The influence of Rooibos (Aspalathus linearis) on adrenal steroidogenic P450 enzymes

by

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Figure 2.1. Rooibos growing in the Cederberg region of South Africa [www.rooibosltd.co.za].

2.3 Processing

Rooibos made a successful transition from a wild to a cultivated crop and is one of the few economically important fynbos plants. Rooibos seeds are sown from February to March and seedlings are replanted during July and August in the Southern hemisphere winter period. Only the top half of the plant is cut, with about 30 cm left above the soil (figure 2.2). It is vital to make sure that healthy leaves remain on the plant after harvesting or else the plant will not survive. In addition during the second year of harvesting, the plant should not be cut below the height harvested the previous year. It is best to harvest slightly higher each year for new growth can come from the previous season's wood. Rooibos is harvested once a year between December and April [www.asnapp.org.za].

As previously mentioned rooibos was harvested with axes by the Khoi-Khoi tribe —bruised, fermented and dried in the sun [Wilson, 2005]. Today rooibos is still processed in a similar

manner, although the process has been mechanized. Processing is carried out in two different ways to produce the two types of tea — after harvesting, the leaves and stems are either bruised and fermented to produce the traditional fermented rooibos or immediately dried to prevent oxidation, producing unfermented rooibos. Before packaging, the dry product is sterilized by steam pasteurization [Standley *et al*., 2001].

Rooibos tea is referred to as fermented tea since the polyphenols are oxidized during the fermentation process, resulting in the changing of the color of the leaves from green to red. The resulting tea is a rich orange/red color and it is this distinctive color that led to the African name *rooibos*, which means "red bush" [McKay and Blumberg, 2007; Erickson, 2003]. The unfermented rooibos, also called green rooibos, contains higher levels of polyphenol antioxidants than its fermented counterpart [Joubert, 1996].

Figure 2.2. Mature rooibos plant [reproduced from Agribussiness in sustainable natural African plant products, Crop profile, www.asnapp.org.za].

