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Differential Modulation of Gene Expression Encoding Hepatic and Renal Xenobiotic Metabolizing Enzymes by an Aspalathin-Enriched Rooibos Extract and Aspalathin

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ABSTRACT

Modulation of the expression of hepatic and renal genes encoding xenobiotic metabolizing enzymes by an aspalathin-enriched green rooibos (*Aspalathus linearis*) extract (GRE) was investigated in the liver and kidneys of F344 rats following dietary exposure of 28 d, as well as selected xenobiotic metabolizing genes in rat primary hepatocytes. In the liver, GRE upregulated genes ($p < 0.05$) encoding aldehyde dehydrogenase, glucose phosphate isomerase, and cytochrome P450 while 17*β*-hydroxysteroid dehydrogenase 2 (*Hsd17β2*) was downregulated. In primary hepatocytes, GRE lacked any effect, while aspalathin downregulated *Hsd17β2*, mimicking the effect of GRE *in vivo,* and upregulated catechol-*O*-methyl transferase and marginally ($p < 0.1$) cytochrome P450 2e1. In the kidneys, GRE upregulated ($p < 0.05$) genes encoding the phase II xenobiotic metabolism enzymes, glutathione-S-transferase mμ and microsomal glutathione-S-transferase, while downregulating genes encoding the ATP binding cassette transporter, cytochrome P450, gamma glutamyltransferase 1, and N-acetyltransferase 1. Differential modulation of the expression of xenobiotic metabolizing genes *in vivo* and *in vitro* by GRE is dose-related, duration of exposure, the tissue type, and interactions between specific polyphenol and/or combinations thereof. Aspalathin is likely to be responsible for the downregulation of estradiol and testosterone catabolism by GRE in the liver. The differential gene expression by GRE in the liver and kidneys could, depending on the duration exposure and dose utilized, determine the safe use of such an extract in humans for specific health and/or disease outcomes.

Introduction

Xenobiotics, such as polyphenols, are foreign substances entering the body and can either be beneficial or harmful, depending on the dose and/or their metabolic fate. They may interact with the cellular metabolic pathways and cellular constituents such as membrane lipids, proteins, and DNA, often leading to the modulation of normal cellular functions [1]. Most xenobiotics undergo metabolism that include phase I, phase II metabolizing enzymes and phase III transporters, present in abundance either at the basal and/or at induced levels following exposure, and these interactions may trigger cellular “stress” responses, leading to an increase in gene expression, ultimately enhancing their elimination [2, 3]. Consequently, these homeostatic responses play a central role in the protection of cells against “environmental” insults such as those elicited by exposure to xenobiotics.

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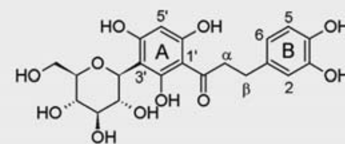
ABBREVIATIONS

Abcb1	ATP binding cassette transporter
Actb	cytoskeletal β-actin
Ald1a1	aldehyde dehydrogenase
Alox15	arachidonate 15-lipoxygenase
BW	body weight
Comt	catechol-O-methyl transferase
Cyp2e1	cytochrome P450 2e1
Cyp4b1	cytochrome P450 4b1
Cyp19a1	cytochrome P450 19a1
GAE	gallic acid equivalents
Ggt1	gamma glutamyltransferase 1
Gpi	glucose phosphate isomerase
Gstm1	glutathione-S-transferase mμ
GRE	green rooibos extract
Gst	glutathione-S-transferase
HED	human equivalent dose
Hk2	hexokinase 2
Hprt1	hypoxanthine guanine phosphoribosyl transferase 1
Hsd17β2	17β-hydroxysteroid dehydrogenase 2
IC_{50}	concentration inhibiting 50% of cell viability
IL-16	interleuken 16
Ldha	lactate dehydrogenase A
LPS	lipopolysaccharide
MEM	minimal essential medium
Mgst1	microsomal glutathione-S-transferase
MPO	myeloperoxidase
Nat1	N-acetyltransferase 1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
nrf2	nuclear factor erythroid 2-related factor 2
qRT-PCR	quantitative reverse transcription polymerase chain reaction
Rpl13a	ribosomal protein L13A
Rplp1	ribosomal protein large P1
Sult	sulfotransferase
TNF-*α*	tumor necrosis factor *α*
TP	total polyphenols
WE	Williams' E medium
UDP	Uridine 5′-diphosphate
Ugt	UDP-glucuronyltransferase



▶ **Fig. 1** Chemical structure of aspalathin, the major dihydrochalcone of unfermented rooibos.

