UCL SCHOOL OF PHARMACY

Evaluation of herb-drug interactions in Nigeria with a focus on medicinal plants used in diabetes management

By: *Ezuruike F. Udoamaka*

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DECLARATION

This thesis describes research conducted in the School of Pharmacy, University College London between October 2010 and January 2015 under the supervision of Dr. Jose M. Prieto-Garcia. It is being submitted for the degree of Doctor of philosophy (PhD), University College London and has not been submitted before for any degree or examination in any other University. I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed: .....this day.....2015

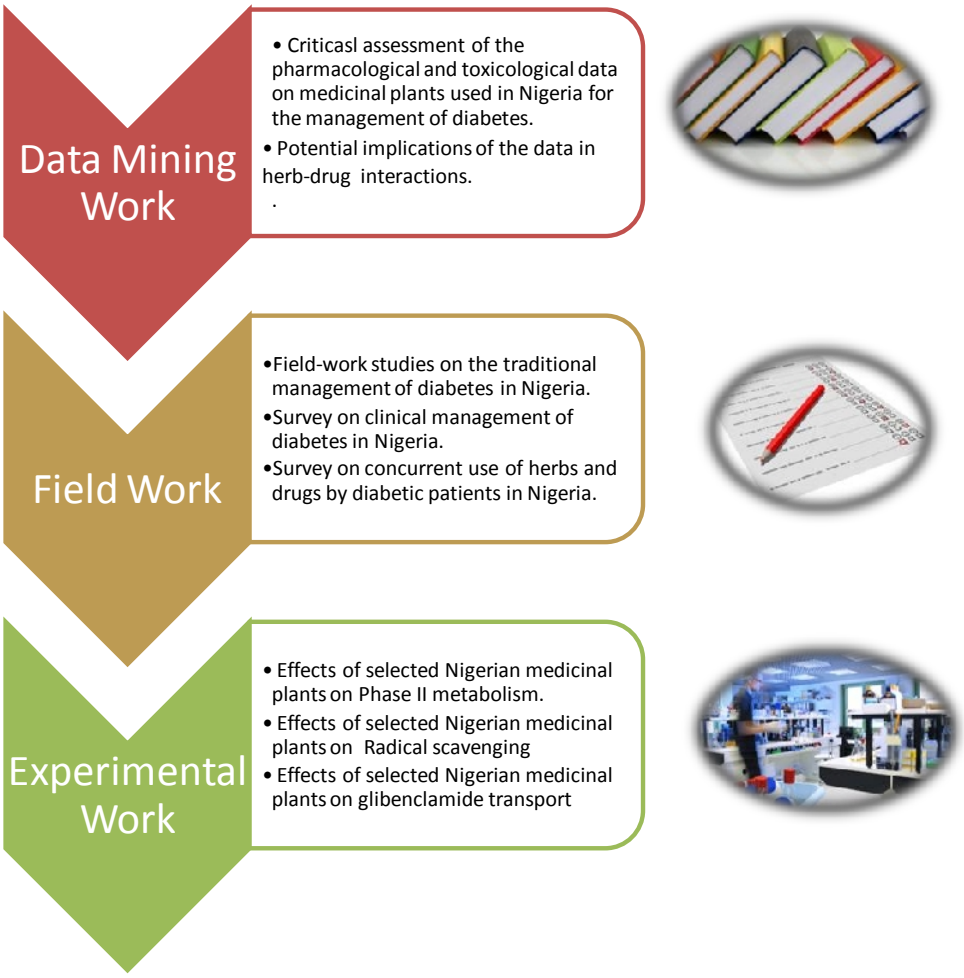
ABSTRACT

Studies have shown an increasing use of herbal medicines alongside conventional drugs by patients in their disease management especially for chronic diseases, with the attendant risks of herb-drug interactions. In order to forestall this, adequate information about the pharmacological and toxicological profile of herbal medicines and how these would in turn affect the bioavailability of the co-administered drug is required.

To evaluate potential herb-drug interactions that could occur in diabetes management in Nigeria- (a) An assessment of available data on the pharmacological and toxicological effects of plants used in diabetes management was conducted as a means of mapping those with identified potential risks for herb-drug interactions; (b) A field work study was carried out in different localities in Nigeria to identify potential pharmacokinetic interactions based on the prescription drugs and herbal medicines co-administered by diabetic patients; and (c) Experimental analysis of plant samples collected during the field work was done to assess their effects on known cell detoxification mechanisms and pharmacokinetic parameters.

The results of the research have confirmed the continued use of a wide range of medicinal plants in diabetes management, many of which have not been thoroughly investigated. In addition, 50% of diabetic patients visiting healthcare facilities in Nigeria routinely manage their diabetes or existing co-morbidities with herbal medicines alongside prescription drugs. Even more worrying is the frequent use of unlabeled herbal preparations which would constitute a huge challenge in the proper identification of herb-drug interactions when they occur. Based on previously available data and the experimental results of this research, a number of these herbal medicines have been identified as having overlapping interactions with prescription drugs. There is therefore a need for better regulation of herbal medicine use alongside pharmacovigilance monitoring in Nigeria in order to forestall the occurrence of clinically relevant untoward herb-drug interactions.

GRAPHICAL ABSTRACT



DEDICATION

To dad, for motivating me to start the PhD; and Nedu for being the backbone on which the work was done. I love you both.

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LIST OF ABBREVIATIONS

ABC	Adenosine triphosphate-binding cassette
ADME	Absorption, Distribution, Metabolism and Excretion
AP	Apical
ATP	Adenosine triphosphate
AUC	Area under the concentration time curve
BCRP	Breast cancer resistance protein
BL	Basolateral
BSO	Buthionine sulfoximine
CAM	Complementary and alternative medicine
Cmax	Peak plasma drug concentration
CYP	Cytochrome
DDI	Drug-drug interactions
DPPH	2, 2-diphenyl-1-picryhydrazyl
DPP-IV	Dipeptidyl peptidase –IV
FD4	Fluorescein isothiocyanate-dextran
FDA	Food and Drug Administration
FPG	Fasting plasma glucose
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide 1
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione-S-transferases
HBSS	Hank's balance salt solution
HDI	Herb Drug Interaction
HPLC-DAD	High Performance Liquid Chromatography with diode array detection
IC_{50}	Inhibitory concentration at 50%
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
MDR	Multidrug resistant protein
MNTC	Maximum non-toxic concentration
MRP	Multidrug resistant associated protein
NAFDAC	National Agency for Food and Drug Administration and Control
NOD	Non-obese diabetes
OAT	Organic anion transporter

OCT	Organic cation transporter
OGTT	Oral glucose tolerance test
*P*app	Apparent permeability
PD	Pharmacodynamic
PET	Polyethylene terephthalate
P-gp	P-glycoprotein
PK	Pharmacokinetic
PL	Product licence
$PPAR_{\gamma}$	Peroxisome proliferator-activated nuclear receptors
PXR	Pregnane X receptor
Rh-123	Rhodamine-123
ROS	Reactive oxygen specie
SJW	St John's Wort
SLC	Solute Carrier
SNP	Sodium nitroprusside
SSA	Sulfosalicylic acid
SULT	Sulfotranferases
SUR	Sulfonylureaa
SXR	Steroid and xenobiotics sensing receptor
TEER	Transepithelial electrical resistance
THR	Traditional Herbal Registration
TMP	Traditional medicine practitioner
UGT	Uridine diphosphoglucuronosyl transferase
VCR	Vincristine
WHO	World Health Organisation
WT	Wild type

1 GENERAL INTRODUCTION

1.1 HISTORY OF THE USE OF HERBAL MEDICINE AND THE INCIDENCE OF HERB-DRUG INTERACTIONS

The findings from excavations of skeletons from the Neanderthal period indicates that knowledge of the medicinal properties of plants dates to as far back as approximately 60,000 years ago (Solecki, 1975). Written records have also shown that physicians of the ancient world utilized botanical medicines for their disease management. These have been mentioned in several medical works of historical significance including the *Sushrita Samhita,* an Ayurvedic textbook written between the 4th and 5th centuries; the Egyptian medical manuscript *Ebers Papyrus,* a comprehensive compendium of drugs and prophylactics derived from animal, plant and mineral sources written about 552 B.C; and Chinese medical texts written as early as 3000 years B.C. (Oubré et al., 1997).

Herbal medicine practice continued to flourish until the 17th century. In the 18th century, its use began to decline in favour of the more 'scientific' pharmacological remedies (Trevelyan, 1993). In the early 1800's, Friedrich Sertűrner isolated the first pharmacologically active pure compound, morphine from seeds of the opium poppy plant *Papaver somniferum* L. (Hamilton and Baskett, 2000, Li and Vederas, 2009). Soon after, many herbal-derived medicines such as cinchona and digitalis as well as synthetic derivatives of plant-based drugs like aspirin were introduced into modern medicine (Saklani and Kutty, 2008).

For digitalis in particular, Withering transformed it from a family recipe for dropsy containing more than twenty substances to a modern drug, after recognising the plant's slim margin of safety as well as the importance of dose (Withering, 1785, Goldman, 2001). This recognition of the ease of producing more active and safer drugs from pure compounds drove the preference for pharmaceutical companies as sources of medicines for healthcare (Rates, 2001). In the early 19th century, scientific methods became more advanced and preferred, and the practice of traditional botanical healing was gradually being dismissed as 'quackery' (Winslow and Kroll, 1998).

The practice of herbal medicine in many countries is based on traditional knowledge of plant use by local communities and passed on through several generations, either through oral or written forms, and recently through the study of ethnopharmacology (Houghton, 1995). Although a large number of conventional drugs are based on herbal medicines, the main difference between the two is that the latter contains a large number of chemicals (consisting of both primary and secondary metabolites) rather than a single pharmacologically active substance, which act on one another to moderate, oppose or enhance an effect (Edwards et al., 2012).

Different pharmaceutical tragedies in the 1950's such as the use of thalidomide during pregnancy coupled with advances in scientific knowledge about natural products and its perceived lack of side effects rekindled consumers' interest in herbal medicines (Brantley et al., 2014). For Africa in particular, economic circumstances made imported techniques and drugs less accessible, thereby pressurizing the authorities to consider the possibility of utilising the hitherto abandoned traditional medicine to improve the health situation. As a result, the African regional committee of the World Health Assembly in its twenty-sixth session in 1976 passed a resolution inviting member states to 'take appropriate steps to ensure the use of essential drugs and medicinal plants of the traditional pharmacopoeia so as to meet the basic needs of communities and ensure the development of the African Pharmaceutical industry' (Bannerman et al., 1983). This formed part of the basis for recommendations by the World Health Organization for the use of traditional plant medicines in fulfilling the unmet needs of modern healthcare systems (He et al., 2010, Trevelyan, 1993).

Unfortunately, this perceived lack of side effects for herbal medicines compared to conventional drugs led to a generalization by the lay public that 'natural means safe' (Markowitz and Zhu, 2012). Numerous plant-derived products containing biologically active phytochemicals became available in different forms for use worldwide. This range from non-standardized preparations of plant-derived raw materials to extracts from different plant sources available either in encapsulated forms, as tablets or as beverages and tinctures. Some extracts have also been included as ingredients in sports/nutrition drinks, powders and 'energy' bars. The fallout of the proliferation of herbal medicine use was that patients began to co-administer them alongside their prescription drugs in a bid to obtain the maximal therapeutic benefit, often without informing their healthcare practitioners (Bruno and Ellis, 2005); thereby giving rise to incidences of herb-drug interactions (HDIs).

HDIs are either pharmacodynamic (PD) or pharmacokinetic (PK) in nature. For the former, this occurs when co-administered herbs and conventional medicines enhance or negate each other's effects as a result of similar or disparate pharmacological activity respectively. PK interactions on the other hand arise from the ability of the herb to modulate the absorption, distribution, metabolism and/or excretion (ADME) of the drug. Changes in drug absorption may be mediated through modulation of intestinal uptake and efflux transporters, while changes in metabolism and excretion occur through the modulation of hepatic/renal uptake and efflux transporters and/or through inhibition/induction of metabolising enzymes. Altered drug distribution as a result of protein binding displacement by herbs has not yet been reported (de Lima Toccafondo Vieira and Huang, 2012).

Prior to the emergence of reports of clinically relevant HDIs between St John's Wort (*Hypericum perforatum* L.) and drugs such as digoxin, warfarin, protease inhibitors and oral contraceptives; very few herb-drug interactions were documented (Williamson, 2003). The generalization that 'natural means safe' soon gave way to the notion that the example of St John's Wort was possibly only the 'tip of the iceberg' given the widespread use of herbal medicines alongside prescription drugs. This resulted in increased efforts to screen numerous herbal medicinal products for potential drug interactions primarily those used by 'at risk' populations such as the elderly and those patients more likely to be on more than one prescription drug ('poly-pharmacy') (Markowitz and Zhu, 2012).

Pharmacodynamic herb-drug interactions require a basic pharmacological knowledge about the herb and drug, but this is often lacking for the herb. If this information is available however, PD HDIs can be more easily predicted and therefore monitored. When herbal medicines are known to be pharmacologically active in a particular disease context for which other prescription medicines are being taken, caution should be applied with monitoring by healthcare practitioners to decipher when this might result in clinically relevant HDIs. Synergistic or additive therapeutic efficacy may lead to unfavourable toxicities and complicate the dosage regimen of long term medications; while antagonistic interactions will result in decreased efficacy and therapeutic failure (Hu et al., 2005).

In instances of synergistic interactions, these might require an adjustment of the dose of the prescription drug. Examples of improved glycaemic control have been shown with the co-administration of *Momordica charantia* L. (Karela) with chlopropramide, with suggestions of a dose reduction for the latter (Aslam and Stockley, 1979, Leatherdale et al., 1981). This is often beneficial as patients especially those taking herbal medicines would be more open to the possibility of a dose adjustment with the concurrent use of their herbal medicines. However, it is important to note that due to variability in plant sources and contents, the pharmacological activity due to the herb may not always be consistent hence the importance of strict regulations for standardized herbal medicines. Evidence of a number of identified pharmacodynamic HDIs of commonly used herbal medicines with different clinically relevant drugs have so far been published (Izzo and Ernst, 2009, Hu et al., 2005, Williamson, 2003, Fugh-Berman and Ernst, 2001).

Pharmacokinetic HDIs are the more difficult to deduce and have so far been the most commonly identified and documented form of clinically-relevant HDIs (de Lima Toccafondo Vieira and Huang, 2012, Delgoda and Westlake, 2004). The interactions of herbs with different pharmacokinetic parameters (transporters and metabolising enzymes) are however often not the cause for concern. Given that plants have always been a rich

source of food and medicines to man, the expression and activity of the multiplicity of transporters and metabolising enzymes present in humans have been phylogenetically shaped by man's exposure to these phytochemicals (Gurley, 2012). It could therefore be argued that the synthetic medications themselves are the sources of interference since the human pharmacokinetic system has already been pre-conditioned to work with secondary products of natural origin (Butterweck et al., 2004). Thus, in the presence of these herbs, these interferences result in HDIs.

The evaluation of these interferences/interactions of new drug entities with different pharmacokinetic parameters is actually a crucial aspect of pre-clinical drug discovery, ultimately responsible for determining the final clinical success of a drug candidate. This is because the ADME properties of drugs need to be pre-determined to ensure acceptable clinical efficacy and safety prior to a drug being approved for use (Li, 2001). The information is also required for predicting contraindications in drug prescribing by healthcare practitioners, thereby avoiding any clinically relevant drug-drug interactions (DDIs).

The biggest challenge in the identification of potential HDIs is that herbal medicines are not subject to the same 'rigid' pre-clinical or clinical assessments and regulations as those carried out with new drug entities (Zhou et al., 2010b). Many herbal medicines are treated as 'food supplements' even though they should not necessarily be regarded as such (Williamson, 2003). As a result, potential risks of HDIs are not identified and/or flagged up prior to its wide circulation to the unsuspecting public.

In Europe, efforts have been made to ensure better monitoring of all herbal medicines in circulation, either through the issuance of a full product licence (PL), which requires sufficient evidence of safety, quality and efficacy, including full clinical trial data; or the issuance of a traditional herbal registration (THR) under the Traditional Herbal Medicinal Products Directive (2004/24/EC). THRs are issued to herbal medicines indicated for relatively minor, self-limiting conditions. For a THR registration to be granted, evidence of traditional use of at least thirty years, (fifteen years in the EU), with acceptable levels of quality and safety is sufficient and supersedes the need for results of clinical trials (Cox and Roche, 2004).

1.2 EXPERIMENTAL TARGETS FOR EVALUATING PHARMACOKINETIC HERB-DRUG INTERACTIONS

Herbal medicines share the same drug metabolizing enzymes and drug transporters with several clinically important drugs. Thus, current methods for evaluating the pharmacokinetics of pharmaceutical drugs have been adapted for the evaluation of herbal medicines (Na et al., 2011); and in so doing are useful for predicting potential herb-drug interactions.

More than 400 drug transporters are responsible for the uptake and efflux of drugs, with high expression in the epithelia of the intestine, liver, kidney and the blood brain barrier (BBB). These exist in two major super families- the Adenosine triphosphate-binding cassette (ABC) and the solute carrier (SLC). Seven of these transporters have compelling evidence of their involvement in drug absorption and disposition as well as drug interactions. These include P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), OAT1, OAT3, OATP1B1 and OATP1B3 from the organic anion transporter family and OCT2 from the organic cation transporter family (Giacomini et al., 2010, Ho and Kim, 2005). Of all the identified HDIs involving transporters, P-gp has been the most implicated, thus more focus will be given to this transporter in this thesis.

Drug metabolism has always been a key aspect of drug development as enzymatic biotransformation is the principal route for the elimination of most drugs from the body. It is also necessary for the activation of some pro-drugs to pharmacologically active entities. The metabolising enzymes are divided into two- phase I metabolising enzymes and phase II metabolising enzymes. Phase I metabolism comprises metabolic oxidation, reduction and hydrolysis, which are catalysed by cytochromes P (CYP) 450s, flavin-containing monoxygenases, monoamine oxidase, carboxylesterase, amidase, epoxide hydrolase and peroxidase. The CYPs are made up of approximately seventy members in eighteen families and are the most important of all the enzymes, catalysing mainly oxidative reactions (Pelkonen et al., 2005).

Phase II metabolism which often follows phase I metabolism, comprises various conjugation reactions whereby hydrophilic species are added to the drug molecule to promote elimination. These include- glucuronidation catalysed by uridine diphosphoglucuronosyl transferases (UGTs), sulfation catalysed by sulfotransferases (SULTs) and glutathione conjugation catalysed by glutathione-S-transferases (GSTs). These enzymes are responsible for the transfer of sugars, sulfates and sulfhydryls respectively to a wide variety of substrates. Methylation and acetylation are also phase II metabolic pathways of elimination for some drugs (Gurley, 2012, Markowitz and Zhu, 2012).

1.2.1 Herb-Drug interactions involving P-glycoprotein

P-gp (also known as MDR1; multi-drug resistant protein or ABCB1) is a member of the human family of ABC transporters. This family of transporters play a major role in the defence of the body against carcinogens and toxins. They do this by the active efflux of substrates against their concentration gradient through the hydrolysis of adenosine triphosphate (ATP) (Li et al., 2010).

P-gp is a 170kDa surface glycoprotein and was first observed in cultured cells, which when exposed to increasing concentrations of cytotoxic drugs developed resistance to their cytotoxic effects (Juliano and Ling, 1976). It was later shown to be present in low levels in most tissues but much higher amounts are found on epithelial cells with excretory roles such as those of the small intestine as well as many cultured cells of mammalian origin. Its role in the intestinal epithelium is the extrusion of certain xenobiotics from the blood to the intestinal lumen as well as to prevent drugs in the lumen from entering the bloodstream, ultimately resulting in decreased absorption & decreased oral bioavailability (Sharom, 2011). While intestinal P-gp limits the absorption of xenobiotics, liver and kidney P-gp (and indeed other transporters) facilitate their elimination through biliary and urinary excretion respectively (Gurley, 2012).

Due to its localization and function, P-gp interacts with a wide range of structurally diverse compounds which are non-polar or weakly amphipatic, including natural products. These compounds interact either as P-gp substrates and/or modulators of P-gp activity (inhibitors, sensitizers or reversers). There is also an overlap of substrates of P-gp and other drug transporters, such as the multidrug resistance associated proteins (MRPs) and the BCRP. Blockade of P-gp or disruption of the MDR1 gene facilitates accumulation of these substrates within previously resistant cells, leading to increased sensitivity to these drugs (Zhou et al., 2004).

In the small intestine, interactions with P-gp are important in determining the bioavailability & 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. ‘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* L. also known as turmeric, a popular spice used in many Asian dishes. It has also been

isolated from other *Curcuma* species except *Curcuma zedoaria* (Christm.) Roscoe (Hou et al., 2008). Curcumin dose-dependently decreased the P-gp mediated efflux and increased the cellular accumulation of Rhodamine-123 (Rh-123), a known P-gp substrate *in vitro,* in both primary cultures of rat hepatocytes and in a P-gp over-expressing cell line, KB-V1 cells. Western blot analysis showed that this was partly due to decreased expression of protein levels of P-gp resulting in the development of an increased resistance to curcumin; as well as a direct interaction of curcumin with P-gp (Zhou et al., 2004).

Similar modulation of P-gp function has been shown *in vitro* with other metabolites of curcumin such as demethoxycurcumin and bisdemethoxycurcumin (Ampasavate et al., 2010)*;* as well as tetrahydrocurcumin (THC), the main metabolite of curcumin *in vivo.* THC inhibited the function of P-gp and subsequently increased the accumulation of rhodamine and calcein-AM in KB-V1 cells but not in wild type cells. It also showed similar inhibitory effects on other ABC drug transporters- multidrug resistance protein 1 (MRP1 or ABCC1) and mitoxantrone resistance protein (MXR or ABCG2) (Limtrakul et al., 2007).

Surprisingly, while curcumin has an inhibitory effect on P-gp, the administration of methanol extracts of *Curcuma* species (*C. longa, C. aromatic* and *C. zedoaria*) had the opposite effect. They all increased Rh-123 efflux dose dependently; as well as enhanced the net efflux of [^{3}H]-digoxin, another well-known P-gp substrate in Caco-2 monolayers. This opposing effect of the curcuma drugs is however not attributed to curcumin since *C. zedoaria* is not known to contain curcumin but showed similar effects with the other *Curcuma* species (Hou et al., 2008).

Several *in vivo* studies have been carried out to evaluate the *in vitro* P-gp modulatory effects of curcumin. In rats given an oral dose of 60mg/kg of curcumin for four days, there was decreased expression of protein levels of P-gp in the small intestine, with increased P-gp levels in the liver, while those of the kidney remain unchanged based on western blot analysis. Other studies have also shown that decreased P-gp expression levels in the small intestine often results in a compensatory over-expression in other organs as seen with the liver. Similarly, the pharmacokinetics of 30mg/kg celiprolol (a beta blocker and also a P-gp substrate) was also affected with the co-administration of curcumin. The peak plasma drug concentration (C_{max}) and the area under the concentration time curve (AUC) levels were increased, while the systemic clearance was reduced. Similar effects were not seen in rats which were pre-administered curcumin only 30mins prior to the administration of the celiprolol, indicating that the effect was due to curcumin's modulation of P-gp expression levels (Zhang et al., 2007).

The absence of a compensatory effect on the plasma concentration levels of celiprolol despite increased P-gp expression in the liver might be because there are no known metabolites for the drug (Hartmann et al., 1990), thus only the effects of the increased P-gp levels in the small intestine were evidenced in the drug's bioavailability. Similar increased bioavailability has been shown with etoposide, another P-gp substrate when administered orally but not with that administered intravenously (Lee et al., 2011); thereby confirming curcumin's modulation of the intestinal absorption of the two drugs due to its inhibitory effect on the P-gp efflux pump in the small intestine.

Paradoxically, when the effect of the P-gp modulatory effects of curcumin was investigated in twelve healthy volunteers administered 300mg/day curcumin for six days prior to the oral administration of 50mg talinolol (a beta-blocker and another P-gp substrate) on the seventh day, decreased bioavailability of talinolol indicated by decreased C_{max} and AUC levels; and increased renal clearance were observed as opposed to the increased bioavailability seen in the experiments with rats. The authors suggest that this might either be as a result of the up-regulation of other ABC transporters, since talinolol is also known to be a substrate of MRP2 or due to an extensive metabolism of curcumin to other metabolites of unknown P-gp effect (Juan et al., 2007). Similar *in vivo* studies have not been carried out with *Curcuma* drugs to find out what the effects and subsequent clinical implication might be when herbal medicines containing whole extracts of *Curcuma* species containing both curcumin and other opposing P-gp modulatory constituents present are co-administered with prescription drugs.

Garlic (*Allium sativum* L.) is another medicinally used herb that has shown low to moderate P-gp inhibtory effects based on the evaluation of various garlic products *in vitro* (Foster et al., 2001). However, decreased bioavailability of the protease inhibitors saquinavir and ritonavir were observed with the co-administration of garlic in humans. This might be due to the inhibitory effects of garlic on CYP3A4 as both drugs are known substrates of both P-gp and CYP3A4 (Zhou et al., 2004). Other herbs that have shown P-gp modulatory effects include green tea (*Camellia sinensis (*L.) Kuntze), grapefruit juice (*Citrus paradisi* Macfad.), milk thistle (*Silybum marianum* (L.) Gaertn.) piperine from *Piper nigrum* L. and *Piper longum* L. species and St John's Wort (SJW). The clinical significance of these interactions on the pharmacokinetics of various pharmaceutically important drugs as well as the constituents responsible for these interactions have been identified for some of them (He et al., 2010, Colalto, 2010, Hu et al., 2005, Zhou et al., 2004, Williamson, 2003).

1.2.2 Herb-drug interactions involving Cytochrome P450 enzymes

Cytochrome enzymes are membrane-bound proteins found in the endoplasmic reticulum of hepatocytes and mucousal cells of the gastrointestinal tract (GIT), indicating that the liver and the GIT play major roles in drug metabolism. Only a few specific isoforms- CYP1A1/2, CYP2A6, CYP2B6, CYP2C8/9/19, CYP2D6, CYP2E1 and CYP3A4/5 from three families are however responsible for the bulk of phase 1 metabolism. CYP3A4 is the most important as it metabolises around 50% of pharmaceutical drugs in use. Inter-individual variation in the expression of CYPs is common, with expression subject to genetic and environmental factors. There is also evidence of genetic polymorphisms for CYP1A2, CYP2A6, CYP2D6, CYP2C9, CYP2C19 and CYP3A resulting in variations in the functional activity of the CYPs in those individuals (Na et al., 2011).

Due to the interaction of a plethora of drugs with various cytochrome P450 enzymes, the potential pharmacological and toxicological impact of early screening for CYP enzyme activity has long been a part of the process of drug development (Delgoda and Westlake, 2004). In fact, the FDA guidance for drug interactions with drug metabolising enzymes were developed long before those for interactions with drug transporters (Giacomini et al., 2010). Various specific drug probes for the different clinically important CYP enzymes such as the benzodiazepine midazolam for CYP3A4, have been selected for their use in experimental evaluations of drug interactions; with guidelines on the degree of variations that should be considered 'clinically significant' (Gurley, 2012).

Interactions with CYP enzymes can either occur through an inhibition (either reversible or irreversible) or an induction of the enzyme. The competitive mechanism of reversible inhibition results in an almost immediate response; while mechanism-based or irreversible inhibition is characterised by a time- and concentration- dependent blockade. The effect of the latter can sometimes persist even after the herbal medicine is withdrawn, since the recovery of the enzyme activity will depend on its replacement by a newly synthesized one (Gurley, 2012, de Lima Toccafondo Vieira and Huang, 2012). When components of herbal medicines inhibit CYPs, they decrease the metabolic clearance of a co-administered drug that is a substrate of the inhibited pathway; while the reverse is the case for induction of CYPs. Induction of CYPs is a slow and regulated process often requiring transcriptional gene activation (Na et al., 2011).

There is overlapping tissue distribution of CYP3A4 and P-gp, as well as a broad spectrum of drugs that interact with both proteins either as substrates, inhibitors or inducers (Zhou et al., 2007). The expression of both proteins has also been shown to be co-ordinately regulated by nuclear receptors, one of which is the pregnane X receptor (PXR), otherwise known as the

steroid and xenobiotics sensing receptor (SXR). In fact, it has been hypothesized that P-gp and CYP3A4 found in the villus of the small intestine act together to limit the absorption of many xenobiotics (Zhang and Lim, 2008). It therefore appears that dual substrates for CYP3A4 and P-gp would have a much higher potential for clinically relevant HDIs (de Lima Toccafondo Vieira and Huang, 2012).

A review of all preclinical and clinical evidence relating to drug interactions with St John's wort has shown that the herb is able to jointly induce the activity of both CYP3A4 and P-gp, possibly through the activation of PXR. This inducing effect of components of the herb form the basis for its interaction with many clinically relevant drugs such as cyclosporine, hormonal contraceptives, anticoagulants and anti-HIV drugs with consequences of treatment failure in many cases, especially for drugs which are substrates of both proteins such as cyclosporin. There is also some evidence that the herb has can also modulate the activity of other CYP enzymes such as CYP2C19. These results are in contrast to the potent inhibitory effects previously shown by SJW in *in-vitro* studies suggesting that components of SJW might actually be CYP substrates rather than CYP inhibitors (Mannel, 2004). Garlic is another herb that has shown modulation of both CYP3A4 and P-gp activity (*Allium sativum* L.) (Gallicano et al., 2003, Piscitelli et al., 2002).

Individual constituents of herbal medicines have sometimes been evaluated independently as modulators of both P-gp and CYP3A4 activity. In a study carried out with eight phytochemical constituents from six commonly consumed herbal spices, only two of the spice components- curcumin and 6-gingerol from *Zingiber officinale* Roscoe showed modulatory effects on both P-gp and CYP3A4; although the other spice components produced some form of modulatory effect of CYP3A4 activity but not P-gp (Zhang and Lim, 2008). In addition to CYP3A4 modulatory effects, a number of herbal medicines have shown interactions with other CYP enzymes based on *in vitro* and/or *in vivo* data, with some of them showing modulatory effects of more than one CYP enzyme. Various extensive reviews covering this subject have so far been published with some of them identifying herbal constituents responsible for these modulatory effects (Brantley et al., 2014, Na et al., 2011, He et al., 2010, Delgoda and Westlake, 2004, Zhou et al., 2003, Ioannides, 2002).

In some cases, the constituents are ubiquitous in a number of herbal medicines or found in food drugs that are consumed in large amounts for dietary purposes such as the polyphenols gallic acid, catechin and quercetin; indole-3-carbinol found in broccoli and other cruciferous vegetables identified as an up-regulator of CYP1A2 activity and bergamottin and its derivatives found in grapefruit and other citrus fruits and identified as a potent inhibitor of CYP3A4 (Ioannides, 2002). These have raised a number of questions about the significance

of extrapolating these modulatory effects of the different herbal medicines to pharmacokinetic HDIs since these will often be all too frequently identified given the overlap in their disposition pathways.

Although more often regarded as negative, some HDIs actually result in beneficial effects in humans. For instance, CYP2E1 catalyzes the activation of certain nitrosamines and other environmental carcinogens. Thus, inhibition of this enzyme could minimize the deleterious effects of these carcinogens. It is thought to be one of the mechanisms by which diets rich in sulphur-containing plant constituents such as isothiocyanates and allyl sulphides in garlic have chemopreventive properties (Gurley, 2012).

Given the various complications encountered in the prediction of HDIs, various authors have recommended that results of pharmacokinetic interactions of herbal medicines or their components should not be evaluated in themselves. On the other hand, they should always be interpreted in the context of co-administered prescription drugs with sufficient information about the bioavailability of the herb itself; and then backed up by evidence from clinical studies (Markowitz and Zhu, 2012, Na et al., 2011, He et al., 2010).

1.2.3 Herb-drug interactions involving Phase II metabolising enzymes

Human UGTs represent a super-family of endoplasmic reticulum-bound enzymes that catalyze glucuronidation. Of all human UGTs, just 10 specific enzymes from two families (UGT1A and 2B) catalyze the bulk of all xenobiotics' conjugations, utilizing glucuronic acid as the donor sugar (Gurley, 2012). The liver and the intestine are the main sites for UGT glucuronidation, with a majority of the enzymes expressed in the liver. Glucuronidation accounts for approximately 35% of all drugs metabolized by phase II enzymes (He et al., 2010, Na et al., 2011).

The SULT super-family are cytosolic enzymes that catalyze the sulfation of a multitude of xenobiotics, therapeutic drugs, bile acids, peptides, lipids, hormones and neurotransmitters. Only members of the SULT 1 & 2 family particularly SULT1A are primarily involved in the sulfation of xenobiotics. They catalyze the addition of sulfates to hydroxyl and monoamine groups on phenolic-type molecules (He et al., 2010). Although sulfation like most conjugation reactions generally renders a substrate less toxic, it has also been shown to activate certain plant-derived procarcinogens such as safrole, thereby resulting in a more toxic product (Gurley, 2012).

GSTs catalyze the nucleophilic addition of a sulfhydryl group (glutathione) to electrophilic substrates. Most GSTs are soluble cytosolic enzymes, which in mammalian species are divided into four families- alpha (α), mu (μ), pi (π) and theta (θ); in addition to a small

family of microsomal GSTs and one identified mitochondrial GST (GST kappa) (Eaton and Bammler, 1999). Many carcinogens such as the epoxides are reportedly detoxified by GSTs. Activation of the enzyme by inducers promote their elimination from the body thereby playing a role in chemoprevention. GST inhibitors on the other hand promote the cytotoxicity of anti-neoplastic agents when co-administered, thereby playing a role in overcoming tumour-induced drug resistance (Mukanganyama et al., 2002, Hatono et al., 1996). Asides the detoxification of exogenous chemicals, another important role of GST is in the detoxification of endogenous products of lipid peroxidation. This underlies its role in protecting the body against oxidative stress (Eaton and Bammler, 1999).

Many phytochemicals are themselves conjugated by the various phase 2 enzymes. Glucuronidation and sulfation have been identified as the main elimination pathway for silymarin, a mixture of polyphenolic flavonoids extracted from milk thistle (*Silybum marianum* (L.) Gaertn.). The glucuronide conjugates account for 55% of the total plasma concentration, while the sulfates account for 28% (Wen et al., 2008). Asides being a substrate, it has also been shown to inhibit UGT 1A1, 1A6, 1A9, 2B7 and 2B15 as well as some members of the CYP family; with UGT 1A1 being the most inhibited of all the metabolising enzymes. Although UGT 1A1 and 1A9 are involved in the metabolic disposition of the anti-cancer drug irinotecan, co-administration with milk thistle did not however affect its pharmacokinetics in a clinical study carried out in six volunteers (van Erp et al., 2005). This is thought to be as a result of the poor bioavailability of silymarin partly due to its extensive pre-systemic metabolism (Gurley et al., 2012).

Finally, some nuclear receptors such as the PXR and the CAR (constitutive androstane receptor) regulate the expression of some of the phase 2 metabolising enzymes. Herbal medicines that activate either of these receptors can therefore bring about an induction of these enzymes resulting in increased metabolic clearance. Hyperforin from SJW and glucosinolates from cruciferous vegetables (after *in vivo* conversion to isothiocyanates) have been found to induce the activity of various GSTs and UGTs (Gurley et al., 2012).

1.3 PREDICTION OF HERB DRUG INTERACTIONS AND ITS IMPLICATION IN DISEASE MANAGEMENT

By their very nature of being composed of plant-based chemicals and thus identified by the human body as xenobiotics, all herbal medicines undergo some form of pharmacokinetic interaction with one or more of the different transporters or metabolising enzymes that constitute the ADME system. The varied nature of these pharmacokinetic interactions undergone by herbal medicines and their constituents indicate that prediction of herb-drug interactions can only best be done in the context of the co-administered prescription drug.

The same herbal medicine can be utilized to produce a beneficial effect on the one hand or a detrimental effect on the other.

For example, a number of plant-based GST inhibitors or inhibitors of ABC transporters have been proposed as chemo-modulators to enhance the cytotoxic effect of anti-cancer drugs due to their relatively non-toxic nature (Hayeshi et al., 2007, Hayeshi et al., 2004, Wu et al., 2011). Prolonged inhibition of these pharmacokinetic parameters can unfortunately bring about increased toxicity if they interfere with the elimination of other toxic xenobiotics that may be present in the body at the same time. Given that most herbal remedies are administered orally in a chronic regimen, the possibility of such prolonged effects should be anticipated.

It is therefore necessary to understand the absorption and disposition pharmacokinetics of herbal medicines as a prerequisite for predicting potential interactions when co-administered with prescription drugs; but also for the purposes of estimating the bioavailability of the 'active' constituents for which the herbal medicine is being taken in the first instance (Brantley et al., 2014).

The majority of drug interaction studies are *in vitro* largely due to its high throughput nature and substantially reduced costs relative to *in vivo* studies (Markowitz and Zhu, 2012). *In vitro* studies also provide mechanistic information about potential interactions. However, the use of 'non-therapeutically' relevant concentrations and the inability to incorporate the true biological fate of the test compound in the assay setup are limitations to their use for the accurate prediction of HDIs (Venkataramanan et al., 2006).

In vivo interaction studies are usually necessary to provide evidence of the clinical importance of previously obtained *in vitro* results. Animal studies give important information on HDIs, but interspecies variations in the substrate specificity, catalytic features and amino acid sequences of the proteins may cause difficulty in extrapolating animal data to humans. Therefore, it may be difficult to predict accurately the effects of herbal preparations in humans based on animal studies, and human studies are usually required to confirm HDIs (Zhou et al., 2003).

When carrying out *in vivo* studies involving herbal medicines, regulatory agencies recommend the identification of one or more marker compounds ('stated ingredients'), which is then used for monitoring the quality, safety and efficacy of the herbal product in clinical studies (Cox and Roche, 2004). In many cases, these marker compound(s) are responsible for some of its known biological effects (Markowitz and Zhu, 2012). An assessment of the phytochemical bioavailability of the marker compounds in biological

fluids would in turn provide evidence of exposure. However, one limitation of this approach is that herbal medicines are a complex mixture of multiple ingredients, many of which remain unidentified and/or uncharacterised. Even if these 'other' constituents do not contribute to the biological effect of the herb, they may still affect its pharmacokinetics and should therefore be monitored.

Evaluation of the clinical evidence of most identified pharmacokinetic herb-drug interactions have shown that only a small percentage of *in vitro* identified HDIs produce a significant clinical effect. One very good example is with milk thistle whereby despite substantial evidence for interactions with transporter proteins, phase 1 and phase 2 metabolising enzymes, a significant clinical effect was only identified with CYP2C9 (Brantley et al., 2014, Hermann and von Richter, 2012).

"Nonetheless, *in vitro* studies still provide much value for identifying specific herbal medicines that may potentially pose an interaction risk and thus serve as a 'signal' that *in vivo* studies may be warranted to confirm clinical relevance" (Markowitz and Zhu, 2012). In addition, it raises an alert to healthcare practitioners thereby encouraging better clinical monitoring (Williamson et al., 2013). It could also provide a mechanistic understanding of the potential herb interaction which enables clinicians make predictions of other drugs that may be similarly affected, especially those with low therapeutic indices. Such clinical studies of drug interactions with herbal medicines should not be arbitrarily carried out with just any therapeutic drug but in the context of the therapeutic use for which it is meant and/or in the presence of drugs that are likely to be co-administered by the patient alongside herbal medicines.

It is important to note that the assessment and interpretation of the clinical evidence of HDIs is in itself fraught with certain limitations. There is very often large variability in the phytochemical content of different herbal preparations of the same botanical specie due to variability in source, cultivation practices and extraction procedures. It is therefore important to carry out such studies with products whose components have been quantitatively and qualitatively analysed; as well as to make sure that the interaction effect(s) seen is not due to any adulterants or contaminants.

Many clinical studies have been identified as having a somewhat 'flawed' study design- in that they are carried out in healthy human volunteers as opposed to compromised subjects without the use of positive reference controls to confirm the magnitude of any observed interaction. Inter-individual variability in the expression of some of these pharmacokinetic parameters as well as genetic variation in drug response due to the existence of polymorphisms can also have a profound effect on the pharmacokinetic profile of the drug

(Gurley, 2012, Venkataramanan et al., 2006). All these factors should therefore be considered when proposing interpretations of any clinically observed HDI.

In this regard, there is a strong advocate for the pharmacovigilance of herbal medicines post-registration to identify previously unidentified or unrecognised risks. Periodic safety update reports (PSUR) are requested at defined time points by regulation bodies such as the UK's MHRA (Medical and healthcare products regulatory agency). Registration licence holders are expected to provide a critical evaluation of the risks and benefits of the product in light of whatever new information that may have become available in the interim since registration.

1.4 DIABETES MANAGEMENT IN NIGERIA AND THE RISK OF HERB-DRUG INTERACTIONS

1.4.1 Overview of Diabetes

Diabetes mellitus is a chronic metabolic disorder of multiple aetiology. It is characterized by uncontrolled high blood glucose levels and disturbances of carbohydrate, protein and fat metabolism. This is either as a result of insufficient endogenous insulin production by the beta cells of the islets of the pancreas, or impaired insulin secretion and/or action. The former condition is known as Type-1 diabetes, while the latter is known as Type-2 diabetes.

In type-1 patients, there is destruction of the beta-cells such that insulin production is compromised. This is often due to an auto-immune disorder indicated by the presence of insulin antibodies in these individuals. Some other type-1 patients are regarded as 'idiopathic', wherein the cause of beta cell destruction is unknown (WHO, 1999). So far, the highest risk factor identified for type-1 diabetes is a genetic predisposition. Epigenetic and environmental factors have also been implicated as being contributory to its onset (Todd, 2010). Administration of exogenous insulin for blood glucose metabolism is mandatory for the survival of type-1 patients, due to this lack of, or insufficient insulin production.

Type-2 diabetes is the more common form of diabetes with an estimated occurrence in almost 90-95% of diabetic patients (ADA, 2011). Although the exact pathophysiology is still unclear, it is increasingly evident that there are various factors which initially predispose individuals to beta cell dysfunction and decreased insulin sensitivity of the peripheral tissues. These in turn, lead to impaired glucose tolerance and subsequently to the development of type-2 diabetes. The factors that have so far been identified are genetic predisposition, age, physical inactivity, obesity or body-fat distribution and diet. In most cases, beta cell dysfunction and/or decreased insulin sensitivity would have been present for

several years before hyperglycaemia begins to manifest in the patient (Kahn, 2003, Scheen, 2003).

There are also other specific but less common types of diabetes, which do not fit into the criteria for either type-1 or type-2 diabetes but are characterised by high blood glucose levels. These include drug-induced or chemical-induced diabetes, diabetes caused by diseases of the exocrine pancreas (such as cystic fibrosis) or by infections, and gestational diabetes (Mayfield, 1998).

Clinical symptoms of diabetes include- frequent urination, frequent thirst, increased hunger, weight loss and fatigue. In addition to high blood glucose levels, there are usually high levels of glucose in the urine (glucosuria). Ketoacidosis is present only in severe cases of diabetes often in type-1 diabetes, which may lead to a diabetic coma and death if not treated. The long term effect of diabetes is the progressive development of diabetes-related vascular complications (WHO, 1999).

Various studies have identified increased oxidative stress as the link between diabetes and vascular complications. There is often a mitochondrial overproduction of reactive oxygen species (ROS) in specific cell types during glucose oxidation due to high blood glucose levels. These ROS cause tissue damage by activating certain intracellular pathways, resulting in vascular diseases such as atherosclerosis, cardiomyopathy, diabetic foot syndrome, retinopathy, nephropathy and diabetic neuropathy. Insulin resistance is also a risk factor for cardiovascular diseases due to the increased release of free fatty acids from stored triglycerides. Oxidation of these free fatty acids results in increased production of ROS (Giacco and Brownlee, 2010, Maritim et al., 2003). These vascular diseases are the common causes of mortality & morbidity amongst type-2 diabetic patients.

1.4.2 Current Therapeutic Options in the Management of Type-2 Diabetes

The prevalence of type 2 diabetes has been increasing in the last decade due to increase in the various identified risk factors, provoked by urbanisation and the resultant lifestyle changes (Guariguata et al., 2014). To mitigate this, the main therapeutic goal in the current treatment of type-2 diabetes is the maintenance of patient's glycaemic levels as close to the non-diabetic range as possible with the aid of pharmacotherapeutic drugs, both to improve symptoms of diabetes as well as prevent diabetes-related complications. This is in addition to the implementation of lifestyle modifications including increased physical activity and diet adjustment to a low-fat diet, of which no more than 10% should be saturated fatty acids (Nyenwe et al., 2011).

A fasting plasma glucose (FPG) concentration of ≥ 7mmol/L (126mg/dL) or ≥ 11.1mmol/L (200mg/dL) 2 hours after the intake of the equivalent of 75g glucose (also known as the oral glucose tolerance test or OGTT); or a glycated haemoglobin value (HbA1c) of ≥ 6.5% is a confirmatory diagnosis for diabetes. Individuals with FPG levels between 6.1-6.9mmol/L (110-125mg/dL) are classified as having impaired fasting glucose (IFG); while those with OGTT values between 7.8-11mmol/L (140-200mg/dL) have impaired glucose tolerance (IGT). These two groups of people have glucose levels outside the normal range and have a higher risk (10 times more than normal patients) of developing clinical diabetes (ADA, 2011, WHO).

The complexity of type-2 diabetes presupposes that different classes of drugs can act as anti-diabetic agents. The choice of drug is therefore determined by the medical needs of the patient, the potency of the agent, the side effect profile of the drug, its ease of administration and cost-effectiveness. As monotherapy, most of these drugs (except insulin) only reduce HbA1c levels by 0.5% to 2%. For a patient with HbA1c of 8.5%, a single agent is therefore unlikely to achieve the therapeutic goal (Nyenwe et al., 2011). In many cases, patients are often on a 'combination therapy' of either two (or more) different types of anti-diabetic drugs or the anti-diabetic medication alongside another medication addressing the other existing risk factor(s) or vascular complication.

Current drugs used in diabetes management can be categorized into three groups (Chehade and Mooradian, 2000, Sheehan, 2003). Drugs in the first group increase endogenous insulin availability. These include the sulfonylureas (SURs) such as glibenclamide, the glinides, insulin analogues, glucagon-like peptide 1 (GLP-1) agonists and dipeptidyl peptidase-IV (DPP-IV) inhibitors. The first two members of this group act on the sulfonylurea receptor in the pancreas to promote insulin secretion; with the SURs becoming increasingly popular and recommended as additional treatment for patients with HbA1c levels ≥ 7% (Nathan et al., 2006).

Glucagon-like peptide 1 (GLP-1) is a naturally occurring, rapidly degraded peptide secreted from the walls of the small intestine which stimulates insulin secretion. It also slows down gastric motility and gastric secretion. Because of its overall benefit in diabetic patients, exenatide a synthetic GLP-1 agonist has been developed for the management of type-2 diabetes. However, it is only available as an injectable which limits its use. GLP-1 is degraded by the dipeptidyl peptidase-4 enzyme (DPP-4). Orally administered DPP-4 inhibitors have been developed for the management of type-2 diabetes, but their modest reduction in HbA1c levels has made its use unpopular (White and Campbell, 2008).

The second group of drugs enhance the sensitivity of insulin. This includes the thiazolidinediones and the biguanide metformin. Biguanides are derivatives of galegine, a guanidine isolated from *Galega officinalis* L. also known as goat's rue or French lilac. The plant is used in the treatment of people with diabetes in Europe. Due to the toxic effects associated with galegine, a structural modification was introduced which led to the development of metformin (dimethyl biguanide) (Figure 1.1); with the synthetic analogue having improved efficacy, less toxicity and better tolerability (Bailey and Day, 2004).

Metformin increases the sensitivity of the liver and peripheral tissues to insulin by modulating insulin's signalling pathway, thereby resulting in decreased gluconeogenesis in the liver as well as increased glucose uptake by peripheral tissues. It has also been shown to be beneficial for other cardiovascular risk factors due to its ability to decrease blood pressure and improve plasma lipid profiles (Goodarzi and Bryer-Ash, 2005). It is currently the most popular oral anti-diabetic medication in most parts of the world and is recommended as first line treatment especially when initiating chemotherapy in newly diagnosed patients (Nathan et al., 2006).

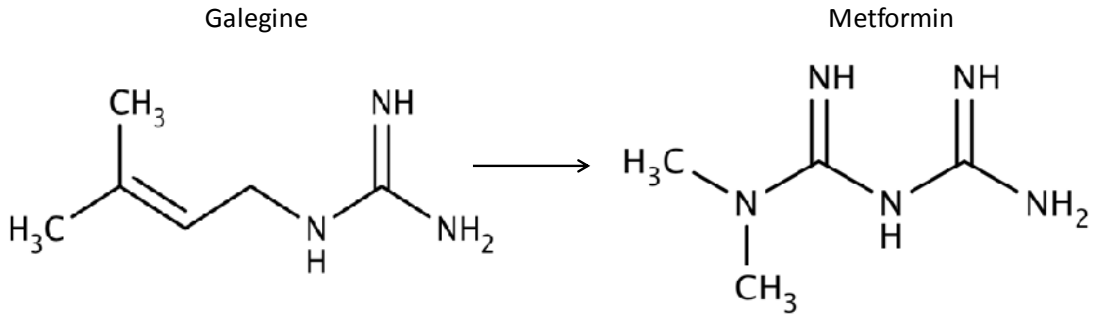

Figure 1.1: Structural modification of Galegine from *Galega officinalis* to the type-2 anti-diabetic drug Metformin

Thiazolidinediones are direct stimulators of the 'gamma' member of the peroxisome proliferator-activated nuclear receptors (PPARɣ). Upon activation, these nuclear receptors stimulate the expression of genes responsible for lipid metabolism resulting in decreased levels of free fatty acids as well as increased carbohydrate metabolism (Hannele Yki-Järvinen, 2004). By improving the action of insulin, this group of drugs help to decrease insulin resistance in peripheral tissues. Thiazolidinediones are however taken with caution by patients who have existing cardiovascular problems as rosiglitazone is thought to increase the risk of ischemic heart failure in such patients (Kaul et al., 2010).

The last group of drugs are those that decrease the patient's need for insulin. Prominent amongst this group are the alpha glucosidase inhibitors, acarbose & miglitol. They inhibit

the alpha glucosidase enzymes present in the brush border of the small intestine, which are responsible for the breakdown of polysaccharides to monosaccharides. This action decreases the rate of absorption of polysaccharides, thereby reducing postprandial glucose levels. All the above mentioned drugs are orally administered and more commonly used by diabetic patients due to their ease of administration.

The administration of recombinant human insulin to decrease blood glucose levels in type-2 diabetic patients is not considered to be the first line of treatment, despite being the most effective since it is only available as injectables. This makes it more cumbersome and less amenable to use by patients. However, when other alternatives do not bring about significant blood glucose reduction in the patient, insulin is considered either as a monotherapy, or in addition to an existing oral anti-diabetic drug (Nathan et al., 2006).

