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The influence of *Aspalathus linearis* (Rooibos) and dihydrochalcones on adrenal steroidogenesis: Quantification of steroid intermediates and end products in H295R cells

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ABSTRACT

The steroid hormone output of the adrenal gland is crucial in the maintenance of hormonal homeostasis, with hormonal imbalances being associated with numerous clinical conditions which include, amongst others, hypertension, metabolic syndrome, cardiovascular disease, insulin resistance and type 2 diabetes. *Aspalathus linearis* (Rooibos), which has been reported to aid stress-related symptoms linked to metabolic diseases, contains a wide spectrum of bioactive phenolic compounds of which aspalathin is unique. In this study the inhibitory effects of Rooibos and the dihydrochalcones, aspalathin and nothofagin, were investigated on adrenal steroidogenesis. The activities of both cytochrome P450 17α-hydroxylase/17,20 lyase and cytochrome P450 21-hydroxylase were significantly inhibited in COS-1 cells. In order to study the effect of these compounds in H295R cells, a human adrenal carcinoma cell line, a novel UPLC–MS/MS method was developed for the detection and quantification of twenty-one steroid metabolites using a single chromatographic separation. Under both basal and forskolin-stimulated conditions, the total amount of steroids produced in H295R cells significantly decreased in the presence of Rooibos, aspalathin and nothofagin. Under stimulated conditions, Rooibos decreased the total steroid output 4-fold and resulted in a significant reduction of aldosterone and cortisol precursors. Dehydroepiandrosterone-sulfate levels were unchanged, while the levels of androstenedione (A4) and 11β-hydroxyandrostenedione (11βOH-A4) were inhibited 5.5 and 2.3-fold, respectively. Quantification of 11βOH-A4 showed this metabolite to be a major product of steroidogenesis in H295R cells and we confirm, for the first time, that this steroid metabolite is the product of the hydroxylation of A4 by human cytochrome P450 11β-hydroxylase. Taken together our results demonstrate that Rooibos, aspalathin and nothofagin influence steroid hormone biosynthesis and the flux through the mineralocorticoid, glucocorticoid and androgen pathways, thus possibly contributing to the alleviation of negative effects arising from elevated glucocorticoid levels.

1. Introduction

In steroidogenesis, the steroid hormone output of the adrenal plays a pivotal role in the maintenance of hormonal homeostasis. Steroidogenic enzymes consisting of steroidogenic cytochrome P450 (P450) enzymes and 3β-hydroxysteroid dehydrogenase II (3βHSD) catalyse the biosynthesis of mineralocorticoids, glucocorticoids and androgen precursors in the adrenal cortex through multiple reactions (Fig. 1). Cytochrome P450 side-chain cleavage (CYP11A1) catalyses the conversion of cholesterol to pregnenolone (PREG) which is further metabolized by both 3βHSD and P450 17α-hydroxylase/17,20 lyase (CYP17A1) at a branch point in the pathway, thus determining the steroidogenic output of the adrenal. CYP17A1 catalyses the hydroxylation of PREG to yield 17-hydroxypregnenolone (17OH-PREG), which in turn is a substrate for the subsequent lyase reaction, yielding the androgen precursor dehydroepiandrosterone

Abbreviations: P450, cytochrome P450; 3βHSD, 3β-hydroxysteroid dehydrogenase II; CYP11A1, cytochrome P450 side-chain cleavage; PREG, pregnenolone; CYP17A1, P450 17α-hydroxylase/1720 lyase; 17OH-PREG, 17-hydroxypregnenolone; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone-sulfate; PROG, progesterone; 17OH-PROG, 17α-hydroxyprogesterone; 16OH-PROG, 16α-hydroxyprogesterone; A4, androstenedione; CYP21, cytochrome P450 21-hydroxylase; CYP11B1, cytochrome P450 11β-hydroxylase; CYP11B2, aldosterone synthase; ALDO, aldosterone; DOC, deoxycorticosterone; CORT, corticosterone; 11-DHC, 11-dehydrocorticosterone; 11βOH-A4, 11β-hydroxyandrostenedione; 11βHSD, 11β-hydroxysteroid dehydrogenases; ACE, angiotensin-converting enzyme; UPLC–MS/MS, ultra performance liquid chromatography/tandem mass spectrometry.

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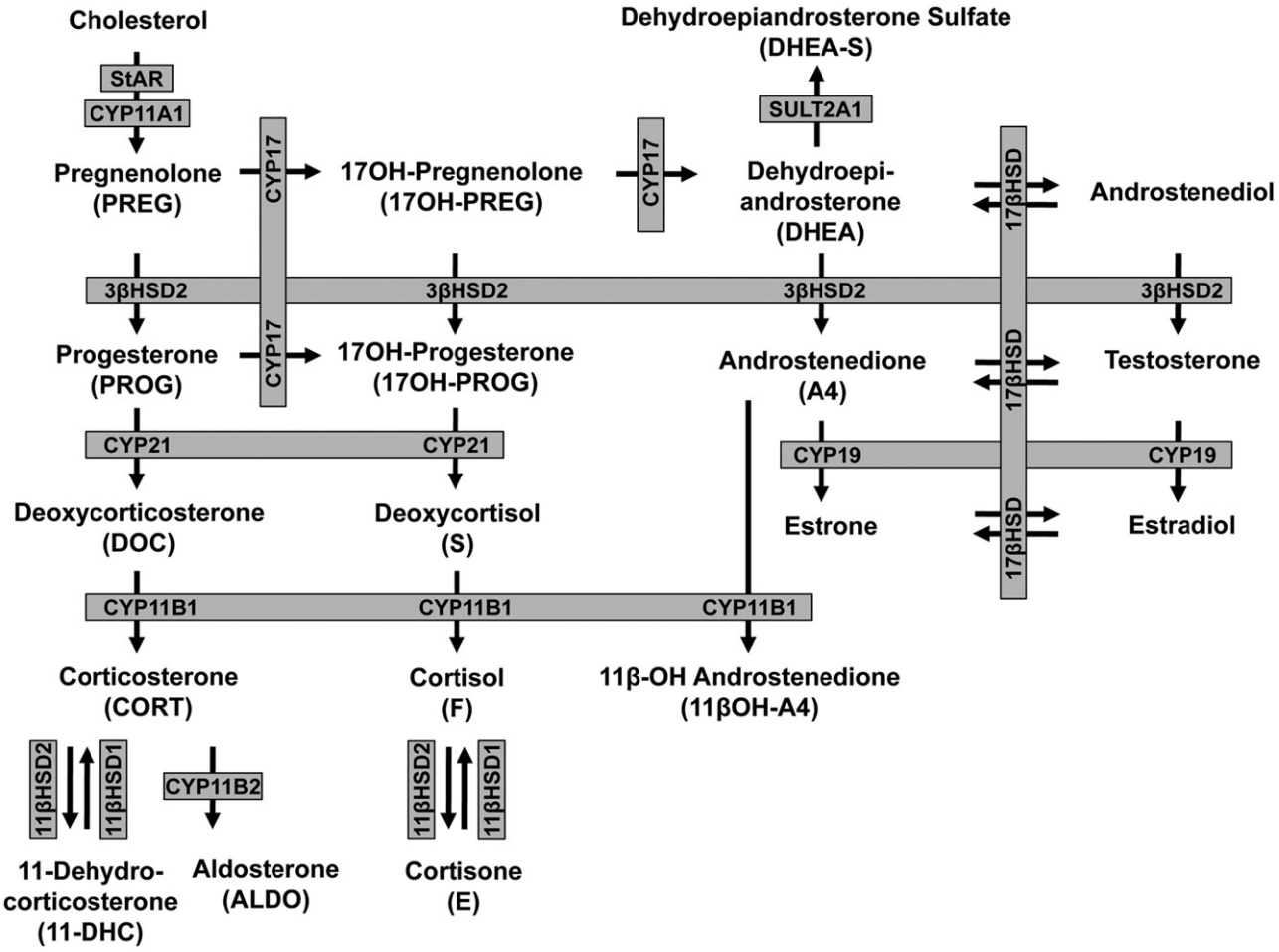

Fig. 1. Human steroid biosynthesis. Major enzymes and steroids involved in mineralocorticoid, glucocorticoid, androgen and estrogen biosynthesis.

(DHEA). In addition, human CYP17A1 converts progesterone (PROG) to 17α-hydroxyprogesterone (17OH-PROG) and 16α-hydroxyprogesterone (16OH-PROG) [1]. The metabolism of the Δ^5-steroids, PREG, 17OH-PREG and DHEA to the respective Δ^4-steroids, PROG, 17OH-PROG and androstenedione (A4), is catalysed by 3βHSD. Cytochrome P450 21-hydroxylase (CYP21) hydroxylates PROG and 17OH-PROG to yield the substrates for cytochrome P450 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2). The latter catalyses the biosynthesis of the mineralocorticoid, aldosterone (ALDO), from deoxycorticosterone (DOC), while CYP11B1 catalyses the conversion of DOC and deoxycortisol to the glucocorticoids, corticosterone (CORT) and cortisol, respectively [2]. In addition to the major steroid hormones, the adrenal also produces, amongst others, sex steroids, albeit at low levels, as well as cortisone, 11-dehydrocorticosterone (11-DHC) and 11β-hydroxyandrostenedione (11βOH-A4), at significantly higher levels [3]. The former two metabolites are products of reactions catalysed by 11β-hydroxysteroid dehydrogenases (11βHSD type 1 and 2) [4], while the latter has been reported to be the product of the 11β-hydroxylation of A4 [5].

Glucocorticoids play a role in the maintenance of the homeostasis of the central nervous system, glucose homeostasis and immune modulation, and are considered stress hormones as their secretion is strongly altered by exposure to environmental stressors. Stress, be it psychological or physical, activates the hypothalamic–pituitary–adrenal (HPA) axis. Activation of the HPA-axis involves the release of adrenocorticotropic hormone (ACTH), resulting in the stimulation of the adrenal cortex with the subsequent elevation in circulating cortisol. Cortisol, released in response to stress, also forms part of the negative feedback system regulating the HPA-axis. During chronic stress, however, the negative feedback regulatory mechanism is overridden, resulting in elevated basal cortisol levels [6,7]. Elevated glucocorticoid levels, resulting from hormonal imbalances, have been associated with numerous clinical conditions which include, amongst others, hypertension, metabolic syndrome, cardiovascular disease, hyperglycemia, insulin resistance, type 2 diabetes, depressed immune function, osteoporosis and suppression of the reproductive system [7–10].