The herbal tea, rooibos (*Aspalathus linearis* [Burm.f.] R.Dahlgren, Leguminosae), has become increasingly popular for its unique flavor and health promoting properties [4]. For the production of nutraceutical extracts, especially those with antidiabetic properties, the "unfermented" product (green rooibos) is preferred due to the high levels of the antioxidant and antidiabetic [5] dihydrochalcone, aspalathin (▶ **Fig. 1**). At present, very little is known about the effects of rooibos on xenobiotic metabolism, especially when utilizing polyphenol-enriched extracts and aspalathin, the major rooibos flavonoid. Studies indicated that rooibos extracts exhibited drug interactions through the modulation of cytochrome P450 (CYP) protein expression in the liver of rats [6]. An extract of unfermented rooibos enhances the activity of the phase II drug metabolizing enzymes, glutathione-S-transferase and UDP-glucuronosyltransferase in the liver of rats [7]. Dietary exposure of rats to a fermented rooibos extract increased the expression of CYP3A4 in the gut accompanied by a significant decrease in the serum concentration of 4-hydroxymidazolam, a drug known to be metabolized by the enzyme [8]. Rooibos flavonoids were shown to modulate steroid hormone levels *in vitro* by inhibiting key enzymes in the adrenal steroidogenic pathway [9], while rooibos extracts and aspalathin inhibit the activity of cytochrome P450 enzymes involved in the metabolism of hypoglycemic and hypolipidemic drugs [10]. These investigations emphasize the importance to elucidate the potential beneficial and/or adverse effects of polyphenol-enriched extracts of rooibos when utilized as nutraceutical supplements.

In the present study, the effect of an aspalathin-enriched GRE on the expression of genes encoding xenobiotic metabolizing enzymes was investigated in rat liver and kidneys. The same extract was utilized demonstrating antidiabetic properties [11] and the modulation of the expression of antioxidant defense and oxidative stress-related genes in the liver of rats [12]. Additional experiments included the modulating role of GRE and aspalathin on the expression of selected xenobiotic metabolizing enzymes in primary rat hepatocytes.

Results

Daily intake of feed supplemented with GRE had no significant effect on BW gain, clinical chemical parameters, and relative liver and kidney weights of rats as reported previously [12]. Furthermore, feed intake was not significantly different between the treated and control rats, averaging respectively 97.3 and 96.5 g/kg BW. This feed intake equaled a daily TP intake of 76 mg GAE/kg BW, an aspalathin intake of 36 mg/kg, and other major flavonoids, including the rooibos flavones and flavonols, of 14 mg/kg BW.

Of the 84 genes analyzed, only four genes were significantly ($p \leq 0.05$) affected in the liver by GRE (▶ **Table 1**). Three genes, *Ald1a1, Cyp19a1,* and *Gpi*, were upregulated, whereas *Hsd17β2* was downregulated. Downregulation of *Hsd17β1* (− 1.81) was not significant. Marginal changes included upregulation of non-muscle cytoskeletal beta actin (*Actb*) and downregulation of *Smarcal1_predicted* (matrix-associated, actin-dependent regulator of chromatin, subfamily a like 1), *Alox* 15, and *MPO.* The major

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▶ **Table 1** Differential gene expression profile of xenobiotic metabolizing enzymes in the liver and kidneys of rats following exposure to GRE for 28 d.

Gene	Liver	Gene	Kidneys
Ald1a1	+ 2.39 (0.05)	Abcb1	− 1.85 (0.01)
Cyp19a1	+ 1.98 (0.02)	Cyp4b1	− 2.19 (0.05)
Gpi	+ 2.48 (0.007)	Ggt1	− 1.69 (0.02)
Hsd17β2	− 2.42 (0.008)	Gstm1	+ 1.57 (0.05)
Alox15	− 1.23 (0.098)	Mgst1	+ 1.29 (0.01)
MPO	− 1.82 (0.1)	Nat1	− 1.22 (0.02)
Actb	*+1.22(0.085)*		
Smarcal1_predicted	− 1.66(0.07)		

The values indicate fold differences. Numbers in brackets signify $p \leq 0.05$; – indicates genes downregulated; + indicates genes upregulated. $0.1 \geq p > 0.05$ indicated marginal differences

CYPs, *Cyp3A* and *1a1/1a2*, nuclear receptors, ATP-binding cassette (ABC) transporters, and phase II transferases genes, *Gst, Sult, Ugt*, and *Nat* were not significantly affected. In the kidneys, only six of the 84 xenobiotic metabolism-associated genes analyzed were significantly ($p \leq 0.05$) affected by GRE (▶ **Table 1**). The genes encoding *Gstm1* and *Mgst1* were upregulated, whereas *Abcb1, Cyp4b1, Ggt1*, and *Nat1* were downregulated.

