



UNIVERSITY  
OF TASMANIA

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

**Adam Douglas Pirie**

**BLWSc, Sydney**

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

**June 2014**

## **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

5<sup>th</sup> June 2014

## **Authority of Access**

This thesis is not to be made available for loan or copying for two years following the date this statement was signed. Following that time the thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.



Adam Douglas Pirie

5<sup>th</sup> June 2014

## **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.

The following people and institutions contributed to the publication or preparation of the work undertaken as part of this thesis:

Candidate: Adam Pirie,

Author 1: Christian Narkowicz<sup>1</sup>,  
Author 2: Glenn Jacobson<sup>1</sup>,  
Author 3: Sergey Shabala<sup>2</sup>,  
Author 4: Michelle Keske<sup>3</sup>,  
Author 5: Dominic Geraghty<sup>4</sup>,  
Author 6: Noel Davies<sup>5</sup>,  
Author 7: James Horne<sup>5</sup>,  
Author 8: Pavel Nesterenko<sup>6</sup>,

Author 9: Kiran Ahuja<sup>4</sup>,  
Author 10: David Parsons<sup>2</sup>,  
Author 11: Murray Adams<sup>4</sup>,  
Author 12: Cecilia Shing<sup>4</sup>,  
Author 13: Jolanda Renggli<sup>1</sup>,  
Author 14: Nynke Jager<sup>1</sup>,  
Author 15: Anton Peristy<sup>6</sup>.

<sup>1</sup>School of Pharmacy, University of Tasmania; <sup>2</sup>School of Agricultural Science University of Tasmania; <sup>3</sup>Menzies Research Institute, University of Tasmania; <sup>4</sup>School of Human and Life Sciences, University of Tasmania; <sup>5</sup>Central Science Laboratory, University of Tasmania; <sup>6</sup>School of Chemistry, University of Tasmania.

## **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 authors 1-3 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 authors 1-3 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.*

We the undersigned agree with the above stated “proportion of work undertaken” for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed: 

Candidate Adam Douglas Pirie

Author 1 \_\_\_\_\_

Author 2 \_\_\_\_\_

Author 3 \_\_\_\_\_

Author 4 \_\_\_\_\_

Author 5 \_\_\_\_\_

Author 6 \_\_\_\_\_

Author 7 \_\_\_\_\_

Author 8 \_\_\_\_\_

Author 9 \_\_\_\_\_

Author 10 \_\_\_\_\_

Author 11 \_\_\_\_\_

Author 12 \_\_\_\_\_

Author 13 \_\_\_\_\_

Author 14 \_\_\_\_\_

Author 15 \_\_\_\_\_

## Acknowledgements

The list of people I owe a debt of gratitude for their involvement in and/or support of this work is long and extensive.

Firstly I would like to thank both of my School of Pharmacy supervisors Dr Glenn Jacobson and Dr Christian Narkowicz for their support and encouragement whilst pursuing such a multi-disciplinary project. To my other supervisors Prof Dominic Geraghty, Dr Michelle Keske and Prof Sergey Shabala, thank you for your enthusiasm and willingness to support such an unconventional project; and for providing the individual expertise and guidance needed to enable this thesis to happen successfully. Your support, insight and guidance, especially when things weren't running as planned was truly appreciated. You are all well and truly been responsible for me going from "soil boy" to "drug-discovery child".

To the Utas CSL staff, especially A/Prof Noel Davies and Dr James Horne, your approachability, ability and willingness to provide mentoring, in addition to your technical prowess, is very much appreciated.

To my other co-authors. Thankyou for your approachability, willingness to be involved in various facets of the project and ability to provide input, expertise and guidance when I knocked on your door needing a technique or equipment to "plug and play" with my samples. Without you, many of the questions asked during this work would still remain unanswered.

Dr Peter Traill, Heather Galloway and Mr Phillip Andrews, Dr Dino Premilovac, Eloise Bradley and the other members of the MRG, in addition to Drs Nuri Güven, Jason Smith, Rahul Patel and Mr Steve Weston. Your assistance, support, provision of logistics for this project has been invaluable as has your tolerance of me asking "rookie" questions when my brain occasionally decided to go on vacation, so thank you.

Mum, Dad, Jarrod and Rhys, thank you for being sounding boards, supporters, listening to me vent and generally letting me know that you care.

Most of all I would like to thank my wonderful fiancé Lucy, without you none of this would have happened and I will be forever grateful for your support. You have no idea how much I am looking forward to our next adventure(s) together.