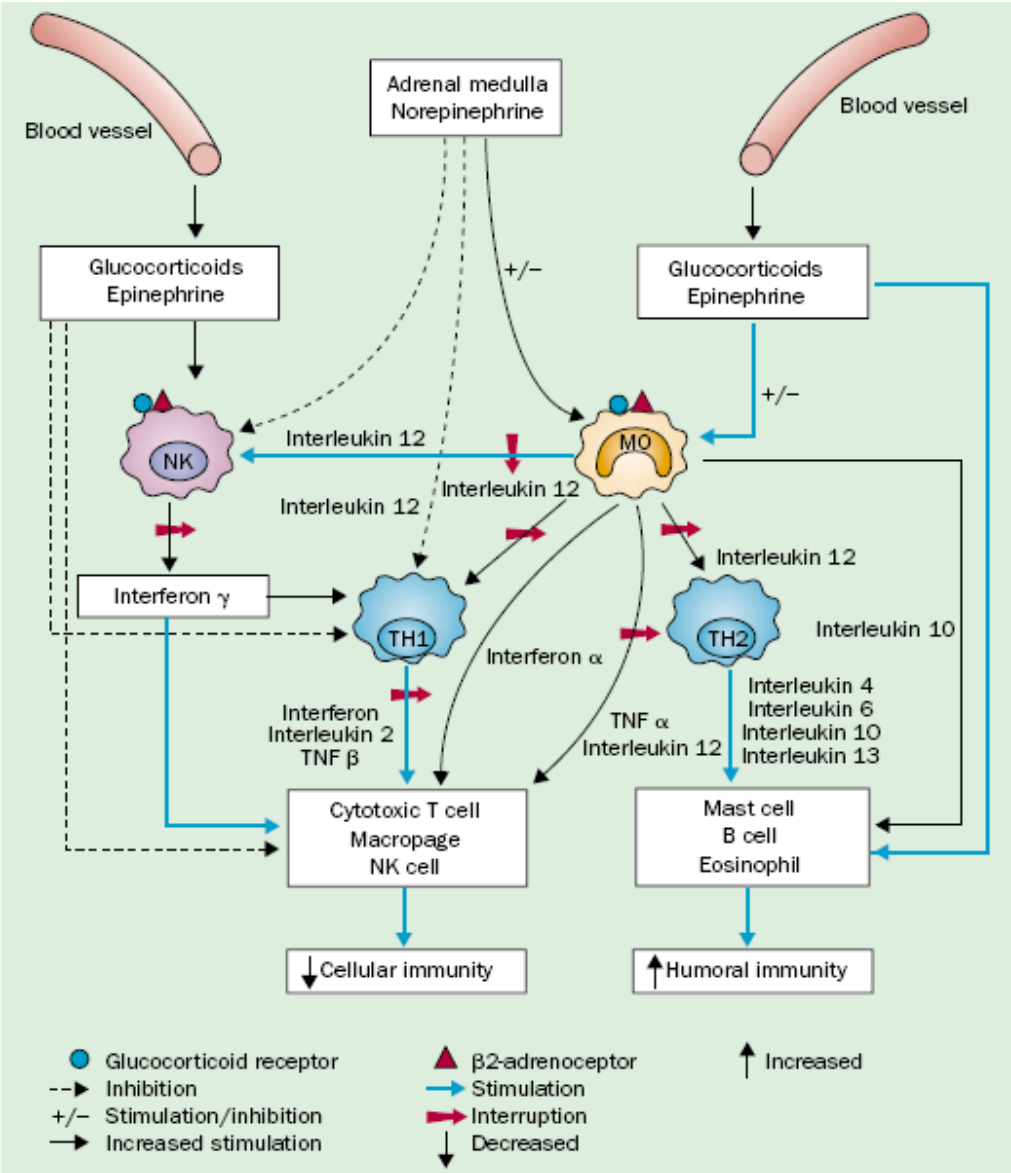

Figure 3.5. Systemic effects of the stress hormones glucocorticoids and catecholamines secreted by the adrenal gland and norepinephrine released by sympathetic nerve terminals. Solid lines indicated stimulation and dashed lines indicated inhibition. NK, natural-killer cells; MO, macrophage; Th1, T-helper lymphocyte type 1 cells; Th2, T-helper lymphocyte type 2 cells; TNF, tumour necrosis factor [Vissoci Reichea *et al.*, 2004].

In this study four extracts, methanol and aqueous extracts of fermented and unfermented rooibos, were prepared and the inhibition of PROG binding to microsomal P450 enzymes subsequently assayed. The extracts inhibited the binding of PROG with the unfermented extracts exhibiting a greater inhibitory effect that the fermented extracts (figure 5.4). The inhibition of PROG binding by the unfermented rooibos methanol and aqueous extracts was greater than 35 % ($P<0.001$).

The lowest inhibition was obtained with the fermented rooibos aqueous extract which inhibited the binding of PROG by 13 % ($P<0.05$) with the fermented rooibos methanol extract inhibiting PROG binding by 27 % ($P<0.01$). The inhibition of PROG binding by the unfermented rooibos aqueous extract was significantly higher ($P<0.001$) than the inhibition of fermented rooibos methanol and aqueous extracts. The final concentration of the unfermented rooibos aqueous and methanol extracts were 0.74 mg/ml and 0.72 mg/ml respectively. The final concentration of the fermented rooibos methanol and aqueous extracts were both 0.73 mg/ml.

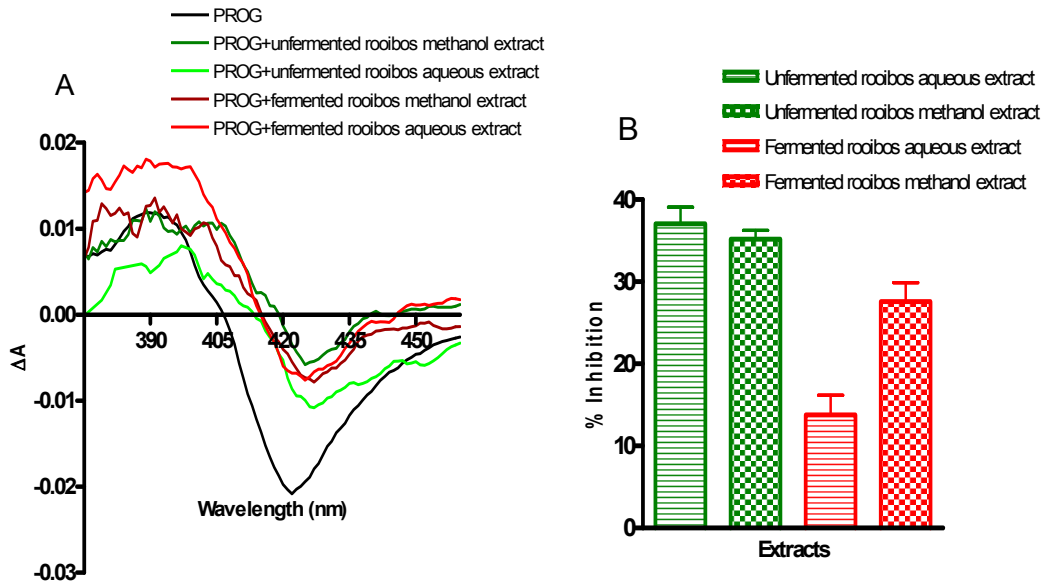

Figure 5.3. A) Inhibition of PROG-induced type I difference spectra in ovine adrenal microsomes ([P450] = 0.8 µM) and B) percentage inhibition of PROG binding in the presence of rooibos extracts. [PROG] = 16 µM; [unfermented rooibos aqueous extract] = 0.74 mg/ml; [unfermented rooibos methanol extract] = 0.72 mg/ml; [fermented aqueous extract] = 0.73 mg/ml, and [fermented rooibos methanol extract] = 0.73 mg/ml. are presented as the mean, error bars represent SEM and n=3. Inhibition of PROG by the extracts was analysed by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

The inhibition of aqueous extracts of the unfermented rooibos on the binding of PROG and 17OH-PROG to adrenal microsomal P450 enzymes was investigated using varying concentrations of substrate (2 – 32 µM) and extracts (final concentrations, 0.184 – 0.736 mg/ml). The inhibition of PROG (2µM) and 17OH-PROG (2µM) binding to microsomal P450 enzymes in the presence of 2µl of the aqueous extract of unfermented rooibos (final concentration of 0.736 mg/ml) was 29.4 % and 10 %, respectively (figure 5.4).