Experiments in primary hepatocytes were conducted at "nontoxic" levels (i.e., concentrations that provided at least 80 % cell viability). These concentrations of GRE and aspalathin were below their IC_{50} levels (▶ **Table 2**). GRE had no effect on the expression of *Comt, Hsd17β2,* and *Cyp2e1* (▶ **Table 3**). Aspalathin, however, significantly ($p \leq 0.05$) increased the expression of *Comt* and downregulated the *Hsd17β2* gene while marginally increased the expression of *Cyp2e1*. Compared to the concentration of aspalathin used, its equivalent concentration in GRE was approximately 40-fold below when considering the IC_{50} for cytotoxicity (0.12 mg/mL) and 30-fold below the level used for the gene expression analyses (0.025 mg/mL). This was based on an aspalathin concentration of 18.4 % in the GRE.

▶ **Table 2** Concentration of GRE and aspalathin required to inhibit 50 % ATP production and levels utilized for gene expression analyses in rat primary hepatocytes.

Compound	Cell viability IC_{50} (mg/mL)*	Gene expression (mg/mL)
GRE	0.12 (0.11–0.13)** (0.05 mM)#	0.03 (0.01 mM)#
Aspalathin	1.91 (1.85–1.97)**	0.31 mM

*Means (95 % confidence intervals) of five replications of two independent experiments. *IC_{50}: inhibition of 50 % of ATP production. #Values represents the equivalent aspalathin concentration in GRE.

▶ **Table 3** Gene expression induction/inhibition of xenobiotic metabolizing enzymes in primary hepatocytes after exposure to GRE and aspalathin at nontoxic levels.

Treatment	*Comt*	*Hsd17β2*	*Cyp2e1*
GRE	0.83 (0.15)	1.18 (0.62)	1.10 (0.21)
Aspalathin	+ 2.05 (0.04)	− 0.68 (0.05)	+ 1.65 (0.07)

Data normalized against the *Hprt1* reference gene. Control (untreated) group set as fold = 1. $p \leq 0.05$ designated as significant and values are given in brackets. – indicates genes downregulated; + indicates genes upregulated.

Discussion

Many plant constituents, and more specifically flavonoids, are known to affect xenobiotic metabolizing enzymes by altering the metabolic fate of drugs [6]. In this regard, specific polyphenol-CYP450 interactions have a significant impact on the efficiency of drug therapies [13]. Studies on rooibos herbal tea extracts indicated that specific herb-drug interactions should be considered [8, 10], as rooibos extracts exhibit various beneficial health promoting effects including antioxidant activity, immune responses, anticancer, and more recently antidiabetic and anti-inflammatory effects [4, 11, 14, 15].

The present study represents the first investigation on the *in vivo* effects of an aspalathin-enriched GRE on the expression of genes encoding xenobiotic metabolizing enzymes in rat liver and kidneys by qRT-PCR array analyses. In the liver, the *Gpi* gene, encoding the enzyme catalyzing the conversion of glucose-6-phosphate to fructose-6-phosphate in the second step of glycolysis, was upregulated by GRE. Malignant cells increase their expression of glycolytic enzymes such as *Gpi* and *Hk2*, as well as glucose uptake resulting in production of ATP and synthesis of nucleic acids and lipids essential for cell survival and division. Normal cells exhibit metabolic flexibility and are capable of regulating their dependence on glycolysis relative to mitochondrial respiration, allowing the cells to adapt with the prevailing nutrient availability or energy demands [16]. Rooibos may therefore, favor anaerobic glycolytic metabolism, which appears to be a natural adaptation to reduce oxygen availability [17]. The redirection of energy metabolism toward glycolysis may also reduce oxidative damage and suppresses apoptosis as reflected in cancer cells [18]. The *Ald1a1*

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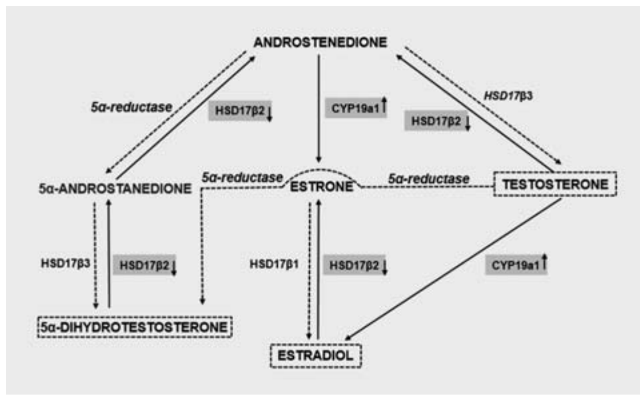