## Contents

Statement of Originality .....	i
Authority of Access.....	i
Statement of Ethical Conduct .....	i
Statement of Co-Authorship.....	ii
Manuscripts arising from this thesis and statement of contribution .....	iii
Acknowledgements .....	vi
PhD overview.....	xx
1.1 The Vascular System.....	1
1.1.1 Cardiovascular system .....	1
1.1.1.1 Vessel Anatomy .....	1
1.1.1.1.1 The Endothelium.....	4
1.1.2 Glucose delivery and uptake .....	7
1.1.2.1 Insulin signalling .....	8
1.1.3 Inflammation and the Disease process .....	9
1.1.3.1 Causes of endothelial dysfunction .....	10
1.1.3.2 Platelets .....	14
1.1.3.3 Lipoproteins .....	14
1.1.3.4 Hyperlipidaemia .....	16
1.1.3.5 Hyperglycaemia.....	17
1.1.4 Treatment .....	18
1.1.4.1 Lowering of low-density lipoprotein .....	21
1.1.4.2 Lowering blood glucose .....	23
1.1.4.3 Reducing Oxidative Stress.....	24
1.1.4.4 Increasing NO production.....	25
1.1.4.5 Alteration of signal transduction and receptors.....	25
1.2 Potential for <i>C. rossii</i> as a nutraceutical product.....	27
1.2.1.1 The plant .....	28
1.3 Plant Metabolism.....	30
1.3.1 ROS generation and function in planta .....	30
1.3.2 ROS induced damage .....	35



1.3.2.1	Low Temperature, high light conditions and exposure to ultra-violet light	38
1.3.2.2	High Sodium and low potassium .....	40
1.3.3	Antioxidants to combat ROS .....	41
1.3.3.1	Flavonoid structure.....	43
1.3.3.2	Role of flavonoids in Planta .....	45
1.4	<i>C. rossii</i> novel flavonoid - pharmaceutical potential.....	46
1.5	PhD Aims .....	48
2	Flavonoid and tannin production of <i>Carpobrotus rossii</i> is modulated by environmental conditions.....	49
2.1	Abstract .....	49
2.2	Introduction .....	50
2.3	Material and Methods.....	53
2.3.1	Sampling methods and location .....	53
2.3.2	Chlorophyll fluorescence .....	53
2.3.3	Soil Sampling .....	54
2.3.4	Stomata density .....	54
2.3.5	Leaf sap nutrient analysis and osmolarity .....	54
2.3.6	Flavonoid concentration by HPLC.....	54
2.3.7	Antioxidant activity assessment.....	55
2.3.8	Glasshouse experiment .....	56
2.3.9	Statistical Analysis .....	57
2.4	Results.....	58
2.4.1	Field Survey .....	58
2.4.2	Biomass and flavonoid production under controlled conditions.....	63
2.4.3	Leaf ionic relations under saline conditions .....	71
2.5	Discussion .....	74
2.6	Conclusion .....	77
2.7	Acknowledgements .....	78
3	Low-temperature and ultraviolet B exposure induce separate and structurally unrelated biochemical responses in <i>Carpobrotus rossii</i> .....	79

3.1 Abstract .....	79
3.2 Introduction .....	80
3.3 Experimental .....	81
3.3.1 Materials and reagents .....	81
3.3.2 Low Temperature experiments .....	82
3.3.2.1 Plant propagation.....	82
3.3.2.2 Sample collection .....	82
3.3.3 Ultraviolet B radiation exposure .....	83
3.3.3.1 Plant propagation and growing conditions- Ultra-violet B exposure.....	83
3.3.3.2 Sample collection .....	83
3.3.4 Statistical analysis.....	83
3.4 Results.....	83
3.4.1 Temperature Experiments.....	83
3.4.1.1 Temperatures and growing conditions.....	83
3.4.1.2 Flavonoid production.....	84
3.4.1.3 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay performance .....	86
3.4.1.4 Antioxidant Activity .....	86
3.4.1.5 Biomass production .....	87
3.4.1.6 Nutrient levels .....	87
3.4.2 Ultra-violet B exposure.....	88
3.4.2.1 Flavonoid and betalain production.....	88
3.4.2.2 DPPH Antioxidant Activity .....	88
3.4.2.3 Effect of UVB on Biomass .....	88
3.4.2.4 Nutrient levels .....	88
3.5 Discussion .....	89
3.5.1 Mechanisms and specificity of ROS generation.....	89
3.5.2 Nutritional levels, sodium and potassium, biomass.....	93
3.5.3 Antioxidant activity .....	95
3.6 Conclusion .....	96
3.6.1 Acknowledgements .....	96