1.4.3 Clinical Management of Diabetes in Nigeria

The International Diabetes federation (IDF) estimates that the prevalence of diabetes in Nigeria for adults (aged 20-79 years) is expected to rise from 5.0% of its population in 2013 to 5.5% in 2035 (Guariguata et al., 2014). A study carried out in the city of Port-Harcourt (one of the coastal cities in Nigeria) in 2000 gave a prevalence of 7.9% (Nyenwe et al., 2003). This high rate, which is greater than the IDF estimated prevalence may be as a result of the survey being conducted in adults ≥ 40years. The middle aged years (between the ages of 35 and 65) have repeatedly been shown to be the most common age bracket for diabetic patients in Nigeria as well as globally (Guariguata et al., 2014, Okoro et al., 2002).

Studies have also showed that the prevalence of diabetes is higher in urban areas than in rural areas and that a high percentage of diabetic patients are undiagnosed and/or asymptomatic. In addition, the greater percentage of the diabetic population in Nigeria are type-2 patients, which constitute more than 80% of diabetic hospital admissions and more than 50% of total hospital admissions. More importantly, type 2 diabetes in Nigeria has a high mortality rate of over 30% mainly due to diabetic foot ulcers (DFU); with ignorance, late diagnosis and poor glycaemic control identified as some of the reasons for the high mortality rate (Chijioke et al., 2010, Ogbera et al., 2007).

A case study carried out at the University of Ilorin teaching hospital located in the south-west of Nigeria showed that the quality of care of diabetic patients receiving treatment in hospital facilities currently falls below the recommended minimum international standards (Okoro et al., 2002). The reason for this non-adherence to recommended guidelines by hospitals in Nigeria may not be too far-fetched. Average percentage contribution by the government to the total health expenditure is less than 30% (less than 2% of GDP is

allocated to health); while the remaining 70% are 'out-of-pocket' expenses borne by the patients themselves or their family members in subsidized public hospitals (WHO, 2000). This greater percentage of health costs being borne by the patient is very likely a huge financial burden for a chronic disease like diabetes. Studies have shown that direct payments by patients prevent many people from seeking care in the first place, and that improving universal health coverage requires systems that raise funds through alternative forms of pre-payment such as taxes and/or insurance (Evans and Etienne, 2010).

There is limited access to healthcare for most patients in Nigeria, characterized by a high doctor to patient ratio (Kengne et al., 2005). In addition, distance and costs of healthcare have been identified as significant determinants to the choice of healthcare and often act as deterrents to healthcare access (Amaghionyeodiwe, 2008). Many patients would rather purchase their drugs from local pharmacies so as to eliminate the costs of consultation fees and the time 'wasted' in visiting health facilities due to long delays. This practice increases the risk of cardiovascular complications as routine blood glucose levels aren't determined. Patients are therefore unaware if adequate glycaemic control is being achieved with their current therapy. As a result, there is a high incidence of diabetic complications and hyperglycaemic emergencies amongst diabetic patients in healthcare facilities in Nigeria (Gill et al., 2009, Ogbera et al., 2007, Ogbera et al., 2009).

The aim of physicians when prescribing existing pharmacological agents is to bring about a more intensive blood glucose control such that HbA1c levels are less than 7%. To achieve this, patients often have to constantly be on medications. Given that most diabetic patients are unable to achieve adequate glycaemic control on monotherapy, polytherapy is more common. In a study carried out in a diabetic clinic in Ibadan Nigeria, over 80% of type 2 diabetic patients were prescribed a combination therapy of metformin and a sulphonylurea to achieve adequate glycaemic control. The average number of drugs prescribed per day increased to an average of four in the presence of other vascular complications. Anti-hypertensives, anti-coagulants & anti-lipidaemics were the medications commonly taken by such patients (Enwere et al., 2006).

The combined costs of these drugs can bring undue financial strain on the patient in the long term given the already existing unstable economic environment of most developing countries. A situation whereby the monthly cost of glibenclamide therapy is equivalent to more than a week's wages would therefore be very unfavourable for the patient (Beran and Yudkin, 2006). Various studies have showed that the need for chronic intake of a large number of drugs with their attendant side effects, in addition to the high cost of purchase have been identified as the reasons for non-adherence to diabetes therapy. As a result of this,

patients often have recourse to alternative forms of therapy such as herbal medicines (Adisa et al., 2009, Yusuff et al., 2008, Enwere et al., 2006).

A recent study evaluating how treatment burden affects the benefits of glycaemic treatment showed that the patient's view of the burden of the specific treatment being considered for the disease management, and not necessarily the intensity of the treatment greatly determines the overall glycaemic control achieved. It therefore advocates for shared decision making whereby patients' preferences are specifically considered prior to implementing a particular glycaemic treatment (Vijan et al., 2014). It should not be overlooked that some of these patients' preferences might involve the concurrent use of herbal medicines. Healthcare practitioners should therefore be aware of this and be open to options whereby maximal therapeutic benefit to the patient can still be achieved.

1.4.4 Potential Risks of Herb-Drug Interactions amongst Diabetic Patients

The use of herbal medicines as part of traditional medicine practice continues to play an important role in healthcare for a large number of the population of developing countries, particularly those living in rural areas. This is primarily because such areas are often severely under-provided with western-type healthcare, thereby bringing about a greater dependence on the more-accessible but also more culturally acceptable traditional medicine for their disease management (Alves and Rosa, 2007, Bodeker and Kronenberg, 2002, Stock, 1983).

Studies have however now shown an increasing use of herbal medicines and indeed other forms of complementary and alternative medicine (CAM) in urban areas. Suggestions as to the reasons for this include- 'that this could either be as a result of increased rural-urban migration'; or 'a means for patients to supplement the high costs of conventional medicines with the 'relatively' cheaper traditional alternative'; or 'simply because of a dissatisfaction with conventional therapy' (van Andel and Westers, 2010, Saydah and Eberhardt, 2006).

Surprisingly, a large subgroup of patients who use herbal medicines do not necessarily do so based on cost-considerations or at the expense of modern healthcare delivery. On the other hand, these patients prefer the autonomy of being in control of their disease management as well as the ability to make treatment decisions; and are willing to try out alternative therapies alongside (Garrow and Egede, 2006, Astin, 1998). Some of the reasons that have been given by patients for their concomitant use of herbs and pharmaceutical drugs include: 'a desire for a more holistic approach to healthcare; a belief that this will produce a better outcome; a desire for safer, more natural treatments at lower costs; and a need for a larger

number of healthcare options than that offered by biomedicine' (Jonas, 2002, Picking et al., 2011).

The presence of chronic diseases including arthritis, cancer, cardiovascular diseases and diabetes represent key disease areas where the use of CAM have been shown to be increasingly prevalent. In a study carried out in Malaysia amongst patients with chronic diseases wherein 60% used CAM for their disease management, over 70% of the patients were diabetic (Hasan et al., 2009). This high prevalence is unfortunately accompanied by poor communication between patients and clinicians. For instance, diabetic patients in the US have been identified as the least likely to inform their healthcare practitioners of their herb use (Saydah and Eberhardt, 2006).

The average worldwide prevalence for the use of CAM alongside conventional medicines amongst diabetes patients has been estimated as 45.5%. This reduces to approximately 30% when looking only at the use of biological based therapies (the use of natural products as opposed to other mind and body practices) (Chang et al., 2007). Given that this prevalence rate is based on studies carried out in only nine countries, most of which are developed ones, this is quite possibly an underestimation. Developing countries like Mexico and India have already shown prevalence rates of over 60% (Argaez-Lopez et al., 2003, Kumar et al., 2006). Furthermore, the prevalence of diabetes is estimated to increase globally, and at an even higher rate in developing countries (Guariguata et al., 2014). This implies that the worldwide prevalence of herbal medicine users amongst diabetic patients is also very likely to increase.

In Nigeria, the prevalence of concomitant use of herbal medicines alongside conventional drugs in diabetes management is about the same as the estimated worldwide prevalence rate. A study carried out amongst diabetic patients in Lagos gave a prevalence of 46% for the use of CAM alongside prescription drugs, most of which were herbal medicines (Ogbera et al., 2010). This high prevalence is seen in urban areas which have relatively better access to modern healthcare facilities, possibly due to high healthcare costs. Another study carried out to estimate herbal use in all the geo-political zones of Nigeria, encompassing both urban and rural areas showed that 41% of respondents use herbal medicines either singly or in combination with conventional medicines as their treatment choice (Osemene et al., 2011).

One of the main challenges of diabetes management is that despite existing pharmacotherapy, attaining adequate glycaemic control especially in type-2 diabetic patients is really difficult due to a steady decline in beta cell function (Robertson et al., 2004, Wallace and Matthews, 2000). Poor glycaemic control increases the risk of diabetic related complications ensuing in more complex treatment plans which the patients have to deal with

for a long time. This characteristic of diabetes lends itself as a condition whose patients are likely to seek out alternative forms of treatment (Chang et al., 2007).

In clinical practice, the use of two or more drugs by patients with chronic diseases or those with co-morbid conditions pose a risk of drug-drug interactions which physicians take into serious consideration while prescribing an appropriate therapeutic regimen for the patient. Herbal medicines also pose a similar risk since they are primarily plant-derived, biologically active chemical constituents. The chronic nature of diabetes means that the concurrent use of herbal medicines together with prescribed drugs could result in undesirable herb-drug interactions. Unfortunately, the use of herbal medicines is still not very well regulated as most times these are self-prescribed by the patients themselves. As a result, healthcare practitioners are unable to provide informed advice to their patients when prescribing to forestall unwanted HDIs.

Now that it has become apparent that herb use is more widespread than previously thought, more focus has been given to evaluating various herbal medicines for possible HDIs. Some studies have focused on evaluating the pharmacodynamic interactions between herbs and drugs taken for the same ailment. It is often assumed that the co-administration of a herb and a drug both having the same therapeutic benefit will result in an additive effect, but this is not always the case. For example, when extracts of pawpaw (*Carica papaya* L.) leaves known for its hypoglycaemic effects (Maniyar and Bhixavatimath, 2012) were co-administered with glimepiride, rather than the expected enhanced hypoglycaemic effect, the leaves decreased the hypoglycaemic effect of the glimepiride due to a decrease in its onset of action, while it produced an enhanced hypoglycaemic effect with metformin (Fakeye et al., 2007). Similarly, the water soluble fractions of okro (*Abelmoschus esculentus* (L.) Moench) significantly decreased the *in vivo* absorption of metformin resulting in a loss of its hypoglycaemic effect (Khatun et al., 2011).

An enhanced hypoglycaemic effect should therefore not always be assumed by patients and physicians alike, as there may be other underlying interactions that may negate this expected effect as shown with okro. A true assessment can however only be possible when *in vivo* experimental studies are carried out or active therapeutic monitoring of patients' blood glucose levels. These are unfortunately beyond the scope of this current PhD research.

Limited information about the pharmacokinetic profile and potential risks of herb-drug interaction of most herbal medicines makes pharmacokinetic HDIs more difficult to predict. Consequently, efforts have been channelled towards investigating the effects of different herbal extracts on known pharmacokinetic parameters. Individual studies involving medicinal plants used in Nigeria, and indeed in West Africa are however limited (Oga et al.,

2012, Agbonon et al., 2010, Appiah-Opong et al., 2008, Deferme et al., 2003). Various reviews of the scientific literature have shown that although pharmacokinetic data involving herbal remedies have significantly increased in recent years, many of these have focused only on a small number of commonly used herbal products; while for the majority of herbal medicines used in traditional medicine, data on their disposition and biological fate in humans is lacking (He et al., 2010).

Efforts have also been made to collate existing information about herbal medicines as a guide to healthcare practitioners, and as a means of forestalling potentially clinically relevant herb-drug interactions (Joint Formulary Committee, 2014, Williamson et al., 2013, Ulbricht et al., 2008). Despite a higher prevalence of herb use in Africa, similar resources collating information about commonly used herbal medicines, particularly those that are indigenous to African countries as a guide for healthcare practitioners are far less common. Only one review covering thirty-five plants was identified (Cordier and Steenkamp, 2011).

Medicinal plants used in diabetes management in Nigeria are utilised in various forms (Figure 1.2) - either as food vegetables incorporated as part of one's diet; as crude herbal preparations; or as manufactured herbal medicinal products, which are more commonly found in pharmacy stores. The herbal preparations and manufactured herbal medicinal products are often multi-component mixtures of two or more plants most likely to produce a 'synergistic effect'.

Crude herbal preparations are the most commonly utilized form of herbal medicine (Fakeye et al., 2009, Olisa and Oyelola, 2009). These are often prepared by a traditional medicine practitioner (TMP). Sometimes, patients purchase plant parts from various herbal markets based on recommendation by the herb seller, friends or family which they then prepare by themselves. Common methods of preparation include maceration with aqueous or alcoholic solution, infusion, decoction and the direct use of powdered plant parts sometimes sprinkled on food.

The expertise acquired by traditional medicine practitioners by prolonged use is such that they are often aware of the right method of preparation and/or the right dosage to avoid the harmful effects of herbal medicines. This expertise is however on the premise that these herbal medicines are being used by themselves for the disease management and not alongside conventional medicines. Thus, there is very little awareness of the risks of herb-drug interactions. These risks are further heightened in patients who self-medicate herbal medicines based on recommendations by friends and/or family with the preconception that 'herbal medicines are natural and hence safe'.

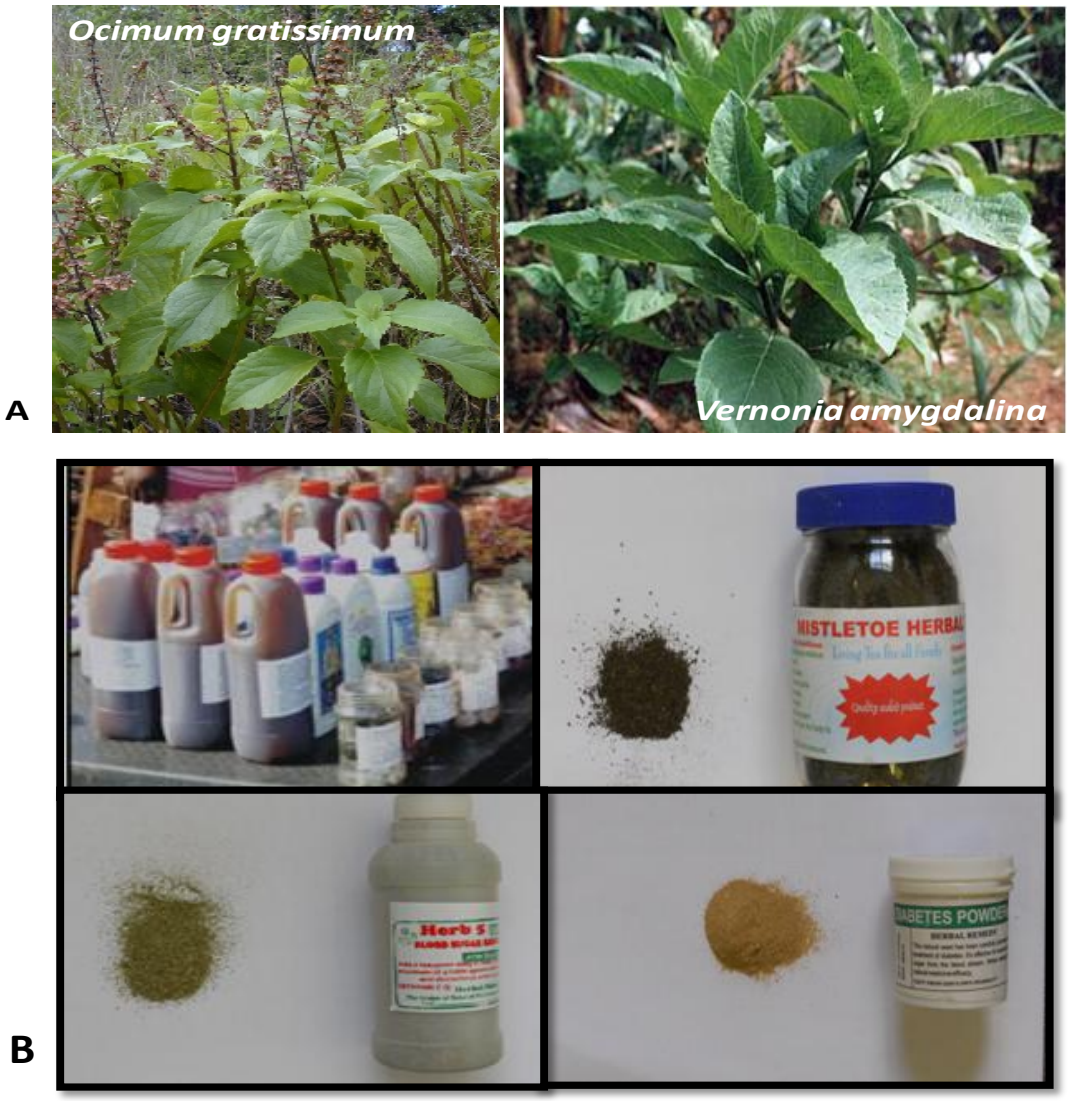

Figure 1.2: Various forms in which medicinal plants are utilised as herbal medicines in Nigeria. (a) *Ocimum gratissimum* and *Vernonia amygdalina* are two anti-diabetic medicinal plants commonly used as food vegetables and often grown in home gardens; (b) Crude herbal preparations prepared by herbalists themselves or sold in herbal shops and/or markets, often comprising unidentified constituents.

Manufactured herbal medicinal products are powdered plant extracts in capsules or compressed as tablets as well as liquid preparations containing a specified dose. These are the only forms of herbal medicine that are currently subject to regulation in Nigeria by the National Agency for Food and Drug Administration and Control (NAFDAC). A registration status renders to the product a permit for it to be sold and distributed outside the locality of production. The quantitative list of ingredients either by their botanical names or common names, evidence of efficacy and safety, as well as the indications and contraindications of the product are some of the requirements for registration. A 'listing status' is initially given

to manufactured herbal medicinal products for a period of two years, which precludes the need for rigorous assessment of good manufacturing practices or laboratory evaluations of efficacy. A full registration licence is then given after a reassessment of the safety of the product based on post market surveillance and laboratory evaluations of safety and efficacy (NAFDAC, 2005).

The absence of an extensive regulatory framework encompassing most of the herbal medicines utilised in Nigeria means that the current system is ill-suited for pharmacovigilance monitoring to help the identification of some of the unwanted effects that may be encountered during disease management. There is therefore a need for an experimental evaluation of the potential for herb-drug interactions for these anti-diabetic plants so that their use in diabetes management can be properly harnessed by the population.

1.5 AIM OF THESIS

The complexity of diabetes and the current therapeutic options available for its management, coupled with the associated treatment burden due to healthcare costs identifies it as a disease with a very high risk for concomitant use of herbal medicines particularly in developing countries like Nigeria. Unfortunately, adequate information about the pharmacological and toxicological profile of most of the herbal medicines used in diabetes management that would enable the prediction and/or identification of potential herb-drug interactions is lacking. In addition, there is the absence of an appropriate regulatory framework for the use of herbal medicines that would ensure pharmacovigilance monitoring, identification and reporting of these herb-drug interactions if and when encountered by healthcare practitioners.

In a bid to address some of these identified problems, the aim of this PhD research is the evaluation of medicinal plants used in diabetes management in Nigeria for possible herb-drug interactions.

1.5.1 Research strategy

To achieve this, the research for this thesis was carried out in three parts:

- An assessment of available data on the pharmacology and toxicology of herbs traditionally used for diabetes management in Nigeria and theoretical predictions of herb-drug interactions that their use may pose. A review collating the results of this assessment and its implications in disease management has now been published (Ezuruike and Prieto, 2014).
- A field work study carried out in different locations of Nigeria with the aim of identifying medicinal plants that are commonly used by diabetic patients in their disease management; and an assessment of the potential risks of pharmacokinetic herb-drug interactions amongst a cohort of diabetic patients in two urban cities in Nigeria based on their co-administered prescription drugs.
- Experimental evaluation of the effects of some medicinal plants collected during the field work on different cell detoxification mechanisms and pharmacokinetic parameters; as a means of identifying potential herb-drug interactions.

2 CRITICAL ASSESSMENT OF PHARMACOLOGICAL AND TOXICOLOGICAL DATA FOR MEDICINAL PLANTS USED IN DIABETES MANAGEMENT IN NIGERIA

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2.1 INTRODUCTION

A number of ethnobotanical surveys have been carried out in different parts of Nigeria to identify plants used in the traditional management of diabetes (Soladoye et al., 2012, Gbolade, 2009, Etuk and Mohammed, 2009, Abo et al., 2008). These surveys have highlighted not only the dependence of a large percentage of the Nigerian population on herbal medicines, but also the use of a large number of plants from diverse plant families. Various reviews have also highlighted the traditional use of a wide range of medicinal plants in diabetes management in other parts of the world (Andrade-Cetto and Heinrich, 2005, Grover et al., 2002, Marles and Farnsworth, 1995, Bailey and Day, 1989, Bever, 1980).

Although a lot of these medicinal plants have been shown to possess a number of beneficial therapeutic effects, they could also be harmful, depending on the dose administered or the method of preparation. Pharmacological and/or toxicological evidence available for these medicinal plants are 'scattered' in various publications, which are sometimes not readily available or accessible to patients, healthcare practitioners and researchers to make a thorough assessment of their beneficial and toxic effects in diabetes management.

This chapter, therefore, aims to provide Nigerian healthcare professionals with a useful resource to enable a thorough assessment of the pharmacological and toxicological profile of plants used in diabetes management so as to ensure better advice and safer use.

2.2 METHOD

Information about medicinal plants traditionally used in the management of diabetes in Nigeria was obtained from published papers and texts on ethnobotanical studies; as well as those investigating the effect of plant(s) used in diabetes management, in which the place of use and/or sample collection was identified as Nigeria. A literature search of electronic databases such as Google Scholar, Pubmed and Scopus up to 2013 was carried out using 'Diabetes' and 'Nigeria' as keywords for the primary searches; and then 'Plant name-accepted or synonyms', 'Constituents', 'Drug interaction' and/or 'Toxicity' for the secondary searches.

In order to highlight medicinal plants traditionally used in diabetes management with the potential for integration into the healthcare system, not all identified plants were included. Only those with (1) more than one reference to its use in diabetes management in Nigeria based on ethnobotanical studies were retained; and/or (2) experimental evidence in one or more diabetes experimental models validating its activity. This analysis is therefore not

exhaustive for all the plants reportedly used traditionally for diabetes management in Nigeria.

2.3 RESULTS AND DISCUSSION

Data for one hundred and fifteen plants traditionally used in diabetes management in Nigeria were obtained; either from previously conducted ethnobotanical studies (Igoli et al., 2004, Igoli et al., 2005, Aiyeloja and Bello, Okoli et al., 2007, Abo et al., 2008, Ajibesin et al., 2008, Olowokudejo et al., 2008, Etuk and Mohammed, Gbolade, Ogbonnia and Anyakora, Lawal et al., 2010); or from primary research papers. These are tabulated according to their accepted Latin Name (based on www.plantlist.org). Synonyms are included for plants which were not identified with their accepted names in the primary research paper. For each of the identified plants, the family name, common name(s), identified region of use in diabetes management, experimental evidence of activity (where available), other medicinal uses, plant part(s) used, traditional method(s) of preparation, identified active constituent(s), other relevant phytochemical constituents, as well as data on interaction and toxicity studies are included (Table 1; Appendix 1).

Out of the 115 plants, only twelve of them have no experimental evaluation of their blood sugar reducing effects, either *in vivo* or *in vitro*. In selecting studies to be included, priority was given to investigations carried out with samples collected in Nigeria. Certain publications were not included if the study design of the experimental evidence wasn't appropriate enough for validating the effect of the plant, such as the absence of a suitable control or the use of improper doses.

Two-thirds of the identified plants with experimental evidence for their biological activity involved samples collected from Nigeria. For the remaining one-third, although the studies were not carried out with plant samples sourced from within Nigeria, these were still included, as the available experimental evidence could provide some information validating their use in diabetes management in Nigeria. However, possible chemical variability would need to be taken into consideration. The ethnobotanical research investigating the local knowledge and geographical distribution of *Moringa oleifera* Lam. in Nigeria (Popoola and Obembe, 2013) provides a rationale for including information from studies carried out in different countries, as some of these locally available plants could have been initially sourced from elsewhere.

In-vitro experimental studies as well as phytochemical studies carried out on the plant species regardless of the source of the plant samples were also included. These together

could provide more insight into the biological activity(s) of the plant, which would in turn help to promote a more rational use of the plant in diabetes management, either in the presence or absence of other co-morbidities. For completeness, reports on the antioxidant properties of many of the identified plants have been included as this has become a popular parameter in assessing the beneficial effects of a plant in diabetes management.

2.3.1 Assessment of the Preclinical Pharmacological Evidence

2.3.1.1 In vivo hypoglycaemic activity

Reducing blood sugar levels is the classical clinical target in all forms of diabetes. Thus, the *in vivo* sugar lowering effect of putative hypoglycaemic plants is therefore a premise to infer their potential clinical efficacy. *In vivo* validation also provides an indication of the relative toxicity of the plant. Although most herbal medicines have a long history of traditional use, only their experimental validation at known doses may give a clearer idea about its safety and efficacy, in line with the objectives of the WHO traditional medicine strategy (WHO, 2013).

Ninety six out of the one hundred and fifteen plants have been evaluated in various *in vivo* animal models of diabetes, mostly using alloxan and/or streptozotocin (STZ)-induced diabetic animals, which are the most frequently used animal diabetes models worldwide (Fröde and Medeiros, 2008). These chemical agents are cytotoxic to the β-cells of the pancreatic islets, generating a state of insulin deficiency (akin to type-1 diabetes) with subsequent hyperglycaemia (Szkudelski, 2001). Other *in vivo* models used include spontaneous diabetic animal models obtained as a result of one or more genetic mutations- such as obese zucker fatty rats, db/db mice and KK-A^y mice; as well as the use of high glucose or fructose-fed animals. This latter group better simulate the development of diabetes from insulin resistance as is more commonly found in patients with type-2 diabetes (Srinivasan and Ramarao, 2007).

Although most plants were only evaluated experimentally in a type-1 diabetes model, some of these have been shown to be effective hypoglycaemic agents in type-2 diabetes patients, such as extracts of *Bridelia ferruginea* Benth. Daily administration of an infusion of its leaves to type-2 diabetic patients previously on insulin injections for eight weeks resulted in a significant decrease in their blood sugar levels (Iwu, 1983).

Only two out of the 96 plant species evaluated *in vivo* were ineffective in their experimental model of study, namely *Zea mays* L. (Suzuki et al., 2005) and *Cucumeropsis mannii* Naudin (Teugwa et al., 2013). Despite its identified *in vitro* PPARα and ɣ agonist activities and α-

glucosidase inhibitory effects (Lee et al., 2010), extracts of *Zea mays* failed to produce a significant hypoglycaemic effect *in vivo* (Rau et al., 2006). This could be due to the absence of the bioactive constituent(s) responsible for the hypoglycaemic effects or their availability in very low amounts as a result of chemical variability in the samples investigated. Nonetheless, the complete absence of an *in-vivo* hypoglycaemic effect would not necessarily preclude its use in the clinical management of diabetes, which also takes into account the presence of co-morbid conditions. In this regard, *Zea mays* could also provide protection against diabetic nephropathy, as it has been shown to improve kidney parameters *in vivo* (Suzuki et al., 2005).

In the case of *Cucumeropsis mannii*, its traditional use involves the ingestion of its ashes or its juice (Gbolade, 2009). This may indicate that its use is based on its oligo-elements and/or vitamins and not necessarily on secondary plant metabolites. The supplementation of elements such as chromium, magnesium and vanadium is actively explored in the treatment of diabetes (Anderson et al., 1997; Halberstam et al., 1996; Rodríguez-Morán and Guerrero-Romero, 2003); some of which have been identified in the seeds of *Cucumeropsis mannii* (Badifu and Ogunsua 1991).

2.3.1.2 In vitro pharmacological evidence

It is recommended that *in vitro* experiments are carried out to ascertain the mechanism of action for the plant. Certain plants produce hypoglycaemia as a side effect of their *in vivo* toxicity (Marles and Farnsworth, 1995). There is also the risk that the hypoglycaemic effect is being mediated –at least in part- through an unwanted physical mechanism, rather than a physiological one, such as was observed with *Gymnema sylvestre* (Retz.) R. Br. Ex Sm (Persaud et al., 1999). This immediately eliminates the potential use of such plant as a therapeutic hypoglycaemic agent. In addition, due to the ethical considerations surrounding animal use (Festing and Wilkinson, 2007), it is advised that validation experiments are 'replaced' with non-animal models where possible.

Over one-third of the plants have been studied for their *in vitro* effects in models that could possibly explain some or all of their mechanism of action. Twenty-nine plants have inhibitory effects against either α-amylase or α-glucosidase enzymes; five plants have agonist activity on the PPARγ receptor; whose activation enhances glucose metabolism; four plants increase insulin release from pancreatic cells; five plants increase glucose uptake in muscles or liver; while two plants increased the expression of the glucose transporter GLUT4, which in turn increases glucose uptake into muscles and adipose tissues. Two

plants were identified as potential DPP-IV inhibitors; while six plants were identified as aldose reductase inhibitors (Figure 2.1).

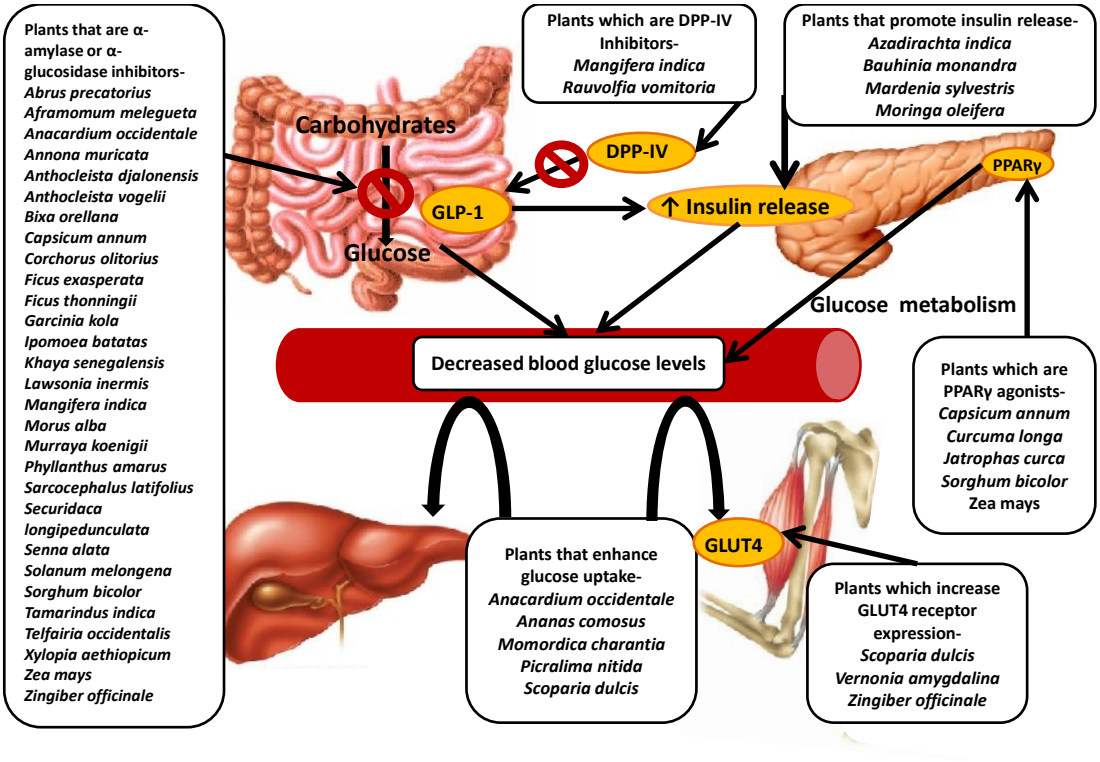


Figure 2.1: Proposed molecular mechanisms of hypoglycaemic effects for the selected species.

In vitro experiments are often designed to 'reflect' the mechanism of existing drugs used in diabetes management. Plants that possess alpha amylase or alpha glucosidase inhibitory effects reflect the action of acarbose; PPARγ agonist activity reflect the thiazolidinediones, while aldose reductase inhibitors are potential agents for preventing diabetic complications like the drug epalrestat. Thus with this identified mechanisms, researchers and healthcare professional alike can immediately identify the potential therapeutic benefit of the plant. This information could contribute to a more rational therapeutic regimen for diabetes patients, possibly benefitting from a synergistic effect with herbal remedies.

The disadvantage of the molecular approach for experimental validation of plant activity is that the biological assays only explore known targets and do not take into account extracts that might be acting on unknown targets, possibly through innovative mechanisms. In addition, herbal medicines are often complex mixtures of various phytochemicals which may work synergistically to achieve a desired therapeutic outcome (Williamson, 2001). In such cases, a single end-point *in vitro* biological assay will not be sufficient in evaluating the clinical effect of the plant.

As an example, the methanol extract of the root and stem of *Gongronema latifolium* Benth. produced a greater anti-hyperglycaemic effect in glucose loaded rats than each of its fractions, possibly indicating a synergistic –if not additive- effect of its constituents even though some of the fractions of the whole extract possessed hyperglycaemic effects. In addition, a number of triterpenes (α & β-amyrin cinnamate, lupenyl cinnamate, lupenyl acetate) possessing *in vitro* insulin stimulating effects were isolated from only one of the active fractions further confirming the synergistic activity of phytoconstituents of herbal medicines (Adebajo et al., 2013).

There is also a holistic approach in the herbal management of diabetes such that plants which are not hypoglycaemic themselves may be included in multi-component preparations because of their benefits in co-morbid conditions. Thus, *in vitro* studies might not immediately indicate the beneficial effect of the plant. A good example is the use of the aphrodisiac plant *Mondia whiteii* (Hook.f.) Skeels (Quasie et al., 2010). Despite not showing *in vitro* hypoglycaemic effect (Etoundi et al., 2010), it is commonly included in multi-component preparations for diabetes management in men since erectile dysfunction is a common complication of the illness (personal communication during field work). This however does not preclude any *in vivo* activity which is yet to be evaluated.

2.3.1.3 Bioactive compounds

Many plant secondary metabolites have been associated with specific beneficial effects in diabetes, which might account for the threrapeutic effect of the herbal drug (Qi et al., 2010). Thus, apart from a bioguided fractionation, the biologically active agent of a plant can also be hypothesised by evaluating the phytochemical constituents that have previously been isolated. These can thereafter be confirmed in specific pharmacologic experiments.

Over forty compounds have been identified from twenty three antidiabetic plants, either through an activity guided fractionation or *in silico* studies as the bioactive constituents responsible for some or all of the plants' beneficial effects in diabetes. Some of these constituents are species-specific such as the alkaloid mahanimbine from *Murraya koenigii* (L.) Spreng (Dineshkumar et al., 2010); while others are known to be present in many plants like the alkaloid trigonelline, which is responsible for the hypoglycaemic effect of *Abrus precatorius* L. (Monago and Nwodo, 2010) and *Trigonella foenum-graecum* L. (fenugreek), a plant whose use in diabetes management is popular across India and Europe (Bailey and Day, 1989).

These compounds are classified according to similar chemical features as follows: compounds containing nitrogen (**1-9**) (Figure 2.2), terpenes (**10-16**) (Figure 2.3), phenolic

compounds (**17-33**) (Figures 2.4) and compounds containing hydroxyl groups including sugars (**34-40**) (Figure 2.5).

2.3.1.3.1 Nitrogen containing compounds

A number of alkaloidal and non-alkaloidal active principles from plants used in diabetes management have been reported. Some of these were isolated from samples not collected from Nigeria such as hypoglycin A (**1**) and B (**2**) - from the fruit of *Blighia sapida* K.D.Koenig., which produced hypoglycaemic effects in rabbits, monkeys rats and mice but not in cats, dogs and pigeons (Chen et al., 1957).

Murraya koenigii leaves are used traditionally in Indian Ayurvedic system to treat diabetes. Mahanimbine (**5**) isolated from Indian plant samples decreased blood glucose levels in STZ-induced diabetic rats and also produced a dose-dependent α-amylase and α-glucosidase inhibitory effect (Dineshkumar et al., 2010). Its cellular mechanism of action is also thought to be mediated by an increase in glucose utilization in 3T3-L1 cells (Dinesh Kumar et al., 2013). On the other hand, mahanimbine and other related carbazole alkaloids isolated from plant samples from Nigeria decreased the glucose-mediated insulin release from INS-1 cells when compared to control, suggesting that its enhanced glucose uptake is not due to increased insulin release (Adebajo et al., 2005).

The alkaloid trigonelline (**4**) isolated from the seeds of *Abrus precatorius* (L.) collected from the eastern part of Nigeria decreased blood glucose levels in alloxan-induced diabetic rats as well as reduced the activity of glucose-6-phosphatase and glycogen phosphorylase, two enzymes important for glucose production (Monago and Nwodo, 2010).

Akuammicine (**3**) isolated from the chloroform extract of the seeds of *Picralima nitida* (Stapf) T.Durand and H.Durand stimulated glucose uptake in 3T3-L1 adipocytes (Shittu et al., 2010). It is also present in plants of the genus *Alstonia* such as *A. boonei* De Wild. and *A. Congensis* Engl. and would probably contribute to their blood glucose lowering activity. Ajmaline (**6**) and isosandwichine (**7**) from *Rauvolfia vomitoria* Afzel. were identified as DPP-IV inhibitors using an *in silico* approach (Guasch et al., 2012).

Garlic and onions are commonly used as part of the diet in many Nigerian households and the hypoglycaemic effect of plant samples collected from Nigeria have also been studied (Eyo et al., 2011). This hypoglycaemic effect is possibly due to the presence of S-methylcysteine sulfoxide (**8**) (SMCS) in onions and S-allylcysteine sulfoxide (**9**) (SACS) in garlic, which have been isolated from Indian plant samples and have been shown to improve

glucose tolerance in alloxan-induced diabetic rats (Sheela et al., 1995). Clinical studies in humans have shown that the supplementation of garlic to diabetic patients in combination with hypoglycaemic drugs improves glycaemic control in addition to the reduction of cardiovascular risk (Sobenin et al., 2008). Although they both contain nitrogen, SMCS and SACS are primarily classed as sulphur containing compounds.

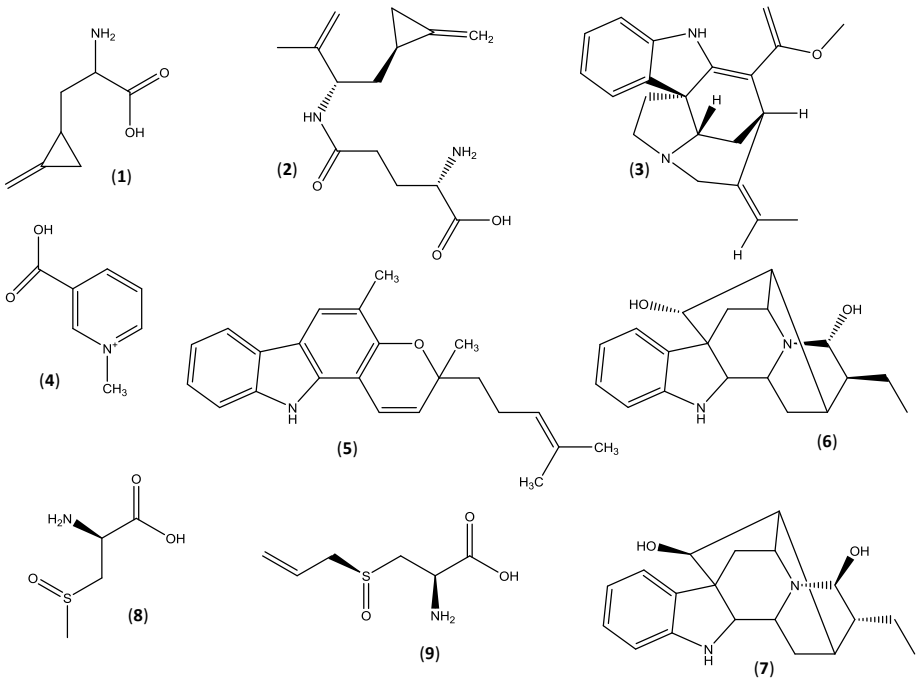


Figure 2.2: Nitrogen containing active principles from plants used in diabetes management.

2.3.1.3.2 Terpenes

A number of terpenes have been isolated as bioactive constituents in plants used for diabetes management (Figure 3). The leaves of *Gongronema latifolium*, otherwise known as 'utazi' or 'madu-maro' in Ibo and Yoruba respectively is commonly used as a food vegetable and is widely recognised for its traditional use in diabetes management. Lupenyl cinnamate (**15**), lupenyl acetate and α- & β-amyrin cinnamates (**16**) isolated from the combined root and stems of locally obtained samples have recently been identified as some of the bioactive compounds, possessing both anti-hyperglycaemic effects in glucose-fasted rats as well as insulin stimulating effects in INS-1 cells (Adebajo et al., 2013).

Foetidin from the whole plant and the unripe fruits of *Momordica foetida* collected in Nigeria also decreased blood glucose levels of normal fasted, but not alloxan-induced rats at only 1mg/kg (Marquis et al., 1977).

Acetylenic glucosides (**10**) and (**11**) isolated from *Bidens pilosa* decreased blood glucose levels in the murine type 2 diabetes model C57BL/Ks-*db/db* mice (Ubillas et al., 2000); and inhibited the spontaneous development of diabetes in non-obese diabetic (NOD) mice by modulating the differentiation of T-helper cells (Chang et al., 2004).

The monoterpenes myrcene (**12**), citral (**13**) and geraniol (**14**) found in *Cymbopogon citratus* were identified as aldose reductase inhibitors using *in-silico* docking methods (Vyshali et al., 2011). They are also components of the essential oil of many medicinal plants used in Nigeria as shown in Table 1 (Appendix 1). This preliminary information warrants further *in vitro* and *in vivo* studies involving plant samples from Nigeria known to contain these compounds, given that the beneficial effect of the essential oil of *Cymbopogon citratus* in the amelioration of glycaemia, insulinaemia & lipid dysmetabolism has now been validated *in vivo* in experimentally induced type-2 diabetic rats (Bharti et al., 2013).

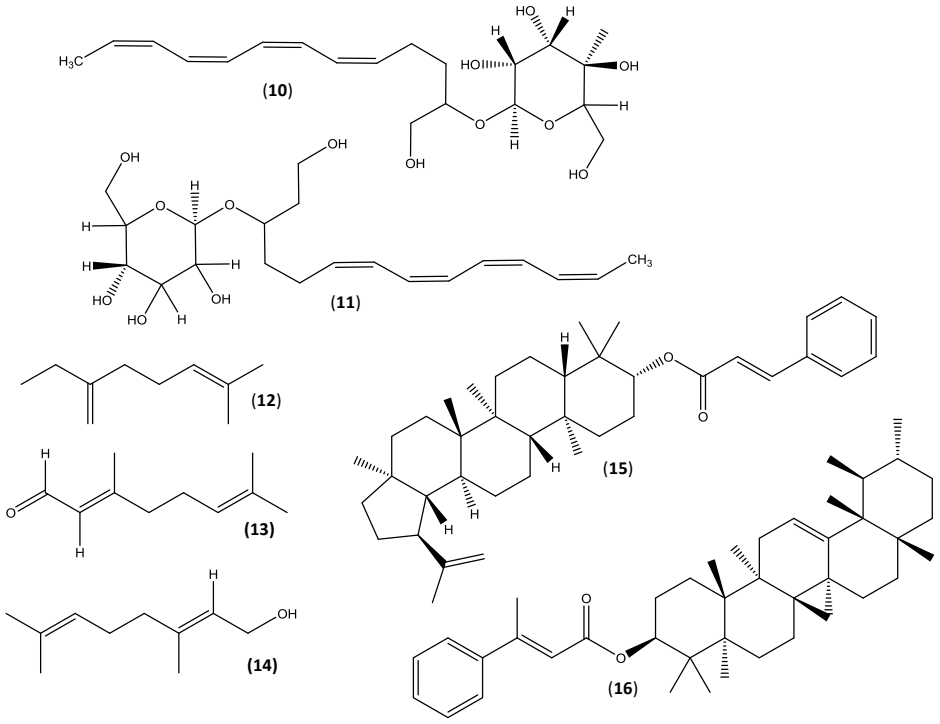


Figure 2.3: Terpenes identified as active principles from plants used in diabetes management.

2.3.1.3.3 Phenolic compounds

A wide range of phenolic compounds have been identified as active principle(s) in some of the plants. Anthraquinone glycosides from *Morinda citrifolia* L, namely damnacanthol-3-*O*-β-D-primeveroside (**18**) and lucidin 3-*O*-β-D-primeveroside (**19**), decreased blood glucose

levels in STZ-induced diabetic mice at 100 mg/kg (Kamiya et al., 2008). Incidentally, this plant is not native to Nigeria and isn't known to grow in Nigeria. However, the use of a registered herbal product of the juice extract, Tahitian noni juice® (TNJ) is quite popular in Nigeria for various ailments including diabetes. Administration of 1ml/150mg body weight of the rats twice daily for four weeks prior to and after the induction of diabetes with alloxan resulted in significant decrease in blood sugar levels, indicating a prophylactic effect of the extract against alloxan-induced diabetes (Horsfal et al., 2008). The presence of these phenolic compounds in the marketed product has however not been confirmed.

Kolaviron (**17**) is a mixture of flavanones (GB1, GB2 and kolaflavanone) isolated from the acetone extract of the edible nuts of *Garcinia kola* Heckel (bitter kola), which is valued in most parts of West Africa. It decreased blood sugar levels in normal and alloxan induced diabetic mice at a dose of 100mg/kg; as well as inhibited rat lens aldose reductase (RLAR) activity (Iwu et al., 1990).

Other phenolic compounds have been identified as bioactive constituents but not from plant samples collected in Nigeria. A diacylated anthocyanin peonidin 3-*O*-[2-*O*-(6-*O*-*E*-feruloyl-β-D-glucopyranosyl)-6-*O*-*E*-caffeoyl-β-D-glucopyranoside]-5-*O*-β-d-glucopyranoside isolated from the root of *Ipomoea batatas* (L.) Poir. showed potent maltase inhibitory effects *in vivo* (Matsui et al., 2002); while ellagic acid (**26)** and 3,5-dicaffeoylquinic acid (**27**) isolated from the hot water extract of the leaves showed potent aldose reductase inhibitory effects (Terashima et al., 1991).

Lawsone (**20**) (a naphtoquinone) and gallic acid (**21**) isolated from the ethanol extract of the aerial parts of *Lawsonia inermis* L. inhibited the formation of advanced glycated end products *in vitro* (Sultana et al., 2009).

Some methoxy phenyl derivatives (**22-25**) isolated from the rhizomes of *Zingiber officinale* Roscoe have been identified as aldose reductase inhibitors both *in vitro* and *in vivo;* suppressing sorbitol accumulation in human erythrocytes as well as lens galactitol accumulation in 30% galactose-fed rats (Kato et al., 2006).

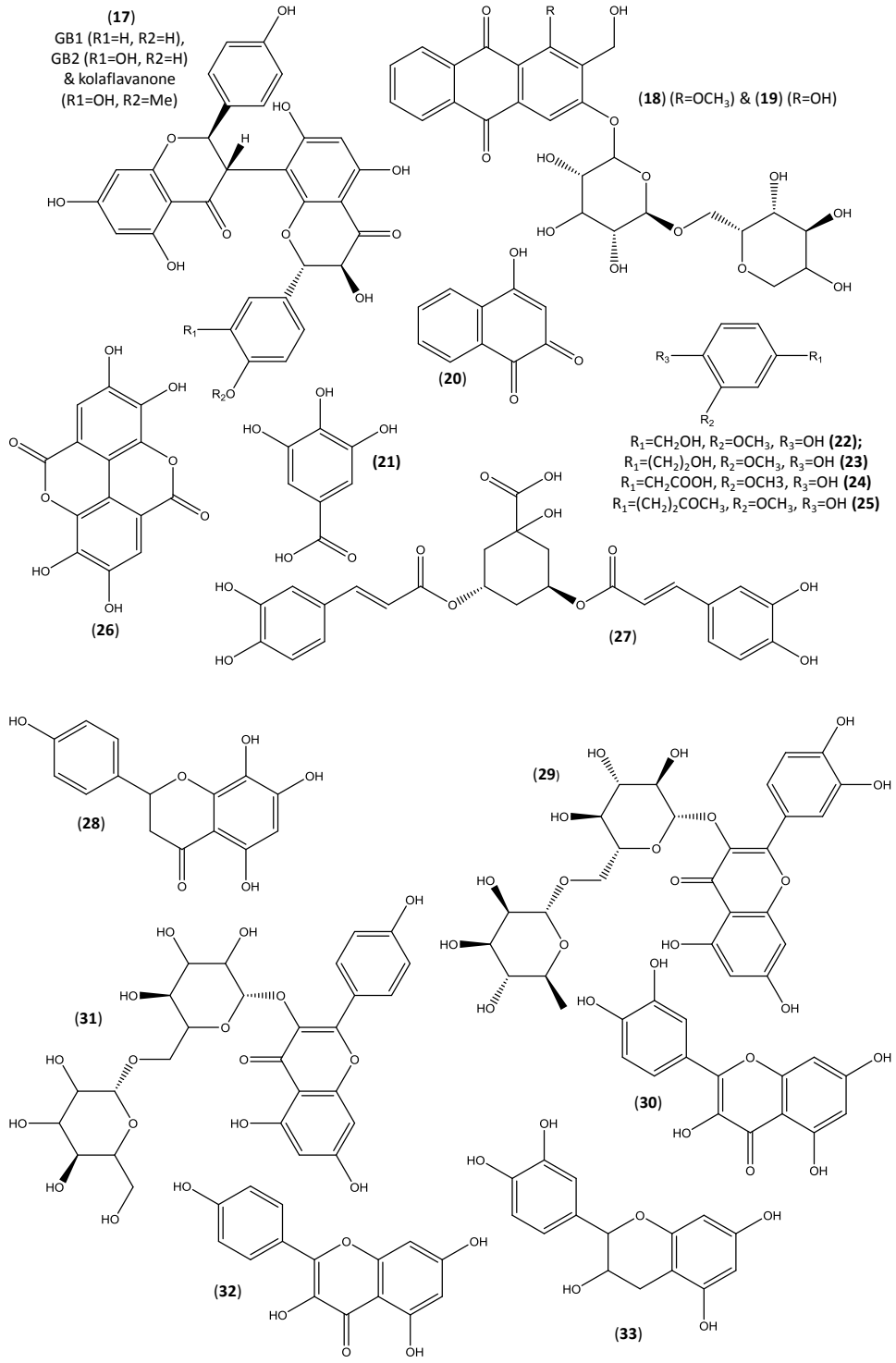


Figure 2.4: Phenolic compounds identified as active principles from plants used in diabetes management.

Several isolated flavonoids have also been identified as bioactive constituents. Isoscutellarein (8-hydroxy apigenin) (**28**) is a flavonoid isolated from the hot water extract of the leaves of *Bixa orellana* L., which was identified as an aldose reductase inhibitor (Terashima et al., 1991).

Rutin (**29**) and quercetin (**30**) were isolated from the leaves of *Bauhinia monandra* Kurz as the anti-hyperglycaemic constituents in alloxan-induced diabetic rats (Alade et al., 2011) (Alade et al., 2012). A bioassay guided fractionation of the stem bark of *Cassia fistula* L. led to the identification of catechin (**33**) as the bioactive agent. It decreased plasma glucose levels in STZ-induced diabetic rats, with direct effects on glucose metabolizing enzymes and expression of the glucose transporter GLUT4 (Daisy et al., 2010).

