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Evaluation of Cytotoxic, Genotoxic and CYP450 Enzymatic Competition Effects of Tanzanian Plant Extracts Traditionally Used for Treatment of Fungal Infections

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Abstract: HIV-infected patients in sub-Saharan countries highly depend on traditional medicines for the treatment of opportunistic oral infections as candidiasis. Previous investigations on antifungal activity of medicinal plant extracts utilized by traditional healers in Tanzania have revealed 12 extracts with potent antifungal activity. Although the plants may be good candidates for new treatment opportunities, they can be toxic or genotoxic and could cause pharmacokinetic interactions when used concomitantly with antiretroviral agents. Therefore, we investigated the cytotoxicity, genotoxicity and cytochrome P450 interaction potential of these medicinal plants. Cytotoxicity was tested by Hoechst 33342, Alamar Blue, calcein-AM, glutathione depletion and O_2-consumption assays and genotoxicity by a Vitotox assay. Competition of the 12 extracts on substrate metabolism by CYP3A4, 2C9, 2C19 and 2D6 was tested with high-throughput CYP inhibition screening. Pregnane X receptor (PXR) activation was tested using Chinese hamster ovary cell lines expressing human PXR. Herbal extracts inducing high human PXR activation were tested for enhanced CYP3A4 mRNA levels with quantitative polymerase chain reaction. Genotoxicity was found for *Jatropha multifida*, *Sterculia africana* and *Spirostachys africana*. All plant extracts showed high cytotoxic effects in almost all tests. Potent competition with CYP3A4, 2D6, 2C9 and 2C19 was found for 75% of the herbal extracts. *Spirostachys africana* did not affect CYP2D6 and for *S. africana* and *Turraea holstii* no effect on CYP2D6 and CYP3A4 (DBF) was found. Nine plant extracts showed significant activation of human PXR, but only *Agaura salicifolia*, *Turraea holstii* and *S. africana* significantly induced CYP3A4 mRNA levels. These results indicate the possibility of potential medicinal plant-antiretroviral interactions.

Traditional medicines are commonly used in sub-Saharan countries like Tanzania, with up to 80% of the population depending on traditional medicines for their primary health care [1]. In Tanzania, up to 21% of the people who seeked care from public health care facilities first consult a traditional healer [2]. Especially, HIV-infected persons who often encounter opportunistic infections during their disease course highly depend on this form of health care. According to *Medicine du Monde*, a French non-governmental organization, in Kagera region, five out of every six HIV-infected patients receive their medical attention from a traditional healer rather than from a hospital or primary health care facility [3]. A survey among 532 HIV patients visiting the HIV clinic of Muhimbili National Hospital, Dar es Salaam, Tanzania, reported use of traditional medicines by 62 patients (11.7%). Focusing on HIV patients with oral lesions, mostly oral Candida infections, a prevalence of traditional medicine use of 40.3% was found [4].

Fungal infections, like oral candidiasis, are commonly seen among HIV-infected patients in sub-Saharan Africa. The problems with effective management of these infections such as high costs, drug resistance and toxicity, drives the search for cheap treatment alternatives. We previously investigated the antifungal activities of plants used by Tanzanian traditional healers for treatment of fungal infections. Twelve medicinal plants were identified with potent antifungal activity, which supports the claims by traditional healers on the antifungal effectiveness of these Tanzanian medicinal plants [5] (table 1). These medicinal plants might offer cheap alternative treatment opportunities for HIV+ patients with opportunistic fungal infections. Furthermore, the active compounds may be interesting candidates for development of new antifungal agents. However, little is known of the safety of these medicinal plant extracts. Some of them were found to contain compounds that can be potentially cytotoxic or genotoxic. For example, *Pteridium aquillinum* contains the carcinogenic compound ptaquiloside [6–10]. A case of *Agaura salicifolia* intoxication, with vomiting, arterial hypotension and bradycardia was reported in a

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Table 1.

Twelve most potent herbal plant parts used for treatment of fungal infections and other diseases in Tanzania.[1]

Family	Species (voucher specimen no.)	Local name	Part used	Life form	Region collected	Preparation	Other uses[2]
Anacardiaceae	*Sclerocarya birrea* Sond (OH8)	Muongozi	Root	Tree	Morogoro	Topical: burned leaves (ashes) mixed with simsim oil and topical applied	Snake poison (boiled roots for drink)
Celastraceae	*Elaeodendron buchananii* (Loes) (OH19)	Muhorachwi	Stem bark (SB)	Tree	Singida	Oral: ddied barks are ground mixed with porridge, or water for drinking or topic	Pneumonia
Dennsstraediaceae	*Pteridium aquilinum* (L.) Kuhn (OH41)	Shilu	Leaves	Herb	Mlalo	Topical: dried grounded mixed with water topical applied	–
Ericaceae	*Agauria salicifolia* Oliv. (OH45)	Mwomboa	Leaves	Tree	Mlalo	Topical: dried grounded mixed with water topical applied	–
Euphorbiaceae	*Jatropha multifida* L. (OH53)	Maugwamwipoli	Stem	Shrub	Coast region	Topical: juice from leaves, stem topical applied	–
	Spirostachys africana Sonder (OH54)	Ormotanga	Stem	Tree	Coast region	Topical: dried roots are grounded mixed with cooking oil and topical applied	–
Meliaceae	*Turraea holstii* Gurk (OH37)	Muhenga	Leaves	Shrub	Mlalo	Oral: boiled with water and drink	Convulsions
Mimosaceae	*Acacia robusta* subsp. usambarensis (Taub) Brenan (OH38)	Mkame	Leaves	Tree	Mlalo	Topical: burned leaves (ashes) mixed with water and topical applied	Convulsions
	Acacia nilotica (L.) Wild ex Del. (OH58)	Kloriti	Stem	Shrub	Coast region	Topical: SB boiled for wash/topical, SB dried grounded and topical applied	–
Rutaceae	*Clausena anisata* Oliv. (OH6)	Mjavikali	SB	Shrub	Morogoro	Oral: boiled with water and drink	Convulsions, gonorrhea
Sterculiaceae	*Sterculia africana* (Lour) Fiori (OH39)	Muhoza	Leaves	Tree	Mlalo	Oral: boiled with water and drink	Convulsions
Vitaceae	*Cyphostemma hildebrandtiii* (Gilg) Desc. (OH14)	Damanyamwili	Leaves	Herb	Morogoro	Topical: leaves burned, and ashes mixed with water and topical applied	–

[1]Conducted from Hamza *et al*. [5].
[2]No other use reported.

28-year-old woman due to ingestion of tea made of the leaves [11]. Therefore, cytotoxicity will be tested by Hoechst 33342, Alamar Blue, calcein-AM, glutathione depletion and O_2-consumption assays, which focus on cellular proliferation, mitochondrial activity, cell membrane integrity, intracellular glutathione status and O_2-consumption, respectively. This battery of toxicity assays was chosen due to its diversity on different aspects of cellular toxicity.

