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# Model development for predicting *in vitro* bio-capacity of green rooibos extract based on composition for application as screening tool in quality control†

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Mounting evidence of the ability of aspalathin to target underlying metabolic dysfunction relevant to the development or progression of obesity and type 2 diabetes created a market for green rooibos extract as a functional food ingredient. Aspalathin is the obvious choice as a chemical marker for extract standardisation and quality control, however, often the concentration of a single constituent of a complex mixture such as a plant extract is not directly related to its bio-capacity, *i.e.* the level of *in vitro* bioactivity effected in a cell system at a fixed concentration. Three solvents (hot water and two EtOH–water mixtures), previously shown to produce bioactive green rooibos extracts, were selected for extraction of different batches of rooibos plant material ($n$ = 10). Bio-capacity of the extracts, tested at 10 μg ml$^{-1}$, was evaluated in terms of glucose uptake by C2C12 and C3A cells and lipid accumulation in 3T3-L1 cells. The different solvents and inter-batch plant variation delivered extracts ranging in aspalathin content from 54.1 to 213.8 g kg$^{-1}$. The extracts were further characterised in terms of other major flavonoids ($n$ = 10) and an enolic phenylpyruvic acid glucoside, using HPLC-DAD. The 80% EtOH–water extracts, with the highest mean aspalathin content (170.9 g kg$^{-1}$), had the highest mean bio-capacity in the respective assays. Despite this, no significant ($P \geq 0.05$) correlation existed between aspalathin content and bio-capacity, while the orientin, isoorientin and vitexin content correlated moderately ($r \geq 0.487$; $P < 0.05$) with increased glucose uptake by C2C12 cells. Various multivariate analysis methods were then applied with Evolution Program-Partial Least Squares (EP-PLS) resulting in models with the best predictive power. These EP-PLS models, based on all quantified compounds, predicted the bio-capacity of the extracts for the respective cell types with RMSECV values ≤ 11.5, confirming that a complement of compounds, and not aspalathin content alone, is needed to predict the *in vitro* bio-capacity of green rooibos extracts. Additionally, the composition of hot water infusions of different production batches of green rooibos ($n$ = 29) at 'cup-of-tea' equivalence was determined to relate dietary supplementation with the extract to intake in the form of herbal tea.

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## Introduction

The dramatic increase in metabolic diseases such as obesity, type 2 diabetes and cardiovascular disease emphasises the importance of addressing poor diet and lifestyle, recognised as the only long-term strategy able to reduce the diabetic burden.[1] Functional foods and beverages provide an option to promote health through the diet as evidence suggests that diet modification should not only focus on macronutrients, but that micronutrients are essential for ensuring metabolic health.[2,3] The beneficial role of plant polyphenols as modulators of glycaemia has garnered much attention, driving the interest in single compounds and complex mixtures such as

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plant extracts containing high levels of specific polyphenols with proven bioactivity.

Mounting evidence of the ability of aspalathin, a *C*-glucosyl dihydrochalcone, to target underlying metabolic dysfunction relevant to the development or progression of obesity, type 2 diabetes and cardiovascular diseases[4,5] fuelled interest in the development of a functional food ingredient extract from green rooibos (*Aspalathus linearis* (Burm.f.) Dahlgr.), used as herbal tea.[6] Green rooibos extracts, containing different levels of aspalathin and/or prepared with different solvents, were shown to have relevant anti-diabetic properties in different *in vitro* and *in vivo* models.[7] Sub-chronic feeding of obese diabetic KK-Ay mice with a cold-water green rooibos extract (GRE; 66 g $kg^{-1}$ aspalathin) suppressed an increase in fasting blood glucose levels.[8] An aspalathin-enriched GRE (184 g $kg^{-1}$ aspalathin), prepared using 80% ethanol–water as solvent, was effective in reducing elevated blood glucose levels of streptozotocin-induced diabetic Wistar rats, following acute oral administration.[9] Treatment of diabetic non-human primates with GRE (128 g $kg^{-1}$ aspalathin) three times a day at 90 mg $kg^{-1}$ lowered plasma low-density lipoprotein (LDL)-cholesterol, oxidised LDL levels and oxidised coenzyme Q10.[10] Compounds present at much lower concentration in these extracts were nothofagin, the 3-deoxy derivative of aspalathin, orientin and isoorientin, several flavonol glycosides such as bioquercetin and rutin, and the enolic phenylpyruvic acid glucoside, *Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid (PPAG).

Following a quality-by-design (QbD) approach, process parameters for producing a hot water extract of green rooibos containing at least 80 g $kg^{-1}$ aspalathin were established[11] as the first step toward extracts with a guaranteed *in vitro* bio-capacity. Hot water extraction was selected in favour of other extraction solvents as it represents the most common solvent for the production of food grade extracts of rooibos. A QbD approach shifts the focus from quality assurance through testing to quality control (QC) by process understanding.[12] Miller *et al.*[11] also identified critical material attributes that would impact on the aspalathin content of the extract, which addressed to some extent one of the key issues that handicaps implementation of QbD for herbal products, *i.e.* quality variation of the herbal raw material.[13] Another key issue of QbD of herbal products is the difficulty in defining acceptable ranges for the critical quality attributes of the product.[13] For a functional food extract, *in vitro* bio-capacity can be added as a critical quality attribute. However, food ingredient extract manufacturers are not normally set up for routine testing of the bio-capacity of extracts. A non-biological analytical assay that is practical and could serve as surrogate measurement of the *in vitro* bio-capacity would be ideal as a first screening tool for QC of the final product, such as spray-dried extract powder.

The present study investigated whether the aspalathin content of GRE alone, given its high concentration, or in combination with other major compounds, could predict its *in vitro* bio-capacity, and thus strengthen its use as a quality parameter of the spray-dried powder. Enhanced glucose uptake in C2C12 myocytes and C3A hepatocytes, and lipid accumulation in 3T3-L1 adipocytes were used to assess the bio-capacity of the extract. These cell-based assays were selected since C2C12, C3A and 3T3-L1 cells represent major tissues involved in maintaining glucose homeostasis *in vivo*.[14,15] Variation in the extract composition was ensured by employing, in the first instance, three extraction solvents, *i.e.* hot water and two ethanol-water mixtures, and secondly, preparing extracts from different batches of raw material. The use of different solvents also served to delineate whether extract type and thus matrix affects *in vitro* bio-capacity. The potential effect of extract type/matrix on the bioavailability of aspalathin was clarified by assessing its intestinal permeability using the Caco-2 model. In addition, the composition of hot water infusions of green rooibos, prepared as for a regular cup of herbal tea, was determined to relate dietary supplementation with extract to intake in the form of herbal tea. For these calculations, the effective dose for diabetic non-human primates[10] and dose translation to humans were taken into account.

