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***In vitro* modulation of glibenclamide transport by P- glycoprotein inhibitory anti-diabetic African plant extracts**[*]

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[*] Dedicated to Professor Dr. Cosimo Pizza 70th birthday in recognition of his outstanding contribution to natural product research.

Abstract

The rise of diabetes incidence in Nigeria enhances the use of popular remedies which may interact with conventional therapies. The aqueous extracts of 27 popular Nigerian 'antidiabetic' plants were tested for their *in vitro* effects on glutathione levels within HepG2 cells, P-glycoprotein (P-gp) mediated Rh-123 efflux activity in Caco2 vincristine-resistant cells and modulation of glibenclamide transport in Caco-2 monolayers. The extract from *Ximenia americana* significantly depleted intracellular glutathione at $100\,\mu g/mL$ similarly to the reference buthionine sulphoximine ($p<0.05$). Other ten extracts raised glutathione levels. Eight extracts inhibiting P-gp efflux in a concentration-dependent manner ($p<0.01$) were selected for further evaluation in a bi-directional transport model across Caco-2 monolayers: *Annona senegalensis*, *Bridellia ferruginea, Cassytha filiformis, Daniellia ogea*, *Khaya ivorensis, Syzygium guineense, Terminalia avicennioides*, and *Ximenia Americana*. When interferences in paracellular transport were discarded, only three of them may be modulating the efflux ratio of glibenclamide (Efflux ratio 2.65 ± 0.13) in the same manner the reference drug Verapamil (Efflux ratio 1.14 ± 0.25; $P<0.01$) does: *Syzygium guineense* (Efflux ratio 1.70 ± 0.23; $P<0.01$), *Terminalia avicennioides* (Efflux ratio 1.80 ± 0.25; $P<0.05$) and *Ximenia Americana* (Efflux ratio 1.66 ± 0.10; $P<0.01$). HPLC-UV analyses for P-gp inhibitors in these extracts revealed several phenolic compounds such as rutin, gallic acid and ellagic acid reported to decrease P-gp expression and/or directly modify its function. In conclusion, some popular herbal medicines used by Nigerian diabetic patients are here shown to potentially affect glibenclamide absorption at concentrations that could be reached in the intestinal tract.

Keywords: Diabetes, Drug Interactions, P-glycoprotein, Glyburide, Glutathione, *Syzygium guineense*. *Terminalia avicennioides*, *Ximenia Americana*.

Introduction

The use of herbal medicines as one of the aspects of traditional medicine practice is increasingly being promoted in developing countries as a means of meeting the unmet healthcare needs of the population [1]. As a result, many diabetic patients utilize herbal medicines as supplementation to their conventional medicines for their disease management. The chronic nature of the treatment for diabetes increases the risk of potential Herb-Drug Interactions (HDI) between traditional herbal products and prescription medicines notably those controlling blood sugar levels [2]. The implications of inhibition of drug absorption can have far-reaching effects, with hypoglycaemia being potentially fatal [3]. Thus, there is a need to anticipate such potential HDIs, which would otherwise present a further burden on the patient and on the country's healthcare system.

In order to forestall this, adequate information about the pharmacological and toxicological profile of these herbal medicines and how these would in turn affect the bioavailability of the co-administered drug is required. We previously reviewed and discussed the evidence behind more than 100 plants with ethnopharmacological records for their use in the management of diabetes in Nigeria [4], as well as their impact in Nigerian patients [5]. Only forty of the plants assessed have records of *in vitro* interactions with known pharmacokinetic parameters, with some interacting with more than one parameter. *Morinda lucida* has been shown to inhibit P- glycoprotein (P-gp) efflux as well as cytochrome and GST enzymes. More importantly, some of these identified plants -*Vernonia amygdalina, Mangifera indica* and *Phyllanthus amarus*- are currently being co-administered by diabetic patients with prescription drugs with overlapping pharmacokinetic effects as identified during the field work [5]. These brings to immediate attention those plants that would require further evaluation to confirm the presence or not of clinically relevant HDIs. In this work we will try to fill the gap by studying the effect of 27 plants on glutathione intracellular levels and P-gp efflux activity.

In the small intestine, interactions with P-gp are important in determining the bioavailability and subsequent oral dosing of the drug, either singly or in combination with other drugs. It also helps in identifying drugs that would be contraindicated, since

a co-administration may bring about altered plasma concentration levels [6]. In both the intestine and the liver, conjugation with glutathione is a fundamental part of the metabolism of xenobiotics. When the conjugate glutathione-xenobiotic is formed, it becomes the substrate for further metabolic reactions with the final product being eliminated from the organism. 'Too low' plasma levels could result in decreased efficacy or no efficacy, while 'too high' levels could result in increased toxicity. Various herbal medicines have been shown to interact with P-gp, some of which have been attributed to the presence of identified secondary plant metabolites in the plant. One of such plant metabolites is curcumin, a natural polyphenol found in the rhizomes of *Curcuma longa,* which are used by hundreds of millions of people all over the World every day both as food and as a medicine [7].

A number of studies have identified glibenclamide as a substrate and inhibitor of P-gp in P- gp overexpressing cell lines [8,9]. In line with this, clarithromycin (a known P-gp inhibitor) altered the pharmacokinetics of glibenclamide in healthy subjects by increasing its peak plasma concentration (Cmax) and area under the plasma concentration-time curve (AUC) by 1.25 and 1.35 fold respectively [10]; indicating a potential risk of P-gp induced DDI. This transport study was therefore carried out to assess the potential for HDIs between the extracts previously identified as P-gp modulators in the Rh-123 efflux assay and glibenclamide when both are co-administered by diabetic patients.

To contribute to the prediction of potential pharmacokinetic herb-drug interactions in Nigeria, we here evaluated for the first time the possible interaction of a number of these medicinal plants used in diabetes management on the efflux activity of P-glycoprotein with further evaluation of its effects on the transport of glibenclamide, itself a P-gp substrate- one of the most commonly administered drugs to diabetic patients.

Results and Discussion

The extracts were obtained in the field and extracted according to popular recipes. The conditioned extracts were chromatographed against a set of 13 pyhtochemicals. A file containing all the chromatograms has been provided as Supporting information. Our extraction protocol mimics the quantities of a typical quantity per cup: some 10 grams of plant material in 200 mL water. According Table 1, average yield of the extracts is

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12% w/w, thus implying some 1.2 g of extractable matter. Therefore a fasting patient could ingest a solution of 1.200mg/200ml = 0.6 mg/mL or 600 µg/mL, in direct contact to the intestinal epithelium. This is equivalent to six times our maximum tested concentration (100 µg/mL).