Beside the safety of these medicinal plant extracts in terms of cytotoxicity or genotoxicity, the question rises whether usage of these medicines may cause pharmacokinetic interactions with concomitantly administered antiretroviral agents (ARV). ARVs are metabolized by enzymes of the cytochrome P450 (CYP450) enzyme system. The protease inhibitors (PI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) are mainly metabolized by CYP3A4 and to a lesser extent by CYP2B6, 2C9, 2C19 and 2D6. Another class of ARVs, the nucleoside reverse transcriptase inhibitors are not metabolized through CYP450 enzymes [12]. Concomitant administration of herbal medicines might induce or inhibit CYP450 enzymes, thereby causing subtherapeutic ARV plasma levels with risk of development of HIV resistance or high ARV plasma levels with risk for toxicity, respectively. For example, St. John's wort has been shown to increase the clearance and oral bioavailability of nevirapine with 35% in HIV patients [13]. This clinical interaction may be due to the inductive capacity of St. John's wort on hepatic and/or intestinal CYP3A4 [14]. Mills *et al*. recently described the potential for interaction of two South African medicines with ARVs metabolized by CYP3A4 [15]. Furthermore, for Indonesian herbal medicines interaction with CYP3A4 and CYP2D6 has recently been reported [16].

With the plans of World Health Organization to provide worldwide access to antiretroviral agents, also HIV-infected patients who formerly depend on traditional medicines will get more access to these Western medications. Therefore, interactions between medicinal plants and antiretroviral medications are a growing concern. The above-mentioned survey in Dar es Salaam found that 36 HIV-infected patients (58%) on ARVs were concomitantly using traditional medicines underlying the need for urgent studies on potential herb-ARV interactions [4]. Nothing is known on the eventual risk for CYP450 enzyme interaction of the potentially useful Tanzanian herbal extracts. Therefore, beside cytotoxicity and genotoxicity, we investigated the extracts of the 12 most antifungal active medicinal plants for their competitive activities on CYP2C9, 2C19, 2D6 and 3A4, the activation of PXR and the induction of mRNA levels of CYP3A4.

Materials and Methods

Materials. Ketoconazole, 3-cyano-7-ethoxycoumarin, furafylline and sulfaphenazole were obtained from Ultrafine Chemicals (Manchester, UK), glucose-6-phosphate (G6P), G6P-dehydrogenase (G6PDH) and $NADP^+$ were purchased from Roche (Mannheim, Germany). $MgCl_2$ and potassium phosphate monobasic were obtained from Merck (Darmstadt, Germany) and potassium phosphate dibasic and dimethyl sulfoxide (DMSO) from J. T. Baker (Deventer, The Netherlands). Doxorubicin, monochlorobimane (MCB), tranylcypromine, quercetin, quinidine, 4-nitrosoquinoline-1-oxide and benzo[a]pyrene were ordered from Sigma-Aldrich (St. Louis, MO, USA). CYP450 supersomes, 7-methoxy-4-methylcoumarin (MFC), 7-benzyloxyquinoline (7-BQ) and dibenzylfluorescein (DBF) were obtained from BD Gentest (Woburn, MA, USA), Hoechst 33342 from Riedel de Haën (Seelze, Germany), Alamar Blue from Sero-Tec (Oxford, UK), calcein-AM from Molecular Probes (Eugene, OR, USA), Trypsin from Gibco-BRL Life Technologies (Rockville, MD, USA), 96-well Nunclon culture plates from Nunc (Roskilde, Denmark), O_2 test kit from Luxcel Biosciences (Cork, Ireland), Vitotox test kit from Thermo (Vantaa, Finland), Tularik 0901317 (NV Organon, Oss, The Netherlands), Superscript II Rnase H reverse transcriptase (Invitrogen, Paisley, UK) and S9 homogenate from NOTOX B.V. (′s-Hertogenbosch, The Netherlands).

The cell culture medium Dulbecco's modified Eagle's medium, nutrient mixture F12 (DMEM/HAM F12 medium in a ratio of 1:1) with phenol red was obtained from Gibco-BRL (Invitrogen, Carlsbad, CA, USA), bovine calf serum from Hyclone (Logan, UT, USA) and 100 IU/100 µg penicillin/streptomycin from Gibco-BRL.

Medicinal plants. Plants traditionally used for treatment of fungal infections were collected from Tanga, Singida, Coast and Morogoro regions of Tanzania in February–March 2004 and voucher samples were deposited at the Herbarium of the Department of Botany, University of Dar es Salaam, Dar es Salaam, Tanzania. Based on a previous study on the antifungal activity of the collected plants, the 12 most potent medicinal plant extracts were selected for this study (table 1) [5].

Preparation of plant extracts. Methanolic extracts of the plant materials were prepared as described previously [5]. Methanol was used because in 80% methanol all lipophilic and water-soluble compounds of the herb, thus including those taken up by the body, can be extracted. In short, 400 g of dried and grounded plant materials were extracted with 80% methanol and filtered after 24 hr. This procedure was repeated three times to ensure exhaustive extraction. The methanol was evaporated under reduced pressure in a rotoevaporator at 40°. The remaining extracts were freeze-dried and stored at –20° until further use. Before the start of the assays, stock solutions of plant extracts of 50 mg/ml in DMSO were prepared for Hoechst 33342, Alamar Blue, calcein-AM, glutathione, O_2-consumption and PXR assays and of 100 mg/ml in DMSO for CYP competition assays and the Vitotox test. Stocks were serially diluted in DMSO by 2-fold. The concentration range was based on the antifungal activities that were found (see Hamza *et al*. [5]).

Preparation of reference compounds. Stock concentrations of 10^{-2} M were prepared for the reference compounds in DMSO and stored at –20°. Test concentrations were prepared in 100% DMSO in the range of 10^{-2} to 10^{-5} M with $\sqrt{10}$ dilutions. These dilutions were transported to a deep-well plate and diluted in phosphate-buffered saline (PBS) to reach the final test concentrations in the plates for cytotoxicity assays in the range of 3.16×10^{-8} to 3.16×10^{-3} M (0.0183 to 18.3 µg/ml), for Vitotox assays of 3.16×10^{-6} to 10^{-4} M, for the CYP450 enzymes of 10^{-7} to 10^{-4} M and for the PXR assay of 3.16×10^{-10} to 10^{-4} M. The final DMSO concentration in the tests was 0.316% for cytotoxicity assays and 0.1% for the other assays.

Cell cultures. HepG2 and HeLa cells were purchased from the American Type Culture Collection (Rockville, MD, USA), while CHO-PXR 8G cells were prepared within Organon. This cell line contained a promoter luciferase read-out system in combination with the human PXR receptor. All three cell lines were cultured in 175 cm^2 Roux flasks in low glucose Dulbecco's modified Eagle's medium and nutrient mixture F-12 supplemented with 10% foetal bovine serum, 2 mM glutamine and 100 U/100 µg Pen/Strep at 37° in a humidified atmosphere and flushed with 5% CO_2 in air. Complete medium was refreshed every 3–4 days with subculturing.

Table 2.

Summary of assay conditions and concentrations of enzyme, substrate, positive controls and buffer.