Fractionation of the methanol extract of the leaves of *Senna alata* (L.) Roxb. (syn- *Cassia alata*), which showed potent α-glucosidase inhibitory effects, identified kaempferol gentiobioside (**31**) and kaempferol (**32**) as the bioactive compounds (Varghese et al., 2013).

Increased translocation of GLUT4 receptors to the plasma membrane of L6 myotubes was also observed with a flavonoid-rich fraction of *Scoparia dulcis* L. (Beh et al., 2010), although the bioactive constituent(s) was not identified.

The presence of aromatic hydroxyl groups in the benzo-ɣ-pyran structure of flavonoids is associated with its antioxidant properties, particularly its free radical scavenging effects. These properties have been shown to protect pancreatic islet cells from oxidative stress as well as help in the regeneration of β-cells as shown with epicatechin found in green tea (Sabu et al., 2002) and quercetin (Coskun et al., 2005). More importantly, they can prevent the formation of advanced glycated end products (AGEs) and other diabetic complications associated with high oxidative stress conditions such as artherosclerosis, nephropathy, neuropathy, retinopathy and erectile dysfunction (Rahimi et al., 2005). Thus, the presence of quercetin and epicatechin as well as other potent antioxidant flavonoids in a wide range of plants such as *Irvingia gabonensis, Khaya senegalensis, Mangifera indica, Securidaca longipedunculata* and *Ocimum gratissimum,* will contribute to -and in some cases may be the basis for- their use in the holistic management of diabetes which includes the prevention of diabetic complications.

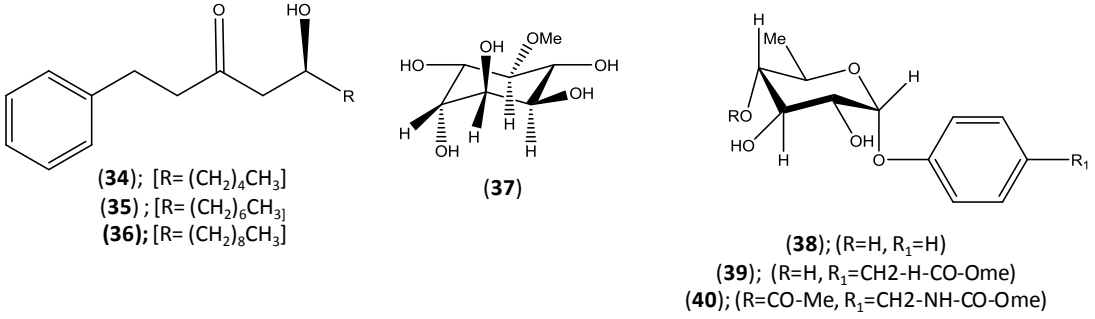
Other flavonoids have also been shown to directly affect specific therapeutic targets in diabetes. For instance, supplementation of mice diet with naringin or hesperidin modulated the activity of glucose metabolizing enzymes, with an increase in hepatic glucokinase activity and decrease in hepatic glucose-6-phosphatase activity in diabetic db/db mice (Jung

et al., 2004) and GK type-2 diabetic rats (Akiyama et al., 2009). These two flavonoids are constituents of all citrus fruits and have also been identified in *Senna alata* (Hennebelle et al., 2009) and *Rauvolfia vomitoria* (Campbell-Tofte et al., 2011) and as such may account for some of their effects. Myricetin is another flavonoid that has shown direct beneficial effects in diabetes through enhanced glycogen metabolism (Ong and Khoo, 2000) and improved insulin sensitivity (Liu et al., 2007). It has been identified in some of the plants either in its aglycone form or as a glycoside and may thus contribute to their anti-diabetic effects. These are the *Allium* species, *Aloe vera, Azadirachta indica, Citrus* species, *Carica papaya, Bryophyllum pinnatum, Cassia sieberiana, Chrysophyllum albidum, Ipomoea batatas* and *Bridelia ferruginea.*

2.3.1.3.4 Hydroxylated compounds including sugars

Some other non- phenolic hydroxylated cyclic compounds have been isolated and identified as bioactive agents. These include 6- (**34**), 8- (**35**) and 10-gingerols (**36**) from *Zingiber officinale,* which were shown to enhance glucose uptake into muscles as a result of a direct increase in the expression of the GLUT4 receptor (Li et al., 2012).

An inositol derivative, D-3-O-methyl chiroinositol (**37**) isolated from the methanol extract of the stem bark of *Bauhinia thonningii* Schum. produced a dose-dependent decrease in blood glucose levels in alloxan-induced diabetic rats (Asuzu & Nwaehujor 2013).



(**34**); [R= $(CH_2)_4CH_3$]
(**35**) ; [R= $(CH_2)_6CH_3$]
(36); [R= $(CH_2)_8CH_3$]

(**37**)

(**38**); (R=H, R_1=H)
(**39**); (R=H, R_1=CH2-H-CO-Ome)
(**40**); (R=CO-Me, R_1=CH2-NH-CO-Ome)

Figure 2.5: Other hydroxylated compounds identified as active principles from plants used in diabetes management.

Finally, a number of benzyl derivatives including carbamates and thiocarbamates have been isolated from fractions of the methanol extract of the fruits of *Moringa oleifera* Lam. These compounds have been shown to possess insulin secretory effects in pancreatic INS-1 cells at the very low dose of 100ppm. Some of these compounds were identified as 1-*O*-phenyl α-L-rhamnopyranoside (**38**), methyl *N*-(4-α-L-rhamnopyranosyl)benzylcarbamate (**39**); and methyl *N*-4-(4'-*O*-acetyl-α-L-rhamnopyranosyl)benzylcarbamate (**40**).

2.3.2 Assessment of the Clinical Evidence

The validation of biologically active plants in randomized, placebo-controlled clinical trials involving human subjects is a necessary step towards the possible integration of traditional herbal products into health systems. For these purposes, isolation of the active constituent may not be necessary. The European Directive of Traditional Herbal Medicinal Products is an example of how reports of traditional use and a sound safety profile are enough to regulate herbal medicines (Cox and Roche, 2004). However, knowing the identity of the active principle would be ideal in order to ensure a better quality control and perhaps a more defined dosage.

Fourteen plants have been clinically evaluated in human subjects, either singly or in combination. These are *Bridelia ferruginea, Citrus aurantium, Gongronema latifolium, Ocimum gratissimum, Rauvolfia vomitoria, Vernonia amygdalina, Carica papaya, Curcuma longa, Ipomoea batatas, Irvingia gabonensis, Gymnema sylvestre, Phyllanthus amarus* and *Solanum aethiopicum* (Table 1; Appendix 1), of which the first six involved plant samples collected from Nigeria. Only *Phyllanthus amarus* did not produce the desired clinical effect (Moshi et al., 2001).

Most of the clinical studies were not randomized, controlled trials but preliminary studies evaluating the therapeutic effect of the plant in human subjects. Exceptions to these were those carried out on *Rauvolfia vomitoria* and *Citrus aurantium* (Campbell-Tofte et al., 2011), *Irvingia gabonensis* (Ngondi et al., 2009) and *Ipomoea batatas* (Ludvik et al., 2004). A meta-analysis by Leung and his colleagues of all clinical studies carried out on *Momordica charantia* identified flaws in most of the study design, despite the extract consistently producing a hypoglycaemic effect (Leung et al., 2009). These imitations in study design hinder their clinical utilization as herbal medicines as appropriate conclusions that will act as guidelines for their use cannot be drawn.

A good knowledge of the traditional use of plants based on ethnobotanical studies is very important in the design of a good clinical study. This is especially important for plants which are used as mixtures, as the individual components may be working synergistically to produce the overall desired effect. An example is the synergistic effect produced by a decoction mix of the leaves of *Gongronema latifolium, Ocimum gratissimum* and *Vernonia amygdalina* in modulating baseline blood glucose levels, which was not observed with the individual plants (Ejike et al., 2013). Given that many of these herbal remedies are currently being taken by diabetic patients alongside their prescription medicines, a concerted effort between clinicians and researchers would be an ideal way to recruit patients to such studies.

To ensure the reliability of conclusions drawn from any clinical study, they should always involve proper planning with appropriate controls and ought to be conducted within a reasonable time frame; in line with the guidelines of the 'Declaration of Helsinki'. In addition, the recommendations for reporting randomized clinical trials, as defined in the 'Consolidated Standards of Reporting Randomized Clinical Trials (CONSORT) statement' (Schulz et al., 2010) should also be followed. Nonetheless, this relatively high 'success' rate amongst the various studies conducted highlights the potential of harnessing ethnobotanical information in enhancing patient therapy.

2.3.3 Assessment of the Toxicological Evidence

The administration of whole plant extracts or fractions consisting of a myriad of compounds, can elicit different biological effects in the body, some of which may be harmful. Sometimes, these toxic effects are only associated with certain parts of the plant. For example, the leaves of *Senna occidentalis* have hepatoprotective effects and are used traditionally for the treatment of liver disorders (Jafri et al., 1999). However, ingestion of toxins found in the seeds (beans) is thought to be the probable cause of acute hepato-myoencephalopathy (HMP) in children (Vashishtha et al., 2009). This risk of toxicity associated with the use of herbal products is one of the main reasons for the hesitance amongst healthcare practitioners towards promoting their integration into healthcare systems.

Adequate knowledge about the traditional use of such plants is very necessary as this often helps to forestall the ingestion of such toxic plants or plant parts. Sometimes the toxic component may have been identified such as abrin, a toxic protein found in the seeds of *Abrus pecatorius,* with an estimated human fatal dose of 0.1-1µg/kg (Kirsten et al., 2003). In rare cases, the hypoglycaemic agent in the plant could also be the toxic agent, such as with hypoglycin from *Blighia sapida* (Sherratt, 1986). Thus, the therapeutic use of such plants as whole extracts is therefore not recommended.

Various plants have been associated with specific organ toxicity. Examples include the nephrotoxic effects of *Alstonia congensis, Aristolochia spp, Cassia sieberiana, Ficus exasperata, Securidaca longipedunculata* and the hepatotoxic effects of *Cassia sieberiana, Ficus exasperata, Morinda citrifolia, Ocimum gratissimum, Picralima nitida, Senna occidentalis* and *Sphenocentrum jollyanum.* The cardiotoxic and neurotoxic effects of some other extracts have also been identified. Sometimes, these toxic effects are only seen at high doses, which would therefore not preclude their continued use as medicinal plants so long as

there is appropriate information about the safe dose ranges. The use of other more toxic plants would however need to be completely discontinued.

A thorough analysis of the plant extracts as well as identified phytochemical constituents with respect to their safety/toxicity profile particularly in humans can ensure a critical assessment of its therapeutic potential. Previously, coumarins which are a component of a wide range of plants were identified as hepatotoxic based on various studies carried out in rodents. However, further studies have showed that certain animal species are resistant to coumarin-induced toxicity. The 7-hydroxylation metabolic pathway is the most favoured in humans leading to the formation of non-toxic metabolites, whereas in rats the most favoured pathway is a 3,4-epoxidation leading to the formation of toxic metabolites. Knowledge of this and a quantitative health risk assessment has now confirmed its safety in humans (Lake, 1999; Felter et al., 2006).

2.3.4 Assessment of the Potential Herb-Drug Interactions

Evaluation of medicinal plants for potential herb-drug interactions is equally as important as its evaluation for efficacy and safety. Out of the one hundred and fifteen plants, thirty of them have shown *in vitro* and/or *in vivo* modulation of the activity of one or more of these ADME parameters (Figure 7). Some of these interactions were on absorption, either by modulating the effect of P-glycoprotein (P-gp), or by direct effects on the intestinal tight junctions. Other pharmacokinetic interactions were on metabolism, by interacting with one or more cytochrome P450 enzymes responsible for phase 1 metabolism or either of the phase 2 metabolic enzymes (Table 1; Appendix 1).

For drugs that are P-gp substrates such as glibenclamide, this effect of the efflux transporter is one of the determinant factors in the recommended dose of the drug to ensure that an adequate therapeutic concentration is achieved in the blood stream. Co-administration of the drug with a herb with inhibitory effects on P-gp such as *Acacia nilotica, Annona senegalensis, Bauhinia thonningii, Bridelia ferruginea, Carica papaya* and *Morinda lucida* might result in increased plasma concentration of the drug.

The activity of CYP enzymes can be modified either by induction or inhibition as seen with the extracts of *Bixa orellana* and *Jatropha curcas* respectively. The biological activity of the xenobiotics metabolized by these enzymes can be greatly altered as a result (Rendic and Carlo, 1997). St John's wort (*Hypericum perforatum*) is a very good example of a herbal product that has produced clinically significant effects as a result of its interactions with P-gp and CYP enzymes (Henderson et al., 2002).

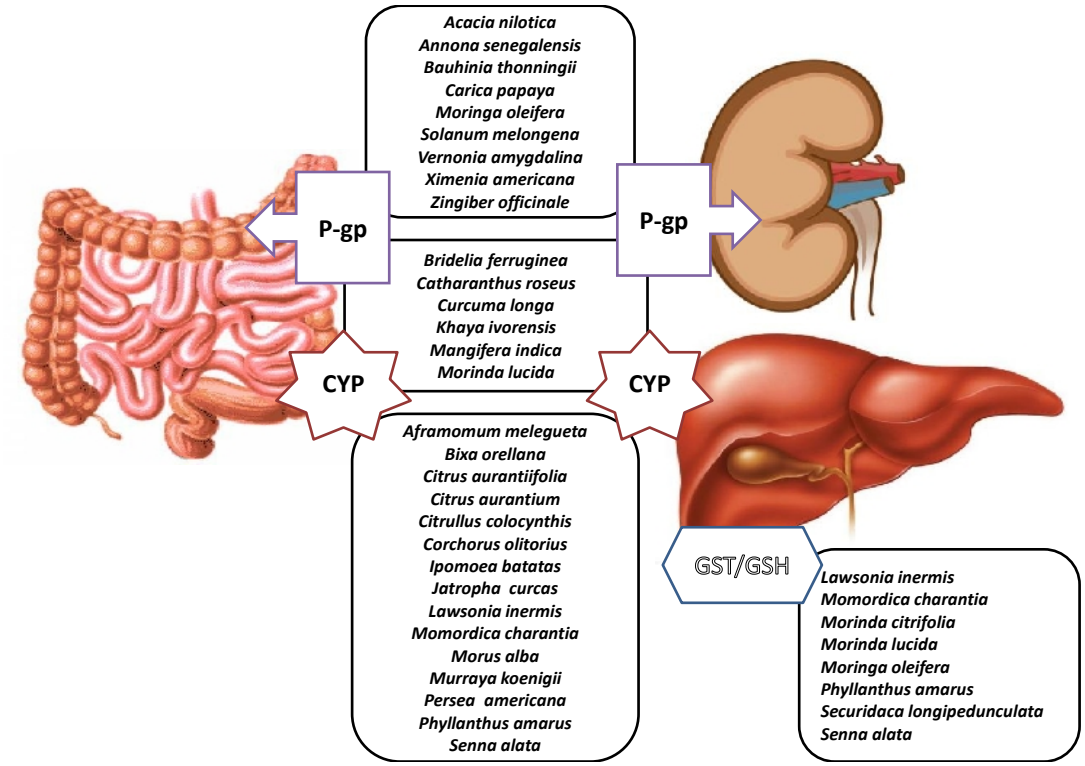


Figure 2.6: Identified *in vitro* pharmacokinetic herb-drug interactions.

In vitro interactions have also been identified with the phase 2 metabolizing enzymes, particularly with the glutathione transferases (GSTs). As with P-gp & CYPs, such interactions can alter the plasma concentration and resulting therapeutic effect of the co-administered substrate drug. In addition, GSTs directly control the levels of glutathione (GSH) within the cell. GSH also acts as an antioxidant within cells, and is particularly important in diabetic conditions characterized by oxidative stress. Unfortunately, plants such as *Securidaca longipedunculata* which decrease GSH levels might be counter-productive in diabetic patients.

For many of the plants shown in Figure 2.6, the phytochemical constituents responsible for the pharmacokinetic interaction are still unknown. Polyphenols present in plants especially flavonoids have been the most implicated in herb-drug interactions, with direct effects seen with specific flavonoids on P-gp and drug metabolizing enzymes (Morris and Zhang, 2006) (Galati and O'Brien, 2004) (Hodek et al., 2002). It should however be taken into account that many of the *in vitro* pharmacokinetic interactions or alarming case studies published in primary literature fail to translate into significant clinical risks for the patients, as seen with many herbal remedies taken in Europe (Williamson et al., 2013). There is still an urgent

need for effective pharmacovigilance of herbal medicines to ensure their safe and effective use in therapeutic management (Shaw et al., 2012).

2.4 CONCLUSIONS

This assessment, which is based on data mining of already existing published data for the selected plant species, shows that there is very good preclinical evidence for the efficacy of most of the plants traditionally used in Nigeria for the management of diabetes, either as hypoglycaemic agents or as useful agents in the management of diabetic complications. The original contribution of this work is in the mapping of (1) their identified pharmacological mechanism/s of action and (2) potential interactions with key parameters of the ADME process that can arise from the use of these plants. This can serve to promote the more rational use of these plants as herbal medicines based on the expected therapeutic outcome and their tabulated toxicological effects.

Given that only twenty three plants have been further explored to identify the active constituents responsible for their biological activity, the pharmacological evidence available for most of the plants can incentivise phytochemists to evaluate those plants with a potential for drug development. Particular emphasis can be given to those with no known toxicological effects but with identified molecular mechanisms of action. These include *Anacardium occidentale, Ananas comosus, Carica papaya, Citrus aurantium, Irvingia gabonensis, Sarcocephalus latifolius, Solanum aethiopicum, Sorghum bicolour, Tamarindus indica, Telfaria occidentalis* and *Vernonia amygdalina.*

Finally, these data can provide evidential support for the clinical development of a number of medicinal plants as adjuvant therapy. The criteria for selection should be based on social acceptance/frequency of use (with particular focus on those commonly encountered as food and vegetables), efficacy and toxicity profile, and availability/sustainability of the supply chain, possibly tailored to each region. A set of quality parameters for the standardisation of these plants as herbal preparations (such as Pharmacopoeial monographs) would be required to ensure the reproducibility of their therapeutic effects. As a means of giving credence to the pre-clinical experimental evidence, intervention or clinical studies with the standardised materials should be carried out in order to validate their usefulness in diabetes management. With this assessment, the therapeutic potential of these medicinal plants may be harnessed towards a possible integration into the healthcare system.

3 ASSESSMENT OF PHARMACOKINETIC HERB-DRUG INTERACTIONS AMONGST DIABETIC PATIENTS- FIELD WORK IN NIGERIA

3.1 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. As a result, a number of diabetic patients utilize herbal medicines as supplementation to their conventional medicines for their disease management.

The chronic nature of the treatment for diabetes however increases the risk of potential herb-drug interactions. The implications of clinically relevant herb-drug interactions can have far-reaching effects for the Nigerian population. Thus, by anticipating potential toxicities or possible herb–drug interactions, clinically relevant HDIs with significant risks which would otherwise present a further burden on the patient and on the country's healthcare system can be avoided.

A fieldwork study to identify the herbal medicines used by diabetic patients in Nigeria in their disease management was carried out. The objective of the study was to assess the potential for pharmacokinetic herb-drug interactions amongst this 'at risk' group based on available information about herbal medicines and the pharmacokinetic profile of the prescribed drugs used by the patients.

3.2 RESEARCH DESIGN AND METHODS

3.2.1 Management of Diabetes in Nigeria by Traditional Medicine Practitioners

In order to obtain information about herbs traditionally used in the management of diabetes in Nigeria, visits were done to five of the six geopolitical zones in Nigeria (Kaduna-North west, Abuja-North central, Imo-South east, Benin- South south and Lagos & Ibadan- South west) between February and April 2011 (Figure 3-1). The North east could not be visited due to political unrest and militant activities in the area.

Based on recommendations by members of the communities visited or visits to local herbal markets (Bode market in Ibadan Oyo state, Mushin market in Lagos and Zaria market in Kaduna and herbal trade fairs in Lagos and Kaduna), a total of thirty persons involved in traditional herbal medicine practice were approached to be interviewed about their traditional management of diabetic patients.

Semi structured interviews based on a sample questionnaire were conducted on those persons who consented. Data obtained from those contacted include- background information about age, sex and occupation, duration of practise as a traditional medicine

practitioner, understanding of diabetes and method(s) employed for treatment including the names of any herbal medicines used.

Information provided about medicinal plants used in diabetes management was verified by consulting different literature to determine the botanical names of plants from their given local names. Identification of collected plant samples 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) for plant samples collected in the north; and by staff of the Forestry Research Institute of Nigeria (FRIN) in Ibadan for samples collected in the south.

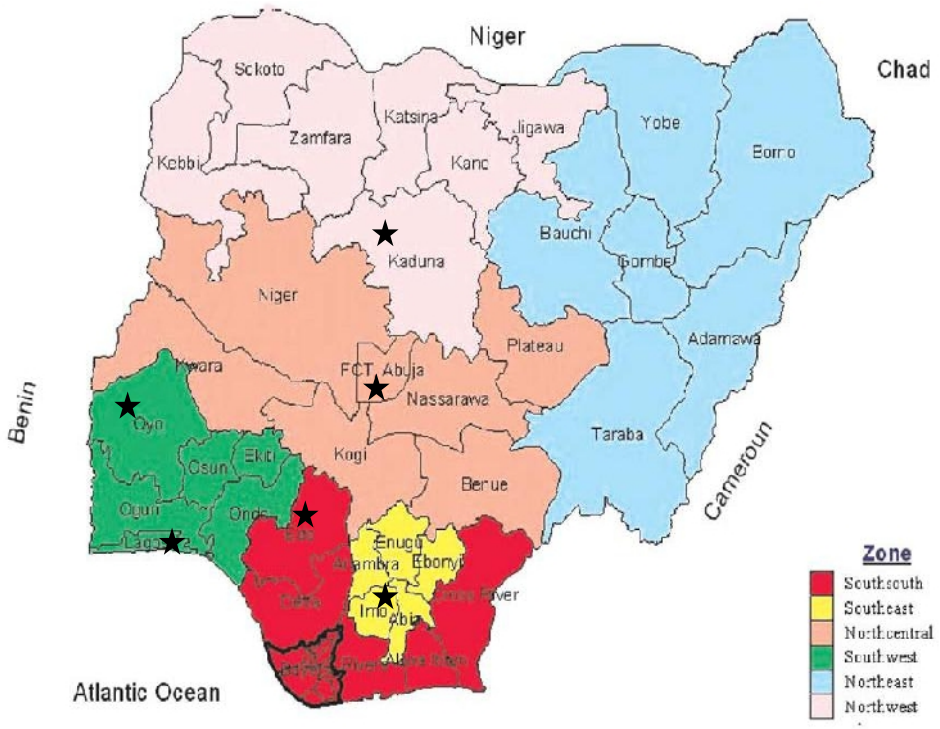


Figure 3-1: Map of Nigeria with stars indicating places visited as part of field work.

Figure 3-2: Aerial photograph of Bode market in Ibadan

Figure 3-3: (a) Close-up photos of some medicinal plant leaves and (b) Fruits of Pseudocolocynth or 'Tagiri' (*Lagenaria breviflora* (Benth.) Roberty) sold in the herbal market. (Pictures taken during fieldwork visit).

3.2.2 Assessing the Risk of Herb-Drug Interactions amongst Diabetic Patients

A follow-up study to obtain information about the use of herbal medicines alongside prescription drugs by diabetic patients in Nigeria was conducted in August and September 2012 in the Pharmacy departments of two secondary health care facilities- General hospital Lagos Island and Central hospital Benin City- two urban cities in the southern part of Nigeria. The study -including the proposed questionnaire to be administered- was approved by the ethics committee of UCL (Appendix 2- Ethics Application 4124/001); and prior consent was also obtained from the hospitals (Appendix 3).

Diabetic patients visiting the Pharmacy departments of the hospitals were selected based on the presence of an anti-diabetic drug on their prescription sheet. All patients meeting the above criteria were approached to be interviewed. However, only those who gave their consent were asked questions assessing their herb use based on a semi-structured questionnaire (Appendix 4). These questions were asked while drugs were being dispensed and medication counselling was given to the patients afterwards.

Information obtained from the questionnaire are demographic background of respondents including age and sex, information on the identity of the herbs (where available) and prescription drugs used either for diabetes or other co-morbidities as well as the monthly cost of prescription. Herbal medicines as used in this study consisted of the following: supplements containing extracts of medicinal plants, un-standardised herbal preparations from herbalists, herbal preparations made by the patients themselves using plant parts purchased in herbal markets or collected from their own gardens.

The descriptive and inferential statistics to determine measures of frequency and relationship between variables were carried out using IBM SPSS statistical package (version 22, IBM U.S.A.). Chi square test was used to test the association of herb use with patients' background characteristics such as gender, age groups, presence of co-morbidities and cost of monthly prescription. Significant relationship between variables was identified if $p < 0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 Management of Diabetes by Traditional Medicine Practitioners

Only twenty of the thirty persons contacted agreed to be interviewed about their use of the herbs in diabetes management. A summary of their socio-demographic characteristics are given in Table 3-1. Most of the respondents were male (85%). Half of them were aged between 45 & 60 years. Their years' of experience ranged from 5 to 50, but did not always

correlate with the age of the practitioner. Those who obtained their knowledge from family members often started practising at an early age; and so might have more years of experience than older respondents.

Eighteen respondents were traditional medicine practitioners (TMPs), one of whom was also a trained ethnobotanist; while two were herbal sellers in local markets. For the former, the conducted interviews were aimed at obtaining information about their management of diabetic patients. The results are shown in Table 3-2. While for the latter, only information about plants used and/or sold for the management of diabetes was obtained. A summary of the plants mentioned by all the respondents is given in Table 3-3.

Table 3-1: Socio-demographic characteristics of TMPs & Herbal sellers

Background characteristics (n=20)	Respondents
Age groups:	
30-45	7
45-60	10
Above 60	3
Gender	
Male	17
Female	3
Geographical location	
South east	4
South west	7
South south	2
North west	4
North central	3
Type of herbal education/training (TMPs)	
Family inheritance only	6
Informal education/Apprenticeship only	0
Formal education	2
Family inheritance & Formal education[§]	4
Family inheritance & Apprenticeship	1
Apprenticeship & Formal education[§]	1
Unknown	4

[§]Formal education undertaken by these respondents was for formulation and packaging of herbal medicines.

A general consensus amongst all the TMPs was that diabetes is a disease characterised by an excessive amount of ‘sugar’ in the body, often as a result of improper diet habits. This understanding of diabetes, may be due to the local names for diabetes in Nigeria; known as ‘ciwan sugar’ in Hausa, ‘oria sugar’ in Igbo and ‘ito-sugar’ in Yoruba. When literally translated to English, these all mean ‘sugar disease’. Other local names for diabetes include ‘atogbe’ in Yoruba, ‘yar maiko’ in Hausa and ‘oboro mammiri’ in Igbo, which in translation represent one of the other mentioned symptoms of diabetes.

Table 3-2: Knowledge of diabetes and its management by herbalists and herbal sellers

TMP's knowledge of diabetes & its management	Respondents (n=18)
Clinical symptom of diabetes*:	
Frequent urination	14
Sugar in the urine/saliva	7
Frequent thirst	6
Increased fatigue	6
Weight loss	6
Erectile dysfunction	4
Increased hunger	4
Dizziness	3
Dry/Itchy/Scaly Skin	2
Body pains	2
Delayed wound healing	2
Coughing	1
Memory loss	1
Yellowing of the eyes	1
Tingling sensation in feet	1
Cardiovascular symptoms	1
Irregular breathing	1
Method of diagnosis:	
Symptoms only	8
Symptoms & diagnostic test	8
Symptoms & traditional test#	1
Unknown	1
Mode of treatment:	
Herbs only	8
Herbs & diet	5
Herbs & non-plant parts	2
Herbs & nutritional supplements	1
Herbs & divinations	1
Herbs, non-plant parts & divinations	1
Duration of treatment:	
≤ 2 weeks	3
2 weeks – 3 months	11
3 – 6 months	3
Unknown	1

*Respondents' made a diagnosis based on one or more of the symptoms indicated, with or without a laboratory test.

#The patient is asked to urinate on a particular local leaf called 'awede' (*Heterotis rotundifolia* commonly known as 'chickweed'); and then a confirmatory diagnosis is given if the leaf changes colour from green to black.

Some of the symptoms used by the TMPs for their diagnosis such as 'yellowing of the eyes', 'memory loss' and 'coughing' are not known clinical symptoms of diabetes. However, these were each mentioned by only one of the herbalists. In addition, most of the respondents mentioned the use of at least one known clinical symptom of diabetes in their diagnosis.

One of the TMP's description of the disease was unrelated to the clinical understanding of diabetes, while another suggested it to have an underlying spiritual basis.

> "The definition of diabetes in the context of sugar isn't correct because diabetes existed long before sugar was known. It is an air-borne disease that is carried by the wind, which affects the liver when inhaled". (MC)

> "Diabetes is a spirit-related disease linked to sugar, which is why it is called 'yar maiko' in Hausa. Although it is associated with starchy diet and sweet foods, these are only contributory factors and not the causative agent". (MM)

The first response might indicate a false diagnosis and pose a risk of the patient not being properly managed for diabetes. Interestingly though, the TMP mentioned the use of only one plant *Anogeissus leiocarpus* (DC.) Guill. and Perr. in his management of diabetic patients, whose hypoglycaemic effects *in vivo* in alloxan-induced diabetic rats has previously been evaluated based on an ethnobotanical study (Etuk and Mohammed, 2009).

The traditional management of diabetes by the herbalists is usually aimed at reducing the high blood sugar levels. Several herbalists posit that 'bitter-tasting' plants are effective treatments for diabetes. The perception of most TMPs is that the use of herbal medicines brings about a complete cure of diabetes, which orthodox medicines fail to do. This is probably because most practitioners consider blood glucose control as the end goal for diabetes treatment. Only five of the respondents associated the disease with an impairment of the function of insulin from the pancreatic beta cells. As such, they were all aware that a reduction in blood glucose levels is not an indication for 'cured diabetes'. For this latter group, their focus in diabetes management is twofold: first for blood glucose control and the second for pancreas regeneration.

When asked about the side effects and possible interactions of herbal medicines, most TMPs considered these medicinal plants to be relatively 'safe' compared to conventional drugs; although one respondent identified possible side effects like dizziness, body weakness and night sweats. Two respondents mentioned that the unwanted effects produced by certain plants are not seen with the leaves but by the stem bark and roots.

> "Although plants can produce certain unwanted effects in people, these interactions are more common with the other parts of the plant like the root and the bark, but not the leaves". (EO)

It however seems that most respondents consider these 'more toxic' plant parts as having greater efficacy as reflected in table 2-3, given that the barks and the roots are the more frequently used plant parts. Most TMPs said that they discourage their patients from using

orthodox medications when undergoing treatment, not necessarily to prevent herb-drug interactions since they weren't aware of that but to prove the efficacy of their treatment.

The full cost of diabetes 'treatment' by the TMPs varied greatly, ranging from 15000 naira (≈ £60) to 250000 naira (≈ £1000). This was often dependent on the severity of the patients' symptoms (based on the blood glucose level at diagnosis) which would in turn determine what plant parts would be used as well as the duration of treatment. For less readily available plants which need to be collected from the forests, incurred transportation costs would contribute towards the treatment costs. A few respondents said that the patients' financial status sometimes determines how much he/she is asked to pay for treatment.

The advantage of herbal medicine use to low income earners is the option of flexible payment to TMPs or self-sourcing of the plant themselves if the identity is known. An alternative is that patients visit herbal markets (Figure 3-2) and the herbal sellers recommend different medicinal plants for the particular ailment of interest, which can either be single or multi-component. The patients then purchase the plant parts and prepare it for themselves at home, either by infusion or decoction.

A total number of fifty eight identified medicinal plants used for the traditional management of diabetes were obtained from the respondents (Table 3-3). Some plants which could not be identified by their local names are not included in the table due to the absence of plant samples/voucher specimens. Forty of the identified medicinal plants were mentioned by only one of the respondents. This may not necessarily be due to the unpopularity of these plants but might be a result of the few number of respondents interviewed. In fact, only two of the identified medicinal plants- *Phaseolus lunatus* L. and *Stachytarpheta indica* (L.) Vahl are herein identified for the first time as being used in diabetes management in Nigeria, although ethnobotanical studies has identified their use in other parts of the world.

Two of the plants- *Picralima nitida* (Stapf) T. Durand & H. Durand (PN) and *Vernonia amygdalina* Delile (VA) were the most frequently used based on informant consensus. They were both mentioned by nine out of the twenty respondents interviewed, with the use of the former by at least one TMP in all of the geopolitical zones visited. Pharmacological evidence of their hypoglycaemic effects has been confirmed, including a study carried out with VA in human subjects (Ejike et al., 2013). The alkaloid akuammicine has also been isolated from the seeds of PN and identified as one of the constituents responsible for its biological activity (Shittu et al., 2010).

Table 3-3: Tabular summary of ethnobotanical survey of plants used for the management of diabetes by herbalists

Plant name(s)*	Family	Common name	Local name(s)	Places where plant is used	No of times mentioned (%)	Part(s) used	Mode of use in diabetes management
***Ageratum conyzoides* (L.) L.**	Compositae	Goat weed	Imi esu (Ibo) Arusansan (Yoruba)	Abuja (NC)	1 (5%)	Whole plant	The fresh plant sample is macerated and the liquid extract taken twice daily. Juice extract from the fresh plant is applied to diabetic foot ulcers to promote wound healing.
***Allium sativum* (L.)**	Amaryllidaceae	Garlic	Aayu (Yoruba) Ayo-ishi (Ibo) Tafarunua (Hausa)	Imo (SE) Kaduna (NW) Lagos(SW)	4 (20%)	Bulb	The fresh bulb is added to liquid herbal preparations containing other medicinal plants.
***Aloe vera* (L.) Burm.f.**	Asparagaceae	Aloe vera		Imo (SE)	1 (5%)	Whole plant	Juice extract is drunk or used as a component of a mixed anti-diabetic herbal preparation for diabetes.
***Alstonia boonei* De Wild.**	Apocynaceae	Stoolwood tree	Ahun Yoruba) Egbu (Igbo)	Lagos (SW)	1 (5%)	Bark	A decoction of the bark (mixed with *Zingiber officinale*) is drunk.
***Anacardium occidentale* (L.)**	Anacardiaceae	Cashew	Kanju (Hausa) Kaju (Yoruba) Sashu (Igbo)	Lagos (SW) Abuja (NC)	3 (15%)	Bark Leaves Gum	A decoction of the leaves (or bark) is taken twice daily. The gum bark can also be chewed.
***Annona senegalensis* Pers.**	Annonaceae	Wild custard apple	Uburu ocha (Ibo) Ogoganto (Esan), Abo (Yoruba), Gwander-daji (Hausa)	Edo (SS)	1 (5%)	Stem-bark	A decoction of the stem bark is drunk as a mixture of barks used in the management of diabetes.
***Anogeissus leiocarpus* (DC.) Guill. & Perr.**	Combretaceae	Axlewood	Marke (Hausa) Orin-odan (Yoruba) Atara (Igbo)	Abuja (NC)	1 (5%)	Stem-bark	A decoction of the stem bark (boiled for several hours) is taken twice daily.
***Anthocleista djalonensis* A. Chev.**	Gentianaceae	Cabbage tree	Uvuru (Igbo) Sapo (Yoruba) Kwari (Hausa) Onigbogbo (Esan)	Lagos (SW) Edo (SS)	2 (10%)	Stem-bark Root	A decoction of the stem bark is drunk, either singly or as a mixture of barks used in the management of diabetes.
***Aristolochia repens* Mill.**	Aristolochiaceae	Dutchman's pipe	Ako-igun (Yoruba)	Lagos (SW) Edo (SS) Kaduna (NW)	5 (25%)	Root	One teaspoon of the powdered root (mixed with *Khaya spp*, *Mondia whitei*) is dissolved in warm water & taken twice daily. Powdered root (with *Picralima nitida* seeds) are mixed in lime juice and taken twice daily. Substitute lime juice with coconut water in patients with ulcer.
***Beta vulgaris* L.**	Amaranthaceae	Beetroot		Imo (SE)	1 (5%)	Root vegetable	Juice extract is drunk or used as a component of herbal preparation for diabetes.
***Bridelia ferruginea* (Benth.) Mull.Arg**	Phyllanthaceae		Oha/Ola (Igbo) Ira (Yoruba) Kimi (Hausa)	Imo (SE) Lagos/Oyo (SW)	1 (5%)	Stem-bark Root	A decoction of the stem bark is drunk, either singly or as a mixture with other barks used in the management of diabetes.

Plant name(s)*	Family	Common name	Local name(s)	Places where plant is used	No of times mentioned (%)	Part(s) used	Mode of use in diabetes management
***Bryophyllum pinnatum* (Lam.) Oken**	Crassulaceae	Africa never die Resurrection plant	Ewe abamoda (Yoruba)	Lagos (SW)	1 (5%)	Leaves	The powdered leaf (mixed with Picralima nitida seeds and the bark of unripe plantain) is given as maintenance medication when the glucose level is controlled.
***Carica papaya* L.**	Caricaceae	Pawpaw	Ibepe (Yoruba) Okworo bekee (Igbo) Gwanda (Hausa)	Kaduna (NC)	1 (5%)	Seeds	Dried powdered seeds are mixed with other plants (see above).
***Cassia sieberiana* DC.**	Leguminosae	Drumstick tree	Ukosei(Esan) Margaa (Hausa) Margaje (Fulani) Aridantooro (Yoruba)	Abuja (NC) Edo (SS)	2 (10%)	Root	A pinch of the powdered root (mixed with *Picralima nitida seeds* and *Diterium senegalensis* stem bark) is taken 2ce daily after food. A glass full of a decoction of the root is taken twice daily.
***Cassytha filiformis* L.**	Lauraceae	Love vine	Rumfargada (Hausa)	Abuja (NC)	1 (5%)	Whole plant	The plant (especially the root) is chewed before meals.
***Chrysophyllum albidum* G.Don**	Sapotaceae	Star apple	Agbalumo (Yoruba) Udala (Igbo)	Imo (SE)	1 (5%)	Stem-bark Seeds Fruit	A decoction of the stem bark is drunk, either singly or as a mixture with other barks used in the management of diabetes.
***Citrullus lanatus* (Thunb.) Matsum. & Nakai**	Cucurbitaceae	Water melon	Bara (Yoruba) Egusi (Igbo)	Kaduna (NC)	1 (5%)	Seeds Fruits	The powdered seeds are used as a condiment in local soups.
***Citrus aurantiifolia* (Christm.) Swingle**	Rutaceae	Lime	Osan wewe (Yoruba) Oroma nkilishi (Igbo)	Lagos (SW) Abuja (NC) Edo (SS)	3 (15%)	Fruit	The juice from the fruit is often used as the vehicle for herbal preparations.
***Cochlospermum planchonii* Hook. F. ex Planch.**	Bixaceae	False cotton	Awo owu (Yoruba) Zunzunaa (Hausa)	Abuja (NC)	1 (5%)	Roots Stem-bark	A decoction of the powdered roots is taken twice daily.
***Costus afer* Ker Gawl.**	Costaceae	Spiral ginger Bush cane	Okpete (Igbo) Kakikuwa (Hausa) Ireke-omode (Yoruba)	Kaduna (NW)	1 (5%)	Leaves Stem Rhizome	An infusion of the leaves is drunk or the powder is sprinkled on food.
***Cucumis sativus* L.**	Cucurbitaceae	Cucumber		Imo (SE)	1 (5%)	Fruit	The fruit is eaten or the juice extract drunk. The juice an ingredient in a multi-component herbal preparation.
***Daniellia ogea* (Harms) Holland**	Leguminosae	Gum copal tree	Ohin (Esan) Abwa (Igbo) Ojia/Iyaa (Yoruba) Dannu (Hausa)	Edo (SS)	1 (5%)	Stem bark	A decoction of the stem bark is drunk as a mixture of barks used in the management of diabetes.
***Daucus carota* L.**	Apiaceae	Carrot		Imo (SE)	1 (5%)	Root vegetable	The root vegetable is eaten fresh or the extract is drunk regularly by diabetic patients.
***Detarium senegalense* J.F.Gmel.**	Leguminosae	Tallow tree Sweet detar	Taurar (Hausa) Ogbogbo (Yoruba)	Abuja (NC)	1 (5%)	Stem bark	A pinch of the powdered stem bark (mixed with *Picralima nitida* seeds and *Cassia sieberiana* root) is taken twice daily after food.

Plant name(s)*	Family	Common name	Local name(s)	Places where plant is used	No of times mentioned (%)	Part(s) used	Mode of use in diabetes management
***Gongronema latifolium* Benth.** † **Botanical synonym: *Marsdenia latifolia* (Benth.)**	Asclepiadaceae		Utazi (Igbo) Madumaro/ Arokeke (Yoruba) Utasi (Efik)	Imo (SE) Lagos (SW)	4 (20%)	Leaves	Powdered dried leaves are taken as a tea infusion. Fresh leaves are blended (mixed with *Vernonia amygdalina*) and the liquid extract is drunk.
***Helianthus annuus* L.**	Compositae	Sunflower		Abuja (NC)	1 (5%)	Leaves	
***Hunteria umbellata* (K.Schum.)**	Apocynaceae		Erin (Yoruba)	Ibadan (SW)	1 (5%)	Stem bark	A decoction of the stem/root is drunk, either singly or as a mixture of barks used in the management of diabetes.
***Isoberlinia doka* Craib & Stapf**	Leguminosae	Doka	Doka (Hausa)	Kaduna (NW)	1 (5%)	Stem Root	A decoction of the stem/root is drunk, either singly or as a mixture of barks used in the management of diabetes.
***Khaya ivorensis* A. Chev.**	Meliaceae	African mahogany	Oganwo (Yoruba) Madachi (Hausa) Ono (Igbo) Okpen (Esan)	Lagos/Ibadan (SW)	2 (10%)	Stem bark	A decoction of the stem bark is drunk, either singly or as a mixture of other barks used in the management of diabetes. A teaspoonful of the powdered stem bark in a mix with other plants is dissolved in warm water and drunk twice daily.
***Khaya senegalensis* (Desv.) A. Juss.**	Meliaceae	African mahogany	Oganwo (Yoruba) Madachi (Hausa) Ono (Igbo) Okpen (Esan)	Abuja (NC) Edo (SS)	2 (10%)	Stem bark	A decoction of the stem bark is drunk, either singly or as a mixture of other barks used in the management of diabetes.
***Mangifera indica* L.**	Anacardiaceae	Mango	Mangoro	Edo (SS)	1 (5%)	Stem bark	A decoction of the stem bark is drunk as a mixture of barks used in the management of diabetes.
***Mondia whiteii* (Hook. F.) Skeels**	Apocynaceae	White ginger	Isirigun (Yoruba)	Lagos (SW)	1 (5%)	Root	The powdered root (with *Aristolochia repens* root and *Khaya spp* bark) is mixed in warm water and the liquid is taken.
***Moringa oleifera* Lam.**	Moringaceae		Zogale (Hausa) Ewe igbale (Yoruba) Okwe oyibo (Igbo)	Imo (SE) Kaduna (NC)	1 (5%)	Leaves	A warm infusion of the powdered leaves (mixed with *Vernonia amygdalina*, *Picralima nitida* and pawpaw seeds) is taken twice daily. Powdered leaves are sprinkled on meals.
Musa spp	Musaceae	Plantain Banana		Imo (SE) Kaduna (NW) Lagos (SW)	4 (20%)	Fruit Root Leaves Trunk	The plant parts are crushed with a mortar and pestle and the juice extract is taken (half a shot 3ce daily with/without honey). The unripe plantain fruit is cut into small pieces and then macerated in water for a few days. The liquid is taken as a drink several times a day.
***Nauclea pobeguinii* (Pobeg. Ex Pellegr.) Merr. Ex E.M.A. Petit**	Rubiaceae		Ubulu-ilu (Igbo)	Imo (SE)	1 (5%)	Bark	A decoction of the stem bark is drunk, either singly or as a mixture with other barks used in the management of diabetes
***Ocimum gratissimum* L.**	Lamiaceae	Scent leaf Mint African basil	Nchonwu (Igbo)	Imo (SE) Edo (SS)	2 (10%)	Leaves	Powdered leaves are drunk as a tea infusion.

Plant name(s)*	Family	Common name	Local name(s)	Places where plant is used	No of times mentioned (%)	Part(s) used	Mode of use in diabetes management
***Persea americana* Mill.**	Lauraceae	Avocado pear		Kaduna (NW) Edo (SS)	2 (10%)	Seed Stem-bark	A decoction of the stem bark/seed is drunk, either singly or as a mixture of other barks used in the management of diabetes.
***Phaseolus lunatus* L.**	Leguminosae	Lima beans	Akidi (Igbo)	Imo (SE)	1 (5%)	Seeds	The seeds are cooked and eaten as a meal. A decoction of the seeds is taken mixed with other plants used for diabetes.
***Picralima nitida* (Stapf) T. Durand & H. Durand** Botanical Synonyms: *Picralima klaineana* Pierre *Picralima macrocarpa* A. Chev. *Tabernaemontana nitida* Stapf	Apocynaceae		Osu-igwe (Igbo) Mkpokiri (Igbo) Abere (Yoruba)	Imo (SE) Abuja (NC) Kaduna (NW) Lagos (SW) Edo (SS)	9 (45%)	Seeds	Powdered seeds are sprinkled on food (alone or mixed with *Cassia sieberiana* root and *Diterium senegalensis* stem bark) before eating. An infusion of the powdered seeds is made with warm water and drunk before meals. Powdered seeds are macerated overnight in water or in lime juice (mixed with mistletoe leaves) and one or two shots of the mix taken thrice daily. The powdered seeds are macerated (in a mixture with *Bryophyllum pinnatum* and the bark of unripe plantain) and the liquid is taken thrice daily. Powdered seeds (with *Aristolochia repens*) are mixed in lime juice and taken twice daily. Substitute lime juice with coconut water in patients with ulcer.
***Piliostigma thonningii* (Schum.) Milne-Redh.**† Synonym: *Bauhinia thonningii* Schum.	Leguminosae	Camel's foot	Kalgo (Hausa)	Abuja (NC)	1 (5%)	Roots	A decoction of the roots is taken thrice daily.
***Piper nigrum* L.**	Piperaceae	Black pepper		Imo (SE)	1 (5%)	Seeds	The seed is an ingredient in a multi-component herbal preparation.
***Rauvolfia vomitoria* Afzel.**	Apocynaceae		Olora/Asofeyeje (Yoruba) Akanta(Igbo) Wadda(Hausa)	Lagos (SW)	1 (5%)	Stem	A decoction of the stem/root is drunk, either singly or as a mixture of barks used in the management of diabetes.
***Sarcocephalus latifolius* (Sm.) E. A. Bruce** Botanical Synonym: *Nauclea latifolia* Sm.	Rubiaceae	African peach	Ubuluinu (Igbo) Opepe (Yoruba)	Abuja (NC)	1 (5%)	Stem bark	A decoction of the stem bark is drunk, either singly or as a mixture with other plant materials used in diabetes management. Alcohol can also be used as the vehicle.
***Scoparia dulcis* L.**	Plantaginaceae		Roma fada (Hausa) Mesenmesen/gogoro (Yoruba)	Abuja (NC)	1 (5%)	Whole plant	A decoction of the whole plant is drunk. Roots are chewed prior to eating (especially sugar containing meals)
***Securidaca longepedunculata* Fresen.**	Polygalaceae		Ipeta (Yoruba) Uwar magunguna/ Sanya (Hausa) Ezeogwu(Igbo)	Lagos (SW)	2 (10%)	Root Leaves	Powdered parts (singly or together) are macerated in warm water and the liquid taken twice daily.
***Sphenocentrum jollyanum* Pierre**	Menispermaceae		Akerejupon (Yoruba)	Lagos (SW)	1 (5%)	Root	The roots are macerated in water for three days. Half a glass of the filtrate is drunk every day.

Plant name(s)*	Family	Common name	Local name(s)	Places where plant is used	No of times mentioned (%)	Part(s) used	Mode of use in diabetes management
***Stachytarpheta indica* (L.) Vahl**	Verbenaceae			Abuja (NC)	1 (5%)	Whole plant	The fresh plant sample is macerated and the liquid extract taken twice daily.
***Strophanthus hispidus* DC.**	Apocynaceae		Sagere (Yoruba), Aguru-ala (Igbo)	Lagos (SW) Imo (SE) Kaduna (NW)	3 (15%)	Stem	A decoction of the stem bark is drunk, either singly or as a mixture with other barks used in the management of diabetes.
***Syzygium guineense* (Willd.) DC.**	Myrtaceae		Malmoo (Hausa) Adere (Yoruba) Ori (Igbo)	Kaduna (NW)	1 (5%)	Stem Root	A decoction of the stem/root is drunk, either singly or as a mixture of barks used in the management of diabetes.
***Tamarindus indica* L.**	Leguminosae		Tsamiya (Hausa) Icheku oyibo (Igbo) Ajagbon (Yoruba)	Kaduna (NW)	1 (5%)	Stem Root	A decoction of the stem/root is drunk, either singly or as a mixture of barks used in the management of diabetes.
***Tapinanthus bangwensis* (Engl. & K. Krause) Danser** Botanical Synonym: *Loranthus bangwensis* (Engl. & K.Krause)	Loranthaceae	African Mistletoe		Imo (SE) Lagos (SW) Abuja (NC)	5 (25%)	Leaves	Powdered leaves are soaked overnight in water and the mixture is heated up the next day and drunk. Powdered leaves are soaked overnight in lime juice (mixed with *Picralima nitida*) and a shot is taken thrice daily. Powdered dried leaves are drunk as a tea infusion. A tablespoonful of the powdered leaves (mixed with equal amount of *Securidaca longepedunculata*) are taken at night.
***Telfairia occidentalis* Hook. f.**	Cucurbitaceae	Fluted pumpkin	Ugwu (Igbo)	Imo (SE)	1 (5%)	Seeds	The seeds are dried for several days and the powdered form is either sprinkled on food or taken as an infusion.
***Terminalia avicennioides* Guill. & Perr.**	Combretaceae		Baushee (Hausa)	Kaduna (NW)	1 (5%)	Stem Root	A decoction of the stem/root is drunk, either singly or as a mixture of barks used in the management of diabetes.
***Tetrapleura tetraptera* (Schum. & Thonn.) Taub.**	Leguminosae		Aridan (Yoruba) Mkpuru oshosho (Igbo)	Abuja (NC) Lagos (SW)	1 (5%)	Fruit Bark	
***Vernonia amygdalina* Delile**	Compositae	Bitter leaf	Olugbu (Igbo) Shikawa (Hausa) Ewuro (Yoruba)	Imo (SE) Abuja (NC) Edo (SS) Lagos (SW)	9 (45%)	Leaves	Fresh leaves are squeezed & the juice drank (could be mixed with garlic and ginger). Powdered dried leaves are drunk as a tea infusion. Powdered dried leaves (teaspoonful) are sprinkled on food.
***Ximenia americana* L.**	Olacaceae		Tsaada (Hausa)	Kaduna (NW)	1 (5%)	Stem Root	A decoction of the stem/root is drunk, either singly or as a mixture of barks used in the management of diabetes.
***Xylopia aethiopica* (Dunal) A. Rich**	Annonaceae	Negro pepper	Unien (Esan) Uda (Igbo)	Edo (SS)	1 (5%)	Fruit	The fruit is an ingredient in a multi-component herbal preparation.
***Zingiber officinale* Roscoe**	Zingiberaceae	Ginger	Atale (Yoruba)	Lagos (SW) Imo(SE) Kaduna (NW)	3 (15%)	Rhizome	The fresh rhizome is added to liquid herbal preparations containing other medicinal plants (such as *Alstonia boonei* and *Vernonia amygdalina*)

***** This column indicates the 'accepted' botanical names of the identified plants according to the website http://www.theplantlist.org.
† This botanical name by which the plant is known in Nigeria is not an 'accepted' name on http://www.theplantlist.org but is a synonym for the name indicated under botanical synonym.