The use of HepG2 cells as a proxy for primary hepatocytes is well-established despite several limitations such as a low expression of CYP enzymes which is reversible upon re-expression of the Nuclear factor C/EBPα [11]. However, HepG2 still conserve glutathione metabolism providing with an accessible in vitro cell model for both physiological and pathological models [12]. Our results on the effects of the plant extracts on the intracellular content of glutathione are shown in Figure 1. Only the extract obtained from *Ximenia americana* significantly depleted intracellular glutathione at 100 µg/mL similarly to 10 µM of the reference buthionine sulphoximine (p<0.01). Interestingly, ten extracts significantly raised glutathione levels including *Anthocliesta djalonensis*, *Aristolochia repens*, *Bridelia ferruginea*, *Cassytha filiformis*, *Isoberlinia doka*, *Khaya senegalensis*, *Moringa oleifera*, *Mondia whitei*, *Rauvolfia vomitoria* and *Tamarindus indica*. The latest could be regarded as a protective effect, but may in turn have the result of favouring conjugation in Phase 2 thus rendering inactive drugs which detoxification heavily relies on this pathway, such as acetaminophen. However, we have to bear in mind that if the plant extracts are also inhibitors of key enzymes such Glutathione transferase (GST), then any increase of glutathione levels would not be effectively translated into conjugation of xenobiotics. This is a limitation in the interpretation of any clinical implication of the results from this assay.

To identify extracts that may modulate P-gp efflux activity, Caco-2 Vincristine-Resistant (overexpressing P-gp) were co-incubated with Rh-123 and 100 µg/mL of each extract for two hours. Cells were also incubated with 20 µM verapamil as positive control while cells with only Rh-123 served as negative control. The intracellular Rh-123 fluorescence determined after cell lysis. The percentage change in Rh-123 fluorescence relative to the fluorescence in the control cells after co-incubation with the extracts is shown in Figure 2.

Eight extracts- *Annona senegalensis*, *Bridelia ferruginea*, *Cassytha filiformis*, *Daniellia ogea*, *Khaya ivorensis*, *Syzygium guineense*, *Terminalia avicennioides* and

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Ximenia americana produced a highly significant elevation (P<0.01) in the intracellular accumulation of Rh-123 (Figure 2). Their effects are largely concentration-dependent as shown in Figure 3. These results may suggest that these plant extracts are acting as drug transport modulators via inhibition of the P-gp efflux. However, the uptake of Rh-123 into cells can be either a passive or an active process: OATP1A2-mediated facilitated transport is predominant at low single digit µM concentrations, whilst double digit µM concentrations occur via micelle-mediated passive diffusion. This and cell line-dependent factors may influence the interpretation of transport assays [13].

The highest observed inhibitory effect was exerted by *Bridelia ferruginea* followed by *Ximenia americana* and *Khaya ivorensis* with 3- to 2- fold that produced by the positive control verapamil at 20 µM. Such high changes in intracellular Rh- 123 accumulation by plant extracts are not uncommon. A similar study carried out with the stem bark extract of *Mangifera indica* produced a 1000% increase in intracellular Rh-123 accumulation [11]. As shown in Figure 3, the extracts produced a concentration-dependent inhibition of P-gp activity, which might indicate a certain degree of specificity in their effect.

HPLC-UV-PDA analyses for ubiquitous P-gp modulators pointed out that the activities of *Bridelia ferruginea,* and *Ximenia americana* may be explained at least in part by the presence of gallic acid. Gallic acid, besides its well- known antioxidant activity, can decrease P-gp expression in HK-2 cells [15] and directly modify its function on KB-C2 cells [16]. *Syzygium guineense* and *Annona senegalensis* contain rutin and ellagic acid, respectively. Rutin and Ellagic acid, have been also described as inhibiting efflux pumps and transporters [17–19].

To evaluate the effects of the selected P-gp inhibitory extracts on the permeability and transport of glibenclamide, three parameters were measured: the TEER value before and after the transport experiment, the apparent permeability of the paracellular marker FD4 across the Caco-2 monolayers and the apparent permeability of glibenclamide across the monolayer. The first two measurements were carried out to monitor the effect of the extracts on the integrity of the monolayer, while the last measurement was for direct estimation of its effect on glibenclamide transport (Table 2).

The maturation of tight junctions during differentiation of Caco-2 cells grown *in vitro*
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is a crucial factor for its use as a model for intestinal drug permeability and transport.

Various studies recommend the use of cells that have been grown in a monolayer for at least 21 days for transport experiments based on evidence of morphological changes related to cellular differentiation [12]. A decrease in the permeability of the cell monolayer is an indicator for tight junction maturation and can be monitored by measuring the TEER values of the monolayer over several days [13].

In bi-directional transport experiments, P-gp substrates are confirmed by comparing apparent permeability in the apical to basolateral (AP-BL) direction with that in the basolateral to apical (BL-AP) direction. Compounds with efflux ratio (*P*app (BL-AP)/*P*app (AP-BL) greater than 2 to 3 are typically identified as P-gp substrates [14]. This study obtained an average efflux ratio of 2.65 ± 0.13 for glibenclamide, thereby confirming it as a P-gp substrate (Table 4.7). As far as we know, this is the first time glibenclamide is experimentally confirmed as a P-gp substrate in such bi-directional transport model.

A typical P-gp inhibitor would decrease the efflux of the P-gp substrate, thereby decreasing the permeability in the efflux direction or secretory permeability or *P*app (BL-AP) while increasing the absorptive permeability *P*app (AP-BL). This change in the *P*app of both directions would ultimately result in a decreased efflux ratio. As shown in table 3, verapamil the control P-gp inhibitor decreased the *P*app (BL-AP) of glibenclamide from 6.3×10^{-5} cm/s to 4.62×10^{-5} cm/s and increased its *P*app (AP-BL) from 2.65×10^{-5} cm/s to 4.03×10^{-5} cm/s, thereby decreasing its efflux ratio from 2.65 to 1.14 (P<0.01).

Interferences in paracellular transport (denoted by a significant *P*app FD4) were suspected for *Cassytha filiformis* and *Khaya ivorensis*. With those discarded, only three of them may be clearly modulating the efflux ratio of glibenclamide in the same manner the reference drug Verapamil (Efflux ratio 1.14 ± 0.25; P<0.01) does: *Syzygium guineense* (Efflux ratio 1.70 ± 0.23; P<0.01)*, Terminalia avicennioides* (Efflux ratio 1.80 ± 0.25; P<0.05) and *Ximenia americana* (Efflux ratio 1.66 ± 0.10; P<0.01). Contrary to expectation that all the active extracts in the Rh-123 efflux assay would interfere with the transport of glibenclamide, only two of them – *Syzygium guineense* and *Terminalia avicennioides* - produced a similar effect as verapamil in this model as seen in Table 2. They both increased the *P*app (AP-BL) of glibenclamide to 3.38 and 3.06×10^{-5} cm/s respectively and decreased the *P*app (AP-BL) of glibenclamide to 5.7

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and 5.56×10^{-5} cm/s respectively. Aside for its possible P-gp inhibitory effect, *Terminalia avicennioides* also produced a greater effect on membrane permeability with possible opening of tight junctions, evidenced by its increased Papp (AP-BL) transport of FD4 and a higher change in TEER values compared to glibenclamide alone. This could in turn contribute to the increased Papp (AP-BL) seen with glibenclamide.