▶ **Fig. 2** Schematic diagram of key enzymes involved in the metabolism of steroid hormones modulated by GRE in the liver of rats. ↓ Indicates gene (*Cyp17β2*) downregulated, ↑ indicates gene (*Ctp19a1*) upregulated and their involvement in the regulation of the steroid hormones likely to be affected in text boxes.

gene, responsible for the catalysis of acetaldehyde to acetate, its less toxic form, and thus important in alcohol metabolism, was upregulated. The concurrent ingestion of GRE and alcohol therefore may be beneficial in the prevention of adverse effects following excessive alcohol consumption. This is in agreement with a study indicating that green tea (*Camellia sinensis*) extract also promotes alcohol metabolism [19].

GRE upregulated the *Cyp19a1* (aromatase) gene in the liver, while the expression of *Hsd17β2* (hydroxysteroid dehydrogenase) was downregulated. These enzymes play a key role in the metabolism of steroid hormones in the liver since *Hsd17β2* encodes for the enzyme converting estradiol, testosterone, and 5α-dihydrotestosterone to their respective less active forms, estrone, androstenedione, and 5α-androstanedione (▶ **Fig. 2**) [20]. Androstenedione, a product of cholesterol catabolism, can be reversibly converted to testosterone by *Hsd17β3* or to estrone by *Cyp19a1*. Estrone and testosterone can be converted to estradiol by *Hsd17β1* and *Cyp19a1*, respectively [21]. In addition to the direct testosterone conversion to 5α-dihydrotestosterone by 5α-reductase, the latter can also be derived from androstenedione via 5α-androstanedione through 5α-reductase and *Hsd17β3*. This pathway also seems to be effected due to the reduced expression of *Hsd17β2*, responsible for the oxidation of 5α-dihydrotestosterone into 5α-androstanedione. It would appear that GRE potentially could regulate gene expression of steroidogenic enzymes resulting in the impairment of the breakdown of estradiol, testosterone, and 5α-dihydrotestosterone in the liver while promoting the conversion of androstenedione into estrone and testosterone to estradiol. Modulation of the expression of these two enzymes may be desirable in normal cells, since it promotes proliferation after cell damage [22], but not in cancer cells where it would promote tumor growth. It is known that increased estrogen levels in the blood and interaction with insulin with the subsequent increase in the insulin growth factor are associated with endometrial cancers in postmenopausal women [23]. The role of hydroxysteroid dehydrogenases in prostate cancer is also of interest as it was shown that *Hsd17β2* was down- and *Hsd17β3* upregulated in the cancer tissue [24]. In this regard, *Hsd17β2* plays an important role in the inactivation of sex steroids in peripheral tissue, specifically testosterone and the reactive metabolite dihydrotestosterone that plays a key role in prostate cancer development [20]. The subsequent downregulation of *Hsd17β2* and upregulation of *Cyp19a1* by GRE in the liver are likely to increase the blood levels of testosterone, dihydrotestosterone, and estradiol. In this regard, the flavone aglycone, apigenin, exhibits anti-aromatase and anti-17*β*-hydroxysteroid dehydrogenase activity [25], which could be of interest as the apigenin glucosides in rooibos, vitexin and isovitexin, could modulate the activity of these enzymes. A recent study indicated that rooibos disrupts glucocorticoid metabolism in humans *in vivo* by increasing the level of cortisone resulting in a decrease in the cortisol : cortisone ratio [26]. In rats, rooibos decreased corticosterone and deoxycorticosterone levels as well as the cortisone : testosterone ratio following a gavage treatment for 10 d. *In vitro* studies indicated that rooibos inhibited the 11*β*-hydroxysteroid dehydrogenase type 1 (11*β*HSD1) resulting in an decrease in the cortisol : cortisone ratio [26]. The disruption of key steroid-metabolizing enzyme, 11*β*HSD1 related to cortisol metabolism as well as the downregulation of *Hsd17β2* expression (current study) by rooibos provide interesting scenarios related to the interaction of rooibos with steroidogenesis and steroid metabolism which should be further explored.

Although only marginally downregulated, *Alox15* and *MPO* genes are of interest as they represent NF-*κ*B target genes related to inflammation, suggesting that rooibos is likely to exhibit anti-inflammatory effects by inhibition of NF-*κ*B activation. Recent studies indicated that rooibos exhibited anti-inflammatory effects in skin keratinocytes [15], while aspalathin and nothofagin was found to reduce LPS-induced vascular inflammatory responses *in vivo* and in endothelial cells *in vitro* by the suppression of TNF-*α* and IL-16 production via the protection against NF-*κ*B activation [27].