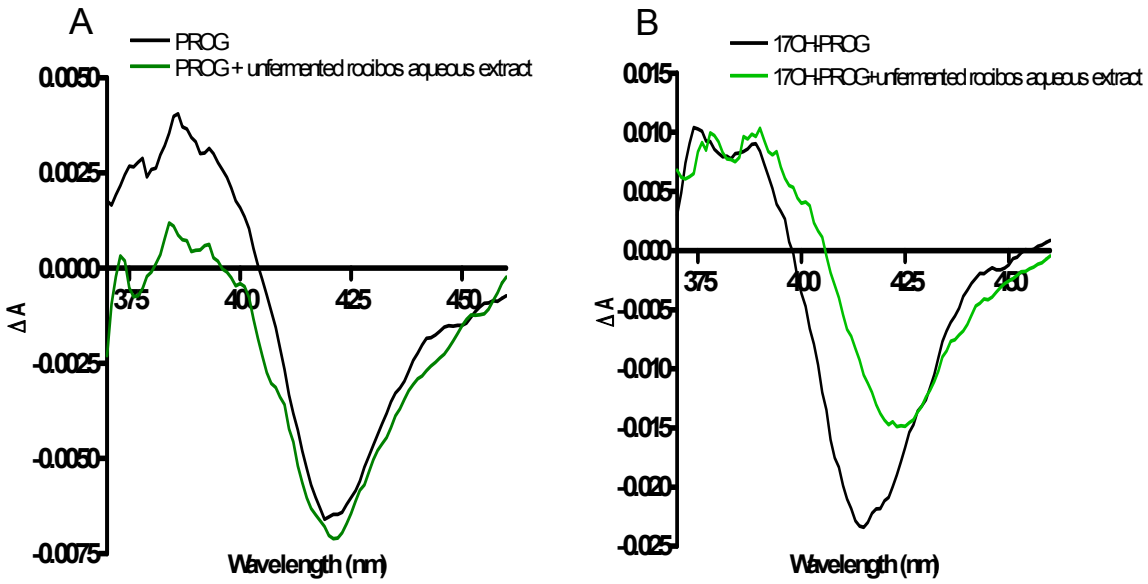

Figure 5.4. Inhibition of steroid-induced type I difference spectra in ovine adrenal microsomes. [P450] = 0.8 µM; A) [PROG] = 2 µM; [unfermented rooibos aqueous extract] = 0.184 mg/ml and B) [17OH-PROG] = 2 µM;[unfermented aqueous extract] = 0.184 mg/ml.

The data obtained from the assays determining the degree of inhibition of PROG and 17OH-PROG at various substrate and extract concentrations generated hyperbolic saturation binding curves (figure 5.5). It can be seen from the data that the maximum substrate binding capacity (B_{max}) of the microsomal enzymes differs markedly. This may be due to the fact that the microsomal preparation contains both CYP17 and CYP 21 enzymes and while PROG and 17OH-PROG are both natural substrates for CYP21, CYP17 catalyses only the conversion of PROG showing negligible catalytic activity towards 17OH-PROG.

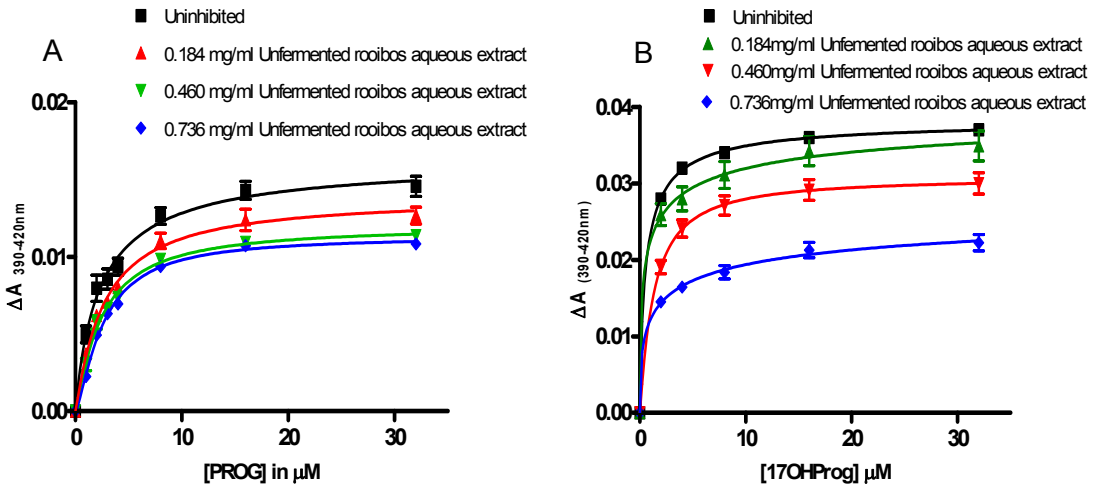

Figure 5.5. Hyperbolic saturation binding curves of die inhibitory effect of unfermented rooibos aqueous extract on A) PROG and B) 17OH-PROG binding to microsomal P450 enzymes. [P450] = 0.8 µM; [Prog] and [17OH-PROG] = 2 – 32 µM; [unfermented rooibos aqueous extracts] = 0.184 – 0.736 mg/ml. Results are presented as the mean, error bars represent SEM and n=3.