Maintaining normal cortisol levels is therefore critical to endocrine function. Therapies aimed at alleviating negative effects arising from the chronic stimulation of the HPA-axis could thus be aimed at decreasing plasma glucocorticoid levels. Strategies employed in the treatment of diseases associated with endocrine disorders involve selective inhibitors of either the enzymes catalysing adrenal steroid biosynthesis or antagonist activity at steroid receptor level. Several compounds have been identified which inhibit steroidogenesis by interfering with one or more enzymes in the steroidogenic pathway [11,12]. One such compound, ketoconazole, which is widely used to treat patients with Cushing's syndrome, inhibits CYP11A1 and CYP11B1 as well as ACTH secretion at therapeutic doses. Ketoconazole, however, has been found to trigger toxicological effects *in vivo*, decreasing A4 and testosterone levels to 60% below basal plasma levels while increasing 17OH-PROG levels. It is therefore critical in the development of pharmaceutical drugs to ensure that the inhibition of specific

steroidogenic enzymes is selective and that endocrine toxicity is avoided [13,14].

In recent years, the shift towards the use of natural and herbal medicinal products has led to attention being placed on the influence of, amongst others, polyphenolic compounds on steroidogenesis. Polyphenolic compounds include the dihydrochalcones, flavones, isoflavones and flavanols, and are commonly referred to as flavonoids. These secondary plant metabolites, abundantly present in soy and soy products, legumes and lentils, have been shown to demonstrate potent anti-oxidant, anti-inflammatory, anti-atherosclerotic and anti-mutagenic properties [15–19]. In addition, numerous studies have shown that flavonoid compounds exhibit phytoestrogenic activity and may aid clinical conditions such as osteoporosis, breast cancer and cardiovascular disease [20–22]. However, it has been suggested that flavonoid compounds may possibly be cytotoxic since they may act as mutagens, pro-oxidants and inhibitors of key enzymes [23]. High doses of genistein have, for example, been shown to result in decreased fertility and sexual dysfunction in experimental animals [24]. The overall health benefits of flavonoids therefore still remain uncertain [25]. The effect of flavonoid compounds on adrenal steroidogenesis has been investigated by various groups. Ohno et al. [26] showed that flavonoid compounds such as diadzein, genistein and 6-hydroxyflavone selectively inhibits key steroidogenic enzymes including 3βHSD, CYP17A1, CYP21 and CYP11B1 in H295R cells. Mesiano et al. [27] demonstrated that both genistein and diadzein inhibit cortisol production in ACTH-stimulated cultured fetal and postnatal adrenal cortical cells as well as in cAMP-stimulated H295 cells. More recently, Ohlsson et al. [28] showed that the flavonoids diadzein and genistein dose-dependently inhibit cortisol, ALDO and testosterone production in H295R cells.

Rooibos (*Aspalathus linearis*) is consumed world-wide as a herbal tea and is anecdotally reported to aid stress-related symptoms. It is a rich source of dietary polyphenols and as such, possesses potent antioxidant activities [29,30]. In addition, Rooibos extracts have been shown to exhibit anti-mutagenic, anti-cancer and immune modulating properties [31–33]. Rooibos consumption has been shown to significantly improve the lipid profiles and redox status in humans at risk for developing cardiovascular disease [34]. In addition, it has been shown by Persson et al. [35] that Rooibos significantly inhibits angiotensin-converting enzyme (ACE) activity after an oral intake of a single dose of Rooibos tea, supporting the potential role of Rooibos in the overall management of cardiovascular and metabolic-related diseases. Rooibos has been shown to have beneficial effects on glucose homeostasis and type 2 diabetes, with aspalathin stimulating glucose uptake in muscle tissues as well as insulin secretion from pancreatic β-cells [36]. Aspalathin is unique to Rooibos, while nothofagin has only been identified in one other species, *Nothofagus fusca* [37,38].

The aim of this study was to determine the influence of Rooibos and the dihydrochalcones, aspalathin and nothofagin, on the catalytic activities of CYP17A1 and CYP21 expressed in non-steroidogenic COS-1 cells, as well as on steroidogenesis in H295R cells, a human adrenal carcinoma cell line. Although previous studies have investigated the inhibitory effect of flavonoid compounds on adrenal enzyme activities, specific metabolites in the respective steroidogenic pathways, and on enzyme expression, the effect of these compounds on steroid hormone biosynthesis has not been fully addressed. Unsatisfactory analytical methods have impeded the efficient analyses and quantification of metabolites produced in adrenal steroidogenesis. In this study we report a novel ultra performance liquid chromatography/tandem mass spectrometry (UPLC–MS/MS) method for the identification and quantification of twenty-one steroid metabolites. This method enabled us to quantify not only the end metabolites of the mineralocorticoid, glucocorticoid and androgen precursor pathways, but also to simultaneously analyse the steroid profile of the intermediate metabolites in basal and forskolin stimulated H295R cells.

2. Materials and methods

2.1. Materials

Unfermented Rooibos was provided by the South African Rooibos Council. Aspalathin and nothofagin were supplied by Prof. W.C.A. Gelderblom (Medical Research Council, Western Cape, South Africa) Nucleobond® AX plasmid preparation kits were purchased from Machery-Nagel (Duren, Germany). COS-1 cells were obtained from the American Type tissue Culture Collection (Manassas, VA, USA) and *Mirus TransIT®*-LT1 transfection reagent was purchased from Mirus Bio Corporation (Madison, WI, USA). Penicillin–streptomycin, fetal calf serum and trypsin–EDTA were obtained from Gibco BRL (Gaithersburg, MD, USA). Deuterated cortisol (9,11,12,12-D4-cortisol) was purchased from Cambridge isotopes (Andover, MA, USA). Steroids, forskolin, trilostane, Dulbecco's modified Eagle's Medium (DMEM) and an MTT assay kit were purchased from Sigma–Aldrich (St. Louis, MA, USA). DMSO was obtained from Merck (Darmstadt, Germany). DMEM/F_{12} and gentamicin were purchased from Invitrogen/Gibco (Grand Island, New York, USA). Cosmic calf serum was supplied by HyClone®, Thermo Scientific (South Logan, Utah, USA). A bicinchoninic acid (BCA) protein determination kit was purchased from Pierce (Rockford, IL, USA). The UPLC BEH C18 column was purchased from Waters and the Kinetex PFP column was purchased from Phenomenex. All other chemicals were of the finest quality and supplied by trustworthy scientific supply houses.

2.2. Methanol extractions of unfermented Rooibos

Rooibos is produced both as a fermented and unfermented product, with the latter subjected to milder processes ensuring oxidative changes to secondary plant metabolites are minimized. A Rooibos extract was prepared by extracting 30 g unfermented plant material with 300 ml chloroform for 8 h using a glass soxhlet extractor fitted with a double wall condenser. The plant material was subsequently extracted with 300 ml methanol for 8 h. The extract was dried on a rotary evaporator and the vacuum released under nitrogen. The dried extract was resuspended in deionised water to a final concentration of 86 mg extract/ml and centrifuged at 6000 × *g* for 5 min. The supernatant was divided into aliquots and stored at −20 °C. The Rooibos extract was protected from light and oxygen at all times to avoid any compositional changes.

2.3. Enzyme assays in transiently transfected COS-1 cells

COS-1 cells were grown at 37 °C and 5% CO_2 in DMEM containing 0.9 g/l glucose, 0.12% $NaHCO_3$, 10% fetal calf serum and 1% penicillin–streptomycin. The cells were plated into 12 well dishes with each well containing 1×10^5 cells in 1 ml, 24 h prior to transfection. Cells were transiently transfected with 0.5 μg DNA (baboon CYP17A1/pCIneo, baboon CYP21/pCIneo and baboon CYP11B1/pTarget) and 1.5 μl *Mirus TransIT®*-LT1 transfection reagent according to the manufacturer's instructions. Control transfection reactions were performed using the pCIneo vector containing no DNA insert. Cells were incubated for 72 h after which the appropriate steroid substrate, PREG, PROG, 17OH-PROG, DOC, deoxycortisol or A4 was added to the medium. Substrate conversion in the presence of Rooibos was assayed by the addition of 50 μl extract (final concentration, 4.3 mg extract/ml). Aspalathin and nothofagin, dissolved in ethanol, were added to a final concentration of 10 μM. At specific time intervals, 500 μl aliquots were removed and the steroids extracted using a 10:1 volume of

dichloromethane to culture medium. The medium was removed and the dichloromethane phase dried under N_2. The steroids were resuspended in 150 μl methanol and analysed. After each experiment, the cells were washed and collected in phosphate buffer (0.1 M, pH 7.4), disrupted via sonication and the total protein content determined by the Pierce BCA method according to the manufacturer's instructions.

2.4. Steroid metabolism in H295R cells

H295R cells were grown to confluency at 37 °C and 5% CO_2 in growth medium, DMEM/F_{12}, supplemented with L-glutamine, 15 mM HEPES, pyridoxine, 1.125 g $NaHCO_3$/l, 1% penicillin streptomycin, 0.01% gentamicin and 10% cosmic calf serum, after which cells were plated into 12 well dishes (1 ml, 4×10^5 cells/ml). PREG metabolism was assayed as follows: after an incubation period of 48 h, the growth medium was aspirated off and replaced with growth medium containing 0.1% cosmic calf serum. Cells were incubated for a further 12 h after which the appropriate treatments were added to the medium. Basal steroid metabolism was assayed in the presence of Rooibos by the addition of 50 μl extract (final concentration, 1 mg extract/ml). Aspalathin and nothofagin were added to a final concentration of 10 μM. Basal steroid metabolism was stimulated with the addition of forskolin to a final concentration of 10 μM. A4 metabolism was assayed as follows: 24 h after plating cells the medium was replaced with growth medium containing 10 μM trilostane. After 24 h, the medium was replaced with growth medium containing 10 μM trilostane and 1 μM A4. Control assays included the addition of 10 μM trilostane and 1 μM cortisol; 10 μM trilostane; and medium only. After 48 h the medium (500 μl) was removed and the steroids extracted as described in Section 2.3. D4-cortisol (15 ng), was added to the dichloromethane/culture medium mixture as an internal standard prior to extraction.