The reasons for these conflicting results between the Rh-123 efflux assay and the bidirectional transport assay for the other extracts might be due to other unidentified interacting effects of the extracts. For instance, although *Khaya ivorensis* increased the Papp (AP-BL) of glibenclamide from $2.65 - 5.6 \times 10^{-5}$ cm/s, the Papp (BL-AP) also increased very slightly from 6.13×10^{-5} cm/s to 6.6×10^{-5} cm/s. One possible suggestion for the increased effect of *Khaya ivorensis* on glibenclamide transport in the absorptive direction might be due to an effect on membrane permeability given the higher change in TEER values but these would need to be clarified. These studies have however highlighted the possibility of obtaining false positives in the identification of P-gp modulators simply based on *in vitro* intracellular accumulation assays.

Although the clinical implication of the P-gp inhibitory effect of these two extracts on glibenclamide bioavailability can only best be assessed in an *in vivo* study in humans, it may be suggested that its effects on glibenclamide bioavailability is likely to be minimal. This is because the extracts only caused a slight increase in the absorptive apparent permeability of glibenclamide. With a borderline efflux ratio of 2.38, glibenclamide would only be considered as a moderate P-gp substrate such that the P-gp inhibition of the extracts did not bring about a significant change in its apparent permeability. As this is the first time the identity of glibenclamide as a P-gp substrate based on a bi-directional transport model is being carried out, a comparison of the effect of well-known P-gp inhibitors on the Papp of glibenclamide alongside other P-gp substrates with higher efflux ratios would therefore need to be carried out to ensure a thorough assessment.

Finally, some of the extracts did not exhibit the expected characteristics of P-gp modulators either in the P-gp screening or the bi-directional transport, they could still modulate intestinal drug absorption through their effects on tight junctions. Further experiments would be needed to confirm this especially as decrease in TEER values

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have been identified as a cytotoxic mechanism that could potentially affect intestinal drug absorption [19].

Glutathione levels may affect/modulate P-gp (a MRP1 protein) mediated transport in this model is a very complex manner. On the one hand, it has been reported that MRP1 transports vincristine in an ATP- and GSH-dependent manner [24], so in principle extracts increasing GSH will positively influence efflux activity. On the other hand, GSH conjugates, GSH and GSH disulfide are actively transported across membranes using the same MRP1 transport protein, which may itself compete with xenobotic efflux [25]. Ballatori and co-workers affirm that this high background rate of GSH transport is a major confounding variable in GSH transport measurements, and severely limits the choice of cells with which this process can be studied. To further complicate matters, GSH tself facilitates membrane transport of endo- and xenobiotics by stimulating MRP-mediated transport [26].

We may conclude that more *in vivo* experiments are warranted to ascertain the effect of the here reported active herbals. In the meantime, we suggest that diabetic patients on glibenclamide, the second most prescribed anti-diabetic drug in Nigeria, using herbal remedies containing *Syzygium guineense Terminalia avicennioides* and *Ximenia americana* should be specially monitored by healthcare professionals, for these herbals modulated P-gp efflux in our models at concentrations that could be reached in the intestinal tract. We also suggest caution to those regularly using *Ximenia americana* if they need to use drugs which detoxification is dependent on glutathione.

Material and Methods

Chemicals

HPLC standards (purity ≥ 95%), Fluorescein isothiocyanate-dextran (FD4), Rhodamine-123 (Rh-123) and glibenclamide (Glib) were purchased from Sigma-Aldrich with purity ≥ 95%. DMEM (Dulbecco's Modified Eagle Media), GlutaMAX, FBS (fetal bovine serum), Penicillin-Streptomycin antibiotic, 0.05% Trypsin/EDTA, trypan blue, PBS (Phosphate saline buffer), pH 7.4 were from Gibco. Verapamil (Securon I.V. 2.5mg/mL), and vincristine 2mg/mL (Hospira) were both purchased from AAH Pharmaceuticals.

Plant Material

Plant materials were either bought in markets or collected from different regions in Nigeria in November 2012 by one of the authors (U.F. Ezuruike). Identification of collected plant samples collected in the North was done by Mr. Ibrahim Muazzam, an ethnobotanist and staff of the Department of Medicinal Plant Research & Traditional Medicine, National Institute for Pharmaceutical Research & Development (NIPRD). Plant samples collected in the South were verified by staff of the Forestry Research Institute of Nigeria (FRIN) in Ibadan. Vouchers were deposited in the Department of Pharmaceutical and Biological Chemistry, University College London School of Pharmacy, United Kingdom.

Extraction procedures

The procedure for extraction of the plant samples (infusion or decoction) was based on the traditional use of the plants as described by the respondents (Table 2-3). For infusion, 300 ml of boiled distilled water was added to 10g of plant material with continuous stirring for 15 minutes on a laboratory hot plate magnetic stirrer (IKA Werke Labortechnik). For decoction, 20 g of plant material was heated continuously under reflux in a round bottom flask containing 400 ml of distilled water for one hour using an Electrothermal 3-in-1 laboratory heating mantle. All extracted plant samples were then allowed to stand until cold before filtering with a Buchner flask. All the filtered extracts were frozen in round bottom flasks and then freeze dried (Edwards Pirani 501 Savant super modulo freeze drier) to obtain the dried extract. All dried plant extracts were then stored in 10 ml glass vials at -80°C until needed.

For analyses and cell assays, plant stock concentrations (50 mg/mL) were prepared by dissolving crude extracts in bidistilled, microfiltered, aseptic water (MiliQ). The solutions were cold sterilised by microfiltration (Millipore disk filters, 0.22 microns) in a laminar flow cabinet into sterile microcentrifuge tubes. These aliquots were at -80°C until further testing.

