenues for future work.

The structure of the predominant flavonoid peak has been determined unequivocally by NMR, and it is highly likely that the proposed structures of the two related compounds proposed in Chapter 4 are correct. There are also another two related flavonoids which contain a ferulic acid substituent, whose structures are yet to be determined and which should be the focus of further characterisation.

As noted in Chapter 6, there appears to be a lipid lowering effect in the hyperlipidaemic rats, but given the pilot nature of this work, the ability to detect significant differences between a commercially prescribed control with known lipid lowering activity and the healthy treatment group was limited. Flavonoid treatment produced strikingly similar effects on total cholesterol, LDL, and LDL:HDL as the simvastatin group, suggestive of a similar effect. However, until these experiments are repeated with larger numbers, the possibility of a lipid lowering activity remains speculative.

Although the use of high-fat fed animals such as mice is an appropriate model of insulin resistance, it lacks the resolution of other methods such as the euglycaemic/hyperinsulaemic clamp (EHC) which is considered the 'gold standard' method of measuring improvements in insulin sensitivity/glucose disposal. The EHC and other relevant techniques are commonly used by members of Menzies Research Institute Tasmania Muscle Metabolism and Diabetes research group. The surgeries associated with these techniques are complex and can take between 6-9 months to learn. Additionally, the experiments take place on individual rats and can take >4 hours to complete, meaning a maximum of 2-3 animals per day can be processed by even the most experienced operator. Achieving proficiency in the EHC under a dramatically reduced timeframe, in addition to completing the other experiments detailed in Chapters 2-6 are beyond the scope of this study; and processing sufficient animals to generate statistically meaningful results would have impacted on the ability to complete several other studies. Additionally, purifying sufficient plant material so that all the experiments could be completed in rats would have exhausted the supply of raw CR plants. No investigation into the mechanism(s) by which the apparent improvement in either glucose disposal or lipid lowering were conducted.

## 7.3 Future directions

A number of questions remain unanswered at the completion of this thesis. Depending upon the relevant field of interest, some of them may be considered worthy of future research. From a plant physiology perspective, the effect of potassium and moisture levels on CR metabolite production remains to be determined. Larger experiments with more cultivars could also be conducted to determine whether the effects reported in Chapters 2 and 3 are seen in a wider CR populations.

From an organic chemistry perspective, several avenues of investigation remain; namely the purification and NMR analysis of the minor flavonoid compounds related to the predominant 784 Da flavonoid. This work could confirm the proposed structures of both the minor flavonoids in Chapter 4, in addition to determining the site of ferulic acid substitution of the other two minor spinacetin-based flavonoids.

Given the pilot nature of the pharmacology experiments reported in Chapter 6, it is recommended that similar experiments are repeated using larger sample groups with suitable statistical power. It would be preferable that such experiments were conducted in rats and that the EHC and other relevant techniques which provide higher sensitivity and more information such as measurement of microvascular recruitment than the 6 hour fast, i.p. glucose injection and monitoring of blood glucose concentrations employed for the studies described in Chapter 6. Further investigations into the mechanism(s) of both the improvement in endothelial function/glucose disposal and lipid lowering should occur.

Suggested routes of investigation are a combination of antibody staining vascular tissue for markers of peroxynitrite and superoxide production, for example nitrotyrosine and 8-Oxo-2'-deoxyguanosine; the use of PI3K and eNOS inhibitors wortmanin and $N_{\omega}$-Nitro-L-arginine methyl ester, to determine if the improved function is nitric oxide related; and assessing the aortic concentration and oxidation status of the eNOS co-factor tetrahydrobiopterin to determine if improved function is related to enhanced eNOS coupling. An *in vitro* verification of the flavonoid(s) ability to inhibit HMG-CoA reductase is also recommended.

In addition to being beneficial for the determination of the minor flavonoid's structure, the large scale fractionation of the CR flavonoids into single pure compounds would enable the relative potency of each flavonoid to be determined and general structure activity relationship(s) to be developed. This information, in conjunction with suitable organic chemistry expertise would enable molecule optimisation, and the generation of potential lead drug candidates.

Given CR's history of use by indigenous Australians and early European settlers and evidence of safety; pilot scale clinical trials of the flavonoid rich extract's ability to treat hyperglycaemia and hyperlipidaemia whilst the more detailed pharmacological experiments described above are undertaken, are justified.

## 7.4 Conclusion

A potential new therapeutic agent for the treatment of a leading cause of human morbidity and mortality, metabolic syndrome, has been identified. It is the authors hope that further clinical investigations of this promising work continue and one day these findings are translated into clinical practice.

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# 9 Appendices

## 9.1 Appendix Chapter 2

**Appendix Table 2.1** Collection location of samples

| Sample Name | Date Collected | Collection Locality | Datum | Easting | Northing | Converted co-ordinates (decimal degrees) | |
|---|---|---|---|---|---|---|---|
| | | | WGS 84 | | | Lat | Long |
| Southport 01 | 15/10/2009 | Burial Grove | | E 43 26.223 | S 146 59.350 | -43.43743 | 146.98876 |
| Southport 02 | 15/10/2009 | | | E 43 26.148 | S 146 58.948 | | |
| Southport 03 | 15/10/2009 | | | E 43 26.148 | S 146 58.948 | | |
| Southport 04 | 15/10/2009 | | | E 43 26.148 | S 146 58.948 | | |
| Southport 05 | 15/10/2009 | Roaring Beach | | E 43 17.423 | S 147 05.330 | | |
| Recherche Bay 01 | 15/10/2009 | | | E 49 34.734 | S 146 53.267 | -43.58076 | 146.88918 |
| Recherche Bay 02 | 15/10/2009 | | | E 49 34.734 | S 146 53.267 | | |
| Recherche Bay 03 | 15/10/2009 | | | E 49 34.734 | S 146 53.267 | | |
| Recherche Bay 04 | 15/10/2009 | | | E 43 34.906 | S 146 53.477 | | |
| Recherche Bay 05 | 15/10/2009 | | | S 43 34.893 | S 146 53.445 | | |
| | | | UTM 55 | | | | |
| 7 Mile Beach 01 | 21/10/2009 | | GDA | 544347 | 5256561 | -42.84174 | 147.54385 |
| 7 Mile Beach 02 | 22/10/2009 | | | 544380 | 5256568 | | |
| 7 Mile Beach 03 | 23/10/2009 | | | 544421/544406 | 5256582/5256635 | | |
| 7 Mile Beach 04 | 24/10/2009 | | | 544416 | 5256549 | | |
| 7 Mile Beach 05 | 25/10/2009 | | | 544511 | 5256614 | | |
| Belrieve Beach 01 | 26/10/2009 | | | 5311172 | 5252556 | -42.87911 | 147.38219 |

| Sample Name | Date Collected | Collection Locality | Datum | Easting | Northing | Converted co-ordinates (decimal degrees) | |
|---|---|---|---|---|---|---|---|
| Belrieve Beach 02 | 27/10/2009 | | | 531129 | 5252562 | | |
| Belrieve Beach 03 | 28/10/2009 | | | 531144 | 5252563 | | |
| Belrieve Beach 04 | 29/10/2009 | | | 531082 | 5252558 | | |
| Belrieve Beach 05 | 21/10/2009 | | | 531063 | 5252565 | | |
| Sloping Main 1 | 27/10/2009 | | | 555795 | 5241754 | -42.97953 | 147.68509 |
| Sloping Main 2 | 27/10/2009 | | | 555805 | 5241728 | | |
| Sloping Main 3 | 27/10/2009 | | | 555800 | 5241711 | | |
| Sloping Main 4 | 27/10/2009 | | | 555848 | 5241305 | | |
| Sloping Main 5 | 27/10/2009 | | | 555854 | 5241308 | | |
| Safety Cove 1 | 27/10/2009 | | | 569553 | 5218328 | -43.16264 | 147.85448 |
| Safety Cove 2 | 27/10/2009 | | | 569534 | 5218366 | | |
| Safety Cove 3 | 27/10/2009 | | | 569472 | 5218441 | | |
| Safety Cove 4 | 27/10/2009 | | | 569397 | 5219190 | | |
| West Shelly Beach 1 | 27/10/2009 | | | 573018 | 5286798 | -42.5677 | 147.88295 |
| West Shelly Beach 2 | 27/10/2009 | | | 573012 | 5286797 | | |
| West Shelly Beach 3 | 27/10/2009 | | | 573871 | 5287013 | | |
| West Shelly Beach 4 | 27/10/2009 | | | 573866 | 5287018 | | |
| West Shelly Beach 5 | 27/10/2009 | | | 573873 | 5287003 | | |
| St Helens 1 | 04/11/09 | | | 608185 | 5419298 | -41.3779 | 148.29071 |
| St Helens 2 | 04/11/09 | | | 608187 | 5419298 | | |
| St Helens 3 | 04/11/09 | | | 608191 | 5419284 | | |
| St Helens 4 | 04/11/09 | | | 608192 | 5419274 | | |
| St Helens 5 | 04/11/09 | | | 608149 | 5419325 | | |
| Bicheno 1 | 04/11/09 | | | 607125 | 5364238 | -41.86962 | 148.29577 |
| Bicheno 2 | 04/11/09 | | | 607125 | 5364238 | | |