2.5. Cell viability

2.5.1. COS-1 cells

The viability of the COS-1 cells was determined by investigating the effect of the test compounds on the ability of the cells to convert testosterone to A4 through endogenous 17β-hydroxysteroid dehydrogenase (17β-HSD) type 2 activity [39]. Testosterone (1 μM) was added to confluent COS-1 cells in the absence and presence of the Rooibos extract (4.3 mg/ml), aspalathin (10 μM) and nothofagin (10 μM). After a 24 h incubation period the steroids were extracted as described in Section 2.3.

2.5.2. H295R cells

Confluent H295R cells were plated out in a 96 well plate (100 μl, 4×10^5 cells/ml) and incubated with the Rooibos extract (1 mg/ml), aspalathin (10 μM), nothofagin (10 μM) as well as forskolin (10 μM) for 48 h. Cell viability was subsequently assayed using a MTT toxicology assay kit according to the manufacturer's instructions.

2.6. Separation and quantification of steroid metabolites in COS-1 cells

Steroid metabolites were analysed by UPLC–MS/MS. Steroid metabolites resulting from the conversion of PREG, PROG, 17OH-PROG, deoxycortisol and A4 in COS-1 cells were separated by UPLC (ACQUITY UPLC, Waters, Milford, USA) using a Waters UPLC BEH C18 (2.1 mm × 50 mm, 1.7 μm) column as previously described [40]. The mobile phases consisted of 1% formic acid (A) and acetonitrile (B). Steroids were eluted at a flow rate of 0.4 ml/min, using a linear gradient from 85% A to 80% B in 3.5 min, followed by a linear gradient from 80% B to 100% B in 0.1 min. The injection volume was 5 μl. A Xevo triple quadrupole mass spectrometer (Waters, Milford, USA) was used for quantitative mass spectrometric detection. All steroids were analysed in multiple reaction monitoring (MRM) mode using an electrospray in the positive ionization mode (ESI+). The following settings were used: capillary voltage of 2.8 kV, cone voltage 15–35 V, collision energy 4–32 eV, source temperature 100 °C, desolvation temperature 500 °C, desolvation gas 1000 l h^{-1} and cone gas 50 l h^{-1}. Calibration curves were constructed by using weighted ($1/x^2$) linear least squares regression. Data was collected with the MassLynx 4.0 software program.

2.7. Separation and quantification of steroid metabolites in H295R cells

2.7.1. Preparation of standards

Stock solutions of PREG, PROG, DOC, CORT, 18OH-CORT, ALDO, 11-DHC, 17OH-PREG, 17OH-PROG, 16OH-PROG, deoxycortisol, cortisol, cortisone, DHEA, DHEA-S, A4, 11βOH-A4, testosterone, DHT and β-estradiol were prepared in ethanol (2 mg/ml). Estrone was dissolved in acetone (2 mg/ml). A series of standards (0.0002, 0.002, 0.02, 0.1, 0.2, 1, 2 and 4 ng/μl) were prepared in methanol from the stock solutions. In addition, each standard contained an internal standard, D4-cortisol (final concentration, 0.1 ng/μl).

2.7.2. UPLC–MS/MS conditions

Steroid metabolites from conversion assays conducted in the H295R cells were separated by UPLC (ACQUITY UPLC, Waters, Milford, USA) using a Phenomenex UPLC Kinetex PFP (2.1 mm × 100 mm, 2.6 μm) column. The mobile phases consisted of 1% formic acid (A) and 49%:49%:2% methanol:acetonitrile: isopropanol (B). Steroid metabolites were eluted at a flow rate of 0.45 ml/min and the injection volume was set to 5 μl. Steroids were quantified using a Xevo triple quadrupole mass spectrometer (Waters, Milford, USA) as described above. The gradient of the LC system is shown in supplementary Table 1, other relevant information including the parent and daughter ions, cone (V) and collision (eV) voltages, retention times and the validation of the UPLC–MS/MS assay is shown in supplementary Table 2.

2.8. Statistical analysis

All experiments were performed in triplicate and results are given as means ± SEM. Statistics were calculated by a one-way ANOVA, followed by a Dunnett's multiple comparison test using GraphPad Prism (version 5) software (GraphPad Software, San Diego, California). A value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

The present study was undertaken to investigate the effect of Rooibos on adrenal steroidogenesis, and in particular, on glucocorticoid production. The influence of an unfermented Rooibos extract and two dihydrochalcone compounds, aspalathin and nothofagin, was investigated on two key adrenal steroidogenic enzymes, CYP17A1 and CYP21, by expression in non-steroidogenic COS-1 cells. We subsequently examined the effect of Rooibos, aspalathin and nothofagin on steroidogenesis in H295R cells.

3.1. Enzyme assays in COS-1 cells expressing CYP17A1 and CYP21

Both CYP17A1 and CYP21 play a central role in adrenal steroidogenesis with CYP17A1 catalysing the biosynthesis of androgen precursors while CYP21 is essential for the biosynthesis of mineralocorticoids and glucocorticoids. The effect of Rooibos (4.3 mg/ml),

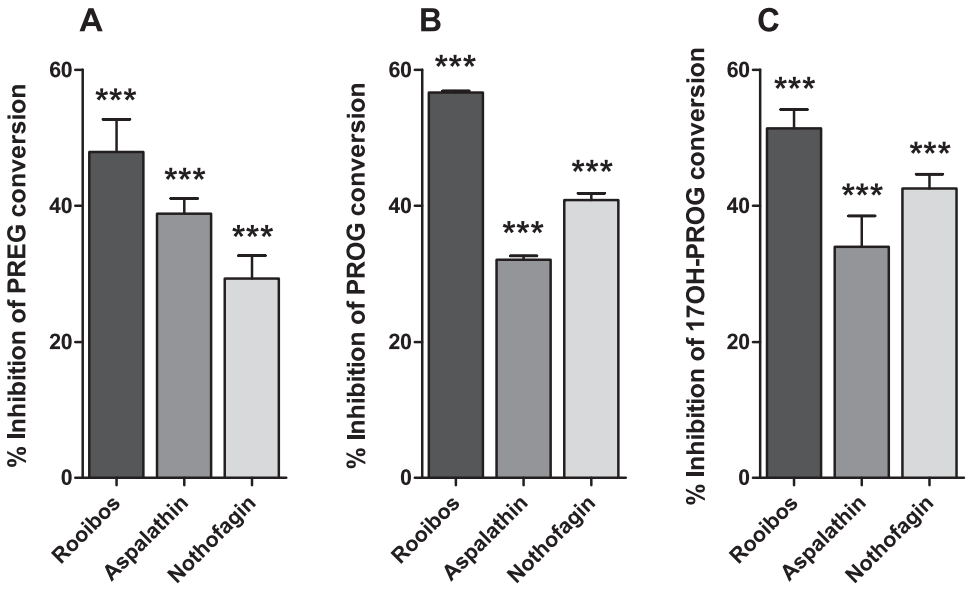

Fig. 2. The influence of Rooibos and dihydrochalcones on substrate conversion in transiently transfected COS-1 cells. (A) Inhibition of PREG (1 μM) conversion by baboon CYP17A1, (B) inhibition of PROG (1 μM) conversion by baboon CYP21 and (C) inhibition of 17OH-PROG (1 μM) conversion by baboon CYP21. Substrates were assayed in the presence of Rooibos (4.3 mg/ml), aspalathin (10 μM) and nothofagin (10 μM) after 4 h. Individual steroids were compared by a one-way ANOVA, followed by a Dunnett's multiple comparison test. Results are expressed as the mean ± SEM (***$P < 0.001$, $n = 3$).

aspalathin and nothofagin (10 μM) were assayed on CYP17A1 activity with PREG (1 μM) as substrate in non-steroidogenic COS-1 cells. After 4 h, PREG conversion was significantly inhibited in the presence of Rooibos (48%), aspalathin (39%) and nothofagin (29%) (Fig. 2A). The magnitude of CYP17A1 inhibition could significantly alter the flux through the steroidogenic pathway. Inhibition of CYP17A1 would likely result in decreased concentrations of glucocorticoids, deoxycortisol and cortisol, and androgen precursors, A4 and DHEA. Ohno et al. [26] previously found that formononetin, genistein and diadzein, at a concentration 25 μM, were unable to inhibit CYP17A1, while 6-hydroxy-flavone inhibited CYP17A1 significantly at the same concentration. Aspalathin and nothofagin are therefore more potent inhibitors, as significant inhibition of PREG conversion is observed at a lower concentration of 10 μM ($P < 0.001$).

The effect of Rooibos (4.3 mg/ml), aspalathin and nothofagin, (10 μM) on the catalytic activity of CYP21 expressed in COS-1 cells with both PROG (1 μM) and 17OH-PROG (1 μM) as substrates was also investigated. After 4 h, significant inhibition of PROG conversion was observed in the presence of Rooibos (57%), aspalathin (32%) and nothofagin (41%) ($P < 0.001$) (Fig. 2B). Similar results were obtained when 17OH-PROG was added as substrate, with Rooibos (51%), aspalathin (34%) and nothofagin (43%) inhibiting 17OH-PROG conversion significantly ($P < 0.001$) (Fig. 2C). In both assays, the presence of Rooibos showed the greatest inhibition, followed by nothofagin and aspalathin. Inhibition of CYP21 would inhibit the production of DOC and deoxycortisol, which in turn will lead to a decrease in the glucocorticoids, CORT and cortisol, as well as the mineralocorticoid, ALDO. Ohno et al. [26] previously demonstrated that the flavonoids 6-hydroxyflavone, diadzein and genistein significantly inhibit CYP21 at concentrations ranging from 12.5 to 25 μM ($P < 0.01$). Subsequent studies confirmed that genistein and diadzein are competitive inhibitors of both 3βHSD and CYP21 [26,41].

