

Interactions between Phytochemical Components of *Sutherlandia frutescens* and the Antiretroviral, Atazanavir *In Vitro*: Implications for Absorption and Metabolism

Adrienne C. Müller¹, Srinivas Patnala^{1,2}, Olena Kis³, Reina Bendayan³, Isadore Kanfer¹

¹ Division of Pharmaceutics, Faculty of Pharmacy, Rhodes University, Grahamstown, South Africa.

² Current address: Faculty of Pharmacy, KLE University, Belgaum, India.

³ Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Canada.

Received, November 09, 2011; Revised, November 30, 2011; Accepted, March 2, 2012; Published, March 5, 2012.

ABSTRACT – Purpose. African traditional medicinal plants, such as *Sutherlandia frutescens* have the potential to interact pharmacokinetically with the protease inhibitor class of antiretrovirals, thereby impacting on their safety and efficacy. The effects of extracts and phytochemical components of *Sutherlandia frutescens*, on the *in vitro* absorption and metabolism of the protease inhibitor, atazanavir were thus investigated. **Methods.** Aqueous and methanolic extracts of *Sutherlandia frutescens* were prepared by freeze-drying of hot water and methanol decoctions of *Sutherlandia frutescens* plant material respectively, whilst crude triterpenoid glycoside and flavonol glycoside fractions were isolated by solvent extraction and subsequent column chromatography. Atazanavir was quantitated in the absence or presence of these compounds as well as commercially available purported constituents of *Sutherlandia frutescens*, namely, L-canavanine, L-GABA and D-pinitol, after a one hour co-incubation in Caco-2 cell monolayers and human liver microsomes. **Results.** The triterpenoid and flavonol glycoside fractions were found to be present in the aqueous and methanolic extracts of *Sutherlandia frutescens* and were shown to contain the sutherlandiosides and sutherlandins known to be present in *Sutherlandia frutescens*. The aqueous extract and D-pinitol significantly reduced atazanavir accumulation by Caco-2 cells, implying a decrease in atazanavir absorption, whilst the opposite was true for the triterpenoid glycoside fraction. Both the aqueous and methanolic extracts inhibited atazanavir metabolism in human liver microsomes, whilst enhanced atazanavir metabolism was exhibited by the triterpenoid glycoside fraction. **Conclusions.** The extracts and phytochemical components of *Sutherlandia frutescens* influenced the accumulation of atazanavir by Caco-2 cells and also affected ATV metabolism in human liver microsomes. These interactions may have important implications on the absorption and metabolism and thus the overall oral bioavailability of atazanavir.

This article is open to **POST-PUBLICATION REVIEW**. Registered readers (see "For Readers") may **comment** by clicking on ABSTRACT on the issue's contents page.

INTRODUCTION

Several surveys detail the use of African traditional medicines (ATMs) by HIV/AIDS patients in South Africa (1-4). Moreover, there have also been anecdotal reports on the use of the indigenous Southern African plants, *Hypoxis hemerocallidea* (African potato) and *Sutherlandia frutescens* (SF) for the treatment of HIV/AIDS (5), which was even recommended at a consultative meeting of representatives of the Ministries of Health of the member states of the Southern African Development Community (SADC), in 2003 (6).

The efflux transporter, P-glycoprotein (P-gp) and CYP450 isoenzymes are expressed in the small intestine and the liver and act on several drug substrates, thereby limiting their oral absorption and facilitating their metabolism (7-9).

Through modulation of CYP isoenzymes and/or P-gp activities, many *in vitro* studies (10-16) allude to potentially significant effects of ATMs on the absorption and metabolism of ARVs. This in turn suggests that concomitant oral administration of the ATMs with ARVs may increase the risk for clinical adverse reactions, such as toxicity as a result of increased absorption and/or reduced metabolism, and lack of efficacy consequent to decreased absorption and/or enhanced metabolism.

The protease inhibitor (PI), atazanavir (ATV) has less severe adverse effects in comparison to lopinavir, therefore it has been included in the South African clinical guidelines for the management of HIV/AIDS in adults and

Corresponding Author: I. Kanfer, Faculty of Pharmacy, Rhodes University, Grahamstown, South Africa. E-mail: I.Kanfer@ru.ac.za

adolescents (17), as an alternative to lopinavir in patients who experience intolerable gastro-intestinal problems, hyperlipidaemia and/or hyperglycaemia. Like other PIs, ATV is a substrate of P-gp (18,19) as well as CYP3A4 and CYP3A5 (20) and may thus be susceptible to pharmacokinetic (PK) interactions with agents able to modulate the activities of this transporter and family of CYP enzymes.

SF is a Southern African plant which has a long history of use in the practice of traditional medicine, particularly by the *isiZulu*, *isiSotho*, *isiXhosa* and Khoi-San people of South Africa (21,22). Aqueous decoctions prepared from this medicinal plant have been used to treat a variety of symptoms and illnesses, such as pain and fever, wounds, stomach ailments, stress and anxiety, cancer and diabetes (21,23). With the advent of the HIV/AIDS crisis in SA, SF has become popular as an “adaptogenic tonic”, which anecdotal reports claim to be useful for alleviating the cachexia (muscle-wasting) (21,22) commonly observed in patients at the end stages of the disease. Pharmaceutical dosage forms, such as tablets and capsules which contain powdered SF leaves are currently readily available in pharmacies, health shops and even online. Triterpenoid glycosides known as Sutherlandiosides A – D (24) and flavonol glycosides, kaempferol and quercetin glycosides known as sutherlandins A – D (25) have been isolated from SF plant material and are known to be present in the plant (26). Moreover, SF is also purported to contain the non-protein amino acid, L-canavanine, the inhibitory neurotransmitter, L-GABA and the sugar, D-pinitol (22). Aqueous and methanolic extracts (100 mg/ml) of SF demonstrated $\geq 50\%$ CYP3A4 inhibitory activity, but $\leq 50\%$ inhibition of P-gp activity (12).

The potential impact of SF on the safety and efficacy of ATV was investigated by determining the effects of aqueous and methanolic extracts as well as phytochemical constituents known or purported to be present in SF, on the accumulation of ATV by Caco-2 cells, an established *in vitro* model of human intestinal epithelium and the metabolism of ATV in human liver microsomes.

MATERIALS AND METHODS

Chemicals and Reagents

Caco-2 cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA) and pooled human liver microsomes were purchased from BD Biosciences (Woburn,