Phytochemical analyses

For phytochemical fingerprinting, chromatograms were obtained in an Agilent 1100 Series (Gradient Quaternary Pump, Online degasser, Photodiode array detector) Software ChemStation. Elution conditions for phytomarkers were as
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described by

Giner et al. [20] on a Phenomenex C18 column (250 × 4.6 mm id, 5 μm). Solvent A (H2O + Acetic Acid 0.2 %) and B (methanol + Acetic Acid 0.2 % were mixed in gradient mode as follows: 0 min 90% A, 0-5 min 80% A, 5-45 min 50% A, 45-55 min 20% A; flow rate 0.8 mL/min. The injection volume, column temperature, and UV wavelength were set at 80 μL, 30°C and 254 nm, respectively.

Cell lines and cell culture

HepG2 cells (Sigma-Aldrich) were cultured in MEM medium (GIBCO) supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL). A Caco-2 cell line resistant to vincristine (Caco-2 VCR) with enhanced Rh-123 efflux activity due to P-gp overexpression was established and kindly provided by Dr E. Chieli (University of Pisa, Italy). Caco2 VCR cells were cultured in DMEM medium (GIBCO) supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), 1% non-essential aminoacid (NEEA) and 20 μM of Vincristine (Securon ampoules). All cells were kept at 37°C in an incubator with a humidified atmosphere of 5%CO_2.

Cell viability assays

Neutral Red (NR) uptake studies were performed according the protocol detailed by Repetto et al. [21]. Briefly, the cells were cultured in 96-well tissue culture plates by adding 200μL/well of a suspension of 2 x 10^4 after 24-hours incubation of Caco-2 cells with the plant extracts, media was removed and 100 μL of NR solution (40μg/mL) pre-warmed at 37° C was added to each well and all plates were placed in the incubator at 37° C for 2h. The cells were rinsed with 150 μL of PBS and the washing solution removed by decanting or gently tapping the plate. 150μL of 'neutral red extracting' solution (96% ethanol, deionised water, glacial acetic acid; 50%:49%:1%) was added to each well of the plate after the washing step. The plate was immediately shaken on a microplate shaker (IKA MS3 basic shaker) for at least 10 minutes until the neutral red had been completely extracted from the cells and formed a homogenous solution. The optical density (O.D.)/absorbance of the extracted neutral red was read out in the plate reader (Tecan) at 540nm. The Maximum Non-Toxic Concentration (MNTC) was calculated as the concentration of treatment not killing more than 10% of the cell population.

Determination of glutathione

This protocol is a modification of enzymatic recycling methodologies reported by Allen et al. [22] and Rahman et al. [23]. HepG2 cells (4×10^4 cells/well) were plated out in transparent flat-bottom 96 well plates and incubated for cell at 37°C with 5% CO2 for 24 hours. Afterwards, the cells were treated with the plant extracts (MNTC or 100 µg/mL, whichever is lower) and the 10 µM of the positive control buthionine sulphoximine (BSO) incubated for 24 hours at same conditions. Subsequently, the medium of each well was aspirated and the wells were washed with 200 µl of PBS. Cells were then lysated with Triton X-100 0.1% solution (50µl) and 25 µl of the cell solution were transferred to another 96 well plate for further protein analysis.

The plates were then treated with 25 µl 5%(v/v) sulfosalicylic acid solution and 25 µl of the solution were aliquoted for analysis. GSH reduced standards at concentrations from 100 to 0.78 µM were prepared and 25 µl were added to the plates. 25 µl of 5% (v/v) sulfosalicylic acid were also added the plate to serve as blank control. The glutathione reaction buffer (7.5 mL of sodium phosphate buffer solution, 2mg/mL of 2.39mM β-NADPH solution and 1mL of 0.01M DTNB (5,5'-Dithiobis-(2-Nitrobenzoic Acid) solution) was added to the plates (125 µl) and the absorbances were read every 30 seconds for 5 minutes at 405nm. Total GSH concentration in the samples was calculated by the following formula: GSH concentration unknown = (GSH ABS ± b)/m, where: GSH ABS is the slope (rate of change) of the DTNB absorbance of the unknown; m is the slope (the rate of change) of the glutathione standard curve; b is the y-intercept of the glutathione standard curve.

Rhodamine-123 uptake assay

Rhodamine-123 (Rh-123) uptake/efflux assays were conducted as described by Chieli et al. [11] with minor modifications. Caco-2 Vincristine-Resistant cells were seeded into 96-well plates for 24 hours to allow for attachment. Then, the growth media was changed to serum free media. Rh-123 (5 µg/mL) was added to the wells already containing MNTC or 100 µg/mL -whichever was lower- of the extracts. 20µM verapamil was used as drug reference. After 2 hours incubation, cells were washed with 20µM verapamil in PBS to stop P-gp efflux activity. Supernatant was taken out and cells lysed with 100 µL of 0.1% Triton X-100 in PBS and the plates were placed in the incubator for 15 minutes. 80 µL of the cell lysates was transferred to a 96-well black

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plate and the fluorescence intensity in each well measured in a Tecan plate reader (Exc-485nm, Em-525nm). The cellular accumulation of Rh-123 for each of the extracts was expressed as the percentage of the accumulation of Rh-123 in the presence of water only.

Drug In vitro Transport in Caco-2 Monolayers

The protocol used for this assay is as described in Hubatsch et al [24] with slight modifications.

Preparation of the cell culture: 24-well cell culture polyethylene terephthalate (PET) inserts with 0.4 µM pore size and 0.33 cm^2 surface area (Millipore-PIHT12R48) pre-loaded in sterile 24-well plates were used for the transport studies. Caco-2 wild type cells from maximally confluent flasks (80- 90%) were trypsinized and then seeded out in the apical layer of the permeable filters at a density of 5 x 10^5 cells/mL in 200µL of complete media (1 x 10^5 cells/insert). The filters were pre-wet with 30µL of complete media prior to seeding. The basolateral chamber of the inserts was each filled with 1 mL of complete cell media. The plates were then placed in the incubator overnight (or for a maximum of 16 hours) to allow for cell attachment. After the incubation period, the medium in the apical layer was replaced with 0.2 mL of fresh complete cell medium so as to remove non-adherent cells as well as to prevent the formation of a multilayer.

Maintenance of the cell culture: Cells were maintained every other day for at least 21days by carefully aspirating off the media in both the apical and basolateral layers and replacing with appropriate volume of fresh medium. The transepithelial electrical resistance (TEER) of the monolayer was measured at least once each week with a Millicell ERS-1 voltammeter (Millipore) to monitor the integrity of the monolayer. Cells were only used for transport experiments if TEER values were $> 300\ \Omega \cdot cm^2$. This was calculated by subtracting the resistance reading from that of a blank filter without cells and multiplying by the surface area of the monolayer. Culture media of the cells were changed 24 hours prior to the transport experiment to prevent exhaustion of the media nutrients.