| Sample Name | Date Collected | Collection Locality | Datum | Easting | Northing | Converted co-ordinates (decimal degrees) | |
|---|---|---|---|---|---|---|---|
| Bicheno 3 | 04/11/09 | | | 606940 | 5364229 | | |
| Bicheno 4 | 04/11/09 | | | 606957 | 5364217 | | |
| Bicheno 5 | 04/11/09 | | | 607109 | 5364255 | | |
| Dolphin Sands 1 | 04/11/09 | | | 593648 | 5339443 | -42.09147 | 148.16917 |
| Dolphin Sands 2 | 04/11/09 | | | 593619 | 5339436 | | |
| Dolphin Sands 3 | 04/11/09 | | | 593582 | 5339432 | | |
| Dolphin Sands 4 | 04/11/09 | | | 593532 | 5339432 | | |
| Dolphin Sands 5 | 04/11/09 | | | 593595 | 5339434 | | |
| Strahan Ocean Beach 1 | 10/11/09 | | | 356508 | 5332378 | -42.14807 | 145.2632 |
| Strahan Ocean Beach 2 | 10/11/09 | | | 356512 | 5332392 | | |
| Strahan Ocean Beach 3 | 10/11/09 | | | 356510 | 5332398 | | |
| Strahan Ocean Beach 4 | 10/11/09 | | | 356544 | 5332389 | | |
| Strahan Ocean Beach 5 | 10/11/09 | | | 356529 | 5332418 | | |
| Arthur River 1 | 11/11/09 | | | 303608 | 5452419 | -41.05832 | 144.66007 |
| Arthur River 2 | 11/11/09 | | | 303609 | 5452422 | | |
| Arthur River 3 | 11/11/09 | | | 303618 | 5452441 | | |
| Arthur River 4 | 11/11/09 | | | 303638 | 5452560 | | |
| Arthur River 5 | 11/11/09 | | | 303635 | 5452568 | | |
| Stanley-Nut 1 | 11/11/09 | | | used Nut 2 in GIS data | used Nut 2 in GIS data | -40.76761 | 145.30578 |
| Stanley-Nut 2 | 11/11/09 | | | 357031 | 5485736 | | |
| Stanley-Nut 3 | 11/11/09 | | | 357027 | 5485742 | | |
| Stanley-Nut 4 | 11/11/09 | | | 357046 | 5485747 | | |
| Stanley-Nut 5 | 11/11/09 | | | 357036 | 5485745 | | |
| Beechford 1 | 10/03/2010 | Beechford | | 495360 | 5485376 | -41.02543 | 146.94265 |
| Beechford 2 | 10/03/2010 | Beechford | | 495360 | 5485376 | | |

| Sample Name | Date Collected | Collection Locality | Datum | Easting | Northing | Converted co-ordinates (decimal degrees) | |
|---|---|---|---|---|---|---|---|
| Beechford 3 | 11/03/2010 | | | 495360 | 5458376 | | |
| Beechford 4 | 11/03/2010 | | | 495784 | 5458316 | | |
| Beechford 5 | 11/03/2010 | | | 495784 | 5458316 | | |
| Bridport 1 | 11/03/2010 | | | 532347 | 5463279 | -40.98158 | 147.38554 |
| Bridport 2 | 11/03/2010 | | | 532323 | 5463290 | | |
| Tomahawk 1 | 11/03/2010 | | | 563822 | 5475912 | -40.86628 | 147.75847 |
| Tomahawk 2 | 11/03/2010 | | " | " | | | |
| Tomahawk 3 | 11/03/2010 | | " | " | | | |
| Tomahawk 4 | 11/03/2010 | | " | " | | | |
| Tomahawk 5 | 11/03/2010 | | " | " | | | |

**Appendix Table 2.2.** Mean soil variables per site, SP 1 and STAN 1-5 sites not removed.

| *Location* | *0-15 cm pH* | *15-30 cm pH* | *30-45 cm pH* | *0-15cm EC µs/cm* | *15-30cm EC µs/cm* | *30-45cm EC µs/cm* | *0-15cm $Na^+$ conc (mM)* | *15-30cm $Na^+$ conc (mM)* | *30-45cm $Na^+$ conc (mM)* | *0-15cm $K^+$ conc (mM)* | *15-30cm $K^+$ conc (mM)* | *30-45cm $K^+$ conc (mM)* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 7MB | 7.64 | 7.77 | 7.92 | 74.94 | 85.12 | 94.42 | 0.33 | 0.36 | 0.42 | 0.08 | 0.09 | 0.09 |
| AR | 8.01 | 7.90 | 7.95 | 73.40 | 77.80 | 72.24 | 0.28 | 0.30 | 0.25 | 0.07 | 0.08 | 0.08 |
| BFD | 6.70 | 6.57 | 6.66 | 69.74 | 80.00 | 70.14 | 0.34 | 0.35 | 0.38 | 0.14 | 0.13 | 0.13 |
| BEL | 4.81 | 4.43 | 4.22 | 29.76 | 32.45 | 39.23 | 0.24 | 0.23 | 0.30 | 0.09 | 0.09 | 0.09 |
| BIC | 6.55 | 7.02 | 7.16 | 56.42 | 46.40 | 41.65 | 0.52 | 0.23 | 0.17 | 0.09 | 0.08 | 0.07 |
| BPT | 6.48 | | | 148.70 | | | 1.12 | | | 0.14 | | |
| DS | 7.11 | 7.54 | 7.68 | 86.33 | 86.93 | 95.20 | 0.44 | 0.48 | 0.47 | 0.07 | 0.08 | 0.08 |
| RB | 7.80 | 7.91 | 8.17 | 72.70 | 80.02 | 79.06 | 0.26 | 0.26 | 0.23 | 0.08 | 0.07 | 0.07 |
| SCB | 5.25 | 4.95 | 4.95 | 14.36 | 18.86 | 12.33 | 0.17 | 0.21 | 0.19 | 0.07 | 0.07 | 0.07 |
| SLO | 5.07 | 5.59 | 5.61 | 15.69 | 21.15 | 23.95 | 0.21 | 0.20 | 0.24 | 0.06 | 0.07 | 0.07 |
| SP | 5.79 | 5.58 | 5.34 | 50.46 | 39.59 | 45.24 | 0.51 | 0.38 | 0.44 | 0.15 | 0.08 | 0.15 |
| STH | 6.39 | 6.50 | 6.59 | 62.46 | 59.58 | 56.78 | 0.20 | 0.20 | 0.20 | 0.08 | 0.08 | 0.07 |
| STAN | 6.81 | | | 1675.25 | | | 14.42 | | | 0.50 | | |
| SOB | 5.91 | 5.68 | 5.29 | 149.74 | 149.86 | 176.02 | 1.50 | 1.40 | 2.15 | 0.12 | 0.09 | 0.14 |
| THK | 8.00 | 8.08 | 8.12 | 57.84 | 61.06 | 60.56 | 0.20 | 0.28 | 0.21 | 0.08 | 0.09 | 0.08 |
| WSB | 6.07 | 6.28 | 6.87 | 155.04 | 241.28 | 370.80 | 0.98 | 1.44 | 2.90 | 0.16 | 0.19 | 0.23 |

**Appendix Table 2.3.** Summary of variation in soil variables with and without clay/colluvium soil sites removed.

| | ***All Samples*** | ***All Stanley samples and Southport 1 removed*** |
|---|---|---|
| Max Soil pH (0-15cm) | 8.1 | 8.1 |
| Min Soil pH (0-15cm) | 4.3 | 4.3 |
| Max Soil pH (15-30cm) | 8.2 | 8.2 |
| Min Soil pH (15-30cm) | 4.3 | 4.3 |
| Max Soil pH (30-45cm) | 8.3 | 8.3 |
| Min Soil pH (30-45cm) | 4.2 | 4.2 |
| | | |
| Max Soil EC (0-15cm) | 2760 | **237** |
| Min Soil EC μS/cm (0-15cm) | 10.36 | 10.36 |
| Max Soil EC μS/cm (15-30cm) | 283 | 283 |
| Min Soil EC μS/cm (15- 30 cm) | 11.43 | 11.43 |
| Max Soil EC μS/cm (30-45cm) | 798 | 798 |
| Min Soil EC μS/cm (30-45cm) | 9.02 | 9.02 |
| | | |
| Max Soil $Na^+$ mM (0-15cm) | 23.64 | **2.46** |
| Min Soil $Na^+$ mM (0-15cm) | 0.13 | 0.13 |
| Max Soil $Na^+$ mM (15-30 cm) | 2.43 | 2.43 |
| Min Soil $Na^+$ mM (15-30 cm) | 0.15 | 0.15 |
| Max Soil $Na^+$ mM (30-45cm) | 4.38 | 4.38 |
| Min Soil $Na^+$ mM (30-45cm) | 0.14 | 0.14 |
| | | |
| Max Soil $K^+$ mM (0-15cm) | 0.70 | **0.22** |
| Min Soil $K^+$ mM (0-15cm) | 0.06 | 0.06 |
| Max Soil $K^+$ mM (15-30 cm) | 0.22 | 0.22 |
| Min Soil $K^+$ mM (15-30 cm) | 0.06 | 0.06 |
| Max Soil $K^+$ mM (30-45cm) | 0.42 | **0.34** |
| Min Soil $K^+$ mM (30-45cm) | 0.06 | 0.06 |

**Appendix Table 2.4.** The best 1 to 4 factor regression models for prediction of flavonoid, tannin and antioxidant production in *C. rossii* leaves.

| Variable | Best 1 Factor models (adjusted $R^2$) | Best 2 Factor model (adjusted $R^2$) | Best 3 Factor model (adjusted $R^2$) | Best 4 Factor model (adjusted $R^2$) |
|---|---|---|---|---|
| Flavonoid | S (36.4) | S + $F_{v/Fm}$ (48.5) | S + $F_{v/Fm}$ + Ks (52.7) | S + Fm + Wf + Ra (56.3) |
| | Fm (17.2) | S + Fm (45.5) | S + $F_{v/Fm}$ + Fm (51.8) | S + Ks + Evd + $W_3$ (54.7) |
| | O (12.5) | S + Ks (45.5) | S + $F_{v/Fm}$ + Nap (50.8) | S + Ks + Evd + Wf (54.4) |
| | $F_{v/Fm}$ (10.2) | S + Ec (40.7) | S + Fm + Wf (50.5) | S + Ks + $F_{v/Fm}$ + Nap (54.2) |
| | $T_6$ (10.1) | S + Np (40.4) | S + $F_{v/Fm}$ + Ec (50.2) | S + Ks + $F_{v/Fm}$ + Evd (53.7) |
| Tannin | Ks (19.3) | Ks + Evd (33.5) | Ks + Evd + S (55.5) | Ks + Evd + S + O (60.1) |
| | $F_{v/Fm}$ (18.6) | Fm + Ra (31.7) | Ks + Evd + $T_6$ (47.7) | Ks + Evd + S + $F_{v/Fm}$ (56.9) |
| | Fm (16.4) | Ks + O (31.6) | Ks + Evd + O (46.1) | Ks + Evd + S + Nas (56.2) |
| | Evd (13.9) | S + Evd (30.7) | Evd + S + Nas (46.0) | Ks + Evd + S + Np (55.6) |
| | O (13.5) | Ec + Evd (30.5) | Ks + Evd + $Ra_6$ (45.7) | Ks + Evd + S + $T_6$ (55.2) |
| Gallic acid equivalents | $F_{v/Fm}$ (29.0) | Evd + Ks (49.4) | Evd + Ks + O (57.3) | Evd + Ks + O + $F_{v/Fm}$ (60.4) |
| | Evd (24.4) | Evd + Ec (43.7) | Evd + Ks +$F_{v/Fm}$ (56.4) | Evd + Ks + S + O (59.9) |
| | O (20.8) | Evd + $F_{v/Fm}$ (42.5) | Evd + Ks + S (54.0) | Evd + Ks + S + $F_{v/Fm}$ (58.1) |
| | $Ev_6$ (17.8) | Evd + Nap (41.6) | Evd + $F_{v/Fm}$ + Ec (54.0) | Evd + Ks + $F_{v/Fm}$ + $W_3$ (57.6) |
| | $Ra_6$ (17.3) | Fm + $Ra_6$ (41.1) | Evd + Ks + Fm (52.2) | Evd + Ks + O + Nap (57.4) |