Massachusetts, USA). [^3H] ATV (3 Ci/mmol) was purchased from Moravek Biochemicals (Brea, California, USA), whilst unlabelled ATV was obtained through the National Institutes of Health AIDS Research and Reference Reagent Programme, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, Maryland, USA). ATV sulphate (100.9 %) was donated by Aspen Pharmacare (Port Elizabeth, South Africa) and diazepam (DIAZ) was obtained from the Biopharmaceutics Research Institute (Rhodes University, Grahamstown, South Africa). Hank's buffered salt solution (HBSS) (containing 1.3 mM CaCl_2 , 0.49 mM MgCl_2 , 0.41 mM MgSO_4 , 5.3 mM KCl, 0.44 mM KH_2PO_4 , 138 mM NaCl, 0.34 mM Na_2HPO_4) as well as 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), bovine serum albumin (BSA), triton X-100, phosphate buffered saline (PBS), β -Nicotinamide adenine dinucleotide 2'-phosphate reduced (NADPH) tetrasodium salt, Bradford reagent for protein assay, L-canavanine, L-GABA and D-pinitol were all purchased from Sigma-Aldrich (St Louis, Missouri, USA). The P-gp inhibitor, PSC833 (i.e., valsopodar) was generously donated by Novartis Pharmaceuticals (Dorval, Quebec, Canada) and ketoconazole (KTZ) was purchased from Oman Chemicals and Pharmaceuticals (Al Buraimi, Sultanate of Oman). A detergent-compatible colourimetric protein assay kit (DC™ Protein Assay) was purchased from Bio-Rad (Mississauga, Ontario, Canada). Cell culture reagents, *viz* Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum, non-essential amino acids, L-glutamine, penicillin, streptomycin, trypsin and ethylene diamine tetraacetic acid (EDTA) were all purchased from Invitrogen (Carlsbad, California, USA). All solvents or acids were obtained from Saarchem (Krugersdorp, South Africa), except HPLC grade acetonitrile which was purchased from Romil Ltd (Cambridge, United Kingdom). PicoFluor 40 scintillation fluid was purchased from PerkinElmer Life and Analytical Sciences (Waltham, Massachusetts, USA). Leaves of *Sutherlandia frutescens* collected in Barrydale, Western Cape, South Africa were verified by the Selmar Schonland Herbarium Grahamstown, South Africa. Sutherlandioside A (98.73%), Sutherlandioside B (99.10%) and combinations of Sutherlandioside C (98.34%) and D (98.53%), Sutherlandin A (93.33%) and B (94.81%) and Sutherlandin C (96.65%) and D (93.46%) were kindly donated by the National Center for Natural Products Research, Research Institute of

Pharmaceutical Sciences at the University of Mississippi, USA.

Preparation of SF Extracts

SF is traditionally prepared for oral administration by the infusion of 2.5 – 5 g dry plant material in a cup of boiling water (22). To be able to examine the effect of the phytochemical constituents which may be present in such a decoction, an aqueous extract of SF was prepared by boiling SF leaves (5 g) in 250 ml water for 20 minutes on a hot plate. The brew was left to cool for 3 hours and was then centrifuged at 10 000 rpm for 15 minutes. The supernatant was transferred to a round-bottomed flask, shell-frozen in liquid nitrogen and freeze-dried. A methanolic extract was similarly prepared, except that SF leaves (5 g) in 250 ml methanol were heated at 50°C. The rationale for the use of the methanolic extract was to be able to determine the effects of phytochemical constituents which are less polar and unlikely to be present in the aqueous extract in any significant quantities. These constituents along with those present in the aqueous extract are likely to be amongst the various phytochemical components in commercial dosage forms which contain SF plant material.

Preparation of SF Flavonol Glycoside and Triterpenoid Glycoside Fractions

Prior to the isolation (24,25) and thus availability of sutherlandiosides and sutherlandins present in SF plant material, crude flavonol and triterpenoid glycoside fractions were prepared for use as indicators of the presence of flavonol and triterpenoid glycosides in SF plant material and extracts thereof.

To prepare the flavonol glycoside and triterpenoid glycoside fractions, SF plant material (100 g) was extracted with ethanol by soxhlet extraction and concentrated under vacuum. The concentrated mass was subsequently extracted with diethyl ether. The ethereal fraction was diluted with alcohol and chromatographed on a silica gel column, where dichloromethane was used to elute an organic fraction which contained the triterpenoid glycosides. The fraction which precipitated on the column and which contained the flavonol glycosides was eluted with ethanol. These crude fractions were further fractionated using a stationary phase of silica gel in dichloromethane and eluted with varying concentrations of ethanol in dichloromethane.

Confirmation of the Presence of Sutherlandiosides and Sutherlandins in SF Triterpenoid and Flavonol Glycoside Fractions and SF Aqueous and Methanolic Extracts

Qualitative HPLC analyses of SF aqueous and methanolic extracts (10 mg/ml), the triterpenoid glycoside fraction (500 µg/ml) and flavonol glycoside fraction (500 µg/ml) were conducted, using a method modified from Prevoo *et al.* 2008 (27). These were the final concentrations of the respective extracts and components used in the *in vitro* studies. Such analyses were also conducted on either individual or combined Sutherlandiosides and sutherlandins at a concentration of 50 µg/ml for each. These qualitative analyses served to confirm that the triterpenoid and flavonol glycoside fractions contained the relevant sutherlandiosides and sutherlandins and that the triterpenoid and flavonol glycoside fractions were present in the methanolic and aqueous extracts.

In Vitro Intestinal Absorption Studies

Cell Culture

Caco-2 cells are human colon adenocarcinoma cells that can undergo spontaneous enterocytic differentiation and polarization once they reach confluency in culture. Completely differentiated monolayers of Caco-2 cells display microvilli and brush border hydrolases on the apical surface, and express endogenous transport systems that are similar to human intestinal epithelium. Because of this structural and functional homology between intestinal epithelium, the primary use of Caco-2 cells has been for transepithelial flux / uptake / accumulation experiments to evaluate transcellular and/or paracellular absorption of drugs. Furthermore, the expression of efflux transporters, such as P-gp, MRPs, and BCRP, and members of the SLC family of influx transporters, such as OCTs and OATP-B have been well characterized (28-30).

Caco-2 cells used from passages 65 to 80 were grown as a monolayer at a density of 2000 cells/cm² on 75 cm² tissue culture flasks in DMEM supplemented with 10% foetal bovine serum, 1% non-essential amino acids, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin, which was replaced every 2 to 3 days, in an incubator set at 37°C, 95% humidity and 5% CO₂. Cells from confluent cultures were harvested using trypsin-EDTA (0.05%) in DMEM and were seeded into 48 well plates at a density of 60 000 cells/cm². Cells were cultured in the plates under the same conditions described above for 21 to 24 days to allow cell differentiation into a tight epithelial monolayer.

Preparation of Conditioning and Incubation Buffers

Conditioning and incubation buffers were prepared using Hank's buffered salt solution (HBSS) containing 1.3 mM CaCl_2 , 0.49 mM MgCl_2 , 0.41 mM MgSO_4 , 5.3 mM KCl , 0.44 mM KH_2PO_4 , 138 mM NaCl , 0.34 mM Na_2HPO_4 , and 5.6 mM D-glucose supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.01% BSA, which was adjusted to pH 7.4. Aqueous and methanolic extracts of SF were prepared in water and methanol respectively to give a final concentration of 10 mg/ml in the conditioning and incubation buffers. This concentration was used to determine whether more dilute extracts of SF than the 100 mg/ml previously investigated (12) were still potent enough to exert an effect on the *in vitro* system of human intestinal absorption. The flavonol and triterpenoid glycoside fractions were similarly prepared in water and methanol respectively. Since these were crude fractions and not individual flavonol and triterpenoid glycosides, the concentration was determined by mass rather than molarity. A final concentration of 500 $\mu\text{g}/\text{ml}$ was expected for each of the two classes of compounds assuming that all 5% w/w of flavonol and of triterpenoid glycosides estimated to be present in SF plant material (26) was extracted when a traditional aqueous decoction of 2.5 g SF plant material in a cup (250 ml) boiling water was prepared. L-canavanine, L-GABA and D-pinitol content in SF appears to be highly variable (22), therefore an estimated average final concentration of 100 μM was chosen for L-canavanine, L-GABA and D-pinitol, which were all dissolved in water. PSC833 (1 μM) dissolved in dimethylsulphoxide (DMSO) was used as the positive control for increased accumulation of ATV in Caco-2 cells. Solvent content was limited to 1% since preliminary experiments showed that at this concentration, water, methanol and DMSO had no effect on ATV accumulation in the cells. In addition, the incubation buffer (but not the conditioning buffer) contained [^3H] ATV (0.1 $\mu\text{Ci}/\text{ml}$) supplemented with unlabelled ATV to give a final concentration of 1 μM ATV.