Transport and Donor solutions: Hank's balanced salt solution (HBSS) at pH 7.4 buffered with 25mM HEPES and 0.35g/l NaHCO3 was prepared as the transport

solution. 10 µM glibenclamide alone or with the selected plant extracts at a concentration of 50 or 100 µg/mL, depending on the estimated MNTC. The positive control consisted on 20µM verapamil added to the transport solution containing glibenclamide. The receiver solution was either the HBSS transport solution alone for glibenclamide transport alone, or the transport solution with either the extracts or verapamil. All donor solutions contained 1mg/mL Fluorescein isothiocyanate-dextran (FD4) for monitoring the integrity of the monolayer during the experiment. All solutions were sterile filtered with 0.22µm Millipore filters and then pre-warmed at 37°C before use.

Transport experiments: The inserts (membrane supports) with the cell monolayers to be used for the transport experiment were pre-washed to remove residual medium by gently replacing the media with the HBSS transport solution. The washing was done under gentle shaking at 60 rpm for 15-20 minutes on a PS-3D laboratory orbital shaker (Grant-bio Instruments) within a humidified incubator without CO2 set at 37°C (Memmert D-91126). After washing, the TEER of the monolayers was measured prior to commencing the transport studies.

Transport studies were carried out in the apical to basolateral (AP-BL) and basolateral to apical (BL-AP) directions. For AP-BL transport studies, the washing solutions were gently removed from the inserts and the 24-well plate and replaced with 1mL of the appropriate receiver solution in the BL layer and 0.2mL of the appropriate donor solution in the AP layer. For BL-AP transport studies, the washing solution was replaced with 0.2mL of the appropriate receiver solution in the AP layer and 1 mL of the appropriate donor solution in the BL layer. An excess of 200 µL was added to all donor solutions when adding them to inserts, which was immediately removed at t = 0 min and used to estimate C0. Sample withdrawals were carried out from the chamber containing the receiver solution at regular time intervals (15, 30, 60, 90 and 120 min) by removing 0.5 mL for AP-BL transport and 0.19 mL for BL-AP transport at each time point. Immediate replacement of the exact volume withdrawn by the appropriate receiver solution was done. The withdrawn samples were placed into microcentrifuge tubes and immediately covered and placed on ice to prevent sample evaporation. Care was taken during sample withdrawals not to disrupt the monolayer of cells. The culture plate with inserts was placed back on the orbital shaker in the incubator in between sample withdrawals to maintain sink conditions.

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Analytical procedures: Two sets of sample analysis were done for the withdrawn samples. An aliquot (50μL) from each time interval was used for spectrophotometric analysis (Ex-490nm, Em-520nm) of the transported FD4. A calibration curve of FD4 was included in each plate and used to estimate the amount of FD4 transported at each time point. The remaining withdrawn samples were analysed using a Perkin Elmer HPLC equipped with an UV-Vis photodiode array detector to measure the amount of glibenclamide transported at each time point. The HPLC conditions used for glibenclamide analysis were as follows: stationary phase was a Phenomenex Synergy Polar-RP80A column (150 x 4.6 mm, I.D = 4 μm) column maintained at 30°C. The mobile phase was an isocratic system consisting of 40% (A) 25 mM potassium phosphate buffer (pH 4.5) and 60% (B) Acetonitrile. The injected volume of sample was 20 μL and the UV-Vis spectrum was monitored at 230 nm with glibenclamide eluting at 2 minutes.

Calculations: the apparent permeability (Papp) of glibenclamide across the monolayers was estimated as follows: Papp = dQ/dt (1/AC0); where dQ/dt is change in the amount of drug (nM) transported per time, A is the surface area of the insert and C0 is the initial concentration of the drug in the donor chamber.

Statistics

Raw data were normalised to percentage with respect to controls. Results are expressed as the mean ± SD, N ≥ 3. Bars graphs were drawn with Excel (Microsoft). Significance of the results was assessed with Instat (GraphPad Software) and expressed as * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$) and **** ($P<0.0001$). In single concentrations studies all treatments were compared to controls using Dunnet Multiple *t*-test. In concentration-dependent studies all values of a treatment were compared to each other using Tukey-Kramer Multiple Comparisons Test.

All numerical data and statistical analyses presented in the graphs are provided in detail as Supporting information.

Abbreviations

Drug-Drug Interactions (DDI); HDI; Herb-Drug Interactions; P-glycoprotein (P-gp); Maximum Non-Toxic Concentration (MNTC); Vincristine Resistant (VCR); Rhodamine-123 (Rh-123); Glibenclamide (Glib).

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Supporting information

Voucher numbers and HPLC Chromatograms for all extracts as well as tabulated numerical data and their statistical analyses are available as Supporting Information.

References

1   Campbell-Tofte J, Mølgaard P, Winther K. Harnessing the potential clinical use of medicinal plants as anti-diabetic agents. Bot Targets Ther 2012; 2: 7

2   May M, Schindler C. Clinically and pharmacologically relevant interactions of antidiabetic drugs. Ther Adv Endocrinol Metab 2016; 7: 69–83

3   Allen, BL; Bush, TM; Elizabeth R. Greyber, MD; Holloway, SW; Kantor, S; Lam, T; Rayburn, KS; Roth, LW; Sanchez-Yamamoto, DS; So, BK; De H. Tran M. Adverse interactions between herbal and dietary substances and prescription. Altern Ther Health Med 2007; 13: 30–35

4   Ezuruike UF, Prieto JM. The use of plants in the traditional management of diabetes in Nigeria: pharmacological and toxicological considerations. J Ethnopharmacol 2014; 155: 857–924

5   Ezuruike U, Prieto JM. Assessment of potential herb-drug interactions among Nigerian adults with type-2 diabetes. Front Pharmacol 2016; 7: 248

6   Zhang D, Luo G, Ding X, Lu C. Preclinical experimental models of drug metabolism and disposition in drug discovery and development. Acta Pharm Sin B 2012; 2: 549–561

7   Bogacz A, Deka-Pawlik D, Bartkowiak-Wieczorek J, Karasiewicz M, Kujawski R, Kowalska A, Chałas A, Czerny B, Grześkowiak E, Mrozikiewicz PM, Bogacz A, Deka-Pawlik D, Bartkowiak-Wieczorek J, Karasiewicz M, Kujawski R, Kowalska A, Chałas A, Czerny B, Grześkowiak E, Mrozikiewicz PM. The effect of herbal materials on the P-glycoprotein activity and function. Herba Pol 2013; 59: 129–141

8   Gedeon C, Behravan J, Koren G, Piquette-Miller M. Transport of glyburide by placental abc transporters: implications in fetal drug exposure. Placenta 2006; 27: 1096–1102