Ec = soil electrical conductivity (15-30cm), Evd= onsite daily evaporation, $Ev_6$= average evaporation occurring at the collection site in the 6 months preceding plant collection, Fm = maximum fluorescence yield, $F_{v/Fm}$= maximum quantum efficiency of Photosystem II , Ks= soil $K^+$ concentration (15-30cm), Nap = $Na^+$ concentration of leaf sap, Nas= soil $Na^+$ concentration (15-30cm), O = osmolarity of tissue sap, Ra = cumulative radiation in the fortnight prior to plant collection, $Ra_6$ = cumulative radiation occurring at the collection site in the 6 months preceding plant collection, S= Stomata density,$T_6$ = average daily maximum temperature experienced in 6 months preceding plant collection, Wf = cumulative rainfall in the fortnight prior to plant collection, $W_3$= cumulative rainfall in the 3 months prior to plant collection.

**Appendix Table 2.5.** Correlations between biomass, flavonoid and antioxidant variables measured at the whole plant scale during NaCl dosing experiment. Correlation assessed with Pearson correlation[a].

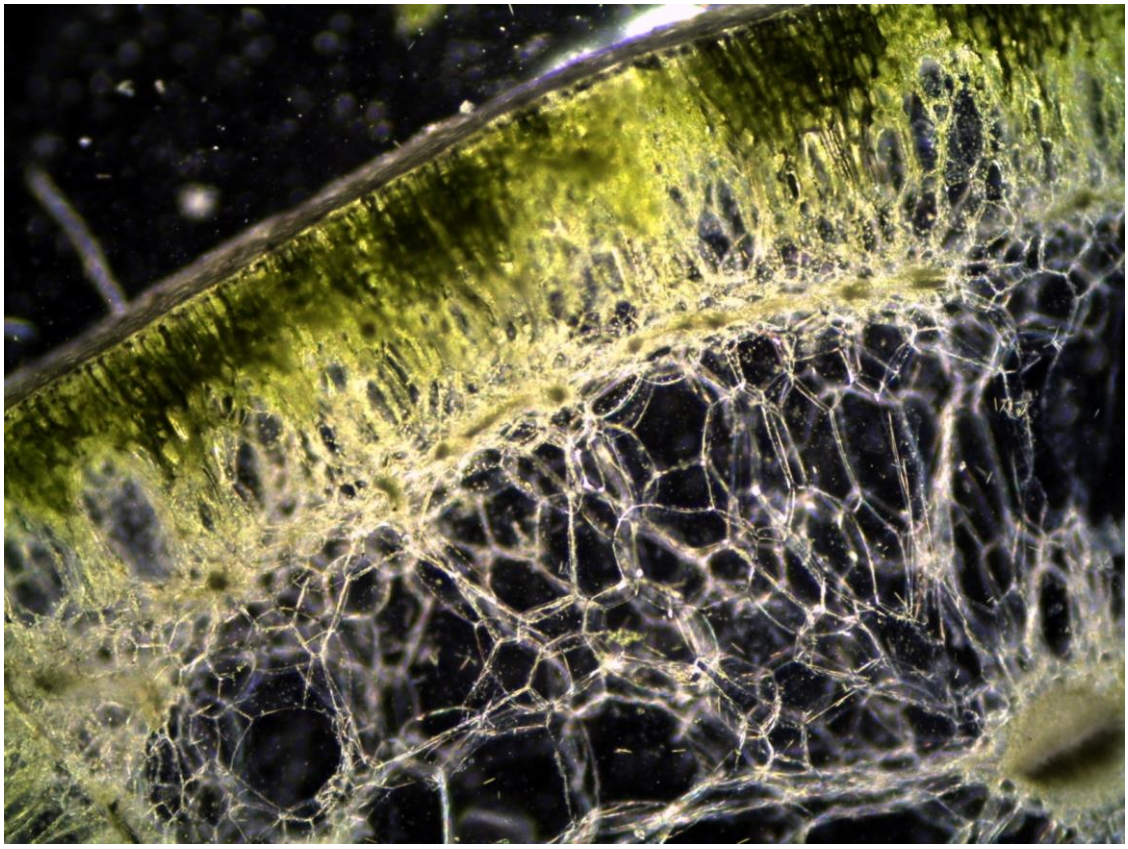
| | **Salt** | **RFW** | **LFW** | **WFW** | **RDW** | **LDW** | **WDW** | **LG** | **RL** | **FC** |
|---|---|---|---|---|---|---|---|---|---|---|
| *RFW* | -0.637 *** | | | | | | | | | |
| *LFW* | -0.688 *** | 0.810 *** | | | | | | | | |
| *WFW* | -0.694 *** | 0.840 *** | 0.999 *** | | | | | | | |
| *RDW* | -0.541 *** | 0.864 *** | 0.824 *** | 0.841 *** | | | | | | |
| *LDW* | -0.455 *** | 0.750 *** | 0.866 *** | 0.888 *** | 0.825 *** | | | | | |
| *WDW* | -0.480 *** | 0.797 *** | 0.900 *** | 0.905 *** | 0.888 *** | 0.992 *** | | | | |
| *LG* | -0.581 *** | 0.627 *** | 0.832 *** | 0.827 *** | 0.752 *** | 0.852 *** | 0.857 *** | | | |
| *RL* | -0.513 *** | 0.638 *** | 0.547 *** | 0.564 *** | 0.568 *** | 0.523 *** | 0.549 *** | 0.536 *** | | |
| *FC* | -0.278 * | 0.135 NS | 0.087 NS | 0.092 NS | -0.061 NS | -0.112 NS | -0.104 NS | -0.194 NS | -0.008 NS | |
| *TFP* | -0.589 *** | 0.649 *** | 0.809 *** | 0.808 *** | 03622 *** | 0.650 *** | 0.663 *** | 0.694 *** | 0.399 ** | 0.415 ** |

[a] (*< 0.05, ** <0.01, *** <0.001, NS = not significant) FC = flavonoid concentration of palisade tissue, LDW = leaf dry weight, LFW = leaf fresh weight, LG = number of leaves gained, RDW = root dry weight, RFW = root fresh weight, RL = root length, TFP = total plant flavonoid production, WDW = whole plant dry weight, WFW = whole plant fresh weight,

**Appendix Table 2.6.** Correlations between biomass, flavonoid and antioxidant variables measured at the individual leaf scale during NaCl dosing experiment. Correlation assessed with Pearson correlation[a].

| | **Salt** | **LW** | **LSA** | **FT** | **$O_P$** | **$O_S$** | **$G_P$** | **$F_P$** | **$G_S$** |
|---|---|---|---|---|---|---|---|---|---|
| *LW* | -0.656 *** | | | | | | | | |
| *LSA* | -0.670 *** | 0.914 *** | | | | | | | |
| *FT* | -0.239 ** | 0.116 NS | 0.176 NS | | | | | | |
| *$O_P$* | 0.965 *** | -0.547 *** | -0.561 *** | -0.149 0.108 | | | | | |
| *$O_S$* | 0.978 *** | -0.588 *** | -0.610 *** | -0.187 * | 0.990 *** | | | | |
| *$G_P$* | 0.253 * | -0.412 *** | -0.376 *** | -0.047 NS | 0.180 NS | 0.167 NS | | | |
| *$F_P$* | 0.109 NS | -0.370 *** | -0.347 *** | 0.812 *** | 0.239 ** | 0.132 NS | 0.256 * | | |
| *$G_S$* | 0.431 *** | -0.341 *** | -0.391 *** | 0.022 NS | 0.370 *** | 0.380 *** | 0.795 *** | 0.362 *** | |
| *SF* | 0.155 NS | -0.189 * | -0.152 NS | 0.051 NS | 0.183 * | 0.152 NS | -0.094 NS | 0.143 NS | -0.032 NS |

[a] (*< 0.05, ** <0.01, *** <0.001, NS = not significant) $F_P$ = palisade leaf tissue flavonoid concentration, $F_S$ = spongy leaf tissue flavonoid concentration, FT = total flavonoid production per leaf, $G_P$ = palisade leaf tissue antioxidant activity (gallic acid equivalents), $G_S$ = spongy leaf tissue antioxidant activity (gallic acid equivalents), LSA = photosynthetic surface area of individual leaves, LW = individual leaf weight, $O_P$ = osmolarity of palisade leaf tissue sap, $O_S$ = osmolarity of spongy leaf tissue sap,



**Appendix Figure 2.1.** Cross section of C. rossii leaf showing the clear segregation between (A) palisade mesophyll and (B) spongy parenchyma.

## 9.2 Appendix Chapter 3

### *9.2.1 HPLC-UV chromatography*

HPLC-UV chromatography was performed on a Dionex UltiMate 3000 HPLC system (Dionex Corporation, Sunnyvale, USA) comprising an UltiMate 3000 Quaternary separation pump, UltiMate 3000 auto-sampler, UltiMate 3000 column thermal compartment and UltiMate 3000 diode array detector. Data acquisition and instrument control was done with Chromeleon 7 software. Each injection volume was 30ul, column temperature was 40°C and an Altima HP (Grace Discovery Science, Deerefield USA) C18-Amide (150×4.6mm×5µm) column was used. Wavelengths 254 nm, 350 nm and 535 nm were monitored and the solvent conditions were as per supplementary table 3.1 below.

