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Original Article

Inhibition of cytochrome P450 3A enzyme by *Millettia aboensis*: its effect on the pharmacokinetic properties of efavirenz and nevirapine

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ABSTRACT

The chronic and comorbid nature of HIV infection necessitate the use of multiple drugs including herbs to relieve symptoms with a possible increase in herb–drug interaction cases. This study was designed to evaluate the effect of *Millettia aboensis* (Hook. f.) Baker, Fabaceae, on cytochrome P450 3A isoenzyme and the influence of this effect on the bioavailability of two antiretroviral agents. *In vitro* effect of ethanol extract of *M. aboensis* on intestinal and liver microsomes extracted from female rats was assessed using erythromycin-*N*-demethylation assay method while *in vivo* effects were determined by estimating simvastatin plasma concentrations in rats. The effect of the extract on pharmacokinetic parameters of orally administered efavirenz (25 mg/kg) and nevirapine (20 mg/kg) was determined in rats divided into groups ($n = 5$). Plasma drug concentrations were assayed using HPLC and pharmacokinetic parameters determined through a non-compartmental analysis as implemented in WinNonlin pharmacokinetic program. The extract inhibited both intestinal and liver microsomal cytochrome P450 3A isoenzyme activities *in vitro* and enhanced simvastatin absorption *in vivo* with possible inhibition of metabolizing enzymes as indicated by significant ($p < 0.05$) increase in maximal concentration, area under curve and mean resident time of the drug. However, further *in vivo* interaction studies in animal model did not produce significant ($p > 0.05$) changes in the pharmacokinetic parameters of efavirenz and nevirapine. HPLC fingerprinting indicated the presence of quercetin and kaempferol in the extract. These findings revealed *M. aboensis* as an inhibitor of cytochrome P450 3A enzyme but, with no significant effect on the bioavailability of orally administered nevirapine and efavirenz.

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Introduction

Polypharmacy in HIV treatment and resultant drug interactions due to the nature of the disease remain an important challenge in HIV treatment (Machtinger and Bangsberg, 2013), in addition to challenges related to poor adherence and intersubject variability in pharmacokinetics (Michaud et al., 2012). Interindividual variability with its crucial role in treatment failure or toxicity is probably, mostly driven by genetic and environmental factors such as drug–drug interactions, drug–food interactions and drug–herb interactions (Michaud et al., 2012), with drug–herb interactions becoming more remarkable because of the current increase in the use of herbal medicines. A report by Rahman and Singhal (2002) estimated that about 65–80% of the population in developing countries rely on herbal medicines as the primary source of treatment with an estimated annual expenditure of over 60 billion US dollars (WHO, 2003). Some herbal constituents have been shown to affect the bioavailability of co-administered drugs (Kang et al., 2009) through various mechanisms including modulation of drug metabolizing enzymes (Dudhatra et al., 2012). Drug metabolizing enzymes play major roles in the bioavailability and elimination of orally administered drugs in the body. Hence, modulation of these enzymes by herbal extracts could lead to alteration in the pharmacokinetic properties of co-administered substrate agents, ultimately affecting their overall therapeutic outcomes.

The plant, *Millettia aboensis* (Hook. f.) Baker, Fabaceae, is considered to be an all-purpose plant in most parts of Africa because of the multiplicity of its use in ethnomedicine (Banzouzi et al., 2008). It is used for the treatment of constipation in children, as laxative, for the treatment of cold and catarrh, diarrhea, headaches, dysentery, chicken pox and measles including respiratory difficulties (Harrison et al., 2011; Borokini and Omotayo, 2012;

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Onyegeme-Okerenta and Okafor, 2014) most of which are associated with HIV/AIDS. Hence, our unpublished survey found an increased use of this plant by people living with HIV/AIDS.

Although the use of Highly Active Antiretroviral Therapy (HAART) has made long-term suppression of HIV a reality, pharmacotherapy of this disease is still associated with some challenges as some of the agents are substrates for cytochrome P450 enzymes predisposing them to drug interactions with enzyme modulators. Also, the drugs are associated with lots of adverse drug events that may result in poor medication.

Nevirapine and efavirenz belong to the non-nucleoside reverse transcriptase inhibitor class of antiretroviral agents approved for the treatment of HIV-1 infection. Although nevirapine is well absorbed after oral administration, a cross-over controlled study in eight healthy adult males indicated significant effects on most of its pharmacokinetic parameters when co-administered with a bioenhancer (Kasibhatta and Naidu, 2007). It undergoes hepatic oxidation by cytochrome P450 isoforms namely CYP3A4 and CYP2B6 to yield several metabolites (Raffanti and Haas, 2001). The most frequent adverse events associated with nevirapine are: rash that may progress to Stevens-Johnson syndrome and elevated liver enzymes (Fagot et al., 2001; Dumond and Kashuba, 2009) and both are dose dependent. Among the antiretroviral agents that are currently in use, nevirapine is the most common cause of serious, clinically apparent acute liver injury (United States National Library of Medicine).

Efavirenz, with a low oral bioavailability of approximately 40–45% and a long half-life of 40–55 h (Dumond and Kashuba, 2009) is also metabolized by CYP3A4 and CYP2B6 enzymes (Veldkamp et al., 2001). It crosses the brain blood barrier attaining 0.5–1.2% of the corresponding plasma concentration in the cerebrospinal fluid causing high rates of neuropsychiatric side effects in >50% of individuals (Veldkamp et al., 2001).

Therefore, since nevirapine and efavirenz are cytochrome P450 enzyme substrates and are susceptible to pharmacokinetic interactions with enzyme modulators, it is imperative to clinically evaluate some of the herbs used by HIV/AIDS patients for possible interactions with the drugs. Hence, this study evaluated the *in vitro* and *in vivo* effects of *M. aboensis* leaf extract on liver and intestinal cytochrome P450 3A isoenzymes. It also evaluated the pretreatment effect of the extract on the bioavailability of two antiretroviral agents – nevirapine and efavirenz in a rat model.

Materials and methods

Drugs

Simvastatin tablets, Teva® (Teva, United Kingdom), and dexamethasone injection, Ecnudexa injection® (Yanzhou, China) were purchased from a registered community pharmacy in Awka, Anambra state, Nigeria. Erythromycin pure sample was purchased from Century Pharmaceuticals Ltd, India. Efavirenz USP and nevirapine anhydrous USP were purchased from Aurobindo Pharma Ltd, India. Ketoconazole USP was purchased from Aarti Drugs Ltd, India. Nevirapine syrup, Nevimune® (Cipla Ltd, India) and efavirenz tablet (Strides Arcolab Ltd, India) were obtained as gifts.