9   Golstein PE, Boom A, van Geffel J, Jacobs P, Masereel B, Beauwens R. P-glycoprotein inhibition by glibenclamide and related compounds. Pflugers Arch 1999; 437: 652–660

10   Lilja JJ, Niemi M, Fredrikson H, Neuvonen PJ. Effects of clarithromycin and grapefruit juice on the pharmacokinetics of glibenclamide. Br J Clin Pharmacol 2007; 63: 732–740

11   Jover R, Bort R, Gómez-Lechón MJ, Castell J V. Re-expression of C/EBPα induces CYP2B6, CYP2C9 and CYP2D6 genes in HepG2 cells. FEBS Lett 1998; 431: 227–230

12   Huang Z-Z, Chen C, Zeng Z, Yang H, Oh J, Chen L, Lu Sc. Mechanism and significance of increased glutathione level in human hepatocellular carcinoma and liver regeneration. FASEB J 2001; 15: 19–21

13   Forster S, Thumser AE, Hood SR, Plant N. Characterization of rhodamine-123 as a tracer dye for use in in vitro drug transport assays. PLoS One 2012; 7: e33253

14   Chieli E, Romiti N, Rodeiro I, Garrido G. In vitro effects of *Mangifera indica* and polyphenols derived on ABCB1/P-glycoprotein activity. Food Chem Toxicol 2009; 47: 2703–2710

15   Chieli E, Romiti N, Rodeiro I, Garrido G. In vitro modulation of ABCB1/P-glycoprotein expression by polyphenols from *Mangifera indica*. Chem Biol Interact 2010; 186: 287–294

17

16   Kitagawa S, Nabekura T, Kamiyama S, Takahashi T, Nakamura Y, Kashiwada Y, Ikeshiro Y. Effects of alkyl gallates on P-glycoprotein function. Biochem Pharmacol 2005; 70: 1262–1266

17   Zhang H-M, Zhao L, Li H, Xu H, Chen W-W, Tao L. Research progress on the anticarcinogenic actions and mechanisms of ellagic acid. Cancer Biol Med 2014; 11: 92–100

18   Tan KW, Li Y, Paxton JW, Birch NP, Scheepens A. Identification of novel dietary phytochemicals inhibiting the efflux transporter breast cancer resistance protein (BCRP/ABCG2). Food Chem 2013; 138: 2267–2274

19   Mohana S, Ganesan M, Agilan B, Karthikeyan R, Srithar G, Beaulah Mary R, Ananthakrishnan D, Velmurugan D, Rajendra Prasad N, Ambudkar S V. Screening dietary flavonoids for the reversal of P-glycoprotein-mediated multidrug resistance in cancer. Mol Biosyst 2016; 12: 2458–2470

20   Hilgers AR, Conradi RA, Burton PS. Caco-2 Cell Monolayers as a Model for Drug Transport Across the Intestinal Mucosa. Pharm Res 1990; 07: 902–910

21   Ranaldi G, Consalvo R, Sambuy Y, Scarino ML. Permeability characteristics of parental and clonal human intestinal Caco-2 cell lines differentiated in serum-supplemented and serum-free media. Toxicol In Vitro 2003; 17: 761–767

22   Balimane P V, Han Y-H, Chong S. Current Industrial Practices of Assessing Permeability and P-Glycoprotein Interaction. 2006; AAPS J. 8(1): E1–E13.

23   Narai A, Arai S, Shimizu M. Rapid decrease in transepithelial electrical resistance of human intestinal Caco-2 cell monolayers by cytotoxic membrane perturbents. Toxicol In Vitro 1997; 11: 347–354

24   Renes J, de Vries EG, Nienhuis EF, Jansen PL, Müller M. ATP- and glutathione-dependent transport of chemotherapeutic drugs by the multidrug resistance protein MRP1. Br J Pharmacol 1999; 126: 681–688

25   Cole SPC, Deeley RG. Transport of glutathione and glutathione conjugates by MRP1. Trends Pharmacol Sci 2006; 27: 438–446

26   Ballatori N, Krance SM, Marchan R, Hammond CL. Plasma membrane

glutathione transporters and their roles in cell physiology and pathophysiology. Mol Aspects Med 2009; 30: 13–28

27   Giner RM, Recio MC, Cuellar MJ, Mai~ez S, Peris JB, Stobing G, Mateu I, Rios J-L. A Taxonomical Study of the Subtribe Leontodontinae Based on the Distribution of Phenolic Compounds. Biochem Syst Ecol 1993; 21: 613–616

28   Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc 2008; 3: 1125–1131

29   Allen S, Shea JM, Felmet T, Gadra J, Dehn PF. A kinetic microassay for glutathione in cells plated on 96-well microtiter plates. Methods Cell Sci 2000; 22: 305–312

30   Rahman I, Kode A, Biswas SK. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. Nat Protoc 2006; 1: 3159–3165

31   Hubatsch I, Ragnarsson EGE, Artursson P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. Nat Protoc 2007; 2: 2111–2119

TABLE 1. Summary of the codes, botanical details, source, extraction and maximum non-toxic concentrations (MNTC) of the selected plant extracts.