Enzyme	CYP2C9	CYP2C19	CYP2D6	CYP3A4	CYP3A4
Enzyme/well (pmol/ml)	10	5	7.5	15	15
Phosphate buffer pH 7.4 (mM)	25	325	325	325	325
Substrate	DBF	DBF	AMMC	DBF	7-BQ
Substrate conc. (μM)	1	2	1.5	1	40
Positive control (reference compound)	Sulfaphenazole	Tranylcypromine	Quinidine	Ketoconazole	Ketoconazole
Positive control conc. (μM)	0.1	0.1	0.1	0.1	0.1
Incubation time (min.)	30	30	30	30	30
Excitation wavelength (nm)	485	485	390	485	409
Emission wavelength (nm)	538	538	460	538	530

AMMC, 3-[2-(N,N-diethyl-N-methylamino) ethyl] 7-methoxy-4-methylcoumarin; 7-BQ, 7-benzyloxyquinoline; DBF, dibenzylfluorescein.

Cytotoxicity assays. The HepG2 cells were trypsinized, counted and resuspended in culture medium to a concentration of 5.3×10^4 cells/ml for Alamar Blue and Hoechst 33342 tests, 1.06×10^5 cells/ml for glutathione depletion tests and 10^4 cells/ml for O_2-consumption tests. For calcein-AM test HeLa cells were trypsinized counted and resuspended in culture medium to a concentration of 5.3×10^4 cells/ml. Then, 190 μl of this cell suspension was seeded per well and the 96-well plate was covered and incubated for 24 hr in a humidified atmosphere at 37° and 5% CO_2. For cytotoxicity assays, an initial plant extract concentration of 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.8 mg/ml DMSO was used. These extracts were five times diluted with PBS and 10 μl was added to the pre-incubated 96-well plate. The final test concentrations ranged from 500 to 8 μg/ml and the final DMSO concentration in the test was 1%. Alamar Blue, Hoechst 33342, calcein-AM and glutathione depletion assays were performed as described by Schoonen *et al*. [17,18]. In the O_2-consumption assay the 96-well plate was also pre-incubated for 24 hr in a humidified atmosphere at 37° and 5% CO_2. Then, the medium was removed and replaced by 190 μl of serum-free medium containing Luxcel A65N-1 oxygen probe (750× diluted). This was followed by the addition of the plant extracts (10 μl) or doxorubicin (10 μl) and 100 μl pre-warmed mineral oil (37°). Plates were sealed and incubated for another 24 hr in a humidified atmosphere at 37° under 5% CO_2. The fluorescence was measured at 340 nm excitation wavelength and 642 nm emission wavelength on a fluorometer at 37° (Victor II, Perkin-Elmer, Groningen, The Netherlands). The minimal toxic dose (MTD) is defined as the lowest plant extract concentration with an inhibition of the fluorescence/luminescence of at least 20% compared to that of doxorubicine at 18.3 μg/ml. The efficacy (EFF) is defined as the percentage of the decrease in fluorescence/luminescence of the plant extract at the highest tested dose (500 μg/ml) compared to the fluorescence/luminescence decrease by doxorubicin at 18.3 μg/ml (100%). The cumulative index (CI) is the cumulative sum of the percentage of inhibition at the seven tested concentrations, in which the effect is above MTD. Each concentration can obtain a maximal score of 80%, leading to a CI between 0 and 560%. A compound is considered toxic when EFF or CI > 50%. Weak toxicity is defined when EFF and CI > 20. Data of reference compounds were calculated in μg/ml according to their molecular weights.

Vitotox test. The Vitotox test was carried out according to the method of Verschaeve *et al*. with slight modifications [19]. The two bacterial strains were cultured according to the manufacturer's instructions. For testing compounds by metabolic activation, a S9 liver homogenate was used from Aroclor 1254-treated rats. Frozen S9 homogenates were diluted in pre-warmed PBS an hour before the start of the experiment. Then, the S9 homogenate is diluted 10 times with the bacterial (RecN2-4 and pr1) strains. Ten microlitres of each plant extract dilution (2-fold) was transported to a second 96-well plate and 90 μl of milli-Q water was added. For references, 25 μl of the dilutions were transported to a deep-well plate and 975 μl milli-Q water was added. Then, 50 μl of these dilutions were transferred to a 96-well plate and 75 μl milli-Q water was added. Then, 9 μl of these extracts and reference dilutions were transported to 384-well plates and 81 μl of RecN2-4 strain or pr1 strain with or without S9 homogenate was added. Herbal extracts were tested in a concentration range from 1–0.0005 mg/ml in 1% DMSO, while for the references a range of 1×10^{-4}–3.16×10^{-6} mol/l √10 dilutions were used in 0.1% DMSO. The plate is sealed and luminescence is measured for each of the wells every 15 min. during 3 hr on a Victor II. The signal to noise ratio (S/N) was calculated for each measurement, being the ratio between the sample value and the corresponding blank value. A sample is considered to be genotoxic when the max S/N (recN2-4)/max S/N (pr1) > 1.5 and when the signal is generated after 45 min. A compound is considered cytotoxic when the S/N ratio for pr1 strain is <0.8. Furthermore, the minimal genotoxic dosage with or without metabolic activation, defined as the lowest compound concentration with or without S9 mixture with a max S/N (recN2-4)/max S/N (pr1) ratio of at least 1.5 is calculated.

CYP450 enzyme competition assays. The competitive activities of the plant extracts on human CYP2C9, 2C19, 2D6 and 3A4 were assayed with the high throughput fluorometric assays from Gentest (Woburn, MA, USA) according to the method of Crespi *et al*. with slight modifications. The assay conditions are summarized in table 2. For CYP3A4 assays, two fluorometric substrates were used. Due to practical issues, the plant extracts were tested in slightly different concentrations of 139.00–1.09 μg/ml with 2-fold dilution steps in a 384-well plate in 0.1% DMSO. The cofactor solutions were prepared in 25 mM phosphate buffer (pH 7.4) containing 1.3 mM $NADP^+$, 3.3 mM G6P, 3.3 mM $MgCl_2$ and 0.4 U/ml G6PDH, except for CYP2D6. For CYP2D6, the cofactor solution contained 0.82 mM $NADP^+$, 0.41 mM G6P, 0.82 $MgCl_2$ and 0.4 U/ml G6PDH.

To the 384-well plates, containing plant extracts and control dilutions, 10 μl of substrate solution was added and the plates were covered with a lid and shaken for 20 min. Then, plates were pre-warmed at 37° in an incubator and 10 μl of enzyme/cofactor solution was added leading to a final volume of 40 μl/well. Thereafter, the plate was put in the Victor II reader, shaked for 20 sec. and pre-incubated for 2 min. at 37°. Then, the plate was measured for 0.1 sec./well after 30 min. of incubation using the excitation and emission wavelengths shown in table 2. Data were exported and analysed using an Excel spreadsheet. The IC_{50} values were calculated by linear interpolation. Data of reference compounds were calculated in μg/ml according to their molecular weights.

PXR induction assay. The plant extracts were serially diluted by 2-fold in PBS and culture medium to obtain final concentrations of 0.5–0.0005 mg/ml in 0.1% DMSO. As reference, the compound

Table 3.

CYP3A4 induction of selected concentrations of herbal medicines that showed high PXR activity.