3.3.2 Assessing the Risk of Herb-Drug Interactions amongst Diabetic Patients

3.3.2.1 Patients' background characteristics and health status

A total number of one hundred and twelve (112) Type-2 diabetic patients, thirty seven (37) from Central hospital Benin-city and seventy five (75) from General hospital Lagos gave their consent and were interviewed for the study. A summary of the background characteristics of the interviewed patients is given in Table 3-4. Five age categories were created: < 30 years, 31-45 years, 46-60 years, 61-75 years and > 75 years. Most of the patients were aged between 61-75 years (50.9%), followed by those between 46-60 years (34.8%). More than 70% of the patients had one or more chronic co-morbidity alongside their diabetes, the most common of which was hypertension. The other identified co-morbidities were arthritis, arteriosclerosis, hypercholesterolemia and neuropathy (Table 3-4).

Exactly 50% of the patients took herbal medicines alongside their prescription drugs either for diabetes, for one of the co-morbidities or for the management of diabetes-related side effects such as erectile dysfunction. Reasons given by the patients for the use of these herbal preparations alongside their prescription drugs were: (a) General perception that herbs are very good especially when taken as part of the patient's diet; (b) To decrease the costs of disease management since herbs are relatively inexpensive. In this case, the herbs are alternated with the drugs thereby resulting in non-adherence to pharmacotherapy; (c) Better feeling of 'wellness' when herbs are taken alongside the orthodox medications.

Fourteen patients had recently discontinued their use of herbal medicines. Reasons given were- 'based on advice by their doctor', 'due to perceived side effects' and 'disappointment at its lack of efficacy'.

In general, for both males and females, approximately 50% used herbal medicines alongside their prescription drugs; and older patients (61-75 years) seemed more likely to use herbs than younger patients (Table 3-4). There was no statistically significant association between gender and herb use ($x^2 = 0.188$, df = 1, p = 0.665); or between age and herb use ($x^2 = 1.555$, df=4, p = 0.817).

Given that the inherent characteristics of the individuals were not a predictor of herb use, it was hypothesised that the presence of one or more chronic co-morbidity as well as a high monthly prescription cost would incentivize more patients to use herbal medicines. These relationships were however not statistically significant ($x^2 = 3.041$, df = 3, p = 0.385) for presence of co-mobidity and ($x^2 = 4.643$, df=3, p = 0.2) for monthly cost of prescription.

Table 3-4: Tabular summary of patient data

Background characteristics		**[+]Patients (%)**	**Herb Use (%)**	
			Yes	**No**
Gender	Male	43 (38.4)	23 (20.5)	20 (17.9)
	Female	69 (61.6)	33 (29.5)	34 (30.4)
Age groups (years)	≤ 30	1 (0.9)	0 (0.0)	1 (0.9)
	31 – 45	9 (8.1)	4 (3.6)	5 (4.5)
	46 – 60	39 (34.8)	19 (16.9)	20 (17.9)
	61 – 75	57 (50.9)	30 (26.8)	25 (22.3)
	> 75	6 (5.4)	3 (2.7)	3 (2.7)
Presence of co-morbidities	Hypertension + Arteriosclerosis + Arthritis	1 (0.9)	0 (0.0)	1 (0.9)
	Hypertension + High cholesterol + Arthritis	6 (5.4)	4 (3.6)	2 (1.8)
	Hypertension + High cholesterol + Arteriosclerosis	1 (0.9)	1 (0.9)	0 (0.0)
	Hypertension + High cholesterol	17 (15.2)	11 (9.8)	6 (5.4)
	Hypertension + Arteriosclerosis	2 (1.8)	1 (0.9)	0 (0.0)
	Hypertension + Arthritis	4 (3.6)	2 (1.8)	1 (0.9)
	Hypertension + Neuropathy	2 (1.8)	1 (0.9)	1 (0.9)
	Hypertension	46 (41.1)	22 (19.6)	24 (21.5)
	High cholesterol	1 (0.9)	1 (0.9)	0 (0.0)
	None	30 (26.8)	13 (11.6)	17 (15.2)
Monthly prescription cost (Naira)*	< 5000	23 (20.5)	9 (8.0)	12 (10.7)
	5001 - 10000	27 (24.1)	17 (15.2)	10 (9.9)
	10001-15000	10 (8.9)	4 (3.6)	6 (5.4)
	>15000	2 (1.8)	0 (0.0)	2 (1.8)

* Data on cost of prescription was not obtained from all the interviewed patients (£1 ≈ 265 Naira, $1≈ 170 naira www.xe.com; Accessed 13th Nov 2014)

[+] Information about herb use in two patients was not obtained.

Based on this study, although there were more herb users within the group of patients with two or more chronic co-morbid conditions than the group with only one or none at all, patients in the former group were not more likely to use herbs than those without. In addition, contrary to expectation, a higher number of the patients who had to spend less money for their drugs (< 10,000 naira) used herbs than those who had to pay more. Finally, none of the individual co-morbidities was identified as a predictor for herb use among diabetic patients (Statistical data not shown).

In trying to understand the opposing relationship between number of co-morbidities or monthly cost of prescription and herb use, it was observed that the prescription cost was not necessarily dependent on the number of co-morbidities the patient was being treated for, but on the choice of medication on the patient's prescription. For instance, a patient might have only diabetes but may have been prescribed gliclazide by his/her doctor, which is more expensive than other types of sulphonylureas. Another patient on the other hand, having diabetes alongside other co-morbidities might be prescribed a cheaper sulphonylurea such as glibenclamide as well as other non-expensive medications for the co-morbidities resulting in lower prescription costs. Thus, patients with the need to purchase more drugs have the option and may request for less expensive alternative brands so as to minimize their drug expenses.

3.3.2.2 Pharmacological management of diabetes

A summary of the drugs used in the pharmacological management of diabetes in the interviewed patients is given in Table 3-5. The biguanide metformin was the most commonly prescribed anti-diabetic medication with over 90% of the interviewed patients on it. Most patients were however on more than one prescription drug, with 75% on metformin taken in combination with another anti-diabetic medication, most commonly a sulphonylurea. This is in addition to the drugs prescribed for other existing co-morbidities. Only three patients were on a single prescription drug for their diabetes management with no co-morbidity.

Insulin was prescribed to less than 10% of the interviewed patients. The high costs of the injection as well as the need for it to be stored in the fridge makes it an unfavourable choice for patients since most of the Nigerian population do not have access to constant electricity. The preference for metformin as the first choice of treatment for diabetic patients in Nigeria is most likely due to its cheap costs (generic brands of metformin can be purchased for as little as 20 Naira – or 0.07 GBP- per tablet). Glibenclamide is another cheaply available oral anti-diabetic agent in Nigeria hence its popularity.

Table 3-5: Tabular summary of the pharmacological management of the patients' based on their prescribed hypoglycaemic agents.

Anti-diabetic Drug(s)	No of Patients	Presence of co-morbidities*
Glibenclamide only	1	1
Insulin only	4	2
Insulin + Gliclazide	1	0
Metformin + Acarbose + Glibenclamide	2	1
Metformin + Acarbose + Gliclazide + Insulin	1	1
Metformin + Glibenclamide	42	25
Metformin + Gliclazide	10	8
Metformin + Gliclazide + Pioglitazone	3	3
Metformin + Glimepiride	19	16
Metformin + Glimepiride + Pioglitazone	2	2
Metformin + Insulin	3	2
Metformin + Insulin + Glibenclamide	2	1
Metformin + Pioglitazone	1	1
Metformin + Vidagliptin	1	1
Metformin only	18	17

* The presence of co-morbidities indicates that there are other non-hypoglycaemic agents on the patients' prescription.

Several studies show that the risk of harmful drug interactions increases with the number of drugs given to a patient (Fakeye et al., 2008). Similarly, the risk of herb-drug interactions is greatly increased for those patients taking herbal medicines who are on more than one prescription drug (Patsalos et al., 2002). Based on the results of this study, it can be stated that diabetic patients are high risk candidates for herb-drug interactions since they are likely to be on a multiple drug regimen of orally administered drugs.

Table 3-6 gives a summary of the pharmacokinetic effects of the different drugs prescribed to the patients, including those for other co-morbidities. In particular, the table highlights the effects of these prescription drugs on enzymes and/or proteins that are common targets for drug interactions.

Table 3-6: Tabular summary of the interaction profile of the prescription drugs used by the surveyed diabetic patients on pharmacokinetic parameters commonly implicated in drug interaction studies

Pharmacokinetic Interaction	Prescription Drug
P-glycoprotein	**Glibenclamide** is a known substrate/inhibitor of P-gp (Lilja et al., 2007) (Golstein et al., 1999) **Metformin** inhibits P-gp expression (Kim et al., 2011) **Vildagliptin** & **sitagliptin** are known substrates for P-gp (He et al., 2007) (Krishna et al., 2007) **Atorvastatin** has been identified as a P-gp substrate *in vitro* & possibly an inhibitor due to interaction with digoxin (Lennernäs, 2003) **Amlodipine** stimulates P-gp efflux activity at low concentrations & inhibits it at higher concentration (Katoh et al., 2000b)
Cytochrome P-450 enzymes	
CYP1A2	Competitive inhibition of CYP 1A2 activity by **Nifedipine** (Katoh et al., 2000a)
CYP2B6	Carriers of a reduced function allele of CYP2B6 tend to have about 15% relative reduction in the active metabolite of **Clopidogrel** (Mega et al., 2009) CYP2B6 catalyzes the metabolism of **Tramadol** to one of its metabolites M2 (Subrahmanyam et al., 2001)
CYP2C8	About 56% of **Pioglitazone** is metabolised to its primary metabolite M-IV by CYP2C8 (Jaakkola et al., 2006)
CYP2C9	**Glibenclamide** & other sulphonylureas are primarily metabolized by CYP2C9. Some alleles of the enzyme metabolise the drug slower resulting in higher blood concentration & either greater therapeutic effect or increased risk of hypoglycaemia (Surendiran et al., 2011, Holstein et al., 2005, Yin et al., 2005). **Losartan** is metabolized by CYP2C9 to its more pharmacologically active metabolite E3174 (Stearns et al., 1995).
CYP2C19	**Clopidogrel** is metabolized by CYP2C19 & genetic polymorphism in this isoenzyme affects its pharmacokinetics & pharmacodynamics. Carriers of a reduced-function allele had about 30% lower levels of the active metabolite of clopidogrel, diminished platelet inhibition, and a higher rate of major adverse cardiovascular events (Mega et al., 2009).
CYP2D6	The opioid effect of **Tramadol** is mediated by its M1 metabolite, which is catalysed by CYP2D6. The analgesic effect of tramadol has been found to be diminished when co-administered with inhibitors of the enzyme (Laugesen et al., 2005).

Pharmacokinetic Interaction	Prescription Drug
CYP2E1	Competitive inhibition of CYP2E1 activity by **Nifedipine** (Katoh et al., 2000a)
CYP3A4	CYP3A4 has been identified as the other main enzyme responsible for the metabolism of **Glibenclamide** (Zhou et al., 2010a) CYP3A4 is responsible for the formation of two active metabolites from the acid and the lactone forms of **Atorvastatin** (Lennernäs, 2003) CYP3A4 is also responsible for the metabolism of **Tramadol** to its M2 metabolite (Subrahmanyam et al., 2001) **Nifedipine** is a known substrate for metabolism by CYP3A4 & prone to drug interactions with inhibitors of the enzyme (Patki et al., 2003) **Indapamide** is excreted after extensive metabolism by CYP3A4 (Sun et al., 2009) **Losartan** is also metabolized by CYP3A4 to its pharmacologically active metabolite E3174 & is also prone to clinically important drug interactions as seen when co-administered with grape fruit juice, a known CYP3A4 inhibitor (Zaidenstein et al., 2001) CYP3A4 is responsible for the metabolism of 37% of **Pioglitazone** (Jaakkola et al., 2006) **Clopidogrel** is also metabolized by CYP3A4 and inter individual variability in enzyme activity can significantly affect its efficacy (Lau et al., 2004)
Increased risk of hepatotoxicity/hepatic impairment	**Atorvastatin** can induce asymptomatic mild elevation of serum transaminases, with the risk of hepatobilliary disease due to significant liver dysfunction in certain cases (Clarke and Mills, 2006) Risk of hepatotoxicity with **Pioglitazone**, thus caution is advised for patients with elevated ALT levels (Marcy et al., 2004).

3.3.2.3 Assessment of potential herb-drug interactions in the surveyed patients

Less than 40% of the herb users were aware of the identity of the herbs used which they either grow or purchase themselves from herbal markets. The remaining 60% of the herb users (over 30% of all the diabetic patients) were unaware of the identity of the herbs used (Table 3-7). This is primarily because these were unlabelled herbal preparations (often multi-component) whose constituents were not divulged by the herbalists. In some instances, the patients said they were not very interested in finding out the identity as long as the preparation was effective. Thus, the risk of herb-drug interactions is even greater amongst this cohort of diabetic patients as they wouldn't be able to give any information to their physicians/healthcare practitioners that can enable them provide appropriate advice with regards to its efficacy and/or safety.

A total number of twelve medicinal plants were identified amongst the patients who knew the composition of the herbal remedies they used for their disease management. The most commonly used medicinal plant were the leaves of *Vernonia amygdalina* (Bitter leaf). It was also one of the most commonly used medicinal plants by the TMP's in their diabetes management (Table 3-3). It is also a commonly used food vegetable particularly in the southern part of Nigeria, which may contribute to its popularity. As such, many diabetic patients easily include it as part of their diet.

The other identified plants used by the diabetic patients are given in Table 3-7. Only four out of the twelve identified plants- *Abelmoschus esculentus*, *Azadirachta indica*, *Terminalia catappa* and *Camellia sinensis* weren't previously mentioned by any of the herbalists contacted (Table 2-3). They are however known for their use in diabetes management based on other ethnobotanical surveys (Ezuruike and Prieto, 2014) (Oboh et al., 2014).

Seven of these twelve plants have previously been identified as having the potential to cause pharmacokinetic herb-drug interactions based on interactions with prescription medicines or known pharmacokinetic parameters. In addition, three of the twelve plants *Azadirachta indica*, *Picralima nitida* and *Terminalia catappa* have also been shown to cause specific organ toxicities in large doses (Table 3-7).

Table 3-7: Tabular summary of the medicinal plants used by the diabetic patients and their known pharmacokinetic profile

Botanical name of herb (Common name)	Number of Patients (%)	Effect on intestinal P-gp or other absorption mechanisms	Effect on cytochromes or other hepatic enzymes (GSTs, ALT, AST)	Other toxic effects
Vernonia amygdalina Delile **(Bitter leaf)**	13 (11.6)	Inhibits P-gp efflux activity (Oga et al., 2012)	_	_
Ocimum gratissimum L. **(Scent leaf)**	5 (4.5)		Dose dependent increase in AST & ALT levels (Ajibade et al., 2012)	
Musa paradisiaca **(Plantain/Banana)**	3 (2.7)	Polyvalent cations in the plant form non-absorbable complexes with certain drugs (Nwafor et al., 2003)	_	_
Mangifera indica L**. (Mango)**	3 (2.7)	Inhibits the P-gp efflux activity (Chieli et al., 2009)	Inhibits CYP 1A1/2 & 3A4 activities in rat liver microsomes (Rodeiro et al., 2009)	_
Persea americana Mill. **(Avocado pear)**	2 (1.8)	_	Inhibits CYP 3A4/5/7 enzymes to different extents (Agbonon et al., 2010)	_
Gongronema latifolium Benth.	1 (0.9)	_	_	_
Abelmoschus esculentus (L.) Moench **(Okro)**	1 (0.9)	Water soluble fractions inhibits metformin absorption *in vivo* (Khatun et al., 2011)	_	_
Anarcadium occidentale L. **(Cashew)**	1 (0.9)	_	_	_
Azadirachta indica A. Juss. **(Neem)**	1 (0.9)	_	_	Pharmacotoxic effects of neem oil in lungs & CNS (Gandhi et al., 1988)
Camellia sinensis (L.) Kuntze **(Tea)**	1 (0.9)	_	_	_
Picralima nitida (Stapf) T.Durand & H.Durand **(Akuamma plant)**	1 (0.9)	_	Elevated AST, ALT & GSH levels (Kouitcheu Mabeku et al., 2008)	Hepatotoxic effects (Fakeye et al., 2004)
Terminalia catappa L. **(Tropical almond)**	1 (0.9)	_	_	Hepatotoxic at high doses due to punicalagin (Lin et al., 2001)
Unknown	36 (31.9)	NA	NA	NA
Discontinued	14 (12.4)	NA	NA	NA

One of the interviewed respondents 'X' was a 41 year old lady who took the squeezed extract of *Vernonia amygdalina* (VA) regularly as well as an overnight decoction of *Mangifera indica* (MI) and had the following drugs on her prescription- Metformin 1g twice daily, Glimepiride 4mg daily, Lisinopril 2.5mg daily, Atorvastatin 20mg daily and Aspirin 75mg daily.

Atorvastatin is a known P-gp substrate and is also metabolized by CYP3A4 enzymes to active metabolites (Lennernäs, 2003) . A combination of the inhibitory effects of VA and MI on P-gp efflux activity as well as the ability of metformin to decrease P-gp expression could bring about an increase in the plasma concentration of atorvastatin beyond the expected level. If the activity of CYP3A4 is however significantly inhibited by MI, then the effects of atorvastatin may not be seen despite its high plasma concentration given that the metabolite is the pharmacologically active entity.

The afore-mentioned scenario is an identified 'potential' herb-drug interaction that may or may not translate to a clinical effect. It is however very useful preliminary information that would enable clinicians undertake better clinical monitoring of a patient's disease management; and hence be more likely to identify clinically relevant herb-drug interactions if encountered.

Table 3-8 represents a schematic map of the different potential pharmacokinetic interactions that can arise between the prescribed drugs and the identified herbs used by the surveyed diabetic patients and for which some form of monitoring should be done. The table shows that all the prescribed drugs could interact with one or more of 7 out of the 12 identified herbs (Table 3-8). Concurrent use of *A. esculentus* (okro) with metformin is a case to highlight, because of the extensive use of the drug and the *in vivo* experimental evidence for a herb-drug interaction. The use of avocado leaves, bitter leaf and mango leaves interfere with efflux pumps or cytochromes 1A2 and 3A4, with the latter potentially interacting with almost all drugs in the table. Finally, consumption of scent leaf, akuamma plant, and/or tropical almond could enhance the adverse hepatic effects of pioglitazone and atorvastatin.

In addition to the possibility of an enhanced therapeutic effect for herbal medicines taken for the same pharmacodynamic effect as the prescription drug (i.e. increased hypoglycaemia); it is important for healthcare practitioners to be aware and on the lookout for any associated pharmacokinetic interactions of co-administered herbal medicines. Such pharmacovigilance monitoring would however only be possible when patients divulge their use of herbal medicines as well as the identity.

Table 3-8: Map of the potential pharmacokinetic interaction of prescribed drugs and herbs used by surveyed diabetic patients

Prescription drug	**Pharmacokinetic effects**										
	Intestinal Absorption	**Efflux**	**Cytochromes**								**Liver Toxicity**
		P-gp	**1A2**	**2B6**	**2C8**	**2C9**	**2C19**	**2D6**	**2E1**	**3A4**	**AST/ALT**
Glibenclamide		a,b VA, MI				a				a MI, PA	
Metformin	**AE**	b									
Pioglitazone										a MI, PA	d OG, PN, TC
Sitagliptin		a VA, MI									
Vidagliptin		a VA									
Amlodipine		b,c									
Indapamide										a MI, PA	
Losartan						a				a MI, PA	
Nifedipine			b MI						b	a MI, PA	
Atorvastatin		a,b VA, MI								a MI, PA	d OG, PN,TC
Clopidogrel				a	a		a			a MI, PA	
Tramadol				a				A		a MI, PA	

Key: For the prescription drugs (a) is an identified substrate of the enzyme/protein; (b) inhibits enzyme/protein activity; (c) enhances enzyme/protein activity; (d) increases plasma levels of transaminases. The two letter codes denotes a herb which also affects the same PK target of the prescription drug: (MI) *Mangifera indica* (Mango); (VA) *Vernonia amygdalina* (Bitter leaf); (OG) *Ocimum gratissimum* L. (Scent leaf); (PN) *Picralima nitida* (Akuamma plant); (AE) *Abelmoschus esculentus* (Okro); (PA) *Persea americana* (Avocado pear); (TC) *Terminalia catappa* (Tropical almond). In bold, preclinical *in vivo* evidence is available for this potential herb-drug interaction.

3.4 CONCLUSIONS

The interviews conducted with TMPs, herbal sellers and diabetic patients in Nigeria have shown that a wide range of plants are still being used in the traditional management of diabetes in Nigeria. Most of these plants have previously been reported for their use in diabetes management with available experimental evidence of their pharmacological activity for all but six of the plants- *Aristolochia repens, Cassia sieberiana, Citrus aurantifolia, Croton lobatus, Curculigo pilosa* and *Khaya ivorensis* (Table 1, Appendix 1).

Surprisingly, *Aristolochia repens* was the third most commonly identified medicinal plant, mentioned by five out of the twenty respondents contacted despite having no experimental evidence for its use. In addition, various species of the *Aristolochia* genus are known to cause nephrotoxic effects suggesting that this may pose a potential health risk; with its use banned in a number of countries (Heinrich et al., 2009).

Although the TMPs purport that using herbal medicines can 'cure' diabetes, with some suggestions that some plants are able to regenerate the pancreas, this is yet to be proven. As such, contrary to the desire of TMPs to have their diabetic patients exclusively treated with herbal medicines, this is unlikely to be the case from the patient's perspective. At the moment, diabetes is not known to have any 'cure', even with prescription drugs. Thus, for most diabetic patients who use herbal medicines, there might be a preference to co-administer these 'safer' alternatives with their prescription drugs to bring about better blood glucose control. This rationale on the other hand can promote the risks of herb-drug interactions amongst these patients.

One of the key findings of our study was that 50% of diabetic patients visiting hospitals in Nigeria use herbal medicines alongside conventional drugs for their disease management (Table 3.4). This result is similar to that obtained from a previous study carried out amongst diabetic patients visiting two different hospitals in Lagos which gave a prevalence of 46% (Ogbera et al., 2010).

Neither the sex of the individual or his/her age was identified as a predictor of herb use within the sample population of diabetic patients interviewed during the fieldwork, even though herb use was more common amongst older patients than younger ones (Table 3-4). In addition, the choice of herbal medicine use was independent of the presence of co-morbidities or the monthly cost of prescriptions. This lack of clinical predictors points towards cultural factors influencing the use of herbal medicines by Nigerian adult diabetic patients. A patient is therefore more likely to use herbal medicine if such practice is common in his/her family and he/she is convinced of the health benefits of the herbal medicine.

As a response to the changing attitudes of patients of Western medicine to the use of herbal medicines, this study is the first one to be carried out in Nigeria with the aim of specifically assessing the risks of herb-drug interactions amongst diabetic patients in Nigeria. The importance of this is highlighted not only by the high percentage of persons using herbal medicines alongside their conventional drugs but also by the high percentage of patients (>30%) who do not know the identity of the herbal medicines being taken, which equates to >60% of herb users (Table 3-7). This poses a huge problem since the toxicity and efficacy of the medicinal plant cannot be assessed, which also constitutes a serious limitation to the identification of herb-drug interactions.

Unfortunately, the issue of 'non-identification' of herbs used by patients in Nigeria is unlikely to be immediately tackled. This is because TMPs who prepare the herbal preparations themselves insist that it is a 'family secret' of which a disclosure will rob them of their business (Ogbera et al., 2010). This is not an exaggerated claim if we take into account the history of "bio-piracy" by companies seeking to patent and profit from their knowledge without sharing benefits (Willcox and Bodeker, 2010). In any case, patients should be advised on the importance of knowing the identity of their herbal medicines especially as this would enable a proper identification of those with true therapeutic benefits.

More importantly, this study highlights the need for an increased emphasis on the evaluation of herbal medicines used in Nigeria for potential pharmacokinetic and pharmacodynamic interactions. Not only because of the paucity of information available but also because of the overlap of a number of pharmacokinetic interactions between some of the herbal medicines and prescription drugs co-administered by diabetic patients as well as the lack of awareness by patients of the potential risks associated with herbal medicine use.

Herbal preparations particularly for diseases like diabetes are often multi-component preparations whereby the management entails not just plants with hypoglycaemic effects, but also medicinal plants that might address the underlying symptom as well as resultant complications of the disease. Thus an increased risk of clinically relevant herb-drug interactions may be seen with these multi-component mixtures which with only one of the constituent plants may have produced an insignificant interaction effect.

There is therefore always a need to follow up pre-clinical *in vitro* evaluations with a clinical assessment in humans utilizing the herbal formulations as they are used in practice. In the presence of sufficient appropriate information, other predictions of herb-drug interactions can be made by healthcare practitioners thereby necessitating appropriate clinical advice to forestall unwanted effects.

4 PHYTOCHEMICAL, BIOCHEMICAL AND BIOLOGICAL ANALYSIS OF PLANTS USED IN DIABETES MANAGEMENT IN NIGERIA

4.1 INTRODUCTION

Identification of pharmacokinetic herb-drug interactions would ideally require *in vivo* experimentation, preferably with human subjects if meaningful results are to be obtained. The ethical issues and high costs associated with this approach are often justified by the existence of case reports, pharmacovigilance data and/or preliminary *in vitro* studies on key targets of the different ADME process.

The experimental evaluation of the pharmacokinetic effects of medicinal plants collected during the field work carried out in this chapter focuses on intestinal absorption and phase 2 metabolism. To this end, a set of *in vitro* assays have been selected including cytotoxicity, modulation of P-gp activity, transport across cell monolayers and modulation of intracellular glutathione levels. The last target also provides information on the potential protective effect against oxidative damage, which is a well known complication in diabetes. To further assess this, the radical scavenging activity of the plant extracts towards nitric oxide will be evaluated.

This chapter also describes the phytochemical analysis of the plant materials. When the identity of individual active compound(s) is not known, a "chemical fingerprint" is the recommended technique to ensure quality standards for plant species used as herbal medicines (Bauer and Tittel, 1996). Although no single high performance liquid chromatography with diode array detection (HPLC-DAD) analysis can provide a full account of all chemicals present in the sample, it is considered as a code of practice for the quality control of herbal extracts. Its sensitivity and low cost has been suggested as an affordable way to complement/corroborate the botanical analysis of African plants (Springfield et al., 2005).

4.2 MATERIALS AND METHODS

4.2.1 Preparation of Plant Materials

4.2.1.1 Selection Criteria and Botanical Identification

The plant samples to be analysed were obtained either from the traditional medicine practitioners or purchased from the herbal markets visited during the field work study. Verification of plant samples was carried out as described in chapter 2. Voucher specimens for samples collected in their natural habitat (Agbede forest) have been deposited at the herbarium of the School of Pharmacy, University College London.

4.2.1.2 Processing of Plant Materials

All plant samples were air-dried for about two weeks (average temperature 30°C) to remove as much moisture as possible to prevent deterioration. Samples were then packed in freezer bags and couriered to the School of Pharmacy, University College London. Upon arrival, they were further dried in a Samas Vickers® laboratory drying chamber electrically heated to 30°C with continuous ventilation. Samples were powdered using a laboratory scale mill (MF 10 Basic IKA WERKE blender) or an industrial size mill (FRITSCH miller), depending on the part of the plant collected and the quantity of plant material.

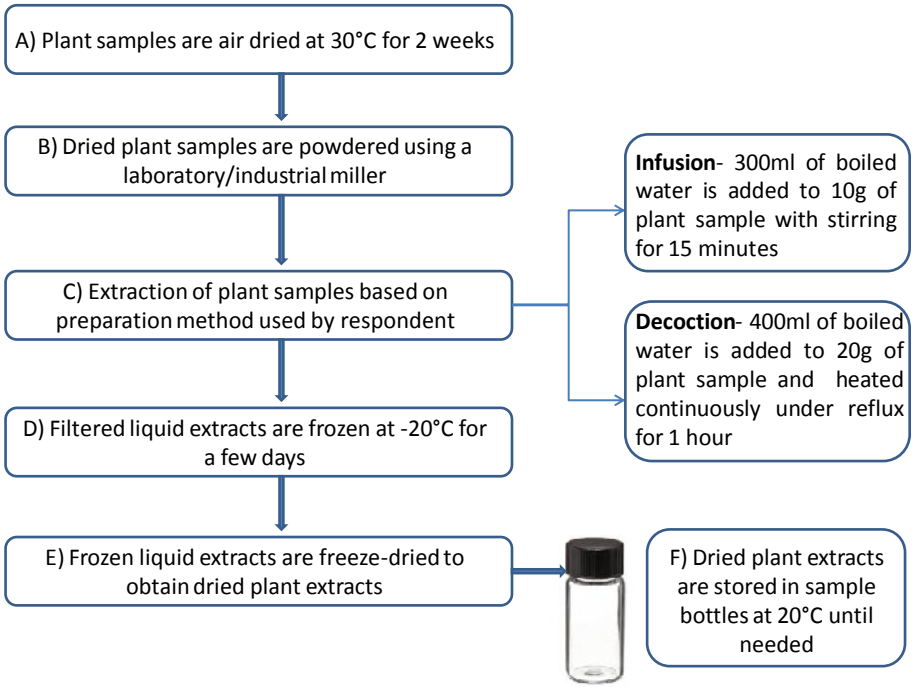


Figure 4.1: Schematic summary of method(s) used to process plant samples

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, 300ml of boiled distilled water was added to 10g of plant material with continuous stirring for 15minutes on a laboratory hot plate magnetic stirrer (IKA WERKE labortechnik electric stirrer). For decoction, 20g of plant material was heated continuously under reflux in a round bottom flask containing 400ml of distilled water for one hour using an Electrothermal® 3-in-1 laboratory heating mantle.

All extracted plant samples were 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 stored in 10ml sample bottles at -20°C until needed. A schematic summary of the steps used in sample preparation is shown in Figure 4.1.

4.2.2 Phytochemical Analysis

Phytochemical analysis of samples of the freeze dried plant extracts was carried out using a high performance liquid chromatography (HPLC) system- Agilent 1200 series equipped with an ultraviolent/visible (UV-Vis) diode array detector to obtain a 'fingerprint' of the samples. The conditions used for the analysis have previously been used for the detection of phenolic compounds in plant samples (Giner et al., 1993).

The stationary phase was a Phenomenex® synergi Polar-RP80A column (250mm x 4.6mm, I.D = 4µm) column maintained at 40°C and protected with a Nova-Pak® C18 guard column. The mobile phase was a binary system consisting of (A) water-glacial acetic acid (99.8:0.2, v/v) and (B) methanol-glacial acetic acid (99.8:0.2, v/v). The gradient elution program for the mobile phase was 10-20% B (0-5mins), 20-50% B (5-65mins), 50-80% B (65-75mins) and 80-100% B (75-80mins) at a flow rate of 1ml/min. The injected volume of sample was 10µl and the UV-Vis spectra were recorded in the range 190 to 400nm, while chromatographic peaks were monitored at 254 and 360nm.

The plant extracts were prepared as 25mg/ml solutions in either micro-filtered double distilled water or HPLC-grade methanol (All solvents were purchased from Fisher Scientific UK). Fifteen plant phenolic compounds: Gallic acid, 2,4-dihydroxybenzoic acid, caffeic acid, epicatechin, epigallocatechin, catechin, vitexin rhamnoside, vitexin, rutin, ellagic acid, quercitrin, hesperidin, quercetin, luteolin and kaempferol were used as standards at concentrations between 0.5 and 1mg/ml in methanol, depending on the intensity of the peak. All standard phenolic compounds were purchased from Sigma Aldrich UK. Samples were filtered through a 0.45µm syringe filter (Millipore Millex® PTFE) before the analysis.

4.2.3 Determination of the Antioxidant Effects of Plant Samples

Aqueous extraction of plant samples results in the preferential extraction of mainly polar compounds such as flavonoid glycosides and tannins (generally known as phenolic compounds) (Seidel, 2006). These phenolic compounds are usually endowed with free-radical scavenging effects due to their labile 'H' atom, and hence are responsible for most of the plant's antioxidant effects. This is because the phenolic groups can accept an electron to form relatively stable phenoxyl radicals, thereby disrupting chain oxidation reactions in cellular components. As a result, plant extracts with potent antioxidant effects are also thought to be beneficial in mitigating hyperglycaemia-induced oxidative stress common in diabetes (Pandey and Rizvi, 2009).

Given that the plant samples were all selected on the basis of their use in the management of diabetes, we wanted to determine if they had good antioxidant activity, which contributes to their benefits in diabetes. Several methods for determining the free radical scavenging effects of compounds have been developed. Two of these, the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay and the nitric oxide scavenging assay were used to evaluate the antioxidant effect of the different extracts.

4.2.3.1 DPPH Radical Scavenging Assay

DPPH is a stable radical compound whose odd valence electron readily becomes paired with either hydrogen or another reducing molecule from a free radical scavenger. This forms the reduced DPPH-H or the corresponding hydrazine as shown in the equation below (Figure 4.2), resulting in a change in the purple colour of the DPPH to the yellow colour of its reduced form. The purple-coloured DPPH gives a maximum absorption at 517nm, thus its signal intensity is inversely proportional to the concentration of the antioxidant present in the system.

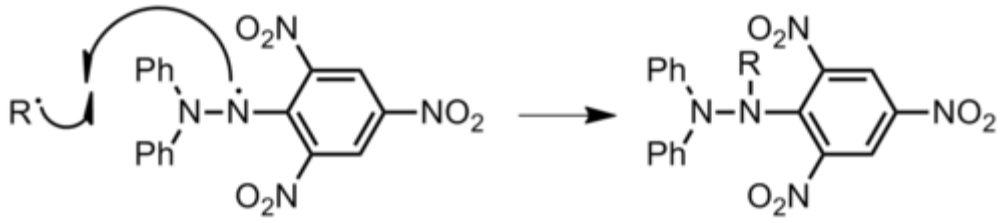


Figure 4.2: Reaction between the free stable radical DPPH and a free radical scavenger (R*) to give reduced DPPH

The method used for this assay was modified for microplate readings from that described by Sharma et al. (Sharma and Bhat, 2009). 200µM stock solution of DPPH (Sigma Aldrich UK**)** was prepared in HPLC-grade methanol buffered with 1mM ammonium acetate (Fisher Scientific UK). The absorbance of DPPH is pH sensitive. The buffered methanol keeps it within a pH range of 5.0-6.0. A stock solution was prepared fresh each week and stored at 4°C wrapped in foil paper to protect from light. 500µM stock solution of caffeic acid (Sigma Aldrich UK) in phosphate buffered saline (Gibco® Invitrogen UK) was also prepared as positive control. A maximum concentration of 5mg/ml dissolved in double distilled water was prepared for all plant extracts. All solutions were filtered through a 0.45µm syringe filter and stored at -20°C until needed.

The assay was carried out in transparent, flat bottom Nunclon® 96 well plates (Thermo Scientific UK). 200µl of the maximum concentration of each of the plant extracts (250µg/ml) and the caffeic acid (100µM) were added in triplicates to the first row of the

plate. Serial dilutions of each sample solution (or the control) was then prepared down the row using the appropriate solvent to a final volume of 100µl in each well. To each of these wells was added 100µl of the DPPH solution (to give a final concentration of 100µM).

A negative control (100µl DPPH solution + 100µl buffered methanol or any other solvent used to dissolve extract) and a blank determination (100µl buffered methanol + 100µl solvent) were also carried out. The covered microtitre plate was wrapped with aluminium foil and incubated for 30mins at room temperature away from any light source. After incubation, the absorbance of each well was measured with a plate reader (Tecan® Infinite M-200) at a wavelength of 517nm.

The percentage of DPPH remaining in each well was calculated with the following equation: **$\%DPPH = 100 X [(A_S - A_{SB})/C]$**; where A_S is the absorbance of the test solution, A_{SB} is the absorbance of the blank and C is absorbance of the negative control. The percentage inhibition is given as **$I (\%) = 100 - \%DPPH$.** The IC_{50} values for each of the samples were interpolated from a graph plot of the different concentrations of the samples against the percentage inhibition.

4.2.3.2 Nitric oxide Scavenging Assay

Nitric oxide is an important physiological messenger and effector molecule in many biological systems including immunological, neuronal and cardiovascular tissues. Under conditions of oxidative stress (such as in hyperglycaemic states), elevated levels of superoxide (O_2^-) and nitric oxide (NO) free radicals are formed. These react to form peroxynitrite ($ONOO^-$), a toxic metabolite which contributes to islet β-cell destruction (Suarez-Pinzon et al., 2001); as well as promotes vascular injury resulting in damage to the cardiovascular system (Förstermann, 2010). An antioxidant that can scavenge NO will therefore be beneficial in hyperglycaemic conditions.

One means of investigating nitric oxide levels in any biological system is to measure nitrite using the Griess reagent. Nitrite (NO_2^-) is one of two primary, stable and non-volatile breakdown products of nitric oxide. When working without cells, a source for nitrite has to be introduced. Sodium nitroprusside (SNP) is a molecule that is highly unstable when introduced into a solution at physiological pH or in the presence of known biological reducing agents, such that it spontaneously decomposes. The decomposition first generates nitric oxide, which then produces nitrite ions through interaction with oxygen. Scavengers of nitric oxide compete with oxygen thereby leading to decreased production of nitrite.

A typical commercial Griess reagent contains 0.2% naphthylenediamine dihydrochloride (NED) and 2% sulphanilamide in 5% phosphoric acid. The nitrite ions enter into an equimolar reaction with sulphanilamide forming a diazonium cation, which then reacts with the NED to produce the azo compound (as shown in Figure 4.3 below). This azo compound is easily quantifiable by measuring the absorbance at 546 nm with a spectrophotometer. By incubating the reaction medium with free-radical scavenging agents, this assay can be used to determine the antioxidant effects of various compounds including plant extracts.

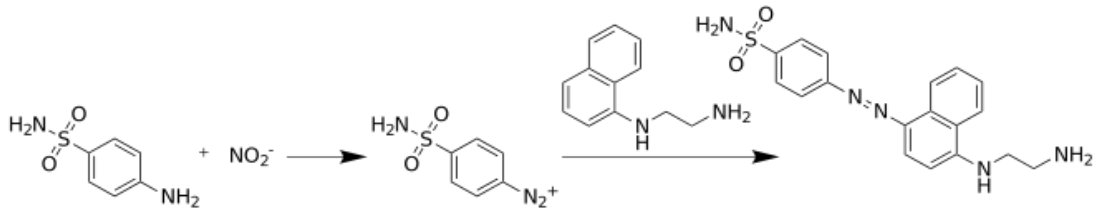


Figure 4.3: Sulphanilamide reacts with the nitrite ions (NO_2^-) to form a diazonium cation which reacts with NED to form the coloured azo compound

Different concentrations of sodium nitrite ($NaNO_2$) (Sigma Aldrich UK) in PBS were prepared in triplicates in different wells of a 96-well microplate up to a maximum concentration of 500µM. 50µl of Griess reagent (Sigma Aldrich UK) was added to each of these wells containing 100 µl of the different $NaNO_2$ concentrations and incubated for 15mins to allow the azo dye to develop. The absorbance of the different wells was then measured using a plate reader (Tecan Infinite M-200 Plate reader) at 546nm and the calibration curve of the different nitrite concentrations against absorbance of the corresponding azo compound was determined using the Magellan® analyzing software.

Serial dilutions of each sample solution and the control were prepared down the row of the 96-well plate (in the DPPH assay) to a final volume of 50µl in each well. 50µl of a 5mM freshly prepared stock solution of SNP in phosphate buffered saline (PBS) was immediately transferred to each of the wells containing either the positive control or the test samples and then placed in the incubator. 90mins after addition of the SNP, 50µl of the Griess reagent was added to each of the wells and incubated for a further 15mins to allow the azo dye to develop. The absorbance of each well was then measured at 546nm in the plate reader.

The concentration of nitrite (NO_2^- sample) remaining in each well was estimated from the $NaNO_2$ calibration curve by comparing each measured absorbance value. Wells containing SNP and vehicle only were taken as the wells with the maximum concentration of nitrite generated (NO_2^- max). The percentage of nitrite scavenged in each well was then estimated as follows: **% Scavenging = (1- (NO_2^- sample/ NO_2^- max))*100**. The IC_{50} values for each

of the samples were interpolated from a graph plot of the different concentrations of the samples against the concentration of nitrite scavenged in each well.

4.2.4 Mammalian Cell Culture Techniques

Two adherent mammalian cell lines, the human hepatocellular carcinoma cell (HepG2) and the human epithelial colorectal adenocarcinoma cell (Caco-2) were used for the biological assays carried out in this thesis. They were both purchased from Sigma Aldrich UK at passages 100 and 43 respectively. A vincristine resistant subline of Caco-2 cells (Caco-2 VCR) was also obtained. This was kindly provided by Dr Elisabeta Chieli of the department of Experimental Biology, University of Pisa, Italy.

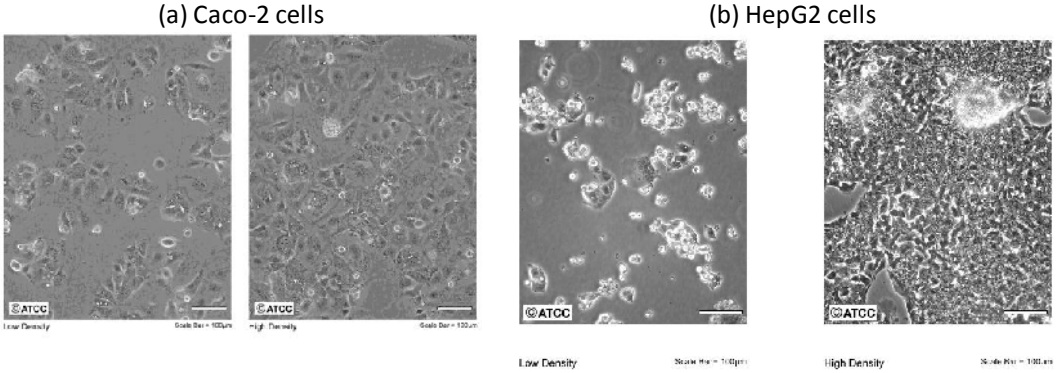


Figure 4.4: Microscopic image of (a) Caco-2 cells and (b) HepG2 cells. Courtesy: American type culture collection (ATCC)

The routine use and maintenance of mammalian cell culture as well as all biological assays involving the different cell lines undertaken in this thesis were carried out under sterile conditions in a laminar flow cabinet (Walker Class 2 laminar air-flow cabinet) unless otherwise stated. Personal protective equipment (gloves, lab coats, masks and hair covers) were worn at all times to reduce the risks of contamination. Gloves were sprayed with 70% ethanol and allowed to air-dry before and after use.

4.2.4.1 Resuscitation of frozen cell stocks

Cell lines are usually stored in frozen form (in liquid nitrogen) when not in use. This is also the form of the cells when purchased. To resuscitate them, the cells were thawed rapidly at 37°C by placing the vial of cells in a pre-heated water bath. The thawed cells were then diluted slowly into a centrifuge tube containing the appropriate warm growth media (see below) to eliminate DMSO, which is toxic to cells. DMSO is used as a cryoprotectant to prevent crystallization, which will otherwise cause cells to lyse during cryopreservation. The tubes were then centrifuged (Biofuge primo R Heraeus centrifuge) for 5 minutes at 1000rpm

to separate the cell pellet from the culture media. Inside the laminar flow cabinet, the media was removed from the centrifuge tube and the cells were properly re-suspended in 15ml of pre-warmed complete media. This was then transferred to a culture flask pre-labelled with the name of the cell line, passage number and date. An inverted microscope (Nikon TMS 10/0.25 lens) was used to check the morphology of the cells before placing in an incubator.

4.2.4.2 Subculture/Maintenance

All cell culture reagents were obtained from Gibco® Invitrogenn UK unless otherwise stated. HepG2 cells were routinely grown in Minimum Essential Medium Alpha + Glutamax. The medium was supplemented with 10% heat inactivated foetal bovine serum (FBS)-EU and 1% penicillin-streptomycin antibiotic (10000units/ml penicillin & 10000 µg/ml streptomycin). Caco-2 cells were maintained in Dulbecco's minimum essential medium with high glucose (4.5g/l) and L-glutamine. The medium was supplemented with 10% FBS, 1% penicillin-streptomycin and 1% non essential amino acids 100X. The Caco-2 VCR cells were maintained in the same media as the wild type Caco-2 cells, but also containing 50nM vincristine (Vincristine 2mg/ml, Hospira UK Limited).

Cells were routinely grown as a monolayer in TPP 75cm^2 tissue culture flasks (T_{75}) containing 15ml of complete media in an incubator at 37°C, 5% CO_2/95% O_2 (Nuaire DH Auto-flow CO_2 air jacketed Incubator). The culture media was changed every 2-3 days until they were approximately 80% confluent. To sub-culture cells, old media was removed and the flask gently rinsed with 5ml of PBS. The monolayer of cells was then detached by adding 1ml of 0.25% Trypsin/EDTA to the flask and placed in the incubator for not more than 5 minutes. Thereafter, 5ml of complete media was added to the flask to dilute and inactivate the trypsin and the cell suspension collected and centrifuged. The media was aspirated off and the cells re-suspended in fresh complete media and then counted. Cells were split into new T_{75} flasks at an appropriate ratio depending on the cell growth rate and then placed back in the incubator.

In order to maintain an available stock of each cell line, cells were re-suspended in freshly prepared freezing medium (culture media for the cells containing 20% FBS & 10% DMSO) ($\approx 10^6$ cells/ml). The cell suspension was aliquoted into sterile cryogenic vials, and transferred to a Nalgene® Cryo Styrofoam freezing container containing 250ml isopropranol (to achieve a -1°C/min cooling rate) and stored overnight at -80°C. The next day, the cryovials were transferred to a liquid nitrogen dewar and stored until needed. All cells were only used for a maximum of fifteen passages before they were discarded and a new cryogenic vial of cells defrosted/resuscitated.

4.2.4.3 Cell Counting

To determine the growth rate of the cells or the exact dilution of cell suspension needed for an assay, a trypan blue dye exclusion method was used to count the number of viable cells. An aliquot of cell suspension (20µl) was taken and diluted with 180µl of 0.4% trypan blue stain in an eppendorf tube. The Neubauer haemocytometer was then carefully loaded with 10µl of the mixed sample on either side. The number of unstained cells was counted with an inverted microscope and the total number of viable cells estimated.

4.2.5 Neutral Red Uptake Assay for Determination of Cell Viability/Cytotoxicity

Prior to the use of the afore-mentioned cell lines in the different biological assays, the toxicity profile of all the extracts were assessed in an *in vitro* cytotoxicity assay. The assay is based on the uptake of neutral red (3-amino-*m*-dimethylamino-2-methylphenazine hydrochloride), a supra-vital dye, and its accumulation in the lysosomes of viable uninjured cells. The uptake of the dye depends on the cell's capacity to maintain pH gradients, through the production of ATP. The dye is weakly cationic and at physiological pH, presents a net charge close to zero, enabling it to penetrate the cell membranes by non-ionic passive diffusion and concentrates in the lysosomes. Inside the lysosome, there is a proton gradient to maintain a pH lower than that of the cytoplasm. Thus the dye becomes charged and is retained, where it binds to anionic and/or phosphate groups of the lysosomal matrix (Repetto et al., 2008).

Extraction of the dye from the viable cells is done using an acidified ethanol solution, and the absorbance of the solubilised dye is quantified spectrophotometrically at 540nm. Quantification of the dye extracted from the cells has been shown to be linear with cell numbers, both by direct cell counts and by protein determinations of cell populations (Weyermann et al., 2005). Given that the toxicity of the extracts is inversely proportional to the viability of the cells, more toxic extracts will result in fewer cells being available for neutral red uptake. The neutral red assay has been identified as a reliable *in vitro* assay that can be used to estimate *in vivo* starting doses for acute toxicity based on its low coefficient of variation from several inter- and intra- laboratory experiments (Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), 2001), hence its selection for this study.

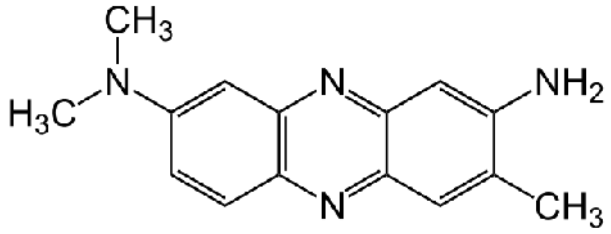


Figure 4.5: Chemical structure of Neutral red

The method used for this assay is as described in Repetto et al. (2008) with slight modifications. Cells to be used for the assay should be ≤ 80% confluent to ensure that they are in their exponential growth phase. After trypsinization, 200 µl of the appropriate cell suspension was plated out in each of the inner wells of a sterile, flat bottom 96-well plate at a density of 1 x 10^5 cells per ml in complete media. The plate was then incubated overnight at 37°C, 5% CO_2/95% O_2. The next day, serial dilutions of the plant extracts to be tested, including the positive control, hydrogen peroxide (BDH laboratory supplies) were made in complete media from the appropriate stock solutions.

On day two, the growth media was carefully aspirated from each well of the microplate and replaced with 200µl of the test solution in replicates. Complete media without drug was added to some of the wells with cells as well as the outer wells without cells to act as positive control and blank respectively. The microplate was then incubated for 24hours. 40µg/ml of neutral red (Sigma Aldrich) in complete media was prepared in a centrifuge tube from a stock solution of 4mg/ml in PBS and incubated overnight, wrapped in foil paper at the same temperature as the cells.

On the third day, the neutral red solution was centrifuged for 10mins at ≈ 1800 rpm to remove precipitated dye crystals. The microplate was then removed from the incubator and briefly examined under the microscope to physically identify the highest tolerated dose (HTD) for each of the extracts. The media in each of the wells was gently aspirated off and replaced with 100µl of the neutral red solution in each well of the plate and then incubated for another 2 hours. After 2 hours, each well of the plate was gently rinsed with 150µl of PBS and the washing solution removed by decanting or gently tapping the plate.

150µl of neutral red de-stain 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 neutral red extract was read out in the plate reader at 540nm. The percentage viability for each of the concentrations of the

drug/extract was estimated as follows: **% viability = ($O.D_S$-$O.D_B$/$O.D_M$-$O.D_B$) x 100;** where $O.D_S$ is the optical density of the sample (either drug or plant extract), $O.D_B$ is the optical density of the blank wells without cells, $O.D_M$ is the optical density of the wells of the positive control containing only complete media. The IC_{50} values for each of the samples were interpolated from a graph plot of the different concentrations of the samples against the percentage viability.