Code	Plant name	Family	Plant Part	Source	Extract	Yield (%w/w)	MNTC (24h; µg/mL) HepG2	Caco2
AB	*Alstonia boonei* De Wild.	Apocynaceae	Stem-bark	Mushin herbal	Decoction	5.03	100	100
AL	*Anogeissus leiocarpa* (DC.) Guill. & Perr.	Combretaceae	Stem-bark	Herbalist in	Decoction	6.21	20	50
AD	*Anthocliesta djalonensis* A.Chev.	Gentianaceae	Stem-bark	Agbede forest	Decoction	3.05	5	>100
AR	*Aristolochia repens* Mill.	Aristolochiaceae	Stem	Mushin herbal	Infusion	18.11	50	>100
AS	*Annona senegalensis* Pers.	Annonaceae	Stem-bark	Agbede forest	Decoction	7.10	50	50
BF	*Bridelia ferruginea* Benth.	Phyllanthaceae	Stem-bark	Ibadan herbal	Decoction	75.86	50	50
CF	*Cassytha filiformis* L.	Lauraceae	Whole plant	Ethnobotanist in	Decoction	12.66	100	>100
CS	*Cassia sieberiana* DC	Leguminosae	Root	Agbede_forest	Decoction	10.27	50	>100
DO	*Daniellia ogea* (Harms) Holland	Leguminosae	Stem-bark	Agbede forest	Decoction	6.05	50	50
GL	*Gongronema latifolium* Benth.	Apocynaceae	Leaves	Mushin herbal	Infusion	8.37	100	>100
ID	*Isoberlinia doka* Craib & Stapf	Leguminosae	Stem	Zaria herbal	Decoction	4.69	100	>100
KI	*Khaya ivorensis* A.Chev.	Meliaceae	Stem-bark	Mushin herbal	Decoction	7.83	100	>100
KS	*Khaya senegalensis* (Desv.) A.Juss.	Meliaceae	Stem-bark	Agbede forest	Decoction	5.02	1	>100
MO	*Moringa oleifera* Lam.	Moringaceae	Leaves	Home garden in	Infusion	35.54	1	>100
MW	*Mondia whitei* (Hook.f.) Skeels	Apocynaceae	Stem	Mushin herbal	Infusion	20.24	1	>100
OG	*Ocimum gratissimum* L.	Lamiaceae	Leaves	Mushin herbal	Infusion	17.04	1	>100
PN	*Picralima nitida* (Stapf) T.Durand & H.Durand	Apocynaceae	Seeds	Ibadan herbal	Infusion	23.71	100	>100
RV	*Rauvolfia vomitoria* Afzel.	Apocynaceae	Stem-bark	Ibadan herbal	Decoction	3.9	1	>100
SD	*Scoparia dulcis* L.	Plantaginaceae	Whole plant	Ethnobotanist in	Decoction	16.35	100	>100
SG	*Syzygium guineense* (Willd.) DC.	Myrtaceae	Stem	Zaria herbal	Decoction	8.01	5	>100
SH	*Strophanthus hispidus* DC.	Apocynaceae	Stem	Ibadan herbal	Decoction	12.2	1	>100
SL	*Securidaca longipedunculata* Fresen.	Polygalaceae	Root	Mushin herbal	Decoction	7.49	10	>100
TA	*Terminalia avicennioides* Guill&Perr.	Combretaceae	Stem	Zaria herbal	Decoction	13.25	100	>100
TB	*Tapinanthus bangwensis* (Engl.&K.Krause) Danser	Loranthaceae	Leaves	Herbalist in Imo	Infusion	32.2	1	>100
TI	*Tamarindus indica* L.	Leguminosae	Stem	Zaria herbal	Decoction	6.05	20	>100
VA	*Vernonia amygdalina* Delile	Compositae	Leaves	Home garden in	Infusion	15.54	100	>100
XA	*Ximenia Americana* L.	Olacaceae	Stem	Zaria herbal	Decoction	11.5	100	>100

Table 2 Effects of P-gp inhibitory plant extracts on the *P*app values of glibenclamide and FD4 and the change in TEER values after 2 hours transport experiments in Caco-2 monolayers. Results show mean ± SD, N ≥ 3. *P<0.05, **P<0.01 according Dunnett Multiple Comparisons Test.