Cellular Accumulation Experiments

These experiments were performed according to well established methods in Dr. R. Bendayan's laboratory (31). To maintain maximum viability of the cells, culture media was replaced 24 hours before the experiment. Accumulation studies were conducted at 37°C and were performed in triplicate for each group on the same cell culture

plate. Culture media was aspirated and cells were initially washed and conditioned for 15 minutes with 250 μl warm (37°C) conditioning buffers. Preliminary experiments showed that 1 hour incubation of Caco-2 cells with SF extracts and components, at these concentrations, under light microscopy at a magnification of 400 X, had no effect on the cell morphology, therefore the viability of the cells was not likely affected by the extracts and constituents of SF for the duration of the incubation time used. The pre-incubation buffers were aspirated and the cells were then incubated with the incubation buffers at 37°C for 1 hour. At the end of the incubation period, the reaction was stopped and the cells were washed twice by the addition of ice-cold (4°C) 0.1 M PBS (500 μl) after aspiration of the incubation buffer. The cells in each well were solubilised with 1% Triton-X (500 μl) at 37°C for 30 minutes before transfer to scintillation vials. Scintillation fluid (2 ml) was added to each vial and the radioactivity of each sample was measured. Three wells of each plate which were only exposed to blank HBSS buffer throughout the experiment were set aside for the determination of protein concentration. Standard solutions of BSA (0.1, 0.25, 0.5, 0.75 and 1 mg/ml) were also prepared. The Bradford method adapted for compatibility with detergents was applied to the samples and standard solutions. Absorbances were measured on a UV spectrophotometer at 750 nm. ATV cellular accumulation was then expressed in pmol/mg protein.

In Vitro Liver Metabolism Studies

Preparation of Microsomes

Pooled human liver microsomes (20 mg/ml protein as purchased) were thawed in a water bath at 37°C and diluted to 2 mg/ml protein with PBS.

Preparation of Incubation Buffers

A stock solution of ATV was prepared by dissolving an accurately weighed amount of ATV sulphate in a mobile phase, *viz* acetonitrile-ammonium formate buffer (10 mM, pH 3) (45:55, v/v) to provide the equivalent of 5 mM ATV. Similarly, a stock solution of NADPH (10 mM) was prepared in PBS. Using these solutions, an incubation buffer containing 0.05 mM ATV and 2 mM NADPH was prepared in PBS. Incubation buffers containing aqueous and methanolic extracts of SF were prepared at concentrations of 20 mg/ml. The flavonol and triterpenoid glycoside fraction buffers had concentrations of 1 mg/ml and those of L-canavanine, L-GABA and D-pinitol had a concentration of 200 μM .

Metabolism Experiments

The microsomes and incubation buffers were pre-incubated at 37°C for 15 minutes in a Labotec (Pty) Ltd model 132 water bath (Johannesburg, South Africa) fitted with a Julabo PC/1 thermostat (Seelbach, Germany). Incubation buffers were then added to the liver microsomes to obtain final incubation concentrations of 1 mg/ml protein, 25 μ M ATV, 1 mM NADPH, 10 mg/ml SF aqueous and methanolic extracts, 100 μ M L-canavanine, L-GABA and D-pinitol, 500 μ g/ml flavonol glycoside and triterpenoid glycoside fractions and 10 μ M KTZ.

Each incubation mixture was prepared in triplicate and incubated for 1 hour at 37°C. The reaction was stopped by addition of ice cold (4 °C) mobile phase (250 μ L), viz, acetonitrile-ammonium formate buffer (10 mM, pH 3) (45:55, v/v) containing DIAZ (2 μ g/ml) used as an internal standard for the HPLC analysis.

HPLC Analysis of ATV in Microsomes

Calibration standards of ATV were prepared by serial dilution of the ATV stock solution with 50:50 PBS: mobile phase, containing 1 μ g/ml DIAZ, as the IS. The chromatographic conditions used were as previously described for the quantitative analysis of ATV in plasma (32). The Bradford method for protein assay was applied to samples of the microsomes and BSA standard solutions as described above and amount of ATV was expressed in nmol/mg protein.

DATA ANALYSIS

The results were reported as the mean $\pm$ SD of values from three independent experiments using different passages of cells and batches of pooled human liver microsomes. To determine the statistical significance of differences observed between control and treatment groups, an unpaired two tailed Student's *t*-test was computed using Graphpad Prism, version 3 (San Diego, California, USA). A *p*-value < 0.05 was considered significant.

RESULTS

The aqueous and methanolic extracts of SF prepared by hot water and methanol decoctions were obtained at yields of 25 % and 15 %, respectively, whilst that of the flavonol and

triterpenoid glycoside fractions was 5 % and 2.3 % respectively.

Figures 1 and 2 show that the isolated sutherlandiosides and sutherlandins, either alone or in combination were all likely present in the triterpenoid glycoside and flavonol glycoside fractions, respectively. In Figure 1 A, Sutherlandioside A eluted at a retention time of 16.6 minutes which corresponded to the presence of a group of unresolved peaks in the triterpenoid glycoside fraction. A similar observation was made for Sutherlandioside B, which eluted at 13.9 minutes (Figure 1 B). In Figure 1 C, a single peak represented the combination of Sutherlandiosides C and D. Nevertheless, the retention time of the peak which represents both of these compounds fell within the same cluster of peaks (13–17 minutes). A similar trend was observed in Figure 2 A, for the combination of Sutherlandins A and B, where one peak between 10 and 11 minutes was present which is within the retention time range of a cluster of peaks (9–11 minutes) in the flavonol glycoside fraction. Two unresolved peaks were evident in Figure 2 B for the combination of Sutherlandins C and D between 9 and 10 minutes which also eluted within the same retention times of the group of peaks in the flavonol glycoside fraction.

From Figure 3, it is evident that both the aqueous (A) and methanolic (B) extracts contain flavonol (peaks at 9–11 minutes) and triterpenoid (peaks at 13–17 minutes) glycosides present in the triterpenoid and flavonol glycoside fractions.

The positive controls, PSC833 (*p* < 0.001) and KTZ (*p* < 0.05) significantly increased ATV accumulation by Caco-2 cells (Figure 4) and human liver microsomes (Figure 5) respectively.

The aqueous extract (10 mg/ml) of SF reduced ATV accumulation (*p* < 0.05) by Caco-2 cells (Figure 4). On the other hand, no effect was exhibited in the presence of the methanolic extract. Figure 5 indicates that both aqueous (*p* < 0.01) and methanolic (*p* < 0.05) extracts (10 mg/ml) of SF significantly increased the amount of ATV present in human liver microsomes after co-incubation with these extracts.

Despite the effects of the aqueous extract of SF, the flavonol glycoside fraction (500 μ g/ml) present therein had no effect on the amount of ATV present in Caco-2 cells (Figure 4) or human liver microsomes (Figure 5).