Animals

Albino rats (127–320 g) of either sex were obtained from the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. All animals were allowed to acclimatize to the new environment before the commencement of the experiment. Feed and water were freely provided and the animals housed according to their sex. Full ethical approval for use of animal subjects was obtained from the Animal Research Ethics Review Board of the University of Nigeria, Nsukka on 28th June 2013. All animal experiments were conducted in line with NIH guide on the use and care of laboratory animals.

Plant material

The leaves of *Millettia aboensis* (Hook. f.) Baker, Fabaceae, Nigeria local names Edo awo, Ukperurumwesi, Mkpukpu manya, were collected from Nsukka, Enugu State, Nigeria, identified and authenticated by a taxonomist, Mr. Alfred Ozioko of Bioresource Development and Conservation Project Center, Nsukka, Enugu State, Nigeria. A voucher specimen was deposited in the Department of Pharmacognosy, of the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University with the herbarium number, PCG/474/A/021. Collected leaves were subsequently cleaned, air-dried under room temperature and pulverized. Pulverized leaves (500 g) were cold macerated in 70% aqueous ethanol for one week with intermittent shaking and changing of solvent every 48 h. The resulting solution was filtered and the filtrate concentrated using rotary evaporator at 40 °C. The resulting extract was properly labeled as EMA and stored in the refrigerator for further use.

Ex vivo *effect of the extract on cytochrome P450 3A (CYP3A) isoenzyme*

The *ex vivo* effects of EMA on CYP3A activity in intestinal and liver microsomes were studied using the method developed by Wrighton et al. (1985) and validated by Umathe et al. (2008). The method is based on the principle that erythromycin is rapidly demethylated by cytochrome P450 3A microsomal enzymes to yield des-*N*-methyl-erythromycin and formaldehyde which produce a yellow color with Nash reagent.

After an overnight fast, female rats were euthanized with pentobarbitone and carefully excised through longitudinal incision in the abdomen to expose the peritoneal cavity. Liver was perfused *in situ* with 10 ml of 0.1 M ice-cold phosphate buffered saline (PBS, pH 7.4) and then, isolated. Similarly, a piece of upper part of the intestine (about 20 cm from the stomach) was also isolated and washed with PBS and the tissues used for further processing.

Preparation of intestinal microsomes

The methods validated by Cotreau et al. (2003), Takemoto et al. (2003) and Umathe et al. (2008) were used. This involved scrapping of the intestinal mucosa with a light plastic slip and the scrapings were mixed followed by successive increasing centrifugation using refrigerated ultracentrifuge (TGL-20M, China) in ice cold histidine-sucrose buffer (HSB, pH 7.0) to obtain a supernatant. The supernatants were combined and then, mixed with 52 mM $CaCl_2$ (0.2 ml of 52 mM $CaCl_2$ to 1 ml of the supernatant), and this was allowed to stand for 20 min to precipitate the microsomes followed by centrifugation at 20,000 × *g* for 15 min to obtain the microsomal pellet which was suspended in 0.5 ml of 0.1 M potassium phosphate buffer containing 20% glycerol and stored at −20 °C until needed.

Preparation of liver microsomes

Liver microsomes were prepared using the methods of Schenkman and Cinti (1978) modified by Umathe et al. (2008). The isolated liver was minced and homogenized in 10 ml 0.25 M sucrose containing 10 mM Tris–HCl (pH 7.4) and then centrifuged at 600 × *g* for 5 min followed by 12,000 × *g* for 10 min to obtain a post-mitochondrial supernatant. The separated post-mitochondrial supernatant was mixed with solid $CaCl_2$ to achieve

a concentration of 8 mM with the supernatant. This was then centrifuged at 20,000 × *g* for 20 min to obtain pellets which were suspended in a mixture of 150 mM KCl-10 mM Tris–HCl, and centrifuged at 20,000 × *g* for 20 min to obtain a pink-colored microsomal pellet. This was suspended in 0.5 ml of 0.1 M potassium phosphate buffer containing 20% glycerol and stored at −20 °C until needed.

*Erythromycin-*N*-demethylation assay*

The microsomal erythromycin *N*-demethylation activity was performed as described by Wrighton et al. (1985). Mixture of microsomal suspension (0.1 ml, 25%), erythromycin (0.1 ml, 10 mM), magnesium chloride (0.1 ml, 150 mM) and potassium phosphate buffer (0.6 ml, 50 mM, pH 7.25) was prepared in duplicate test tubes. These tubes were pre-incubated for 3 min at 37 °C along with the plant extract at two different doses (0.1 ml, 50 and 100 μg) and for negative and positive controls containing Tween 20 (0.1 ml, 1%) and ketoconazole (0.1 ml, 5 μM) respectively. Reaction between these agents was initiated by adding NADPH (0.1 ml, 10 mM), and terminated after 10 min by adding ice-cold trichloroacetic acid (0.5 ml, 12.5%, w/v) solution. The tubes were centrifuged at 1740 × *g* for 10 min to remove proteins. To 1 ml of the supernatant, 1 ml of freshly prepared Nash reagent (2 M ammonium acetate (30 g), 0.05 M glacial acetic acid (0.4 ml), and 0.02 M acetylacetone (0.6 ml)) were added and then heated in a water bath at 50 °C with intermittent shaking for 30 min. After cooling, their absorbances were read at 412 nm. The erythromycin *N*-demethylation activity was calculated from standard (0–100 μM formaldehyde) prepared and ran under the same conditions. The CYP3A4 activity was expressed as nM of formaldehyde obtained per milligram of protein per min calculated from the equation;

$$\text{CYP 3A activity} = \text{Amount of CHO produced (n/mol)} \times \frac{1}{25\ \text{mg protein}} \times \frac{1}{10\ \text{min}}$$

In vivo *cytochrome P450 3A activity assay*

In vivo, CYP3A activity was assessed using an adapted method developed by Kanazu et al. (2004) and validated by Umathe et al. (2008) in rat models. This method is based on the principle that the concentration of a CYP3A substrate agent when orally administered will inversely reflect on the plasma concentration of the agent on modulation of the enzyme. Simvastatin was used as the probe substrate of choice in the experiment according to Hu et al. (2007) and in line with the findings of Foti et al. (2010). CYP3A activity was artificially induced by dexamethasone pretreatment so that the inhibitory effect of the extract would better reflect on simvastatin level.