While CYP21 is unique to the adrenal and essential for mineralocorticoid and glucocorticoid biosynthesis, the dual catalytic activity of CYP17A1 and its competition for substrates with 3βHSD, places this enzyme in a pivotal position in determining the steroidogenic output of the adrenal cortex. Although the hydroxylation of PREG and the subsequent lyase reaction of the intermediate, catalysed by CYP17A1, were not assayed as individual reactions, the data clearly shows an inhibitory effect on the end products of the Δ^5-pathway. Both PREG and 17OH-PREG are substrates for 3βHSD which catalyses the biosynthesis of CYP21 substrates. PROG and 17OH-PROG are thus channelled into the mineralocorticoid and glucocorticoid pathways by CYP21. The role of 3βHSD, however, cannot be ignored. Ohno et al. [41] showed significant inhibition of 3βHSD by genistein, diadzein and quercetin at a concentration of 10 μM ($P < 0.01$). We found significant inhibition of PREG conversion by 3βHSD using the same concentration of aspalathin ($P < 0.01$) and nothofagin ($P < 0.001$) (data not shown).

The inhibitory effects observed on the catalytic activities of these steroidogenic enzymes by Rooibos and the two dihydrochalcone compounds at the tested concentrations was not due to the cells being compromised. The metabolism of testosterone was not impaired in the presence of the Rooibos extract (4.3 mg/ml), aspalathin (10 μM) or nothofagin (10 μM), confirming that the observed effects resulted from inhibition of the catalytic activities of the steroidogenic enzymes (data not shown).

3.2. Steroid metabolism in H295R cells

The H295R cell line is the first established cell line capable of producing all the steroids from the three adrenal cortex zones which include the mineralocorticoids, glucocorticoids and adrenal androgen precursors. In addition, these cells also express 17βHSD and aromatase (CYP19), and are thus capable of producing testosterone, estrone and β-estradiol, making this cell line an excellent model system for studying adrenal steroidogenesis [3,42]. The inter-conversion of the inactive keto-forms of estrogens and androgens to their respective active hydroxyl-forms are catalysed by various 17βHSD enzymes which vary in substrate specificity and tissue expression [43,44]. The accurate detection and quantification of the intermediates and end products in the steroidogenic pathways, however, still remains a challenge. In the most recent study to date, three different LCMS methods were used to identify the steroid metabolites produced in H295R cells [3]. In order to quantify the steroid metabolites of interest, we developed a novel UPLC–MS/MS method enabling the separation and quantification of twenty-one steroid metabolites, using a single chromatographic separation

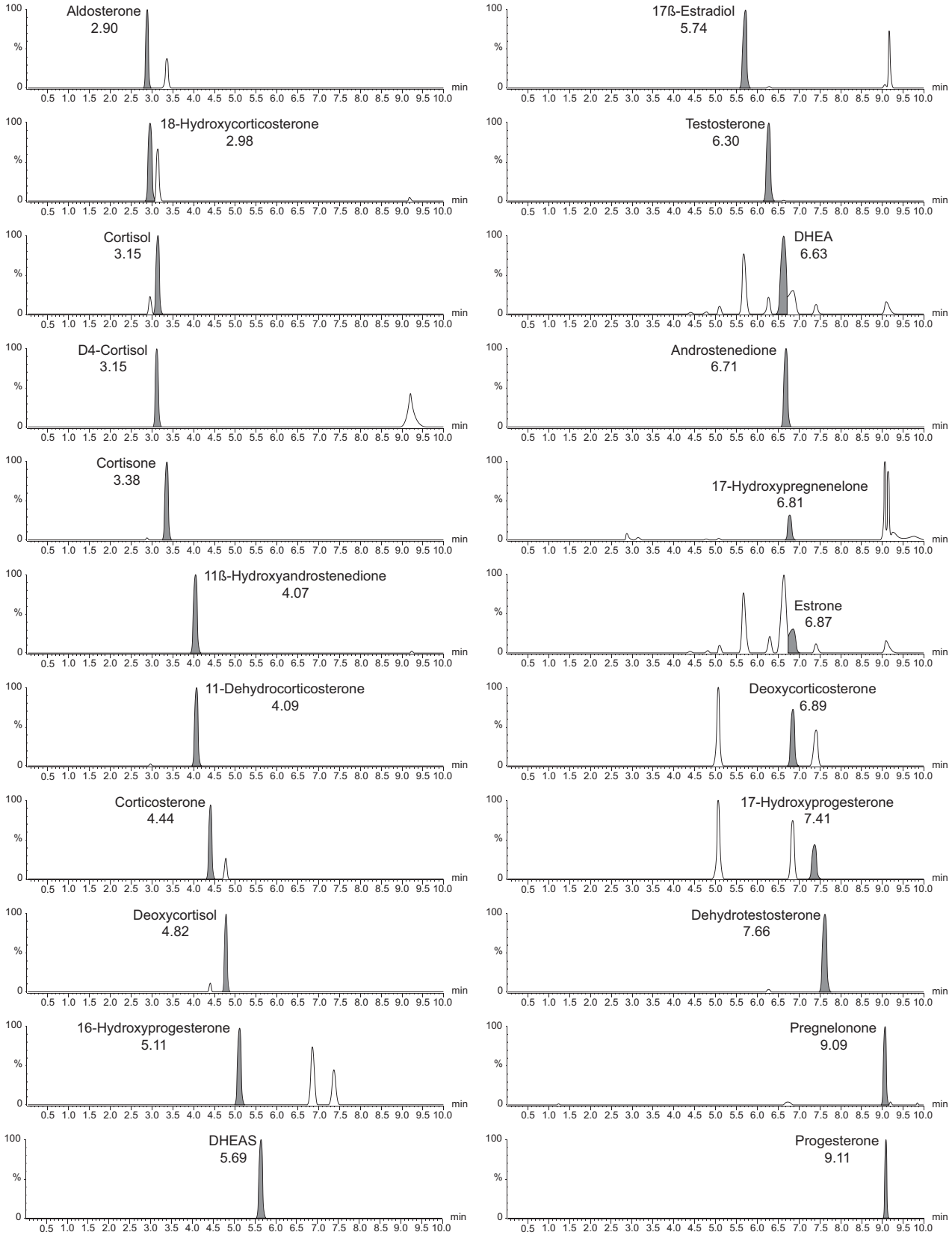

Fig. 3. UPLC–MS/MS chromatographic separation of 21 steroids. Chromatograms of steroid metabolites (5 μl each of a 2 μg/ml standard solution) and D4-cortisol are shown in multiple reaction monitoring (MRM) mode. Retention times of steroid metabolites (shaded peaks) are indicated on the chromatograms.

Table 1
Steroids produced in H295R cells. Cells were incubated in the absence and presence of forskolin (10 μM) for 48 h. Steroids were quantified by UPLC–MS/MS. Percentage of each steroid ± SEM was calculated by dividing the amount of individual steroid by the total steroid. Fold change ± SEM in response to forskolin treatment, was calculated from the changes in absolute values of individual steroids (not shown) compared to basal values. *P* values were calculated using a one-way ANOVA with a Dunnett's post test.

Steroid metabolite	Basal	+ Forskolin	
	% Total ± SEM	% Total ± SEM	Fold Change ± SEM
PREG	2.8 ± 1.7	0.7 ± 0.2	
PROG	1.2 ± 1.1	0.0 ± 0.0	
DOC	2.8 ± 0.2	3.0 ± 0.2	↑3.5 ± 1.1**
CORT	6.2 ± 0.5	18.6 ± 0.4	↑9.1 ± 2.0***
18OH-CORT	0.2 ± 0.0	0.9 ± 0.0	↑11.9 ± 2.8***
ALDO	0.1 ± 0.0	0.2 ± 0.0	↑14.1 ± 5.6***
11-DHC	0.3 ± 0.1	0.1 ± 0.0	
17OH-PREG	ND	ND	
17OH-PROG	1.5 ± 0.1	0.3 ± 0.0	
16OH-PROG	1.7 ± 0.0	0.6 ± 0.0	
Deoxycortisol	44.4 ± 1.4	24.6 ± 1.0	↑1.7 ± 0.5*
Cortisol	14.6 ± 1.0	34.2 ± 1.2	↑7.2 ± 1.9***
Cortisone	0.2 ± 0.1	0.1 ± 0.0	
DHEA	ND	ND	
DHEA-S	0.3 ± 0.2	0.0 ± 0.0	
A4	20.5 ± 0.3	12.5 ± 0.4	↑1.9 ± 0.6***
11BOH-A4	2.3 ± 0.1	3.5 ± 0.3	↑4.5 ± 0.7***
Testosterone	1.0 ± 0.1	0.5 ± 0.0	
DHT	ND	ND	
Estrone	ND	ND	
β-Estradiol	ND	ND	
Total steroid (nM)	3947	11141	↑2.8***

ND, not detectable ($n = 3$).
* $P < 0.01$.
** $P < 0.005$.
*** $P < 0.001$.

without prior derivatisation. The chromatographic separation of the steroid metabolites is shown in Fig. 3. The conditions and validations of this method are shown in supplementary material. This method enabled the detection and quantification of sixteen steroid metabolites from the medium of H295R cells after 48 h under basal conditions and in response to forskolin treatment. Forskolin was used as a general inducer of steroidogenesis since H295R cells are insensitive to ACTH [3]. Forskolin is a diterpene which mimics the effects of ACTH via the activation of adenyl cyclase (cAMP) pathways in adrenal cells [45]. While we did not detect DHEA, β-estradiol, estrone or 17OH-PREG, for which Xing et al. [3] detected low levels, we were however, able to detect and quantify ALDO, 11-DHC, cortisone, 16OH-PROG, 18OH-CORT and DHEA-S after 48 h under basal and forskolin-stimulated conditions in H295R cells.