Solvent A- 2% Acetic acid in $H_2O$, Solvent B- 2% Acetic acid in MeOH,

**Appendix Table 3.1.** Solvent conditions for HPLC-UV chromatography.

| Time (min) | Flow rate (ml/min) | %A | %B |
|---|---|---|---|
| 0 | 0.6 | 60 | 40 |
| 2.40 | 0.4 | 60 | 40 |
| 2.51 | 0.4 | 45 | 55 |
| 14.90 | 0.4 | 45 | 55 |
| 15.00 | 0.6 | 0 | 100 |
| 16.00 | 0.8 | 0 | 100 |
| 18.00 | 0.8 | 0 | 100 |
| 19.00 | 0.6 | 60 | 40 |
| 20.00 | 0.6 | 60 | 40 |
| 22.00 | 0.6 | 60 | 40 |

### *9.2.2 UPLC-MS/MS chromatography*

#### *9.2.2.1 Betalains:*

Typically 5µl aliquots were injected using a Waters Acquity H-series UPLC coupled to a Waters Acquity PDA detector in series with a Xevo triple quadrupole mass spectrometer (Waters Corporation, Milford, USA). A Waters Acquity UPLC BEH C18 column (2.1 x 100mm x 1.7 µm particles) was used, with mobile phases A= 5% formic acid and B= acetonitrile. The column was held at 35°C, flow rate was 0.35 mL/min, with 100% A 0% B for 2 minutes followed by a linear gradient to 50% A and 50% B at 20 minutes, then 3 minutes re-equilibration to original conditions. The PDA was monitored continuously over the range 230 to 500nm at a resolution of 1.2nm.

The mass spectrometer was operated in positive ion electrospray ionization mode, with full scan acquisition over the range *m/z* 300 to 850 every 0.25 seconds with a cone voltage of 20V. Selected ion channels were also monitored simultaneously for *m/z* 347.1 (cone 25V) and *m/z* 551.15 (cone 35V) with 240ms dwell per channel. The ion source temperature was 130°C, the desolvation gas was nitrogen at 950 L/hr, the desolvation temperature was 450°C and the capillary voltage was 2.7KV.

#### *9.2.2.2 Flavonoids:*

Flavonoids were analysed using the same instrument and column as above. Typical injection volume was 40ul. Solvent A was 1% acetic acid in water and solvent B was acetonitrile. The gradient was from 100% A to 90%A:10%B at 0.5 minutes, followed by a linear ramp to 70%A:30%B at 20mins, then 3 minutes re-equilibration to starting conditions. Flow rate was 0.35mL/min and column temperature was 35°C. The PDA was monitored continuously over the range 230 to 500nm at a resolution of 1.2nm

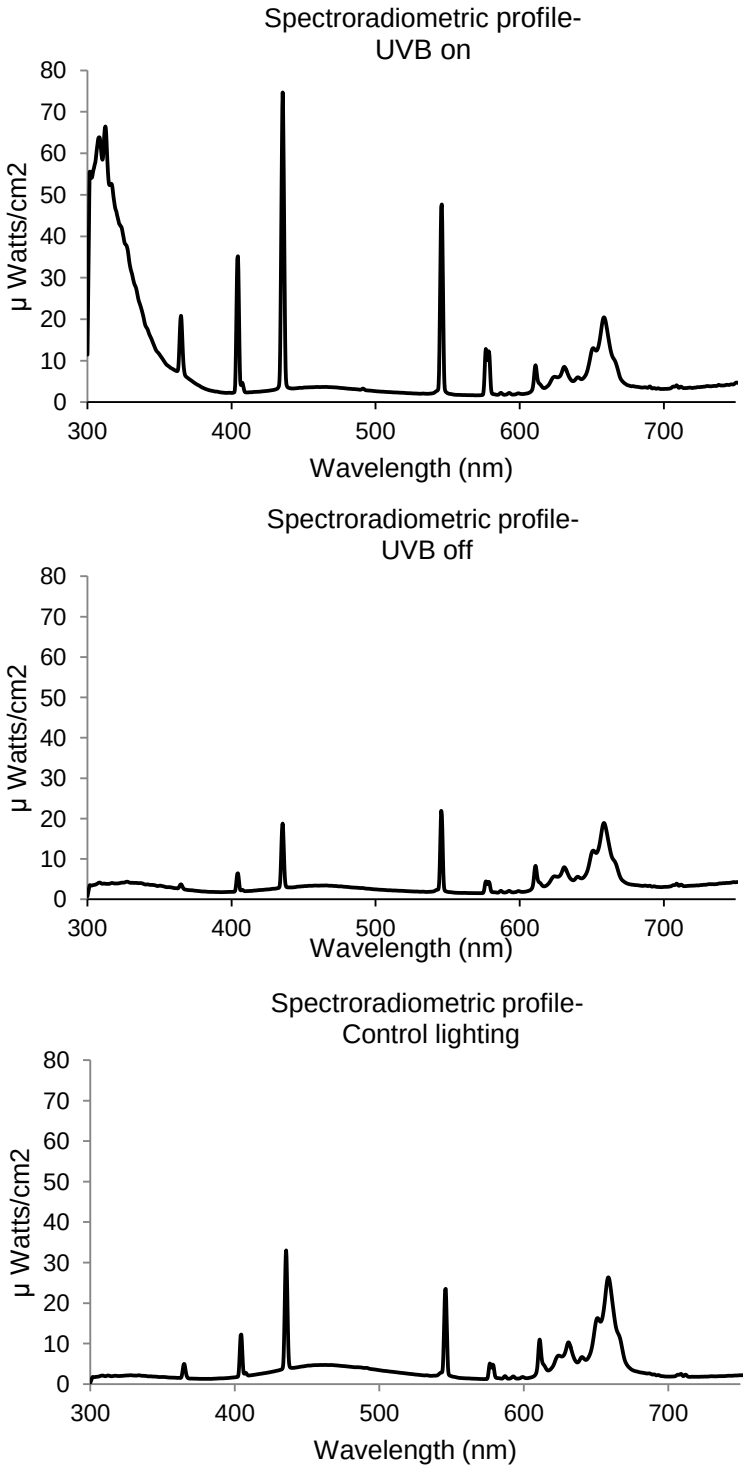
The mass spectrometer was operated in negative ion electrospray ionization mode. Full 'survey scans' were acquired over the range *m/z* 600 to 1200 every 0.3 seconds, with a cone voltage of 55V, followed by data-dependent daughter scans from the strongest ion in each spectrum over the range *m/z* 300 to 1000 at two collision energies (32V and 45V) at 2000 *m/z* per second. Other ion source conditions were as for betalain analyses.

### *9.2.3 Ion Chromatography*

A Dionex ICS-2000 (Dionex Corporation, Sunnyvale, USA) ion chromatograph equipped with Dionex AS autosampler was used to analyse the $Na^+$ and $K^+$ concentrations of the samples. Separations were achieved with a Dionex-CS12A cation exchange column (4x250mm) and Dionex-CG12A guard column (4x50mm). The injection volume was 25µl, the mobile phase was a water solution of 33mM methanesulfonic acid (flow rate 0.5ml/min, 30°C) and run times were 30 minutes per sample. Suppressed conductivity detection was applied using Dionex CSRS 300 (2mm) suppressor and Dionex heated Conductivity cell. Chromeleon 6.8 software was used for instrument control, data collection and analysis.

### *9.2.4 Spectra and radiometric data*

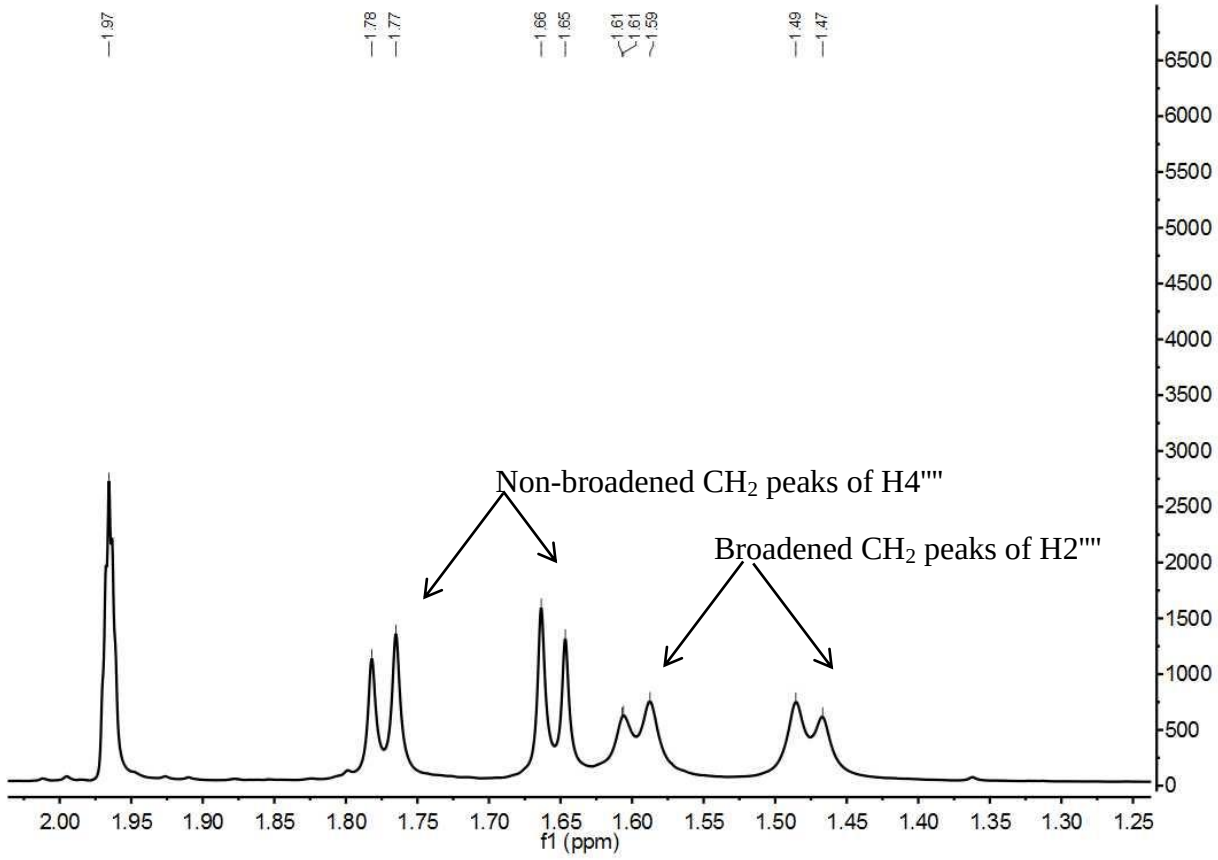
Spectra were recorded with an Apogee spectroradiometer model EEP2000 (Apogee Instruments, Logan, USA) results are the average of two reading per growth tray.