Herbs	Tested concentration (μg/ml)	CYP3A4 fold induction (mean ± S.D.)[1]
Agauria salicifolia	31	0.97 ± 0.21
	63	**2.00 ± 0.64**
Clausena anisata	63	–
	125	–
Cyphostemma hildebrandtii	31	0.37 ± 0.10
Elaeodendron buchananii	31	0.95 ± 0.32
	63	–
Jatropha multifida	250	–
	500	–
Sclerocarya birrea	16	1.49 ± 0.19
	31	1.76 ± 0.50
Sterculia africana	63	1.34 ± 0.06
	125	**2.01 ± 0.96**
Turraea holstii	63	0.39 ± 0.04
	125	**3.95 ± 1.15**
Rifampicin	10^{-4}	**5.38 ± 0.19**
	10^{-5}	**2.79 ± 0.59**
	10^{-6}	1.73 ± 0.38
Tularik 0901317[2]	10^{-5}	**4.89 ± 0.03**
	10^{-6}	**3.76 ± 0.84**
	10^{-7}	**2.22 ± 0.43**

[1]Induction >2 is considered significant.
[2]In M.
– Toxic concentration for HepG2 cells.

Tularik 0901317 was used, which is a potent LXR ligand that is also capable of activating human PXR [20–22]. Ten microlitres of the diluted plant extracts or reference were added to a 384-well culture plate. Finally, 40 μl of CHO-PXR 8G cell suspension (1.2×10^4 cells/well) were added to each well. After 16 hr of incubation at 37° and 5% CO_2, 25 μl of LucLite were added to the wells and cell lysis was allowed for 30 min. at room temperature. Subsequently, the luciferase light signal was measured in a 384-well Topcount NXT (Perkin-Elmer). The luminescence is expressed as counts per second. The EC_{50} values of the herbal extracts are calculated by using the spline 4-parameter calculation. Efficacy of test compounds is defined in percentage by means of the maximal compound stimulation at 0.5 mg/ml divided by the maximal stimulation of Tularik 0901317 at 1×10^{-4} M multiplied by 100. In addition, the induction factor of the tested herbal extracts is calculated by means of the maximal induction of the luminescence signal at the most active concentration of the 11 tested concentrations with respect to the blank signal.

CYP3A4 mRNA induction. Eight herbal extracts that showed high PXR activation were tested for CYP3A4 induction. The herbs and concentrations are shown in table 3. HepG2 cells were seeded on Petri dishes with a density of 4500 cells/cm^2 in culture medium with 10% defined supplemented bovine calf serum and cultured for 24 hr. Then, herbs, and positive controls rifampicin and Tularik 0901317, or vehicle alone were added and incubated for 24 hr. Final test concentration of DMSO was 0.1% for reference compounds rifampicin and Tularik 0901317 and herbs, with exception of *Jatropha multifida* for which the final concentration was 1%. The cells were then yielded and RNA was isolated using trizol reagent and RNA was precipitated with isopropanol. Thereafter, cDNA synthesis was performed by adding 0.5 μg random hexamer primer (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) to 3 μg RNA. The mixture was heated for 10 min. at 70° and quickly chilled on ice for 2 min. cDNA was then synthesized in a total volume of 25 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs and 200 U SuperScript II Rnase H reverse transcriptase. After incubation for 1 hr at 42°, cDNA was diluted to a concentration equivalent to 5 ng/ml RNA.

Quantitative polymerase chain reaction (PCR) was performed using an ABI PRISM 7900 HT sequence detection system was used for quantitative PCR (Applied Biosystems Inc., Foster City, CA, USA). Specific primers were designed using Primer Express software (version 2.0, Applied Biosystems Inc.). To avoid the influence of DNA contamination, primer pairs were designed over an intron and exon boundary. For CYP3A4 and β-actin, the following forward and reverse primers were used respectively, CAGGAGGAAATTGATGCAGTTTT and GTCAAGATACTCCATCTGTAGCACAGT and CTGGCACCCAGCACAATG and GCCGATCCACACGGAGTACT. The PCR reaction consisted of cDNA equivalent to 30 ng RNA in a total volume of 25 μl PCR mix containing 37.5 μM CYP3A4 primers or β-actin primers and 1× SYBRgreen PCR Mix (Applied Biosystems Inc.). The program used was 10 min. at 95°, 40 cycles of 15 sec. at 95° and 1 min. at 60° 100%, followed by a dissociation curve step. Expression levels were normalized by β-actin.

Results

Extracts of all 12 plant materials were obtained in methanol: water (v:v; 4:1) as in this way most of the lipophilic and water-soluble compounds and derivatives will become extracted. These obtained extracts were already tested on their specific biological inhibitory potential of proliferation of *Candida* species. Thus, the used concentrations (μg/ml) in the tests below can be calibrated on their biological merits with respect to inhibition of proliferation of *Candida*. This implies that a large dilution of extract may be indicative for very high concentrations of compounds in the extract or that only one or a few very potent and strong growth inhibitors for *Candida* are present in this extract. A ratio score between the growth inhibitory concentration in *Candida versus* human toxicity, receptor activation or CYP3A4 induction levels may than lead to a ranking of the herbs with the largest potential for *Candida* treatment and a reduction in side effects.

Cytotoxicity.

The effects of the 12 medicinal plant extracts on mitochondrial activity, cellular proliferation, damage to the cellular membrane, glutathione depletion and the electron transport chain activity were tested with Alamar Blue, Hoechst 33342, calcein-AM uptake, glutathione depletion and O_2-consumption assays, respectively. All plant extracts were tested in a concentration range from 8 to 500 μg/ml. This range was based on the minimal concentrations (MIC_0) of the herbal extracts that inhibited growth of *Candida* sp. by 100%. The results of the cytotoxicity assays are shown in table 4 and for a selection of herbs the dose–response curves are shown in fig. 1. In fig. 1F, the interpretation of the results in table 4 is explained by the example curve for doxorubicin in Alamar Blue test.

All herbal extracts, except *P. aquillinum* and *Turraea holstii*, reduced the NADPH content with Alamar Blue. *Eleaodendron buchannanii* had the highest effect on mitochondrial activity with a MTD of 62.5 μg/ml, while *P. aquillinum* and *T. holstii*

Table 4.

Overview of minimal inhibitory concentrations (MIC_0), Efficacy (EFF in %), minimal toxic dosage (MTD, µg/ml) and cumulative index (CI = 0–560) of 12 Tanzanian herbal medicines in cytotoxicity tests ranked from high to low toxicity.

