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

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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) and disrupted 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).

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 which 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-methylglutarric 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 acetocacete to HMG-CoA (Moorjani and Lupien, 1977).

Polyphenolic plant compounds (especially flavonoids) have been shown to offer protection against atherosclerosis and metabolic disorder (hyperlipidemia, hyperglycaemia, hypercholesterolaemia) 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 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.

2. Materials and methods

2.1. 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 (Rompun®; JP Industrial, CA, USA) was purchased for anaesthesia. Sodium pentobarbitone, acetylcholine, sodium nitroprusside and noradrenaline were kept refrigerated at 4°C until used. Sodium pentobarbitone was prepared with 60 mg/kg sodium pentobarbitone, which had their common carotid artery cannulated and were exsanguinated into 100 μl aliquots and stored at −80°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.

2.2. 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 diode array detection (HPLC-DAD) ultra violet chromatograms of the extract with leaf homogenate from authenticated voucher specimens (HO 529461, HO 526062) held at the Tasmanian Herbarium (TAS, AUS). Once dry, the plant extract was reconstituted in water at 100 mg/ml. The CR extract contained a suite of flavonoids apparent from the UV chromatograms, with acid hydrolysis of the extract yielding a common flavonoid aglycone; spinacetin which was identified by LC–MS/MS (Fig. 1). LC–MS/MS data from the unhydrolysed extract was indicative of several closely related acylated flavonol glycosides based on the spinacetin aglycone; spinacetin which was identified by LC–MS/MS (Fig. 1). LC–MS/MS (Supplementary material 1). The LC–MS/MS data also suggested the presence of an HMG substituent (Supplementary data 1).

2.2.2. Animal treatment

Animal experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes – 7th 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).

2.2.3. 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 K3000i (Sysmex Corporation, Kobe, Japan), whilst the lithium heparin blood was transferred to Eppendorf® tubes, centrifuged for 15 min at 850g, divided into 100 μl aliquots and stored at −80°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.

2.2.4. 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, MgCl2 1.65, NaHCO3 22.6, CaCl2 1.8, glucose 5.5, ascorbic acid 0.28 and Na2EDTA 0.05), cleared of adventitial tissue, sectioned into rings (3 × 4 mm) and mounted in 5 ml 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. Endothelial dependant (sodium nitroprusside, SNP) and independent (acetylcholine, ACh) relaxation was measured in aortic segments previously contracted with NA.

2.2.5. Lipid, cholesterol, glucose analysis

Plasma glucose, triglycerides, cholesterol and high density lipoprotein (HDL) were measured by spectrophotometric enzymatic methods (Kone lab 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).

2.2.6. 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 (375 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 s. Glacial acetic acid (20 μl) was then added to inhibit protein binding and facilitate protein precipitation, the sample vortex mixed and centrifuged at 15,000g for 10 min. 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 (Agilent UPLC-Xevo triple quadrupole MS, Waters Corporation, MA, USA). Full LC and MS conditions are given as Supplementary material 1. 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 (R² = 0.9999, RSD = 0.46%, accuracy = 0.08%, n = 6 at 600 ng/g) generated using ‘blank’ kidney homogenates spiked with known HMG concentrations and prepared under identical conditions to the experimental samples.

2.3. 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.

3. 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 (Fig. 2).

There were no differences in the weight of any weighed internal organs or blood parameters between the two groups (Table 1).

Table 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.

The effects of NA, SNP and ACh on aortic rings from control and CR-treated rats are presented in Fig. 3 and Table 3. Concentration–response curves for NA, SNP and ACh were similar for both groups of rats and no statistically significant differences in EC50 values were obtained.

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 (Fig. 4), consistent with the presence of a flavonoid HMG substituent as suggested by LC–MS/MS analysis of the CR extract.