Double reciprocal plots, a convenient linearization of Michaelis-Menten data, were plotted to visualize the inhibition data. It appears that in the presence of unfermented rooibos aqueous extracts, the binding of PROG to the P450 enzymes is being competitively inhibited as only the K_i (binding inhibition constant) changes (figure 5.6). It is possible for compounds in the extracts to bind to the enzymes or to the enzyme-substrate complex and thus mixed inhibition of binding was also analysed. The secondary plots are shown in figure 5.7. Analysis of the data revealed K'_i (apparent inhibition constant for the binding of E + I) and K_i (inhibition constant for the binding of ES + I), to be 1.3 mg/ml (figure 5.7 A and B). The binding of PROG to the microsomal enzymes appears to be competitively inhibited in the presence of the concentrations assayed for unfermented rooibos aqueous extracts.

The double reciprocal plot of 17OH-PROG binding shows that 17OH-PROG is being competitively inhibited at low concentrations of extract with uncompetitive or mixed inhibition occurring at higher concentrations (figure 5.8). Analysis of the data (figure 5.9 A and B) revealed that the K_i and the K'_i values, 0.08 and 1.9 mg/ml do indicate mixed inhibition of the binding of 17OH-PROG to the microsomal enzymes by unfermented rooibos aqueous extracts.

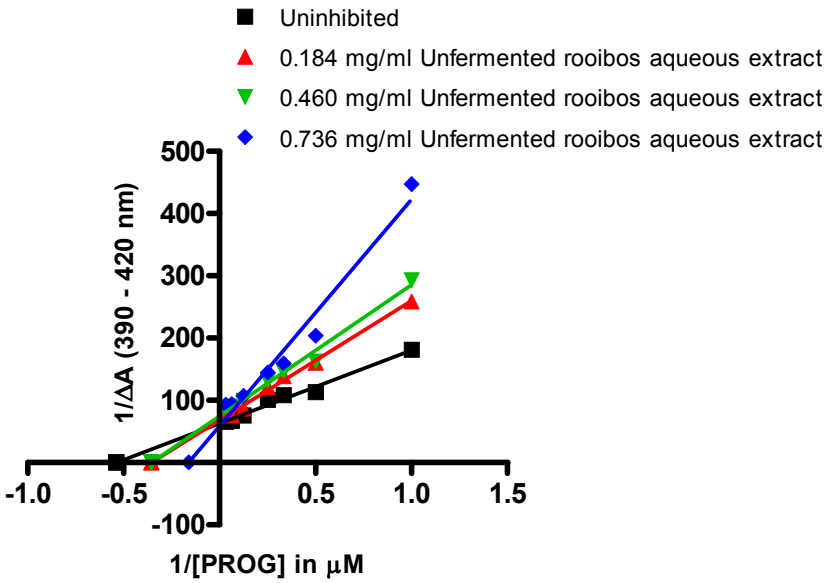

Figure 5.6. Double reciprocal plot of the inhibitory effect of unfermented rooibos aqueous extract on PROG binding. [P450] = 0.8 µM; [PROG] = 2 – 32 µM; unfermented rooibos aqueous extract = [0.184 – 0.736 mg/ml]. Results are presented as the mean of 3 independent experiments.

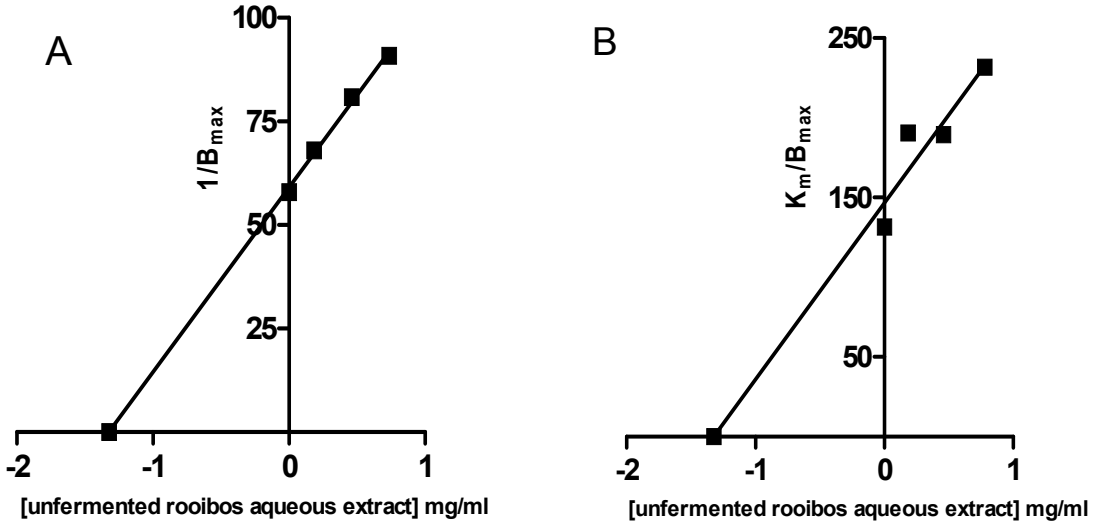

Figure 5.7. Secondary plots for inhibition of PROG binding to microsomal P450 enzymes in the presence of unfermented rooibos aqueous extract A) plot of $1/B^{app}_{max}$ against extract concentration and B) plot of K_m/B^{app}_{max}. [P450] = 0.8 µM; [17OH-PROG] = 2 – 32 µM; unfermented rooibos aqueous extract [0.184 – 0.736 mg/ml]. Results are presented as the mean of 3 independent experiments.