Of the selected genes monitored in primary rat hepatocytes, none were affected by GRE, while aspalathin significantly upregulating the *Comt* and marginally the *Cyp2e1* genes while downregulating the expression of *Hsd17β2,* similar to GRE in the liver. This would imply that aspalathin is likely to contribute in the downregulation of the *Hsd17β2* gene by GRE. No effect was noticed for GRE on the expression of *Hsd17β2* in primary hepatocytes, which could be related to a masking effect by other polyphenolic constituents exhibiting an opposite effect, short duration of exposure or the low dose of exposure. The aspalathin concentration associated with GRE was 30-fold lower when compared to the concentration of pure aspalathin used. A similar effect was noticed for the *Comt* and *Cyp2e1* gene expression by GRE, which could also be related to the low equivalent dose level of aspalathin. These enzymes play an important role in the deactivation of catechol estrogen metabolites, lowering their DNA damaging potential [28]. Of interest is that, further to the effect on steroid metabolism, polyphenols also inhibit *Comt*-mediated *O*-methylation of catechol estrogens due to its higher affinity for the enzyme, which could further adversely impair the activity of the enzyme [29]. The upregulation of *Cyp2e1* is of interest as the gene encodes for the enzyme involved in quinol formation of estrogens, which also has the potential to interact with DNA [30]. The *Cyp2e1 gene* also represents one of

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the major hepatic CYP450 enzymes in the liver responsible for metabolizing toxins and carcinogens, such as carbon tetrachloride, N-nitrosodimethylamine, and acetaminophen [31]. It is involved in the metabolism of alcohol into acetaldehyde and suggested to play an important role in alcohol-induced liver damage via oxidative DNA damage [32]. However, the upregulation of *Ald1a1* by GRE *in vivo* in the liver is likely to effect by the removal of acetaldehyde which further support the view that rooibos alters alcohol metabolism.

Metabolism of long chain fatty acids via ω-1 hydroxylation by Cyp2E1, among other CYPs, results in the formation of hydroxy metabolites, products that constrict arterioles, elevates blood pressure, and promotes inflammation responses by modulating cell signaling pathways related to diabetes, obesity, and carcinogenesis [33]. The cytochrome is also involved in epoxygenase activity of docosahexaenoic and eicosapentaenoic acids with products that exhibit opposite effects compared to that derived from arachidonic acid. Overexpression of *Cyp2e1* and related carcinogen reactions may inadvertently produce reactive oxygen species and, when not regulated properly, could result in negative physiological effects [34]. A recent *in vitro* study indicated that fermented and unfermented rooibos extracts and aspalathin exhibit specific herb drugs-interactions by inhibiting CYP450s involved in the metabolism of the hypoglycemic and dyslipidemic drugs [10].

In the kidneys, GRE modulated expression of several genes– that is, upregulation of *Gstm1* and *Mgst1* enzyme genes and downregulation of the carcinogen-activating cytochrome P450, *Cyp4b1,* and *Nat1* genes. *Gst* enzymes play an important role in the detoxification of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins, and products of oxidative stress by conjugation with glutathione in different organs [35]. Therefore, GRE may play a protective role against the development of carcinogen and/or oxidative stress-induced DNA damage. However, the upregulation of these phase II enzymes relates to the nrf2-regulated genes via antioxidant-responsive element activation, which could imply a responsive effect to pro-oxidant or electrophile effects induced by GRE in the kidneys. Of interest is that both the *Cyp4b1* and *Nat1* genes are associated with the development of bladder cancer, with the former involved in the activation of 2-aminofluorene [36] and the latter in the activation of arylamine metabolites through *O*-acetylation and *N,O*-acetylation [37]. In this regard, GRE seems to play a protective role in counteracting the generation of these reactive metabolites in the kidneys. The downregulation of the glutathione-depletion enzyme gene, *Ggt1,* further suggest that GRE could protect against carcinogens when utilizing the glutathione conjugation deactivation pathway. Glutathione degradation occurs in the proximal tubule at both the luminal [38] and basolateral membrane [39], a process that is modulated by GRE, presumably due to changes in GSH metabolism.