Under basal conditions, the major steroid metabolites detected after 48 h were deoxycortisol (44.4%), A4 (20.5%), cortisol (14.6%) and CORT (6.2%) (Table 1). The higher concentration of deoxycortisol compared to that of cortisol is in accordance with data by Xing et al. [3] suggesting low expression of CYP11B1. However, in comparison, DOC levels are significantly lower than CORT levels, possibly due to metabolism of DOC by both CYP11B1 and CYP11B2. Forskolin stimulation significantly increased the production of ALDO (14.1-fold), 18OH-CORT (11.9-fold), CORT (9.1-fold), cortisol (7.2-fold), 11βOH-A4 (4.5-fold), DOC (3.5-fold), A4 (1.9-fold) and deoxycortisol (1.7-fold). After stimulation, cortisol (34.2%) was the most abundant steroid metabolite, followed by deoxycortisol (24.6%), CORT (18.6%) and A4 (12.5%). These results are in agreement with those obtained by Xing et al. [3] under similar experimental conditions. However, although cortisol and deoxycortisol were the most abundant metabolites under stimulated conditions, analyses of the steroid metabolites showed that there was a greater fold increase in the intermediates of the mineralocorticoid pathway, ultimately resulting in the 14-fold increase observed in ALDO levels.

In addition to altering the steroid profile of the cells, stimulation of forskolin resulted in a 2.8-fold increase in the total amount of steroid detected when compared to basal levels, with the total steroid concentration increasing from 4.0 μM to 11.1 μM (Table 1). It has previously been reported that the majority of agents that stimulate steroid hormone biosynthesis also upregulate steroid acute regulatory (StAR) protein expression. StAR is responsible for the transportation of cholesterol to the inner mitochondrial membrane where CYP11A1 metabolizes it to PREG [46]. King et al. [47] demonstrated that StAR mRNA levels increased by 260% after 24 h in the presence of 40 μM forskolin. Our data shows that the steroid flux through the glucocorticoid pathway is greater than the flux through the androgen precursor pathway under basal conditions, with even fewer metabolites being channelled through the mineralocorticoid pathway. Although the flux through the glucocorticoid pathway remains the highest upon forskolin stimulation, quantification of the intermediates shows that ALDO precursors are present in higher concentrations than that of the C19 metabolites.

Forskolin-stimulation also resulted in a significant increase in 11βOH-A4 production (0.09–0.39 μM) (Table 1). Rainey et al. [48] previously showed 11βOH-A4 to be one of the major steroids produced following forskolin stimulation. It is possible that 11βOH-A4 may be produced in the adrenal by either the lyase of cortisol or the hydroxylation of A4, with early studies favouring the hydroxylation of A4 [5,49]. *In vivo* and *in vitro* studies conducted by Axelrod et al. [50] showed A4 to be the major precursor of 11βOH-A4 in human and baboon adrenals. Conversion assays in human and baboon tissue showed that 1.3 and 1.4% ^{3}H-cortisol and 32 and 46% ^{3}H-A4 was incorporated into 11βOH-A4, respectively. Liakos et al. [51] later showed that transforming growth factor β1 (TGFβ1) inhibited the expression of CYP11B1 and CYP11B2 in H295R cells, resulting in a decrease in ALDO, cortisol and 11βOH-A4 production.

In this study we investigated the biosynthesis of 11βOH-A4 by H295R cells in the absence and presence of trilostane (10 μM), a selective inhibitor of 3βHSD. Neither A4 nor 11βOH-A4 was detected after 48 h in the presence of trilostane. The addition of A4 (1 μM) together with trilostane (10 μM) resulted in a significant increase in 11βOH-A4 levels, indicating that this metabolite is a product of the 11β-hydroxylation of A4 (Fig. 4). No 11βOH-A4 was detected following the addition of cortisol (1 μM) together with trilostane (10 μM). In addition, we assayed the conversion of cortisol in COS-1 expressing recombinant human CYP17A1, in the absence and presence of cytochrome b_5, which augments the lyase reaction, and did not detect 11βOH-A4 after 8 h by UPLC MS/MS analyses (data not shown). While the conversion of A4 to 11βOH-A4 by CYP11B1 has been demonstrated for non-primate species [52,53], to our knowledge, the 11β-hydroxylation of A4 by primate CYP11B1 has only been indirectly implied. We therefore assayed the metabolism of A4, deoxycortisol and DOC in COS-1 cells transiently co-transfected with baboon CYP11B1 and human adrenodoxin (ADX). The data clearly shows that CYP11B1 catalyses the conversion of A4 to 11β-OHA4 with negligible substrate remaining after 8 h (Fig. 5). In the assay in which both deoxycortisol (1 μM) and A4 (1 μM) were added together, the substrates did not appear to inhibit the conversion of each other after 8 h, although the rate of the conversions are yet to be determined. A conversion profile similar to that of deoxycortisol and A4 was obtained when DOC was added as substrate, with negligible substrate remaining after 8 h (results not shown). From our data it is clear that the production of 11βOH-A4 observed in the H295R cells could be attributed to the conversion of A4 by CYP11B1. Furthermore, the 4.5-fold increase observed in 11βOH-A4 levels under forskolin-stimulated conditions (Table 1) is therefore

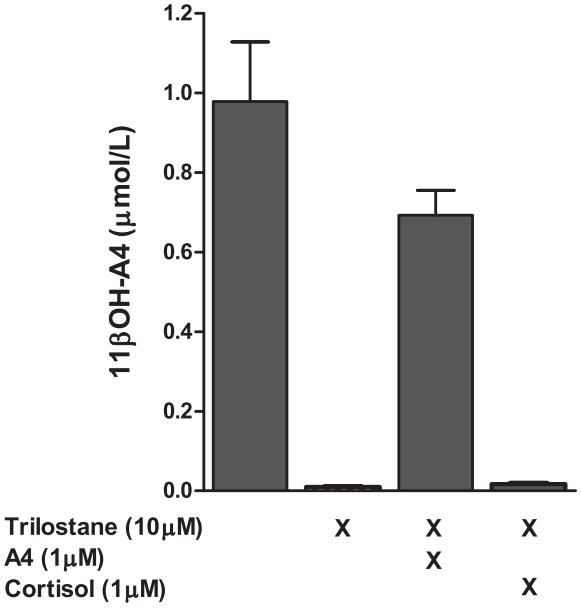

Fig. 4. Analysis of 11βOH-A4 production in H295R cells. Basal 11βOH-A4 production was assayed in the absence and presence of trilostane (10 μM) while the conversion of A4 (1 μM) and cortisol (1 μM) to 11βOH-A4 was assayed in the presence of trilostane after 48 h. Results are expressed as the mean ± SEM ($n = 3$).

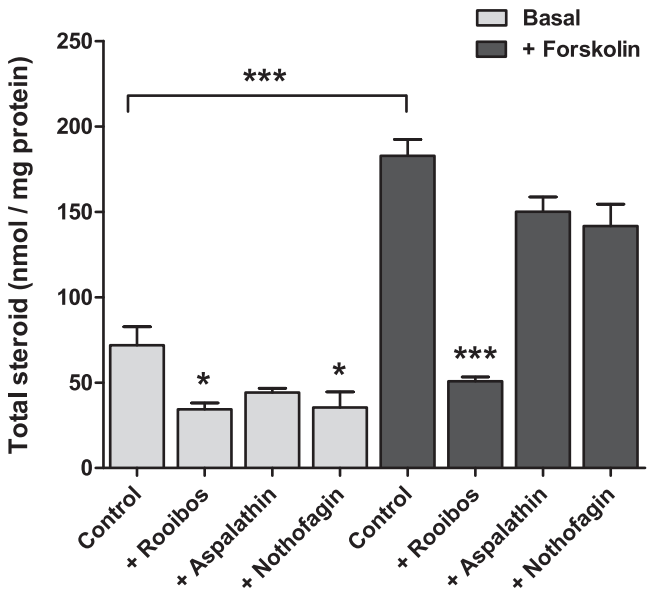

Fig. 6. Basal and forskolin-stimulated steroid production in H295R cells. Steroids were assayed with and without forskolin (10 μM) stimulation, in the presence of Rooibos (1 mg/ml), aspalathin (10 μM) and nothofagin (10 μM) after 48 h. Data was analysed by a one-way ANOVA, followed by a Dunnett's multiple comparison test. Results are expressed as the mean ± SEM ($^{*}P < 0.05$, $^{***}P < 0.001$, $n = 3$).

to be expected since forskolin upregulates not only CYP11B1 mRNA but also the hydroxylase activity in H295R cells [51,54]. In addition, the significant increase in A4 levels in the H295R cells suggests that CYP17A1 activity was stimulated in the presence of forskolin, as was previously reported by Rainey et al. [48], thereby increasing the precursor metabolite for the biosynthesis of 11βOH-A4.

3.2.1. *The influence of Rooibos and flavonoids on H295R metabolism*

Since the goal of this study was to investigate the effect of Rooibos on the outcome of adrenal steroidogenesis, and in particular, on glucocorticoid production, PREG metabolism was investigated in H295R cells. We assayed the effect of Rooibos (1 mg/ml), aspalathin (10 μM) and nothofagin (10 μM) on both basal and forskolin (10 μM) stimulated steroid metabolism. The total amount of steroid detected, decreased significantly under basal conditions in the presence of Rooibos (2-fold), aspalathin (1.6-fold) and nothofagin (2-fold) (Fig. 6). Similarly, under forskolin stimulated conditions, treatment with Rooibos, aspalathin and nothofagin decreased the total amount of steroid detected by 4.0-, 1.4- and 1.4-fold, respectively (Fig. 6). MTT assays conducted in the presence of 1 mg/ml Rooibos extract, 10 μM dihydrochalcone compounds and 10 μM forskolin showed that the inhibitory effects observed in the H295R assays were not due to a decrease in cell viability (data not shown). The greater inhibition observed by the addition of the Rooibos extract in the presence of forskolin may be attributed to compounds which could affect upstream processes. Genistein together with diadzein, have been shown to inhibit ACTH-stimulated cortisol production in cultured fetal and postnatal adrenal cortical cells at concentrations ranging from 0.4 to 40 μM. Both genistein and diadzein also inhibited cAMP-stimulated cortisol synthesis in H295 cells [27].