	Dose range of MIC_0 against *Candida* sp.[1]	Highest test dose	Alamar Blue (HepG2)			Hoechst 33,342 (HepG2)			Glutathione depletion (HepG2)			Calcein-AM uptake (HeLa)			O_2-consumption (HepG2)			
Herbs	(µg/ml)	(µg/ml)	EFF	CI	MTD	EFF	CI	MTD	EFF	CI	MTD	EFF	CI	MTD	EFF	CI	MTD	Ranking[2]
Doxorubicin[3]			**100**	**282**	**0.058**	**100**	**494**	**0.0058**	**100**	**252**	**0.183**	**100**	**253**	**0.183**	**100**	**196**	**0.58**	5
Acacia nilotica	31–1000	500	**97**	**152**	**250**	**50**	**73**	**125**	**219**	**428**	**<7.81**	**93**	**106**	**250**	**116**	**272**	**62.5**	4.5
Clausena anisata	63–4000	500	**112**	**134**	**250**	24	25	250	**137**	**200**	**<7.81**	**89**	**108**	**250**	**110**	**227**	**62.5**	4
Jatropha multifida	250–1000	500	**90**	**119**	**250**	23	5	250	**114**	**204**	**<7.81**	**114**	**194**	**31.3**	**90**	**131**	**250**	4
Acacia robusta ssp. Usambarensis	31–1000	500	**95**	**78**	**125**	**95**	**75**	**500**	**174**	**453**	**<7.81**	**72**	**70**	**62.5**	**136**	**320**	**62.5**	3.5
Sterculia africana	63–1000	500	**111**	**225**	**125**	36	**69**	**125**	**171**	**409**	**<7.81**	**133**	**225**	**125**	20	0	250	3.5
Elaeodendron buchananii	63–250	500	**107**	**215**	**62.5**	33	18	250	**193**	**355**	**62.5**	**98**	**97**	**250**	**119**	**196**	**125**	3.5
Agauria salicifolia	500–4000	500	**91**	**125**	**250**	26	**51**	**125**	**111**	**117**	**125**	0	10	250	**97**	**156**	**31.3**	3.5
Cyphostemma hildebrandtii	250–1000	500	**96**	**89**	**125**	**65**	**86**	**62.5**	**58**	**175**	**<7.81**	**70**	**50**	**250**	**66**	46	**250**	3
Spirostachys africana	1000–2000	500	**95**	**75**	**500**	**54**	34	**500**	**169**	**199**	**<781**	**100**	**92**	**250**	21	0	500	2.5
Pteridium aquillinum	500	500	–38	0	>500	18	0	>500	**56**	**122**	**<7.81**	–3	0	>500	**103**	**102**	**250**	2
Sclerocarya birrea	63–250	500	**73**	**62**	**125**	34	14	250	**66**	**85**	**125**	**69**	**49**	**250**	**90**	**92**	**125**	1.5
Turraea holstii	63–1000	500	–44	0	>500	14	0	>500	–3	0	>500	36	16	>500	**85**	**65**	**250**	0.5

MIC_0, minimal inhibitory concentration: concentration (µg/ml) that visually showed no growth and percentage growth less than 5% spectrophotometrically of the *Candida* sp.

[1]Dose or dose range of each herbal extract wherein MIC_0 against the different tested *Candida* sp. was found (see article [5]).

[2]Herbs are ranked according to the following system: the herb receives a score per assay; when 50 < CI < 100 the test score is 0.5 and when CI > 100 the test score is 1. The sum of the test score defines the final rank of the herb. A herb is considered toxic when EFF or CI is ≥50 (the values are marked bold). A herb has weak toxicity when EFF and CI are both >20 (also the MTD was marked bold).

[3]In fig. 1F, an example of the doxorubicine curve in Alamare Blue test is given with an explanation of the efficacy, minimal toxic dosage and cumulative index.

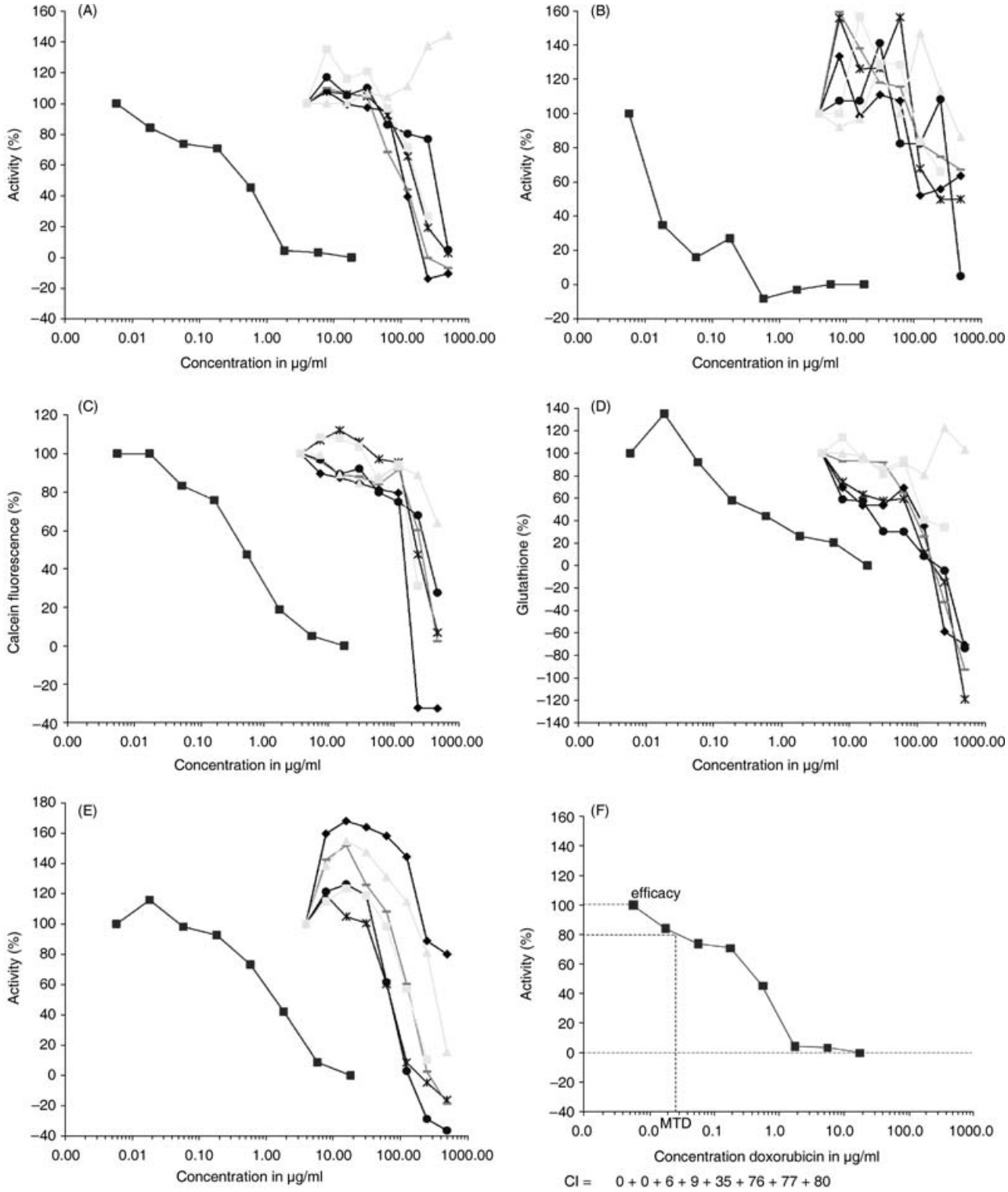

Fig. 1. Effects of *Acacia nilotica* (–✳–), *Sterculia africana* (–◆–), *Elaeodendron buchananii* (–▬–), *Pteridium aquillinum* (–●–), *Sclerocarya birrea* (–■–), *Turraea holstii* (–▲–) and reference compound doxorubicin (–■–) on mitochondrial activity (A), cellular proliferation (B), damage to cellular membrane (C), glutathione depletion (D) and the electron transport chain activity (E) as measured with Alamar Blue, Hoechst 33342, calcein-AM uptake, glutathione depletion and O_2-consumption assays, respectively. In (F), an example curve of doxorubicine in Alamar Blue assay is given for interpretation of efficacy, MTD and CI (see also table 4). Herbs were tested in dose range of 500 to 8 µg/ml and doxorubicin in 0.0058 to 18.3 µg/ml. Samples are indexed to 100% at 0.0058 µg/ml point for doxorubicin and 8 µg/ml point for the plant extracts. The mean of duplicate analysis is shown.