GRE downregulated the *Abcb1* gene in the kidneys. The ABC transporters form part of the phase III drug transporters and constitute a ubiquitous superfamily of integral membrane proteins responsible for the translocation of many substances across membranes. Although this may be advantageous in the treatment of cancer during chemotherapy, the protection of normal cells against toxic compounds may be impaired. Cancer cells are known to increase the expression of ATP-dependent efflux pumps, the ABC transporters including P-glycoprotein (*Abcb1*), which are associated with drug resistance [40]. Therefore, ABC transporters are one of the most critical targets in the treatment of cancer, especially those exhibiting drug resistance. It would appear that GRE could selectively alter these pathways and may therefore be used to sensitize the susceptibility of cancer cells to anticancer drugs in the kidneys. However, GRE may also prevent the transport of its polyphenols from the kidneys. Of interest is that the expression of the phase II enzymes is predominantly affected in the kidneys compared to the liver, which is regarded as the major organ for drug metabolism. However, the kidneys play a major role in the metabolism of drugs, hormones, and xenobiotics, and in some cases, such as the enzymes involved in the processing of glutathione conjugates to their mercapturic acids, biotransformations occur at a faster rate in the kidneys [41].

When extrapolating the average dietary TP intake of 76 mg GAE/kg BW/d to a HED [42], it amounts to 12.3 mg GAE/kg BW/d. The aspalathin intake of 36 mg/kg BW/d by the rats equals a HED of approximately 6 mg/kg BW/d. Based on 1.2 mg aspalathin/200 mL of a fermented rooibos infusion equaling 1 cup [43], humans (70 kg BW) consuming 6 cups of rooibos per day, as suggested by Marnewick et al. [44], have a daily intake of 0.1 mg aspalathin/kg. When consuming 6 cups of green rooibos infusion at 31 mg aspalathin/200 mL [5], the daily intake of aspalathin will equal 2.7 mg/kg BW. This is approximately 13-fold lower when compared to the current exposure in rats and two-fold lower when considering the HED. However, specific safe levels of exposure should be considered as the current study indicated that the expression of drug metabolic enzymes is altered and that a longer duration of exposure also alters the expression of oxidative stress and antioxidant-related defense genes in the liver, as well as reducing iron absorption [12]. In this regard, specific tolerable uptake levels of exposure to plant bioactive nutrients to address health benefits and safety/toxicity have been suggested in order to define thresholds of exposure, thereby minimizing adverse chronic effects [45].

In conclusion, the genes involved in alcohol, energy, and phase I enzymes related to steroid metabolism in the liver were altered. Differences as well as similarities were observed between the *in vivo* study in rats and in primary hepatocytes with respect to the expression of genes related to estrogen and alcohol metabolism. This could be due to variations in the dose level and duration of exposure to GRE and aspalathin. In the kidneys, however, mostly phase II genes, transporters and only certain phase I genes were altered. As the kidney is the main organ for excretion of drugs, hormones, and xenobiotics, modulation of these enzymes could have important functional implications following chronic exposure to polyphenol-enriched pharmaceutical products. Detailed investigations assessing changes in the expression profiles of the genes at a protein level would provide more information about the validity of these alterations at a cellular level.

HPLC-DAD chromatogram of ARC2 at 288 nm (**1**, *Z*-2-(*β*-D-glucopyranosyloxy)-3-phenylpropenoic acid, 0.49 %; **2**, isoorientin, 2.05 %; 3, orientin, 1.05 %; **4**, aspalathin, 18.44 %; **5**, quercetin-3-O-robinobioside, 1.05 %; **6**, vitexin, 0.27 %; **7**, hyperoside, 0.27 %; **8**, rutin, 0.54 %; **9**, isovitexin, 0.39 %; 10, isoquercitrin, 0.38 %; **11**,

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nothofagin, 1.29%) is available as Supporting Information. Insert shows chromatogram of sample at 288 nm with y-axis adjusted to show full aspalathin peak height.

Materials and Methods

Chemicals

Aspalathin (3′-*C*-*β*-D-glucopyranosyl-2′,3,4,4′,6′-pentahydroxy-dihydrochalcone) was purified from unfermented rooibos at the Institute of Biomedical and Microbial Biotechnology, Bellville, South Africa, to a purity of > 95%. WE, HBSS, HEPES, rat tail collagen type 1, collagenase type 4, insulin, sodium-pyruvic acid, L-glutamine, L-proline, ethylene glycol tetraacetic acid, DMSO, Eagle's minimal essential medium, and molecular biology grade *β*-mercaptoethanol (*β*-ME) were purchased from Sigma-Aldrich. FBS was obtained from Invitrogen. Penicillin, streptomycin, fungizone (amphotericin B), trypan blue, and MEM nonessential amino acid solution were obtained from Lonza. Analytical grade methanol, ethanol, and hydrochloric acid were purchased from Merck Chemicals.