## Materials and methods

### Chemicals and consumables

Authentic flavonoid reference standards (purity > 95%) were provided by the Medical Research Council of South Africa (SAMRC, Cape Town, South Africa; aspalathin and nothofagin) and purchased from Extrasynthese (Genay, France; isoorientin, orientin, isovitexin, hyperoside), Karl Roth (Karlsruhe, Germany; vitexin, luteoloside), Sigma-Aldrich (St Louis, MA, USA; isoquercitrin) and Transmit (Gießen, Germany; rutin). *Z*-2-(β-D-Glucopyranosyloxy)-3-phenylpropenoic acid (PPAG) was from the compound library of the Plant Bioactives Group of the Agricultural Research Council (Infruitec-Nietvoorbij, Stellenbosch, South Africa). High-performance liquid chromatography (HPLC) gradient grade acetonitrile and formic acid (98–100%) were obtained from Merck (Darmstadt, Germany), ethanol (99.9%) from Servochem (Cape Town, South Africa) and activated carbon (Norit, particle size < 150 μm) from Sigma-Aldrich. Analytical grade solvents, reagents and chemicals for cell culture were supplied by Sigma-Aldrich. All other cell culture reagents unless specified were obtained from Lonza (Basel, Switzerland). Hyclone® foetal bovine serum was purchased from Thermo Scientific (Waltham, MA, USA) and new-born calf serum from Gibco (Paisley, Scotland). Cell culture consumables were purchased from Corning (New York, NY, USA) unless specified. For the *in vitro* intestinal epithelial transport study, SPLInsert™ Hanging 6-well transwell plates were purchased from SPL Life Sciences (Gyeonggi-do, Korea).

### Preparation of GREs and 'cup-of-tea' infusions

For preparation of the GREs, samples of 10 production batches of green rooibos were obtained from a commercial producer (Rooibos Ltd, Clanwilliam, South Africa). The plant material was used as received (*i.e.* not refined by sieving). Hot water aqueous extracts were prepared, using conditions for optimum

extraction of aspalathin.[11] Briefly, the plant material (50 g) was extracted in a 1 : 10 solid : solvent ratio (m/v) for 30 min with hot water (93 °C), followed by filtration through Whatman no. 4 filter paper (Maidstone, UK) and freeze-drying. Ethanol–water extracts (60% and 80%, v/v) of the same batches of plant material were prepared in a similar manner, but with extraction performed at 40 °C. Additionally, the 80% EtOH–water filtrate was treated with 0.3% activated carbon (m/v) for 20 min to remove chlorophyll and filtered. The ethanol–water filtrates were concentrated under vacuum and the aqueous residues freeze-dried. For comparative purposes, GRE-SB1 (also known as ARC2; supplied by Raps Foundation, Germany), previously shown to have significant antidiabetic activity *in vitro* and *in vivo*,[9,16] was included as a reference extract. GRE-SB1, produced using a patented process,[17] was similarly prepared as the GRE-80 extracts, except that the extract powder, obtained through vacuum-drying, was washed with ethyl acetate to reduce its chlorophyll content.

Hot water infusions, at 'cup-of-tea' strength, were prepared in duplicate from other commercial production batches of green rooibos ($n$ = 29), also supplied by Rooibos Ltd. Briefly, 2.5 g plant material was infused without agitation for 5 min in 200 mL freshly boiled deionised water, the standard recommended procedure. The infusions were filtered through Whatman no. 4 filter paper and aliquots stored at −20 °C until HPLC analysis.

### HPLC analysis of GREs and infusions and quantification of the soluble solids content of infusions

Quantification of the compounds was performed according to a validated HPLC-diode-array detection (DAD) method for rooibos, developed by Walters *et al.*[18] In the case of GRE-SB1, the compositional data, previously reported by Muller *et al.*,[9] was used. Its luteoloside content, previously not quantified, was determined, using the method of Walters *et al.*[18] Peak areas for dihydrochalcones and PPAG were determined at 288 nm and those of the flavones and flavonols at 350 nm.

The soluble solids content of each filtrate, following preparation of the infusions, was determined gravimetrically on triplicate 20 mL aliquots.[19]

### Determination of bio-capacity

Murine skeletal muscle cells (C2C12, ECACC cat. no. 91031101) were purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). Human hepatocellular carcinoma cells (HepG2/C3A, ATCC cat. no. CRL-10741) and murine preadipocytes (3T3-L1, ATCC cat. no. CL-173) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Aliquots of stock solutions of the extracts, prepared in dimethylsulfoxide (DMSO), were prepared and stored at −20 °C. On the day of the experiment, the frozen aliquots were thawed and fresh working solutions of 10 μg mL$^{-1}$ prepared in Krebs-Ringer bicarbonate-HEPES buffer or phenol red free Dulbecco's Modified Eagle's Medium (DMEM). The working solutions containing a final concentration of 0.01% DMSO were sterile filtered into sterile tubes, using sterile 0.22 μm pore sized Millex-GP low-protein binding syringe filters (Merck).

**C2C12 skeletal muscle cells.** Murine C2C12 skeletal muscle cells were seeded at a density of 2.5 × 10$^4$ cells per well in 24-well plates and cultured for two days under standard culture conditions (37 °C, 5% $CO_2$ in humidified air) as previously described by Mazibuko *et al.*[16] C2C12 cells were differentiated in DMEM supplemented with 2% horse serum to induce myotubule formation for a further two days. C2C12 muscle cells were serum-starved for 1 h and subsequently treated in pyruvate-free DMEM containing 8 mM glucose over 4 h with either 10 μg mL$^{-1}$ GRE, 0.01% DMSO (vehicle control) or 1 μM insulin (positive control). After 4 h, glucose uptake was estimated by adding 0.5 μCi mL$^{-1}$ 2-deoxy-[3*H*]-D-glucose per well for 15 min. The cells were lysed with 0.1 M NaOH and 2-deoxy-[3*H*]-D-glucose activity determined by liquid scintillation (2220 CA, Packard Tri-Carb series, PerkinElmer, IL, USA).