4	Pirieol A from <i>Carpobrotus rossii</i> , a novel spinacetin glycoside containing apiose and HMG moieties.....	97
4.1	Abstract.....	97
4.2	Introduction.....	98
4.3	Material and methods .....	99
4.3.1	General experimental procedures.....	99
4.4	Results and Discussion.....	101
4.5	Acknowledgements .....	109
5	Hypolipidaemic effect of crude extract from <i>Carpobrotus rossii</i> (pigface) in healthy rats	110
5.1	Abstract.....	110
5.2	Introduction.....	111
5.3	Materials and Methods .....	112
5.3.1	Materials and Reagents.....	112
5.3.2	Preparation and flavonoid content of <i>C. rossii</i> extract.....	112
5.3.3	Animal treatment .....	113
5.3.4	Blood and tissue collection .....	113
5.3.5	In Vitro vascular responsiveness.....	114
5.3.6	Lipid, cholesterol, glucose analysis .....	114
5.3.7	Determination of kidney HMG levels.....	115
5.3.8	Statistical analysis.....	115
5.4	Results.....	116
5.5	Discussion .....	120
5.6	Conclusion .....	122
5.7	Acknowledgements .....	123
6	Flavonoids from <i>Carpobrotus rossii</i> improve glucose clearance in insulin resistant animals.....	124
6.1	Abstract.....	124
6.2	Introduction.....	125
6.3	Materials and Methods .....	127
6.3.1	Materials and Reagents.....	127

6.3.2	Preparation of crude and flavonoid-rich <i>C. rossii</i> extracts.....	127
6.3.3	Animal models.....	129
6.3.3.1	Animal Housing .....	129
6.3.3.2	Insulin resistance model (high fat diet C57/BL6 mice).....	129
6.3.3.3	Hyperlipidaemia model (high cholesterol diet rats) .....	130
6.3.3.4	Blood collection and measurement .....	131
6.3.4	Statistical analysis .....	131
6.4	Results.....	132
6.4.1	Insulin resistant (C57/BL6) mice .....	132
6.4.1.1	Body weight gain, food and water consumption .....	132
6.4.1.2	Glucose clearance.....	133
6.4.1.3	Blood lipids .....	134
6.4.2	Hyperlipidaemic rats.....	134
6.4.2.1	Body weight gain, food and water consumption .....	134
6.4.2.2	Blood lipids .....	135
6.5	Discussion .....	136
6.5.1	C57/BL6 mice body weight gain, energy and water consumption. ....	136
6.5.2	C57/BL6 Mice glucose tolerance.....	136
6.5.3	C57/BL6 Mice blood lipids .....	137
6.5.4	Sprague-Dawley weight gain, energy and water consumption.....	138
6.5.5	Sprague-Dawley at blood lipids .....	138
6.6	Conclusion .....	139
6.7	Acknowledgements .....	139
6.8	Declaration of interests.....	139
6.9	Contributors statement.....	139
7	Thesis overview and direction of future work .....	140
7.1	Overview .....	140
7.2	Thesis Limitations .....	140
7.3	Future directions.....	142
7.4	Conclusion .....	144
8	References .....	145

9	Appendices.....	173
9.1	Appendix Chapter 2.....	173
9.2	Appendix Chapter 3.....	183
9.2.1	HPLC-UV chromatography .....	183
9.2.2	UPLC-MS/MS chromatography .....	184
9.2.2.1	Betalains:.....	184
9.2.2.2	Flavonoids:.....	184
9.2.3	Ion Chromatography .....	185
9.2.4	Spectra and radiometric data.....	186
9.3	Appendix Chapter 4.....	187
9.3.1	NMR studies.....	187
9.3.2	Preparative HPLC conditions.....	193
9.3.3	UPLC-MS/MS chromatography .....	193
9.3.4	Flavonoids: .....	194
9.4	Appendix Chapter 5 Flavonoid analysis of crude leaf extract.....	195
9.4.1	Flavonoids .....	195
9.4.2	Kidney HMG analysis .....	198
9.4.3	Chromatograms of crude extract for animal studies .....	200