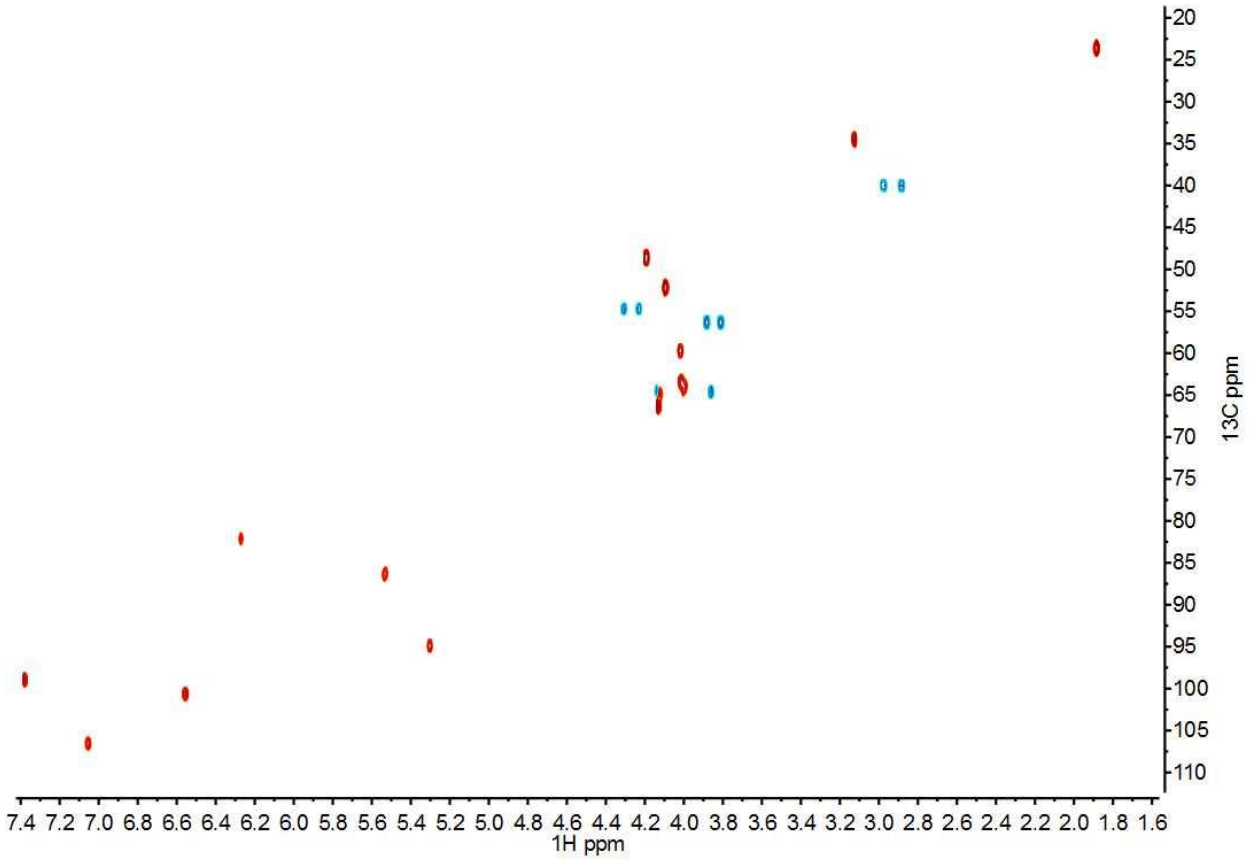

**Appendix Figure 3.1.** Spectroradiometric profile of UVB experimental plants. (a) UVB lamps on, (b) UVB lamps off, (c) control lighting.