### Table 1

<table>
<thead>
<tr>
<th>Organ weight (mg/100 g body weight)</th>
<th>Water ($n = 8$)</th>
<th>CR ($n = 8$)</th>
<th>$p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder</td>
<td>26 ± 2</td>
<td>29 ± 3</td>
<td>0.436</td>
</tr>
<tr>
<td>Testes</td>
<td>913 ± 27</td>
<td>883 ± 26</td>
<td>0.453</td>
</tr>
<tr>
<td>Kidneys</td>
<td>780 ± 17</td>
<td>750 ± 24</td>
<td>0.334</td>
</tr>
<tr>
<td>Liver</td>
<td>4420 ± 62</td>
<td>4521 ± 125</td>
<td>0.482</td>
</tr>
<tr>
<td>Heart</td>
<td>275 ± 4</td>
<td>276 ± 6</td>
<td>0.930</td>
</tr>
<tr>
<td>Spleen</td>
<td>186 ± 6</td>
<td>182 ± 8</td>
<td>0.733</td>
</tr>
<tr>
<td>Lungs</td>
<td>367 ± 9</td>
<td>336 ± 26</td>
<td>0.283</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Water ($n = 8$)</th>
<th>CR ($n = 8$)</th>
<th>$p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC ($10^{12}$/L)</td>
<td>8.72 ± 0.33</td>
<td>8.48 ± 0.10</td>
</tr>
<tr>
<td>HGB (g/L)</td>
<td>153 ± 6</td>
<td>150 ± 2</td>
</tr>
<tr>
<td>HCT %</td>
<td>0.44 ± 0.12</td>
<td>0.44 ± 0.01</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>50.9 ± 0.58</td>
<td>51.8 ± 0.65</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>17.6 ± 0.14</td>
<td>17.7 ± 0.14</td>
</tr>
<tr>
<td>MCHC (g/L)</td>
<td>300 ± 43</td>
<td>343 ± 3</td>
</tr>
<tr>
<td>Platelets ($10^9$/L)</td>
<td>995 ± 27</td>
<td>967 ± 46</td>
</tr>
<tr>
<td>WBC ($10^{9}$/L)</td>
<td>4.49 ± 0.44</td>
<td>4.46 ± 0.54</td>
</tr>
<tr>
<td>Neutrophils ($10^9$/L)</td>
<td>0.77 ± 0.23</td>
<td>0.59 ± 0.25</td>
</tr>
<tr>
<td>Lymphocytes ($10^9$/L)</td>
<td>3.14 ± 0.38</td>
<td>2.57 ± 0.67</td>
</tr>
<tr>
<td>Monocytes ($10^9$/L)</td>
<td>0.28 ± 0.10</td>
<td>0.29 ± 0.08</td>
</tr>
<tr>
<td>Eosinophils ($10^9$/L)</td>
<td>0.07 ± 0.02</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>Basophils ($10^9$/L)</td>
<td>0.12 ± 0.12</td>
<td>0.10 ± 0.10</td>
</tr>
<tr>
<td>Cholesterol (mg/ml)</td>
<td>1.94 ± 0.09</td>
<td>1.57 ± 0.07</td>
</tr>
<tr>
<td>HDL (mg/ml)</td>
<td>0.66 ± 0.04</td>
<td>0.62 ± 0.04</td>
</tr>
<tr>
<td>Triglycerides (mg/ml)</td>
<td>0.85 ± 0.24</td>
<td>0.97 ± 0.18</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>11.2 ± 1.6</td>
<td>11.7 ± 1.3</td>
</tr>
<tr>
<td>Atherogenic cholesterol (mg/ml)</td>
<td>1.29 ± 0.05</td>
<td>1.05 ± 0.08</td>
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4. 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 in rats which resulted in no mortalities and only a minor reduction in serum iron levels recorded after treatment for 90 days

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Fig. 2. 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).

$p < 0.05$. 

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Morita et al. (2009) reported levels of safety in rats for the green tea catechins of 1200 mg/kg BW/day (male) and 400 mg/kg BW/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 in HMG in the kidney after CR may have been

Table 3

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Water EC_{50} (nM, 95% CI)</th>
<th>CR extract EC_{50} (nM, 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td>45 (36–55)</td>
<td>48 (39–59)</td>
</tr>
<tr>
<td>SNP</td>
<td>35 (22–54)</td>
<td>52 (28–98)</td>
</tr>
<tr>
<td>ACh</td>
<td>116 (51–265)</td>
<td>213 (146–312)</td>
</tr>
</tbody>
</table>

Fig. 3. Representative plots and Mean ± SEM response of control (water) and C. rossii (CR) supplemented animals to (a and b) noradrenalina (NA) (n = 7, 5); (c and d) sodium nitroprusside (SNP) (n = 7, 6) and (e and f) acetylcholine (ACh) (n = 4, 6).

Fig. 4. 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).
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 findings: 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 et al. (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 its 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. Conclusion

CR extract improved the lipoprotein profile, but had no beneficial effects on other measured cardiovascular parameters. 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 CR extracts cardioprotective properties in disease models and determine which of the compound(s) impart this protection.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jfct.2014.01.034.

References