**HepG2/C3A liver cells.** Human HepG2/C3A liver cells were seeded at a density of 11 × 10$^4$ cells per well in 24-well plates. Cells were cultured in EMEM supplemented with 10% foetal bovine serum and 2 mM L-glutamine for four days at standard cell culture conditions preceding glucose uptake experiments as described for the C2C12 skeletal muscle cells.

**3T3-L1 derived adipocytes.** The procedure described by Sanderson *et al.*[20] was used to prepare and treat the cell culture. Murine 3T3-L1 preadipocytes were seeded at a density of 0.4 × 10$^4$ cells per well in 96-well plates in preadipocyte growth medium (DMEM supplemented with 10% new-born calf serum). After 3 days of culture, the preadipocytes were differentiated using a cocktail of adipocyte differentiation medium, comprising of DMEM supplemented with 10% foetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone and 1 μg mL$^{-1}$ insulin. The cells were then incubated at standard cell culture conditions for 3 days and on the third day, the medium was gently aspirated and replaced with DMEM supplemented with 10% foetal bovine serum and 1 μg mL$^{-1}$ insulin for a further five days. On day 8, the fully differentiated adipocytes were serum-starved for 1 h and treated for 4 h with 10 μg mL$^{-1}$ GRE, 0.01% DMSO (vehicle control) or 1 μM insulin (positive control) in phenol red free DMEM containing 25 mM glucose supplemented with 0.1% BSA. Intracellular lipid content was determined according to a modified Oil-Red-O method, described by Sanderson *et al.*[20] Briefly, the amount of intracellular lipid was calculated by measuring the Oil-Red-O stain intensity (absorbance at 490 nm), normalised against cell density estimated by crystal violet stain intensity (absorbance at 570 nm).

### Intestinal permeability

Caco-2 cells (human colon adenocarcinoma cell line) (ECACC cat. no. 86010202), originating from the ECACC and purchased from Sigma-Aldrich, were used for the Caco-2 monolayer cell culture model, as described by Bowles *et al.*[21] Monolayer integrity was confirmed by transepithelial electrical resistance

 

(TEER) >300 Ω[22] and passage of Lucifer Yellow < 3% across the monolayer when co-incubated with the extracts.[23] TEER values were measured, using a Millicell-ERS volt ohmmeter (Merck).

The extracts were prepared as concentrated stock solutions in 10% DMSO and diluted using Hank's Balanced Salt Solution (HBSS) buffer (pH 6.0) to contain a final concentration of 0.125% DMSO. The extract concentrations were adjusted to an equivalent aspalathin concentration of 150 μM, based on previous experiments.[21] Transport experiments were performed in the apical to basolateral direction. Briefly, 1.5 mL of the diluted extract solution was added to the apical side of the Caco-2 monolayer and 2.4 mL of HBSS buffer (pH 7.4) was added to the basolateral compartment. Samples (1.2 mL) were taken from the basolateral side at 0, 30, 60, 90 and 120 min and replaced with an equal volume of buffer to maintain sink conditions. Samples were snap-frozen in liquid nitrogen after the addition of ascorbic acid (1% final concentration) and stored at −65 °C. HPLC-DAD analysis of samples from the apical and basolateral compartments was performed in duplicate on an Agilent 1200 system as previously described.[24]

### Statistical analysis and prediction model selection

Unless otherwise stated, bio-capacity data were expressed as the means of three independent experiments, with three technical replicates per experiment and reflected as standard error of the mean. Significant differences ($P < 0.05$) between groups were assessed using one-way analysis of variance (ANOVA), with the Tukey–Kramer multiple comparison test. Data were analysed using GraphPad Prism Software (version 6.0, La Jolla, CA, USA). Pearson's correlation coefficients were determined to find individual compound content significantly ($P < 0.05$) correlating with bio-capacity (XLStat 2016, Addinsoft, New York, NJ, USA).

The bio-capacity parameters, *i.e.* the glucose uptake in C2C12 and C3A cells and lipid accumulation in 3T3-L1 cells, were modelled in terms of the flavonoid and PPAG content of the extracts by first applying the Partial Least Squares (PLS) method[25] to the data after auto-scaling. Given that the obtained PLS models were unsatisfactory, other methods with an embedded feature selection and/or nonlinear approach (Uninformed Variable Elimination (UVE)-PLS, Classification and Regression Trees (CART), Random Forest (RF), kernel-PLS, Neuro-Fuzzy Systems (NFS)) were also tested. Acceptable results were only achieved for the robust versions of PLS, namely Partial Robust M-regression (PRM)[26] and Evolution Program (EP)-PLS,[27] with EP-PLS resulting in models with the best predictive power. Within the framework of EP-PLS, a model is constructed for the assumed fraction of the non-contaminated data ($p$), and the data outliers are identified based on their standardised residuals from the assumed robust model.[28] After identification and rejection of the outliers, the final PLS model is constructed and validated. A detailed description of EP can be found in Walczak.[27] Briefly, this method is based on a subset selection algorithm (evolution program), which maintains a population of potential solutions and incorporates problem-specific knowledge. EP starts with $k$ randomly selected subsets of $m$ initial objects in the studied data set, binary coded as strings of length $m$. The subset presented by the particular string represents the model set (containing $m_{\text{model}}$ objects, where $m_{\text{model}}$ should be in the range $[m_{\min}$–$m_{\max}]$, where $m_{\min}$ denotes the maximal number of factors to be considered for model construction, and $m_{\max} = m_{\min} + [m(1-p) - m_{\min}]/2)$, where $p$ denotes the assumed fraction of data contamination. The fitness function of each string is defined as: fitness $(f)$ = 1/RMSE $(f)$ (where RMSE denotes Root Mean Squared Error estimated for the $g$ objects sorted according to the absolute value of their residuals for the model with $f$ factors, where $g$ denotes number of objects in the uncontaminated subset ($g = m(1-p)$). The number of factors is calculated based on the Root Mean Square Error of Prediction (RMSEP) for the first $(g - m_{\text{model}})$ objects from the test set sorted according to the absolute value of their residuals. If $m_{\text{model}}$ objects are selected that do not contain outliers, $(g - m_{\text{model}})$ good objects are left and they can be used to estimate model complexity. These objects are identified by sorting the remaining $(m - m_{\text{model}})$ objects according to their deviation from the constructed model. The outlying objects are expected to be at the end of this list. Then the $i$-th string $s_i$ is replaced by string $s_i'$ containing $g$ objects with the lowest residuals from the optimal model (model for which RMSE was calculated). To construct the next generation of $k$ strings, subsets of $s'$ is considered. The new population is again evaluated and all procedures are repeated until a stop criterion is reached. Outliers are diagnosed based on the standardised residuals from a robust model, as proposed by Rousseeuw and Leroy.[28]