4.2.6 Estimation of Intracellular Glutathione Levels in HepG2 Cells

Glutathione (GSH) is a ubiquitous tri-peptide thiol (L-γ-glutamyl-L-cysteinylglycine) or α-amino acid present in various mammalian cells in millimolar range (0.5-10mM). It is known to function directly or indirectly in many important biological phenomena, including the synthesis of proteins and DNA, transport, enzyme activity, metabolism and protection of cells (Meister and Anderson, 1983). Of all the functions of GSH, two of these are relevant in the process of cell detoxification- (1) its role as an antioxidant, and (2) its role in the phase 2 metabolism of xenobiotics. In general, both of these are as a result of the general function of GSH to protect cells against oxygen toxicity (Fahey and Sundquist, 2006).

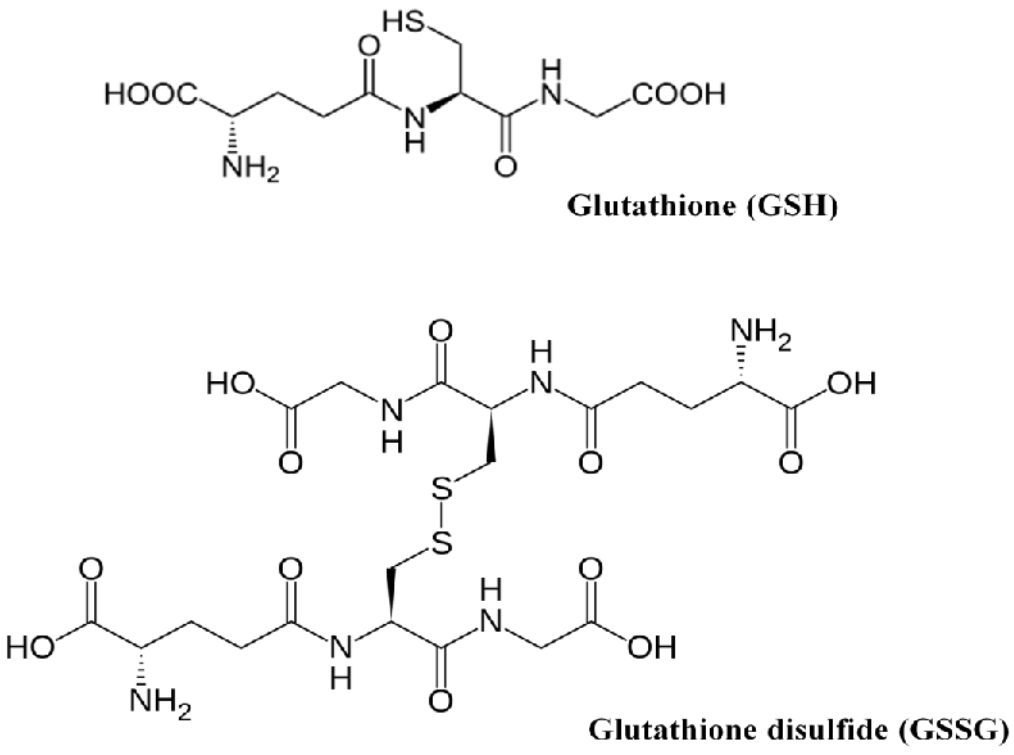


Figure 4.6: Chemical structures of the oxidized (GSH) and reduced (GSSG) forms of glutathione.

GSH exists in two different forms within cells: the reduced sulfhydryl form (GSH) and the glutathione disulfide (GSSG) (Figure 4.6). In healthy cells, GSSG is less than 0.2% of the total GSH concentration. Oxidative stress has a profound effect on the cellular thiol balance and can lead to a decreased GSH/GSSG ratio in many body organs. Reactive oxygen species ($O_2^{\cdot-}$, $OH^{\cdot}$, H_2O_2 & R-OOH) as well as xenobiotics are neutralized by GSH through a cascade of detoxification mechanisms involving the enzymes glutathione peroxidases (GPx), glutathione-S-transferases (GST) and glutathione reductase (GR) (Rahman et al., 2007). Thus, disease conditions or xenobiotics that induce oxidative stress would in turn affect intracellular GSH concentrations. By measuring the intracellular GSH levels in the presence of the plant extracts, we can determine if the extracts would enhance or diminish the detoxification effects of GSH.

The method used in the intracellular determination of GSH levels was adapted from that described by Rahman et al., (2007) and Allen et al., (Allen et al., 2000) with slight modifications. The assay is based on the reaction of GSH with DTNB (5, 5'-dithio-bis (2-nitrobenzoic acid)), also known as Ellman's reagent to produce TNB (5-thio-2-nitrobenzoic acid), a yellow coloured chromophore which can be measured spectrophotometrically at 405nm, and an oxidized glutathione-TNB adduct (GS-TNB). The GS-TNB adduct is reduced by GR in the presence of NADPH to form GSH and TNB, with the former being recycled back into the system to produce more TNB (Rahman et al., 2007) (Figure 4.7).

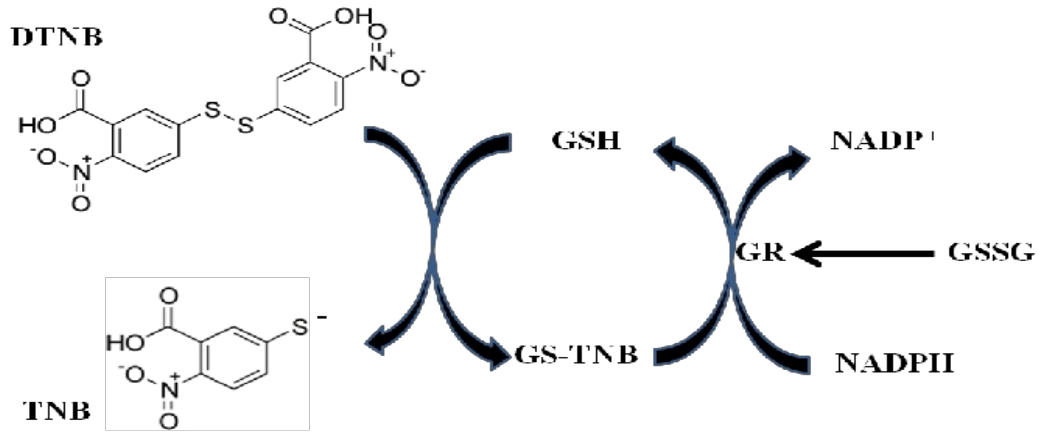


Figure 4.7: Enzymatic recycling of glutathione (GSH) from glutathione disulfide (GSSG) by glutathione reductase (GR) in the presence of NADPH. GSH reacts with DTNB to GS-TNB and TNB, with subsequent reduction of the former to GSH. TNB can be measured at 405 nm. (Adapted from Rahman et al., 2007).

The GSSG present in the sample is also reduced by GR to form two molecules of GSH. Thus, the amount of glutathione measured represents the sum of reduced and oxidized GSH in the sample. The rate of change in absorbance of TNB at 405nm is linearly proportional to the total concentration of GSH in the sample. The concentration of an unknown sample can therefore be determined by calculating from the linear equation or the regression curve generated from several standard concentrations of GSH.

All chemicals and reagents used in this assay were purchased from Sigma Aldrich UK, unless otherwise stated. HepG2 cells were seeded out in sterile, flat bottom 96-well plates at a density of 1 x 10^5 cells per ml (2 x 10^4 cells per well). Only the wells of the inner last three columns of the plate (columns 9-11) of the plate were used as shown in the coloured wells of figure 3.6 below, and the plate was incubated overnight to allow for cell attachment. The next day, the cells were treated with the plant extracts to be tested in triplicates, at their maximum non toxic concentration as determined by the neutral red assay. Three wells were not treated with any drug and served as media control, while three other wells were treated with 10μM buthionine sulphoximine (BSO) as the positive control. All drug/extract incubations were done for 24 hours.

Prior to commencing the GSH assay, the following solutions ought to be prepared- phosphate buffer solution containing 168ml of a 286mM solution of sodium phosphate dibasic in distilled water, 32ml of a 286mM solution of sodium phosphate monobasic and 6.3mM EDTA; 5% sulfosalicylic acid in double distilled water (SSA); 0.1% Triton X-100; 1mM stock solution of reduced glutathione in 5% SSA solution and 10mM DTNB in phosphate buffer solution (this solution can be stored in aliquots at -20°C, protected from light for up to two months). Apart from the DTNB, all the other solutions can be stored in the fridge at 2-4 °C for up to a week. 2.39mM or 2mg/ml β-NADPH in phosphate buffer solution was freshly prepared on the day of the assay.

24 hours after incubation with the extract/drug, the cells were washed with PBS (200μl per well) and the PBS aspirated off. 60μl of 0.1% Triton X-100 was added to each well of the plate containing cells and then shaken on a microplate shaker for 2 minutes to lyse the cells. 25μl of the cell lysate from each well was transferred to another microplate with cover, wrapped in foil to prevent evaporation and set aside for protein assay determination. The UV light of the laminar flow hood was turned off for the rest of the assay to minimize GSH oxidation.

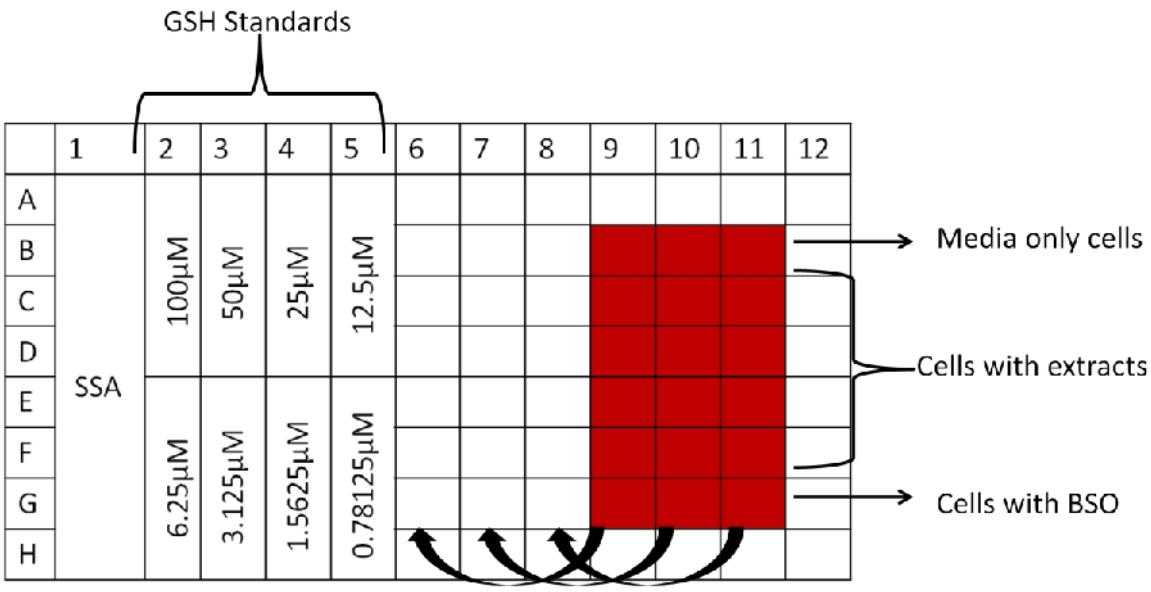


Figure 4.8: Schematic representation of layout of 96-well plate for GSH assay

To the remaining cell lysate, 25 µl of 5% sulfosalicylic acid solution (Sigma Aldrich UK) was added and the plate shaken for another 2 minutes. After shaking, 25 µl of the lysed cell solution was transferred to the wells of the next three inner columns of the plate (columns 6 - 8). 25 µl of the 5% SSA solution was added to each well of the first column of the plate as blank. A double dilution of a standard solution of reduced glutathione in 5% SSA, starting from 100µM GSH as the highest concentration was then prepared (often in the last column of the plate-column 12). 25µl of each concentration of the standard was then transferred in triplicates to the wells of Columns 2-5 (Figure 4.8).

The glutathione reaction buffer needed for the assay was prepared by adding 1ml of the freshly prepared β-NADPH and 3µl of glutathione reductase (equivalent to 2 units of GR) to 7.5ml of the phosphate buffer solution for each 96-well plate. This is vortexed for 5 seconds and then 1 ml of DTNB solution is immediately thawed and added to the reaction buffer and then vortexed for another 5 minutes. All solutions to be used were kept on ice until needed. 125µl of the reaction buffer was added to each well of the 96-well plate containing either 25µl of the blank SSA solution, 25µl of the GSH standards or 25µl of the cell lysate solution. The microplate was immediately covered and then wrapped in foil paper and then shaken in the microplate for 15 seconds. Immediately after, the absorbance of the wells of the microplate was read in a kinetic cycle in the TECAN plate reader every 30 seconds for 5 minutes at 405nm (11 readings).

The slope of the curve obtained from the 11 readings of each of the GSH standard concentrations (otherwise known as the I-slope), which represents the rate of change of GSH levels at that concentration is plotted against the GSH concentration to give a regression curve (otherwise known as the F-slope curve). The concentration of GSH in the samples from the cell lysate solutions is then estimated by substituting the average I-slope values for each of the extract/drug into the equation of the F-slope curve.

Cell protein estimation was carried out using the *DC*® protein assay kit (Bio-Rad, -500-0116). The kit consists of an alkaline copper tartrate solution (reagent A), dilute Folin reagent (reagent B) and a solution of sodium dodecyl sulphate (reagent S). A serial dilution of bovine serum albumin in 0.1% triton X-100 was prepared from a 1mg/ml stock solution. 25µl of each of the standards was transferred in replicates to different wells of the microplate containing the cell lysate that was set aside for protein estimation. 25µl of reagent A' (reagent A containing 2% reagent S) was added to each well followed by 200µl of reagent B. The contents of the microplate were mixed in the plate reader for 5 seconds and the absorbance read after 15minutes at 750nm.

Statistical analysis was carried out using GraphPad Prism Version 6.0 (GraphPad software Inc., San Diego, CA). A paired student's t-test between the intracellular GSH concentration obtained for the control cells and that obtained with the cells treated with either extracts or BSO was carried out. A change in intracellular GSH levels was considered statistically significant when $p < 0.05$.

4.2.7 Modulation of P-glycoprotein Dependent Rhodamine-123 Efflux in Caco-2 Cells

Rhodamine-123 (Rh-123) is a cationic hydrophilic fluorescent P-gp substrate, which has been used as a probe to assess the functional activity of P-gp in a variety of cell lines and assays (Lee et al., 1994). In the intestine, the absorptive transport of Rh-123 occurs primarily by a passive diffusion process via the paracellular pathway. However after absorption, Rh-123 is secreted back into the lumen, first of all via a transcellular uptake process through the basolateral membrane followed by P-gp mediated efflux through the apical membrane (Troutman and Thakker, 2003).

Caco-2 cells and Caco-2 VCR cells were used for this drug accumulation/efflux assay where the amount of the Rh-123 retained by the cells after an incubation period (in the presence or absence of inhibitors) is measured spectrofluorometrically (Chieli and Romiti, 2008). Compounds that interact with the P-gp transporter either as substrates or inhibitors as

depicted in Figure 4.9 below will modulate the P-gp mediated efflux of Rh-123 and can therefore be identified.

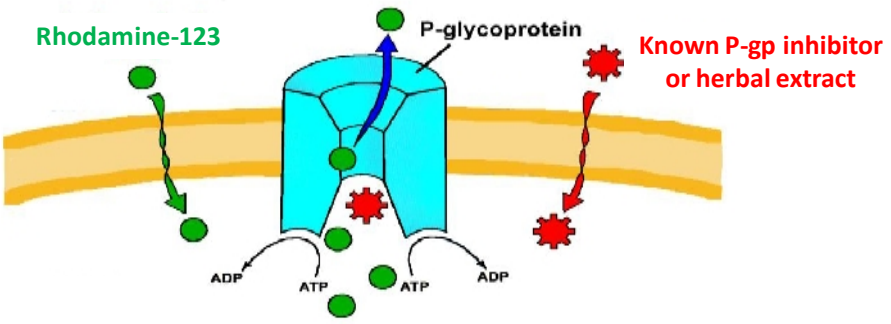


Figure 4.9: Schematic representation of the inhibition of Rhodamine-123 efflux by modulators of the P-glycoprotein transporter

The protocol used for this assay is as described in Chieli et al., (Chieli et al., 2009) with slight modifications. Both Caco-2 cells to be used are routinely grown in the appropriate complete growth media in 75cm2 tissue culture flasks as described in section 3.4.2. The culture media of the cells were usually changed a day before plating out to ensure that the cells were in their exponential stage of growth. The media of Caco-2 VCR cells was replaced with complete media without vincristine at this point.

80 to 90% confluent culture flask of cells were trypsinized and then plated out in the inner wells of a sterile 96-well plate at a density of 7.5 x 10^4 cells/ml (1.5 x 10^4 cells per well) in full culture media (without vincristine). The cells were then placed in the incubator overnight to allow for attachment. The next day, the growth media of the cells was changed to serum free media (without 10% FBS). This was to ensure a more uniform condition for the majority of the cell population as well as to prevent adsorption of drugs to the serum proteins. The microplates were then replaced back in the incubator for 24 hours.

On the day of the assay 5µg/ml Rhodamine-123 (Sigma Aldrich UK) solution in serum free media sufficient for the assay was prepared from a 5mg/ml stock solution in DMSO (solution A). 100µg/ml of all the extracts to be tested were then prepared in solution A (solution B), while 20µM verapamil (Securon® I.V. 2.5mg/ml, Abbot Laboratories Limited) was also prepared in solution A as positive control (solution C). The media in each of the wells of the microplate was then aspirated off and then replaced with either solution A, B or C in replicates. The microplates were placed back in the incubator for 2hours to allow for

intracellular rhodamine accumulation within the cells. At the same time, 20µM verapamil in PBS (solution D) was also prepared and stored in the fridge at 4°C.

After the two hours of incubation, the media in the wells are all aspirated off and each well was washed three times, gently but quickly with 200µl each of the ice cold solution D to stop the P-gp efflux activity. 100µl of 0.1% Triton X-100 in PBS was then added to each well and then placed in the incubator for fifteen minutes to lyse the cells and completely solubilise the intracellular rhodamine. 80µl of each of the cell lysates in each well was transferred to a 96-well black plate and the fluorescence intensity of each well was 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 measured for rhodamine only (solution A), that is under control conditions.

4.2.8 Effects of the P-gp Modulatory Extracts on Glibenclamide Transport in Caco-2 Monolayers

Caco-2 cells grown in monolayers on permeable filters are used for the *in vitro* prediction of intestinal drug permeability and transport. Many of the brush border enzyme and transport proteins that mediate the active uptake or efflux of drugs in the intestine are functional in Caco-2 cells (Hubatsch et al., 2007); and maintenance of the cells on permeable filter inserts has been shown to facilitate cell differentiation and polarity. As a result, over the years, Caco-2 cells have become the best established model of the intestinal absorptive epithelium and have been extensively utilised to study the transport and toxicity of nutrients (Ranaldi et al., 2003).

Sulphonylurea receptor 1 (SUR1), the binding site of action for glibenclamide is a member of the ABC (ATP binding cassette) family of transporters, which also includes P-gp and cystic fibrosis transmembrane conductance regulator protein (CFTR). Glibenclamide has been shown to also interact with these latter members of the ABC transporters. The interaction of glibenclamide with P-gp was confirmed by its ability to enhance cellular accumulation of [^{3}H]colchicine, a known substrate of P-gp in cells which express significant amounts of P-gp as well as an enhanced [^{3}H]glibenclamide accumulation in the presence of other known P-gp substrates/inhibitors (Golstein et al., 1999). The aim of this experiment was therefore to elucidate the effects of the plant extracts previously identified as P-gp modulators on the intestinal transport of glibenclamide in Caco-2 cells.

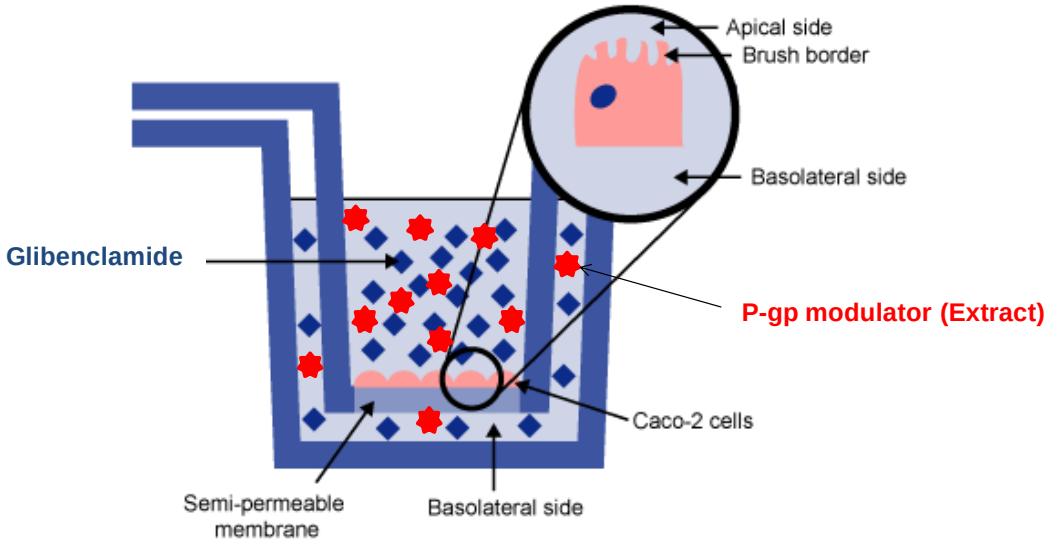


Figure 4.10: Schematic representation of the transport of glibenclamide across a monolayer of Caco-2 cells grown on a permeable filter in the presence of an identified P-gp modulator.

The protocol used for this assay is as described in Hubatsch et al., (Hubatsch et al., 2007) with slight modifications. 24-well cell culture polyethylene terephthalate (PET) inserts with 0.4µM pore size and 0.33cm^2 surface area (Millipore UK, -PIHT12R48) pre-loaded in sterile 24-well plates were used for the transport studies. Caco-2 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 were each filled with 1ml of complete media and the plates with the filter support were then placed in the incubator overnight (for a maximum of 16hours to allow for cell attachment). After the incubation period, the medium in the apical layer was replaced with 0.2ml of fresh medium so as to remove non-adherent cells as well as prevent the formation of a multilayer.

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 voltohmmeter (Millipore UK) to monitor the integrity of the monolayer. Cells were only used for transport experiments if TEER values were > 300Ωcm^2 (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 must be changed at most 24 hours prior to the transport experiment itself to prevent an exhaustion of the media nutrients.

On the day of the experiment, the physiological transport solution- Hank's balanced salt solution (HBSS) was prepared by dissolving one packet of the powder sufficient for making one litre (Sigma Aldrich) in double distilled water and buffered with 25mM HEPES and 0.35g/l $NaHCO_3$ and the pH adjusted to 7.4. The donor solution was prepared by adding 10μM glibenclamide (Sigma Aldrich) to the transport solution alone or with the plant extracts identified as modulators (at a concentration of 50 or 100μg/ml depending on the estimated NR cytotoxicity). 20μM verapamil added to the transport solution containing glibenclamide was used as positive control. 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) (Sigma Aldrich) for monitoring the integrity of the monolayer during the course of the experiment. All solutions were sterile filtered with 0.22μm Millipore filters and then pre-warmed at 37°C before use.

The filter 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 r.p.m. for 15-20 minutes on a PS-3D laboratory orbital shaker (Grant-bio Instruments, Cambridge UK) within a humidified incubator without CO_2 set at 37°C (Memmert D-91126, Schwabach FRG Germany). 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 1ml of the appropriate donor solution in the BL layer. An excess of 200μl was added to all donor solutions when adding them to inserts, which was immediately removed at t=0mins and used to estimate C0.

Sample withdrawals were carried out from the chamber containing the receiver solution at regular time intervals (15mins, 30mins, 1hr, 1hr 30mins and 2hours) by removing 0.5ml for AP-BL transport and 0.19ml 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 eppendorf tubes and immediately covered and placed on ice to prevent sample evaporation. Care must be 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.

Sample analysis- 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 ultraviolent/visible (UV-Vis) diode 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® synergi Polar-RP80A column (150mm x 4.6mm, I.D = 4µm) column maintained at 30°C. The mobile phase was an isocratic system consisting of 40% (A) 25mM potassium phosphate buffer (pH 4.5) and 60% (B) Acetonitrile. The injected volume of sample was 20µl and the UV-Vis spectrum was monitored at 230nm with glibenclamide eluting at 2minutes.

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

4.3 RESULTS AND DISCUSSION

4.3.1 Preparation of Plant Materials

A total number of thirty plant samples comprising twenty seven species from seventeen families were collected from either the herbalists or bought from the herbal markets during the field work for *in vitro* analysis of their risks of herb-drug interactions. Their identification was carried out as described in chapter 2. Six species were from the Apocynaceae family while four were from the Leguminosae family. Meliaceae and Combretaceae families had two species each, while the other families had only one each.

Eight samples were extracted by infusing with hot water, while the remaining twenty two were extracted by decoction. The yields of the different plant samples varied greatly from as low as 3% w/w to as high as over 75% w/w. The yield of the samples were independent of the plant part used or the method of extraction since the highest and lowest observed yields were from the stem-bark of two different samples (*Bridelia ferruginea* and *Anthocleista djalonensis*), which were extracted by the same decoction method (Table 4.1).

Picralima nitida used in diabetes management- (a) Seeds purchased from Ibadan market (PN1), (b) Fruit collected from a tree growing in a compound in Akabo, Imo state and (c) herbal preparation containing the seeds purchased from a herbalist (PN2)

Anogeissus leiocarpus (a) tree, (b) stem in close view and (c) stem-bark sample (AL1) obtained from herbalist

Figure 4.11: Pictures of some of the plant samples collected during field work.

Table 4.1: Tabular summary of plant samples- Collection & Extraction

Plant name	Family	Plant Part	Sample ID	Place of collection/purchase	Extraction method	Yield (%w/w)
***Alstonia boonei* De Wild.**	Apocynaceae	Stem-bark	AB1	Mushin herbal market	Decoction	5.03
***Annona senegalensis* Pers.**	Annonaceae	Stem-bark	AS1	Agbede forest	Decoction	ND
***Anogeissus leiocarpa* (DC.) Guill. & Perr.**	Combretaceae	Stem-bark	AL1	Herbalist in Abuja	Decoction	ND
***Anthocleista djalonensis* A.Chev.**	Gentianaceae	Stem-bark	AD1	Agbede forest	Decoction	3.05
		Root	AD2	Mushin herbal market	Decoction	15.38
***Aristolochia repens* Mill.**	Aristolochiaceae	Stem	AR1	Mushin herbal market	Infusion	18.11
***Bridelia ferruginea* Benth.**	Phyllanthaceae	Stem-bark	BS1	Ibadan herbal market	Decoction	75.86
***Cassia sieberiana* DC.**	Leguminosae	Root	CS1	Agbede forest	Decoction	10.27
		Stem-bark	CS2	Agbede forest	Decoction	ND
***Cassytha filiformis* L.**	Lauraceae	Whole plant	CF1	Ethnobotanist in Abuja	Decoction	12.66
***Daniellia ogea* (Harms) Holland**	Leguminosae	Stem-bark	DO1	Agbede forest	Decoction	6
***Gongronema latifolium* Benth.**	Apocynaceae	Leaves	GL1	Mushin herbal market	Infusion	8.37
***Isoberlinia doka* Craib & Stapf**	Leguminosae	Stem	ID1	Zaria herbal market	Decoction	4.69
***Khaya ivorensis* A.Chev.**	Meliaceae	Stem-bark	KS1	Mushin herbal market	Decoction	7.83
		Stem-bark	KS3	Ibadan herbal market	Decoction	3.77
***Khaya senegalensis* (Desv.) A.Juss.**	Meliaceae	Stem-bark	KS2	Agbede forest	Decoction	5.02
***Mondia whitei* (Hook.f.) Skeels**	Apocynaceae	Stem	MW1	Mushin herbal market	Infusion	20.24
***Moringa oleifera* Lam.**	Moringaceae	Leaves	MO1	Home garden in Imo	Infusion	35.54
***Ocimum gratissimum* L.**	Lamiaceae	Leaves	OG1	Mushin herbal market	Infusion	17.04
***Picralima nitida* (Stapf) T.Durand & H.Durand**	Apocynaceae	Seeds	PN1	Ibadan herbal market	Infusion	23.71
***Rauvolfia vomitoria* Afzel.**	Apocynaceae	Stem-bark	RV1	Ibadan herbal market	Decoction	3.9
***Scoparia dulcis* L.**	Plantaginaceae	Whole plant	SD1	Ethnobotanist in Abuja	Decoction	16.35
***Securidaca longipedunculata* Fresen.**	Polygalaceae	Root	SL1	Mushin herbal market	Decoction	7.49
***Strophanthus hispidus* DC.**	Apocynaceae	Stem	SH1	Ibadan herbal market	Decoction	12.2
***Syzygium guineense* (Willd.) DC.**	Myrtaceae	Stem	SG1	Zaria herbal market	Decoction	8.01
***Tamarindus indica* L.**	Leguminosae	Stem	TI1	Zaria herbal market	Decoction	6.05
***Tapinanthus bangwensis* (Engl. & K.Krause) Danser**	Loranthaceae	Leaves	ML1	Herbalist in Imo	Infusion	32.2
***Terminalia avicennioides* Guill. & Perr.**	Combretaceae	Stem	TA1	Zaria herbal market	Decoction	13.25
***Vernonia amygdalina* Delile**	Compositae	Leaves	VA1	Home garden in Lagos	Infusion	15.54
***Ximenia Americana* L.**	Olacaceae	Stem	XA1	Zaria herbal market	Decoction	11.5

4.3.2 Phytochemical Analysis

The elution conditions previously described in section 4.2.2 allowed for a good separation of all the fifteen phytochemical standards, which were detectable at 254nm and/or 360nm. The chromatogram obtained is shown below (Figure 4.12). A HPLC-DAD fingerprint for each of the plant extracts was also obtained under the same chromatographic conditions (Appendix 5). Based on this fingerprint, some of the phytochemical standards were identified in the plant samples by comparing the retention time (Rt) and UV absorption spectra of each identified peak with those of the known standards.

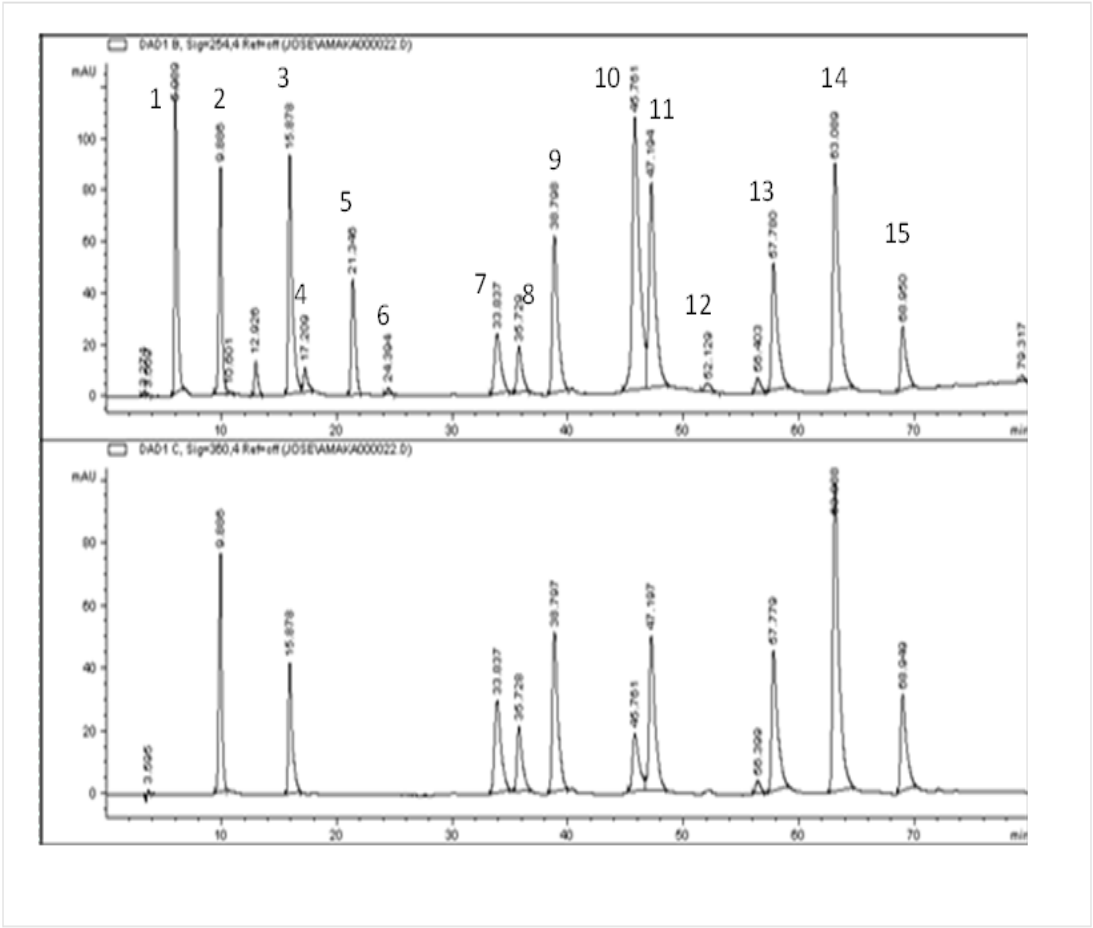


Figure 4.12: HPLC-UV chromatogram of phytochemical standards at 254 nm (up) and 360 nm (down).

(1) Gallic acid, (2) 2,4-dihydroxybenzoic acid, (3) Caffeic acid, (4) Epicatechin, (5) Epigallocatechin, (6) Catechin, (7) Vitexin rhamnoside, (8) Vitexin, (9) Rutin, (10) Ellagic acid, (11) Quercitrin, (12) Hesperidin, (13) Quercetin, (14) Luteolin and (15) Kaempferol. Each standard was present in the cocktail at a final concentration between 0.5 & 1mg/ml.

Table 4-2: Tabular summary of phenolic compounds present in plant samples

Plant name (Sample ID)	Phenolic standards detected in sample based on HPLC-DAD analysis*	Phenolic standards undetected in the sample but known to be present in specie (Ref: Ezuruike and Prieto, 2014)	Other phenolic compounds known to be present in specie (Ref: Ezuruike and Prieto, 2014)
***A. boonei* (AB1)**	**Quercitrin**	None	None
***A. senegalensis* (AS1)**	**Epigallocatechin, Gallic acid**	Catechin, Epicatechin, Quercetin, Rutin	Quercitin-3-*O*-glucoside (Isoquercetin)
***A. leiocarpa* (AL1)**	**Epigallocatechin,** Ellagic acid, Gallic acid	None	Castalagin, Chlorogenic acid, Flavogallonic acid, *p*-Coumaric acid, Protocatechuic acid (3,4-dihydroxybenzoic acid),
***A. djalonensis* (AD1/AD2)**	None	None	None
***A. repens* (AR1)**	None	None	None
***B. ferruginea* (BS1)**	**Epicatechin, Gallic acid**	Kaempferol, Quercitrin, Vitexin,	Apigenin, Ferrugin (Tri-*O*-methylmyricetin), Gallocatechin-(4'-*O*-7)-epigallocatechin, Isoquercetin, Myricetin, Myricetin-3-rhamnoside, Quercetin-3-neohesperidoside, Tetra-*O*-methylmyricetin, Rutisin (Tetra-*O*-methylquercetin), 3-*O*-methylquercetin
***C. sieberiana* (CS1/CS2)**	Epicatechin (CS1), None (CS2)	Quercitrin (Quercetin-3-*O*-rhamnoside)	Epiafzelechin
***C. filiformis* (CF1)**	**Gallic acid, Vitexin rhamnoside**	Rutin (Quercetin-3-*O*-rutinoside)	Isorhamnetin, Isorhamnetin-3-*O*-β-glucoside, Isorhamnetin-3-*O*-rutinoside, Isorhamnetin-3-*O*-robinobioside, Quercetin-3-*O*-robinobioside, Isovanillin, Kaempferol-3-*O*-robinobioside, Vanillin
***D. ogea* (DO1)**	**Epicatechin, Gallic acid**	None	None
***G. latifolium*(GL1)**	**Caffeic acid, Vitexin rhamnoside**	None	None
***I. doka* (ID1)**	None	None	None
***K. ivorensis* (KS1/KS3)**	None	None	None
***K. senegalensis* (KS2)**	None	Catechin, Quercitrin, Rutin	Procyanidin
***M. whitei* (MW1)**	None	None	Isovanillin (2-hydroxy-4-methoxybenzaldehyde), Vanillin (3-methoxy-4-hydroxybenzaldehyde), 6-methoxy-7- hydroxy coumarin, 6-methoxy-7,8-dihydroxy coumarin
***M. oleifera* (MO1)**	None	None	Chlorogenic acid, Isoquercetin, Kaempferol glycoside, Rhamnetin, Vanillin

Plant name (Sample ID)	Phenolic standards detected in sample based on HPLC analysis*	Phenolic standards undetected in the sample but known to be present in specie (Ref: Ezuruike and Prieto, 2014)	Other phenolic compounds known to be present in specie (Ref: Ezuruike and Prieto, 2014)
O. gratissimum (OG1)	Caffeic acid	Rutin, Vitexin	Apigenin-7-*O*-glucosdie, Caffeoyl derivatives, Cichoric acid, Circiliol, Cirsimaritin, Isoquercetin, Isothymusin, Isovitexin, Kaempferol-3-*O*-rutinoside, Luteolin-5-*O*-glucoside, Luteolin-7-*O*-glucoside, Nevadensin, Rosmarinic acid, Vicenin-2
P. nitida (PN1)	None	None	None
R. vomitoria (RV1)	**Gallic acid**	None	Apigenin rhamnoside, Naringenin
S. dulcis (SD1)	**Vitexin**	Luteolin, Quercetin	Apigenin, Hispidulin, *p*-Coumaric acid
S. longipedunculata (SL1)	None	Gallic acid, Caffeic acid, Epicatechin, Quercetin, Rutin	Apigenin, Cinnamic acid, Chlorogenic acid, *p*-Coumaric acid, Sinapic acid, 3,4,5-tri-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeic acid
S. hispidus (SH1)	None	None	None
S. guineense (SG1)	**Gallic acid**	None	None
T. indica (TI1)	None	Luteolin, Vitexin	Apigenin, Naringenin, Procyanidin B2, Vanillin
T. bangwensis (ML1)	**Gallic acid**	Catechin, Epicatechin, Rutin	Catechin-3-*O*-rhamnoside, Catechin-7-*O*-rhamnoside, Epicatechin gallate, Peltatoside, Quercetin-3-*O*-*β*-D-glucopyranoside, 4-methoxy-catechin-7-*O*-rhamnoside
[§]T. avicennioides (TA1)	None	Ellagic acid	Ellagic acid rhamnoside, Flavo-gallonic acid, Punicalagin (Shuaibu et al., 2008); Trimethyl gallic acid (Ekong and Idemudia, 1967)
V. amygdalina (VA1)	None	Luteolin, Rutin	Apigenin glucuronide, Caffeoylquinic acid, Chlorogenic acid, Luteolin-7-*O*-glucosdie, Luteolin-7-*O*-glucuronide, Luteolin-7-*O*-rutinoside, Luteolin-4-*O*-rutinoside
X. americana (XA1)	Gallic acid	Quercetin, Quercitrin	Kaempferol-3-*O*-(6”-gallyolyl)-*β*-glucopyranoside, Quercetin-3-*O*-(6”-gallyolyl)-*β*-glucopyranoside, Quercetin-3-*O*-*β*-xylopyranoside

The compounds highlighted in bold have not previously been reported in the plant species.

[§] TA1 isn’t reviewed in {Ezuruike and Prieto, 2014) so the references for its identified phenolic compounds are included in the table.

A preliminary identification of phenolic compounds present in each plant sample was carried out by comparison of the Rt and UV spectra (Table 4.2). They were however a number of unidentified peaks present in most plant samples due to the absence of the appropriate phenolic standard (Appendix 5). Many of the phenolic standards detected in our plant samples are being reported in those species for the first time to the best of our knowledge (as indicated in bold in Table 4.2). At the same time, some phenolic standards which have previously been shown to be present in some of the plants were not detected in our samples. For example, HPLC analysis identified the presence of catechin and epicatechin in the leaves of *A. senegalensis* collected from Burkina Faso; and isoquercetin and rutin in samples collected from Togo (Potchoo et al., 2008). These four phenolic compounds were however not present in our stem-bark extract.

It is very well known that the chemical composition of a plant including those of phenolic compounds vary greatly from one plant sample to another as well as between one plant part and another of the same sample. This is primarily because of the influence of a combination of genetic, environmental and edaphic factors such as condition of the soil, weather, season or time of day of harvesting, place and method of storage; as well as the extraction procedure employed (Peschel et al., 2013, Pandey and Rizvi, 2009). The absence of some of the previously identified standards could therefore be due to any of these reasons. In particular, the extraction method chosen for our samples was based on its traditional use and would not necessarily ensure the optimum extraction of polyphenols from the plant.

The most common phenolic compound(s) detected in the plant samples was gallic acid and/or derivatives of gallic acid. One plausible reason for this might be that the detected compound could either be that of gallic acid itself as found in the plant species or a hydrolysis product of gallocatechins present in the plant. For example, from our analysis, some plant samples in which epigallocatechin was detected- *Annona senegalensis* and *Anogeissus leiocarpus* were also found to contain gallic acid. In addition, although previous reports have confirmed the presence of an epigallocatechin gallate in samples of *Bridelia ferruginea,* the phytochemical analysis of our sample identified only its hydrolysis products- gallic acid and epicatechin.

Some plant samples – such as *Anthocleista djalonensis, Aristolochia repens* and *Picralima nitida*- have so far not been reported to contain any phenolic compounds. Our studies shown that indeed, their chromatograms present very few peaks consistent with phenolic compounds. This however does not imply that these species do not contain any phenolic compounds, but that these may be in very low amounts and yet to be identified.

4.3.3 Antioxidant Effects of Plant Samples

The maximum concentration tested for each of the plant extracts was 500µg/ml. At this concentration, only five plant extracts- AD1, AR1, CF1, MW1 and VA1 did not show any antioxidant effect in the two assays (Table 4.3). Most of the extracts (sixteen of them) produced some antioxidant activity in the DPPH assay but not in the nitric oxide scavenging assay; while the remaining eight extracts- AS1, BS1, DO1, KS1, KS3, KS2, ML1 and SG1 produced good antioxidant activity in both the DPPH assay and the NO assay.

All the eight extracts with activity in the NO assay produced greater antioxidant effect in the DPPH assay with lower IC_{50} values (< 20µg/ml), except for KS2 whose IC_{50} was 47.3 µg/ml. These results are not surprising given that the NO scavenging assay is a more selective assay compared to the DPPH assay. Whereas the presence of compounds with free labile hydrogen atoms is sufficient to produce good antioxidant effects in the DPPH assay, NO scavenging requires the presence of compounds that can out-compete its reaction with oxygen to form nitrite ions.

It could therefore be proposed based on our results, that plant extracts with good NO scavenging effects such as the aforementioned eight extracts (and/or superoxide scavenging effects) are likely to be more beneficial antioxidants in hyperglycaemic states than those without. The relevance of NO in diabetes will be further discussed in Chapter 5.

Four of the five extracts which did not produce any antioxidant effects in the DPPH or the NO assay- AD1, AR1, MW1 and VA1 were also samples in which no phenolic compounds were detected based on our phytochemical analysis (Table 4.2). As mentioned previously, this might be because the extraction method utilized did not ensure extraction of its antioxidant components. For instance, although our sample did not produce any antioxidant activity, *in vitro* antioxidant effects of polyphenol rich fractions of *Vernonia amygdalina* in free radical scavenging assays have previously been shown (Fasakin et al., 2011, Igile et al., 1994).

To further buttress this effect of extraction method on biological activity, the methanol extract of the leaves of *Ocimum gratissimum* has previously been shown to possess nitric oxide scavenging effects at < 250µg/ml as well as a higher DPPH radical scavenging activity with an IC_{50} of 12.3µg/ml (Awah and Verla, 2010). The aqueous extract of our sample on the other hand did not show any NO scavenging effect at > 500µg/ml and had a higher DPPH scavenging IC_{50} of 59µg/ml.

Table 4.3: Tabular summary of IC_{50} of plant samples in Antioxidant assays

Sample Name	Sample ID	IC_{50} [µg/ml] in DPPH Mean ± SD (n = 3)	IC_{50} [µg/ml] in NO Mean ± SD (n = 3)
Alstonia boonei	AB 1	83.06 ± 2.63	> 500
Annona senegalensis	AS 1	17.15 ± 4.76	39.50 ± 4.45
Anogeissus leiocarpa	AL 1	80.19 ± 27.93	> 500
Anthocleista djalonensis **bark**	AD 1	> 500	> 500
Anthocleista djalonensis **root**	AD 2	214.44 ± 40.45	> 500
Aristolochia repens	AR 1	> 500	> 500
Bridelia ferruginea	BS 1	13.29 ± 6.29	43.34 ± 9.23
Cassia sieberiana	CS 1	51.79 ± 2.00	> 500
Cassytha filiformis	CF 1	> 500	> 500
Daniellia ogea	DO 1	8.57 ± 1.11	168.49 ± 8.16
Gongronema latifolium	GL 1	143.34 ± 10.84	> 500
Isoberlinia doka	ID 1	40.14 ± 5.11	> 500
Khaya ivorensis	KS1	10.87 ± 1.05	66.68 ± 3.94
Khaya ivorensis	KS 3	10.35 ± 2.42	41.88 ± 8.41
Khaya senegalensis	KS 2	47.30 ± 13.09	118.49 ± 20.41
Tapinanthus bangwensis	ML1	15.40 ± 4.21	91.93 ± 13.07
Mondia whitei	MW 1	> 500	> 500
Moringa oleifera	MO 1	71.35 ± 6.53	> 500
Ocimum gratissimum	OG 1	59.02 ± 3.30	> 500
Picralima nitida	PN 1	138.85 ± 15.95	> 500
Rauvolfia vomitoria	RV 1	122.94 ± 26.69	> 500
Scoparia dulcis	SD 1	80.00 ± 2.86	> 500
Securidaca longipedunculata	SL 1	291.50 ± 14.74	> 500
Strophanthus hispidus	SH 1	117.45 ± 21.06	> 500
Syzygium guineense	SG 1	15.10 ± 6.23	34.44 ± 3.95
Tamarindus indica	TI 1	70.01 ± 7.64	> 500
Terminalia avicennioides	TA 1	33.18 ± 1.86	> 500
Vernonia amygdalina	VA 1	> 500	> 500
Ximenia americana	XA 1	82.21 ± 2.59	> 500
Caffeic Acid	CA	0.41 ± 0.09	10.67 ± 2.30
Gallic Acid	GA	0.85 ± 0.14	20.875 ± 1.27

Surprisingly, although we identified the presence of vitexin rhamnoside and gallic acid in *Cassytha filiformis* (CF1); in addition to other phenolic compounds that have been detected in the species, the plant sample did not produce any antioxidant effect at 500µg/ml. A comparison of the results of the phytochemical analysis and the antioxidant assays carried out on the plant samples therefore indicate that there is no correlation between the number of phenolic compounds detected in the sample and the observed antioxidant effects. One reason for this might be that the phenolic compounds though present, may be in such low amounts that no antioxidant effects may be seen. No quantification of the phenolic compounds was however carried out in this study to confirm this.

It is however worthwhile to note that a higher amount of phenolic compounds in a plant sample may not necessarily translate to higher antioxidant activity. Another study in which a quantification of the phenolic compounds detected in different samples was carried out did not see a proportional relationship between the quantity of phenolic compounds detected and the antioxidant potential of the plant sample (Proestos et al., 2005).

KS1 was the most active of all the samples in both antioxidant assays, but yet none of the phenolic standards was detected in our sample. There have also not been any previous reports of the identification of any phenolic compounds in that specie, although a phytochemical screening confirmed the presence of phenols in the leaves but not in the bark; and the absence of flavonoids in both the stem-bark and the leaves (Ibrahim et al., 2006). On the other hand, *Khaya* spp are known to contain a large number of limonoids, a class of triterpenoids (Roy and Saraf, 2006, Adesida et al., 1971). The antioxidant effects of limonoids found in *Citrus spp*. have been reported (Poulose et al., 2005, Yu et al., 2005). Thus, the presence of limonoids may account for the good antioxidant effects observed for *Khaya ivorensis* (KS1 and KS3) as well as *Khaya senegalensis* (KS2).

These considerations further highlight the absence of a clear relationship between polyphenolic compounds and antioxidant effects.

4.3.4 Neutral Red Uptake Assay for Determination of Cell Viability/Cytotoxicity

The viability of the cells was determined after 24 hours incubation with either the plant extracts or drugs to be used in the different assays. 24 hours was selected as the maximum time of incubation since subsequent cellular assays to be conducted with the extracts were ≤ 24 hours.

Prior to evaluation of the extracts, the viability of the cells in the presence of different concentrations of hydrogen peroxide (H_2O_2) and/or dimethyl sulfoxide (DMSO) were determined (Figure 4.13). Polyphenols under certain cell culture conditions are known to bring about a generation of hydrogen peroxide (H_2O_2) in the culture media (Bellion et al., 2009, Halliwell, 2003), which in themselves can be toxic to cells. DMSO on the other hand is commonly used a vehicle for drugs to be used in biological assays. It is however known to be toxic to cells above a given concentration.

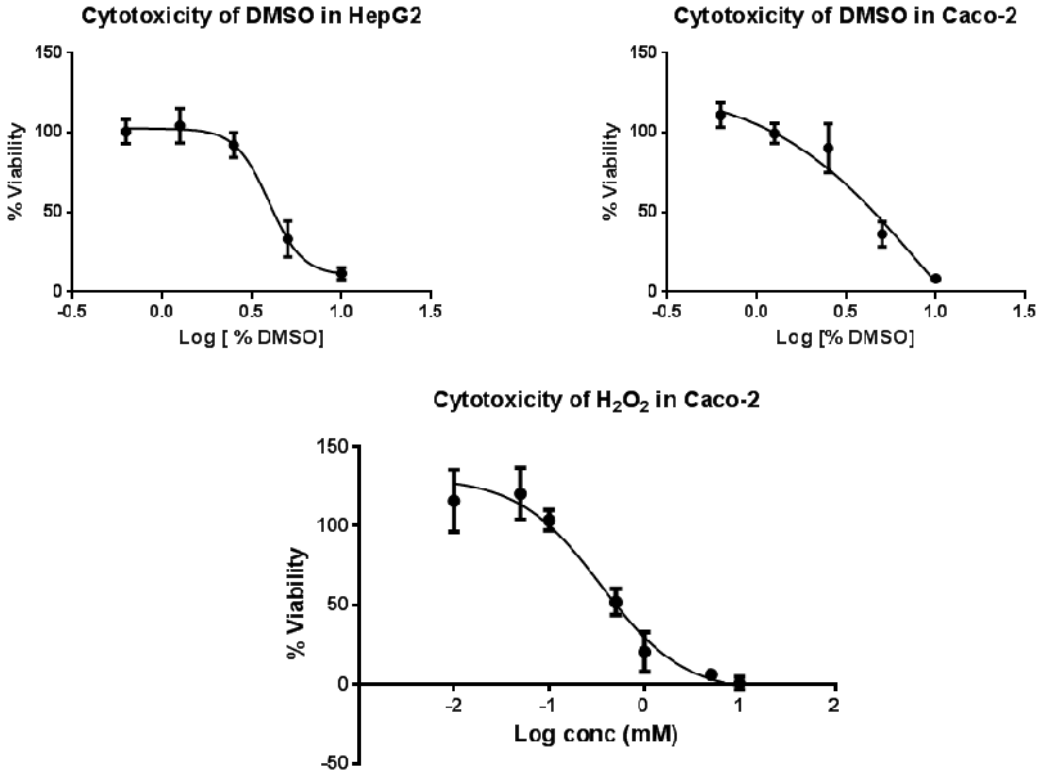


Figure 4.13: Viability of HepG2 & Caco-2 cells in the presence of different concentrations of H_2O_2 and/or DMSO. (Results show mean ± SEM, N ≥ 3)

The cytotoxicity of the two compounds was therefore carried out in order to estimate the non-toxic concentration range. Either of these chemicals was subsequently included as positive controls during the cytotoxicity estimation of the extracts as validation for the assay.