## 9.3 Appendix Chapter 4

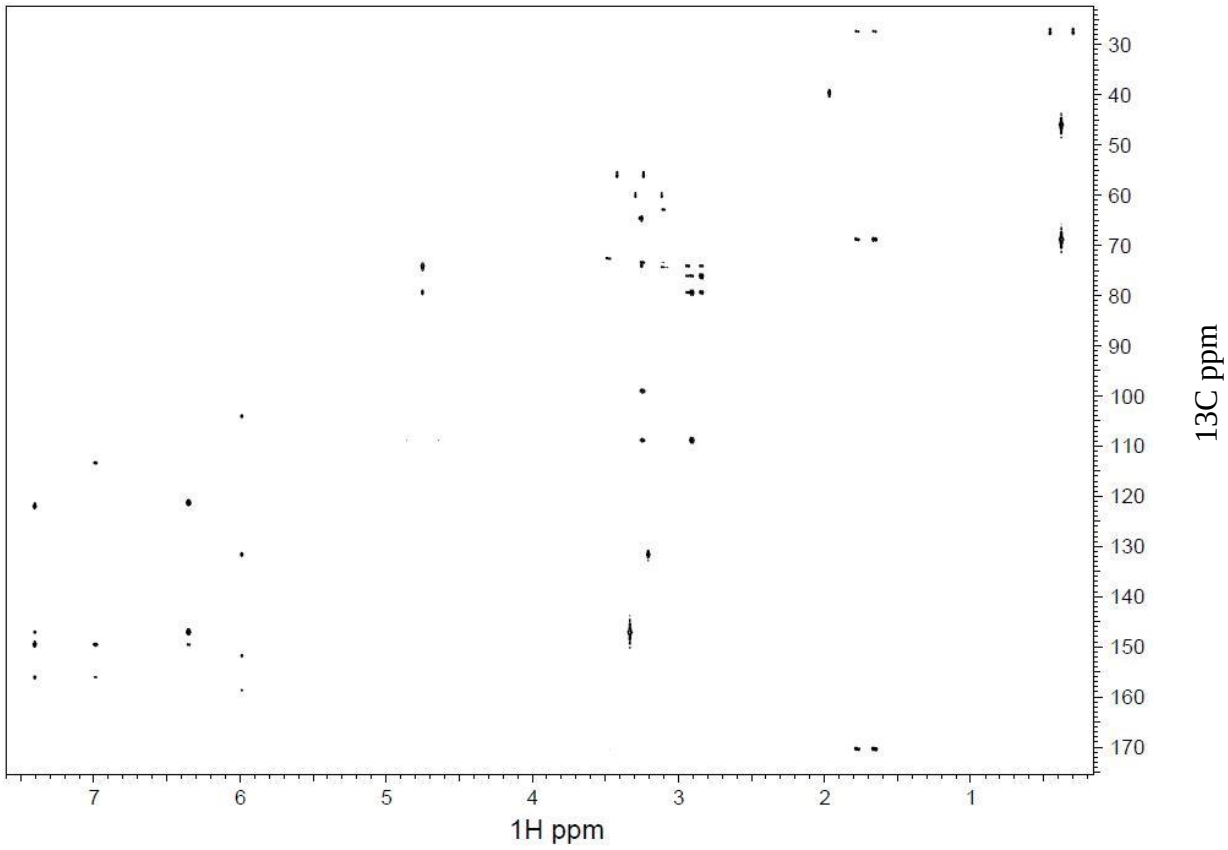
### *9.3.1 NMR studies*



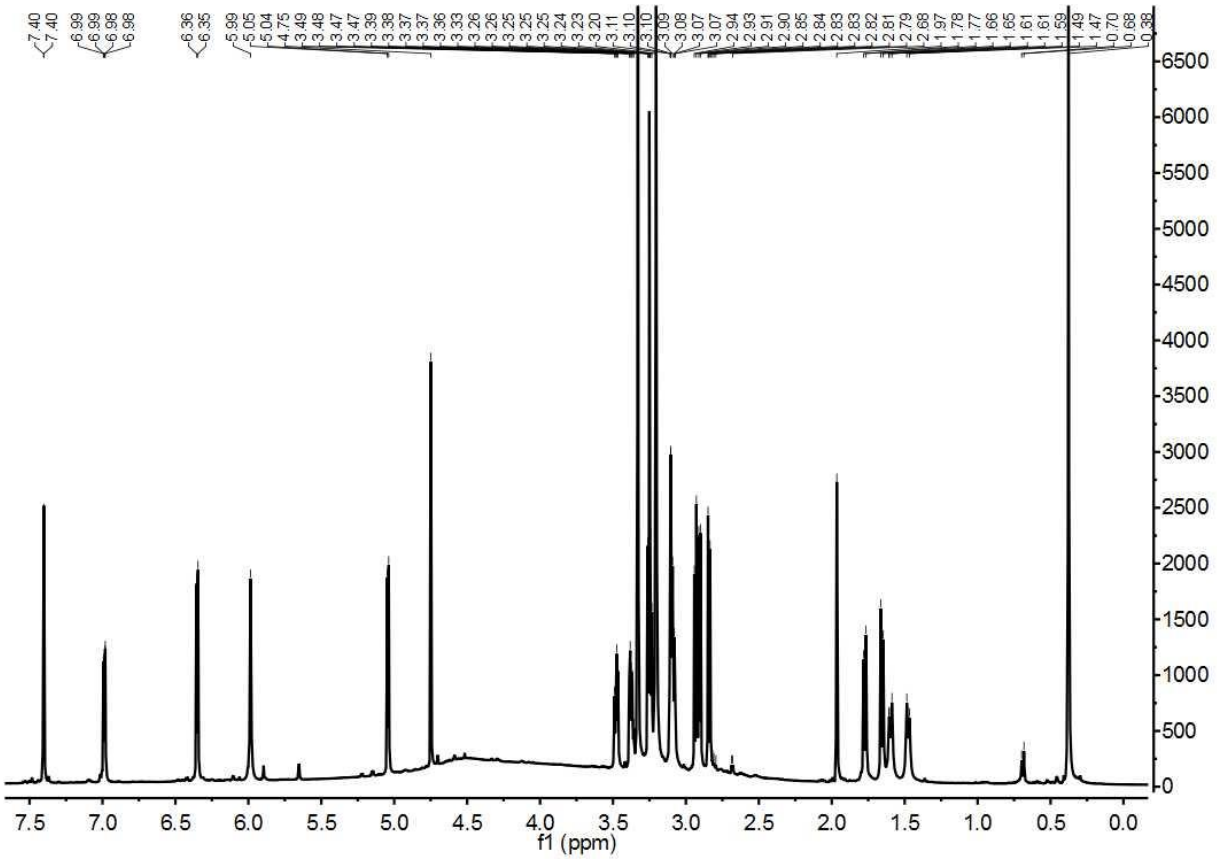

**Appendix Figure 4.1.** Compound 1 – evidence of line broadening in HMG methylene resonances.



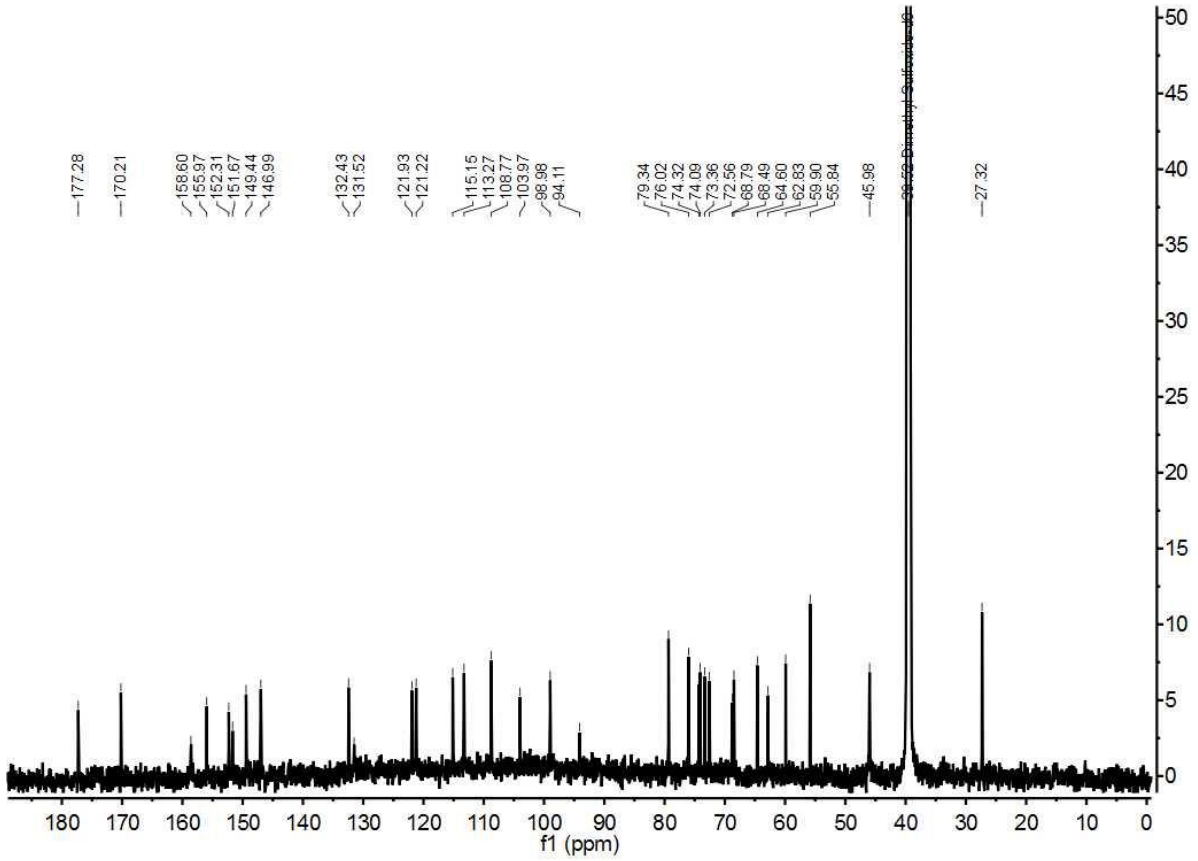

**Appendix Figure 4.2.** Compound 1 1H-13C HSQC.



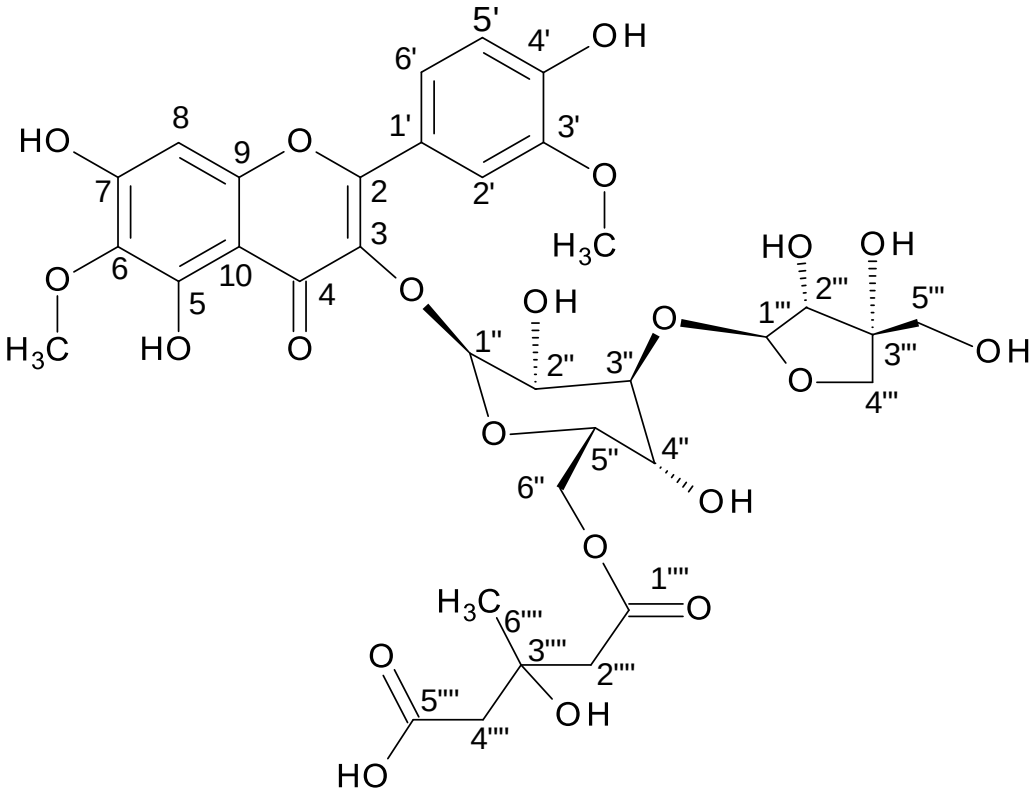

**Appendix Figure 4.3.** Compound 1 1H-13C HMBC.




**Appendix Figure 4.4.** Compound 1 1H-1D




**Appendix Figure 4.5.** Compound 1 13C-1D




**Appendix Figure 4.6.** The structure of Compound 1 (Pirieol A) as determined by NMR experimentation.

### *9.3.2 Preparative HPLC conditions.*

HPLC preparatory was performed on a Water 2695 Chromatographic station coupled to Waters 2487 Dual wavelength photometric detector with detection at 254 and 350 nm, and a Waters Differential Refractometer model 210 (Waters Corporation, Milford, USA). Data acquisition and control was done with Empower 3 software. Each injection comprised 100 µl of saturated extract solution in solvent A, column temperature was 40°C, and an Apollo (Grace Discovery Science, Deerefield USA) C18 (250 × 10 mm, ID 5µm) column was used.

Solvent A composed of 396 mL methanol, 600 mL water and 4.0 mL of glacial acetic acid.

Solvent B: 100% methanol

**Appendix Table 4.1.** Preparative HPLC conditions for the purification of the 784Da flavonoid

| Time (min) | Flow rate (ml/min) | %A | %B |
|---|---|---|---|
| 0 | 2.5 | 100 | 0 |
| 60 | 2.5 | 100 | 0 |
| 61 | 2.5 | 0 | 100 |
| 65 | 2.5 | 0 | 100 |
| 72 | 2.5 | 100 | 0 |
| 75 | 2.5 | 100 | 0 |

### *9.3.3 UPLC-MS/MS chromatography*

UPLC-MS/MS was performed using a Waters Acquity H-series UPLC coupled to a Waters Acquity PDA detector in series with a Xevo triple quadrupole mass spectrometer (Waters Corporation, Milford, USA). A Waters Acquity UPLC BEH C18 column (2.1 x 100mm x 1.7 µm particles)

### *9.3.4 Flavonoids:*

Flavonoids were analysed using the same instrument and column as above. Typical injection volume was 40ul. Solvent A was 1% acetic acid in water and solvent B was acetonitrile. The gradient was from 100% A to 90%A:10%B at 0.5 minutes, followed by a linear ramp to 70%A:30%B at 20mins, then 3 minutes re-equilibration to starting conditions. Flow rate was 0.35mL/min and column temperature was 35°C. The PDA was monitored continuously over the range 230 to 500nm at a resolution of 1.2nm