Extract and drug administration

Female rats (20) were divided into four groups of five animals per group. Three groups received dexamethasone (80 mg/kg *i.p.*) daily in three divided doses for three consecutive days, while one group received the vehicle and served as dexamethasone untreated control group. After 24 h of last dose of dexamethasone and vehicle treatment, EMA at the dose of 400 mg/kg [based on established LD_{50} (Ajaghaku et al., 2012), and unpublished ED_{50} results] was administered to one group, while ketoconazole, 5 mg/kg (positive control) was orally administered to another group. The remaining two groups received the vehicle (1% Tween 20). After 1 h of the last set of administrations, simvastatin (20 mg/kg, *p.o.*) was administered to all the groups and blood samples were collected in heparinized tubes at 0.5, 1, 2, 4, 8, 12 and 24 h interval post simvastatin administration. Blood samples were immediately centrifuged at 1740 × *g* for 10 min to obtain plasma which were stored at −20 °C for further analysis.

Sample preparation and HPLC analysis of simvastatin

Simvastatin in the prepared samples was analyzed by the method of Eggadi et al. (2013) with modifications. Plasma samples were prepared by adding 10% trichloroacetic acid about three times the amount of the plasma and then, mixed to precipitate protein. The mixture was left in a cool place for 15 min followed by centrifugation at 1700 × *g* for 10 min to obtain a supernatant which was filtered through a 0.45-μm disk membrane filter. The filtrate (10 μl) was injected into the HPLC and analyzed using C18 (4 μm, 30 mm × 4.60 mm) as the stationary phase and acetonitrile:water:orthophosphoric acid in respective ratios of 65%:35%:0.1% (v/v) as the mobile phase at a flow rate of 1 ml/min and a UV detection wavelength of 235 nm with a retention time of 0.24 min. Different concentrations of pure simvastatin were prepared to yield varying concentrations which were filtered through the membrane filter and used to obtain the standard curve for simvastatin.

***In vivo* interaction studies**

In vivo *effect of* Millettia aboensis *extract on efavirenz and nevirapine pharmacokinetics*

Albino rats were divided into four groups ($n = 5$). Feed and water were withdrawn 12 h before the commencement of the experiment. Animals in group 1 and 2 received an oral dose of efavirenz alone (25 mg/kg) and nevirapine alone (20 mg/kg) respectively. Group 3 received EMA (400 mg/kg) 1 h before the administration of nevirapine (20 mg/kg) and Group 4 received EMA (400 mg/kg) 1 h prior to efavirenz (25 mg/kg) administration. Blood samples were collected from the animals across the groups through ocular vein puncture using capillary tubes into EDTA tubes at time intervals of 0, 0.5, 1, 2, 4, 8, and 12 h. The blood samples were centrifuged at 1740 × *g* for 10 min for plasma separation and the plasma frozen at −20 °C until assayed.

HPLC analysis of efavirenz

Plasma efavirenz concentrations were assayed using HPLC according to the methods of Sailaja et al. (2007). Chromatography was performed with C18 (4 μm, 30 mm × 4.60 mm) analytical column and 50:50 acetonitrile–phosphate buffer (pH 3.5) as mobile phase at UV detection of 247 nm at a flow rate of 0.8 ml/min and retention time of 0.2 min.

HPLC analysis of nevirapine

Nevirapine concentrations in the plasma were detected using the HPLC method developed by Kumar et al. (2010). The chromatography was carried out on a C18 column (4 μm, 30 mm × 4.60 mm) using a mixture of ammonium acetate buffer (pH 4.0) and acetonitrile (85:15, v/v) as the mobile phase at 254 nm UV detection at a flow rate of 1.2 ml/min and retention time of 0.17 min.

Determination of pharmacokinetic parameters

Different pharmacokinetic parameters were determined using a non-compartmental method using WinNonLin pharmacokinetic programs (version 5.0) (Pharsight Corporation, Mountain View,

CA). The pharmacokinetic parameters determined included: T_{max}, C_{max}, C_{last}, AUC, AUMC, MRT, $t_{1/2}$, V_z and Cl.

T_{max}	time taken for drugs to attain maximal plasma concentration
C_{max}	maximal drug plasma concentration
C_{last}	last measurable drug plasma concentration
AUC	area under curve from the time of dosing to the time of the last observation
AUMC	area under moment curve from the time of dosing to the time of last measurable concentration
MRT	mean residence time
$t_{1/2}$	terminal half-life
V_z	volume of distribution based on the terminal phase
Cl	total body clearance

HPLC fingerprint analysis of Millettia aboensis *extract*

The extract (EMA, 2 mg) was reconstituted with 2 ml of HPLC grade methanol. The mixture was sonicated for 10 min and thereafter centrifuged at 1740 × *g* for 5 min. The dissolved sample (100 μg) was mixed with 500 μl of HPLC grade methanol. HPLC analysis was done using Dionex P580 HPLC system coupled to photo diode array detector (UVD 340S, DionexSofttron GmbH, Germany). Detection was at 235, 254 and 340 nm. The separation column (125 mm × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nano pure water (adjusted to pH 2 by the addition of formic acid). Methanol was used as eluent. Compounds were detected using diode array and identified based on similarity with data in the inbuilt library.

Statistical analysis

All results were presented as mean ± SEM. The data were subjected to *t*-test and one-way analysis of variance (ANOVA) test with group differences determined using *post hoc* Dunnett's multiple comparisons test using SPSS. Results were considered statistically significant at $p < 0.05$.

Results

Ex vivo *assessment of CYP3A activity*

Effect of Millettia aboensis *extract on intestinal microsomes*

The result of the *ex vivo* effects of the extract on intestinal CYP3A activity is presented in Fig. 1. From the result, whereas the 50 μg concentrations of the extract could not produce reductions in intestinal CYP3A activity the 100 μg concentration achieved a significant inhibition of the enzyme activity ($p = 0.040$) similar to ketoconazole at 5 μM.

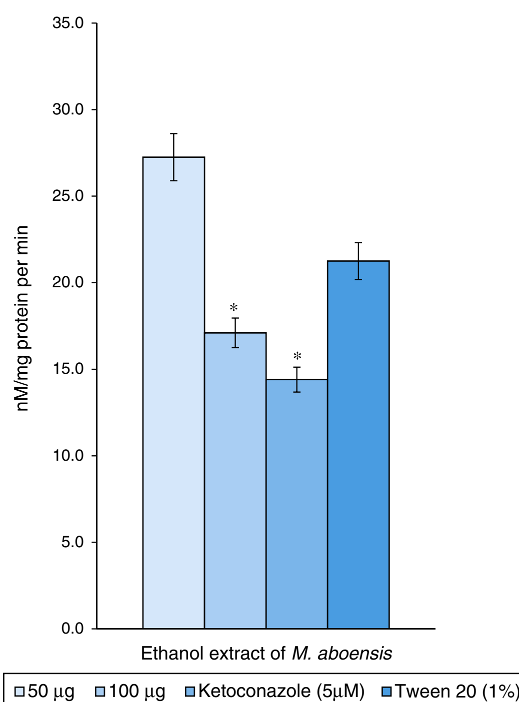

Fig. 1. Effect of *Millettia aboensis* extract on CYP3A activity in intestinal microsomes. *$p < 0.05$ compared to the negative control.