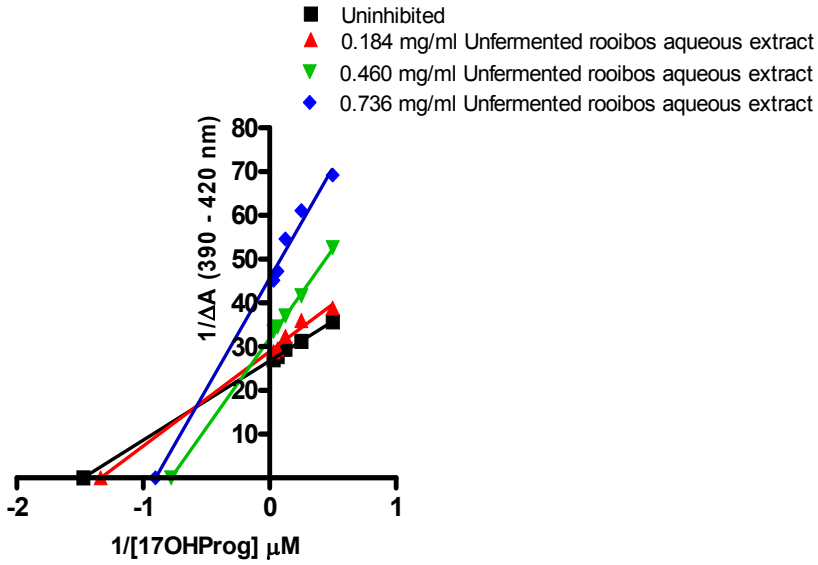

Figure 5.8. Double reciprocal plot of the inhibitory effect of unfermented rooibos aqueous extract on 17OH-PROG binding. [P450] = 0.8 µM; [17OH-PROG] = 2 – 32 µM; unfermented rooibos aqueous extract = [0.184 – 0.736 mg/ml]. Results are presented as the mean of 3 independent experiments.

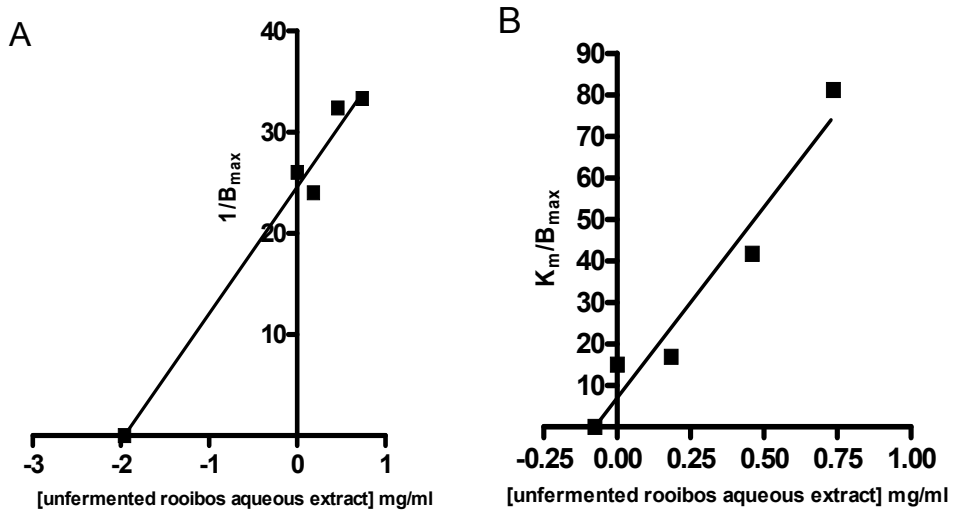

Figure 5.9. Secondary plots for mixed inhibition of 17OH-PROG binding to microsomal P450 enzymes in the presence of unfermented rooibos aqueous extract A) plot of $1/B^{app}_{max}$ against extract concentration and B) plot of K_m/ B^{app}_{max}. [P450] = 0.8 µM; [0.184 – 0.736 mg/ml]. Results are presented as the mean of 3 independent experiments.