| Sample	Direction of transport	% of Initial TEER	*P*app Glib (x 10^{-5} cm/s)		Efflux ratio (Glib)		*P*app FD4 (x 10^{-6} cm/s)	
Gliblenclamide (10 µM)	AP-BL	67 ± 17	2.65 ± 0.13	-	2.38 ± 0.23	-	5.62 ± 0.32	-
	BL-AP	63 ± 20	6.30 ± 0.33	-			5.05 ± 0.51	-
Glib (10µM) + Verapamil (20 µM)	AP-BL	63 ± 38	4.06 ± 0.23	**	1.14 ± 0.25	**	-	-
	BL-AP	64 ± 11	4.62 ± 0.26	**			-	-
Glib (10µM) + *A. senegalensis* (50 µg/ml)	AP-BL	48 ± 13	2.36 ± 0.14	ns	2.17 ± 0.19	ns	6.20 ± 0.54	ns
	BL-AP	23 ± 7	5.13 ± 0.25	**			5.64 ± 0.49	ns
Glib (10µM) + *B. ferruginea* (50 µg/ml)	AP-BL	44 ± 13	1.66 ± 0.08	**	2.51 ± 0.16	ns	4.90 ± 1.88	ns
	BL-AP	17 ± 6	4.16 ± 0.23	**			5.42 ± 2.08	ns
Glib (10µM) + *C. filiformis* (100 µg/ml)	AP-BL	70 ± 28	1.83 ± 0.12	**	1.60 ± 0.15	**	2.93 ± 0.38	ns
	BL-AP	49 ± 36	2.93 ± 0.18	**			2.11 ± 0.28	*
Glib (10µM) + *D. ogea* (50 µg/ml)	AP-BL	45 ± 31	1.17 ± 0.06	**	4.97 ± 0.20	**	4.40 ± 1.86	ns
	BL-AP	23 ± 9	5.82 ± 0.33	ns			3.95 ± 1.67	ns
Glib (10µM) + *K. ivorensis* (100 µg/ml)	AP-BL	26 ± 9	5.60 ± 0.32	**	1.19 ± 0.34	**	0.77 ± 0.09	*
	BL-AP	18 ± 6	6.66 ± 0.36	ns			1.87 ± 0.21	*
Glib (10µM) + *S. guineense* (100 µg/ml)	AP-BL	53 ± 23	3.38 ± 0.16	**	1.70 ± 0.23	**	5.80 ± 1.60	ns
	BL-AP	20 ± 5	5.76 ± 0.29	ns			2.99 ± 0.83	ns
Glib (10µM) + *T. avicennioides* (100 µg/ml)	AP-BL	25 ± 17	3.09 ± 0.19	*	1.80 ± 0.25	*	9.67 ± 2.59	ns
	BL-AP	19 ± 5	5.56 ± 0.32	*			2.41 ± 0.65	ns
Glib (10µM) + *X. americana* (100 µg/ml)	AP-BL	28 ± 13	1.37 ± 0.07	**	1.66 ± 0.10	**	9.33 ± 3.97	ns
	BL-AP	25 ± 27	2.27 ± 0.12	**			3.37 ± 1.44	ns
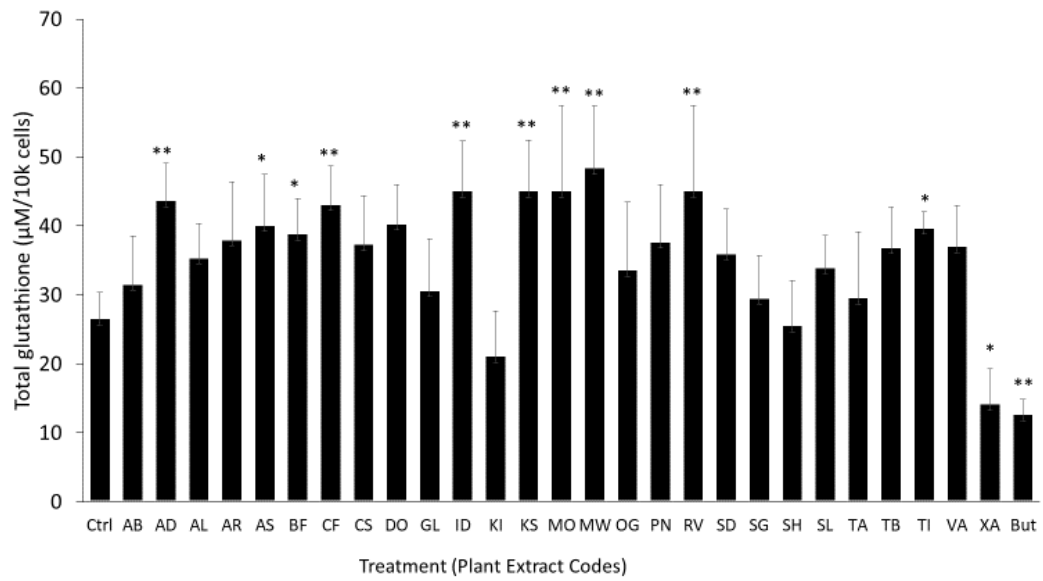
Figure 1: Intracellular concentration of glutathione in HepG2 cells after treatment with either extracts or 10µM buthionide sulfoximine (But) with respect to the Control (water). Results show mean ± SD, N ≥ 3. * P<0.05, ** P<0.01, according Dunnett Multiple Comparisons Test. Plant codes as per Table 1.
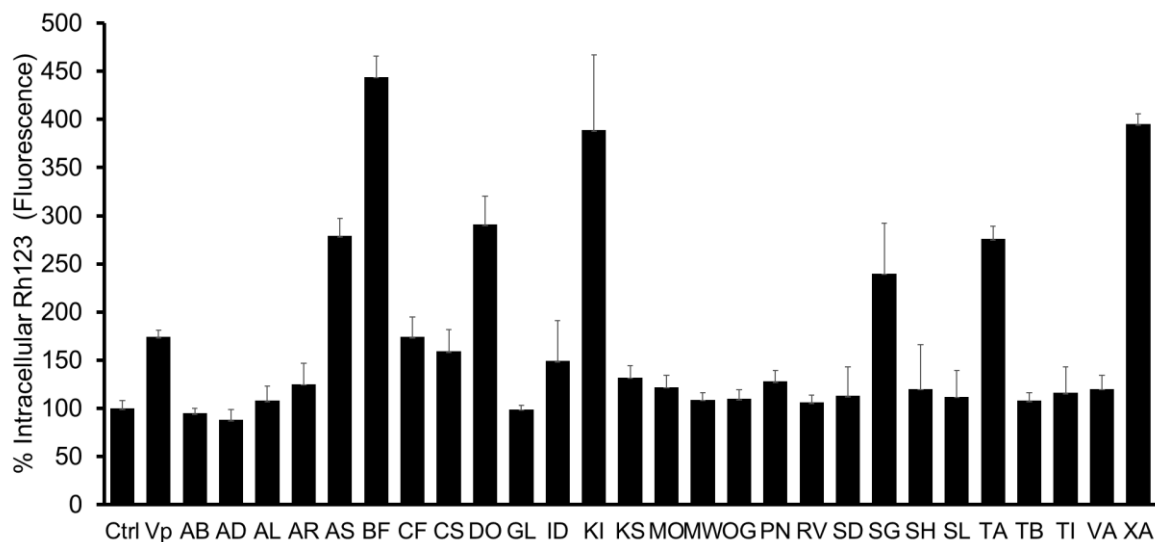
Figure 2: Percentage of intracellular Rh-123 concentration in Caco-2 Vincristine-Resistant cells in the presence of either extracts or 20μM verapamil (Vp) with respect to the Control (water). Results are the mean ± SD, N ≥ 3. * P<0.05 and ** P<0.01, according Dunnett Multiple Comparisons Test. Plant codes as per Table 1.
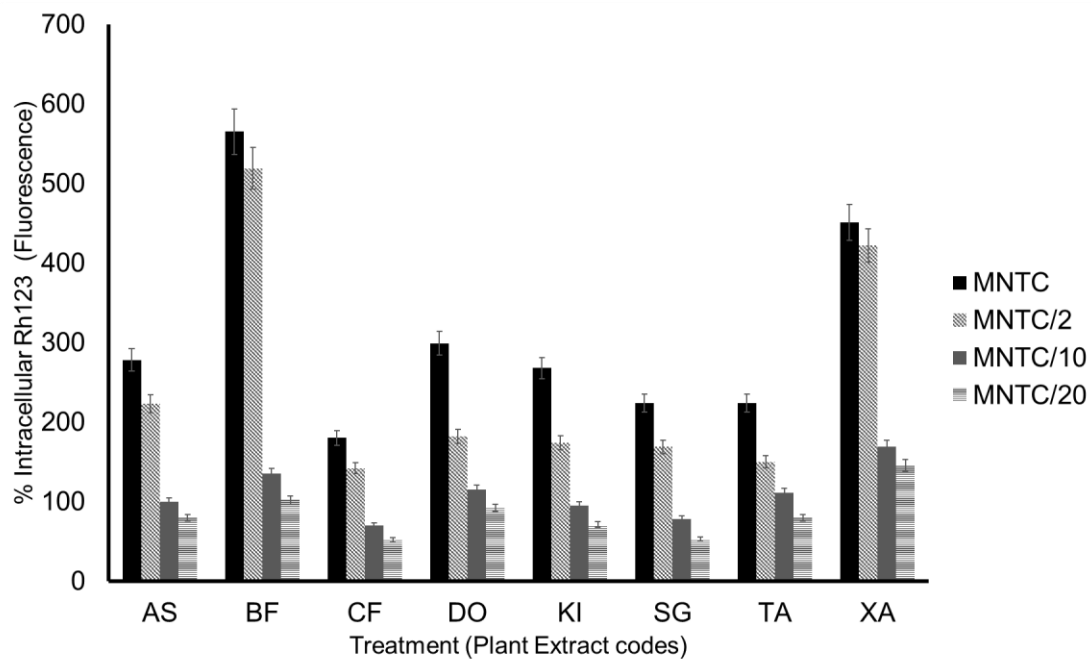
Figure 3: Dose-dependent inhibition of Intracellular Rh-123 concentration in Caco-2 Vincristine Resistant cells in the presence of serial dilutions of the extracts with respect to the Control (water). Plant codes and maximum non-toxic concentrations (MNTC) as per Table 1. Results are the mean ± SD, N = 3. [ns] P>0.05, * P<0.05, ** P<0.01, according Tukey-Kramer Multiple Comparisons Test.