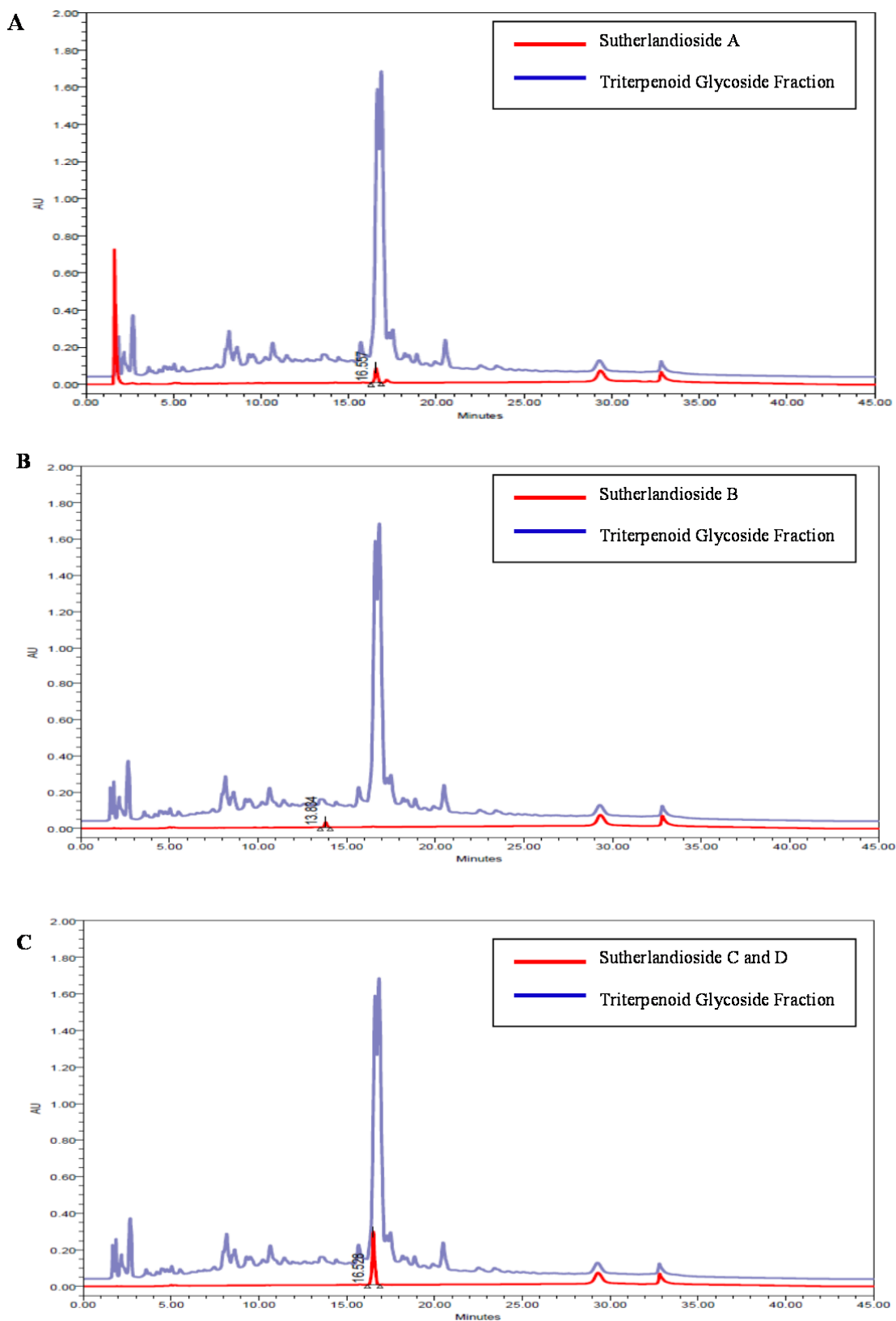


Figure 1: Overlay of chromatograms to show (A) Sutherlandioside A (B) Sutherlandioside B and (C) a combination of Sutherlandioside C and D are present in the triterpenoid glycoside fraction.

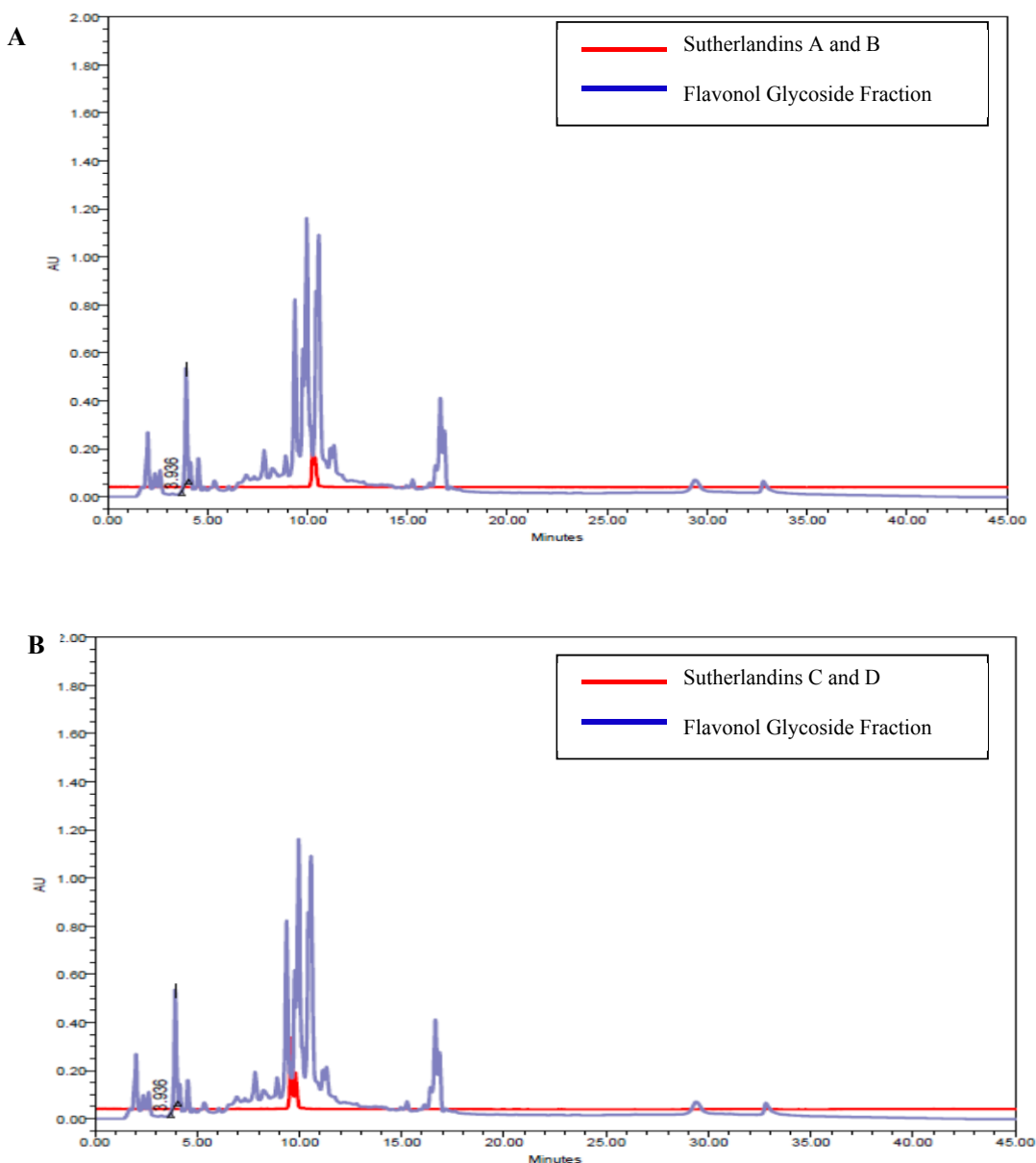


Figure 2: Overlay of chromatograms to show that (A) a combination of Sutherlandin A and B and (B) a combination of Sutherlandin C and D are present in the flavonol glycoside fraction.