Effect of Millettia aboensis *extract on liver microsomes*

The extract showed a direct dose dependent liver CYP3A inhibition (Fig. 2). While one way ANOVA showed significance difference with the extract, *post hoc* analysis indicated that the extract was significant at 100 μg but not at 50 μg.

Assessment of in vivo *CYP3A activity*

The pharmacokinetic parameters of simvastatin administered to albino rats with EMA to both dexamethasone and vehicle pretreated and then, to simvastatin control groups are presented in Table 1. The plasma concentration–time graphs used for the estimation of the pharmacokinetic parameters are presented in Fig. 3. One way analysis of variance showed significant difference in all the parameters with further subgroup analysis (using LSD) indicating that pretreatment with dexamethasone and vehicle resulted in a significant decrease in C_{max} ($p = 0.005$), AUC ($p = 0.001$), and AUMC ($p = 0.005$) while $t_{1/2}$, V_z ($p = 0.001$) of simvastatin and Ke were increased compared to the non-dexamethasone treated control group. Pretreatment with EMA significantly ($p < 0.05$) increased T_{max}, $t_{1/2}$, AUC, AUMC and MRT of simvastatin with a significant decrease in clearance (Cl) and volume of distribution while the other parameters were not significantly changed compared to the dexamethasone and vehicle treated group.

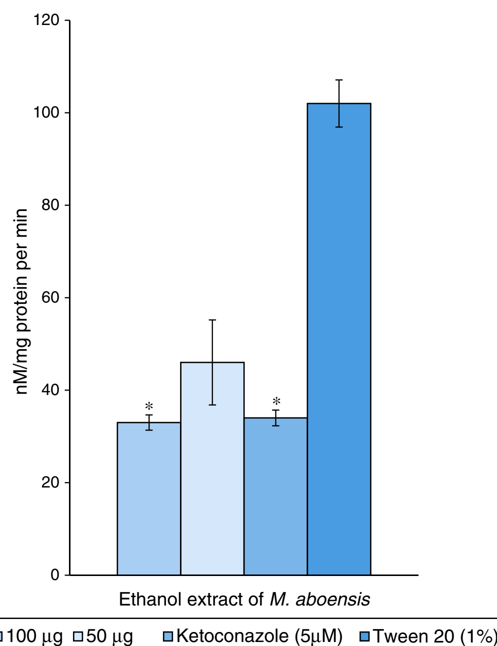

Fig. 2. Effect of *Millettia aboensis* extract on CYP3A activity in liver microsomes. *$p < 0.05$ compared to the negative control.

Table 1
Comparative effect of *Millettia aboensis* extract on the pharmacokinetic parameters of single oral dose of simvastatin.

Parameter	Vehicle + SIMVA	DX + EMA + SIMVA	DX + KETO + SIMVA	DX + vehicle + SIMVA
T_{max} (h)	8.00 ± 1.000	8.00 ± 0.000[a]	4.00 ± 0.000	1.00 ± 0.000
C_{max} (μg/ml)	5.92 ± 0.013	3.38 ± 0.017	5.42 ± 0.014[a]	2.22 ± 0.021[b]
AUC (μg/ml/h)	83.3 ± 0.487	69.6 ± 0.636[a]	58.4 ± 0.115[a]	29.5 ± 0.345[b]
$t_{1/2}$ (h)	11 ± 1.170	133 ± 85.770[a]	10 ± 0.547	26 ± 2.375
V_z (ml/kg)	2.75 ± 0.362	5.48 ± 1.145[a]	3.75 ± 0.809[a]	11.84 ± 2.071[b]
Cl (ml/kg/h)	0.17 ± 0.014	0.08 ± 0.052[a]	0.26 ± 0.019[a]	0.31 ± 0.049
AUMC (μg/ml/h^2)	955.3 ± 4.151	842.2 ± 10.498[a]	534.2 ± 6.030[a]	311.7 ± 4.881[b]
MRT (h)	11.47 ± 0.107	12.07 ± 0.406[a]	9.15 ± 0.184[a]	10.54 ± 0.423
Ke (1/h)	0.044 ± 0.004	0.014 ± 0.011	0.070 ± 0.004[a]	0.027 ± 0.002

DX, dexamethasone (80 mg/kg, *i.p.*); SIMVA, simvastatin (20 mg/kg, *p.o.*); EMA, ethanol extract of *Millettia aboensis* (400 mg/kg, *p.o.*); KETO, ketoconazole (5 mg/kg).
[a] $p < 0.05$ compared to DX + vehicle + SIMVA group.
[b] $p < 0.05$ compared to vehicle + SIMVA group.

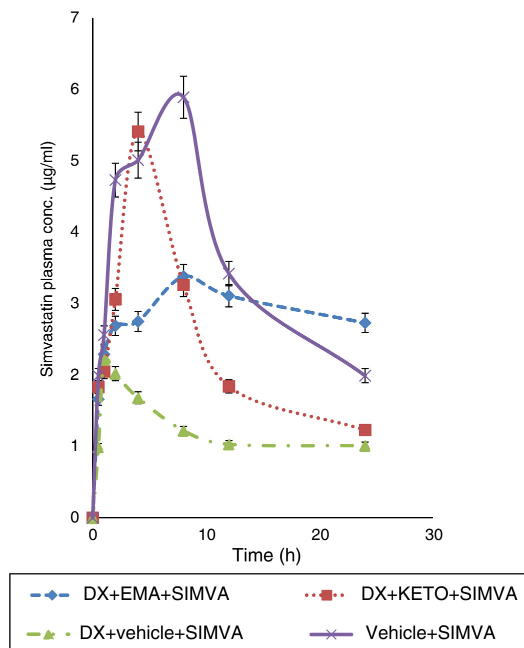

Fig. 3. Plasma concentration–time curve of simvastatin administered with ethanol extracts of *Millettia aboensis* to dexamethasone/vehicle pretreated rats. $n = 5$ represented as mean ± SEM; DX, dexamethasone (80 mg/kg, *i.p.*); SIMVA, simvastatin (20 mg/kg, *p.o.*); EMA, ethanol extract of *M. aboensis* (400 mg/kg, *p.o.*); KETO, ketoconazole (5 mg/kg).