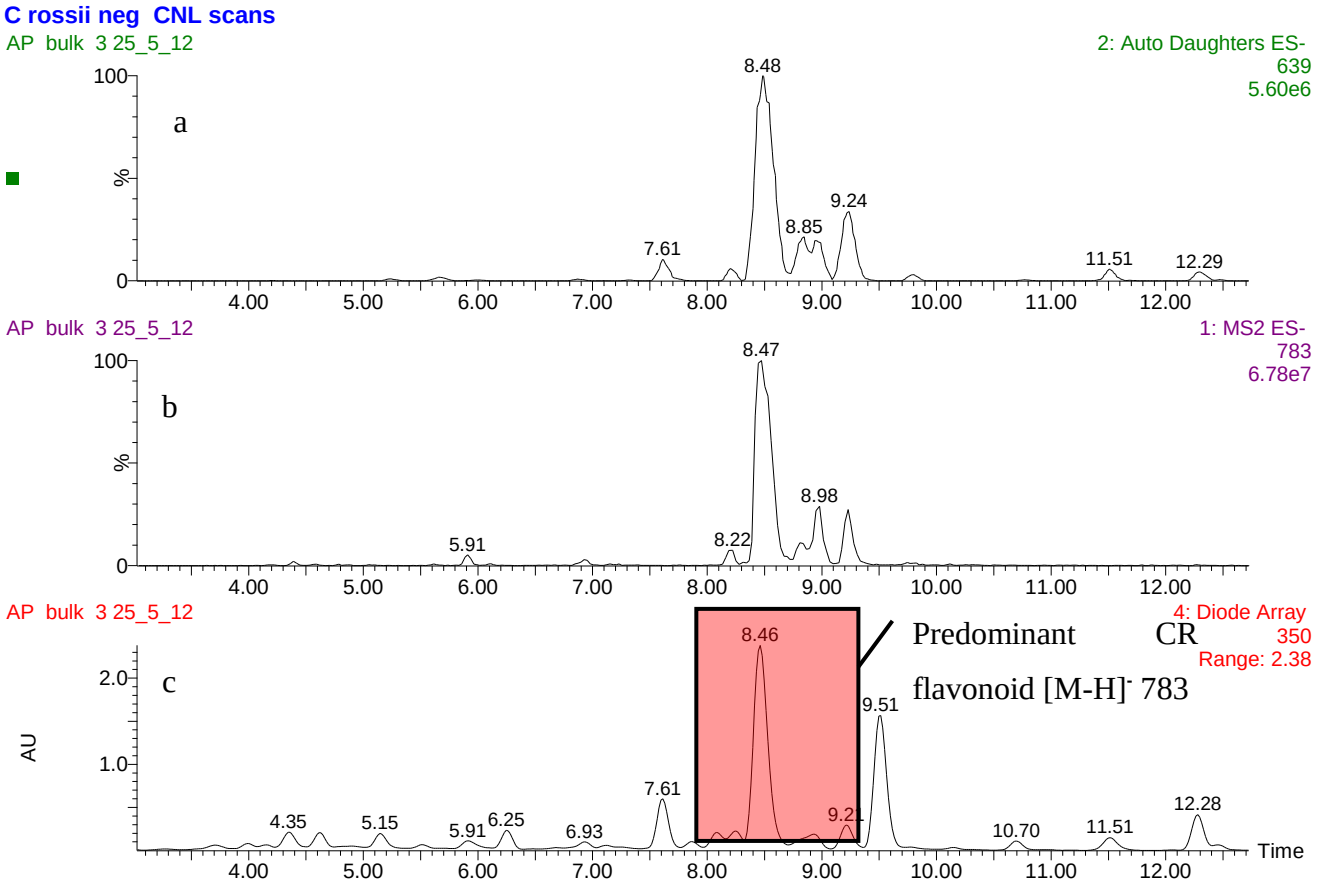
The mass spectrometer was operated in negative ion electrospray ionization mode. Full 'survey scans' were acquired over the range *m/z* 600 to 1600 every 0.3 seconds, with a cone voltage of 55V, followed by data-dependent daughter scans from the strongest ion in each spectrum over the range *m/z* 300 to 1000 at two collision energies (32V and 45V) at 2000 *m/z* per second with 240ms dwell per channel. The ion source temperature was 130°C, the desolvation gas was nitrogen at 950 L/hr, the desolvation temperature was 450°C and the capillary voltage was 2.7KV.

## 9.4 Appendix Chapter 5 Flavonoid analysis of crude leaf extract

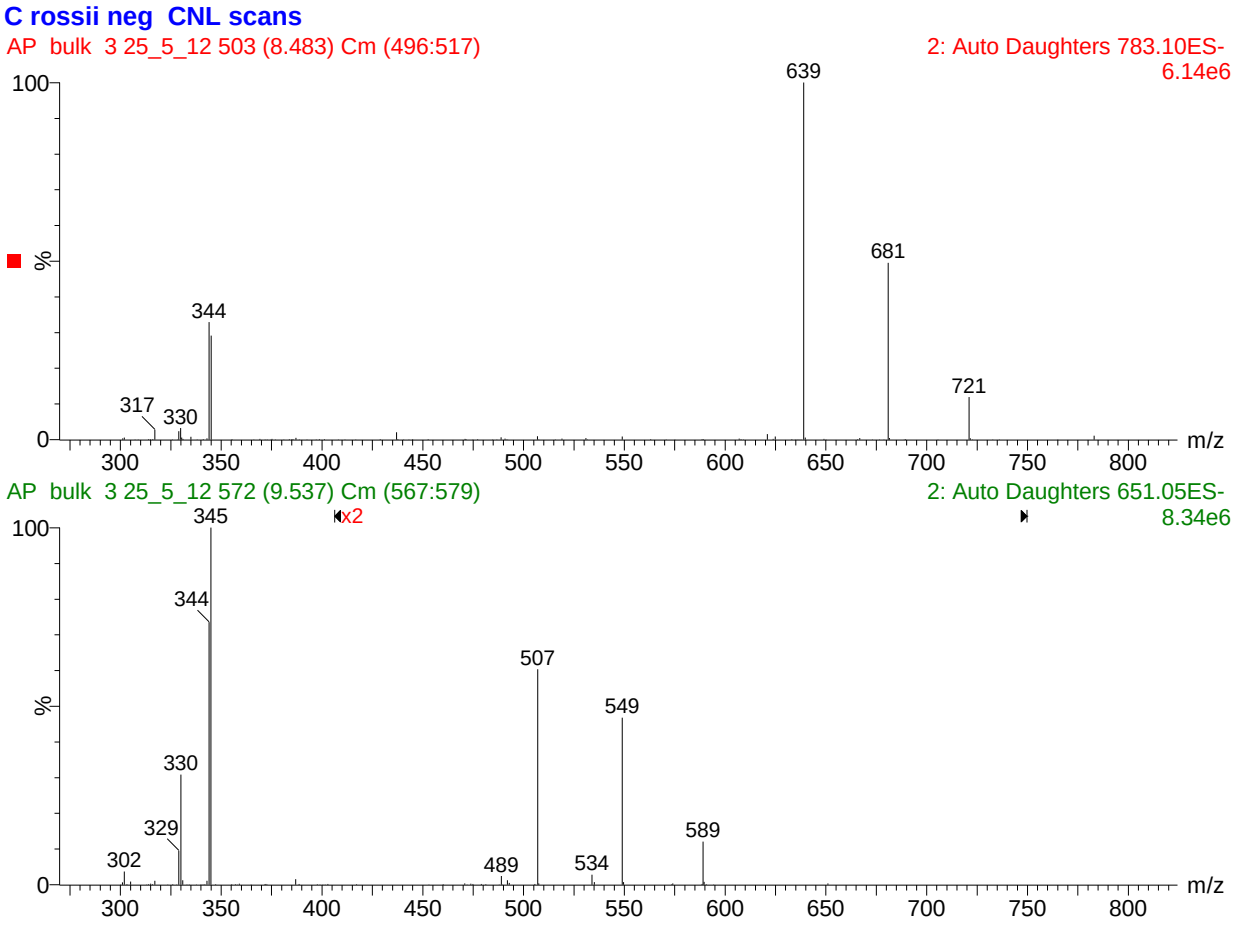
### *9.4.1 Flavonoids*

Flavonoids samples were analyzed using a Waters Acquity H-series UPLC coupled to a Waters Xevo triple quadrupole mass spectrometer. Typical injection volume was 40ul. Solvent A was 1% acetic acid in water and solvent B was acetonitrile. The gradient was from 100% A to 90%A:10%B at 0.5 minutes, followed by a linear ramp to 70%A:30%B at 20mins, then 3 minutes re-equilibration to starting conditions. Flow rate was 0.35mL/min and column temperature was 35°C. The PDA was monitored continuously over the range 230 to 500nm at a resolution of 1.2nm

The mass spectrometer was operated in negative ion electrospray ionization mode. Full 'survey scans' were acquired over the range *m/z* 600 to 1200 every 0.3 seconds, with a cone voltage of 55V, followed by data-dependent daughter scans from the strongest ion in each spectrum over the range *m/z* 300 to 1000 at two collision energies (32V and 45V) at 2000 *m/z* per second. with 240ms dwell per channel. The ion source temperature was 130°C, the desolvation gas was nitrogen at 950 L/hr, the desolvation temperature was 450°C and the capillary voltage was 2.7KV.




**Appendix Figure 5.1.** (a) UPLC-MS/MS Chromatogram of flavonoids generating product ions at m/z 639, (b) UPLC-MS/MS chromatogram of flavonoid [M-H]- 783 isomers, (c) UPLC-DAD chromatogram at 350nm indicating the presence of flavonoid compounds. The loss of 144 daltons from from the various $[M-H]^-$ 783 flavonoids to generate *m/z* 639 product ions between 8.22-9.24 minutes was consistent with the loss of dehydrated HMG.

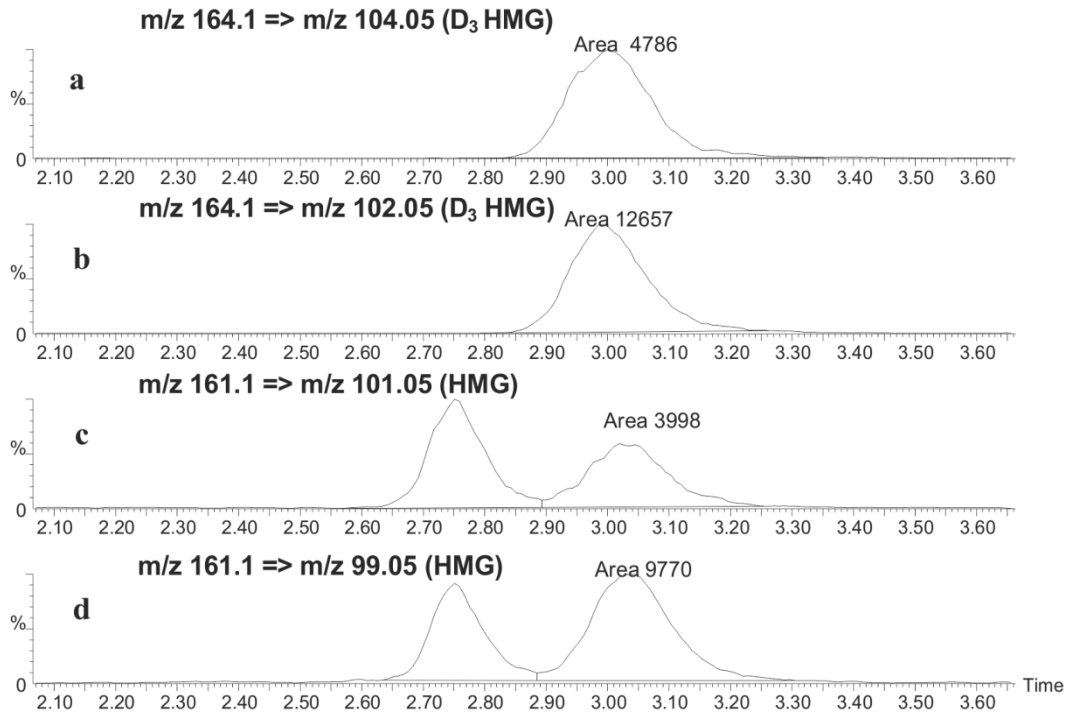



**Appendix Figure 5.2.** MS/MS fragmentation of CR representative flavonoids. (a) $[M-H]^-$ 783 showing the clear loss of 144 daltons (consistent with the loss of a dehydrated HMG moiety) to generate *m/z* 639 and aglycone (*m/z* 344, 345) product ions, and (b) $[M-H]^-$ 651 showing the clear loss of 144 daltons (consistent with the loss of a dehydrated HMG moiety) to generate *m/z* 507 and aglycone (*m/z* 344, 345) product ions