Table 5.

IC_{50} values (μg/ml)[1] of the Tanzanian herbal medicines on CYP2C9, 2C19, 2D6, 3A4 enzymes.

Herbs	Dose range of MIC_0 against *Candida* sp. (μg/ml)	CYP2C9	CYP2C19	CYP2D6	CYP3A4 (7BQ)	CYP3A4 (DBF)	Ranking[2]
Acacia nilotica	31–1000	1.03	5.98	22.6	3.37	3.30	2
Acacia robusta ssp. Usambarensis	31–1000	3.78	9.42	29.1	22.3	6.03	3
Agauria salicifolia	500–4000	3.12	14.34	28.6	10.2	6.74	4
Clausena anisata	63–4000	19.93	39.82	5.02	15.2	78.2	8
Cyphostemma hildebrandtii	250–1000	4.37	6.09	2.96	1.26	2.69	1
Elaeodendron buchananii	63–250	3.78	12.66	37.95	33.7	3.30	5
Jatropha multifida	250–1000	15.42	4.26	79.4	54.7	60.1	9
Pteridium aquillinum	500	4.98	12.06	70.5	8.05	16.8	7
Sclerocarya birrea	63–250	5.78	22.18	39.41	10.58	14.39	6
Spirostachys africana	1000–2000	20.00	32.22	>139	35.2	61.9	10
Sterculia africana	63–1000	22.75	66.96	>139	114	>139	12
Turraea holstii	63–1000	35.58	6.30	>139	16.0	>139	11
Positive controls		0.146	0.498	0.103	0.236	0.161	

MIC_0, minimal inhibitory concentration: concentration (μg/ml) that visually showed no growth and percentage growth less than 5% spectrophotometrically.
[1]IC_{50}: concentration of herbal medicine (μg/ml) whereby CYP activity is inhibited with 50% in comparison to that of reference compound.
[2]Ranking according to IC_{50} values.

Table 6.

Inductive activities of Tanzanian medicinal plants on human pregnane X receptor (hPXR).

Herbs	Dose range of MIC_0 against *Candida* sp. (μg/ml)	hPXR EC_{50} (μg/ml)[1]	hPXR induction factor[2]	Highest PXR induction level (μg/ml)
Acacia nilotica	31–1000	250	0.11	–
Acacia robusta ssp. Usambarensis	31–1000	250	0.44	–
Agauria salicifolia	500–4000	31.25	**5.44**	63
Clausena anisata	63–4000	93.75	**3.91**	125
Cyphostemma hildebrandtii	250–1000	15.63	**2.41**	–
Elaeodendron buchananii (Loes)	31–250	46.88	**4.49**	63
Jatropha multifida	250–1000	62.50	**5.90**	500
Pteridium aquillinum	500	93.75	**3.58**	250
Sclerocarya birrea Sond	63–250	39.10	**3.33**	16
Spirostachys africana	1000–2000	250	0.27	–
Sterculia africana	63–1000	31.25	**2.16**	125
Turraea holstii	63–1000	31.25	**4.12**	63
Tularik 0901317 (mol/l in 0.1% DMSO)		1.00×10^{-6}	6.41	1.00×10^{-4}

[1]EC_{50}, lowest plant extract concentration whereby hPXR activity is inhibited by 50% compared to reference compound.
[2]Stimulation factor is defined by means of maximal induction of the PXR activity at the most active concentration of the 11 tested concentrations with respect to the blanc signal.
An induction factor >2 is considered significant (bolt).

showed no effect at concentrations up to 500 μg/ml. Seven medicinal plant extracts (*Acacia nilotica*, *Acacia robusta*, *Agauria salicifolia*, *Cyphostemma hildebrandtii*, *Elaeodendron buchannanii*, *J. multifida*, *Spirostachys africana*, *Sterculia africana*) reduced DNA levels with Hoechst 33342. *C. hildebrandtii* had the highest effect on cellular proliferation with a MTD of 62.5 μg/ml, while again for *P. aquillinum* and *T. holstii* no effects were found. Eleven medicinal herbal extracts induced glutathione depletion, while for *T. holstii* no effect on glutathione depletion was found. Eight even scored an activity below 7.18 μg/ml. Nine medicinal plant extracts induced cellular membrane damage with calcein-AM. *J. multifida* had the strongest effect on the membrane integrity with a MTD of 31.3 μg/ml. Except for *Ster. africana* and *Spir. africana*, all herbal extracts showed toxicity in this O_2-consumption test. The highest effect was found for *A. salicifolia* with a MTD of 31.3 μg/ml.

Vitotox test.

Genotoxic effects were found for *Ster. africana*, *J. multifida* and *Spir. africana* with minimal genotoxic dosages of 1.00, 0.5 and 1.00 mg/ml, respectively. However, after metabolic activation (addition of S9), no genotoxicity was measured. In addition, the results also indicated that many of the plant extracts were toxic according to criteria mentioned (see M&M). This toxicity could mask the genotoxic response.