In vivo *drug interaction results*

In vivo *effect of* Millettia aboensis *extract on efavirenz pharmacokinetics*

Table 2 shows the pharmacokinetic parameters obtained from rats following single oral administrations of efavirenz alone and in the presence of EMA while Fig. 4 shows their plasma concentration–time profiles. The results showed a non-significant increase in C_{max} from 3.88 ± 0.108 μg/ml in efavirenz only group to 4.18 ± 0.000 μg/ml in EMA pretreated group. Area under curve showed little or no change in the extract pretreated group compared to the group that received only drug. AUMC increased from 257.5 ± 3.221 μg/ml/h^2 when efavirenz was administered alone to 262.9 ± 0.328 μg/ml/h^2 in the presence of EMA, though, not significant. Time to reach maximum concentrations (T_{max}) showed a non-significant decrease in the presence of EMA similar to half-life ($t_{1/2}$) and mean resident time (MRT). The volume of distribution and clearance were both decreased ($p > 0.05$) in the pretreated group compared to the control.

Table 2
Comparative effect of *Millettia aboensis* extract on the pharmacokinetic parameters of single oral dose of efavirenz.

Parameter	EFV alone	EMA + EFV
T_{max} (h)	2.67 ± 0.667	2.00 ± 0.000
C_{max} (μg/ml)	3.88 ± 0.108	4.18 ± 0.000
AUC (μg/ml/h)	42.8 ± 0.046	44.17 ± 0.054
$t_{1/2}$ (h)	83.20 ± 33.048	61.29 ± 18.847
V_z (ml/kg)	5.06 ± 2.317	4.90 ± 1.970
Cl (ml/kg/h)	0.06 ± 0.024	0.05 ± 0.005
AUMC (μg/ml/h^2)	257.5 ± 3.221	262.9 ± 0.328
MRT (h)	6.01 ± 0.034	5.95 ± 0.003
Ke (1/h)	0.012 ± 0.005	0.013 ± 0.003

EMA, ethanol extract of *M. aboensis* (400 mg/kg, *p.o.*); EFV, efavirenz (25 mg/kg, *p.o.*).

In vivo *effect of* Millettia aboensis *extract on oral nevirapine pharmacokinetics*

Table 3 shows the pharmacokinetic parameters obtained from rats following oral administration of nevirapine alone and in EMA pretreated rats with their plasma concentration–time profiles presented in Fig. 5. From this table, it is evident that the co-administration of EMA extract influenced most of the parameters including T_{max}, C_{max}, $t_{1/2}$, and AUMC however, with only $t_{1/2}$ effect being significant ($p = 0.002$).

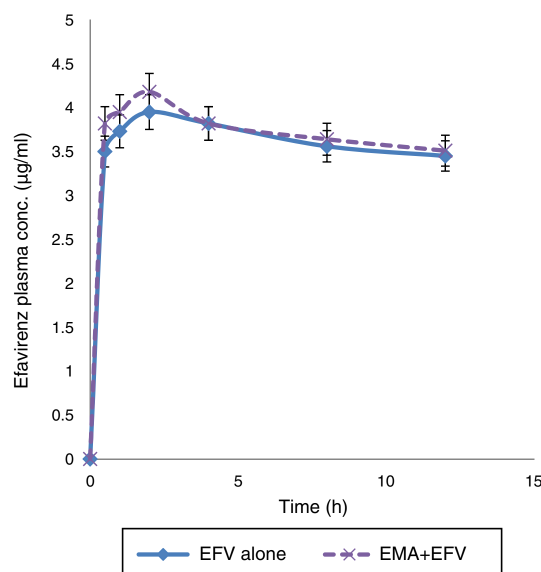

Fig. 4. Plasma concentration–time profile of efavirenz in ethanol extract of *Millettia aboensis* pretreated rats. $n = 5$ represented as mean ± SEM; EMA, ethanol extract of *M. aboensis* (400 mg/kg, *p.o.*); EFV, efavirenz (25 mg/kg, *p.o.*).

Table 3
Comparative effect of *Millettia aboensis* extract on the pharmacokinetic parameters of single oral dose of nevirapine.

Parameter	NVP alone	EMA + NVP
T_{max} (h)	2.17 ± 1.014	0.67 ± 0.167
C_{max} (μg/ml)	2.82 ± 0.070	3.23 ± 0.020
AUC (μg/ml/h)	31.02 ± 0.514	31.79 ± 0.750
$t_{1/2}$ (h)	75.70 ± 1.915	29.75 ± 0.450[a]
V_z (ml/kg)	10.39 ± 0.545	8.07 ± 0.295
Cl (ml/kg/h)	0.06 ± 0.045	0.10 ± 0.046
AUMC (μg/ml/h^2)	186.99 ± 4.659	192.62 ± 1.201
MRT (h)	6.13 ± 0.065	6.07 ± 0.113
Ke (1/h)	0.014 ± 0.002	0.034 ± 0.001

EMA, ethanol extract of *M. aboensis* (400 mg/kg, *p.o.*); NVP, nevirapine (20 mg/kg, *p.o.*).
[a] $p < 0.05$ compared to NVP alone.

HPLC fingerprint result

The chromatogram (Fig. 6) obtained from the HPLC profile of EMA shows five major peaks numbered A, B, C, D and E. Peak numbers A and B correspond to a simple phenolic derivative – flavonoid glycosides based on their UV curves. Peak number C (the major compound) exhibited a UV curve typical of quercetin glycoside, suggested because the HPLC-UV absorbance maxima were achieved at 256 and 352 nm which is a characteristic spectrum of quercetin glycoside. Further liquid chromatographic-electrospray ionization mass spectroscopic analysis revealed strong peaks that showed loss of hexose sugars. The ion peak at m/z 303.2 was another diagnostic indication of quercetin aglycone. Peak numbers D and E exhibited UV curves typical of kaempferol glycosides.