### *9.4.2 Kidney HMG analysis*

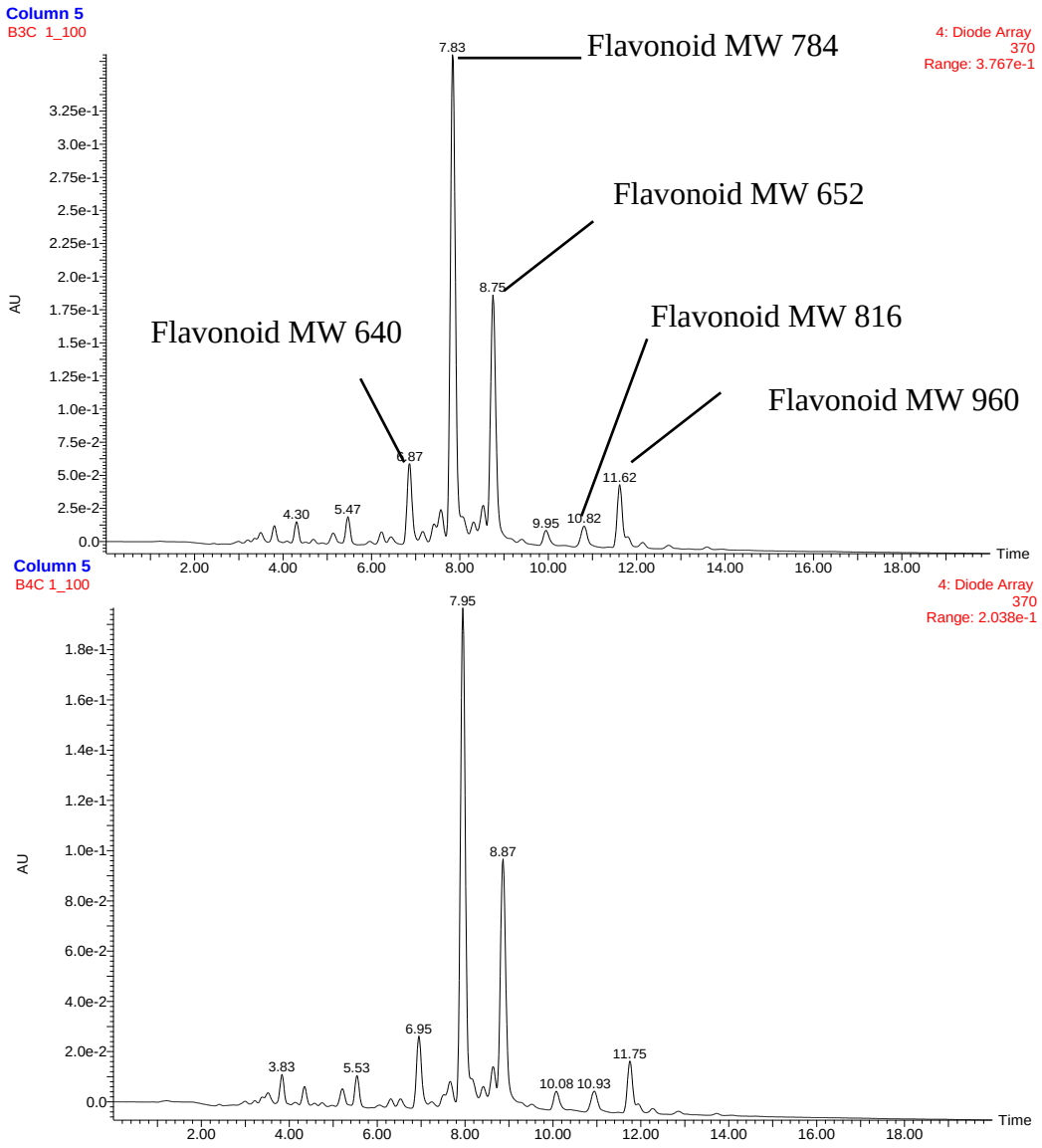
Samples were analyzed using the same instrument and column as above. 5uL injections were made onto a Waters Acquity UPLC BEH C18 column (2.1 x 100mm x 1.7 micron particles), mobile phases A= 1% acetic acid in water and B= 100% acetonitrile. The column was held at 40ºC, the flow rate was 0.3 mL/min, using 100% A for the first 1.5 minutes, followed by an immediate switch to 40% A/60% B which was held for a further 3.5 minutes before re-equilibration to original conditions for 3 minutes. Under these conditions HMG eluted at 3.0 minutes.

The mass spectrometer was operated in negative ion electrospray multiple reaction monitoring (MRM) mode. The MRM transitions were *m/z* 161.1 to *m/z* 99.05 and *m/z* 101.05 for HMG, and *m/z* 164.1 to *m/z* 102.05 and *m/z* 104.05 for the D3 HMG internal standard. The cone voltage was 20V, the collision energy 11V and the dwell time was 120ms per channel. The ion source temperature was 130°C, the desolvation gas was nitrogen at 950 L/hr, whilst the desolvation temperature was 450°C and the capillary voltage was 2.8KV.

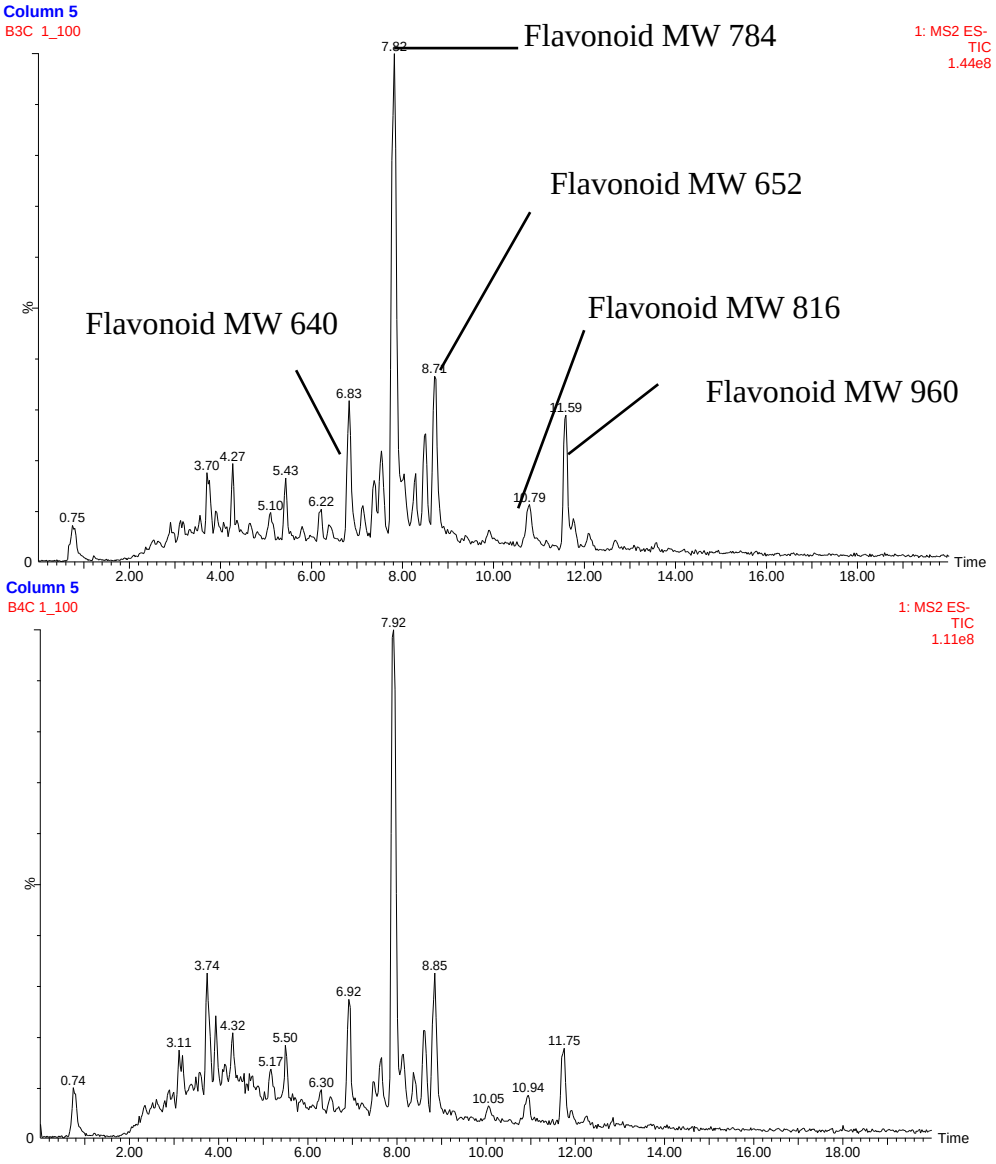



**Appendix Figure 5.3.** UPLC-MS/MS chromatogram of two MRM channels used for monitoring the presence of HMG and deuterated HMG ($D_3$ HMG) in samples of rat kidney. Panels A and B are from the $D_3$ HMG standard, with panels C and D from non-deuterated (endogenous) HMG. The ratio of HMG areas between MRM channels is consistent for both standard (A:B = 0.38) and kidneys (C:D = 0.41), with a characteristic retention time offset for the deuterated standard (~ 3.00 min versus 3.04 min). Another endogenous compound undergoing the same mass losses is observed at 2.75 min in HMG chromatograms.

### *9.4.3 Chromatograms of crude extract for animal studies*




**Appendix Figure 5.4.** 370nm chromatogram of the crude *Carpobrotus rossii* extracts used for the *in vivo* studies with relevant flavonoid peaks identified.




**Appendix Figure 5.5** Total ion chromatogram (TIC) of the crude *Carpobrotus rossii* extracts used for the *in vivo* studies with relevant flavonoid peaks identified.