From the inhibitory dose response curve obtained for hydrogen peroxide in Caco-2 cells, if the extracts generate H_2O_2 levels above 100µM during the course of their incubation in cell culture, this might induce cytotoxic effects in the cells. However, Bellion et al. (2009) showed that 100µg/ml of the polyphenolic extracts of fruits generated ≤ 50 µM of H_2O_2 after 24hours incubation in culture. One could therefore safely infer that the H_2O_2 generated by the incubation of ≤ 100µg/ml of any of the extracts would not be sufficient as to induce a cytotoxic effect on the Caco-2 cells. This is in addition to the presence of antioxidants in the samples that could also contribute to the breakdown of H_2O_2 further decreasing its toxicity. Similarly, the use of DMSO concentrations ≤ 1% would also not induce cytotoxic effects in the cells (Figure 4.13).

The IC_{50} and MNTC values were determined for all the plant samples in the two different cell lines- HepG2 and Caco-2. This was to provide an indication of the cytotoxic nature of the collected plant samples. Given that the extracts are complex mixtures containing a myriad of phytoconstituents, the use of an inhibitory dose response curve to estimate both the IC_{50} and MNTC values would result in an over-estimation, in which the complex mixture is being subjected to a model intended for single pure compounds. Thus, the results obtained for the samples are expressed as range values as shown in Table 4.4, from which an approximate MNTC concentration was determined.

A number of studies have identified the neutral red (NR) cytotoxicity assay as a sensitive *in vitro* assay for detecting early toxicity in cells (Fotakis and Timbrell, 2006, Weyermann et al., 2005). In addition, IC_{50} values obtained from *in vitro* NR assays gave a good correlation with *in vivo* LD_{50} values for some identified chemicals, with suggestions that the former can be a good predictor for *in vivo* acute toxicity (Konsoula and Barile, 2005). Thus, at IC_{50} values of ≥ 100µg/ml and estimating a plasma volume of about 16L in an adult, most of the extracts would need to be taken in amounts greater than 1g in order to produce a toxic effect *in vivo* (Houghton et al., 2007).

Most of the plant extracts were non-cytotoxic in HepG2 cells with approximate IC_{50} values ≥ 200µg/ml except DO1, KS1, KS3, SH1 and TA1 which had IC_{50} values > 50µg/ml. Possible hepatotoxic effects of these five extracts have not been previously evaluated. However punicalagin which is a known component of TA1 has been shown to be hepatotoxic at high doses, bringing about elevation of serum AST and ALT levels (Lin et al., 2001).

Table 4-4: Tabular summary of cytotoxicity of plant extracts in HepG2 and Caco-2 cells

Plant name(s)	Sample ID	Cytotoxicity HepG2 (µg/ml)		Cytotoxicity Caco-2 (µg/ml)	
		IC_{50}	MNTC	IC_{50}	MNTC
A. boonei	**AB1**	> 500	> 500	$31.25 < IC_{50} < 62.5$	< 10
A.senegalensis	**AS1**	≈ 500	≥ 3.5	$15.625 < IC_{50} < 62.5$	< 10
A.leiocarpa	**AL1**	$200 < IC_{50} < 500$	15 < MNTC < 30	> 125	10 < MNTC < 31.25
A.djalonensis	**AD1**	> 500	1 < MNTC < 10	> 500	< 250
	AD2	> 500	1 < MNTC < 10	> 500	< 1
A. repens	**AR1**	> 500	31.25 < MNTC < 62.5	> 500	≥ 500
B. ferruginea	**BS1**	$250 \leq IC_{50} \leq 500$	≥ 3.5	$250 < IC_{50} < 500$	≤ 5
C. sieberiana	**CS1**	> 500	31.25 < MNTC < 62.5	≥ 250	≤ 1
C. filiformis	**CF1**	> 500	7.5 < MNTC < 15	> 500	≤ 30
D. ogea	**DO1**	$31.25 < IC_{50} < 62.5$	< 2	> 500	< 0.1
G. latifolium	**GL1**	> 500	62.5 < MNTC < 125	≤ 10	< 0.1
I.doka	**ID1**	> 500	< 1	> 500	> 500
K. ivorensis	**KS1**	$125 < IC_{50} \leq 250$	62.5 < MNTC < 125	> 500	≥ 100
	KS3	$62.5 < IC_{50} < 125$	31.25< MNTC < 62.5	$IC_{50} > 500$	> 500
K. senegalensis	**KS2**	>500	≤ 5	$100 < IC_{50} < 500$	≤ 50
M. whitei	**MW1**	>500	< 2	$IC_{50} \geq 500$	≤ 100
M. oleifera	**MO1**	>500	< 2	$250 < IC_{50} < 500$	< 5
O. gratissimum	**OG1**	>500	< 2	$125 < IC_{50} < 500$	< 0.5
P. nitida	**PN1**	$250 < IC_{50} < 500$	125 < MNTC < 250	$250 < IC_{50} < 500$	125 < MNTC < 250
	PN2	$250 < IC_{50} < 500$	62.5< MNTC<125	$IC_{50} \geq 500$	≤ 1

Plant name(s)	**Sample ID**	**Cytotoxicity HepG2 (µg/ml)**		**Cytotoxicity Caco-2 (µg/ml)**	
		IC_{50}	**MNTC**	**IC_{50}**	**MNTC**
R.vomitoria	**RV1**	$125 < IC_{50} < 250$	< 2	$250 < IC_{50} < 500$	≤ 50
S. dulcis	**SD1**	>500	> 500	≥ 500	≤ 100
S. hispidus	**SH1**	$31.25 < IC_{50} < 125$	< 2	≤ 50	≤ 1
S. guineense	**SG1**	>500	$1 < MNTC < 10$	$250 < IC_{50} < 500$	$125 < MNTC < 250$
T. indica	**TI1**	>500	$1 < MNTC < 30$	$IC_{50} \geq 500$	≤ 100
T. bangwensis	**ML1**	>500	< 2	≥ 500	≤ 50
T. avicennioides	**TA1**	$125 < IC_{50} < 250$	$1 < MNTC < 10$	$250 < IC_{50} < 500$	≤ 125
V.amygdalina	**VA1**	>500	$62.5 < MNTC < 125$	> 500	≤ 0.5
X. americana	**XA1**	$250 < IC_{50} < 500$	$125 < MNTC < 250$	> 500	≤ 50

On the other hand, although samples of *Cassia sieberiana* (CS1), *Ocimum gratissimum* (OG1) and *Picralima nitida* (PN1) did not show cytotoxic effects in the HepG2 cells, the stem bark and pods of CS1 (Obidah et al., 2009, Toma et al., 2009); the leaves of OG1 (Ajibade et al., 2012, Onaolapo and Onaolapo, 2012); and stem-bark and fruit rind of PN1 (Kouitcheu Mabeku et al., 2008, Fakeye et al., 2004) have been shown to produce hepatotoxic effects *in vivo* either at high doses or after chronic administration.

Most of the extracts seemed to be more toxic to the Caco-2 cells, producing lower IC_{50} values than that obtained in HepG2 cells. The only exceptions to this were *Khaya ivorensis* (KS1 & KS3) and *Terminalia avicennioides* (TA1) whose IC_{50} values were lower in Caco-2 cells compared to HepG2 cells. However their IC_{50} values were not low enough as to suggest specific toxicity for hepatic cells compared to intestinal cells. Nonetheless, only four extracts- AB1, AS1, GL1 and SH1 produced IC_{50} values $\leq$ 100µg/ml in Caco-2 cells indicating a relative non-toxic profile for more than twenty of the collected plant samples.

Although IC_{50} values are often the parameter used to estimate toxicity, one cannot overlook the obtained maximum non-toxic concentration (MNTC) values, particularly for extracts with values $\leq$ 1µg/ml, which is equivalent to approximately 10mg *in vivo*. It therefore requires that such plant extracts would need to be ingested in amounts less than 10mg so as not to produce any toxic effect. It is important to note here that several limitations (as mentioned in Chapter 1) can affect the estimation of the *in vivo* toxic profile of these extracts based on *in vitro results.* In addition, the cell lines used in this study are rapidly proliferating cancer cell lines and similar results may not be obtained when an evaluation of the toxicity profile is done in normal cells.

In addition to being used to evaluate the toxic profile of the plant samples, the NR cytotoxicity assay was also used to estimate the concentration of the plant extracts to be used in subsequent assays. For extracts which gave MNTC concentrations $\geq$ 100µg/ml, this was used as the test concentration for the GSH assay in HepG2 cells, while the nearest lower approximate value was used for those with MNTC < 100µg/ml (Table 4.5).

For cellular assays involving Caco-2 cells, all extracts were tested at 100µg/ml for the Rh-123 efflux assay. Extracts which produced MNTC concentrations < 100µg/ml after a 24 hour incubation were re-tested in a 4 hour incubation assay since the Rh-123 efflux assay and the transport assay require a two hour incubation of the extracts. At the end of the four hour incubation period, most extracts produced > 90% cell viability except AS1, AL1, BS1 and DO1 which produced approximately 50% viability (Data not shown).

4.3.5 Estimation of Intracellular Glutathione Levels in HepG2 Cells

The rate of change in the kinetic absorbance of TNB for the different GSH standard concentrations is linearly proportional to the amount of GSH in each sample. Thus, a graph plot of the slopes of each GSH standard concentration (I-slopes) against the GSH concentration, otherwise known as the F-slope curve can be used to estimate the amount of GSH in the treated wells given their individual I-slopes (Figure 4.14).

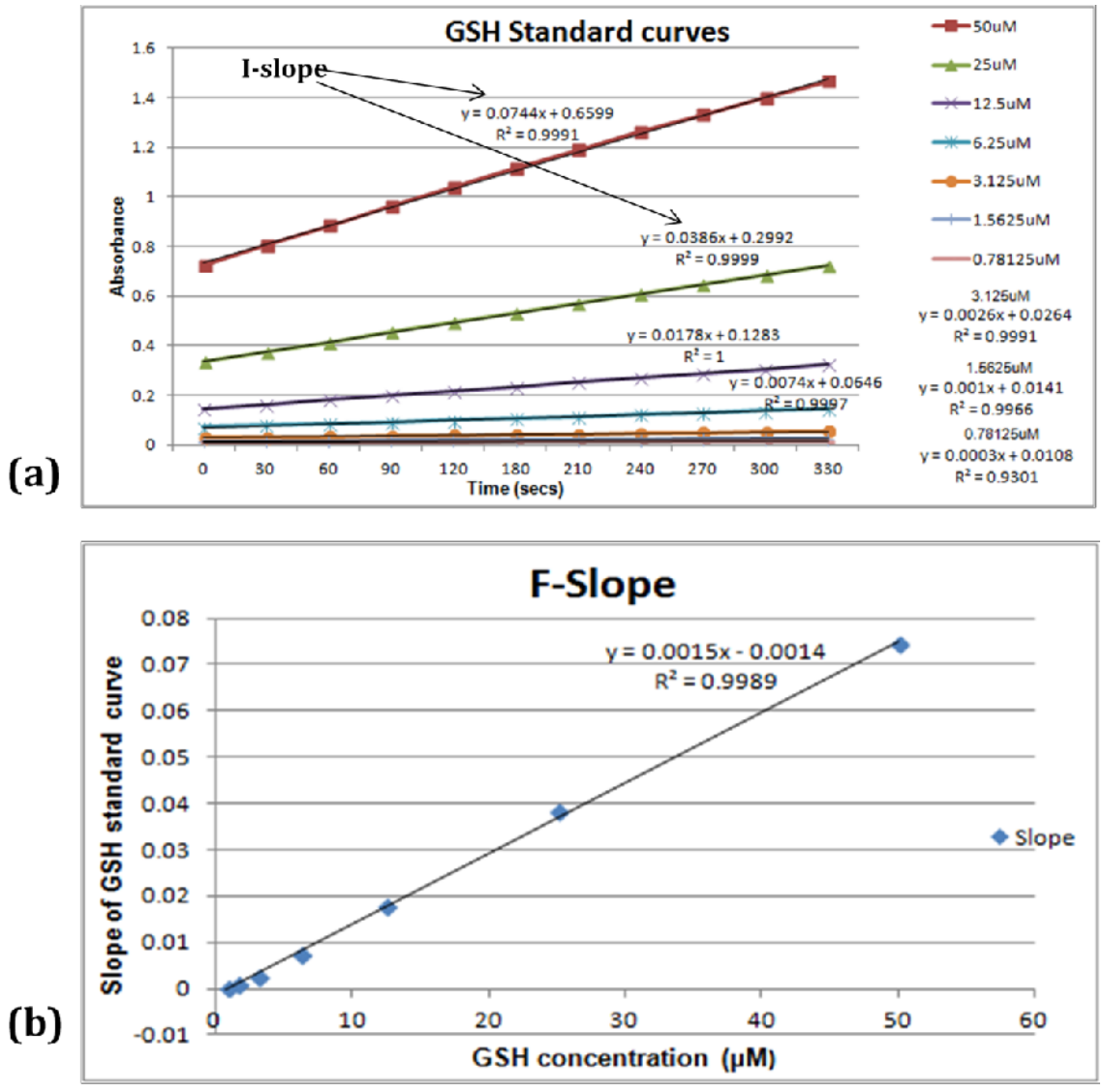


Figure 4.14: Graph plot of the rate of change in TNB absorbance at the different GSH standard concentrations (a), which is used to estimate the amount of GSH in each sample from the obtained f-slope curve (b)

The glutathione concentration obtained for the control cells was 26.66 ± 3.74 µM. Three of the extracts- the stem-bark of *Khaya ivorensis* obtained from Lagos and Ibadan (KS1 & KS3) as well as the stem-bark of *Ximenia americana* (XA1) decreased the intracellular GSH concentration beyond levels seen with the control cells to 21.23 ± 6.45 µM, 18.98 ± 7.66 µM and 17.84 ± 6.03 µM respectively. This decrease was found to be statistically significant ($P<0.05$). However, none of the decreases produced by the extracts were as much as that produced by the positive control BSO, which decreased intracellular GSH levels to 12.76 ± 2.08 µM ($P<0.0001$) (Figure 4.15 and Table 4.5).

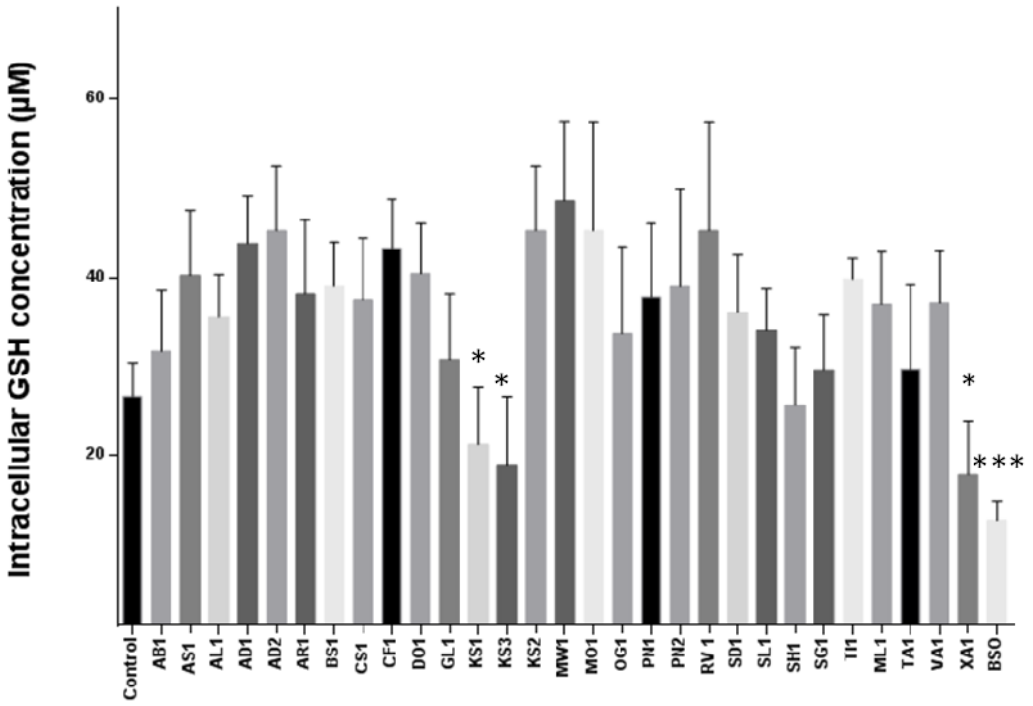


Figure 4.15: Effect of 24-hour incubation of extracts at MNTC values on the intracellular GSH concentration in HepG2 cells. Only three extracts KS1, KS3 and XA1 produced a statistically significant ($P<0.05$) decrease in GSH concentrations based on a paired student's t-test. (Results show Mean ± SEM, $n \geq 3$)

Table 4-5: Tabular summary of intracellular GSH concentrations in HepG2 cells in the presence and/or absence of extracts. (Results show mean ± SEM, n ≥ 3)

Plant/Drug name (Sample ID)	Conc (µg/ml)	GSH (µM /10^5 cells)
Control (Water)	-	26.66 ± 3.74
***Alstonia boonei* (AB1)**	100	31.66 ± 6.86
***Annona senegalensis* (AS1)**	5	40.21 ± 7.29
***Anogeissus leiocarpa* (AL1)**	20	35.49 ± 4.83
***Anthocleista djalonensis* (AD1)**	5	43.78 ± 5.28
(AD2)	5	45.2 ± 7.1
***Aristolochia repens* (AR1)**	50	38.12 ± 8.3
***Bridelia ferruginea* (BS1)**	5	38.96 ± 4.98
***Cassia sieberiana* (CS1)**	50	37.45 ± 6.91
***Cassytha filiformis* (CF1)**	10	43.23 ± 5.45
***Daniellia ogea* (DO1)**	1	40.43 ± 5.58
***Gongronema latifolium* (GL1)**	100	30.74 ± 7.38
***Khaya ivorensis* (KS1)**	100	21.23 ± 6.45
(KS3)	50	18.98 ± 7.66
***Khaya senegalensis* (KS2)**	1	45.2 ± 7.19
***Mondia whitei* (MW1)**	1	48.53 ± 8.94
***Moringa oleifera* (MO1)**	1	45.2 ± 12.22
***Ocimum gratissimum* (OG1)**	1	33.72 ± 9.71
***Picralima nitida* (PN1)**	100	37.78 ± 8.22
(PN2)	100	38.93 ± 10.9
***Rauvolfia vomitoria* (RV1)**	1	45.2 ± 12.22
***Scoparia dulcis* (SD1)**	100	36.06 ± 6.4
***Securidaca longipedunculata* (SL1)**	10	34.06 ± 4.61
***Strophanthus hispidus* (SH1)**	1	25.66 ± 6.41
***Syzygium guineense* (SG1)**	5	29.64 ± 6.1
***Tamarindus indica* (TI1)**	20	39.83 ± 2.25
***Tapinanthus bangwensis* (ML1)**	1	37 ± 5.81
***Terminalia avicennioides* (TA1)**	5	29.71 ± 9.43
***Vernonia amygdalina* (VA1)**	100	37.13 ± 5.8
***Ximenia americana* (XA1)**	100	17.84 ± 6.03
Buthionine sulfoximine (BSO)	10µM	12.76 ± 2.08

Reduced glutathione plays a very important role in decreasing xenobiotics-induced toxicity in cells. Thus depletion of intracellular GSH levels can result in an exacerbation of toxicity in the cells. Buthionine sulfoximine (BSO) which was used as the positive control in the assay brings about a depletion of intracellular GSH levels by inhibiting γ-glutamylcysteine synthetase (γ-GCS), one of the enzymes needed for intracellular GSH synthesis (Drew and Miners, 1984). As expected, BSO (10µM) depleted the intracellular GSH levels in the HepG2 cells by over 50% compared to control (Table 4.5).

Although the mechanism by which KS1, KS3 and XA1 depleted the intracellular GSH levels is not known, their presence in cells have the potential to enhance xenobiotics-induced toxicity, especially when co-administered with drugs requiring glutathione-S-transferases (GSTs) for their detoxification. In addition, they also pose an additional risk of worsening hyperglycaemia-induced oxidative stress conditions in diabetic patients. This would however depend on the overall effect of the extracts on other intracellular antioxidant enzymes.

The intracellular GSH-depletion effect of KS1 & KS3 was quite surprising given that they have previously been shown to be potent nitric oxide (NO) scavengers (Table 4.3). It has previously been shown that depletion of intracellular GSH levels significantly increases NO-mediated cytotoxicity (Walker et al., 1995). In this regard, one would have expected an increase in GSH levels to be partly responsible for its NO-scavenging effects but this was not the case. Thus the expected theoretical protection of KS1 and KS3 against this radical is offset by the depletion of intracellular GSH levels. This highlights the risk of minimalistic marketing approaches focusing on the idea that "antioxidant plants" are healthy without a better understanding of the other biological effects of the plant.

Apart from KS1, KS3 and XA1, the other extracts increased the intracellular GSH concentration above the levels seen with the control cells after 24 hours incubation, although these increases were not statistically significant. It has previously been mentioned that incubation of extracts containing polyphenols brings about an increase in extracellular H_2O_2 levels in cell culture but not *in vivo*, which could in turn result in an induction of intracellular antioxidant enzymes (Bellion et al., 2009). It is therefore possible that the observed increased GSH levels may be partly due to this and should not necessarily be extrapolated to *in vivo* conditions without further investigation.

4.3.6 Modulation of P-Glycoprotein Dependent Rhodamine-123 Efflux in Caco-2 Cells

By culturing wild type Caco-2 cells in increasing concentrations of vincristine over a period of time, P-gp expression can be induced in cells with previously low levels of P-gp (Eneroth et al., 2001). The intracellular accumulation of P-gp substrates is inversely proportional to the activity of the transporter in these P-gp expressing cells. Cells with higher P-gp expression have a higher efflux activity for P-gp substrates resulting in lower intracellular concentration. Similarly, the addition of a P-gp inhibitor will limit its efflux activity with a resultant increase in intracellular concentration of the P-gp substrate.

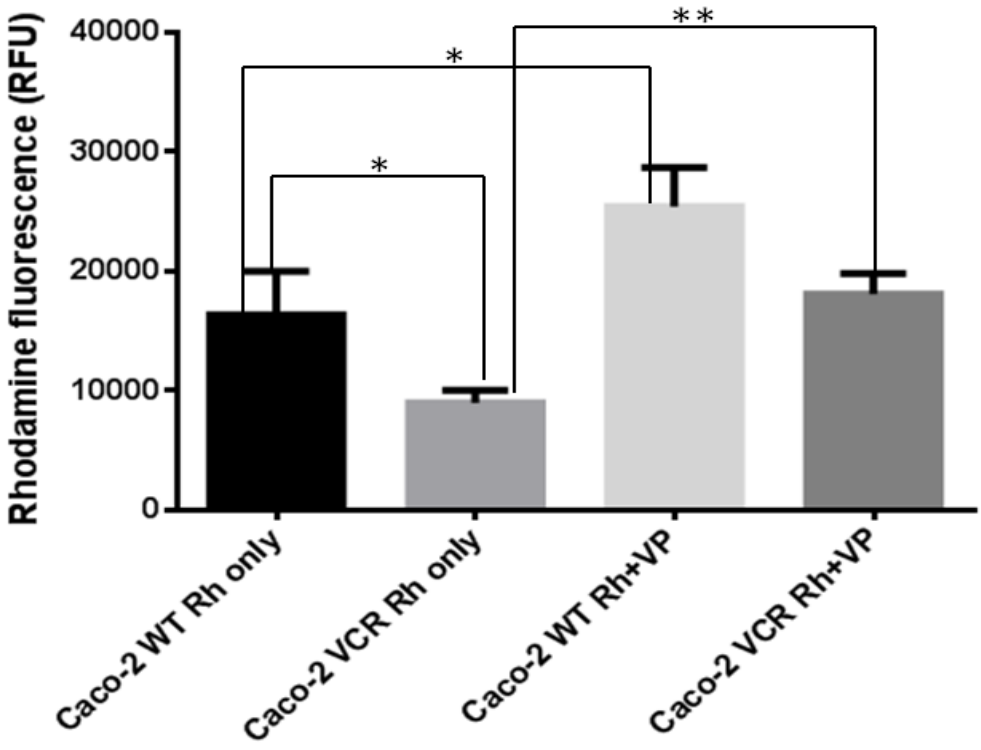


Figure 4.16: Effect of increased P-gp expression and presence of a P-gp inhibitor on the intracellular concentration of a P-gp substrate in Caco-2 WT and Caco-2 VCR cells. (Results show mean ± S.D, N ≥ 2). Statistical significance based on a paired T-test.

To confirm this, we measured the intracellular rhodamine (Rh-123) fluorescence in both Caco-2 WT and Caco-2 VCR cells incubated for two hours with 5µg/ml Rh-123 in the presence or absence of 20µM verapamil as described in section 4.2.7. As shown in Figure 4.16, the intracellular Rh-123 fluorescence measured in relative fluorescence units (RFU) was lower in the Caco-2 VCR cells compared to Caco-2 WT cells. Addition of verapamil brought about an increase in intracellular Rh-123 fluorescence in both the Caco-2 VCR and the Caco-2 WT cells.

Table 4-6: Effect of plant extracts on intracellular Rh-123 accumulation in Caco-2 VCR and Caco-2 WT cells.

Plant/Drug name (Sample ID)	**Intracellular Rh-123 Fluorescence (% of control)**	
	Caco-2 VCR (N ≥ 3)	**Caco-2 WT (N ≥ 2)**
VERAPAMIL	176 ± 47	163 ± 32
***Alstonia boonei* (AB1)**	95 ± 5	87 ± 3
***Anthocliesta djalonensis* (AD1)**	88 ± 11	106 ± 44
***Anogeissus leiocarpus* (AL1)**	108 ± 15	80 ± 20
***Aristolochia repens* (AR1)**	125 ± 22	118 ± 10
***Annona senegalensis* AS1)**	278 ± 136	185 ± 91
***Bridelia ferruginea* (BS1)**	564 ± 124	397 ± 81
***Cassytha filiformis* (CF1)**	180 ± 10	150 ± 40
***Cassia sieberiana* (CS1)**	159 ± 23	138 ± 35
***Daniellia ogea* (DO1)**	299 ± 39	274 ± 95
***Gongronema latifolium* (GL1)**	99 ± 4	79 ± 2
***Isoberlinia doka* (ID1)**	165 ± 42	89 ± 47
***Khaya ivorensis* (KS1)**	285 ± 54	208 ± 89
***Khaya ivorensis* (KS3)**	288 ± 53	228 ± 72
***Khaya senegalensis* (KS2)**	126 ± 10	89 ± 13
***Moringa oleifera* (MO1)**	122 ± 12	88 ± 88
***Mondia whitei* (MW1)**	109 ± 7	113 ± 32
***Ocimum gratissimum* (OG1)**	110 ± 9	99 *
***Picralima nitida* (PN1)**	121 ± 7	115 ± 34
***Picralima nitida* (PN2)**	122 ± 4	91 ± 1
***Rauvolfia vomitoria* (RV1)**	106 ± 8	101 ± 23
***Scoparia dulcis* (SD1)**	113 ± 30	92 ± 0
***Syzygium guineense* (SG1)**	224 ± 52	251 ± 49
***Strophanthus hispidus* (SH1)**	120 ± 46	98 ± 26
***Securidaca longipedunculata* (SL1)**	112 ± 27	105*
***Tamarindus indica* (TI1)**	116 ± 27	90 ± 13
***Tapinanthus bangwensis* (ML1)**	116 ± 5	121*
***Terminalia avicennioides* (TA1)**	224 ± 49	142 ± 27
***Vernonia amygdalina* (VA1)**	107 ± 17	112 ± 15
***Ximenia americana* (XA1)**	447 ± 97	412 ± 54

* Results for these extracts are based on data from only one experiment.

To identify extracts that modulate P-gp efflux activity, Rh-123 was co-incubated with 100µg/ml of each extract for two hours in both Caco-2 WT and Caco-2 VCR cells, and the intracellular Rh-123 fluorescence determined after cell lysis. Cells were also incubated with 20µM verapamil as positive control while cells with only Rh-123 served as negative control. The percentage change in Rh-123 fluorescence relative to the fluorescence in the control cells after co-incubation with most of the extracts was greater in Caco-2 VCR cells compared to Caco-2 WT cells (Table 4.6). This indicates increased P-gp efflux activity in the vincristine resistant cells, such that modulation of the efflux transporter resulted in a greater change in intracellular Rh-123 accumulation.

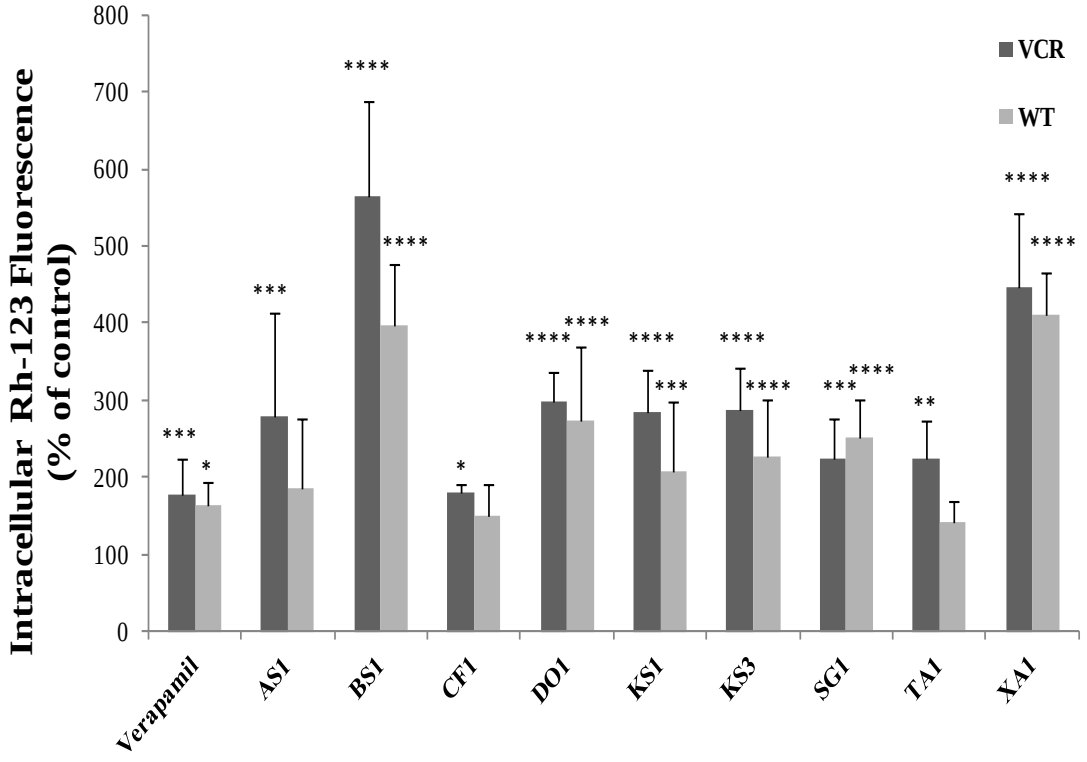


Figure 4.17: Increased intracellular Rh-123 concentration in Caco-2 cells (WT and VCR) in the presence of either extracts or 20µM verapamil. (Resuts show mean ± SD, N ≥ 3. One-way ANOVA followed by Bonferroni's post test was carried out to determine significance of extracts. (*, **, *, **** represent P<0.05, P<0.01, P<0.001 and P<0.0001 respectively).**

Nine extracts- *Annona senegalensis* (AS1), *Bridelia ferruginea* (BS1), *Cassytha filiformis* (CF1), *Daniellia ogea* (DO1), *Khaya ivorensis* (KS1 & KS3), *Syzygium guineense* (SG1), *Terminalia avicennioides* (TA1) and *Ximenia americana* (XA1) produced a change in intracellular accumulation of Rh-123 which was significantly different from control in the Caco-2 VCR cells but not necessarily in the Caco-2 WT cells. Similar to the effect of the positive control verapamil, these extracts increased the percentage Rh-123 fluorescence (Figure 4.17) which suggests that all the extracts are acting as P-gp inhibitors.

In Caco-2 VCR cells, the highest observed inhibitory effect was produced by BS1 with more than 500% increase in Rh-123 accumulation compared to control cells followed by XA1 with more than 400% increase. These inhibitory effects of BS1 and XA1 were 3-fold and 2-fold that produced by the positive control verapamil. 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 (Chieli et al., 2009).

Not all of the nine extracts however produced a significant change in Rh-123 accumulation in Caco-2 WT cells. AS1, CF1 and TA1 produced a P-gp inhibitory effect in the VCR resistant cells but not in the WT cells. These results are not surprising as various studies have only been able to identify modulators of the P-gp efflux transporter when using modified cell lines with increased P-gp activity but not with the wild type cells (Taub et al., 2005, Lee et al., 1994). The modulatory effect of SG1 was however quite surprising given that it produced a greater change in intracellular rhodamine accumulation in the WT cells than in the VCR cells, both of which were significant. The reason for this is still unclear although it is quite possible that the increased intracellular Rh-123 accumulation due to SG1 (and possibly for some of the extracts) may not be as a result of a direct inhibition of P-gp as other tests are necessary to confirm this.

Finally, different concentrations (100µg, 50 µg, 10 µg and 5 µg) of the active extracts were co-incubated with Rh-123 to determine if the proposed interaction with P-gp was dose-dependent. As shown in Figure 4.18, most of the extracts except CF1 and SG1 produced a dose-dependent inhibition of P-gp activity, although the intracellular Rh-123 fluorescence at 5µg/ml and 10µg/ml for all the extracts were not significantly different from those of the control cells at 100% (statistical error bars not shown).

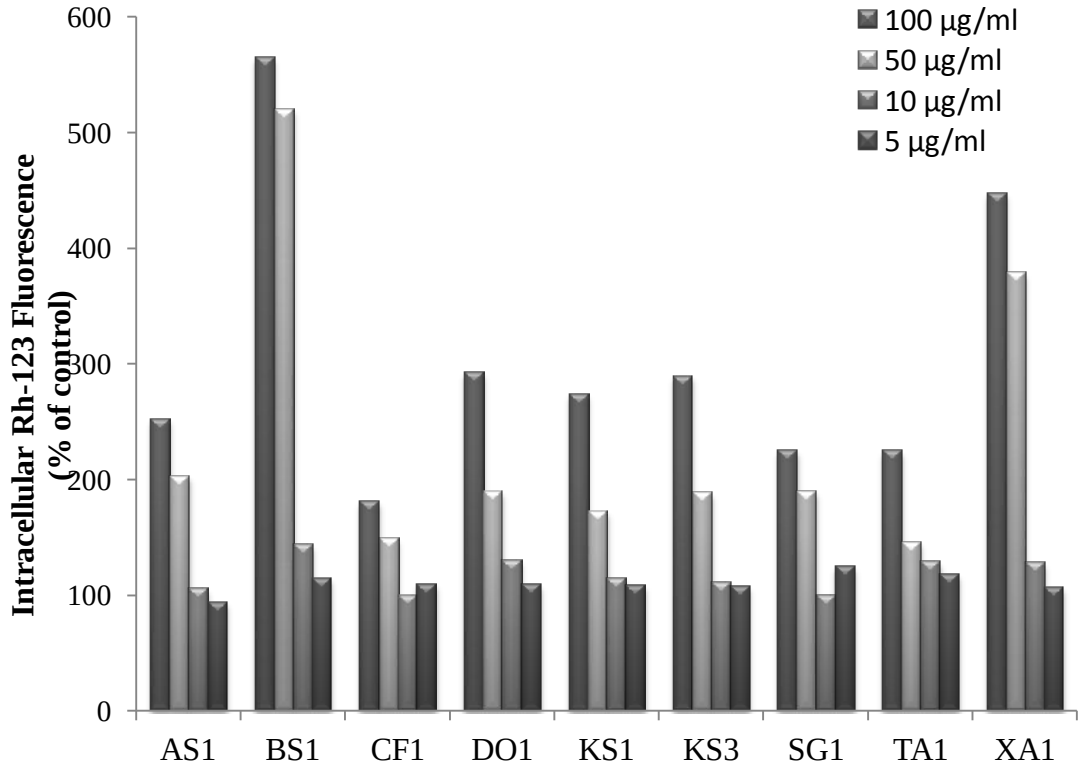


Figure 4.18: Effect of different concentrations of extracts on the intracellular Rh-123 fluorescence in Caco-2 VCR cells.

4.3.7 Effects of the P-Gp Modulatory Extracts on Glibenclamide Transport in Caco-2 Monolayers

A number of studies have identified glibenclamide as a substrate and inhibitor of P-gp in P-gp over expressing cell lines (Gedeon et al., 2006, Golstein et al., 1999). 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 (Lilja et al., 2007); 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 accumulation assay and glibenclamide when both are co-administered by diabetic patients.

To evaluate the effects of the co-incubated extracts on the permeability and transport of glibenclamide, three parameters were measured- the TEER value before and after the transport experiment and the apparent permeability of the paracellular marker FD4 across the Caco-2 monolayers; as well as 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, which could in turn affect glibenclamide transport; while the last measurement was for direct estimation of its effect on glibenclamide transport.

The maturation of tight junctions during differentiation of Caco-2 cells grown *in vitro* 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 (Hilgers et al., 1990). 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 (Ranaldi et al., 2003).

To confirm tight junction formation and maturation as well as readiness of the monolayer for transport experiments, TEER values were monitored at least once a week up to Day 26. As shown in Figure 4.19, a steady increase in TEER values was observed for the Caco-2 cells grown on monolayers with values of over $400\Omega cm^2$ eighteen days post–seeding. Only monolayers whose TEER values were $\geq 300\Omega cm2$ after growing for a minimum of 21 days were subsequently used for the assay.

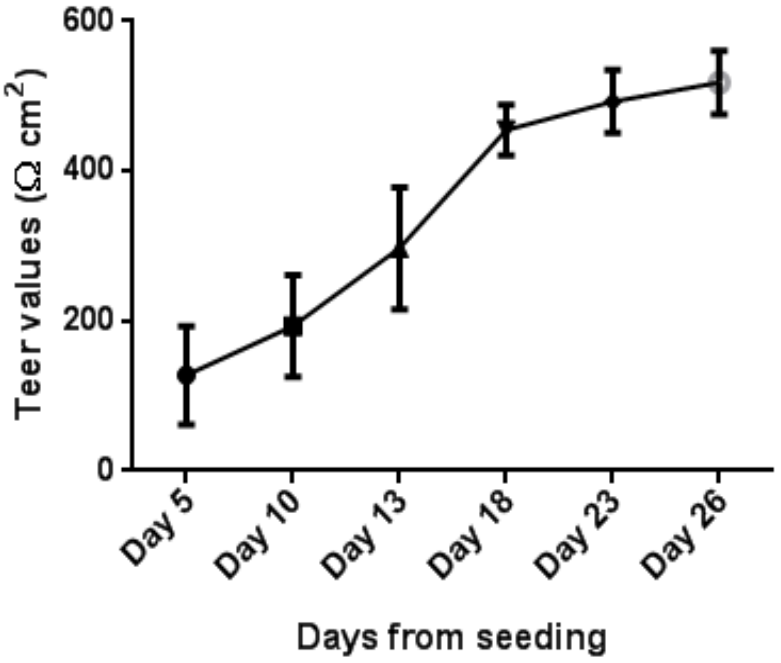


Figure 4.19: TEER values of Caco-2 cells during differentiation when grown in monolayers. (Each point represents the mean ± SD, N ≥ 10 for cells plated out on two different days).

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 (Balimane et al., 2006). This study obtained an average efflux ratio of 2.4 for glibenclamide, thereby confirming it as a P-gp substrate (Table 4.7). This is the first time glibenclamide is experimentally confirmed as a P-gp substrate in a 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 4.7, verapamil the control P-gp inhibitor decreased the *P*app (BL-AP) of glibenclamide from 6.3 x 10^{-5} cm/s to 4.62 x 10^{-5} cm/s, and increased its *P*app (AP-BL) from 2.65 x 10^{-5} cm/s to 4.03 x 10^{-5} cm/s, thereby decreasing its efflux ratio from 2.38 to 1.13, thus confirming its effect in a bi-directional transport model as a P-gp inhibitor.

Table 4-7: Tabular summary of the effects of 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.

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)
Glib only (10µM)	AP-BL	67 ± 17	2.65	2.38	5.62
	BL-AP	63 ± 20	6.3		9.05
Glib (10µM) + VP (20µM)	AP-BL	63 ± 38	4.06	1.13	ND
	BL-AP	64 ± 11	4.62		ND
Glib 10µM + AS1(50µg/ml)	AP-BL	48 ± 13	2.36	2.17	6.2
	BL-AP	23 ± 7	5.13		5.64
Glib (10µM) + BS1 (50µg/ml)	AP-BL	44 ± 13	1.66	2.5	4.9
	BL-AP	17 ± 6	4.16		5.42
Glib (10µM) + CF1 (100µg/ml)	AP-BL	70 ± 28	1.83	1.6	2.93
	BL-AP	49 ± 36	2.93		2.11
Glib (10µM) + DO1 (50µg/ml)	AP-BL	45 ± 31	1.17	4.97	4.4
	BL-AP	23 ± 9	5.82		3.95
Glib (10µM) + KS1 (100µg/ml)	AP-BL	26 ± 9	5.6	1.2	0.77
	BL-AP	18 ± 6	6.66		1.87
Glib (10µM) + SG1 (100µg/ml)	AP-BL	53 ± 23	3.38	1.7	5.8
	BL-AP	20 ± 5	5.76		2.99
Glib (10µM) + TA1 (100µg/ml)	AP-BL	25 ± 17	3.09	1.8	9.67
	BL-AP	19 ± 5	5.56		2.41
Glib (10µM) + XA1 (100µg/ml)	AP-BL	28 ± 13	1.37	1.66	9.33
	BL-AP	25 ± 27	2.27		3.37

ND- Not determined

Contrary to expectation that all the modulated extracts would be identified as P-gp inhibitors since they increased intracellular Rh-123 concentration, only two of the extracts- SG1 and TA1 produced a similar effect as verapamil. They both increased the *P*app (AP-BL) of glibenclamide to 3.38 and 3.06 x 10-5 cm/s respectively and decreased the *P*app (AP-BL) of glibenclamide to 5.7 and 5.56 x 10-5 cm/s respectively. Asides for its possible P-gp inhibitory effect, TA1 also produced a greater effect on membrane permeability with possible opening of tight junctions, evidenced by its increased *P*app (AP-BL) transport of FD4 and a higher change in TEER values compared to glibenclamide alone. This would in turn contribute to the increased *P*app (AP-BL) seen with glibenclamide.

Although it was assumed that an increase in intracellular Rh-123 concentration was possibly due to a P-gp modulatory effect, it has now been shown using the bidirectional transport assay that this may not necessarily be so. An increase in paracellular permeability due to an opening of tight junctions as evidenced by an increase in TEER values and/or increase in *P*app (AP-BL) of a marker for paracellular transport such as FD4 could also result in increased intracellular concentration. This might be a possible explanation for the previously 'assumed' P-gp modulatory effects for some of the extracts which were not confirmed in the bidirectional transport assay.

For instance, although KS1 increased the *P*app (AP-BL) of glibenclamide from 2.65– 5.6 x 10^{-5} cm/s, the *P*app (BL-AP) also increased very slightly from 6.13 x 10^{-5} cm/s to 6.6 x 10^{-5} cm/s; and there was also a decrease in TEER values to approximately 20% of initial value with KS1 as opposed to approximately 60% of initial value with glibenclamide alone. However, these assumptions would need to be clarified using other appropriate assays. 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.

5 GENERAL DISCUSSION

5.1 BACKGROUND

Despite increasing drug production by the pharmaceutical industry, the availability of conventional medicines continues to remain one of the challenges of healthcare provision all over the world. This is primarily because of the grave inequity in medicine access between countries due to cost-related factors, particularly for new medicines wherein the price of the drug is determined by the patent holder (Laing et al., 2003). At the same time, a large percentage of the world's population including those living in urban areas with better access to modern healthcare systems do not consider conventional medicines as the 'sole' solution to their healthcare problems due to their cultural beliefs and values (van Andel and Westers, 2010). In these contexts, the use of traditional systems of medicine in disease management will continue to flourish.

Although there has been increasing advocacy for the integration of traditional medicine practice into modern healthcare systems, there are several identified dilemmas that make this very challenging, one of which is the selection of medicinal plants with the greatest potential as herbal medicines. A number of ethnobotanical surveys previously carried out in different parts of Nigeria have highlighted the availability of a large number of plants for diabetes management in Nigeria. The pharmacological and/or toxicological evidence available for these medicinal plants were however 'scattered' and not readily available to patients, healthcare practitioners and researchers to make a thorough assessment of their beneficial and toxic effects in diabetes management.

A data-mining of a hundred and fifteen plants which met the criteria for either ethnobotanical and/or pharmacological evidence was carried out to provide a useful resource for harnessing the therapeutic benefits of these herbal medicines as a first step towards the possible integration of traditional medicine in the Nigerian healthcare system. This resource is freely available as an open access paper which would be beneficial in Nigeria but also in the wider continent because of the overlap between plant use within and outside Nigeria in the management of various diseases (as shown in Table 1, Appendix 1).

In conventional systems of medicine, the risks of drug-drug interactions (DDIs) have become higher with the practice of poly-pharmacy. A prudent approach which is now routinely adopted in drug development is to obtain sufficient mechanistic knowledge about individual drugs that would in turn enable better prediction of these DDIs and forestall adverse reactions (Delgoda and Westlake, 2004). Similarly, for patients whose preference would be to integrate the use of herbal medicines alongside their conventional treatment, the risk of HDIs is an inevitable feature that should be considered. Just as the awareness of the potential risk of DDIs does not prohibit the practice of poly-pharmacy, the potential risk

HDIs should also not preclude the co-administration of herbal medicines alongside prescription drugs by patients who choose to do so. On the other hand, a thorough assessment of the benefits and limitations of integrating both forms of treatment as it relates to the overall therapeutic goal needs to be considered.

This PhD thesis is therefore a step forward to evaluate potential herb-drug interactions in Nigeria with a focus on diabetic patients, given that accurate prediction of HDIs is best done in the context of co-administered prescription drugs with a view to promoting their integration into the healthcare system. A thorough evaluation of herb-drug interactions in diabetes management in Nigeria will be incomplete if it only sought to identify their potential harmful risks, more so since the same herbal medicine could bring about a positive HDI with one drug and a negative HDI with another. The implications of the results of this PhD research will therefore be discussed both in the context of the beneficial outcomes of the identified HDIs as well as their possible risks.

5.2 CLINICAL IMPLICATIONS OF IDENTIFIED HERB DRUG INTERACTIONS

A number of *in vivo* and/or *in vitro* experimental evidence of the hypoglycaemic effects validating the use of most of the medicinal plants used in diabetes management in Nigeria are available. There are however much fewer reports about clinical studies on these plants which would be an important confirmatory evidence of its clinical benefit in humans.

The clinical study of *Phyllanthus amarus* is a case in point whereby the expected hypoglycaemic effect was not observed in type-2 diabetic patients when extracts of the plant were administered as a substitute to their oral hypoglycaemic agents (Moshi et al., 2001). On the other hand, clinical evidence of improved glycaemic control in patients who co-administered a hypoglycaemic herbal medicine alongside their oral hypoglycaemic drug compared to those on oral hypoglycaemic drugs alone have been observed for *Momordica charanthia* (Leatherdale et al., 1981); and a combination of *Rauvolfia vomitoria* and *Citrus aurantium* (Campbell-Tofte et al., 2011). It is therefore possible that better glycaemic control might also be observed in patients who co-administer *Phyllanthus amarus* with their oral hypoglycaemic agents, but this needs to be clinically evaluated.

These two studies provide some evidence wherein a patient can obtain a beneficial HDI when a herbal medicine is co-administered with conventional drugs. It is important to emphasize that these would not necessarily be the case for all hypoglycaemic herb-drug combinations. On the other hand, while some might result in negative pharmacodynamic HDI due to hypoglycaemia, others may not even produce any improved glycaemia compared

to the intake of oral hypoglycaemic drugs alone. It is on this basis that suggestions have been made in Chapter 2 that a concerted effort should be made to clinically validate the beneficial effects of these herbal medicines with particular focus on those commonly taken by patients as part of their diet. One strategy that can be employed in the design of such studies is to take into consideration the identified mechanism of action as a means of incorporating a possible synergistic effect. By doing this, untoward pharmacodynamic HDI can also be identified, which could be addressed with an appropriate dose adjustment.

The assessment of potential pharmacokinetic herb-drug interactions that may arise from the combined use of herbal medicines and prescription drugs in diabetes management is equally as important as the evaluation of its clinical efficacy. Approximately forty of the plants assessed in Chapter 2 have *in vitro* interactions with known pharmacokinetic parameters, with some interacting with with more than one parameter. *Morinda lucida* in particular has been shown to inhibit 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 (Chapter 3). These brings to immediate attention those plants that would require further evaluation to confirm the presence or not of clinically relevant HDIs.

The results of the field work also provide evidence that the use of more than one prescription drug in the therapeutic management of diabetes either for glycaemic control or the management of other diabetes-related co-morbidities is quite common. There are multiple implications of these- both for the additional burden that this could cause to these patients in the long term particularly in regard to prescription costs; as well as the associated risk of untoward HDIs for patients who co-administer herbal medicines alongside.

This work also highlights the paucity of experimental evidence for potential pharmacokinetic HDIs for herbal medicines used in Nigeria as well as the high prevalence of use of unidentified herbal medicines, another factor that can hamper the identification and/or prediction of clinically relevant HDIs. One of the means undertaken in this PhD research to address this was to evaluate the effect of a number of medicinal plants collected during the field work in a number of *in vitro* assays that would provide information about potential pharmacokinetic interaction and/ or toxicological risks.

The hypothesis of oxidative stress as the pathogenic mechanism linking insulin resistance with dysfunction of both beta cells and endothelium, and eventually leading to clinical diabetes and cardiovascular disease has been gaining credibility during the last decade. It has also been suggested as a possible reason wherein treating cardiovascular risk with drugs

such as calcium channel blockers, ACE inhibitors, AT-1 receptor antagonists, and statins - compounds which show intracellular preventive antioxidant activity- have resulted in a decrease in the onset of new cases of diabetes (Ceriello and Motz, 2004, Robertson et al., 2004).