GRE

The aspalathin-enriched GRE (GRC2) was prepared according to a patented process and supplied by Raps Foundation [46]. In short, the plant material was extracted with 80% ethanol-water mixture and dried under vacuum-drying. The chlorophyll of the dried extract extracted was removed with ethyl acetate and the residue vacuum-dried and stored at − 20 °C until used. The aspalathin-enriched GRE contained 18.4% aspalathin, 1.3% nothofagin, 3.8% flavones (orientin, isoorientin, vitexin, and isovitexin), and 2.2% flavonols (quercetin glycosides) as detailed by HPLC-DAD analysis [11]. A detailed HPLC chromatogram is provided as a supplementary file. The TP in the extract, according to the Folin-Ciocalteu method, was 39.2 g GAE/100 g extract [12].

Animal study design

Details of the animal study have been described in detail elsewhere [12] and was approved by the Ethics Committee for Research on Animals of the South African Medical Research Council (Ref No. 05/07; April 17, 2007). Seven- to eight-week-old male Fischer (F344) rats (n = 10), weighing between 150 and 200 g were divided into two groups: the control group receiving a normal diet (Epol Ltd.) and a treatment group receiving the same diet supplemented with GRE (0.2%) for 28 d. This GRE dose was selected based on HED consuming six cups of green rooibos per day (details in the Discussion section). All the rats had free access to the feed and water. Upon termination, rats were fasted overnight, sacrificed by cervical dislocation, and tissue sections of the liver and kidneys harvested and (200–400 mg) immediately snap frozen in liquid nitrogen and stored at − 80 °C until gene expression analyses of the xenobiotic metabolizing enzymes. Five liver and kidney samples were randomly selected of the rats from each treatment and control groups.

qRT-PCR

Total RNA was isolated from the liver and kidney tissue (20–25 mg) of three rats in each treatment and control group using the RNeasy mini kit according to the manufacturer's instructions (Qiagen). Tissue samples were homogenized in a Tissuelyser II (Qiagen) for 40 s and the RNA eluted with RNase-free water (50 µL). Genomic DNA contamination was removed using the Ambion Turbo DNA-free kit (Applied Biosystems) according to the manufacturer's recommendations. RNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific) and the RNA integrity confirmed by determining the RNA 28S:18S ratio using a Bioanalyzer 2100 (Agilent Technologies). The isolated RNA samples were stored at − 80 °C. First strand complimentary deoxyribonucleic acid (cDNA) synthesis was carried out using the RT^2 First Strand Kit (Qiagen) according to the manufacturer's protocol and samples stored at − 20 °C until further use.

qRT-PCR array analyses of the samples were done in triplicate. The cDNA (20 µL) was further diluted in nuclease-free water to a final volume of 111 µL. An aliquot (102 µL) was mixed with a twice concentrated RT^2 qPCR SYBR green I master mix (550 µL) (Qiagen), containing HotStart DNA polymerase and nuclease-free water (448 µL) to a final volume of 1100 µL. The cDNA mixture (10 µL) was transferred to a PCR profiler array (Qiagen) containing 84 genes, coded for xenobiotic metabolizing enzymes and transporters, five reference genes, and quality control parameters (http://www.sabiosciences.com/PCRArrayPlate.PhP). Data were generated using an ABI 7900HT RT-PCR apparatus (Applied Biosystems) with a two-step cycling program (1 cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 sec and then at 60 °C for 1 min). The data were analyzed using the Microsoft Excel-based PCR array data analysis template from Qiagen (http://www.superarray.com/pcrarraydata analysis.php). All samples were analyzed in triplicate. The mean Ct value for the genomic DNA control (GDC) parameter of each sample was determined and a gene-by-gene analysis for each gene of interest (GOI) was done using the equation $\Delta Ct_{(GOI)}$ = Ct (GDC) – Ct (GOI). Five reference genes (*Rplp1, Hprt1, Rpl13a, Ldha*, and *Actb*) were used for normalization. The relative number of transcripts in the treated and control was calculated according to the comparative Ct (ΔΔCt) method. The fold-change for each treated sample relative to the control sample was calculated using the formula of $2^{-\Delta\Delta Ct}$. Statistical calculations were conducted, based on the ΔCt values, using a two-tailed t-test and $p \leq 0.05$ was designated as significant.