Discussion

This study indicated that the ethanol extract of *M. aboensis* exhibited good cytochrome P450 enzyme inhibitory property both

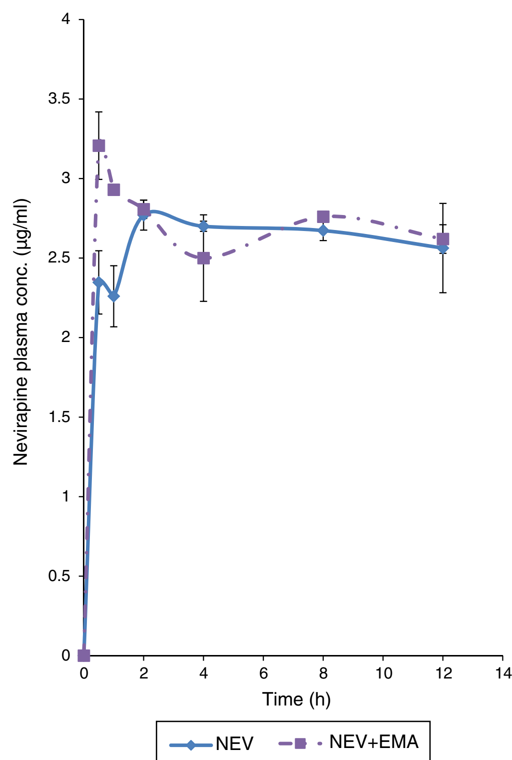

Fig. 5. Plasma concentration–time profile of nevirapine in ethanol extract of *Millettia aboensis* pretreated rats. $n = 5$ represented as mean ± SEM; EMA, ethanol extract of *M. aboensis* (400 mg/kg, *p.o.*); NVP, nevirapine (20 mg/kg, *p.o.*).

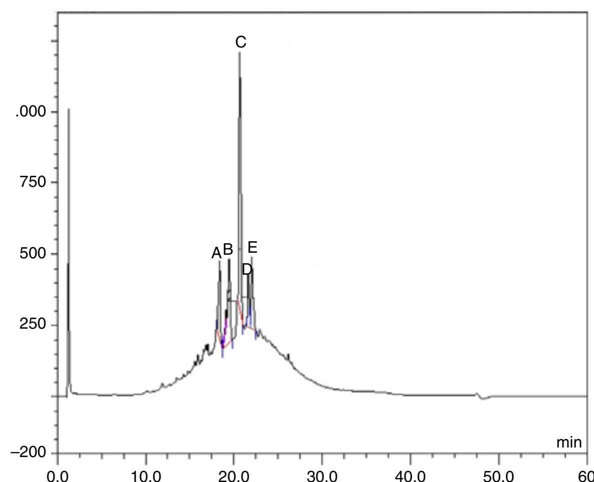

Fig. 6. HPLC fingerprint of ethanol extract of *Millettia aboensis*. (A and B) Simple phenolic derivatives: flavonoid glycosides; (C) quercetin glycoside; (D and E) kaempferol type glycosides.

in vitro and *in vivo*. Further interaction studies to determine the effect of the extract on efavirenz and nevirapine pharmacokinetics demonstrated that the extract did not show significant effect on the pharmacokinetic profile of these drugs.

Drug interactions involving cytochrome P450 enzymes are mostly mediated through enzyme inhibition or induction mechanisms. Enzyme inhibition is an immediate phenomenon and occurs more in practice. The therapeutic importance of enzyme inhibition could be seen in practice when, the co-administration of an inhibitor with a therapeutic agent lead to an increase in the bioavailability of the parent drug or a decrease in the elimination of compounds with resultant substance accumulation and toxicity. This process is referred to as a "bioenhancing effect". Many herbal inhibitors of cytochrome P450 enzymes and bioenhancers have been reported (Randhawa et al., 2011; Nduka et al., 2013; Mazzari and Prieto, 2014).

The effect of the ethanol extract of *M. aboensis* on intestinal microsomes indicated variable concentration effects on intestinal CYP3A enzymes activity. The extract at a lower concentration (50 μg) acted as an activator of the enzymes while inhibitory effect was evident at higher concentration (100 μg). This finding may be due to the nature of enzyme inhibition where competitive inhibition of complex enzymes at low inhibitor concentrations allows a shift in the equilibrium to the activated form. The small intestine is shown to be involved in first pass processes similar to the liver, though, to a much smaller degree (Mitschke et al., 2008). However, some researchers have suggested that the role of intestinal metabolism may be greater than hepatic metabolism irrespective of the lower quantities of CYP enzymes in the small intestine (Lin et al., 1999; Paine et al., 1997). Similarly, the result of the study on the liver microsomes further indicated that the extract of *M. aboensis* exhibited a dose dependent liver CYP3A inhibition. This result, like that of the intestinal microsomal inhibition, indicated the possibility of drug interactions occurring with co-administration of the plant extract with CYP3A substrate agents. Ketoconazole served as the reference standard in this study because of its established *in vitro* and *in vivo* inhibitory effects on CYP450 3A activity (Greenblatt et al., 2011) which was also observed in this study.

The result of erythromycin-*N*-demethylation assay in the intestinal and the liver microsomes indicated that the intestinal microsomal CYP3A activity was inhibited to a greater extent with more variable inhibition than liver microsomal activity similar to the findings of Umathe et al. (2008). This might probably be due

to the higher concentrations of CYP enzymes in liver microsomes compared to intestinal microsomes (Lin et al., 1999).

The *in vivo* assay method is based on the principle that the suppression of a major metabolizing enzyme responsible for the metabolism of a substrate drug will lead to an increase in the plasma concentration of the substrate drug when orally administered. Female rats were used for the study because dexamethasone treated female rat liver microsomes have been shown to have properties similar to those of humans and hence, a useful model for evaluating drug interactions involving cytochrome P450 3A enzyme inhibition (Kanazu et al., 2004). Simvastatin was chosen as the substrate of choice in line with studies by Hu et al. (2007). Different probe substrates are available for use including midazolam, nifedipine and testosterone among others with midazolam being mostly used. However, a study by Foti et al. (2010) revealed buspirone and simvastatin to be relevant clinically as CYP3A probe substrates with similar or greater sensitivity than midazolam.

The significant increases in AUC, AUMC and slight increase in C_{max} of simvastatin in the extract treated group were indicative of enhanced absorption and bioavailability of the drug. According to Jambhekar and Breen (2009), AUC and C_{max} are used in the measurement of extent of drug bioavailability which *inter alia* is dependent on first pass effect. Therefore, the higher values of AUC, AUMC and MRT in the extract-treated rats were indicative of enhanced systemic availability of simvastatin and possible suppression of CYP3A which is the enzyme responsible for the metabolism of simvastatin both in the intestine and liver. Therefore, the concurrent use of this herb with drugs that are substrates of cytochrome P450 3A may be exposing the patient(s) to drug interactions and severe adverse drug effects which may impair adherence, patient safety and clinical outcomes.