The triterpenoid glycoside fraction significantly increased the accumulation of ATV ($p < 0.001$) by Caco-2 cells (Figure 4) but significantly reduced the ATV present ($p < 0.001$) in human liver microsomes (Figure 5).

Overall, the purported constituents of SF, *viz* L-canavanine, D-pinitol and L-GABA had no effect on the amount of ATV present in Caco-2 cells (Figure 4) and human liver microsomes (Figure 5) respectively, except for a significant reduction in ATV accumulation ($p < 0.01$) in Caco-2 cells observed in the presence of D-pinitol (Figure 4).

DISCUSSION

The presence of the individual or combined sutherlandiosides and sutherlandins in the triterpenoid and flavonol glycoside fractions, as well as the aqueous and methanolic extracts of SF suggest that these phytochemical constituents of SF (24,25) may have contributed at least in part to any effects on the amount of ATV present after co-treatment of Caco-2 cells and human liver microsomes with ATV and each fraction and/or extract.

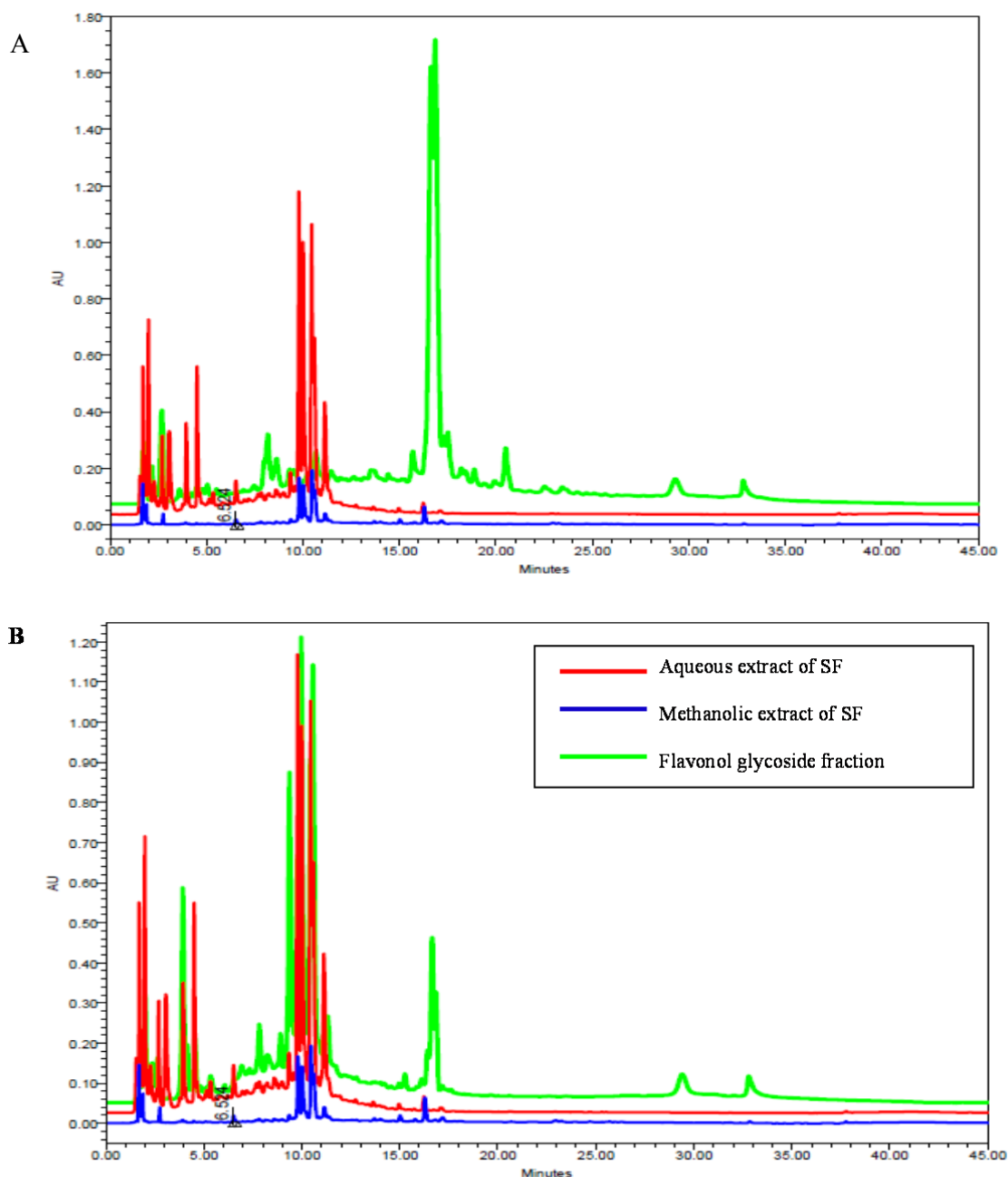


Figure 3: Overlay of chromatograms to show that (A) the triterpenoid glycosides (peaks at 13 – 17 minutes) and (B) the flavonol glycosides (peaks at 9 – 11 minutes) are present in the aqueous and methanolic extracts of SF.

The accumulation of ATV by Caco-2 cells may have been curtailed by the aqueous extract of SF by reducing the activity of influx transporters of ATV or enhancing the activity of efflux transporters of ATV, such as P-gp, which are present in Caco-2 cells and in the human intestinal epithelium. Through one or both of these mechanisms, the extract may have had the

potential to limit ATV absorption and thus ATV bioavailability. Brown *et al.* 2008 (33) found that a higher concentration of an aqueous extract (20 mg/ml) of SF than that used in this study (10 mg/ml) produced no change in transepithelial flux of the ARV, nevirapine in Caco-2 cell monolayers.