In vitro studies utilizing primary rat hepatocyte cultures

Primary hepatocytes were isolated from male Fischer 344 rats (ca. 200 g/rat) according to the collagenase perfusion technique [47]. Cell viability was determined using trypan blue exclusion stain and hepatocyte cultures yielding a viability of greater than 80% were used. Cells were plated in WE containing 10% FBS, insulin (20 U/L), L-glutamine (2 mM), HEPES (10 mM), penicillin (100 U/ml), and streptomycin (100 µg/mL) for 3 h at 37 °C. Cells were washed twice with HBSS and supplemented with modified WE (200 µL in 96-well plates or 2 mL in 60 mm plates) containing 0.5% FBS, L-proline (2 mM), and sodium pyruvate (10 mM), in addition to the other components described above, and incubated for 24 h at 37 °C in

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▶ **Table 4** Primer sequences of selected genes for qPCR analyses in primary hepatocytes.

Gene	Genebank	Sense	Antisense
		5′→3′	5′→3′
Comt	NM_012531	CCATGGAGATGAACCCTGACTAC	TAGGCCTGCAAAGTTCAGCATT
Cyp2e1	NM_031543	TTGGCCGACCTGTTCTTTG	TTTCTGGGTATTTCATGAGGATCA
Hsd17β2	NM_024391	CGCAGAGAAGATACAAGACAAAGG	AAAGTGCAAGACCCCAGCAT
Hprt1	NM_012583	TCAAGCAGTACAGCCCCAAAAT	CAACACTTCGAGAGGTCCTTTTC

air/carbon dioxide (95 : 5). Different concentrations of GRE or aspalathin (100 µL) were added to the respective wells of a 96-well opaque-walled microtiter plate and the plate incubated for 24 h at 37 °C in air/carbon dioxide (95 : 5).

Cell viability assay

Cells were plated at a density of 2×10^4 viable cells in collagen-coated 96-well opaque-walled plates for the cell viability assay. GRE dilutions (0.2, 0.1, 0.05, 0.025 mg/mL) and aspalathin (2.0, 1.25, 0.63, 0.31, 0.16 mM) were prepared in modified WE culture medium to a final DMSO concentration of 1 %. A 1 % DMSO culture medium solution was used as control. Cell viability was determined based on the assessment of ATP production as a measure of metabolically active cells, using the CellTitre-Glo assay (Promega) according to the manufacturer's protocol. The luminescence signal was recorded using a Veritas Microplate Luminometer (Turner Biosystems). The percentage ATP inhibition was determined and the respective IC_{50} values (concentration of GRE and aspalathin required to inhibit 50 % ATP production in the cell) calculated using GraphPad Prism 5 software. The assay was repeated five times to obtain average IC_{50} values for GRE and aspalathin.

Gene expression analyses in primary hepatocytes

Hepatocytes (≥ 80 % viability) were plated at a density of 3×10^6 viable cells in collagen-coated culture dishes (60 mm) for RNA extractions. The cells were exposed to nontoxic levels below the IC_{50} values of GRE (0.025 mg/mL) and aspalathin (0.31 mM) (▶ **Table 2**) for 24 h at 37 °C as describe above. Each treatment and control were repeated four times in a total volume of 2 mL per tissue culture dish. The hepatocyte cultures were washed with ice-cold phosphate buffered saline (pH7.4) and collected in RLT buffer (350 µL/dish) (Qiagen), containing guanidine isothiocyanate and *β*-mercaptoethanol. The cells in four tissue culture dishes were combined into clean nuclease-free tubes and lysed in liquid nitrogen. Total RNA was isolated, and cDNA synthesized as described above. The gene expression of three xenobiotic metabolizing genes (*Comt, Cyp2e1, Hsd17β2*) were analyzed by qRT-PCR with the hypoxanthine guanine phosphoribosyltransferase 1 (*Hprt1*) gene as an endogenous control. Gene-specific qPCR primers (▶ **Table 4**) were designed using the Primer Express software v2.5 (Applied Biosystems) and synthesized by Integrated DNA Technologies Inc. (IDT). The qRT-PCR array and statistical analyses were conducted as described above.

Supporting Information

HPLC-DAD chromatogram of ARC2 at 288 nm (**1**, *Z*-2-(*β*-D-glucopyranosyloxy)-3-phenylpropenoic acid, 0.49 %; **2**, isoorientin, 2.05 %; 3, orientin, 1.05 %; **4**, aspalathin, 18.44 %; **5**, quercetin-3-O-robinobioside, 1.05 %; **6**, vitexin, 0.27 %; **7**, hyperoside, 0.27 %; **8**, rutin, 0.54 %; **9**, isovitexin, 0.39 %; 10, isoquercitrin, 0.38 %; **11**, nothofagin, 1.29 %). Insert shows chromatogram of sample at 288 nm with y-axis adjusted to show full aspalathin peak height.

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Conflict of Interest

The authors declare no conflicts of interest

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