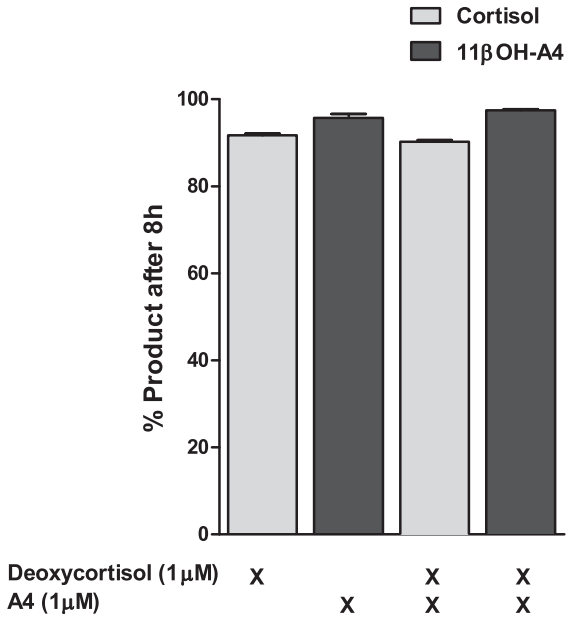

Fig. 5. Cortisol and 11βOH-A4 formation in transiently transfected COS-1 cells. Conversion of deoxycortisol and A4 was assayed after 8 h in COS-1 cells co-expressing baboon CYP11B1 and human ADX. Results are expressed as the mean ± SEM ($n = 3$).

It is possible that the decrease in steroid production by Rooibos may be due to the inhibition of either CYP11A1 or the transport of cholesterol to the inner mitochondrial membrane. We have shown that Rooibos, aspalathin and nothofagin significantly inhibit CYP17A1 and CYP21 in COS-1 cells and inhibition of these key enzymes would ultimately influence the steroid outcome of the adrenal gland. Under basal conditions, the addition of Rooibos and the dihydrochalcone compounds resulted in a small increase in the production of the mineralocorticoid, ALDO (Table 2). Significant reductions were, however, observed in the levels of A4 (Rooibos, 2.7-fold; aspalathin, 2.4-fold and nothofagin, 3.5-fold) and testosterone (Rooibos, 8.8-fold; aspalathin, 4.3-fold and nothofagin, 7.1-fold). The reduction of A4, and consequently of testosterone, is most likely due to inhibition of 3βHSD as human CYP17A1 does not readily catalyse the lyase of 17OH-PROG to A4. In addition, increased DHEA-S production (1.6-fold) was observed in the presence of Rooibos, which could also imply inhibition of 3βHSD. The increase in PREG and 17OH-PROG levels in the presence of

Table 2
Steroids produced in H295R cells under basal conditions in the presence of Rooibos and dihydrochalcones. Cells were incubated for 48 h with Rooibos (1 mg/ml), aspalathin (10 μM) and nothofagin (10 μM). Steroids were quantified by UPLC–MS/MS. Percentage of each steroid ± SEM was calculated by dividing the amount of individual steroid by the total steroid. Fold change ± SEM in response to Rooibos, aspalathin and nothofagin treatment, was calculated from the changes in absolute values of individual steroids (not shown) compared to basal values. *P* values were calculated using a one-way ANOVA with a Dunnett's post test.

Steroid metabolite	Basal	+ Rooibos		+ Aspalathin		+ Nothofagin	
	% Total ± SEM	% Total ± SEM	Fold change ± SEM	% Total ± SEM	Fold change ± SEM	% Total ± SEM	Fold change ± SEM
PREG	2.8 ± 1.7	5.4 ± 1.7		2.0 ± 0.4		0.9 ± 0.5	
PROG	1.2 ± 1.1	0.5 ± 0.1		0.2 ± 0.0		0.3 ± 0.1	
DOC	2.8 ± 0.2	2.8 ± 0.1		9.0 ± 0.8		11.6 ± 1.5	
CORT	6.2 ± 0.5	7.7 ± 0.4		10.4 ± 0.6		11.3 ± 1.0	
18OH-CORT	0.2 ± 0.0	0.7 ± 0.3		0.6 ± 0.1		0.7 ± 0.2	
ALDO	0.1 ± 0.0	0.3 ± 0.0		0.2 ± 0.0		0.2 ± 0.0	
11-DHC	0.3 ± 0.1	0.5 ± 0.0		0.2 ± 0.0		0.3 ± 0.0	
17OH-PREG	ND	ND		ND		ND	
17OH-PROG	1.5 ± 0.1	4.5 ± 0.1	↑1.6 ± 0.4*	0.7 ± 0.2	↓3.6 ± 1.1**	0.7 ± 0.2	↓4.8 ± 1.3**
16OH-PROG	1.7 ± 0.0	0.7 ± 0.1	↓5.0 ± 0.7***	1.2 ± 0.0	↓2.3 ± 0.4***	1.0 ± 0.1	↓3.6 ± 0.7***
Deoxycortisol	44.4 ± 1.4	27.2 ± 1.6	↓3.5 ± 0.6**	44.3 ± 0.3		42.4 ± 0.6	↓2.3 ± 0.6*
Cortisol	14.6 ± 1.0	23.8 ± 2.0		14.8 ± 0.1		15.3 ± 1.4	
Cortisone	0.2 ± 0.1	0.3 ± 0.0		0.1 ± 0.0		0.1 ± 0.0	
DHEA	ND	ND		ND		ND	
DHEA-S	0.3 ± 0.2	0.5 ± 0.1	↑1.6 ± 0.7**	0.1 ± 0.0		0.1 ± 0.0	
A4	20.5 ± 0.3	16.3 ± 0.4	↓2.7 ± 0.6**	13.9 ± 0.9	↓2.4 ± 0.4**	13.0 ± 0.4	↓3.5 ± 0.8***
11BOH-A4	2.3 ± 0.1	8.7 ± 0.4	↑1.9 ± 0.4**	1.8 ± 0.1		1.7 ± 0.2	↓3.0 ± 0.6*
Testosterone	1.0 ± 0.1	0.3 ± 0.0	↓8.8 ± 2.0***	0.4 ± 0.1	↓4.3 ± 1.0***	0.3 ± 0.0	↓7.1 ± 2.6***
DHT	ND	ND		ND		ND	
Estrone	ND	ND		ND		ND	
β-Estradiol	ND	ND		ND		ND	
Total steroid (nM)	3947	1890	↓2.1*	2429	↓1.6	1952	↓2.0*

ND, not detectable ($n = 3$).
* $P < 0.01$.
** $P < 0.005$.
*** $P < 0.001$.

Rooibos under stimulated conditions, suggest the inhibition of 3βHSD, CYP17A1 and CYP21. 16OH-PROG, a dead end product, was also reduced in the presence of Rooibos, suggesting either a reduction of PROG levels due to 3βHSD inhibition or inhibition of CYP17A1. Under basal conditions, cortisol and cortisone levels were not significantly affected, while basal deoxycortisol levels decreased significantly in the presence of Rooibos (3.5-fold) and nothofagin (2.3-fold), possibly due to inhibition of CYP21. Under basal conditions, both aspalathin and nothofagin significantly reduced 17OH-PROG, 16OH-PROG, A4 and testosterone levels, confirming our findings in COS-1 cells.

The effect of Rooibos was notably more pronounced during forskolin treatment than under basal conditions. Rooibos significantly decreased the levels of the glucocorticoids, cortisol (4.9-fold), cortisone (5.2-fold) and CORT (5.2-fold), as well as the glucocorticoid precursors, deoxycortisol (5.1-fold) and DOC (3.4-fold) under stimulated conditions (Table 3). Aspalathin and nothofagin also decreased cortisol levels by 1.3-fold and 1.7-fold, respectively. While no effect was observed in ALDO levels in the presence of Rooibos, there was a significant reduction of the precursor metabolites. Androgen precursor production was significantly affected by Rooibos, aspalathin and nothofagin under stimulated conditions. A4, testosterone and 11βOH-A4 levels decreased, while DHEA-S levels remained unchanged. A4 is the primary precursor of testosterone and A4 produced in the adrenal contributes to testosterone biosynthesis either by secretion or peripheral conversion of the precursor [3]. A reduction in circulating levels of A4 and testosterone by Rooibos may have clinical implications. However, in a study conducted in human test subjects, an increase in testosterone levels after Rooibos consumption for 6 weeks was observed. In addition, DHEA-S levels in men remained unchanged, while a considerable increase in DHEA-S levels were detected in women (results unpublished).

To date the function of 11βOH-A4 in human adrenal steroidogenesis remains uncertain. In a recent study investigating the inhibition of estrogen biosynthesis in gonadal masculinization of rainbow trout, Vizziano et al. [55] showed that masculinization was induced by the inhibition of CYP19 in genetic all-female populations following the administration of 11βOH-A4. The production of this steroid metabolite, exhibiting weak androgenic activity [56] and inhibitory effects on CYP19, which catalyses the biosynthesis of estrone and β-estradiol, could have implications in the output of adrenal steroidogenesis. In this study we found that 11βOH-A4 comprised only 2.3% of the total steroids assayed while A4 comprised 20.5% under basal conditions (Table 1). Upon forskolin stimulation, the production of 11βOH-A4 significantly increased (4.5-fold), comprising 3.5% of the total steroids. Although Xing et al. [3] reported a 5-fold increase in 11βOH-A4 levels upon forskolin stimulation, they found that in adult adrenal cells under basal conditions, A4 and 11βOH-A4 levels comprised 4- and 9% of the total steroids respectively. However, a 17-fold increase was observed in 11βOH-A4 levels following ACTH stimulation. Nevertheless, 11βOH-A4 appears to be stimulated by Rooibos, with a significant increase (1.9-fold) being detected under basal conditions. The production of this metabolite is significantly inhibited by nothofagin (3-fold) only, demonstrating the complex nature of the extract. Following forskolin stimulation, 11βOH-A4 production was inhibited significantly by Rooibos and dihydrochalcone compounds, with a concomitant decrease in A4 levels.

The data shows that while aspalathin and nothofagin are of the most abundant flavonoids in Rooibos [57] and demonstrated similar inhibitory effects, they did not in all cases reflect the effects brought about by Rooibos, clearly indicating that other compounds contribute to the effects of Rooibos on adrenal steroidogenesis. The observation that Rooibos is able to significantly reduce glucocorticoid production during forskolin treatment, but not under basal conditions, may have therapeutic applications for Rooibos in the management of stress-related conditions. In addition, Rooibos resulted in a much greater inhibition (4-fold) in the total amount of steroids detected under forskolin stimulated conditions

Table 3
Steroids produced in forskolin-stimulated H295R cells in the presence of Rooibos and dihydrochalcones. Cells were incubated for 48 h with forskolin (10 μM), Rooibos (1 mg/ml), aspalathin (10 μM) and nothofagin (10 μM). Steroids were quantified by UPLC–MS/MS. Percentage of each steroid ± SEM was calculated by dividing the amount of individual steroid by the total steroid. Fold change ± SEM in response to Rooibos, aspalathin and nothofagin treatment, was calculated from the changes in absolute values of individual steroids (not shown) compared to forskolin values. P values were calculated using a one-way ANOVA with a Dunnett's post test.