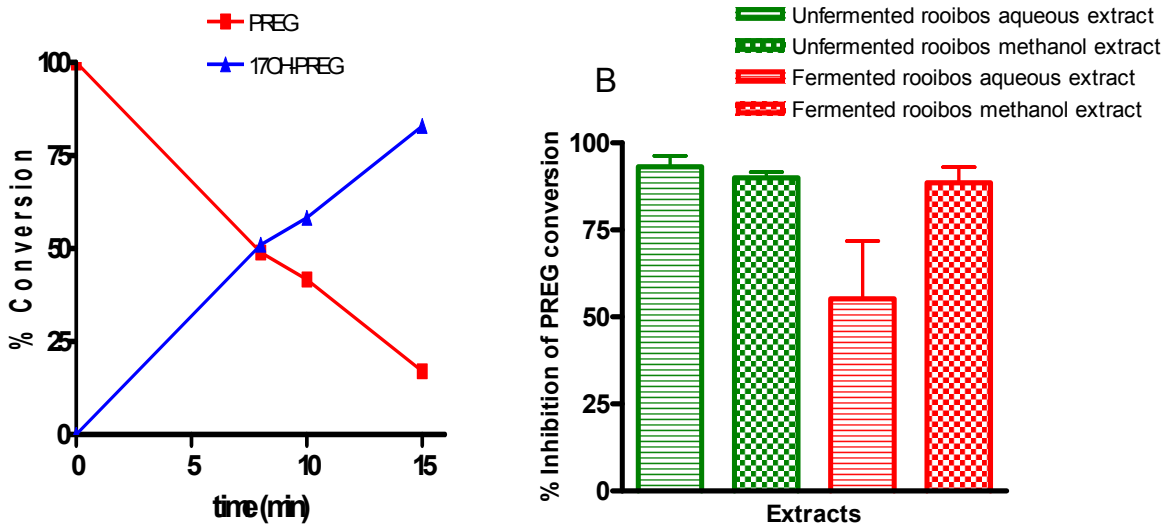

Figure 5.10. Metabolism of PREG (10 µM) by ovine adrenal microsomal P450 enzymes ([P450] = 0.35 µM) after initiation with NADPH (1 mM). (A) Percentage conversion in the absence of rooibos extracts and (B) percentage inhibition of PREG (after 15 min) in the presence of rooibos extracts. Unfermented rooibos: [aqueous extract] = 2.3 mg/ml, methanol extract = 2.3 mg/ml); fermented rooibos: [aqueous extract] = 2.3 mg/ml and methanol extract = 2.3 mg/ml. Results are presented as the mean, error bars represent SEM and n=3. Inhibition of PREG metabolism by the extracts was analysed by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

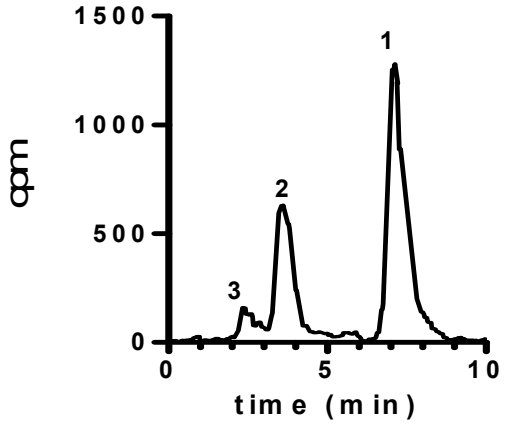

Figure 5.11. HPLC analysis of PREG (10 µM) metabolism by ovine adrenal microsomes at 10 min. Peak 1, PREG (7.5 min); peak 2, 17OH-PREG (3.5 min); and peak 3, DHEA (2.2 min).

In adrenal microsomes, the conversion of PROG to its subsequent metabolites is catalyzed by both CYP17 and CYP21. The conversion of PROG to 17OH-PROG is catalyzed by CYP17