The AUC, AUMC, C_{max} and T_{max} are important pharmacokinetic parameters used to assess the degree of absorption and bioavailability of drugs (Labaune, 1989; Proudfoot, 1999; Jambhekar and Breen, 2009). The non-significant changes seen with these parameters in the extract pretreated group compared to the groups that received only efavirenz or nevirapine were indicative of little or no effect on the bioavailability of the drugs by the extract. These observations might be related to the properties of these drugs and the enzyme(s) involved in their metabolism. It has been demonstrated that in the absence of an important detoxifying system such as CYP3A, organisms can still metabolize some xenobiotics as a result of overlying substrate specificity of P450 enzymes and potential activation of alternative enzymes involved in their metabolism (van Waterschoot and Schinkel, 2011). Since efavirenz and nevirapine are substrates to both CYP 3A4 and 2B6 enzymes, inhibition of CYP3A by the extract may not have significantly altered their bioavailabilities due to possible adaptation mechanisms that may have utilized the other route(s) of drug metabolism. This inhibition may also have activated the alternative metabolic pathway (CYP2B6) similar to the reports of van Waterschoot in midazolam metabolism in CYP3A knockout rats (van Waterschoot and Schinkel, 2011). Moreover, some researchers have reported CYP2B6 as the major enzyme involved in efavirenz metabolism (Ward et al., 2003). The significant reduction in the half-life of nevirapine when co-administered with the extract suggests possible effect of the extract on nevirapine metabolism which may lead to nevirapine accumulation and toxicity with time.

Fingerprinting construction is an important quality control tool for herbal samples which is accepted by the World Health Organization and used for identifying plant extracts' constituents (Ciesela, 2012). The constituents suggested to be present in our extract were mainly of flavonoid origin and most known 'bioenhancers' of plants origin are mainly alkaloids, saponins and flavonoids. Quercetin found in citrus fruit was reported to achieve its bioenhancing activity through inhibition of cytochrome P450 3A4 and P-glycoprotein mechanisms (Randhawa et al., 2011). It has been found to increase the AUC and C_{max} of diltiazem, tamoxifen and digoxin (Randhawa et al., 2011).

The major limitations of this study were the inability of the study to establish the enzyme kinetics of the studied extract; secondly, the study neither isolated nor characterized the different constituents in the extract to evaluate their effects on the isoenzymes of interest; in addition, this study only focused on cytochrome P450 3A enzymes without considering the other isoenzymes which may also be involved in the interactions.

Conclusion

This study established *in vitro* and *in vivo* inhibition of cytochrome P450 3A enzyme by *M. aboensis* without a significant effect on the disposition of nevirapine and efavirenz. However, possible accumulation of nevirapine with time was observed. Although no significant interactions were observed between the plant extract and the used antiretroviral agents, co-administration of this plant with drugs that depend solely on CYP3A for their metabolism may lead to significant interactions. Therefore, other interaction studies between this plant extract and other drugs are recommended.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors contribution

SON and MJO designed the study. SON and DA were responsible for execution of the project and conducted data analysis. SON, KCA and CVU drafted the manuscript. All the authors reviewed and approved the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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References

Ajaghaku, D.L., Ilodigwe, E.E., Obi, H.I., Uzodimma, S.U., 2012. Toxicological evaluation of ethanol leaf extract of *Milletia aboensis* (Hook. f) Baker. IJPI J. Pharmacol. Toxicol. 2, 1–8.

Banzouzi, J.T., Prost, A., Rajemiarimiraho, M., Ongoka, P., 2008. Traditional uses of the African *Millettia species* (Fabaceae). Int. J. Bot. 4, 406–420.

Borokini, T.I., Omotayo, F.O., 2012. Phytochemical and ethnobotanical study of some selected medicinal plants from Nigeria. J. Med. Plants Res. 6, 1106–1118.

Ciesela, L., 2012. Biological fingerprinting of herbal samples by means of liquid chromatography. Chromatogr. Res. Int., http://dx.doi.org/10.1155/2012/532418.

Cotreau, M.M., von Moltke, L.L., Beinfeld, M.C., Greenblatt, D.J., 2003. Methodologies to study the induction of rat hepatic and intestinal cytochrome P450 3A at the mRNA, protein, and catalytic activity level. J. Pharmacol. Toxicol. Methods 33, 45–55.

Dudhatra, G.B., Mody, S.K., Awale, M.M., Patel, H.B., Modi, C.M., Kumar, A., Kamani, D.R., Chauhan, N.B., 2012. Comprehensive review on pharmacotherapeutics of herbal bioenhancers. Sci. World J. 2012, 1–33.

Dumond, J.B., Kashuba, A.D.M., 2009. Pharmacotherapy of human immunodeficiency virus infection. In: Koda-Kimble, M.A., Young, L.Y., Alldredge, B.K., Corelli, R.L., Guglielino, B.J., Kradjan, W.A., Williams, B.R. (Eds.), Applied Therapeutics. The Clinical Use of Drugs. , 9th ed. Lippincott Williams and Wilkins, New York, pp. 61-1-24.

Eggadi, V., Ponna, S.K., Kankanala, S.R., Sheshagiri, S.B.B., Gaddam, S.R., 2013. Determination of simvastatin and diltiazem in rat plasma by HPLC and pharmacokinetic studies. Int. J. Pharm. Sci. Res. 21, 53–56.

Fagot, J.P., Mockenhaupt, M., Bouwes-Bavinck, J.N., 2001. Nevirapine and the risk of Stevens-Johnson syndrome or toxic epidermal necrolysis. AIDS 15, 1843–1848.

Foti, R.S., Rock, D.A., Wienkers, L.C., Wahlstrom, J.L., 2010. Selection of alternative CYP3A4 probe substrates for clinical drug interaction studies using *in vitro* data and *in vivo* simulation. Am. Soc. Pharmacol. Exp. Ther. 38, 981–987.

Greenblatt, D.J., Zhao, Y., Venkatakrishnan, K., Duan, S.X., Harmatz, J.S., Parent, S.J., Court, M.H., von Moltke, L.L., 2011. Mechanism of cytochrome P450 3A inhibition by ketoconazole. J. Pharm. Pharmacol. 63, 214–221.

Harrison, J.J.E.K., Dankyi, E., Kingsford-Adaboh, R., Ishida, H., 2011. In search of new leads: a closer look at the therapeutic potential of the constituents of *Millettia thonningii*, *Millettia pachycarpa* and their structural analogues. Int. J. Pharm. Pharm. Sci. 3, 71–81.

