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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), 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.

1. Introduction

Carpobrotus species are large trailing succulent perennials which grow on the Pacific coast of the Americas, South Africa, and Australia. The fruit of *Carpobrotus edulis* is eaten in South Africa, and *Carpobrotus rossii* ((Haw.) Schwantes, 1928) 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 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-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)

Abbreviations: ACh, acetylcholine; CR, *Carpobrotus rossii*; EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoprotein; HMG, 3-hydroxy-3-methylglutaric acid; HMG-CoA, HMG-Coenzyme A; NA, noradrenaline; SNP, sodium nitroprusside; SBP, systolic blood pressure; UPLC–MS/MS, ultra-performance liquid chromatography tandem mass spectrometry.

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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 was purchased from Boehringer Ingelheim (NSW, AUS). All other standard reagents and materials were of analytical grade.

2.2. Methods

2.2.1. 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 529462) 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 flavonols apparent from the UV chromatograms, with acid hydrolysis of the extract yielding a common flavonol 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. It would be reasonable to assume that acid hydrolysis of the glycosides in the gut would also yield the spinacetin aglycone. Crude CR extract was found to consist of 31% w/v acylated spinacetin glycosides measured by HPLC-DAD (with these spinacetin glycosides greater than 95% purity). 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).

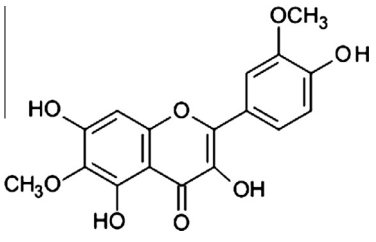

Fig. 1. Structure of the flavonol aglycone, spinacetin.

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 1000i (Sysmex Corporation, Kobe, Japan), whilst the lithium heparin blood was transferred to Eppendorf® tubes, centrifuged for 15 min at 850*g*, 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, $MgCl_2$ 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 × 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. Endothelium dependant (sodium nitropruside, 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 (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).

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 (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 s. Glacial acetic acid (20 µl) was then added to inhibit protein binding and facilitate protein precipitation, the sample vortex mixed and centrifuged at 15,000*g* 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 (Aquity 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 ($R^2 = 0.9999$, 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