Steroid metabolite	Forskolin	Forskolin + Rooibos		Forskolin + aspalathin		Forskolin + nothofagin	
	% Total ± SEM	% Total ± SEM	Fold change ± SEM	% Total ± SEM	Fold change ± SEM	% Total ± SEM	Fold change ± SEM
PREG	0.7 ± 0.2	8.7 ± 1.1	↑4.2 ± 1.6*	0.6 ± 0.2		1.1 ± 0.1	
PROG	0.0 ± 0.0	0.9 ± 0.1		0.0 ± 0.0		0.1 ± 0.0	
DOC	3.0 ± 0.2	3.7 ± 0.2	↓3.4 ± 0.6**	3.2 ± 0.1		5.8 ± 0.3	
CORT	18.6 ± 0.4	14.1 ± 0.4	↓5.2 ± 0.3***	20.8 ± 0.8		21.6 ± 0.4	
18OH-CORT	0.9 ± 0.0	1.8 ± 0.0	↓2.1 ± 0.3***	1.0 ± 0.0		1.1 ± 0.1	
ALDO	0.2 ± 0.0	0.6 ± 0.0		0.3 ± 0.0		0.4 ± 0.0	
11-DHC	0.1 ± 0.0	0.3 ± 0.0		0.1 ± 0.0		0.1 ± 0.0	
17OH-PREG	ND	ND		ND		ND	
17OH-PROG	0.3 ± 0.0	6.3 ± 0.1	↑4.9 ± 0.2***	0.3 ± 0.0		0.2 ± 0.1	
16OH-PROG	0.6 ± 0.0	0.8 ± 0.1	↓3.1 ± 0.4***	0.7 ± 0.0		0.7 ± 0.0	
Deoxycortisol	24.6 ± 1.0	19.4 ± 0.1	↓5.1 ± 0.6***	23.7 ± 1.8		28.5 ± 0.4	
Cortisol	34.2 ± 1.2	27.8 ± 1.2	↓4.9 ± 0.2***	34.7 ± 1.3	↓1.3 ± 0.1***	28.8 ± 0.6	↓1.7 ± 0.2***
Cortisone	0.1 ± 0.0	0.1 ± 0.0	↓5.2 ± 1.2**	0.1 ± 0.0		0.1 ± 0.0	↓2.6 ± 0.4*
DHEA	ND	ND		ND		ND	
DHEA-S		0.1 ± 0.0					
A4	12.5 ± 0.4	9.3 ± 0.5	↓5.5 ± 0.7***	10.3 ± 0.2	↓1.7 ± 0.2***	9.1 ± 0.3	↓2.0 ± 0.2***
11BOH-A4	3.5 ± 0.3	6.0 ± 0.5	↓2.3 ± 0.0***	3.8 ± 0.2	↓1.3 ± 0.1**	2.1 ± 0.1	↓2.5 ± 0.4***
Testosterone	0.5 ± 0.0	0.1 ± 0.0	↓22.8 ± 5.2***	0.4 ± 0.0	↓1.7 ± 0.2***	0.3 ± 0.0	↓2.4 ± 0.2***
DHT	ND	ND		ND		ND	
Estrone	ND	ND		ND		ND	
β-Estradiol	ND	ND		ND		ND	
Total steroid (nM)	11,141	2788	↓4.0***	8248	↓1.4*	7786	↓1.4*

ND, not detectable ($n = 3$).
* $P < 0.01$.
** $P < 0.005$.
*** $P < 0.001$.

compared to the 2-fold inhibition observed under basal conditions. A recent study by Beltrán-Debóna et al. [58] showed negligible effects of Rooibos in animals with no metabolic disturbance, while significant reductions in serum cholesterol, triglyceride and free fatty acid concentrations were observed in hyperlipemic mice.

Although the addition of Rooibos led to a reduction in overall biosynthesis of steroid hormones, the data shows that under basal conditions the steroid metabolites in the mineralocorticoid pathway are not significantly affected by the extract or by the two dihydrochalcones, while a decrease in the flux is evident upon forskolin stimulation in the presence of the extract only. While cortisol and cortisone levels are unchanged, the glucocorticoid intermediates are decreased under basal conditions by Rooibos and both compounds. However, upon stimulation with forskolin the inhibitory effect of Rooibos on the flux through the pathway, which results in significant inhibition of cortisol and cortisone production, is greater than that of aspalathin and nothofagin. Similarly, it would appear that a reduction of the flux through the androgen precursor pathway is more pronounced in the case of forskolin stimulation in the presence of the Rooibos extract.

In conclusion, our data indicates that Rooibos and the flavonoid compounds, aspalathin and nothofagin, interact with, and inhibit the steroidogenic enzymes influencing the shunt of metabolites in the mineralocorticoid, glucocorticoid and androgen pathways. The UPLC–MS/MS method developed for this study can be applied in the analysis and accurate quantification of adrenal steroid metabolites and the steroid flux through these precursor pathways. In addition, this method would be applicable in the assessment of inhibition profiles of compounds impacting steroidogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2011.11.003.

References

[1] P. Swart, A.C. Swart, M.R. Waterman, R.W. Estabrook, J.I. Mason, Progesterone 16a hydroxylase activity is catalyzed by human cytochrome P450 17a-hydroxylase, J. Clin. Endocrinol. Metab. 77 (1993) 98–102.
[2] C.R. Jefcoate, Cytochrome P-450 enzymes in sterol biosynthesis and metabolism, in: P.R. Ortiz de Montellano (Ed.), Cytochrome P450: Structure, Mechanism and Biochemistry, Plenum Press, New York, 1986, pp. 387–428.
[3] Y. Xing, M.A. Edwards, C. Ahlem, M. Kennedy, A. Cohen, C.E. Gomez-Sanchez, W.E. Rainey, The effects of ACTH on steroid metabolomic profiles in human adrenal cells, J. Endocrinol. 209 (2011) 327–335.
[4] G. Albertin, C. Tortorella, L.K. Malendowicz, F. Aragona, G. Neri, G.G. Nussdorfer, Human adrenal cortex and aldosterone secreting adenomas express both 11beta-hydroxysteroid dehydrogenase type 1 and type 2 genes, Int. J. Mol. Med. 9 (2002) 495–498.
[5] G.L. Cohn, P.J. Mulrow, Androgen release and synthesis *in vitro* by human adult adrenal glands, J. Clin. Invest. 42 (1963) 64–78.
[6] N.A.T.M. Huizenga, J.W. Koper, P. De Lange, H.A.P. Pols, R.P. Stolk, D.E. Grobbee, F.H. De Jong, S.W.J. Lamberts, Interperson variability but intraperson stability of baseline plasma cortisol concentrations, and its relation to feedback sensitivity of the hypothalamo–pituitary–adrenal axis to a low dose of dexamethasone in elderly individuals, J. Clin. Endocrinol. Metab. 83 (1998) 47–54.
[7] G.P. Chrousos, P.W. Gold, Editorial: a healthy body in a healthy mind—and *vice versa*—the damaging power of "uncontrollable" stress, J. Clin. Endocrinol. Metab. 83 (1998) 1842–1845.
[8] J.W. Tomlinson, P.M. Stewart, 11β-Hydroxysteroid dehydrogenase type 1 as a therapeutic target in the metabolic syndrome, Drug Discov. Today: Ther. Strateg. 2 (2005) 93–96.
[9] G.P. Vinson, Angiotensin II, corticosteroids, type II diabetes and the metabolic syndrome, Med. Hypotheses 68 (2007) 1200–1207.
[10] T.B VanItallie, Stress: a risk factor for serious illness, Metabolism 51 (2002) 40–45.