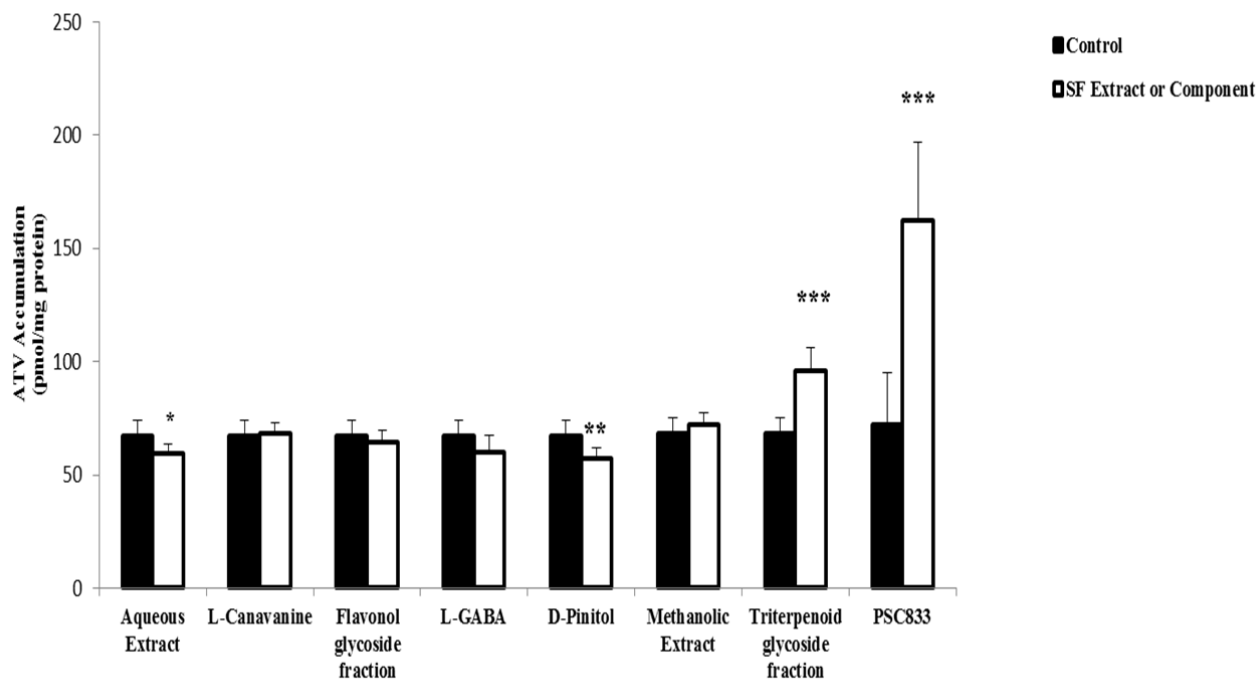


Figure 4: Effect of extracts and components of SF on ATV (1 μ M) accumulation in Caco-2 cells, *in vitro*. PSC833 (1 μ M) was used as a positive control. Each bar represents mean $\pm$ SD; n = 3. * (p < 0.05), ** (p < 0.01) and *** (p < 0.001) in comparison to control (Student's *t*-test).

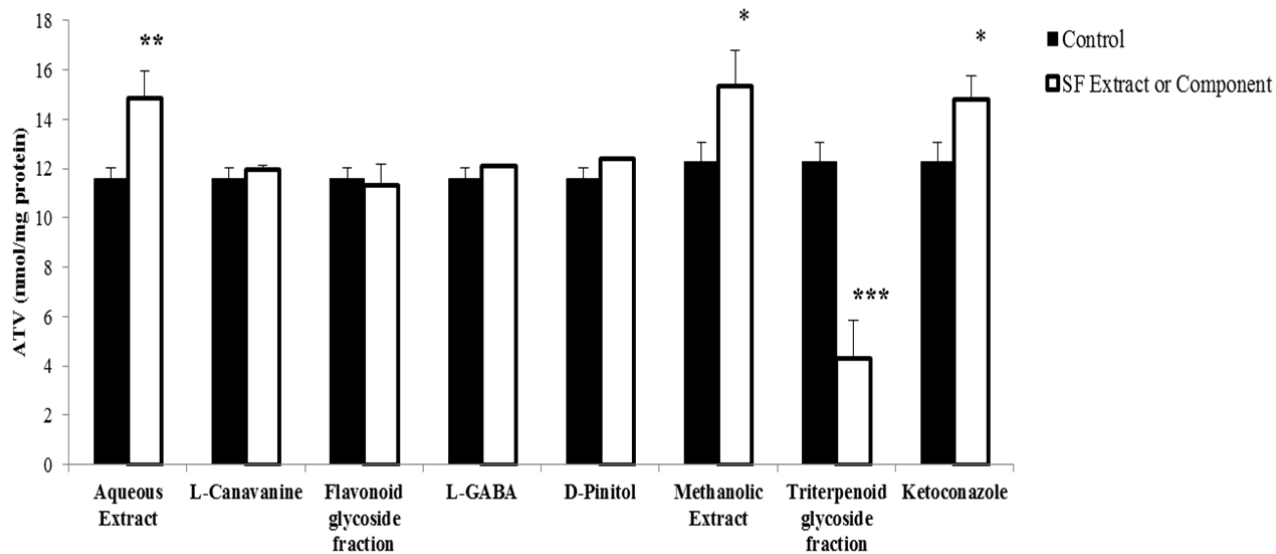


Figure 5: Effect of extracts (10mg/ml) and components of SF on ATV (25 μ M) metabolism in human liver microsomes, *in vitro*. KTZ (10 μ M) is used as a positive control. Each bar represents mean $\pm$ SD; n = 3. * (p<0.05), ** (p<0.01) and *** (p<0.001) in comparison to control (Student's *t*-test).

Unlike ATV, nevirapine is however not a substrate of P-gp (34), which may possibly indicate that the aqueous extract of SF elicited its effect on drug absorption, in Caco-2 cells and possibly in the human intestinal epithelium too,

via P-gp. Mills *et al.* 2005 (12) found that a 100 mg/ml aqueous extract of SF exhibited inhibition of efflux of a fluorescent probe via inhibition rather than activation of basal human P-gp ATPase activity, further indicating that the effect

of the aqueous extract of SF on P-gp may be substrate-dependent. The aqueous and methanolic extracts inhibited ATV metabolism as evidenced by the increase in ATV after co-treatment with each extract in human liver microsomes, indicating elimination was decreased and the bioavailability of ATV may be increased by these extracts. It is hypothesized that the effect of the SF extracts on ATV metabolism may be CYP3A4-mediated since observations by others (12) demonstrated that 100 mg/ml aqueous and methanolic extracts of SF inhibited CYP3A4 activity by $\geq 50\%$ and ATV is a known CYP3A4 substrate (20).

The observation that the flavonol glycoside fraction exhibited no effect on the amount of ATV in the Caco-2 cells and human liver microsomes indicates that the flavonol glycosides, such as Sutherlandins A – D present in SF (25) were not likely responsible for the effects demonstrated by the aqueous and methanolic extracts of SF. The transepithelial flux of cimetidine in Caco-2 cells was altered by 4 flavonol aglycones, but not by 3 out of 4 of the corresponding flavonol glycosides (100 μM) (35) and the flavonol aglycones, quercetin and kaempferol are known to inhibit CYP3A4-mediated metabolism (36-38). It may thus be concluded that glycosylation of flavonols may limit the capability of these compounds to modulate transepithelial flux in Caco-2 cells and CYP3A4-mediated metabolism in microsomes. Since deglycosylation of flavonol glycosides may occur in the gastro-intestinal tract (39), before these reach the site of intestinal absorption and the liver, changes in the bioavailability of ATV which may be observed *in vivo* after concurrent administration of an aqueous decoction of SF may potentially be related to the flavonol glycosides present therein. However deglycosylation of flavonol-3-glycosides appeared to be impeded in cell-free extracts from human intestines and livers (40), thus it is not clear whether the effects of the SF flavonol glycosides on ATV absorption and metabolism would be significant *in vivo*. A flavonol glycoside fraction from SF plant material similarly demonstrated no effect on progesterone and pregnenolone metabolism in sheep adrenal microsomes (27).