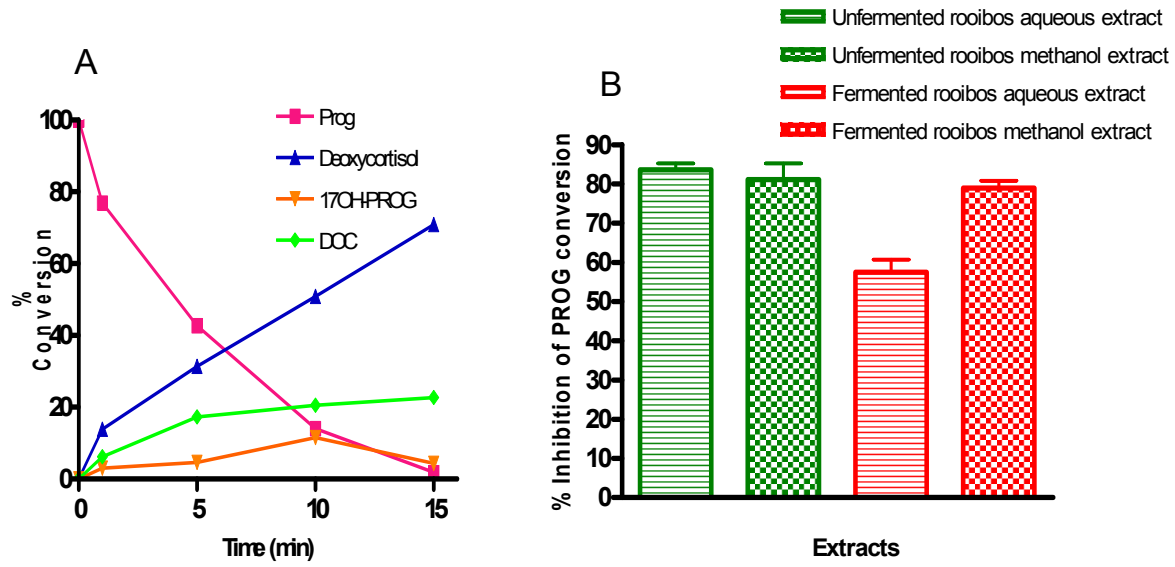

Figure 5.12. Metabolism of PROG (10 µM) by CYP17 and CYP21 ([P450] = 0.35 µM) in ovine adrenal microsomes after initiation with NADPH (1 mM) to deoxycortisol, DOC and 17OH-PROG metabolites. (A) Percentage conversion in the absence of rooibos extracts and (B) percentage inhibition of PROG conversion (after 15 min) in the presence of rooibos extracts. Unfermented rooibos: [aqueous extract] = 2.275 mg/ml, methanol extract = 2.25 mg/ml); fermented rooibos: [aqueous extract] = 2.3 mg/ml and methanol extract = 2.3 mg/ml. Results are presented as the mean, error bars represent SEM and n=3. Inhibition of PROG metabolism by the extracts was analysed by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

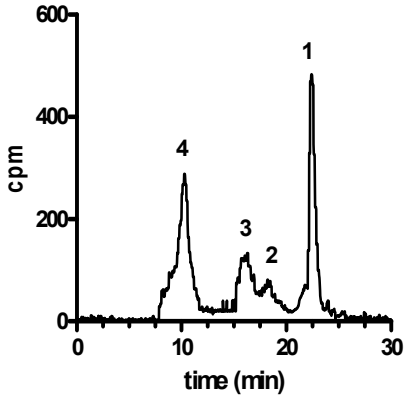

Figure 5 .13. HPLC analysis of PROG (10 µM) metabolism by ovine adrenal microsomes at 10 min. Peak 1, PROG (23 min); peak 2, 17-OH-PROG (19.5 min); peak 3, DOC (16.8 min); and peak 4, deoxycortisol (10 min).

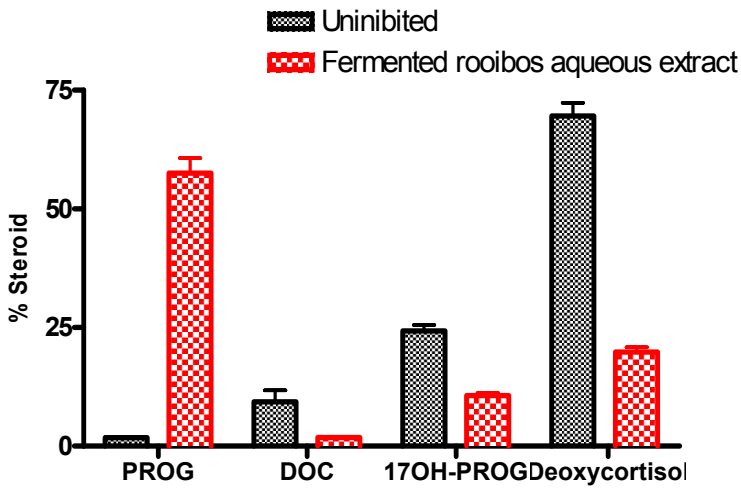

Figure 5.14. Conversion of PROG (10 µM) to steroid metabolites DOC, 17OH-PROG and deoxycortisol in the absence and presence of fermented rooibos aqueous extract in ovine adrenal microsomes [P450] = 0.35 µM) after initiation with NADPH (1 mM); [Fermented rooibos aqueous extract] = 2.3 mg/ml. Results are presented as the mean, error bars represent SEM and n=3.

5.3.4 COS1 conversion assay

The influence of rooibos extracts on the catalytic activity of the individual enzymes was investigated by expressing CYP17 and CYP21 in COS1 cells.

Baboon CYP17 expressed in COS1 cells, in the absence of rooibos extracts, converted 97 % PROG to 17OH-PROG and negligible amounts 4-androstenedione. In the presence of the unfermented rooibos methanol extract, the conversion of PROG to its metabolites was significantly inhibited ($P<0.001$) with 41 % being converted. PROG conversion in the presence of the fermented rooibos methanol extract was inhibited to a lesser degree, 39 % ($P<0.01$). Results are illustrated in figure 5.15 A.

In COS1 cells expressing baboon CYP21 70 % PROG was converted to DOC in the absence of rooibos extracts. Both unfermented and fermented rooibos methanol extracts inhibited the conversion of PROG to DOC significantly ($P<0.001$), with 30% DOC being formed (figure 5.15 B).