## Results

### Flavonoid and PPAG content of GREs and infusions

Table 1 summarises the individual flavonoid and PPAG content of the GREs. Their aspalathin content was substantially higher than that of the other compounds, irrespective of extract type. The GRE-80 extracts had the highest aspalathin content, varying between 115.5 to 213.8 g $kg^{-1}$ extract. As the same batches of plant material were used to prepare the different types of extract, large variation in aspalathin content was also evident for GRE-60 and GRE-W extracts. The other compounds were present at much lower quantities in the extracts, totalling 79.7, 68.2 and 51.3 g $kg^{-1}$ of GRE-80, GRE-60 and GRE-W, respectively. Their content also showed large variation as a result of batch-to-batch variation of the raw material. The individual flavonoid and PPAG content of GRE-SB1 (reference extract; prepared with 80% ethanol–water) was within the ranges observed for GRE-80.

Table 2 summarises the individual flavonoid and PPAG content of infusions prepared from a large number of commercial batches of green rooibos ($n = 29$). The mean aspalathin concentration was 179.2 mg $L^{-1}$ with a range of 76 to

**Table 1** Phenolic and PPAG content (g $kg^{-1}$) of the reference extract (GRE-SB1) and extracts prepared by using different plant material batches ($n$ = 10) and extraction solvents

| | | GRE-80 | | GRE-60 | | GRE-W | |
|---|---|---|---|---|---|---|---|
| Compounds | GRE-SB1 | Mean ± SEM | Range | Mean ± SEM | Range | Mean ± SEM | Range |
| Aspalathin | 184.4 | 170.9 ± 8.4 | 115.5–213.8 | 125.4 ± 7.9 | 85.8–161.7 | 95.2 ± 5.9 | 54.1–115.5 |
| Nothofagin | 12.9 | 15.0 ± 1.3 | 8.2–21.6 | 10.6 ± 1.1 | 5.6–16.6 | 7.7 ± 0.7 | 3.6–11.8 |
| Isoorientin | 20.5 | 15.6 ± 0.4 | 13.9–18.7 | 13.8 ± 0.6 | 10.0–17.9 | 9.1 ± 0.3 | 7.2–10.6 |
| Orientin | 10.5 | 12.9 ± 0.3 | 11.6–15.3 | 10.8 ± 0.4 | 8.1–13.3 | 8.6 ± 0.2 | 7.4–9.7 |
| Bioquercetin | 10.5 | 10.7 ± 0.5 | 8.1–14.1 | 10.3 ± 0.8 | 5.6–15.4 | 8.0 ± 0.5 | 5.6–11.5 |
| Vitexin | 2.7 | 2.5 ± 0.1 | 2.2–3.2 | 2.0 ± 0.1 | 1.4–2.6 | 1.6 ± 0.1 | 1.2–1.8 |
| Hyperoside | 2.7 | 4.1 ± 0.3 | 2.9–6.5 | 3.8 ± 0.4 | 1.7–6.8 | 2.3 ± 0.2 | 1.5–4.0 |
| Rutin | 5.4 | 4.3 ± 0.2 | 3.1–9.9 | 4.0 ± 0.3 | 2.0–5.9 | 3.3 ± 0.2 | 2.1–4.7 |
| Isovitexin | 3.9 | 3.5 ± 0.1 | 2.8–4.0 | 3.1 ± 0.2 | 2.0–3.6 | 1.9 ± 0.1 | 1.5–2.2 |
| Isoquercitrin | 3.8 | 5.3 ± 0.6 | 3.1–9.9 | 4.8 ± 0.7 | 1.7–10.2 | 2.7 ± 0.3 | 1.4–5.4 |
| Luteoloside | 0.0 | 0.7 ± 0.0 | 0.5–0.8 | 0.6 ± 0.0 | 0.5–0.7 | 0.7 ± 0.0 | 0.5–0.8 |
| PPAG | 4.9 | 5.1 ± 0.1 | 4.3–5.8 | 4.4 ± 0.1 | 3.9–4.9 | 5.3 ± 0.2 | 4.7–6.1 |

Abbreviations: GRE-60, 60% ethanol-water green rooibos extract; GRE-80, 80% ethanol-water green rooibos extract; GRE-SB1, reference green rooibos extract; GRE-W, aqueous green rooibos extract; PPAG, *Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid.

**Table 2** Phenolic, PPAG and soluble solids content (mg $L^{-1}$) of green rooibos infusions ($n$ = 29)

| Parameter | Min | Max | Mean | SEM |
|---|---|---|---|---|
| Aspalathin | 76.0 | 255.2 | 179.2 | 8.14 |
| Nothofagin | 7.02 | 24.86 | 15.18 | 0.85 |
| Isoorientin | 7.58 | 21.18 | 14.71 | 0.57 |
| Orientin | 7.57 | 21.30 | 14.98 | 0.58 |
| Bioquercetin | 4.58 | 18.95 | 12.98 | 0.69 |
| Vitexin | 1.18 | 3.29 | 2.43 | 0.09 |
| Hyperoside | 1.77 | 7.18 | 4.77 | 0.28 |
| Rutin | 2.90 | 8.15 | 6.03 | 0.23 |
| Isovitexin | 1.64 | 4.36 | 2.95 | 0.11 |
| Isoquercitrin | 2.22 | 10.20 | 6.68 | 0.36 |
| PPAG | 3.58 | 11.55 | 7.98 | 0.32 |
| Σ value | 119 | 375 | 268 | 11 |
| Soluble solids | 1.09 | 1.90 | 1.50 | 0.04 |

Abbreviations: PPAG, *Z*-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid.

255 mg $L^{-1}$. The mean content values of the other "major" compounds, *i.e.* nothofagin, isoorientin, orientin and bioquercetin, varied between 12.98 and 15.18 mg $L^{-1}$. The soluble solids content of the infusions, an indication of the strength of the infusion, also reflect the variation in the quality of the production batches.

### Effect of extract on glucose uptake and lipid accumulation

The extracts were evaluated for their effect on glucose uptake in C2C12 muscle (Fig. 1a) and C3A liver cells (Fig. 1b). All the extracts significantly ($P < 0.05$) increased glucose uptake in these cells compared to the control with GRE-80 more effective than GRE-60 and GRE-W in the muscle cells, but not in the liver cells. In addition, lipid accumulation in adipocytes was assessed in 3T3-L1 adipocytes. All extracts improved lipid accumulation significantly compared to the control ($P < 0.05$). GRE-80 and GRE-60 were more effective than GRE-W and GRE-SB1 (Fig. 1c).