The increase in accumulation of ATV by Caco-2 cells in the presence of the triterpenoid glycoside fraction alludes to the potential for the sutherlandiosides and/or sutherlandins present therein to have enhanced absorption and thus bioavailability of ATV, either by inhibition of

efflux transporters of ATV, such as P-gp or increased activity of influx transporters of ATV, which are present in Caco-2 cells and human intestinal epithelium. Madgula *et al.* 2008 (41) found that Sutherlandioside B, the most abundant of the known triterpenoid glycosides (Sutherlandiosides) in SF, is a potential P-gp and MRP substrate. This triterpenoid glycoside and possibly one or more of the others, may therefore have exhibited competitive inhibition of P-gp and/or MRP2-mediated transport of ATV, thereby enhancing ATV accumulation. Reduced ATV levels in human liver microsomes after treatment with this fraction implies that ATV metabolism was increased and the bioavailability of ATV was therefore limited. Activation of CYP3A4 occurs via positive co-operativity (42,43), and since ATV is a CYP3A4 substrate (20), it is likely through this CYP450 isoenzyme that activation of ATV metabolism was elicited. Interestingly, Sutherlandioside B has been shown to inhibit CYP3A4 metabolism of a fluorescent substrate with an IC_{50} of 20 μM (41) and a three-fold lower concentration of the triterpenoid glycoside fraction than that used in this study inhibited CYP450 17 α -hydroxylase and CYP450 21 hydroxylase involved in steroidogenesis in the adrenal gland (27). The discrepancies between the studies may, as previously suggested for the aqueous extract, be due to differences in the substrates utilised.

A clinical study in healthy human volunteers has shown that the bioavailability of a single dose of atazanavir was decreased by chronic dosing of tablets containing *Sutherlandia frutescens* (44). This indicates that SF may have the potential to reduce the efficacy of ATV during routine administration to patients in the clinical setting. These findings corroborate the effect of the aqueous extract and D-pinitol on ATV accumulation in the Caco-2 cells and the effect of the triterpenoid glycoside fraction on ATV metabolism in the human liver microsomes, indicating that these constituents and extract of SF may primarily be involved in the drug-drug interaction.

CONCLUSION

The aqueous extract and D-pinitol significantly reduced ATV accumulation in Caco-2 cells, an established model of human intestinal epithelial cells, implying a decrease in ATV absorption and bioavailability, whilst the opposite effect would be expected after treatment with the triterpenoid

glycoside fraction, which increased ATV accumulation. Both the aqueous and methanolic extracts inhibited ATV metabolism in human liver microsomes, suggesting enhanced ATV bioavailability, whilst the converse was true for the triterpenoid glycoside fraction. The effects of the aqueous extract and D-pinitol in the Caco-2 cells and the triterpenoid glycoside fraction in the human liver microsomes were in agreement with the results of a clinical study in healthy human volunteers (44), therefore, it may be concluded that SF has the potential to alter the absorption and metabolism of ATV in the clinical setting.

ACKNOWLEDGEMENTS

IK gratefully acknowledges financial support from the Medical Research Council (MRC) of South Africa, and ACM thanks the Atlantic Philanthropies Foundation of Rhodes University and the National Research Foundation for scholarships. Christelle and Robby Gass of Zizamele Herbs are acknowledged for providing the authors with a sample of *Sutherlandia frutescens* and Drs Khan, Smillie and Avula of the National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences at the University of Mississippi, USA, are thanked for supplying individual or combined Sutherlandiosides and sutherlandins.

REFERENCES

- Babb DA, Pemba L, Seatlanyane P, Charalambous S, Churchyard GJ, Grant AD. Use of traditional medicine in the era of antiretroviral therapy: experience from South Africa. *Int Conf AIDS*, 2004; 15: B10640.
- Langlois-Klassen D, Kipp W, Jhangri GS, Rubaale T. Use of traditional herbal medicine by AIDS patients in Kabarole District, western Uganda. *Am J Trop Med Hyg*, 2007; 77: 757-763.
- Malangu N. Self-reported use of traditional, complementary and over-the-counter medicines by HIV-infected patients on antiretroviral therapy in Pretoria, South Africa. *Afric J Trad Complement Altern Med*, 2007; 4: 273-278.
- Peltzer K, Preez NFD, Ramlagan S, Fomundam H. Use of traditional complementary and alternative medicine for HIV patients in KwaZulu-Natal, South Africa. *BMC Public Health*, 2008; 8: 255-268.
- Watson RR, Stanaway JD. African Medicinal Plants for the Treatment of HIV/AIDS. In: Watson RR, Preedy VR (eds.), *Botanical Medicine in Clinical Practice*. Wallingford: GAB International, pp 13-30, 2008.
- Giraldo R. Brief report on SADC ministerial consultative meeting on nutrition and HIV/AIDS, 2003. http://www.robertogiraldo.com/eng/papers/SADC_Report_2003.html Accessed on 5 February 2008.
- Chan LMS, Lowes S, Hirst BH. The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. *Eur J Pharm Sci*, 2004; 21: 25-51.
- Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KLR, Chu XY, Dahlin A, Evers R, Fischer V, Hillgren KM, Hoffmaster KA, Ishikawa T, Keppler D, Kim RB, Lee CA, Niemi M, Polli JW, Sugiyama Y, Swaan PW, Ware JA, Wright SH, Yee SW, Zamek-Gliszczynski MJ, Zhang L, International T. Membrane transporters in drug development. *Nat Rev Drug Discov*, 2010; 9: 215-236.
- Kis O, Robillard K, Chan GNY, Bendayan R. The complexities of antiretroviral drug-drug interactions: role of ABC and SLC transporters. *Trends Pharmacol Sci* 2010; 31: 22-35.
- Agbonon A, Eklun-Gadegbeku K, Aklikokou K, Gbeassor M, Akpagana K, Tam TW, Arnason JT, Foster BC. *In vitro* inhibitory effect of West African medicinal and food plants on human cytochrome P450 3A subfamily. *J Ethnopharmacol*, 2010; 128: 390-394.
- Gwaza L, Wolfe AR, Benet LZ, Guglielmo BJ, Chagwedera TE, Maponga CC, Masimirembwa CM. *In vitro* inhibitory effects of *Hypoxis obtusa* and *Dicoma anomala* on CYP450 enzymes and P-glycoprotein. *Afr J Pharm Pharmacol*, 2009; 3: 539-546.
- Mills E, Foster BC, van Heeswijk R, Phillips E, Wilson K, Leonard B, Kosuge K, Kanfer I. Impact of African herbal medicines on antiretroviral metabolism. *AIDS*, 2005; 19: 95-97.
- Monera T, Wolfe A, Maponga C, Benet L, Guglielmo J. *Moringa oleifera* leaf extracts inhibit 6 β -hydroxylation of testosterone by CYP3A4. *J Infect Dev Count*, 2008; 2: 379-383.
- Nair VDP, Foster BC, Thor Arnason J, Mills EJ, Kanfer I. *In vitro* evaluation of human cytochrome P450 and P-glycoprotein-mediated metabolism of some phytochemicals in extracts and formulations of African potato. *Phytomedicine*, 2007; 14: 498-507.
- Usia T, Watabe T, Kadota S, Tezuka Y. Cytochrome P450 2D6 (CYP2D6) Inhibitory constituents of *Catharanthus roseus*. *Biol Pharm Bull*, 2005; 28 (6): 1021-1024.
- van den Bout-van den Beukel C, Hamza OJM, Moshi MJ, Matee MIN, Mikx F, Burger DM, Koopmans PP, Verweij PE, Schoonen WGEJ, van der Ven AJAM. Evaluation of cytotoxic, genotoxic and CYP450 enzymatic competition effects of Tanzanian plant extracts traditionally used for treatment of fungal infections. *Basic Clin Pharmacol Toxicol*, 2008; 102: 515-526.