High glucose levels have been shown to increase nitric oxide (NO) synthesis and superoxide (O_2^-) production through the activation of protein kinase C both *in vitro* and in an *in vivo* experimental diabetes model (Hink et al., 2001). These two radicals (NO and O_2^-) react very quickly to form peroxynitrite resulting in nitrosative stress. The toxic effect of peroxynitrite is dependent on the presence of carbon dioxide (CO_2) which is readily present in physiological conditions. Its rate of reaction with CO_2 is so fast to prevent sufficient scavenging of the molecule itself. Therefore, it has been proposed that scavenging peroxynitrite is not realistic and a more rational approach would be to prevent superoxide and NO reacting in the first place (Squadrito and Pryor, 1998).

It could therefore be proposed that extracts with specific NO (as identified in Table 4.3, such as the stem bark of *Syzygium guineense*) and/or superoxide scavenging effects could mitigate against this toxicological risk and be beneficial antioxidants in hyperglycaemic conditions. It is important to note that the benefits of nitric oxide scavengers would only be in conditions of oxidative stress whereby they readily react with superoxide to form peroxynitrite. This is because NO also known as endothelium derived relaxing factor (EDRF)- is an important biological messenger necessary for maintaining the integrity of blood vessels by vasodilatation (Hink et al., 2001). Thus NO scavenging in non-diabetic conditions may be counterproductive. Further work would also look at the ability of these plant extracts to scavenge superoxide as this has been proposed as a more suitable way to prevent peroxynitrite formation (Squadrito and Pryor, 1998).

In addition, GSH in concert with GSH-dependent enzymes have been identified as one of the body's mechanism of ensuring a regulated defence against oxidative stress (Hayes and McLellan, 1999). In this regard, although none of our extracts produced significant increase in intracellular GSH levels, the intake of those which decreased intracellular GSH levels should be discouraged (Table 4.5). Extracts of *Khaya ivorensis* (KS1 and KS3) is a case in point, which although identified as good NO scavengers also depleted intracellular GSH levels and would not be recommended as being beneficial for oxidative stress.

Thus, the true benefit of extracts with good NO and/or O_2^- scavenging effects in oxidative stress conditions in diabetic patients would need to be evaluated in the context of their effects on other physiologic antioxidant defence mechanisms. By doing this, the possible role of the co-administration of these herbal medicines not just to enhance glycaemic control

in patients but also to limit the incidence of these vascular related complications can be assessed. This is particularly important in Nigeria since studies have shown that the high mortality amongst diabetic patients is largely due to poor blood glucose control and the associated vascular related complications (Chijioke et al., 2010).

Furthermore, the potential pharmacokinetic interaction of the collected medicinal plants on the efflux activity of P-glycoprotein; as well as a further evaluation of their effects on intestinal glibenclamide transport, a known P-gp substrate and the second most prescribed anti-diabetic drug in Nigeria was undertaken. Nine extracts were originally identified as possible P-gp modulators based on the intracellular Rh-123 accumulation assay (Table 4.6, Figure 4.18). However, further evaluation in a bi-directional transport model across Caco-2 monolayers indicates that only two of these- *Syzygium guineense* (SG1) and *Terminalia avicennioides* (TA1) could actually be modulating P-gp activity in an inhibitory capacity similar to verapamil.

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 could 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 *P*app of glibenclamide alongside other P-gp substrates with higher efflux ratios would therefore need to be carried out to ensure a thorough assessment.

Finally, although the other extracts did not exhibit the expected characteristics of P-gp modulators in the bi-directional transport, they could still modulate intestinal drug transport through their effects on tight junctions as monitored by TEER values and paracellular permeability of FD4. *Ximenia americana* (XA1) is a case in point which further decreased TEER to less than 30% of its initial value as well as increased the apparent permeability of the paracellular marker FD4 (Table 4.7). Further experiments would be needed to confirm this especially as decrease in TEER values have been identified as a cytotoxic mechanism that could potentially affect intestinal drug absorption (Narai et al., 1997).

5.3 THE WAY FORWARD

Given that only a small percentage of *in vitro* identified pharmacokinetic HDIs have so far produced a significant clinical effect, the evaluation carried out in this thesis is meant to serve as a guide for active therapeutic monitoring by healthcare practitioners in order to obtain clinical evidence of these HDIs when and if they occur. For this to be achieved, the extension of the pharmacovigilance monitoring system in Nigeria to herbal medicines, with the co-operation of both healthcare practitioners and traditional medicine practitioners (TMPs) is warranted (Shaw et al., 2012).

A very detailed study investigating views about the safety of herbal medicines and hence the need for pharmacovigilance monitoring for herbal medicines amongst TMPs and those involved in the sale of herbal medicinal products in different parts of Lagos state was carried out. Unfortunately, almost 90% of the respondents (including Pharmacists) considered herbal medicines to be safe and without side effects (Awodele et al., 2013). This isn't very surprising given that the highest percentage of herbal medicines used in Nigeria is preparations by the herbalists themselves and TMPs would be unlikely to attribute any harmful effects to their preparations.

On the other hand, studies have shown that the increase in popularity or interest in alternative/herbal medicine for the prevention and treatment of various illnesses has also brought some concerns and fears over the professionalism of practitioners, as well as on the quality, efficacy and safety of the 'natural' formulations available in the market with reports of adulteration. There is therefore a need to not only promote the awareness but also improve channels of communication for pharmacovigilance reporting especially since the knowledge and practice of pharmacovigilance monitoring in Nigeria amongst healthcare providers in general is below average (Osakwe et al., 2013). Community pharmacists in particular can play a very useful role in this regard given that they are usually patients' first point of contact as a cost- and time-saving alternative to visiting hospitals.

Finally, there is an urgent need for better regulation of herbal medicines beyond the standards that are currently in practice in Nigeria. This would entail increased registration of herbal medicinal products as well as the regulation of the TMPs themselves especially with regards to their prepared formulations and the various channels of distribution. The verification of identified clinical evidence of HDIs can only be done alongside a qualitative and quantitative analysis of the phytochemical content of the herbal medicine in question. Thus, these aforementioned improved regulation steps would help to enhance the quality and safety of herbal medicines in circulation.

6 CONCLUSION

It is well known that a large number of medicinal plants are available and are also being utilised as herbal medicines in Nigeria for diabetes management. This PhD research is however the first time that a data mining of all the experimental evidence available for these herbal medicines is being carried out to highlight not only their pharmacological benefits, but also their toxicological effects. This has been done to provide a useful resource for harnessing the therapeutic benefits of these herbal medicines as a first step towards the possible integration of traditional medicine in the Nigerian healthcare system.

Secondly, this is the first time a field work study has been carried out in Nigeria specifically aimed at assessing the risks of pharmacokinetic herb-drug interactions amongst diabetic patients in Nigeria. By doing this, herbal medicines and prescription drugs with overlapping pharmacokinetic effects which are currently being co-administered by patients have been identified. Further monitoring would therefore be required to confirm the presence or not of clinically relevant HDIs.

Thirdly, this work has also highlighted the paucity of experimental evidence for predicting potential pharmacokinetic herb-drug interactions in Nigeria; and to address this has evaluated for the first time the possible interaction of a select number of these medicinal plants on the efflux activity of P-glycoprotein with further evaluation of its effects on glibenclamide, a P-gp substrate commonly administered to diabetic patients. A lot more work is still required to fully evaluate medicinal plants used in Nigeria for potential risks of pharmacokinetic HDIs. Focus should however be given to those most commonly used by the populace.

7 REFERENCES

ABO, K. A., FRED-JAIYESIMI, A. A. & JAIYESIMI, A. E. A. 2008. Ethnobotanical studies of medicinal plants used in the management of diabetes mellitus in South Western Nigeria. *Journal of Ethnopharmacology,* 115**,** 67-71.

ADA, A. D. A. 2011. Standards of Medical Care in Diabetes—2011. *Diabetes Care* 34**,** S11-S61.

ADEBAJO, A. C., OLAYIWOLA, G., VERSPOHL, J. E., IWALEWA, E. O., OMISORE, N. O. A., BERGENTHAL, D., KUMAR, V. & ADESINA, S. K. 2005. Evaluation of the Ethnomedical Claims of *Murraya koenigii*. *Pharmaceutical Biology,* 42**,** 610-620.

ADESIDA, G. A., ADESOGAN, E. K., OKORIE, D. A., TAYLOR, D. A. H. & STYLES, B. T. 1971. The limonoid chemistry of the genus *Khaya* (Meliaceae). *Phytochemistry,* 10**,** 1845-1853.

ADISA, R., ALUTUNDU, M. B. & FAKEYE, T. O. 2009. Factors contributing to nonadherence to oral hypoglycemic medications among ambulatory type 2 diabetes patients in Southwestern Nigeria. *Pharmacy Practice,* 7**,** 163-169.

AGBONON, A., EKLU-GADEGBEKU, K., AKLIKOKOU, K., GBEASSOR, M., AKPAGANA, K., TAM, T. W., ARNASON, J. T. & FOSTER, B. C. 2010. *In vitro* inhibitory effect of West African medicinal and food plants on human cytochrome P450 3A subfamily. *Journal of Ethnopharmacology,* 128**,** 390-394.

AIYELOJA, A. A. & BELLO, O. A. 2006. Ethnobotanical potentials of common herbs in Nigeria: A case study of Enugu State. *Educational Research and Review,* 1**,** 16-22.

AJIBADE, A. J., FAKUNLE, P. B., EHIGIE, L. O. & AKINRINMADE, A. O. 2012. Sub-chronic hepatotoxicity in adult wistar rats following administration of *Ocimum gratissimum* aqueous extract. *European Journal of Medicinal Plants,* 2**,** 19-30.

AJIBESIN, K. K., EKPO, B. A., BALA, D. N., ESSIEN, E. E. & ADESANYA, S. A. 2008. Ethnobotanical survey of Akwa Ibom State of Nigeria. *Journal of Ethnopharmacology,* 115**,** 387-408.

AKIYAMA, S., KATSUMATA, S.-I., SUZUKI, K., NAKAYA, Y., ISHIMI, Y. & UEHARA, M. 2009. Hypoglycemic and hypolipidemic effects of hesperidin and cyclodextrin-clathrated hesperetin in goto-kakizaki rats with type 2 diabetes. *Bioscience, Biotechnology, and Biochemistry,* 73**,** 2779-2782.

ALLEN, S., SHEA, J. M., FELMET, T., GADRA, J. & DEHN, P. F. 2000. A kinetic microassay for glutathione in cells plated on 96-well microtiter plates. *Methods in Cell Science,* 22**,** 305-312.

ALVES, R. R. & ROSA, I. M. 2007. Biodiversity, traditional medicine and public health: where do they meet? *Journal of Ethnobiology and Ethnomedicine* 3.

AMAGHIONYEODIWE, L. A. 2008. Determinants of the choice of health care provider in Nigeria. *Health Care Management Science,* 11**,** 215-227.

AMPASAVATE, C., SOTANAPHUN, U., PHATTANAWASIN, P. & PIYAPOLRUNGROJ, N. 2010. Effects of *Curcuma* spp. on P-glycoprotein function. *Phytomedicine,* 17**,** 506-512.

ANDRADE-CETTO, A. & HEINRICH, M. 2005. Mexican plants with hypoglycaemic effect used in the treatment of diabetes. *Journal of Ethnopharmacology,* 99**,** 325-348.

APPIAH-OPONG, R., COMMANDEUR, J. N. M., AXSON, C. & VERMEULEN, N. P. E. 2008. Interactions between cytochromes P450, glutathione S-transferases and Ghanaian medicinal plants. *Food and Chemical Toxicology,* 46**,** 3598-3603.

ARGAEZ-LOPEZ, N., WACHER, N. H., KUMATE-RODRIGUEZ, J., CRUZ, M., TALAVERA, J., RIVERA-ARCE, E. & LOZOYA, X. 2003. The use of complementary and alternative medicine therapies in type 2 diabetic patients in Mexico. *Diabetes Care* 26**,** 2470-2471.

ASLAM, M. & STOCKLEY, I. H. 1979. Interaction Between Curry Ingredient (Karela) And Drug (Chlorpropamide). *The Lancet,* 313**,** 607.

ASTIN, J. A. 1998. Why patients use alternative medicine: Results of a national study. *JAMA,* 279**,** 1548-1553.

AWODELE, O., DANIEL, A., POPOOLA, T. D. & SALAMI, E. F. 2013. A study on pharmacovigilance of herbal medicines in Lagos West Senatorial District, Nigeria. *The International Journal of Risk and Safety in Medicine,* 25**,** 205-217.

BAILEY, C. J. & DAY, C. 1989. Traditional plant medicines as treatments for diabetes. *Diabetes Care,* 12**,** 553-564.

BAILEY, C. J. & DAY, C. 2004. Metformin: its botanical background. *Practical Diabetes International,* 21**,** 115-117.

BALIMANE, P., HAN, Y.-H. & CHONG, S. 2006. Current industrial practices of assessing permeability and P-glycoprotein interaction. *The AAPS Journal,* 8**,** E1-E13.

BANNERMAN, R. H., BURTON, J. & WEN-CHIEH, C. E. 1983. *Traditional medicine and healthcare coverage: A reader for health administrators and practitioners,* Geneva, Switzerland, World Health Organization.

BEH, J. E., LATIP, J., ABDULLAH, M. P., ISMAIL, A. & HAMID, M. 2010. *Scoparia dulcis* (SDF7) endowed with glucose uptake properties on L6 myotubes compared insulin. *Journal of Ethnopharmacology,* 129**,** 23-33.

BELLION, P., OLK, M., WILL, F., DIETRICH, H., BAUM, M., EISENBRAND, G. & JANZOWSKI, C. 2009. Formation of hydrogen peroxide in cell culture media by apple polyphenols and its effect on antioxidant biomarkers in the colon cell line HT-29. *Molecular Nutrition & Food Research,* 53**,** 1226-1236.

BERAN, D. & YUDKIN, J. S. 2006. Diabetes care in sub-Saharan Africa. *The Lancet,* 368**,** 1689-1695.

BEVER, B. O. 1980. Oral hypoglycaemic plants in West Africa. *Journal of Ethnopharmacology,* 2**,** 119-127.

BHARTI, S. K., KUMAR, A., PRAKASH, O., KRISHNAN, S. & GUPTA, A. K. 2013. Essential oil of *cymbopogon citratus* against diabetes: validation by *in vivo* experiments and computational studies. *Journal of Bioanalysis & Biomedicine,* 5**,** 194-203.

BODEKER, G. & KRONENBERG, F. 2002. A public health agenda for traditional, complementary, and alternative medicine. *American Journal of Public Health,* 92**,** 1582-1591.

BRANTLEY, S. J., ARGIKAR, A. A., LIN, Y. S., NAGAR, S. & PAINE, M. F. 2014. Herb–drug interactions: challenges and opportunities for improved predictions. *Drug Metabolism and Disposition,* 42**,** 301-317.

BRUNO, J. J. & ELLIS, J. J. 2005. Herbal use among us elderly: 2002 national health interview survey. *Annals of Pharmacotherapy,* 39**,** 643-648.

BUTTERWECK, V., DERENDORF, H., GAUS, W., NAHRSTEDT, A., SCHULZ, V. & UNGER, M. 2004. Pharmacokinetic herb-drug interactions: are preventive screenings necessary and appropriate? *Planta Medica,* 70**,** 784-791.

CAMPBELL-TOFTE, J. I. A., MØLGAARD, P., JOSEFSEN, K., ABDALLAH, Z., HANSEN, S. H., CORNETT, C., MU, H., RICHTER, E. A., PETERSEN, H. W., NØRREGAARD, J. C. & WINTHER, K. 2011. Randomized and double-blinded pilot clinical study of the safety and anti-diabetic efficacy of the Rauvolfia-Citrus tea, as used in Nigerian Traditional Medicine. *Journal of Ethnopharmacology,* 133**,** 402-411.

CERIELLO, A. & MOTZ, E. 2004. Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 24**,** 816-823.

CHANG, H.-Y., WALLIS, M. & TIRALONGO, E. 2007. Use of complementary and alternative medicine among people living with diabetes: literature review. *Journal of Advanced Nursing,* 58**,** 307-319.

CHANG, S.-L., CHANG, C. L.-T., CHIANG, Y.-M., HSIEH, R.-H., TZENG, C.-R., WU, T.-K., SYTWU, H.-K., SHYUR, L.-F. & YANG, W.-C. 2004. Polyacetylenic compounds and butanol fraction from *bidens pilosa* can modulate the differentiation of helper t cells and prevent autoimmune diabetes in non-obese diabetic mice. *Planta Medica* 70**,** 1045-1051.

CHEHADE, J. & MOORADIAN, A. 2000. A rational approach to drug therapy of type 2 diabetes mellitus. *Drugs,* 60**,** 95-113.

CHEN, K. K., ANDERSON, R. C., MCCOWEN, M. C. & HARRIS, P. N. 1957. Pharmacologic action of Hypoglycin A and B. *Journal of Pharmacology and Experimental Therapeutics,* 121**,** 272-285.

CHIELI, E. & ROMITI, N. 2008. Kidney proximal human tubule HK-2 cell line as a tool for the investigation of Pglycoprotein modulation by natural compounds. *Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas,* 7**,** 282-295.

CHIELI, E., ROMITI, N., RODEIRO, I. & GARRIDO, G. 2009. In vitro effects of *Mangifera indica* and polyphenols derived on ABCB1/P-glycoprotein activity. *Food and Chemical Toxicology,* 47**,** 2703-2710.

CHIJIOKE, A., ADAMU, A. N. & MAKUSIDI, A. M. 2010. Mortality pattern among type 2 diabetes patients in Ilorin, Nigeria. *Journal of Endocrinology, Metabolism and Diabetes of South Africa,* 15**,** 1-4.

CLARKE, A. T. & MILLS, P. R. 2006. Atorvastatin associated liver disease. *Digestive and Liver Disease,* 38**,** 772-777.

COLALTO, C. 2010. Herbal interactions on absorption of drugs: Mechanisms of action and clinical risk assessment. *Pharmacological Research,* 62**,** 207-227.

CORDIER, W. & STEENKAMP, V. 2011. Drug interactions in African herbal remedies. *Drug Metabolism and Drug Interactions,* 26**,** 53-63.

COSKUN, O., KANTER, M., KORKMAZ, A. & OTER, S. 2005. Quercetin, a flavonoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and β-cell damage in rat pancreas. *Pharmacological Research,* 51**,** 117-123.

COX, P. & ROCHE, D. 2004. Directive 2004/24/EC of the European parliament and of the council amending, as regards traditional herbal medicinal products, Directive 2001/83/EC on the Community code relating to medicinal products for human use. *In:* UNION, T. E. P. A. T. C. O. T. E. (ed.).

DAISY, P., BALASUBRAMANIAN, K., RAJALAKSHMI, M., ELIZA, J. & SELVARAJ, J. 2010. Insulin mimetic impact of Catechin isolated from *Cassia fistula* on the glucose oxidation and molecular mechanisms of glucose uptake on Streptozotocin-induced diabetic Wistar rats. *Phytomedicine,* 17**,** 28-36.

DE LIMA TOCCAFONDO VIEIRA, M. & HUANG, S.-M. 2012. Botanical-drug interactions: a scientific perspective. *Planta Medica,* 78**,** 1400-1415.

DEFERME, S., KAMUHABWA, A., NSHIMO, C., DE WITTE, P. & AUGUSTIJNS, P. 2003. Screening of Tanzanian plant extracts for their potential inhibitory effect on P-glycoprotein mediated efflux. *Phytotherapy Research,* 17**,** 459-464.

DELGODA, R. & WESTLAKE, A. G. 2004. Herbal Interactions Involving Cytochrome P450 Enzymes. *Toxicological Reviews,* 23**,** 239-249.

DINESH KUMAR, B., KRISHNAKUMAR, K., KUMAR, J. S. & MANDAL, M. 2013. Effect of mangiferin and mahanimbine on glucose utilization in 3T3-L1 cells. *Pharmacognosy Magazine,* 9**,** 72-75.

DINESHKUMAR, B., MITRA, A. & MAHADEVAPPA, M. 2010. Antidiabetic and hypolipidemic effects of mahanimbine (carbazole alkaloid) from *Murraya koenigii* (Rutaceae) leaves. *International Journal of Phytomedicine,* 2**,** 22-30.

DREW, R. & MINERS, J. O. 1984. The effects of buthionine sulphoximine (BSO) on glutathione depletion and xenobiotic biotransformation. *Biochemical Pharmacology,* 33**,** 2989-2994.

EATON, D. L. & BAMMLER, T. K. 1999. Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicological Sciences,* 49**,** 156-164.

EDWARDS, S., DA-COSTA-ROCHA, I., LAWRENCE, M. J., CABLE, C. & HEINRICH, M. 2012. A reappraisal of herbal medicinal products. *Nursing times,* 108**,** 24-7.

EJIKE, C. E. C. C., AWAZIE, S. O., NWANGOZI, P. A. & GODWIN, C. D. 2013. Synergistic postprandial blood glucose modulatory properties of *Vernonia amygdalina* (Del.), *Gongronema latifolium* (Benth.) and *Occimum gratissimum* (Linn.) aqueous decoctions. *Journal of Ethnopharmacology,* 149**,** 111-116.

EKONG, D. E. U. & IDEMUDIA, O. G. 1967. Constituents of some West African members of the genus *Terminalia*. *Journal of the Chemical Society (C)***,** 863-864.

ENEROTH, A., E., A., HOOGSTRAATE, J., SCHRENK, D., CONRAD, S., KAUFFMANN, H.-M. & GJELLAN , K. 2001. Evaluation of a vincristine resistant Caco-2 cell line for use in a calcein AM extrusion screening assay for P-glycoprotein interaction. *European Journal of Pharmaceutical Sciences,* 12**,** 205-214.

ENWERE, O. O., SALAKO, B. L. & FALADE, C. O. 2006. Prescription and Cost Consideration at a Diabetic Clinic in Ibadan, Nigeria: A Report. *Annals of Ibadan Postgraduate Medicine,* 4**,** 35-39.

ETOUNDI, C. B., KUATÉ, D., NGONDI, J. L. & OBEN, J. 2010. Anti-amylase, anti-lipase and antioxidant effects of aqueous extracts of some Cameroonian spices. *Journal of Natural Products,* 3**,** 165-171.

ETUK, E. U. & MOHAMMED, B. J. 2009. Informant consensus selection method: A reliability assessment on medicinal plants used in north western Nigeria for the treatment of diabetes mellitus. *African Journal of Pharmacy and Pharmacology,* 3**,** 496-500.

EVANS, D. B. & ETIENNE, C. 2010. Health systems financing and the path to universal coverage. *Bulletin of the World Health Organization,* 88**,** 402-403.

EZURUIKE, U. F. & PRIETO, J. M. 2014. The use of plants in the traditional management of diabetes in Nigeria: Pharmacological and toxicological considerations. *Journal of Ethnopharmacology,* 155**,** 857-924.

FAHEY, R. C. & SUNDQUIST, A. R. 2006. Evolution of glutathione metabolism. *Advances in Enzymology and Related Areas of Molecular Biology.* John Wiley & Sons, Inc.

FAKEYE, T. O., ADISA, R. & MUSA, I. E. 2009. Attitude and use of herbal medicines among pregnant women in Nigeria. *BMC Complementary and Alternative Medicine,* 53.

FAKEYE, T. O., AWE, S. O., ODELOLA, H. A., OLA-DAVIES, O. E., ITIOLA, O. A. & OBAJULUWA, T. 2004. Evaluation of toxicity profile of an alkaloidal fraction of the stem bark of *Picralima nitida* (Fam. Apocynaceae). *Journal of Herbal Pharmacotherapy,* 4**,** 37-45.

FAKEYE, T. O., OLADIPUPO, T., SHOWANDE, O. & OGUNREMI, Y. 2007. Effects of coadministration of extract of *Carica papaya* linn (family cariaceae) on activity of two oral hypoglycemic agents. *Tropical Journal of Pharmaceutical Research,* 6**,** 671-678.

FAKEYE, T. O., TIJANI, A. & ADEBISI, O. 2008. A Survey of the Use of Herbs Among Patients Attending Secondary-Level Health Care Facilities in Southwestern Nigeria. *Journal of Herbal Pharmacotherapy,* 7**,** 213-227.

FASAKIN, C. F., UDENIGWE, C. C. & ALUKO, R. E. 2011. Antioxidant properties of chlorophyll-enriched and chlorophyll-depleted polyphenolic fractions from leaves of *Vernonia amygdalina* and *Gongronema latifolium*. *Food Research International,* 44**,** 2435-2441.

FÖRSTERMANN, U. 2010. Nitric oxide and oxidative stress in vascular disease. *Pflugers Archiv European Journal of Physiology,* 459**,** 923-939.

FOSTER, B. C., FOSTER, M. S., VANDENHOEK, S., KRANTIS, A., BUDZINSKI, J. W., ARNASON, J. T., GALLICANO, K. D. & CHOUDRI, S. 2001. An *in vitro* evaluation of human cytochrome P450 3A4 and P-glycoprotein inhibition by garlic. *Journal of Pharmacy & Pharmaceutical sciences,* 4**,** 176-84.

FOTAKIS, G. & TIMBRELL, J. A. 2006. *In vitro* cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters,* 160**,** 171-177.

FRÖDE, T. S. & MEDEIROS, Y. S. 2008. Animal models to test drugs with potential antidiabetic activity. *Journal of Ethnopharmacology,* 115**,** 173-183.

FUGH-BERMAN, A. & ERNST, E. 2001. Herb–drug interactions: Review and assessment of report reliability. *British Journal of Clinical Pharmacology,* 52**,** 587-595.

GALATI, G. & O'BRIEN, P. J. 2004. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radical Biology and Medicine,* 37**,** 287-303.

GALLICANO, K., FOSTER, B. & CHOUDHRI, S. 2003. Effect of short-term administration of garlic supplements on single-dose ritonavir pharmacokinetics in healthy volunteers. *British Journal of Clinical Pharmacology,* 55**,** 199-202.

GANDHI, M., LAL, R., SANKARANARAYANAN, A., BANERJEE, C. K. & SHARMA, P. L. 1988. Acute toxicity study of the oil from *Azadirachta indica* seed (neem oil). *Journal of Ethnopharmacology,* 23**,** 39-51.

GARROW, D. & EGEDE, L. E. 2006. Association between complementary and alternative medicine use, preventive care practices, and use of conventional medical services among adults with diabetes. *Diabetes Care,* 29**,** 15-19.

GBOLADE, A. A. 2009. Inventory of antidiabetic plants in selected districts of Lagos State, Nigeria. *Journal of Ethnopharmacology,* 121**,** 135-139.

GEDEON, C., BEHRAVAN, J., KOREN, G. & PIQUETTE-MILLER, M. 2006. Transport of glyburide by placental ABC transporters: Implications in fetal drug exposure. *Placenta,* 27**,** 1096-1102.

GIACCO, F. & BROWNLEE, M. 2010. Oxidative stress and diabetic complications. *Circulation Research,* 107**,** 1058-1070.

GIACOMINI, K. M., HUANG, S.-M., TWEEDIE, D. J., BENET, L. Z., BROUWER, K. L. R., CHU, X., DAHLIN, A., EVERS, R., FISCHER, V., HILLGREN, K. M., HOFFMASTER, K. A., ISHIKAWA, T., KEPPLER, D., KIM, R. B., LEE, C. A., NIEMI, M., POLLI, J. W., SUGIYAMA, Y., SWAAN, P. W., WARE, J. A., WRIGHT, S. H., YEE, S. W., ZAMEK-GLISZCZYNSKI, M. J. & ZHANG, L. 2010. Membrane transporters in drug development. *Nature Reviews Drug Discovery,* 9**,** 215-236.

GILL, G. V., MBANYA, J. C., RAMAIYA, K. L. & TESFAYE, S. 2009. A sub-Saharan African perspective of diabetes. *Diabetologia,* 52**,** 8-16.

GINER, R. M., RECIO, M. C., CUELLAR, M. J., MÁÑEZ, S., PERIS, MATEU, I. & RÍOS, J.-L. 1993. A taxonomical study of the subtribe leontodontinae based on the distribution of phenolic compounds. *Biochemical Systematics and Ecology,* 21**,** 613-616.

GOLDMAN, P. 2001. Herbal Medicines Today and the Roots of Modern Pharmacology. *Annals of Internal Medicine,* 135**,** 594-600.

GOLSTEIN, P. E., BOOM, A., VAN GEFFEL, J., JACOBS, P., MASEREEL, B. & BEAUWENS, R. 1999. P-glycoprotein inhibition by glibenclamide and related compounds. *Pflügers Archiv,* 437**,** 652-660.

GOODARZI, M. O. & BRYER-ASH, M. 2005. Metformin revisited: re-evaluation of its properties and role in the pharmacopoeia of modern antidiabetic agents. *Diabetes, Obesity and Metabolism,* 7**,** 654-665.

GROVER, J. K., YADAV, S. & VATS, V. 2002. Medicinal plants of India with anti-diabetic potential. *Journal of Ethnopharmacology,* 81**,** 81-100.

GUARIGUATA, L., WHITING, D. R., HAMBLETON, I., BEAGLEY, J., LINNENKAMP, U. & SHAW, J. E. 2014. Global estimates of diabetes prevalence for 2013 and projections for 2035. *Diabetes Research and Clinical Practice,* 103**,** 137-149.

GUASCH, L., SALA, E., OJEDA, M. J., VALLS, C., BLADÉ, C., MULERO, M., BLAY, M., ARDÉVOL, A., GARCIA-VALLVÉ, S. & PUJADAS, G. 2012. Identification

of novel human dipeptidyl peptidase-iv inhibitors of natural origin (part ii): *In silico* prediction in antidiabetic extracts. *PLOS ONE,* 7**,** e44972.

GURLEY, B. J. 2012. Pharmacokinetic herb-drug interactions (part 1): origins, mechanisms, and the impact of botanical dietary supplements. *Planta Medica,* 78**,** 1478-1489.

GURLEY, B. J., FIFER, E. K. & GARDNER, Z. 2012. Pharmacokinetic Herb-Drug Interactions (Part 2): Drug Interactions Involving Popular Botanical Dietary Supplements and Their Clinical Relevance. *Planta Medica,* 78**,** 1490-1514.

HALLIWELL, B. 2003. Oxidative stress in cell culture: an under-appreciated problem? *FEBS Letters,* 540**,** 3-6.

HAMILTON, G. & BASKETT, T. 2000. In the arms of morpheus: the development of morphine for postoperative pain relief. *Canadian Journal of Anesthesia,* 47**,** 367-374.

HARTMANN, C., FRÖLICH, M., KRAUß, D., SPAHN-LANGGUTH, H., KNAUF, H. & MUTSCHLER, E. 1990. Comparative enantioselective pharmacokinetic studies of celiprolol in healthy volunteers and patients with impaired renal function. *European Journal of Clinical Pharmacology,* 39**,** 573-576.

HASAN, S. S., AHMED, S. I., BUKHARI, N. I. & LOON, W. C. W. 2009. Use of complementary and alternative medicine among patients with chronic diseases at outpatient clinics. *Complementary Therapies in Clinical Practice,* 15**,** 152-157.

HATONO, S., JIMENEZ, A. & WARGOVICH, M. J. 1996. Chemopreventive effect of S-allylcysteine and its relationship to the detoxification enzyme glutathione S-transferase. *Carcinogenesis,* 17**,** 1041-1044.

HAYES, J. D. & MCLELLAN, L. I. 1999. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radical Research,* 31**,** 273-300.

HAYESHI, R., MUKANGANYAMA, S., HAZRA, B., ABEGAZ, B. & HASLER, J. 2004. The interaction of selected natural products with human recombinant glutathione transferases. *Phytotherapy Research,* 18**,** 877-883.

HAYESHI, R., MUTINGWENDE, I., MAVENGERE, W., MASIYANISE, V. & MUKANGANYAMA, S. 2007. The inhibition of human glutathione S-transferases activity by plant polyphenolic compounds ellagic acid and curcumin. *Food and Chemical Toxicology,* 45**,** 286-295.

HE, S. M., G. LI, C., LIU, J. P., CHAN, E., DUAN, W. & ZHOU, S. F. 2010. Disposition pathways and pharmacokinetics of herbal medicines in humans. *Current Medicinal Chemistry,* 17**,** 4072-4113.

HE, Y.-L., SABO, R., SUNKARA, G., BIZOT, M.-N., RIVIERE, G.-J., LEON, S., LIGUEROS-SAYLAN, M., DOLE, W. P. & HOWARD, D. 2007. Evaluation of Pharmacokinetic Interactions Between Vildagliptin and Digoxin in Healthy Volunteers. *The Journal of Clinical Pharmacology,* 47**,** 998-1004.

HEINRICH, M., CHAN, J., WANKE, S., NEINHUIS, C. & SIMMONDS, M. S. J. 2009. Local uses of *Aristolochia* species and content of nephrotoxic aristolochic acid 1 and 2—A global assessment based on bibliographic sources. *Journal of Ethnopharmacology,* 125**,** 108-144.

HENNEBELLE, T., WENIGER, B., JOSEPH, H., SAHPAZ, S. & BAILLEUL, F. 2009. *Senna alata*. *Fitoterapia,* 80**,** 385-393.

HERMANN, R. & VON RICHTER, O. 2012. Clinical Evidence of Herbal Drugs As Perpetrators of Pharmacokinetic Drug Interactions. *Planta Medica,* 78**,** 1458-1477.

HILGERS, A., CONRADI, R. & BURTON, P. 1990. Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa. *Pharmaceutical Research,* 7**,** 902-910.

HINK, U., LI, H., MOLLNAU, H., OELZE, M., MATHEIS, E., HARTMANN, M., SKATCHKOV, M., THAISS, F., STAHL, R. A. K., WARNHOLTZ, A., MEINERTZ, T., GRIENDLING, K., HARRISON, D. G., FORSTERMANN, U. & MUNZEL, T. 2001. Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circulation Research,* 88**,** e14-e22.

HO, R. H. & KIM, R. B. 2005. Transporters and drug therapy: Implications for drug disposition and disease. *Clinical Pharmacology & Therapeutics,* 78**,** 260-277.

HODEK, P., TREFIL, P. & STIBOROVÁ, M. 2002. Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. *Chemico-Biological Interactions,* 139**,** 1-21.

HOLSTEIN, A., PLASCHKE, A., PTAK, M., EGBERTS, E. H., EL-DIN, J., BROCKMÖLLER, J. & KIRCHHEINER, J. 2005. Association between CYP2C9 slow metabolizer genotypes and severe hypoglycaemia on medication with sulphonylurea hypoglycaemic agents. *British Journal of Clinical Pharmacology,* 60**,** 103-106.

HOU, X.-L., TAKAHASHI, K., TANAKA, K., TOUGOU, K., QIU, F., KOMATSU, K., TAKAHASHI, K. & AZUMA, J. 2008. Curcuma drugs and curcumin regulate the expression and function of P-gp in Caco-2 cells in completely opposite ways. *International Journal of Pharmaceutics,* 358**,** 224-229.

HOUGHTON, P. J. 1995. The Role of Plants in Traditional Medicine and Current Therapy. *The Journal of Alternative and Complementary Medicine,* 1**,** 131-143.

HOUGHTON, P. J., HOWES, M. J., LEE, C. C. & STEVENTON, G. 2007. Uses and abuses of in vitro tests in ethnopharmacology: Visualizing an elephant. *Journal of Ethnopharmacology,* 110**,** 391-400.

HU, Z., YANG, X., HO, P. C. L., CHAN, S. Y., HENG, P. W. S., CHAN, E., KOH, H. L. & ZHOU, S. 2005. Herb-Drug Interactions. *Drugs,* 65**,** 1239-1282.

HUBATSCH, I., RAGNARSSON, E. G. E. & ARTURSSON, P. 2007. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nature Protocols,* 2**,** 2111-2119.

IBRAHIM, J. A., AYODELE, E. A., JEGEDE, A. I. & KUNLE, Y. F. 2006. Comparative studies on Khaya. A. Juss (Meliaceae) in Nigeria. *African Journal of Biotechnology* 5**,** 1154-1160.

IGILE, G. O., OLESZEK, W., JURZYSTA, M., BURDA, S., FAFUNSO, M. & FASANMADE, A. A. 1994. Flavonoids from *Vernonia amygdalina* and their antioxidant activities. *Journal of Agricultural and Food Chemistry,* 42**,** 2445-2448.

IGOLI, J. O., IGWUE, I. C. & IGOLI, N. P. 2004. Traditional medicinal practices among the Igede people of Nigeria. *Journal of Herbs, Spices & Medicinal Plants,* 10**,** 1-10.

IGOLI, J. O., OGAJI, O. G., TOR-ANYIIN, T. A. & IGOLI, N. P. 2005. Traditional medicine practice amongst the Igede people of Nigeria Part II *African Journal of Traditional, Complementary and Alternative Medicines* 2**,** 134 - 152.

INTERAGENCY COORDINATING COMMITTEE ON THE VALIDATION OF ALTERNATIVE METHODS (ICCVAM) 2001. Guidance document on using *in vitro* data to estimate *in vivo* starting doses for acute toxicity NIH Publication No. 01-4500. *In:* SCIENCES, N. I. O. E. H. (ed.). Research triangle park, North Carolina.

IOANNIDES, C. 2002. Pharmacokinetic interactions between herbal remedies and medicinal drugs. *Xenobiotica,* 32**,** 451-478.

IWU, M. M. 1983. The hypoglycaemic properties of *Bridelia ferruginea*. *Fitoterapia,* 54**,** 243-248.

IWU, M. M., IGBOKO, O. A., OKUNJI, C. O. & TEMPESTA, M. S. 1990. Antidiabetic and aldose reductase activities of biflavanones of *Garcinia kola*. *Journal of Pharmacy and Pharmacology,* 42**,** 290-292.

IZZO, A. & ERNST, E. 2009. Interactions between herbal medicines and prescribed drugs. *Drugs,* 69**,** 1777-1798.

JAAKKOLA, T., LAITILA, J., NEUVONEN, P. J. & BACKMAN, J. T. 2006. Pioglitazone is Metabolised by CYP2C8 and CYP3A4 in vitro: Potential for Interactions with CYP2C8 Inhibitors. *Basic & Clinical Pharmacology & Toxicology,* 99**,** 44-51.

JAFRI, M. A., SUBHANI, M. J., JAVED, K. & SINGH, S. 1999. Hepatoprotective activity of leaves of *Cassia occidentalis* against paracetamol and ethyl alcohol intoxication in rats. *Journal of Ethnopharmacology,* 66**,** 355-361.

JOINT FORMULARY COMMITTEE 2014. *British National Formulary 67th edition,* London BMJ Group and Pharmaceutical Press.

JONAS, W. B. 2002. Policy, the Public, and Priorities in Alternative Medicine Research. *Annals of the American Academy of Political and Social Science,* 583**,** 29-43.

JUAN, H., TERHAAG, B., CONG, Z., BI-KUI, Z., RONG-HUA, Z., FENG, W., FEN-LI, S., JUAN, S., JING, T. & WEN-XING, P. 2007. Unexpected effect of concomitantly administered curcumin on the pharmacokinetics of talinolol in healthy Chinese volunteers. *European Journal of Clinical Pharmacology,* 63**,** 663-668.

JULIANO, R. L. & LING, V. 1976. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* 455**,** 152-162.

JUNG, U. J., LEE, M.-K., JEONG, K.-S. & CHOI, M.-S. 2004. The Hypoglycemic Effects of Hesperidin and Naringin Are Partly Mediated by Hepatic Glucose-Regulating Enzymes in C57BL/KsJ-db/db Mice. *The Journal of Nutrition,* 134**,** 2499-2503.

KAHN, S. E. 2003. The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. *Diabetologia,* 46**,** 3-19.

KAMIYA, K., HAMABE, W., HARADA, S., MURAKAMI, R., TOKUYAMA, S. & SATAKE, T. 2008. Chemical Constituents of *Morinda citrifolia* Roots Exhibit Hypoglycemic Effects in Streptozotocin-Induced Diabetic Mice. *Biological and Pharmaceutical Bulletin,* 31**,** 935-938.

KATOH, M., NAKAJIMA, M., SHIMADA, N., YAMAZAKI, H. & YOKOI, T. 2000a. Inhibition of human cytochrome P450 enzymes by 1,4-dihydropyridine calcium antagonists: prediction of in vivo drug–drug interactions. *European Journal of Clinical Pharmacology,* 55**,** 843-852.

KATOH, M., NAKAJIMA, M., YAMAZAKI, H. & YOKOI, T. 2000b. Inhibitory Potencies of 1,4-dihydropyridine Calcium Antagonists to P-glycoprotein-Mediated Transport: Comparison with the Effects on CYP3A4. *Pharmaceutical Research,* 17**,** 1189-1197.

KENGNE, A. P., AMOAH, A. G. B. & MBANYA, J.-C. 2005. Cardiovascular Complications of Diabetes Mellitus in Sub-Saharan Africa. *American Heart Association,* 112**,** 3592-3601.

KHATUN, H., RAHMAN, A., BISWAS, M. & ISLAM, A. U. 2011. Water-soluble Fraction of *Abelmoschus esculentus* L Interacts with Glucose and Metformin Hydrochloride and Alters Their Absorption Kinetics after Coadministration in Rats. *International Scholarly Research Network (ISRN) Pharmaceutics,* 2011.

KIM, H. G., HIEN, T. T., HAN, E. H., HWANG, Y. P., CHOI, J. H., KANG, K. W., KWON, K.-I., KIM, B.-H., KIM, S. K., SONG, G. Y., JEONG, T. C. & JEONG, H. G. 2011. Metformin inhibits P-glycoprotein expression via the NF-κB pathway and CRE transcriptional activity through AMPK activation. *British Journal of Pharmacology,* 162**,** 1096-1108.

KONSOULA, R. & BARILE, F. A. 2005. Correlation of in vitro cytotoxicity with paracellular permeability in Caco-2 cells. *Toxicology in Vitro,* 19**,** 675-684.

KOUITCHEU MABEKU, L. B., KOUAM, J., PAUL, A. & ETOA, F. X. 2008. Phytochemical screening and toxicological profile of methanolic extract of *Picralima nitida* fruit-rind (Apocynaceae). *Toxicological & Environmental Chemistry,* 90**,** 815-828.

KRISHNA, R., BERGMAN, A., LARSON, P., COTE, J., LASSETER, K., DILZER, S., WANG, A., ZENG, W., CHEN, L., WAGNER, J. & HERMAN, G. 2007. Effect of a Single Cyclosporine Dose on the Single-Dose Pharmacokinetics of Sitagliptin (MK-0431), a Dipeptidyl Peptidase-4 Inhibitor, in Healthy Male Subjects. *The Journal of Clinical Pharmacology,* 47**,** 165-174.

KUMAR, D., BAJAJ, S. & MEHROTRA, R. 2006. Knowledge, attitude and practice of complementary and alternative medicines for diabetes. *Public Health,* 120**,** 705-711.

LAING, R., WANING, B., GRAY, A., FORD, N. & T HOEN, E. 2003. 25 years of the WHO essential medicines lists: progress and challenges. *The Lancet,* 361**,** 1723-1729.

LAU, W. C., GURBEL, P. A., WATKINS, P. B., NEER, C. J., HOPP, A. S., CARVILLE, D. G. M., GUYER, K. E., TAIT, A. R. & BATES, E. R. 2004. Contribution of Hepatic Cytochrome P450 3A4 Metabolic Activity to the Phenomenon of Clopidogrel Resistance. *Circulation,* 109**,** 166-171.

LAUGESEN, S., ENGGAARD, T. P., PEDERSEN, R. S., SINDRUP, S. H. & BROSEN, K. 2005. Paroxetine, a Cytochrome P450 2D6 Inhibitor, Diminishes the Stereoselective O-demethylation and Reduces the Hypoalgesic Effect of Tramadol[ast]. *Clinical Pharmacology & Therapeutics,* 77**,** 312-323.

LAWAL, I. O., UZOKWE, N. E., IGBOANUGO, A. B. I., ADIO, A. F., AWOSAN, E. A., NWOGWUGWU, J. O., FALOYE, B., OLATUNJI, B. P. & ADESOGA, A. A. 2010. Ethno medicinal information on collation and identification of some medicinal plants in Research Institutes of South-west Nigeria. *African Journal of Pharmacy and Pharmacology,* 4**,** 001-007.

LEATHERDALE, B. A., PANESAR, R. K., SINGH, G., ATKINS, T. W., BAILEY, C. J. & BIGNELL, A. H. 1981. Improvement in glucose tolerance due to *Momordica charantia* (karela). *British Medical Journal,* 282**,** 1823-1824.

LEE, C.-K., KI, S.-H. & CHOI, J.-S. 2011. Effects of oral curcumin on the pharmacokinetics of intravenous and oral etoposide in rats: possible role of intestinal CYP3A and P-gp inhibition by curcumin. *Biopharmaceutics & Drug Disposition,* 32**,** 245-251.

LEE, J. S., PAULL, K., ALVAREZ, M., HOSE, C., MONKS, A., GREVER, M., FOJO, A. T. & BATES, S. E. 1994. Rhodamine efflux patterns predict P-glycoprotein substrates in the National Cancer Institute drug screen. *Molecular Pharmacology,* 46**,** 627-638.

LENNERNÄS, H. 2003. Clinical Pharmacokinetics of Atorvastatin. *Clinical Pharmacokinetics,* 42**,** 1141-1160.

LI, A. P. 2001. Screening for human ADME/Tox drug properties in drug discovery. *Drug Discovery Today,* 6**,** 357-366.

LI, J. W.-H. & VEDERAS, J. C. 2009. Drug Discovery and Natural Products: End of an Era or an Endless Frontier? *Science,* 325**,** 161-165.

LI, Y., REVALDE, J. L., REID, G. & PAXTON, J. W. 2010. Interactions of dietary phytochemicals with ABC transporters: possible implications for drug disposition and multidrug resistance in cancer. *Drug Metabolism Reviews,* 42**,** 590-611.

LILJA, J. J., NIEMI, M., FREDRIKSON, H. & NEUVONEN, P. J. 2007. Effects of clarithromycin and grapefruit juice on the pharmacokinetics of glibenclamide. *British Journal of Clinical Pharmacology,* 63**,** 732-740.

LIMTRAKUL, P., CHEARWAE, W., SHUKLA, S., PHISALPHONG, C. & AMBUDKAR, S. 2007. Modulation of function of three ABC drug transporters, P-glycoprotein (ABCB1), mitoxantrone resistance protein (ABCG2) and multidrug resistance protein 1 (ABCC1) by tetrahydrocurcumin, a major metabolite of curcumin. *Molecular and Cellular Biochemistry,* 296**,** 85-95.

LIN, C.-C., HSU, Y.-F., LIN, T.-C. & HSU, H.-Y. 2001. Antioxidant and hepatoprotective effects of punicalagin and punicalin on acetaminophen-induced liver damage in rats. *Phytotherapy Research,* 15**,** 206-212.

LIU, I. M., TZENG, T.-F., LIOU, S.-S. & LAN, T.-W. 2007. Myricetin, a naturally occurring flavonol, ameliorates insulin resistance induced by a high-fructose diet in rats. *Life Sciences,* 81**,** 1479-1488.

MANIYAR, Y. & BHIXAVATIMATH, P. 2012. Antihyperglycemic and hypolipidemic activities of aqueous extract of *Carica papaya* Linn. leaves in alloxan-induced diabetic rats. *Journal of Ayurveda and Integrative Medicine,* 3**,** 70-74.

MANNEL, M. 2004. Drug Interactions with St John's Wort. *Drug Safety,* 27**,** 773-797.

MARCY, T. R., BRITTON, M. L. & BLEVINS, S. M. 2004. Second-Generation Thiazolidinediones and Hepatotoxicity. *Annals of Pharmacotherapy,* 38**,** 1419-1423.

MARITIM, A. C., SANDERS, R. A. & WATKINS, J. B. 2003. Diabetes, Oxidative Stress, and Antioxidants: A Review. *Journal of Biochemistry and Molecular Toxicology,* 17**,** 24–38.

MARKOWITZ, J. S. & ZHU, H.-J. 2012. Limitations of In Vitro Assessments of the Drug Interaction Potential of Botanical Supplements. *Planta Med,* 78**,** 1421-1427.

MARLES, R. J. & FARNSWORTH, N. R. 1995. Antidiabetic plants and their active constituents. *Phytomedicine,* 2**,** 137-189.

MARQUIS, V. O., ADANLAWO, T. A. & OLANIYI, A. A. 1977. The effect of foetidin from *Momordica foetida* on blood glucose level of Albino rats. *Planta Medica,* 31**,** 367-374.

MATSUI, T., EBUCHI, S., KOBAYASHI, M., FUKUI, K., SUGITA, K., TERAHARA, N. & MATSUMOTO, K. 2002. Anti-hyperglycemic Effect of Diacylated Anthocyanin Derived from *Ipomoea batatas* Cultivar Ayamurasaki Can Be Achieved through the α-Glucosidase Inhibitory Action. *Journal of Agricultural and Food Chemistry,* 50**,** 7244-7248.

MAYFIELD, J. 1998. Diagnosis and classification of diabetes mellitus: new criteria. *American family physician,* 58**,** 1355-62, 1369-70.

MEGA, J. L., CLOSE, S. L., WIVIOTT, S. D., SHEN, L., HOCKETT, R. D., BRANDT, J. T., WALKER, J. R., ANTMAN, E. M., MACIAS, W., BRAUNWALD, E. & SABATINE, M. S. 2009. Cytochrome P-450 Polymorphisms and Response to Clopidogrel. *New England Journal of Medicine,* 360**,** 354-362.

MEISTER, A. & ANDERSON, M. E. 1983. Glutathione. *Annual Review of Biochemistry,* 52**,** 711-760.

MONAGO, C. C. & NWODO, O. F. C. 2010. Antidiabetic Effect of Crude Trigonelline of *Abrus precatorius* Linn Seed in Alloxan Diabetic Rabbits. *Journal of Pharmacy Research,* 3**,** 1916-1919.

MORRIS, M. E. & ZHANG, S. 2006. Flavonoid–drug interactions: Effects of flavonoids on ABC transporters. *Life Sciences,* 78**,** 2116-2130.

MOSHI, M. J., LUTALE, J. J. K., RIMOY, G. H., ABBAS, Z. G., JOSIAH, R. M. & SWAI, A. B. M. 2001. The effect of *Phyllanthus amarus* aqueous extract on blood glucose in non-insulin dependent diabetic patients. *Phytotherapy Research,* 15**,** 577-580.

MUKANGANYAMA, S., WIDERSTEN, M., NAIK, Y. S., MANNERVIK, B. & HASLER, J. A. 2002. Inhibition of glutathione S-transferases by antimalarial drugs possible implications for circumventing anticancer drug resistance. *International Journal of Cancer,* 97**,** 700-705.

NA, D., JI, H., PARK, E., KIM, M., LIU, K.-H. & LEE, H. 2011. Evaluation of metabolism-mediated herb-drug interactions. *Archives of Pharmacal Research,* 34**,** 1829-1842.

NAFDAC 2005. Guidelines for registration/listing of herbal medicines and related products NAFDAC/RR/006/00. *In:* AFFAIRS, D. O. R. A. R. (ed.). Lagos, Nigeria: National Agency for Food and Drug Administration and Control (NAFDAC).