### Effect of extract on membrane permeability of aspalathin

Differences in extract matrix due to extraction solvent did not affect the $P_{app}$ value of aspalathin in the Caco-2 cell monolayer (mean $P_{app} = 0.73 \times 10^{-6}$ cm $s^{-1}$).

### Development of prediction models for bio-capacity

Firstly, the Pearson correlation coefficients for all individual compounds and the different bio-capacity values were determined. The aspalathin content did not correlate significantly with bio-capacity ($P \geq 0.05$), irrespective of the cell-based assay, although the GRE-80 with the highest mean aspalathin content (170.9 g $kg^{-1}$) (Table 1) had the highest bio-capacity in the three assays (Fig. 1a, b and c). Of the other compounds, only the orientin ($r = 0.571$), isoorientin ($r = 0.515$) and vitexin ($r = 0.487$) content correlated significantly ($P < 0.05$) with the enhancement in glucose uptake by C2C12 cells.

The next step was to evaluate a variety of multivariate models for prediction of the respective bio-capacities, based on the flavonoid and PPAG content of the extracts. The most suitable model for each bio-capacity was constructed using EP-PLS and an assumed fraction of contamination of 0.3 (Fig. 2–4). The identified outliers are marked on subplots a, whereas the $y$ observed *vs.* $y$ predicted plots for the model and the cross-validation are shown in subplots b and c, respectively. The RMSE describes the model fit, whereas the Root Mean Square Error of Cross-Validation (RMSECV) describes its predictive power, evaluated based on cross-validation. The best predictive power was observed for enhancement of glucose uptake in C2C12 (RMSECV = 6.73; Fig. 2) and C3A (RMSECV = 5.96; Fig. 3) cells and the worst for lipid accumulation in 3T3-L1 cells (RMSECV = 11.50; Fig. 4). All models are acceptable, taking into account the experimental error of the measured bio-capacity. In light of the known bioactivity of aspalathin and correlation of isoorientin, orientin and vitexin with the extent of glucose uptake in the C2C12 cell model, a prediction model using EP-PLS with an assumed fraction of contami-

 

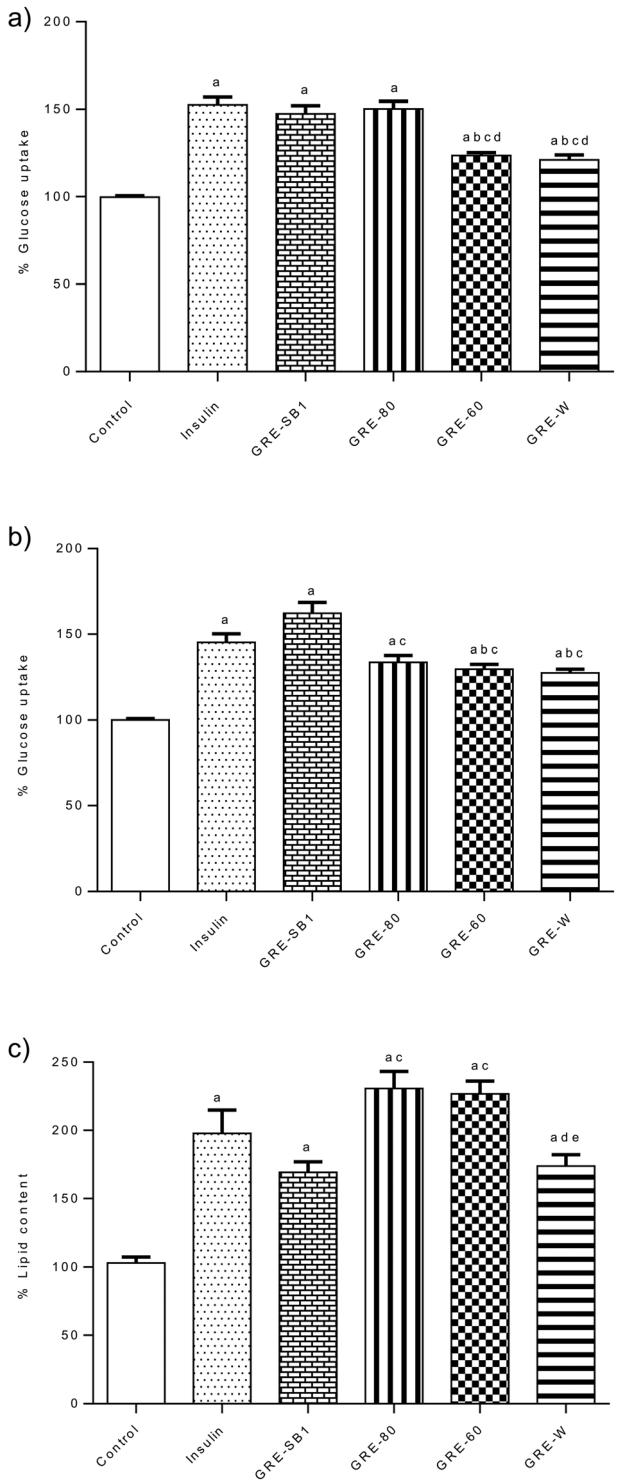


**Fig. 1** Glucose uptake in (a) differentiated C2C12 myocytes and (b) C3A hepatocytes and (c) lipid accumulation in 3T3-L1 adipocytes treated with GRE-80, GRE-60 and GRE-W (10 μg $ml^{-1}$). GRE-SB1 served as reference extract and insulin as positive control. Data are representative of three experiments, each with three technical repeats ($n$ = 9), presented as mean ± SEM. Bars with different letters denote statistical significance between results (One-way ANOVA; $P < 0.05$). a denotes significant difference to the control; b denotes significant difference to insulin; c denotes significant difference to GRE-SB1; d denotes significant difference to GRE-80; e denotes significant difference to GRE-60.