## List of Figures

Figure 1.1. Vessels of the vascular system. ....	1
Figure 1.2. The variation in physiology of different blood vessel types .....	3
Figure 1.3. a) Tetrahydrobiopterin and b) NOS mediated generation of Nitric Oxide .....	5
Figure 1.4. Characteristics of the functioning healthy endothelium.....	6
Figure 1.5. Insulin and exercise result in increased capillary recruitment and glucose ( ) uptake in skeletal muscle tissue.....	7
Figure 1.6. Cellular activities mediated by insulin signalling .....	8
Figure 1.7. The feedback loop of persistent endothelial damage. ....	13
Figure 1.8. The digestion, absorption and cycling of cholesterol and lipids through the vasculature and bloodstream. ....	15
Figure 1.9. The cholesterol synthesis pathway, products and the HMG CoA reductase inhibition by statins. ....	22
Figure 1.10. 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.....	27
Figure 1.11. Location of the major sites of ROS production in photosynthetic cells. ....	31
Figure 1.12. An overview of the photosynthetic process .....	32
Figure 1.13. Potential electron acceptors of photosynthesis. ....	32
Figure 1.14. Schematic of ROS generation mechanisms .....	34
Figure 1.15. The interaction of ROS generation/signalling and damage under unstressed and stressed conditions. ....	35
Figure 1.16. Mechanisms of ROS cellular damage.....	37
Figure 1.17. Potential ROS generation mechanisms of transition metals.....	42
Figure 1.18. (a) The primary flavonoid skeleton, (b) flavonoid families. ....	44
Figure 1.19. Variation in substituents to yield various flavonol aglycones.....	45
Figure 1.20. Substituents of the <i>C. rossii</i> flavonoid.....	47
Figure 2.1. Field survey locations, showing sites of soil and plant collection.....	59
Figure 2.2. Individual plant flavonoid and tannin production highlighting the intra and inter-site variation in metabolite production between plants. ....	60
Figure 2.3. (a) Dendrogram of cluster analysis showing a small group of high flavonoid and tannin producing plants with a larger population of low producing plants.....	61

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. ....	62
Figure 2.5. Effect of salt (NaCl) treatment on plant biomass production. ....	65
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. ....	66
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 spongy 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. ....	69
Figure 2.8. Determination of optimal flavonoid production conditions. ....	70
Figure 2.10. Osmotic and Ionic response to NaCl concentration. ....	71
Figure 2.11. Specific ion response of leaf sap to NaCl treatment (a) palisade Na <sup>+</sup> , (b) palisade K <sup>+</sup> , (c) spongy Na <sup>+</sup> , (d) spongy K <sup>+</sup> . ....	73
Figure 3.1. (a) Statistical summary of control and low-temperature treatments (Mean ± SEM), (b) temperature logs of control and low-temperature treatments. ....	85
Figure 3.2. Analysis of low and control temperature CR leaf palisade tissue. ....	86
Figure 3.3. Na <sup>+</sup> and K <sup>+</sup> levels of low and control temperature mesophyll and palisade tissue ....	87
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. ....	89
Figure 3.5. Anthocyanin (a) and the Betalain aglycone (b) both possess a permanent positive charge as well as visible pigmentation. ....	91
Figure 3.6. (a) The generalised naming structure of flavonoid aglycones (Adapted from Crozier et al. (2009)), and b) spinacetin. ....	92
Figure 4.1. UPLC chromatogram of CR leaf extract ....	99
Figure 4.2. Pirieol A, Key HMBC ( ) and NOESY ( - - ) correlations. ....	107
Figure 4.3. Proposed structures related to compound 1. ....	108

Figure 5.1. Control (water) and <i>C. rossii</i> 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.....	117
Figure 5.2. Representative plots and Mean $\pm$ SEM vascular response of control (water) and <i>C. rossii</i> supplemented animals .....	119
Figure 5.3. Level of free HMG in the kidney tissue of control (water) and <i>C. rossii</i> supplemented rats.....	120
Figure 6.1. (a) Animal weight gain, (b) daily water consumption, and (c) daily energy consumption of C57/BL6 mice.....	132
Figure 6.2. Plasma glucose concentration following a 2mg/kg ip bolus injection of glucose .....	133
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...	135