Hu, O.Y., Hsiong, C.H., Kuo, B.P., Pao, L., 2007. Cytochrome P450 3A inhibitors and enhancers. United States Patent, US 7,169763 B2.

Jambhekar, S.S., Breen, P.J., 2009. Basic Pharmaceutics: Extravascular Routes of Drugs Administration. Pharmaceutical Press, London.

Kanazu, T., Yamaguchi, Y., Okamura, N., Baba, T., Koike, M., 2004. Model for the drug–drug interaction responsible for CYP3A enzyme inhibition. II: establishment and evaluation of dexamethasone-pretreated female rats. Xenobiotica 34, 403–413.

Kang, M.J., Cho, J.Y., Shim, B.H., Kim, D.K., Lee, J., 2009. Bioavailability enhancing activities of natural compounds from medicinal plants. J. Med. Plants Res. 3, 1204–1211.

Kasibhatta, R., Naidu, M.U.R., 2007. Influence of piperine on the pharmacokinetics of nevirapine under fasting conditions: a randomized crossover placebo controlled study. Drug Dev. Res. 8, 383–391.

Kumar, C.H., Kumar, D.A., Rao, J.V.L.N.S., 2010. A new validated RP-HPLC method for the determination of nevirapine in human plasma. Eur. J. Chem. 7, 821–826.

Labaune, J.P., 1989. Handbook of Pharmacokinetics. Ellis Harwood Ltd., Chichester.

Lin, J.H., Chiba, M., Baillie, T.A., 1999. Is the role of the small intestine in first pass metabolism overemphasized? Am. Soc. Pharmacol. Exp. Ther. 51, 135–157.

Machtinger, E.L., Bangsberg, D.R., 2013. Adherence to HIV Antiretroviral Therapy. HIV InSite Knowledge Base Chapter. UCSF Center for HIV Information.

Mazzari, A.L.D.A., Prieto, J.M., 2014. Herbal medicines in Brazil: pharmacokinetic profile and potential herb–drug interactions. Front. Pharmacol. 5, 1–12.

Michaud, V., Bar-Magen, T., Turgeon, J., Flockhart, D., Desta, Z., Wainberg, M.A., 2012. The dual role of pharmacogenetics in HIV treatment: mutations and polymorphisms regulating antiretroviral drug resistance and disposition. Pharm. Rev. 64, 803–833.

Mitschke, D., Reichel, A., Fricker, G., Moenning, U., 2008. Characterisation of cytochrome P450 protein expression along the entire length of the intestine of male and female rats. Drug Metab. Dispos. 36, 1039–1045.

Nduka, S.O., Okonta, J.M., Esimone, C.O., 2013. Effects of *Zingiber officinale* on the plasma pharmacokinetics and lung penetrations of ciprofloxacin and isoniazid. Am. J. Ther. 20, 501–513.

Onyegeme-Okerenta, B.M., Okafor, U.A., 2014. Antimicrobial properties of ethanol leaf extract of *Millettia aboensis* on some selected clinical isolates. Univ. J. Plant Sci. 2, 97–101.

Paine, M.F., Khalighi, M., Fisher, J.M., 1997. Characterisation of interintestinal and intraintestinal variations in human CYP3A-dependent metabolism. J. Pharmacol. Exp. Ther. 283, 1552–1562.

Proudfoot, S.G., 1999. Factors influencing bioavailability: factors influencing absorption from the gastrointestinal tract. In: Aulton, M.E. (Ed.), Pharmaceutics: The Science of Dosage Form Designs. Churchill Livingston, London, pp. 135–137.

Raffanti, S., Haas, D.W., 2001. Antimicrobial agents: antiretroviral agents. In: Hardman, J.C., Limbird, L.E. (Eds.), The Pharmacological Basis of Therapeutics. , 10th ed. McGraw-Hill, US, pp. 3–30.

Rahman, S., Singhal, K., 2002. Problems in Pharmacovigilance of Medicinal Products of Herbal Origin and Means to Minimize Them. Uppsala Reports January Supplement, Uppsala.

Randhawa, G.K., Kullar, J.S., Kumar, R., 2011. Bioenhancer from mother nature and their applicability in modern medicine. Int. J. Appl. Basic Med. Res. 1, 5–10.

Sailaja, A.L., Kumar, K.K., Kumar, D.V.R., Yugandhar, N.M., Srinubabu, G., 2007. Development and validation of a liquid chromatographic method for determination of efavirenz in human plasma. Chromatographia 65, 359–361.

Schenkman, J.B., Cinti, D.L., 1978. Preparation of microsomes with calcium. Methods Enzymol. 52, 83–89.

Takemoto, K., Yamazaki, H., Tanaka, Y., Nakajima, M., Yokoi, T., 2003. Catalytic activities of cytochrome P450 enzymes and UDP-glucuronosyl transferases involved in drug metabolism in rat everted sacs and intestinal microsomes. Xenobiotica 33, 43–55.

Umathe, S.N., Dixit, P.V., Kumar, V., Bansod, K.U., Wanjari, M.M., 2008. Quercetin pretreatment increases the bioavailability of pioglitazone in rats: involvement of CYP3A inhibition. Biochem. Pharmacol. 75, 1670–1676.

van Waterschoot, R.A.B., Schinkel, A.H., 2011. A critical analysis of the interplay between cytochrome P450 3A and P-glycoprotein: recent insight from knockout and transgenic mice. Am. Soc. Pharmacol. Exp. Ther. 63, 390–408.

Veldkamp, A.I., Harris, M., Montaner, J.S.G., 2001. The steady state pharmacokinetics of efavirenz and nevirapine when used in combination in human immunodeficiency virus type 1 infected persons. J. Infect. Dis. 184, 37–42.

Ward, B.A., Gorski, J.C., Jones, D.R., Hall, S.D., Flockhart, D.A., Desta, Z., 2003. The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz metabolism: implication for HIV/AIDS therapy and utility of efavirenz as substrate marker of CYP 2B6 catalytic activity. J. Pharmacol. Exp. Ther. 306, 287–300.

WHO, 2003. Traditional Medicine Facts Sheet. World Health Organisation, http://www.who.int/mediacentre/factsheets/2003/fs134/en/ (accessed August 2015).

Wrighton, S.A., Schuetz, E.G., Watkins, P.B., Maurel, P., Barwick, J., Bailey, B.S., 1985. Demonstrations in multiple species of inducible hepatic cytochrome P-450 and their mRNAs related to the glucocorticoid-inducible cytochrome P-450 of the rat. Mol. Pharmacol. 28, 312–321.