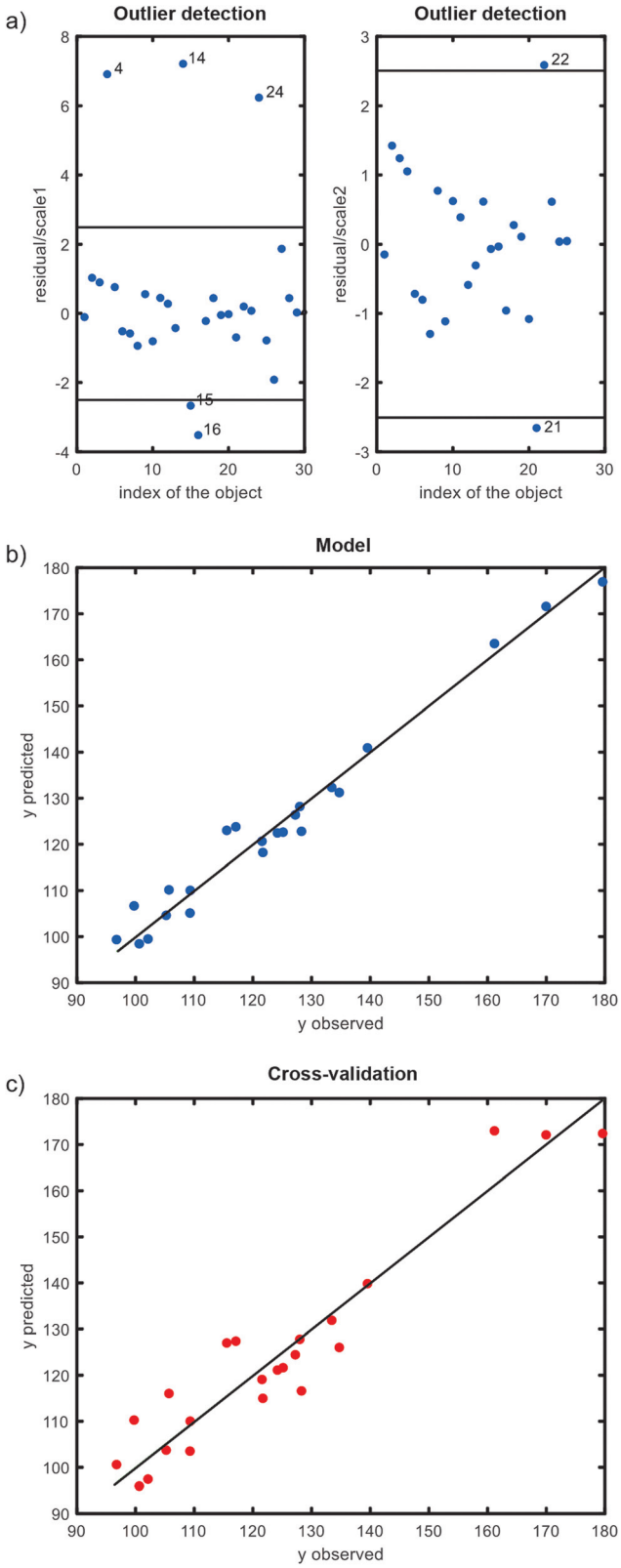


**Fig. 2** EP-PLS model for predicting glucose uptake in differentiated C2C12 myocytes ($y$) based on flavonoid and PPAG content of green rooibos extracts: (a) outliers marked based on their standardised residuals from the model, (b) $y$ observed *vs.* $y$ predicted for the model set and (c) $y$ observed *vs.* $y$ predicted based on cross-validation. RMSE = 3.54; RMSECV = 6.73.

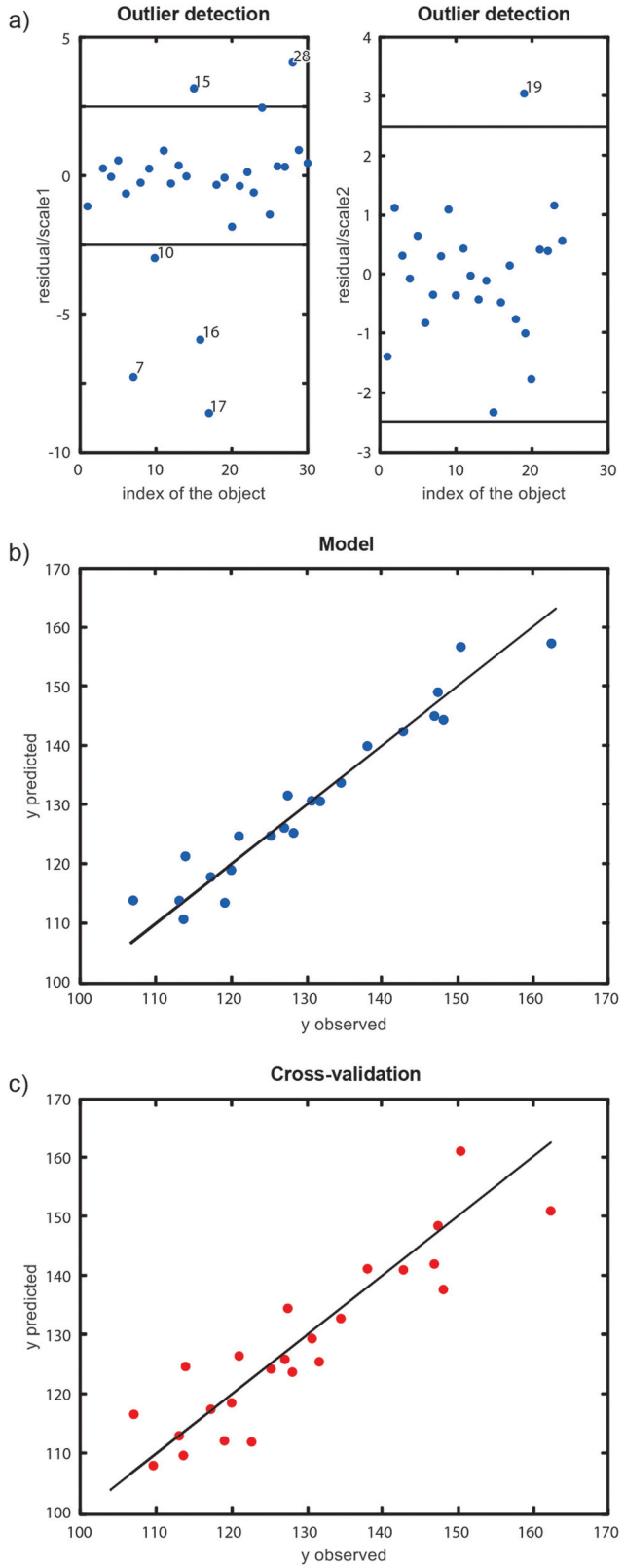


Fig. 3 EP-PLS model for predicting glucose uptake in differentiated C3A hepatocytes (*y*) based on flavonoid and PPAG content of green rooibos extracts: (a) outliers marked based on their standardised residuals from the model, (b) *y* observed *vs.* *y* predicted for the model set and (c) *y* observed *vs.* *y* predicted based on cross-validation. RMSE = 3.44; RMSECV = 5.96.