## List of Tables

Table 1.1. A comparison of the cardiovascular protective effects provided by statin and flavonoid compounds .....	20
Table 1.2. Reactive Oxygen and Nitrogen Species .....	34
Table 1.3. Approximate cost of replacing damaged cellular components .....	38
Table 1.4. Plant antioxidant systems .....	41
Table 2.1. HPLC conditions for flavonoid and tannin detection. ....	55
Table 2.2. Frequency (%) of prediction variable occurrence in the 10 best 4-factor prediction models for flavonoid, tannin and antioxidant production in <i>C. rossii</i> leaves. ....	64
Table 2.3. Relative contribution of inorganic osmolytes ( $K^+$ , $Na^+$ , $Cl^-$ ) towards overall osmotic adjustment in parenchyma and mesophyll leaf tissue of <i>C. rossii</i> at various salinity treatments. ....	72
Table 3.2. Biomass production of control and low-temperature grown plants (n = 15, # n=30) .....	87
Table 4.1. UV Spectrum analysis of the <i>C. rossii</i> flavonoid aglycone. ....	102
Table 4.2. NMR Spectroscopic Data Structure 1 aglycone (800 MHz, d6-DMSO) .....	105
Table 4.3. NMR Spectroscopic Data Structure 1 Substituents (800 MHz, d6-DMSO) .....	106
Table 5.1. Organ weights of control (water) and <i>C. rossii</i> supplemented (CR) animals .....	116
Table 5.2. Full blood analysis and blood biochemistry of control (water) and <i>C. rossii</i> supplemented (CR) animals .....	118
Table 5.3. $EC_{50}$ 's and 95% confidence intervals (CI) for noradrenaline (NA)-, sodium nitroprusside (SNP)- and acetylcholine (ACh)-induced <i>in vitro</i> vascular (aortic) responses from control (water) and <i>C. rossii</i> (CR) treated animals. ....	118
Table 6.1. Macronutrient dietary composition of insulin resistant (HFD) and normal (NDM) diets used in insulin resistant C57/BL6 mice. ....	129
Table 6.2. Macronutrient dietary composition of hyperlipidaemic (HL) and normal (NDR) diets used in hyperlipidaemic Sprague-Dawley rats. ....	130
Table 6.3. Blood lipids of C57/BL6 mice. ....	134
Table 6.4. Blood lipids of Sprague-Dawley rats. ....	136

## List of Appendix Figures

Appendix Figure 2.1. Cross section of <i>C. rossii</i> leaf showing the clear segregation between (A) palisade mesophyll and (B) spongy parenchyma. ....	182
Appendix Figure 3.1. Spectroradiometric profile of UVB experimental plants. (a) UVB lamps on, (b) UVB lamps off, (c) control lighting. ....	186
Appendix Figure 4.1. Compound 1 – evidence of line broadening in HMG methylene resonances. ....	187
Appendix Figure 4.2. Compound 1 <sup>1</sup> H- <sup>13</sup> C HSQC. ....	188
Appendix Figure 4.3. Compound 1 <sup>1</sup> H- <sup>13</sup> C HMBC. ....	189
Appendix Figure 4.4. Compound 1 <sup>1</sup> H-1D. ....	190
Appendix Figure 4.5. Compound 1 <sup>13</sup> C-1D. ....	191
Appendix Figure 4.6. The structure of Compound 1 (Pirieol A) as determined by NMR experimentation. ....	192
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] <sup>-</sup> 783 isomers, (c) UPLC-DAD chromatogram at 350nm indicating the presence of flavonoid compounds. ...	196
Appendix Figure 5.2. . MS/MS fragmentation of CR representative flavonoids. ....	197
Appendix Figure 5.3. UPLC-MS/MS chromatogram of two MRM channels used for monitoring the presence of HMG and deuterated HMG (D <sub>3</sub> HMG) in samples of rat kidney. ....	199
Appendix Figure 5.4. 370nm chromatogram of the crude <i>Carpobrotus rossii</i> extracts used for the <i>in vivo</i> studies with relevant flavonoid peaks identified. ....	200
Appendix Figure 5.5. Total ion chromatogram (TIC) of the crude <i>Carpobrotus rossii</i> extracts used for the <i>in vivo</i> studies with relevant flavonoid peaks identified. ....	201

## List of Appendix Tables

Appendix Table 2.1 Collection location of samples .....	173
Appendix Table 2.2. Mean soil variables per site, SP 1 and STAN 1-5 sites not removed. .	177
Appendix Table 2.3. Summary of variation in soil variables with and without clay/colluvium soil sites .....	178
Appendix Table 2.4. The best 1 to 4 factor regression models for prediction of flavonoid, tannin and antioxidant production in <i>C. rossii</i> leaves. ....	179
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 <sup>a</sup> .....	180
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 <sup>a</sup> .....	181
Appendix Table 3.1 Solvent conditions for HPLC-UV Chromatography.....	183
Appendix Table 5. Preparative HPLC conditions for the purification of the 784Da flavonoid .....	193



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 production 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