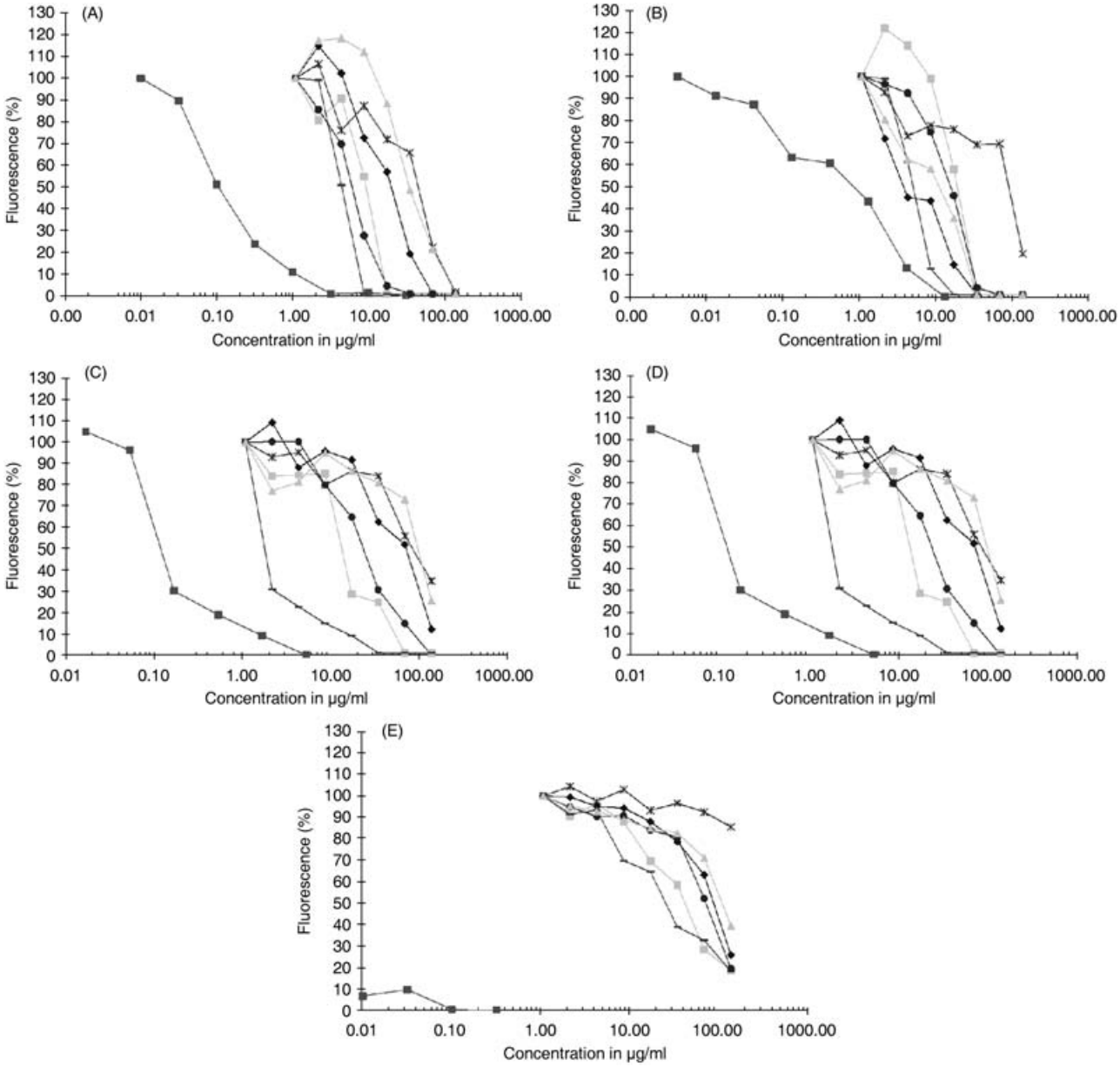

Fig. 2. Competitive activities of *Sclerocarya birrea* (–■–), *Sterculia africana* (–✳–), *Cyphostemma hildebrandtii* (–▬–), *Jatropha multifida* (–◆–), *Pteridium aquillinum* (–●–), *Turraea holstii* (–▲–) and reference compounds [sulfaphenazole (CYP2C9); tranylcypromine (CYP2C19); ketoconazole (CYP3A4) and quinidine (CYP2D6)] (–■–) on CYP2C9 (A), 2C19 (B), 3A4 7BQ (C), 3A4 DBF (D) and 2D6 (E) in a dose range study from 139.00 to 2.00 µg/ml for plant extracts and 0.010–31.40 µg/ml for sulfaphenazole, 0.004–13.30 µg/ml for tranylcypromine, 0.010–32.40 µg/ml for quinidine and 0.017–53.10 µg/ml for ketoconazole. Reference compound concentrations were converted to µg/ml according to their molecular weights. The mean of duplicate analysis is shown.

CYP450 enzyme competition assays.

The IC_{50} values for the 12 medicinal plant extracts are shown in table 5. In fig. 2, the dose–response curves were demonstrated for a representative set of six plant extracts (i.e. *A. nilotica*, *C. hildebrandtii*, *J. multifida*, *Sclerocarya birrea*, *Ster. africana* and *T. holstii*).

All herbal extracts showed dose-dependent competition on the metabolism mediated by CYP2C9, 2C19, 3A4 (7BQ and DBF) and 2D6. All herbal extracts were potent competitors with IC_{50} values of less than 100 µg/ml against CYP2C9 and CYP2C19. The most potent competitor of CYP2C9 was *A. nilotica* (1.03 µg/ml), while *J. multifida* is the most potent competitor of CYP2C19 (4.26 µg/ml). No competitive activity on CYP2D6 was found for *Spir. africana*, *Ster. africana* and *T. holstii*, while the IC_{50} values of the other nine herbal extracts ranged from 2.96–79.4 µg/ml. The most potent competitor of CYP2D6 was *C. hildebrandtii* (2.96 µg/ml). Except for *Ster. africana*, all herbal extracts were potent competitors of CYP3A4 when 7BQ was used as substrate. Furthermore, all herbal extracts with exception of *Ster. africana* and *T. holsti*, were competitive for CYP3A4 when DBF was used as substrate. *C. hildebrandtii* was also found to be the

most potent competitor of CYP3A4 when DBF and 7BQ were used as substrate (1.26 and 2.69 μg/ml, respectively).

PXR assay.

In table 6, the activation of the Tanzanian medicinal plants on PXR is shown. Significant PXR activation with an induction factor >2.00 was found for *A. salicifolia* (5.44), *C. hildebrandtii* (2.41), *E. buchannanii* (4.49), *J. multifida* (5.9), *P. aquillinum* (3.58), *S. birrea* (3.33), *Ster. africana* (2.16) and *T. holstii* (4.12). The highest activity was found for *A. salicifolia* with a stimulation factor of 5.44 at a dosage of 63 μg/ml.

CYP3A4 mRNA induction.

The concentrations of the medicinal plants that showed the highest PXR activation were used to measure the induction of the mRNA levels of CYP3A4. Significant induction of mRNA of CYP3A4 was found for *A. salicifolia*, *T. holstii* and *Ster. africana* (table 3).

Discussion

The Vitotox data indicated that *Ster. africana*, *Spir. africana* and *J. multifida* were genotoxic. However, many plant extracts also showed a strong toxic response on the bacterial cell line *Salmonella typhimurium*. Furthermore, all plant extracts showed a relative strong cellular toxicity in one or more assays. The extract of *A. nilotica* had the highest toxicity, whereas that of *T. holstii* had the lowest toxicity. Additionally, almost all extracts potently competed with CYP2C9, 2C19, 2D6 and 3A4. While 75% of the plant extracts potently activated PXR, only for *A. salicifolia*, *T. holstii* and *Ster. africana* a high induction of mRNA of CYP3A4 could be verified.