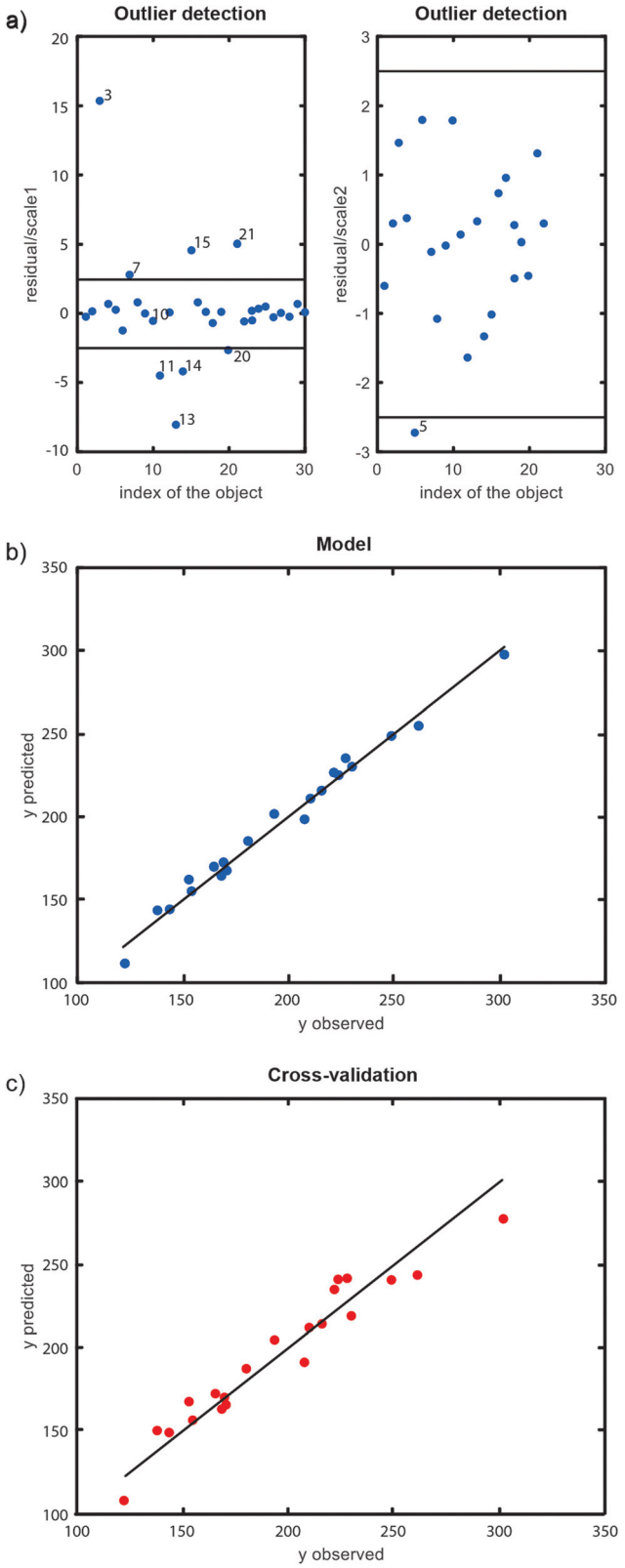


Fig. 4 EP-PLS model for predicting lipid accumulation in 3T3-LI adipocytes (*y*) based on flavonoid and PPAG content of green rooibos extracts: (a) outliers marked based on their standardised residuals from the model, (b) *y* observed *vs.* *y* predicted for the model set and (c) *y* observed *vs.* *y* predicted based on cross-validation. RMSE = 5.15; RMSECV = 11.50.

nation of 0.3 was also constructed using only these compounds, however, its predictive power was much poorer (RMSECV = 12.80; data not shown).

## Discussion

The only long-term strategy that is considered effective in reducing the diabetic burden faced by developing countries such as South Africa are modification of causal factors such as diet and lifestyle. The beneficial effect of aspalathin and green rooibos extracts as modulators of glycaemia has received much attention,[4,7] meriting the present investigation to provide a quality assessment tool for the *in vitro* bio-capacity of a functional green rooibos extract. Such an extract can be used in formulation of food products specifically aimed at diabetics. Although a specific level of activity in relevant cell-based assays does not guarantee efficacy *in vivo*, it would rise the standard for the production of functional plant extracts. Production issues such as extract type and plant material variation were incorporated into the experimental design to clarify their impact on the *in vitro* bio-capacity of the extract.

Rooibos and aspalathin not only improve glucose uptake *in vivo*,[9,29,30] but they also improve insulin resistance, a major causal factor underlying the development of type 2 diabetes. This effect was demonstrated in C2C12 myocytes, 3T3-L1 adipocytes and C3A liver cells.[5,16,31,32] By restoring insulin signalling and activating AMP-activated protein kinase in insulin-resistant cells, rooibos extract and aspalathin improve glucose and lipid metabolism. The study in high fat fed diabetic vervet monkeys[10] supports the potential of GRE not only to improve glycaemia and high-density lipoprotein (HDL)/LDL cholesterol profiles, but also the plasma oxidative status by decreasing oxidised coenzyme Q10 and oxidised LDL levels, thereby mitigating major risk factors for cardiovascular disease.