17. Department of Health, South Africa. (2010). Clinical guidelines for the management of HIV & AIDS in adults and adolescents. http://www.doh.gov.za/docs/factsheets/guidelines/adult_art.pdf Accessed on 2 February 2010.
18. Bousquet L, Roucairol C, Hembury A, Nevers MC, Creminon C, Farinotti R, Mabondzo A. Comparison of ABC transporter modulation by atazanavir in lymphocytes and human brain endothelial cells: ABC transporters are involved in the atazanavir-limited passage across an *in vitro* human model of the blood-brain barrier. *AIDS Res Hum Retrovir*, 2008; 24: 1147-1154.
19. Janneh O, Anwar T, Jungbauer C, Kopp S, Khoo SH, Back DJ, Chiba P. P-glycoprotein, multidrug resistance-associated proteins and human organic anion transporting polypeptide influence the intracellular accumulation of atazanavir. *Antivir Ther*, 2009; 14: 965-974.
20. Le Tiec C, Barrail A, Goujard U, Taburet AM. Clinical pharmacokinetics and summary of efficacy and tolerability of atazanavir. *Clin Pharmacokin*, 2005; 44: 1035-1050.
21. van Wyk BE, Gericke N. Tonic Plants. In: Smit J (ed.), *People's Plants: A Guide to Useful plants of Southern Africa*. Pretoria, South Africa: Briza Publications, Pretoria, pp 139-154, 2000.
22. van Wyk BE, Albrecht C. A review of the taxonomy, ethnobotany, chemistry and pharmacology of *Sutherlandia frutescens* (Fabaceae). *J Ethnopharmacol*, 2008; 119: 620-629.
23. van Wyk BE. A broad review of commercially important southern African medicinal plants. *J Ethnopharmacol*, 2008; 119: 342-355.
24. Fu X, Li XC, Smillie TJ, Carvalho P, Mabusela W, Syce J, Johnson Q, Folk W, Avery MA, Khan IA. Cycloartane glycosides from *Sutherlandia frutescens*. *J Nat Prod*, 2008; 71: 1749-1753.
25. Fu X, Li XC, Wang YH, Avula B, Smillie TJ, Mabusela W, Syce J, Johnson Q, Folk W, Khan IA. Flavonol glycosides from the South African Medicinal Plant *Sutherlandia frutescens*. *Planta Med*, 2010; 76: 178-181.
26. Avula B, Wang YH, Smillie TJ, Fu X, Li XC, Mabusela W, Syce J, Johnson Q, Folk W, Khan IA. Quantitative determination of flavonoids and cycloartanol glycosides from aerial parts of *Sutherlandia frutescens* (L.) R. BR. by using LC-UV/ELSD methods and confirmation by using LC-MS method. *J Pharm Biomed Anal*, 2010; 52: 173-180.
27. Prevoo D, Swart P, Swart AC. The influence of *Sutherlandia frutescens* on adrenal steroidogenic cytochrome P450 enzymes. *J Ethnopharmacol*, 2008; 118: 118-126.
28. Pintoc M, Robine-Leon S, Appay M, Kedingler M, Triadou N, Dussaulx E, Lacroix B, Simon-Assmann P, Haffen K, Fogh J, Zweibaum A. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell*, 1983; 47:323-330.
29. Hidalgo IJ, Raub TJ, Borchardt RT. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology*, 1989; 96: 736-749.
30. Hidalgo IJ, Jibin L. Carrier-mediated transport and efflux mechanisms in Caco-2 cells. *Adv Drug Deliv Rev*, 1996; 22: 53-66.
31. Kis, O, Zastre, JA, Ramaswamy, M, Bendayan, R. pH dependence of organic anion transporting polypeptide 2B1 in Caco-2 Cells: potential role in antiretroviral drug oral bioavailability and drug-drug interactions. *J Pharmacol Exp Ther*, 2010; 334: 1009-1022.
32. Müller AC, Kanfer I. An efficient HPLC method for the quantitative determination of atazanavir in human plasma suitable for bioequivalence and pharmacokinetic studies in healthy human subjects. *J Pharm Biomed Anal*, 2010; 53: 113-118.
33. Brown L, Heyneke O, Brown D, van Wyk JPH, Hamman JH. Impact of traditional medicinal plant extracts on antiretroviral drug absorption. *J Ethnopharmacol*, 2008; 119: 588-592.
34. Janneh O, Chandler B, Hartkoorn R, Kwan WS, Jenkinson C, Evans S, Back DJ, Owen A, Khoo SH. Intracellular accumulation of efavirenz and nevirapine is independent of P-glycoprotein activity in cultured CD4 T cells and primary human lymphocytes. *J Antimicrob Chemother*, 2009; 64: 1002-1007.
35. Taur JS, Rodriguez-Proteau R. Effects of dietary flavonoids on the transport of cimetidine via P-glycoprotein and cationic transporters in Caco-2 and LLC-PK1 cell models. *Xenobiotica*, 2008; 38: 1536-1550.
36. He N, Edeki T. The inhibitory effects of herbal components on CYP2C9 and CYP3A4 catalytic activities in human liver microsomes. *Am J Ther*, 2004; 11: 206-212.
37. Ho PC, Saville DJ, Wanwimolruk S. Inhibition of human CYP3A4 activity by grapefruit flavonoids, furanocoumarins and related compounds. *J Pharm Pharm Sci*, 2001; 4: 217-227.
38. Tsujimoto M, Horie M, Honda H, Takara K, Nishiguchi K. The structure-activity correlation on the inhibitory effects of flavonoids on cytochrome P450 3A activity. *Biol Pharm Bull*, 2009; 32: 671-676.
39. Walle T, Browning AM, Steed LL, Reed SG, Walle UK. Flavonoid glucosides are hydrolyzed and thus activated in the oral cavity in humans. *J Nutr*, 2005; 135: 48-52.
40. Day AJ, DuPont MS, Ridley S, Rhodes M, Rhodes MJC, Morgan MRA, Williamson G. Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver β -glucosidase activity. *FEBS Letters*, 1998; 436: 71-75.

41. Madgula VLM, Avula B, Fu X, Li XC, Smillie TJ, Mabusela W, Syce J, Johnson Q, Folk W, Khan IA, Khan SI. Absorption, metabolic stability and protein binding properties of sutherlandioside B from *Sutherlandia frutescens*. *Planta Med*, 2008; 74: 60.
42. Domanski TL, He YA, Khan KK, Roussel F, Wang Q, Halpert JR. Phenylalanine and tryptophan scanning mutagenesis of CYP3A4 substrate recognition site residues and effect on substrate oxidation and co-operativity. *Biochemistry*, 2001; 40: 10150-10160.
43. Korzekwa KR, Krishnamachary N, Shou M, Ogai A, Parise RA, Rettie AE, Gonzalez FJ, Tracy TS. Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. *Biochemistry*, 1998; 37: 4137-4147.
44. Müller A, Patnala S, Kis O, Bendayan R, Kanfer I. The effect of *Sutherlandia frutescens* on the bioavailability of the protease inhibitor, atazanavir. AAPS Meeting and Exposition, November 2011; Abstract No. W5383.