Genotoxicity of the plant extracts was evaluated with the Vitotox test. This test is based on *S. typhimurium* TA104 recN2-4 that contains a *lux* operon followed by the luciferase gene of *Vibrio fischeri*. Another control strain *S. typhimurium* TA 104*pr*1, which has a constitutively expressed luciferase gene, is used for detection of false-positive responses due to a toxic response of the tested compounds [19]. Our results indicate that most of the plant extracts had a toxic effect. These toxic effects may be due to the beneficial chemical compounds, but may also be the result of impurities with an unwanted effect. Furthermore, bactericidal activity of the plant extracts against *S. typhimurium* may cause the toxicity. In addition, another study testing African plant extracts in the Vitotox test found a high toxic response, which was proposed to be caused by interaction of the extracts with the *lux* operon [23]. Although we found three plant extracts with genotoxicity, the toxic response of the other extracts might mask the genotoxic effects. This may have been the case for *A. nilotica* [24], *Clausena anisata* [25] and *P. aquillinum* [7,26] for which previously genotoxicity has been reported.

With the cytotoxicity assays, the effects of the 12 herbal extracts on cellular toxicity were studied in human cell lines. These assays are a reliable method for high-throughput screening of toxicity effects of potential medicinal compounds [17,18]. We found a high toxicity for almost all herbal extracts at concentrations that were based on the antifungal activity of the herbs found in our previous study [5]. This high toxicity may suggest that the potent antifungal activities of these herbal extracts are due to a general toxic effect of extract ingredients, rather than to a specific growth inhibitory or killing effect on the fungi. However, most of the herbal extracts inhibited the growth of *Candida* sp. by 100%, but had no killing effect [5]. Furthermore, each plant extract had different antifungal effects on the diverse *Candida* sp. tested. For example, all herbal extracts potently inhibited *C. krusei*, while *C. albicans* was only inhibited by *C. anisata*, *S. birrea*, *Spir. africana* and *T. holstii*. Thus, the toxicity effects of the herbal extracts may be more species-specific.

According to the cellular toxicity effects as measured in the five assays, the herbal extracts were ranked from high to low toxicity in table 5. Overall, *A. nilotica* was shown to have the highest toxicity. It was previously reported that in rats that were fed an 8% *A. nilotica* diet for up to 4 weeks their body weights were significantly reduced and serum cholesterol and serum total protein significantly decreased. Symptoms reversed 1 week after treatment termination, leading the authors to conclude that *A. nilotica* had a high toxicity potential [27], but this toxic effect was not irreversible. These findings illustrate that *in vitro* test results can be extrapolated directly to *in vivo* effects. The results of the glutathione depletion assay demonstrate that most of the plant extracts show a dramatic effect on the glutathione levels. These effects can become dramatic *in vivo*, but can be prevented by treatment with N-acetylcysteine for instance after excessive paracetamol use. Furthermore, such a treatment should be in time to prevent liver necrosis. In consistence with the *in vitro* data for *C. anisata* and *P. aquillinum*, toxicity was reported in animal studies and for *A. salicifolia*, *J. multifida* and *P. aquillinum* toxicity has been reported in human beings [7,11,26,28–30]. For *Acacia robusta*, *E. buchananii* and *Ster. africana*, no earlier reports on toxicity have been found.

Interestingly, the two extracts of *S. birrea* and *T. holstii* that showed no cytotoxicity, were among the only four plant extracts that inhibited the growth of *C. albicans*, which is the most common cause of oral candidiasis [5]. Moreover, these two extracts exhibited the strongest inhibitory activities against *C. albicans* and had a broad spectra of antifungal activity [5]. Therefore, these plant extracts may be interesting candidates for further fractionation and isolation of the active compound.

In a recent review, potential interactions between commonly used herbal extracts and antiretroviral agents have been described [31]. These interactions can be based on inhibition or induction of CYPs involved in ARV metabolism. Our results revealed that 75% of the herbs potently compete with CYP3A4, CYP2C9/19 and CYP2D6. A study on 30 Indonesian medicinal plants also revealed that 63% gave significant competition with CYP3A4 and CYP2D6 [16]. In the aforementioned review, it was shown that indeed many herbs can inhibit CYP enzymes. For example, for 33% of the

American 20 Top-selling herbal extracts, the inhibition of CYP's involved in ARV metabolism was shown, and thus potential for interaction with ARVs is present [31]. CYP3A4 is the most important enzyme involved in the metabolism of all PIs and NNRTIs. Our results revealed that, with exception of *Ster. africana* and *T. holstii* when DBF is used as substrate, all plant extracts potently competed with CYP3A4. Therefore, usage of these plant extracts concomitant with ARVs may possibly lead to high plasma levels and hence toxicity. Moreover, also potent competitors of CYP2C9, 2C19 and 2D6 were found and these enzymes might be involved in potential interactions with atazanavir, nelfinavir and ritonavir, respectively.

Turraea holstii and *Ster. africana* extracts were shown to be free of competitors for CYP2D6, while extract of *T. holstii* only potently competed with CYP3A4 with 7BQ as substrate. Remarkably, although nine plant extracts showed PXR activation, only *A. salicifolia*, *T. holstii* and *Ster. africana* showed induction of CYP3A4 mRNA. Usage of these herbs may possibly lead to induction of the CYP3A4, resulting in subtherapeutic plasma levels and development of resistant HIV. To date, the only clinically significant interactions with ARVs were found for garlic, St John's wort and milk thistle and were suspected to be based on CYP3A4 induction [31].

Results of *in vitro* studies might however not correspond with the *in vivo* activity. For St John's wort, for example, some *in vitro* studies reported that St John's wort extract was a potent inducer of CYP2B6 [32], 2C9, 2C19 [33] and 3A4 [14,32–34]. Contradictory, crude St John's wort extracts have been shown to inhibit the activities of CYP2D6, 2C9, 2C19 and 3A4 [35]. In HIV patients, St John's wort led to a 35% increase in the clearance of the ARV nevirapine [13]. In addition, St John's wort has been shown to decrease plasma concentrations of the ARV indinavir with 57% in healthy volunteers [36]. Both interactions are suspected to be caused by induction of CYP3A4.

Additionally, the preparation (oral or topical) of each herbal extract influences the *in vivo* toxicity and interaction potential. The potent competition of CYP might be of less importance for topical agents, but is very relevant when the herbal extract is used orally.

Another important consideration remains that the *in vivo* concentrations of these medicinal plant extracts are unknown. However, the *in vitro* tested concentrations showed antifungal activity, in consistence with the claims of the traditional healers and, therefore, it contains the correct balance of active component(s). Furthermore, at these concentrations toxicity and CYP interactions were also found in this study. Therefore, these medicinal plant extracts might potentially give toxic effects and interactions when used by traditional healers.

In conclusion, the Vitotox test seems less appropriate for testing crude plant extracts for genotoxicity. Maybe a separation by analytical techniques into the active components will lead to a better prediction. Furthermore, almost all plant extracts have been shown to have a relative strong cytotoxicity and high CYP inhibitory potential, which may result in toxic side effects and toxic ARV plasma levels, respectively. *A. salicifolia*, *T. holstii* and *Ster. africana* were the only plant extracts found to significantly induce CYP3A4. Usage of these herbs by patients on ARVs might possibly lead to subtherapeutic plasma levels and development of drug resistant HIV strains. These results indicate that more research on the interaction potential of (African) herbal medicines is urgently needed. Furthermore, the awareness of HIV/AIDS health care workers and the general public for the risks of medicinal plant-ARV interactions needs more attention.

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