Other metabolically active phenolic compounds present in significant amounts in the rooibos extracts and shown to demonstrate hypoglycaemic activity include orientin, isoorientin[33] and PPAG.[34] Rutin was previously shown to enhance the glucose uptake efficacy of aspalathin in C2C12 myocytes.[9] Treatment of streptozotocin-induced diabetic rats with hyperoside (quercetin-3-*O*-galactoside), another glycoside of quercetin, reduced blood glucose levels by improving islet function.[35]

In terms of the *in vitro* bio-capacity of the different types of extract, GRE-80 was the most effective in increasing glucose uptake and lipid accumulation in the respective cell models, comparable to that of the reference extract, GRE-SB1 (Fig. 1). These findings are consistent with results for glucose uptake described by Muller *et al.*,[9] who demonstrated enhanced glucose uptake for C2C12 cells over a broad concentration range (0.05–5 μg $mL^{-1}$) for GRE-SB1. The same extract also enhanced glucose uptake in palmitate-induced insulin-resistant C2C12 skeletal muscle cells.[16] GRE-SB1 and aspalathin significantly increased insulin-stimulated activation of AKT (Ser 473),[32] a key effector enzyme of the insulin pathway. In the C3A cell model, GRE-W appeared to be as effective as GRE-60 and GRE-80 in improving glucose uptake (Fig. 1b). In terms of glucose uptake, C2C12 myocytes in comparison to C3A liver cells were more responsive to extract type. In the liver, glucose uptake is relevant to the modulation of postprandial glucose levels. However, the effect of the extracts on hepatic glucose production, relevant to diabetes, was not assessed.

In adipose cells, under hyperglycaemic conditions (25 mM glucose) as employed in the present study, increased intracellular glucose is converted to lipid. This process is essential for energy homeostasis, as these fat cells act as energy reservoirs by converting excess glucose to triglycerides and releasing fatty acids during periods of fast.[36] As such, adipose tissue along with skeletal muscle plays an essential role in maintaining normoglycaemia.[37] Previous studies demonstrated that the effects of green rooibos extracts were similar to that of the thiazolidinedione antidiabetic drug, pioglitazone, that is specifically effective in the treatment of insulin resistance by sensitising adipose tissue lipid synthesis in type 2 diabetics.[38,39] All extracts improved lipid accumulation to a varying degree with GRE-60 and GRE-80 being similarly effective (Fig. 1c), in spite of GRE-80 having a substantially higher aspalathin content.

In view of the proven biological activity of aspalathin and its naturally high levels in green rooibos, the focus in this study was drawn toward aspalathin as potential predictor of the *in vitro* bio-capacity of green rooibos extract, yet the aspalathin content of the extracts did not correlate significantly ($P \geq 0.05$) with bio-capacity, irrespective of cell model. The equivalent aspalathin concentration range in the tested extracts was 1.2 to 4.73 μM, previously shown to fall within the concentration range eliciting a dose response in C2C12[9] and L6 cells.[29] Moderate (orientin, isoorientin and vitexin) to no correlation (other compounds) between individual compound content and the extent of glucose uptake by C2C12 cells and no correlation with bio-capacity, using the C3A and 3T3-L1 cells, indicate that single compounds, even in the case of aspalathin that is present at high levels, are not suitable as predictors of extract bio-capacity. This is in line with many other studies on plant extracts as their multi-component nature opens the potential for synergistic effects.[40] Other factors also come into play when testing the bio-capacity of a complex mixture such as the physicochemical properties of compounds, their individual molecular structures and effect on different mechanisms. Furthermore, the vast number of uncharacterised constituents in a plant extract add a large degree of uncertainty.

Following a multivariate analysis approach, the content of all quantified compounds ($n$ = 12) was used to build the respective robust PLS models (EP-PLS models) to predict the bio-capacity, based on the different cell models. RMSECV values ≤ 11.50 obtained for the respective prediction models are within the experimental error for the cell-based assays, making the models suitable for prediction of bio-capacity despite the small sample set available. Unfortunately, all models built using variable selection approaches showed poor predictive power (data not shown) compared to the models that included all flavonoid and PPAG content values, thus pre-

cluding identification of a single compound as predictor. A larger sample set with independent validation is needed to confirm the results. Another approach to confirm the effect of the selected compounds on the bio-capacity of the extract, as followed by Fujimura *et al.*,[41] is to add the compounds to a 'non-bioactive' extract. This will elucidate the dose response, as well as allow for interplay between compounds.

The advantage of an extract *vs.* a single compound such as aspalathin as functional ingredient is the contribution of other compounds to bioactivity. The GRE used by Orlando *et al.*[10] to treat diabetic vervet monkeys is not only similar in composition to GRE-60 (aspalathin: 127.8 *vs.* 125.4 g $kg^{-1}$; total quantified compounds: 210.5 *vs.* 193.4 g $kg^{-1}$), but it was effective in improving parameters related to diabetes and obesity when dosed at 90 mg $kg^{-1}$, 3 times per day. Based on the body surface area normalisation method[42] a human of 70 kg would be required to take 2 g extract 3 times per day. A single dose of 2 g extract contains 421 mg of the quantified compounds, which translates into the consumption of between 6 and 18 cups of green rooibos infusion (one cup = 200 mL). This assumption is based on the minimum and maximum content values obtained for the infusions prepared from the 29 batches of commercial green rooibos. If only the aspalathin content of the GRE used to treat the diabetic vervet monkeys is taken into account, 7 to 17 cups of green rooibos infusions must be consumed at a time. None of the quantities is a realistic target for ingestion. However, it should be considered that the animals were maintained on a high fat diet containing 40% fat as energy throughout the treatment period. Combined with lifestyle intervention, it could potentially allow for lower efficacious doses when consuming the extract in a suitable food or beverage product. In a human study, 6 cups of traditional (oxidised) rooibos infusion taken daily over a six-week period significantly reduced the blood cholesterol levels and improved the oxidative status of serum in healthy participants deemed at risk of developing metabolic disease.[43] No compositional data were provided for the latter infusions, but based on the data obtained for the 'cup-of-tea' infusions of a large sample set ($n$ = 114) of traditional rooibos,[19] intake of a substantially lower total flavonoid and PPAG content ($\Sigma$ = 58 mg $L^{-1}$) than for green rooibos (Table 2) is to be expected. Given that an effect was observed in the study by Marnewick *et al.*[43] despite the lower flavonoid and PPAG content of the traditional rooibos infusions, suggests that the efficacious dose could be much lower and at a more realistic level. However, further studies are needed to establish the quantities of extract or infusion required to achieve the desired therapeutic outcome.

## Conclusions

The findings of this study suggests that the chemical complexity of crude green rooibos extracts precludes attributing bio-capacity to a single phenolic compound such as aspalathin, even though it is present in high levels and shown to have bioactivity. Robust PLS models based on 12 compounds, including aspalathin, showed reasonable prediction of bio-capacity, *i.e.* enhancement of glucose uptake by muscle and liver cells and lipid accumulation in fat cells at a fixed extract concentration. Further research is needed to validate one or more of the models before their use as QC screening tools for *in vitro* bio-capacity of functional food extracts could be considered.

## Conflicts of interest

There are no conflicts of interest to declare.

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