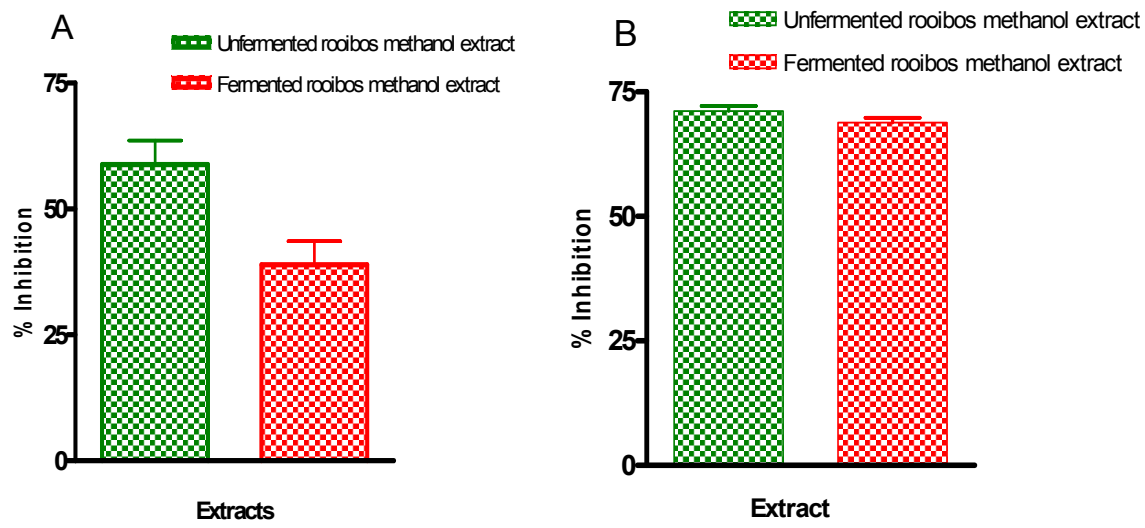

Figure 5.15. Percentage inhibition of PROG (1 µM) metabolism in COS1 cells expressing A) baboon CYP17 and B) baboon CYP 21 in the presence of methanol extracts of unfermented and fermented rooibos. [Unfermented rooibos methanol extract] = 4.5 mg/ml; [fermented rooibos methanol extract] = 4.6 mg/ml. Results are presented as the mean, error bars represent SEM and n=3. Inhibition of PROG metabolism by the extracts was analysed by a one-way ANOVA, followed by Bonferroni's multiple comparison test.

Steroid metabolites were analysed using LC-MS. The elution profile of PROG and DOC standards is shown in figure 5.16. Stock solutions of PROG and DOC (2 mg/ml ethanol) were used to prepare a series of standards, (2, 20, 200, and 2000 ng/ml) in methanol from the stock solutions. A standard curve was generated for each steroid with concentrations ranging from 10 to 1000 ng/ml. The calibration curves were linear over these concentration ranges (r^2 better than 0.99).

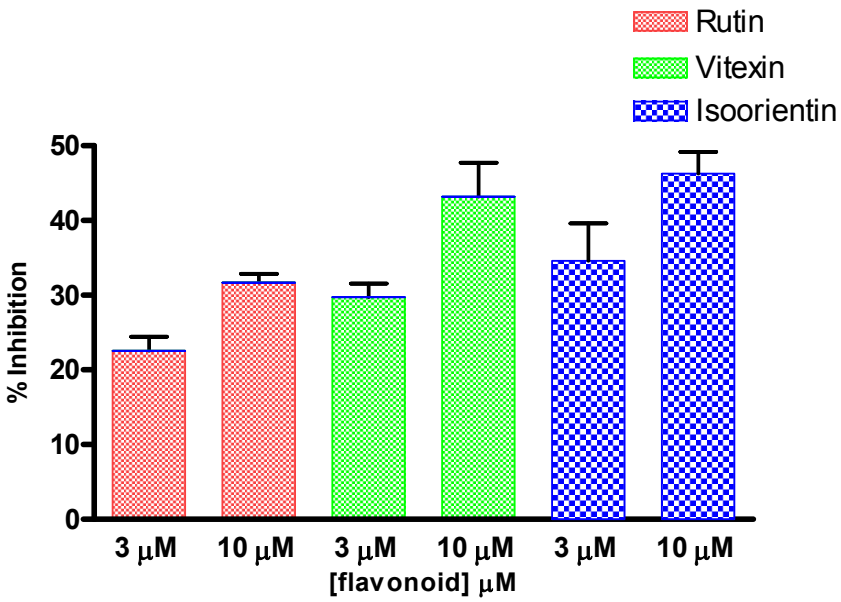

Figure 5.22. Percentage inhibition of PROG (1 μM) metabolism in COS1 cells expressing baboon CYP21 in the presence of rooibos flavonoid compounds rutin, vitexin and orientin. [Rutin] = 3 and 10 μM; [vitexin] = 3 and 10 μM; [orientin] = 3 and 10 μM. Results are presented as the mean, error bars represent SEM and n=3. Inhibition of PROG metabolism by the compounds was analysed by a one-way ANOVA, followed by Bonferroni's multiple comparison test ($p < 0.001$).

5.4 Discussion

The bioactivity of unfermented and fermented rooibos extracts was investigated by determining the influence on P450 enzymes, CYP17 and CYP21. These enzymes are at a branch point in adrenal steroidogenesis and their inhibition would affect the outcome of adrenal steroidogenesis. The binding of endogenous substrate to the enzymes and subsequent substrate conversion were thus assayed. Once activity had been established, the methanol extract of unfermented rooibos was fractionated and individual fractions were assayed to determine the inhibition of the binding of substrates to CYP17 and CYP21 as well as the catalytic activity of these enzymes. The influence of flavonoid compounds present in the fractions on the catalytic activity of CYP21 was subsequently assayed.