[11] G.W. Liddle, D. Island, E.M. Lance, A.P. Harris, Alterations of adrenal steroid patterns in man resulting from treatment with a chemical inhibitor of 11 beta-hydroxylation, J. Clin. Endocrinol. Metab. 18 (1958) 906.
[12] D.S. Loose, P.B. Kan, M.A. Hirst, R.A. Marcus, D. Feldman, Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P450-dependent enzymes, J. Clin. Invest. 71 (1983) 1495–1499.
[13] R.J. Santen, H. Van den Bossche, J. Symoens, J. Brugmans, R. DeCoster, Site of action of low dose ketoconazole on androgen biosynthesis in men, J. Clin. Endocrinol. Metab. 57 (1983) 732–736.
[14] Y. Nishizato, S. Imai, M. Yabuli, H. Kido, S. Komuro, Development of relevant assay system to identify steroidogenic enzyme inhibitors, Toxicol. In Vitro 24 (2010) 677–685.
[15] E. Joubert, W.C.A. Gelderblom, A. Louw, D. de Beer, South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides* – a review, J. Ethnopharmacol. 119 (2008) 376–412.
[16] D.L. McKay, J.B. Blumberg, A review of the bioactivity of South African herbal teas: Rooibos (*Aspalathus linearis*) and Honeybush (*Cyclopia intermedia*), Phytother. Res. 21 (2007) 1–16.
[17] R.J. Nijveldt, E. van Noord, D.E.C. van Hoorn, P.G. Boelens, K. van Norren, P.A.M. van Leeuwen, Flavonoids: a review of probable mechanisms of action and potential applications, Am. J. Nutr. 74 (2001) 418–425.
[18] H. Wei, R. Bowen, Q. Cai, S. Barnes, Y. Wang, Antioxidant and antipromotional effects of the soybean isoflavone genistein, Proc. Soc. Exp. Biol. Med. 208 (1995) 124–130.
[19] E.A. Kirk, P. Sutherland, S.A. Wang, A. Chait, R. LeBoeuf, Dietary isoflavones reduce plasma cholesterol and atherosclerosis in C57BL/6 mice but not LDL receptor-deficient mice, J. Nutr. 128 (1998) 954–959.
[20] B.H. Arjmandi, L. Alekel, B.W. Hollis, D. Amin, M. Stacewiez-Sapuntzakis, P. Guo, S.C. Kukreja, Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis, J. Nutr. 126 (1996) 161–167.
[21] D. Ingram, K. Sanders, M. Kolybaba, D. Lopez, Case–control study of phytoestrogens and breast cancer, Lancet 350 (1997) 990–994.
[22] M.S. Anthony, T.B. Clarkson, C.L. Hughes, T.M. Morgan, G.L. Burke, Soybean isoflavones improve cardiovascular risk factors without affecting the reproductive system of peripubertal rhesus monkeys, J. Nutr. 126 (1996) 43–50.
[23] C.F. Skibola, M.T. Smith, Potential health impacts of excessive flavonoid intake, Free Radic. Biol. Med. 29 (2000) 375–383.
[24] L. You, M. Sar, E.J. Bartolucci, B.S. McIntyre, R. Sriperumbudur, Modulation of mammary gland development in prepubertal male rats exposed to genistein and methoxychlor, Toxicol. Sci. 66 (2002) 216–225.
[25] B. Halliwell, Dietary polyphenols: good, bad, or indifferent for your health? Cardiovasc. Res. 73 (2007) 341–347.
[26] S. Ohno, S. Shinoda, S. Toyoshima, H. Nakazawa, T. Makino, S. Nakajin, Effects of flavonoid phytochemicals on cortisol production and on activities of steroidogenic enzymes in human adrenocortical H295R cells, J. Steroid Biochem. Mol. Biol. 80 (2002) 355–363.
[27] S. Mesiano, S.L. Katz, J.Y. Lee, R.B. Jaffe, Phytoestrogens alter adrenocortical function: genistein and daidzein suppress glucocorticoid and stimulate androgen production by cultured adrenal cortical cells, J. Clin. Endocrinol. Metab. 84 (1999) 2443–2448.
[28] Å. Ohlsson, E. Ullerås, N. Cedergreen, A. Oskarsson, Mixture effects of dietary flavonoids on steroid hormone synthesis in the human adrenocortical H295R cell line, Food Chem. Toxicol. 48 (2010) 3194–3200.
[29] T. Yoshikawa, Y. Naito, H. Oyamada, S. Ueda, T. Tanigawa, T. Takemura, S. Sugino, M. Kondo, Scavenging effects of *Aspalathus linearis* (rooibos tea) on active oxygen species, Exp. Med. Biol. 264 (1990) 171–174.
[30] J.L. Marnewick, E. Joubert, P. Swart, F. Van der Westhuizen, W.C.A. Gelderblom, Modulation of hepatic drug metabolizing enzymes and oxidative status by green and black (*Camellia sinensis*), rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*) teas in rats, J. Agric. Food Chem. 51 (2003) 8113–8119.
[31] J.L. Marnewick, W.C.A. Gelderblom, E. Joubert, An investigation on the antimutagenic properties of South African herbal teas, Mut. Res. 471 (2000) 157–166.
[32] P.W. Snijman, S. Swanevelder, E. Joubert, I.R. Green, W.C.A. Gelderblom, The antimutagenic activity of the major flavonoids of rooibos (*Aspalathus linearis*)—some dose–response effects on mutagen activation–flavonoid interactions, Mut. Res. 613 (2007) 111–123.
[33] R. Hendricks, E.J. Pool, The in vitro effects of Rooibos and black tea on immune pathways, J. Immunoassay Immunochem. 31 (2010) 169–180.
[34] J.L. Marnewick, F. Rautenbach, I. Venter, H. Neethling, D.M. Blackhurst, P. Wolmarans, M. Macharia, Effects of rooibos (*Aspalathus linearis*) on oxidative stress and biochemical parameters in adults at risk for cardiovascular disease, J. Ethnopharm. 133 (2011) 46–52.
[35] I.A.-L. Persson, K. Persson, S. Hägg, R.G.G. Andersson, Effects of green tea, black tea and Rooibos tea on angiotensin converting enzyme and nitric oxide in healthy volunteers, Public Health Nutr. 13 (2010) 730–737.
[36] A. Kawano, H. Nakamurab, S. Hatab, M. Minakawaa, Y. Miuraa, K. Yagasakia, Hypoglycemic effect of aspalathin, a rooibos tea component from *Aspalathus linearis*, in type 2 diabetic model db/db mice, Phytomedicine 16 (2009) 437–443.
[37] E. Joubert, P. Winterton, T.J. Britz, W.C.A. Gelderblom, Antioxidant and pro-oxidant activities of aqueous extracts and crude polyphenolic fractions of rooibos (*Aspalathus linearis*), J. Agric. Food Chem. 53 (2005) 10260–10267.
[38] W.E. Hillis, T. Inoue, The polyphenols of Nothofagus species. II: the heartwood of *Nothofagus fusca*, Phytochemistry 6 (1967) 59–67.
[39] J.M. Trant, Isolation and characterization of the cDNA encoding the channel catfish (*Ictalurus punctatus*) form of cytochrome P450arom, Gen. Comp. Endocrinol. 95 (1994) 155–168.
[40] K.-H. Storbeck, N.W. Kolara, M. Stander, A.C. Swart, D. Prevoo, P. Swart, The development of an ultra performance liquid chromatography coupled atmospheric pressure chemical ionization mass spectrometry assay for seven adrenal steroids, Anal. Biochem. 372 (2008) 11–20.
[41] S. Ohno, N. Matsumoto, M. Watanabe, S. Nakajin, Flavonoid inhibition of overexpressed human 3β-hydroxysteroid dehydrogenase type II, J. Steroid Biochem. Mol. Biol. 88 (2004) 175–182.
[42] W.E. Rainey, I.M. Bird, J.I. Mason, The NCI-H295 cell line: a pluripotent model for human adrenocortical studies, Mol. Cell. Endocrinol. 100 (1994) 45–50.
[43] G. Moeller, J. Adamski, Integrated view on 17betahydroxysteroid dehydrogenases, Mol. Cell. Endocrinol. 301 (2009) 7–19.
[44] W.L. Miller, R.J. Auchus, The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders, Endocr. Rev. 32 (2011) 81–151.
[45] K.B. Seamon, W. Padgett, J.W. Daly, Forskolin: unique diterpene activator of adenylate cyclase in membranes and intact cells, Proc. Natl. Acad. Sci. U.S.A. 78 (1981) 3363–3367.
[46] D.M. Stocco, StAR protein and the regulation of steroid hormone biosynthesis, Annu. Rev. Physiol. 63 (2001) 193–213.
[47] S.R. King, P.R. Manna, T. Ishii, P.J. Syapin, S.D. Ginsberg, K. Wilson, L.P. Walsh, K.L. Parker, D.M. Stocco, R.G. Smith, D.J. Lamb, An essential component in steroid synthesis, the steroidogenic acute regulatory protein, is expressed in discrete regions of the brain, J. Neurosci. 22 (2002) 10613–10620.
[48] W.E. Rainey, I.M. Bird, C. Sawetawan, N.A. Hanley, J.L. McCarthy, E.A. McGee, R. Wester, J.I. Mason, Regulation of human adrenal carcinoma cell (NCI-H295) production of C19 steroids, J. Clin. Endocrinol. Metab. 77 (1993) 731–737.
[49] C.P. Lantos, H. Traikov, M.K. Birmingham, In vitro biosynthesis of steroids by adrenals of macaca mulatta, Steroids 11 (1968) 733–748.
[50] L.R. Axelrod, D.C. Kraemer, J. Burdett, J.W. Goldzieher, Biosynthesis of 11β-hydroxyandrostenedione by human and baboon adrenals, Acta Endocrinol. 72 (1973) 545–550.
[51] P. Liakos, D. Lenz, R. Bernhardt, J.-J. Feige, G. Defaye, Transforming growth factor β1 inhibits aldosterone and cortisol production in the human adrenocortical cell line NCI-H295R through inhibition of *CYP11B1* and *CYP11B2* expression, J. Endocrinol. 176 (2003) 69–82.
[52] H. Sato, N. Ashida, K. Suhara, E. Itagaki, S. Takemor, M. Katagir, Properties of an adrenal cytochrome P-450 (P-$450_{11\beta}$) for the hydroxylations of corticosteroids, Arch. Biochem. Biophys. 190 (1978) 307–314.
[53] H.E. Bülow, R. Bernhardt, Analyses of the CYP11B gene family in the guinea pig suggest the existence of a primordial CYP11B gene with aldosterone synthase activity, Eur. J. Biochem. 269 (2002) 3838–3846.
[54] K. Denner, W.E. Rainey, V. Pezzib, I.M. Bird, R. Bernhardt, J.M. Mathis, Differential regulation of 11β -hydroxylase and aldosterone synthase in human adrenocortical H295R cells, Mol. Cell. Endocrinol. 121 (1996) 87–91.
[55] D. Vizziano, D. Baron, G. Randuineau, S. Mahe, C. Cauty, Y. Guiguen, Rainbow trout gonadal masculinization induced by inhibition of estrogen synthesis is more physiological than masculinization induced by androgen supplementation, Biol. Reprod. 78 (2008) 939–946.
[56] B. Bélanger, J. Fiet, A. Bélanger, Effects of adrenocorticotropin on adrenal and plasma 11β-hydroxyandrostenedione in the guinea pig and determination of its relative androgen potency, Steroids 58 (1993) 29–34.
[57] A. Stalmach, W. Mullen, M. Pecorari, M. Serafini, A. Crozier, Bioavailability of *C* linked dihydrochalcone and flavanone glucosides in humans following ingestion of unfermented and fermented Rooibos teas, J. Agric. Food Chem. 57 (2009) 7104–7111.
[58] R. Beltrán-Debóna, A. Rulla, F. Rodríguez-Sanabriaa, I. Iswaldib, M. Herranz-López, G. Aragonèsa, J. Campsa, C. Alonso-Villaverded, J.A. Menéndeze, V. Micolc, A. Segura-Carreterob, J. Jove, Continuous administration of polyphenols from aqueous Rooibos (*Aspalathus linearis*) extract ameliorates dietary induced metabolic disturbances in hyperlipidemic mice, Phytomedicine 18 (2011) 414–424.