NARAI, A., ARAI, S. & SHIMIZU, M. 1997. Rapid decrease in transepithelial electrical resistance of human intestinal Caco-2 cell monolayers by cytotoxic membrane perturbents. *Toxicology in Vitro,* 11**,** 347-354.

NATHAN, D. M., BUSE, J. B., DAVIDSON, M. B., HEINE, R. J., HOLMAN, R. R., SHERWIN, R. & ZINMAN, B. 2006. Management of Hyperglycemia in Type 2 Diabetes: A Consensus Algorithm for the Initiation and Adjustment of Therapy: A consensus statement from the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes Care,* 29**,** 1963-1972.

NWAFOR, S., ESIMONE, C., AMADI, C. & NWORU, C. 2003. In vivo interaction between ciprofloxacin hydrochloride and the pulp of unripe plantain (*Musa paradisiaca*). *European Journal of Drug Metabolism and Pharmacokinetics,* 28**,** 253-258.

NYENWE, E. A., JERKINS, T. W., UMPIERREZ, G. E. & KITABCHI, A. E. 2011. Management of type 2 diabetes: evolving strategies for the treatment of patients with type 2 diabetes. *Metabolism- Clinical and Experimental,* 60**,** 1-23.

NYENWE, E. A., ODIA, O. J., IHEKWABA, A. E., OJULE, A. & BABATUNDE, S. 2003. Type 2 diabetes in adult Nigerians: a study of its prevalence and risk factors in Port Harcourt, Nigeria. *Diabetes Research and Clinical Practice,* 62**,** 177-185.

OBIDAH, W., SA'AD, U. A. & WUROCHEKKE, A. U. 2009. Toxic effects of aqueous stem bark extract of *Cassia sieberiana* on some biochemical parameters in rats. *African Journal of Biochemistry Research,* 3**,** 229-231.

OBOH, G., OGUNRUKU, O. O., OGIDIOLU, F. O., ADEMILUYI, A. O., ADEDAYO, B. C. & ADEMOSUN, A. O. 2014. Interaction of Some Commercial Teas with Some Carbohydrate Metabolizing Enzymes Linked with Type-2 Diabetes: A Dietary Intervention in the Prevention of Type-2 Diabetes. *Advances in Preventive Medicine,* Article ID 534082**,,** 7 pages.

OGA, E. F., SEKINE, S., SHITARA, Y. & HORIE, T. 2012. P-glycoprotein mediated efflux in Caco-2 cell monolayers: The influence of herbals on digoxin transport. *Journal of Ethnopharmacology,* 144**,** 612-617.

OGBERA, A., DADA, O., ADELEYE, F. & JEWO, P. 2010. Complementary and Alternative Medicine Use in Diabetes Mellitus. *West African Journal of Medicine,* 29**,** 158-161.

OGBERA, A. O., AWOBUSUYI, J., UNACHUKWU, C. & FASANMADE, O. 2009. Clinical features, predictive factors and outcome of hyperglycaemic emergencies in a developing country. *BMC Endocrine Disorders,* 9.

OGBERA, A. O., CHINENYE, S., ONYEKWERE, A. & FASANMADE, O. 2007. Prognostic indices of diabetes mortality. *Ethnicity & Disease,* 17**,** 721-725.

OGBONNIA, S. & ANYAKORA, C. 2009. Chemistry and Biological Evaluation of Nigerian Plants with Anti-Diabetic Properties. *African Natural Plant Products: New Discoveries and Challenges in Chemistry and Quality.* American Chemical Society.

OKOLI, R. I., AIGBE, O., OHAJU-OBODO, J. O. & MENSAH, J. K. 2007. Medicinal Herbs Used for Managing Some Common Ailments among Esan People of Edo State, Nigeria. *Pakistan Journal of Nutrition,* 6**,** 490-496.

OKORO, E. O., ADEJUMO, A. O. & OYEJOLA, B. A. 2002. Diabetic care in Nigeria: report of a self-audit. *Journal of Diabetes and Its Complications,* 16**,** 159-164.

OLISA, N. S. & OYELOLA, F. T. 2009. Evaluation of use of herbal medicines among ambulatory hypertensive patients attending a secondary health care facility in Nigeria. *International Journal of Pharmacy Practice,* 17**,** 101-105.

OLOWOKUDEJO, J. D., KADIRI, A. B. & TRAVIH, V. A. 2008. An Ethnobotanical Survey of Herbal Markets and Medicinal Plants in Lagos State of Nigeria. *Ethnobotanical Leaflets,* 12**,** 851-865.

ONAOLAPO, A. Y. & ONAOLAPO, O. J. 2012. *Ocimum Gratissimum* Linn Causes Dose Dependent Hepatotoxicity in Streptozotocin-Induced Diabetic Wistar Rats. *Macedonian Journal of Medical Sciences,* 5**,** 17-25.

ONG, K. C. & KHOO, H.-E. 2000. Effects of myricetin on glycemia and glycogen metabolism in diabetic rats. *Life Sciences,* 67**,** 1695-1705.

OSAKWE, A., OREAGBA, I., ADEWUNMI, A. J., ADEKOYA, A. & FAJOLU, I. 2013. Impact of training on Nigerian healthcare professionals' knowledge and practice of pharmacovigilance. *The International Journal of Risk and Safety in Medicine,* 25**,** 219-227.

OSEMENE, K. P., ELUJOBA, A. A. & ILORI, M. O. 2011. A Comparative Assessment of Herbal and Orthodox Medicines in Nigeria. *Research Journal of Medical Sciences,* 5**,** 280-285.

OUBRÉ, A. Y., CARLSON, T. J., KING, S. R. & REAVEN, G. M. 1997. From plant to patient: an ethnomedical approach to the identification of new drugs for the treatment of NIDDM. *Diabetologia,* 40**,** 614-617.

PANDEY, K. B. & RIZVI, S. I. 2009. Plant Polyphenols as Dietary Antioxidants in Human Health and Disease. *Oxidative Medicine and Cellular Longevity,* 2.

PATKI, K. C., VON MOLTKE, L. L. & GREENBLATT, D. J. 2003. In Vitro Metabolism Of Midazolam, Triazolam, Nifedipine, And Testosterone By Human Liver

Microsomes And Recombinant Cytochromes P450: Role Of Cyp3A4 And Cyp3A5. *Drug Metabolism and Disposition,* 31**,** 938-944.

PATSALOS, PHILIP N., FRÖSCHER, W., PISANI, F. & VAN RIJN, CLEMENTINA M. 2002. The Importance of Drug Interactions in Epilepsy Therapy. *Epilepsia,* 43**,** 365-385.

PELKONEN, O., TURPEINEN, M., UUSITALO, J., RAUTIO, A. & RAUNIO, H. 2005. Prediction of Drug Metabolism and Interactions on the Basis of *in vitro* Investigations. *Basic & Clinical Pharmacology & Toxicology,* 96**,** 167-175.

PERSAUD, S., AL-MAJED, H., RAMAN, A. & JONES, P. 1999. *Gymnema sylvestre* stimulates insulin release in vitro by increased membrane permeability. *Journal of Endocrinology,* 163**,** 207-212.

PESCHEL, W., PRIETO, J. M., KARKOUR, C. & WILLIAMSON, E. M. 2013. Effect of provenance, plant part and processing on extract profiles from cultivated European *Rhodiola rosea* L. for medicinal use. *Phytochemistry,* 86**,** 92-102.

PICKING, D., YOUNGER, N., MITCHELL, S. & DELGODA, R. 2011. The prevalence of herbal medicine home use and concomitant use with pharmaceutical medicines in Jamaica. *Journal of Ethnopharmacology,* 137**,** 305-311.

PISCITELLI, S. C., BURSTEIN, A. H., WELDEN, N., GALLICANO, K. D. & FALLOON, J. 2002. The Effect of Garlic Supplements on the Pharmacokinetics of Saquinavir. *Clinical Infectious Diseases,* 34**,** 234-238.

POULOSE, S. M., HARRIS, E. D. & PATIL, B. S. 2005. Citrus Limonoids Induce Apoptosis in Human Neuroblastoma Cells and Have Radical Scavenging Activity. *The Journal of Nutrition,* 135**,** 870-877.

PROESTOS, C., CHORIANOPOULOS, N., NYCHAS, G. J. E. & KOMAITIS, M. 2005. RP-HPLC Analysis of the Phenolic Compounds of Plant Extracts. Investigation of Their Antioxidant Capacity and Antimicrobial Activity. *Journal of Agricultural and Food Chemistry,* 53**,** 1190-1195.

QI, L.-W., LIU, E. H., CHU, C., PENG, Y.-B., CAI, H.-X. & LI, P. 2010. Anti-Diabetic Agents from Natural Products- An Update from 2004 to 2009. *Current Topics in Medicinal Chemistry,* 10**,** 434-457.

QUASIE, O., MARTEY, O. N. K., NYARKO, A. K., GBEWONYO, W. S. K. & OKINE, L. K. N. 2010. Modulation of penile erection in rabbits by *Mondia whitei*: Possible mechanism of action. *African Journal of Traditional Complementary Alternative Medicines,* 7**,** 241 - 252.

RAHIMI, R., NIKFAR, S., LARIJANI, B. & ABDOLLAHI, M. 2005. A review on the role of antioxidants in the management of diabetes and its complications. *Biomedicine & Pharmacotherapy,* 59**,** 365-373.

RAHMAN, I., KODE, A. & BISWAS, S. K. 2007. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nature Protocols,* 1**,** 3159-3165.

RANALDI, G., CONSALVO, R., SAMBUY, Y. & SCARINO, M. L. 2003. Permeability characteristics of parental and clonal human intestinal Caco-2 cell lines differentiated in serum-supplemented and serum-free media. *Toxicology in Vitro,* 17**,** 761-767.

RATES, S. M. K. 2001. Plants as source of drugs. *Toxicon,* 39**,** 603-613.

RENDIC, S. & CARLO, F. J. D. 1997. Human Cytochrome P450 Enzymes: A Status Report Summarizing Their Reactions, Substrates, Inducers, and Inhibitors. *Drug Metabolism Reviews,* 29**,** 413-580.

REPETTO, G., PESO, A. D. & ZURITA, L. J. 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature protocols,* 3**,** 1125-1131.

ROBERTSON, R. P., HARMON, J., TRAN, P. O. T. & POITOUT, V. 2004. β-Cell Glucose Toxicity, Lipotoxicity, and Chronic Oxidative Stress in Type 2 Diabetes. *Diabetes,* 53**,** S119-S124.

RODEIRO, I., DONATO, M. T., JIMENEZ, N., GARRIDO, G., MOLINA-TORRES, J., MENENDEZ, R., CASTELL, J. V. & GÓMEZ-LECHÓN, M. J. 2009. Inhibition of

human P450 enzymes by natural extracts used in traditional medicine. *Phytotherapy Research,* 23**,** 279-282.

ROY, A. & SARAF, S. 2006. Limonoids: Overview of Significant Bioactive Triterpenes Distributed in Plants Kingdom. *Biological and Pharmaceutical Bulletin,* 29**,** 191-201.

SABU, M. C., SMITHA, K. & RAMADASAN, K. 2002. Anti-diabetic activity of green tea polyphenols and their role in reducing oxidative stress in experimental diabetes. *Journal of Ethnopharmacology,* 83**,** 109-116.

SAKLANI, A. & KUTTY, S. K. 2008. Plant-derived compounds in clinical trials. *Drug Discovery Today,* 13**,** 161-171.

SAYDAH, S. H. & EBERHARDT, M. S. 2006. Use of complementary and alternative medicine among adults with chronic diseases: United States 2002. *Journal of Alternative and Complementary Medicine (New York, N.Y.),* 12**,** 805-12.

SCHEEN, A. J. 2003. Pathophysiology of type 2 diabetes. *Acta Clinica Belgica,* 58**,** 335-341.

SEIDEL, V. 2006. Initial and bulk extraction. *In:* SARKER, S. D., LATIF, Z. & GRAY, A. I. (eds.) *Natural Product Isolation.* 2nd Edition ed. Totowa, New Jersey Humana Press Inc. .

SHARMA, O. P. & BHAT, T. K. 2009. DPPH antioxidant assay revisited. *Food Chemistry,* 113**,** 1202-1205.

SHAROM, F. J. 2011. The P‐glycoprotein multidrug transporter. *Essays in Biochemistry,* 50**,** 161-178.

SHAW, D., GRAEME, L., PIERRE, D., ELIZABETH, W. & KELVIN, C. 2012. Pharmacovigilance of herbal medicine. *Journal of Ethnopharmacology,* 140**,** 513-518.

SHEEHAN, M. T. 2003. Current Therapeutic Options in Type 2 Diabetes Mellitus: A Practical Approach. *Clinical Medicine & Research,* 1**,** 189-200.

SHEELA, C. G., KUMUD, K. & AUGUSTI, K. T. 1995. Anti-Diabetic Effects of Onion and Garlic Sulfoxide Amino Acids in Rats. *Planta Medica,* 61**,** 356-357.

SHERRATT, H. S. A. 1986. Hypoglycin, the famous toxin of the unripe Jamaican ackee fruit. *Trends in Pharmacological Sciences,* 7**,** 186-191.

SHITTU, H., GRAY, A., FURMAN, B. & YOUNG, L. 2010. Glucose uptake stimulatory effect of akuammicine from *Picralima nitida* (Apocynaceae). *Phytochemistry Letters,* 3**,** 53-55.

SHUAIBU, M., WUYEP, P., YANAGI, T., HIRAYAMA, K., TANAKA, T. & KOUNO, I. 2008. The use of microfluorometric method for activity-guided isolation of antiplasmodial compound from plant extracts. *Parasitology Research,* 102**,** 1119-1127.

SOBENIN, I., NEDOSUGOVA, L., FILATOVA, L., BALABOLKIN, M., GORCHAKOVA, T. & OREKHOV, A. 2008. Metabolic effects of time-released garlic powder tablets in type 2 diabetes mellitus: the results of double-blinded placebo-controlled study. *Acta Diabetologica,* 45**,** 1-6.

SOLADOYE, M. O., CHUKWUMA, E. C. & OWA, F. P. 2012. An 'Avalanche' of Plant Species for the Traditional Cure of Diabetes mellitus in South-Western Nigeria. *Journal of Natural Products and Plant Resources,* 2**,** 60-72.

SOLECKI, R. S. 1975. Shanidar IV, a Neanderthal Flower Burial in Northern Iraq. *Science,* 190**,** 880-881.

SPRINGFIELD, E. P., EAGLES, P. K. F. & SCOTT, G. 2005. Quality assessment of South African herbal medicines by means of HPLC fingerprinting. *Journal of Ethnopharmacology,* 101**,** 75-83.

SQUADRITO, G. L. & PRYOR, W. A. 1998. Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. *Free Radical Biology and Medicine,* 25**,** 392-403.

SRINIVASAN, K. & RAMARAO, P. 2007. Animal models in type 2 diabetes research: An overview. *Indian Journal of Medical Research,* 125**,** 451-472.

STEARNS, R. A., CHAKRAVARTY, P. K., CHEN, R. & CHIU, S. H. 1995. Biotransformation of losartan to its active carboxylic acid metabolite in human liver microsomes. Role of cytochrome P4502C and 3A subfamily members. *Drug Metabolism and Disposition,* 23**,** 207-215.

STOCK, R. 1983. Distance and the utilization of health facilities in rural Nigeria. *Social Science & Medicine,* 17**,** 563-570.

SUAREZ-PINZON, W. L., MABLEY, J. G., STRYNADKA, K., POWER, R. F., SZABÓ, C. & RABINOVITCH, A. 2001. An Inhibitor of Inducible Nitric Oxide Synthase and Scavenger of Peroxynitrite Prevents Diabetes Development in NOD Mice. *Journal of Autoimmunity,* 16**,** 449-455.

SUBRAHMANYAM, V., RENWICK, A. B., WALTERS, D. G., YOUNG, P. J., PRICE, R. J., TONELLI, A. P. & LAKE, B. G. 2001. Identification of Cytochrome P-450 Isoforms Responsible for cis-Tramadol Metabolism in Human Liver Microsomes. *Drug Metabolism and Disposition,* 29**,** 1146-1155.

SULTANA, N., CHOUDHARY, M. I. & KHAN, A. 2009. Protein glycation inhibitory activities of *Lawsonia inermis* and its active principles. *Journal of Enzyme Inhibition and Medicinal Chemistry,* 24**,** 257-261.

SUN, H., MOORE, C., DANSETTE, P. M., KUMAR, S., HALPERT, J. R. & YOST, G. S. 2009. Dehydrogenation of the Indoline-Containing Drug 4-Chloro-N-(2-methyl-1-indolinyl)-3-sulfamoylbenzamide (Indapamide) by CYP3A4: Correlation with in Silico Predictions. *Drug Metabolism and Disposition,* 37**,** 672-684.

SURENDIRAN, A., PRADHAN, S. C., AGRAWAL, A., SUBRAHMANYAM, D. K. S., RAJAN, S., ANICHAVEZHI, D. & ADITHAN, C. 2011. Influence of CYP2C9 gene polymorphisms on response to glibenclamide in type 2 diabetes mellitus patients. *European Journal of Clinical Pharmacology,* 67**,** 797-801.

SUZUKI, R., OKADA, Y. & OKUYAMA, T. 2005. The Favorable Effect of Style of *Zea mays* L. on Streptozotocin Induced Diabetic Nephropathy. *Biological and Pharmaceutical Bulletin,* 28**,** 919-920.

SZKUDELSKI, T. 2001. The Mechanism of Alloxan and Streptozotocin Action in B Cells of the Rat Pancreas. *Physiological Research,* 50**,** 536-546.

TAUB, M. E., PODILA, L., ELY, D. & ALMEIDA, I. 2005. Functional assessment of multiple P-glycoprotein (P-gp) probe substrates: influence of cell line and modulator concentration on p-gp activity. *Drug Metabolism and Disposition,* 33**,** 1679-1687.

TEUGWA, C. M., BOUDJEKO, T., TCHINDA, B. T., MEJIATO, P. C. & ZOFOU, D. 2013. Anti-hyperglycaemic globulins from selected Cucurbitaceae seeds used as antidiabetic medicinal plants in Africa. *BMC Complementary and Alternative Medicine,* 13.

TODD, J. A. 2010. Etiology of Type 1 Diabetes. *Immunity,* 32**,** 457-467.

TOMA, I., KARUMI, Y. & GEIDAM, M. A. 2009. Phytochemical screening and toxicity studies of the aqueous extract of the pods pulp of Cassia sieberiana DC. (Cassia Kotchiyana Oliv.). *African Journal of Pure and Applied Chemistry,* 3**,** 026-030.

TREVELYAN, J. 1993. Herbal medicine. *Nuursing times,* 89**,** 36-38.

TROUTMAN, M. & THAKKER, D. 2003. Rhodamine 123 Requires Carrier-Mediated Influx for Its Activity as a P-Glycoprotein Substrate in Caco-2 Cells. *Pharmaceutical Research,* 20**,** 1192-1199.

UBILLAS, R. P., MENDEZ, C. D., JOLAD, S. D., LUO, J., KING, S. R., CARLSON, T. J. & FORT, D. M. 2000. Antihyperglycemic Acetylenic Glucosides from *Bidens pilosa*. *Planta Medica,* 66**,** 82-83.

ULBRICHT, C., CHAO, W., COSTA, D., RUSIE-SEAMON, E., WEISSNER, W. & WOODS, J. 2008. Clinical Evidence of Herb-Drug Interactions: A Systematic Review by the Natural Standard Research Collaboration. *Current Drug Metabolism,* 9**,** 1063-1120.

VAN ANDEL, T. & WESTERS, P. 2010. Why Surinamese migrants in the Netherlands continue to use medicinal herbs from their home country. *Journal of Ethnopharmacology,* 127**,** 694-701.

VAN ERP, N. P. H., BAKER, S. D., ZHAO, M., RUDEK, M. A., GUCHELAAR, H.-J., NORTIER, J. W. R., SPARREBOOM, A. & GELDERBLOM, H. 2005. Effect of Milk Thistle (*Silybum marianum*) on the Pharmacokinetics of Irinotecan. *Clinical Cancer Research,* 11**,** 7800-7806.

VARGHESE, G. K., BOSE, L. V. & HABTEMARIAM, S. 2013. Antidiabetic components of Cassia alata leaves: Identification through α-glucosidase inhibition studies. *Pharmaceutical Biology,* 51**,** 345-349.

VASHISHTHA, V. M., JOHN, T. J. & KUMAR, A. 2009. Clinical & pathological features of acute toxicity due to *Cassia occidentalis* in vertebrates. *Indian Journal of Medical Research,* 130**,** 23-30.

VENKATARAMANAN, R., KOMOROSKI, B. & STROM, S. 2006. *In vitro* and *in vivo* assessment of herb drug interactions. *Life Sciences,* 78**,** 2105-2115.

VIJAN, S., SUSSMAN, J. B., YUDKIN, J. S. & HAYWARD, R. A. 2014. Effect of patients' risks and preferences on health gains with plasma glucose level lowering in type 2 diabetes mellitus. *JAMA Internal Medicine,* 174**,** 1227-34.

WALKER, M. W., KINTER, M. T., ROBERTS, R. J. & SPITZ, D. R. 1995. Nitric Oxide-Induced Cytotoxicity: Involvement of Cellular Resistance to Oxidative Stress and the Role of Glutathione in Protection. *Pediatric Research,* 37**,** 41-49.

WALLACE, T. M. & MATTHEWS, D. R. 2000. Poor glycaemic control in type 2 diabetes: a conspiracy of disease, suboptimal therapy and attitude. *Quarterly Journal of Medicine,* 93**,** 369-374.

WEN, Z., DUMAS, T. E., SCHRIEBER, S. J., HAWKE, R. L., FRIED, M. W. & SMITH, P. C. 2008. Pharmacokinetics and Metabolic Profile of Free, Conjugated, and Total Silymarin Flavonolignans in Human Plasma after Oral Administration of Milk Thistle Extract. *Drug Metabolism and Disposition,* 36**,** 65-72.

WEYERMANN, J., LOCHMANN, D. & ZIMMER, A. 2005. A practical note on the use of cytotoxicity assays. *International Journal of Pharmaceutics,* 288**,** 369-376.

WHITE, J. R. & CAMPBELL, K. R. 2008. *ADA/PDR medications for the treatment of diabetes,* Washington, Thomson Reuters.

WHO. 1999. Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications. *Part 1: Diagnosis and Classification of Diabetes Mellitus* [Online]. Available: http://whqlibdoc.who.int/hq/1999/WHO_NCD_NCS_99.2.pdf?ua=1 [Accessed 29th December 2014].

WHO. 2000. The World Health Report 2000. *Health Systems: Improving Performance* [Online]. Available: http://www.who.int/whr/2000/en/whr00_en.pdf?ua=1 [Accessed 29th December 2014].

WHO. 2006. Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: Report of a WHO/IDF consultation. Available: http://whqlibdoc.who.int/publications/2006/9241594934_eng.pdf?ua=1 [Accessed 29th December 2014].

WHO 2013. WHO Traditional Medicine Strategy 2014-2023. *In:* ORGANIZATION, W. H. (ed.). Geneva, Switzerland: WHO Press, World Health Organization, Geneva Switzerland.

WILLCOX, M. L. & BODEKER, G. 2010. The ethics of improving African traditional medical practice: A response. *Acta Tropica,* 115**,** 163-164.

WILLIAMSON, E. 2001. Synergy and other interactions in phytomedicines. *Phytomedicine,* 8**,** 401-409.

WILLIAMSON, E. 2003. Drug interactions between herbal and prescription medicines. *Drug safety,* 26**,** 1075-1092.

WILLIAMSON, E., DRIVER, S. & BAXTER, K. 2013. *Stockley's Herbal Medicines Interactions- A guide to the interactions of herbal medicines,* London, United Kingdom, Pharmaceutical Press.

WINSLOW, L. C. & KROLL, D. J. 1998. Herbs as Medicines. *Archives of Internal Medicine,* 158**,** 2192-2199.

WITHERING, W. 1785. *An account of the foxglove, and Some of its Medical Uses: with practical remarks on dropsy, and other diseases.*, M. Swinney for G.G.J. and J. Robinson, Paternoster-Row, London.

WU, C.-P., OHNUMA, S. & V. AMBUDKAR, S. 2011. Discovering Natural Product Modulators to Overcome Multidrug Resistance in Cancer Chemotherapy. *Current Pharmaceutical Biotechnology,* 12**,** 609-620.

YIN, O. Q. P., TOMLINSON, B. & CHOW, M. S. S. 2005. CYP2C9, but not CYP2C19, polymorphisms affect the pharmacokinetics and pharmacodynamics of glyburide in Chinese subjects. *Clinical Pharmacology and Therapeutics,* 78**,** 370-377.

YU, J., WANG, L., WALZEM, R. L., MILLER, E. G., PIKE, L. M. & PATIL, B. S. 2005. Antioxidant Activity of Citrus Limonoids, Flavonoids, and Coumarins. *Journal of Agricultural and Food Chemistry,* 53**,** 2009-2014.

YUSUFF, K., OBE, O. & JOSEPH, B. 2008. Adherence to anti-diabetic drug therapy and self management practices among type-2 diabetics in Nigeria. *Pharmacy World & Science,* 30**,** 876-883.

ZAIDENSTEIN, R., SOBACK, S., GIPS, M., AVNI, B., DISHI, V., WEISSGARTEN, Y., GOLIK, A. & SCAPA, E. 2001. Effect of Grapefruit Juice on the Pharmacokinetics of Losartan and Its Active Metabolite E3174 in Healthy Volunteers. *Therapeutic Drug Monitoring,* 23**,** 369-373.

ZHANG, W. & LIM, L.-Y. 2008. Effects of Spice Constituents on P-Glycoprotein-Mediated Transport and CYP3A4-Mediated Metabolism in Vitro. *Drug Metabolism and Disposition,* 36**,** 1283-1290.

ZHANG, W., TAN, T. M. C. & LIM, L.-Y. 2007. Impact of Curcumin-Induced Changes in P-Glycoprotein and CYP3A Expression on the Pharmacokinetics of Peroral Celiprolol and Midazolam in Rats. *Drug Metabolism and Disposition,* 35**,** 110-115.

ZHOU, L., NARAHARISETTI, S. B., LIU, L., WANG, H., LIN, Y. S., ISOHERRANEN, N., UNADKAT, J. D., HEBERT, M. F. & MAO, Q. 2010a. Contributions of human cytochrome P450 enzymes to glyburide metabolism. *Biopharmaceutics & Drug Disposition,* 31**,** 228-242.

ZHOU, S.-F., ZHOU, Z.-W. & HUANG, M. 2010b. Pharmacokinetics and Disposition of Plant Natural Products in Humans and the Clinical and Toxicological Implications. *Current Organic Chemistry,* 14**,** 1833-1868.

ZHOU, S.-F. M. D. P., XUE, C. C. P., YU, X.-Q. M. D. P., LI, C. P. & WANG, G. P. 2007. Clinically Important Drug Interactions Potentially Involving Mechanism-based Inhibition of Cytochrome P450 3A4 and the Role of Therapeutic Drug Monitoring. *Therapeutic Drug Monitoring,* 29**,** 687-710.

ZHOU, S., GAO, Y., JIANG, W., HUANG, M., XU, A. & PAXTON, J. W. 2003. Interactions of Herbs with Cytochrome P450. *Drug Metabolism Reviews,* 35**,** 35-98.

ZHOU, S., LIM, L. Y. & CHOWBAY, B. 2004. Herbal Modulation of P-Glycoprotein. *Drug Metabolism Reviews,* 36**,** 57-104.

8 APPENDICES

APPENDIX 1:

JOURNAL OF ETHNOPHARMACOLOGY PAPER

APPENDIX 4:

QUESTIONNAIRE USED TO INTERVIEW DIABETIC PATIENTS AND TRADITIONAL MEDICINE PRACTITIONERS

GENERAL INFORMATION FROM ALL RESPONDENTS

- Name of Individual:
- Sex of Individual:
- Town of birth/Tribe:
- Town where they live:
- Age of Individual (15-30, 30-45, 45-60, Above 60):
- Languages spoken:
- Occupation (main and others)

DIABETIC PATIENTS

- When did you start receiving treatment for diabetes?
- How do you prefer to treat diabetes (with medications and/or remedies)?
- What medications do you take?
- How do you obtain the medications that you take?
- What herbal remedies do you take?
- How do you obtain the herbal remedies that you take?
- Do you sometimes skip doses of any of the above mentioned means of treatment?
- Why if at all do you skip doses?
- What treatment do you consider more effective (or do you prefer)?
- Have you (or someone you know) had any adverse reaction during diabetes treatment OR what is your most common complaint during diabetes treatment?
- Any co-morbidity?
- What is your average monthly cost of either treatment?

GENERAL INFORMATION FROM ALL RESPONDENTS

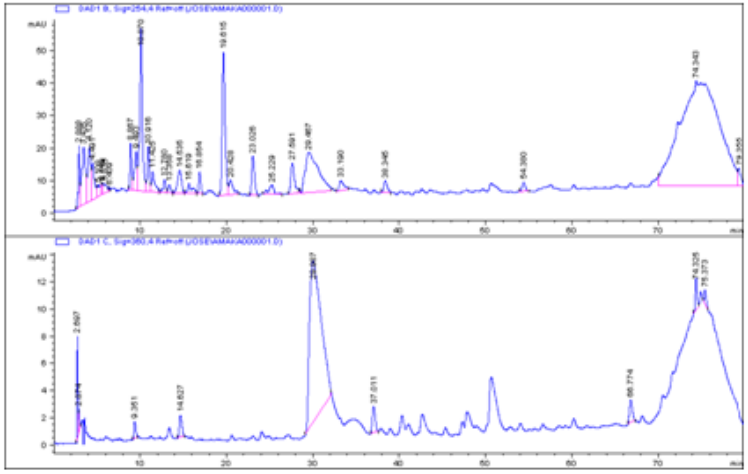
- Name of Individual:
- Sex of Individual:
- Town of birth:
- Town where they live:
- Age of Individual (15-30, 30-45, 45-60, Above 60):
- Languages spoken:
- Occupation (main and others):

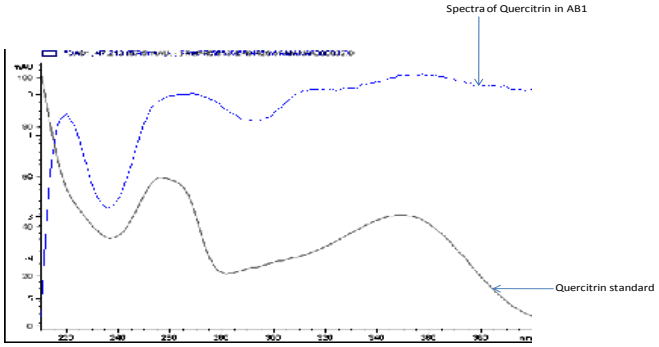
QUESTIONS FOR HERBALISTS

- What is your understanding of diabetes?
- What are the symptoms that you associate with diabetes or that you look out for before you consider an individual as a patient?
- What are the plant parts effective against diabetes & How do you treat it (with plants, medications and/or rituals)?
- What is the form of preparation for these anti-diabetic formulations (Raw, decoction, maceration, infusion, Toast/Roast, Fermentation, Others (specify)?
- How is the preparation administered (oral, topical, injectable)?
- Are there other measures/conditions that are important for the success of your treatment (such as diet, spiritual well-being etc)?
- What is the duration of treatment on the average for each patient? What is the cost of treatment?

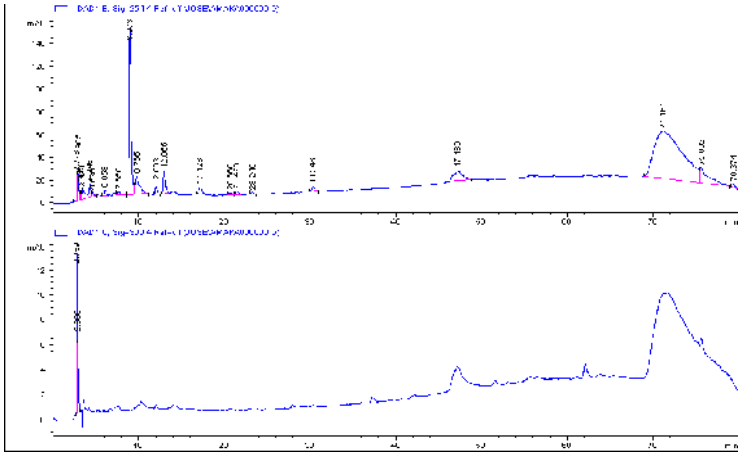
APPENDIX 5: HPLC UV/VIS OF ANALYSED PLANT SAMPLES SHOWING THE FINGERPRINT CHROMATOGRAMS AND SPECTRA OF IDENTIFIED PHENOLIC COMPOUNDS

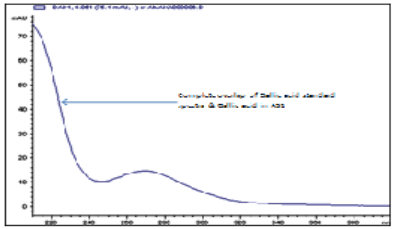
Alstonia Boonei bark

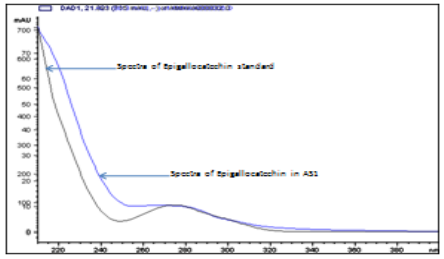




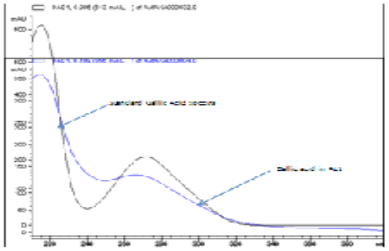
Annona senegalensis bark AS1

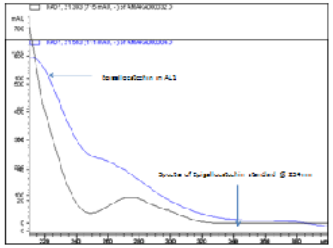


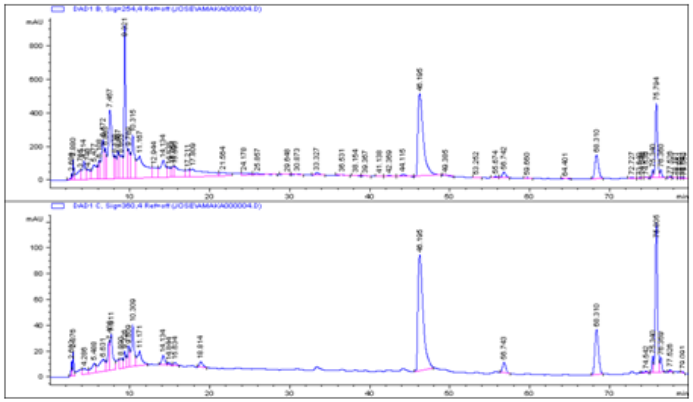


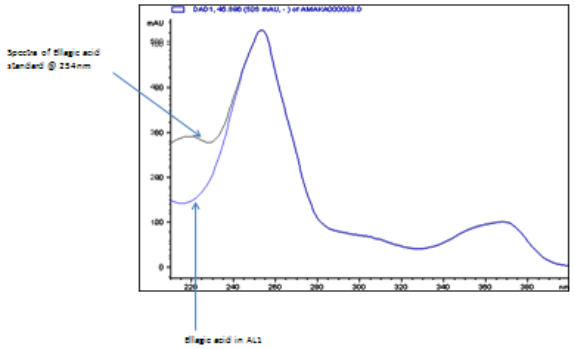


Anogeissus leiocarpus bark (AL1)

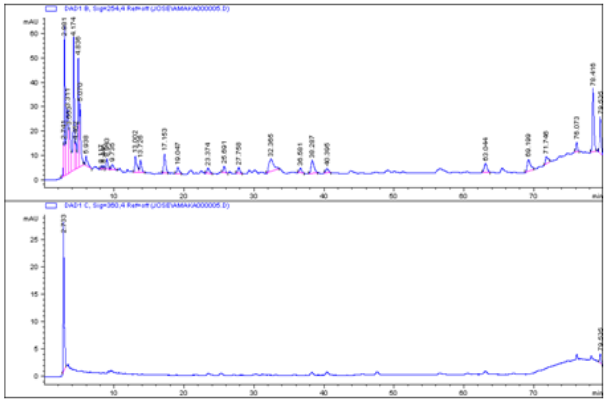




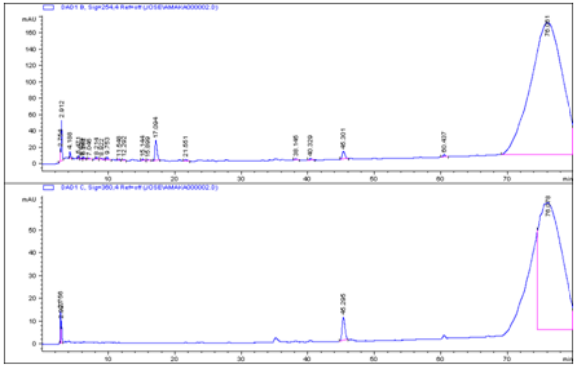




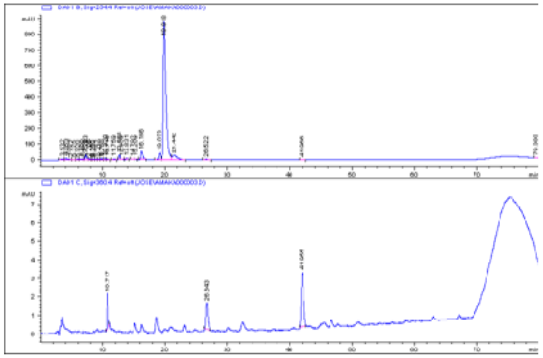
Aristolochia repens stem AR1



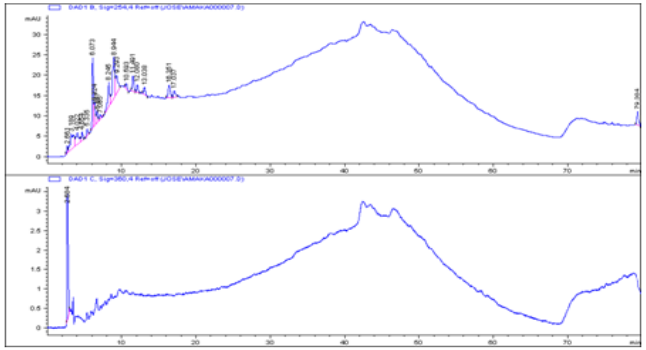
Anthocleista djalonensis bark

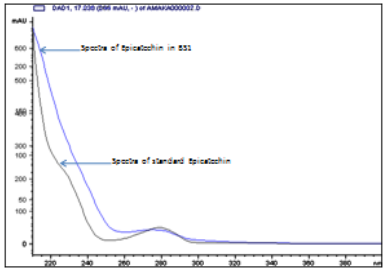


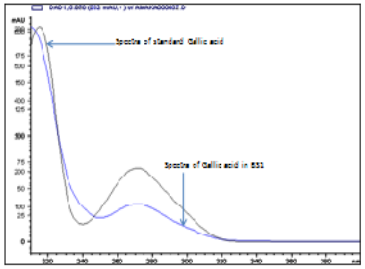
Anthocleista Djalonensis Root



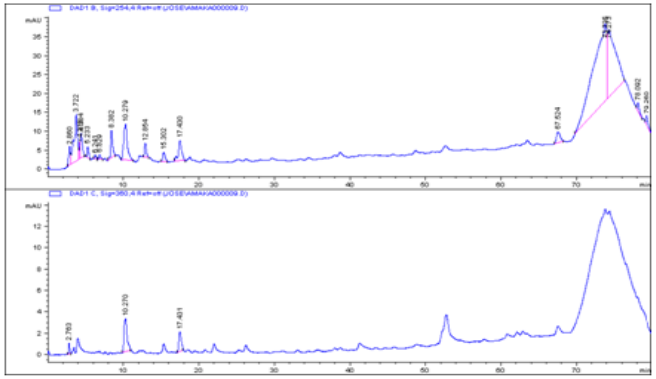
Bridelia ferruginea bark (BS1)

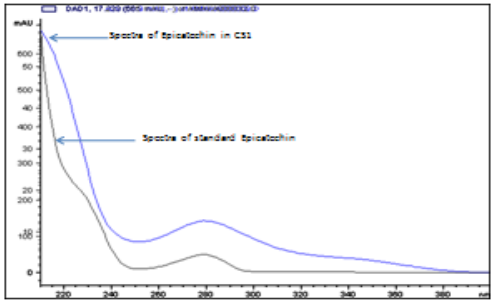




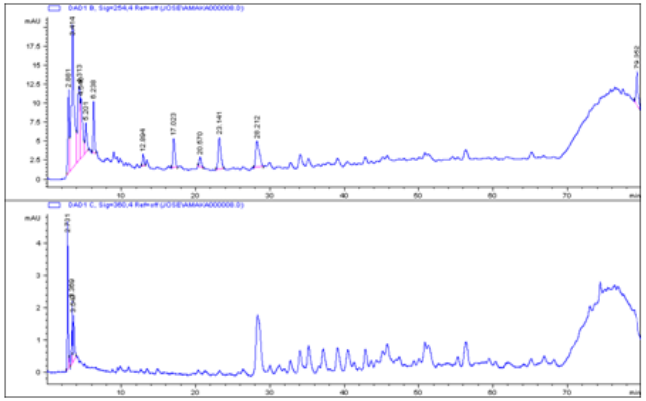


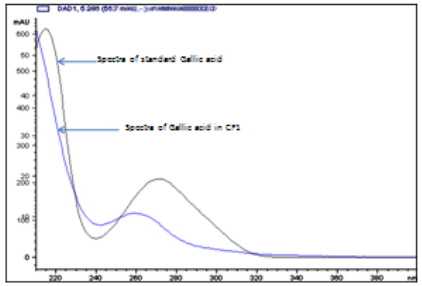
Cassia sieberiana CS1

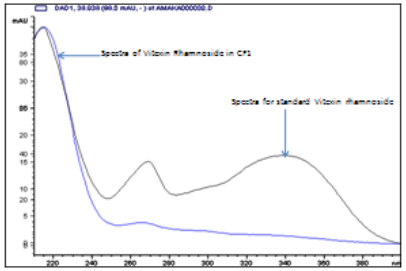




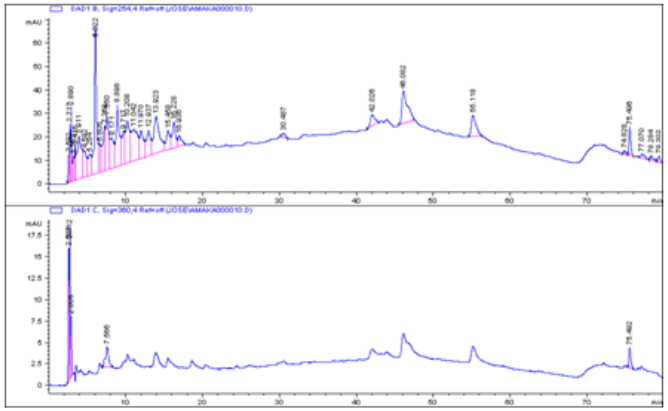
Cassytha filiformis stem (CF1)

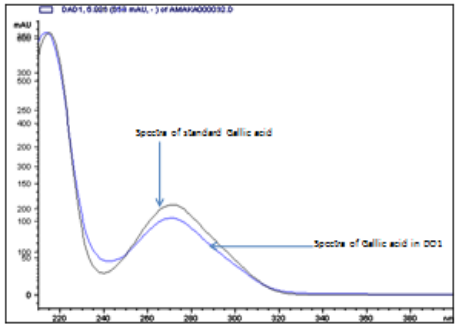


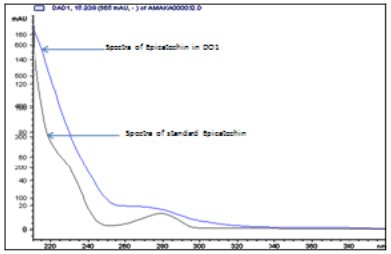




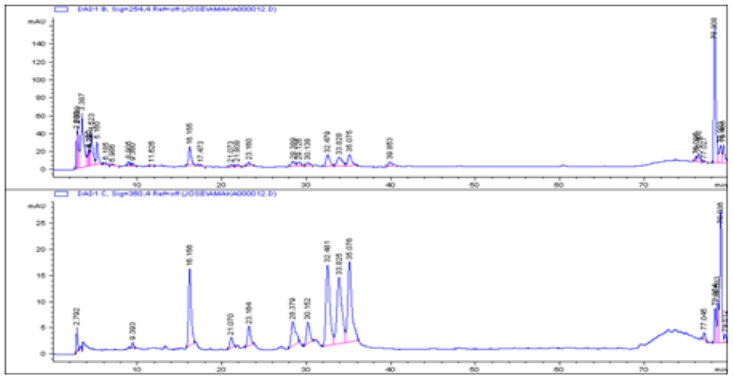
Daniellia ogea bark (DO1)

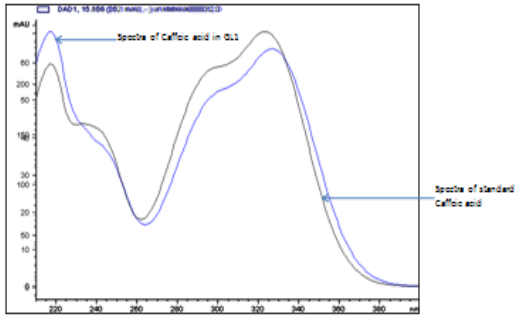


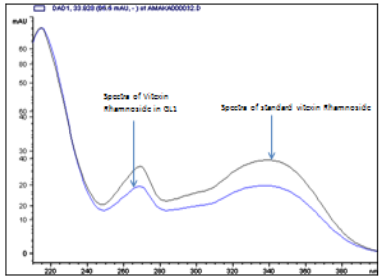




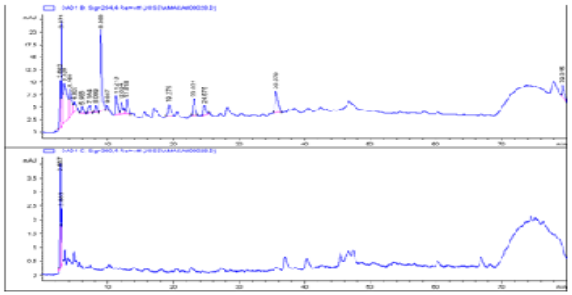
Gongronema latifolium (GL1)



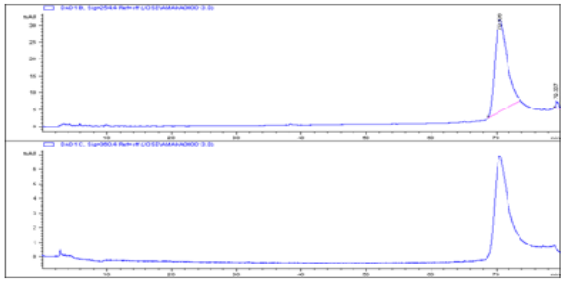




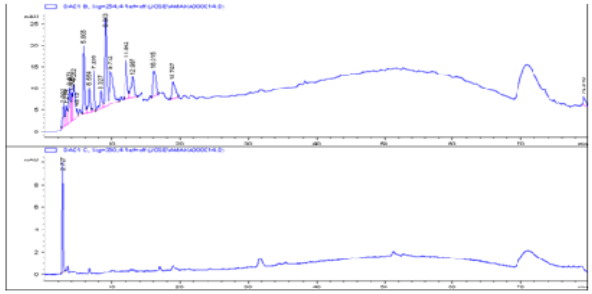
Isoberlinia doka stem (ID1)



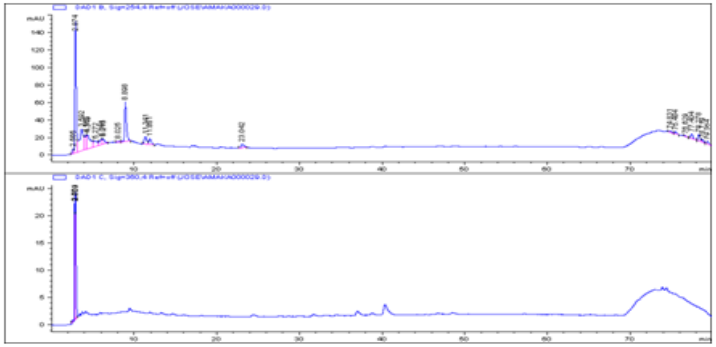
Khaya spp from Mushin (KS1)



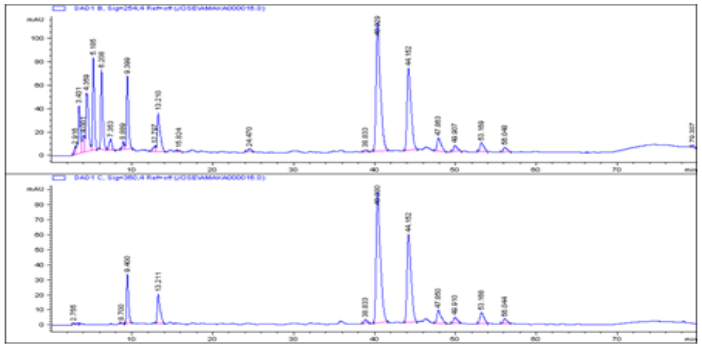
Khaya spp from Ibadan (KS3)



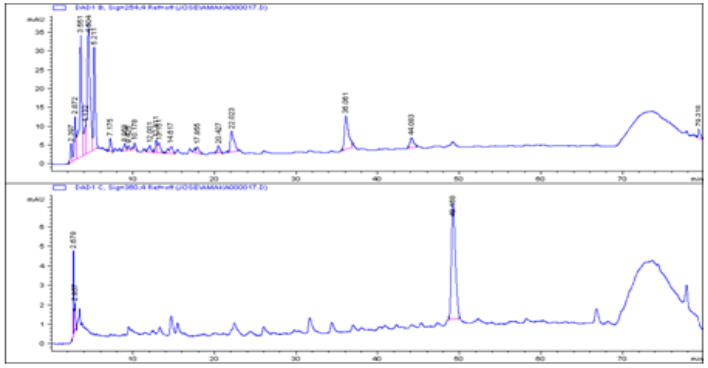
Khaya spp from Agbede (KS2)



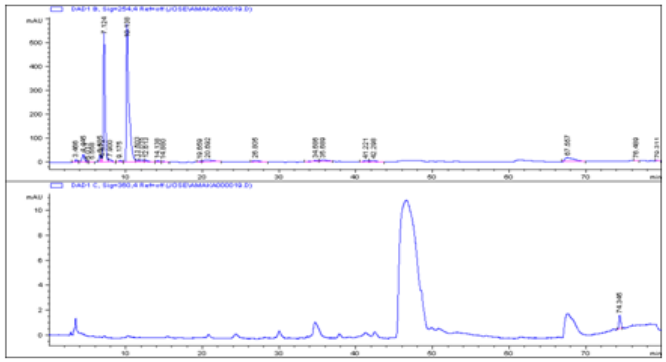
Moringa oleifera leaves (MO1)



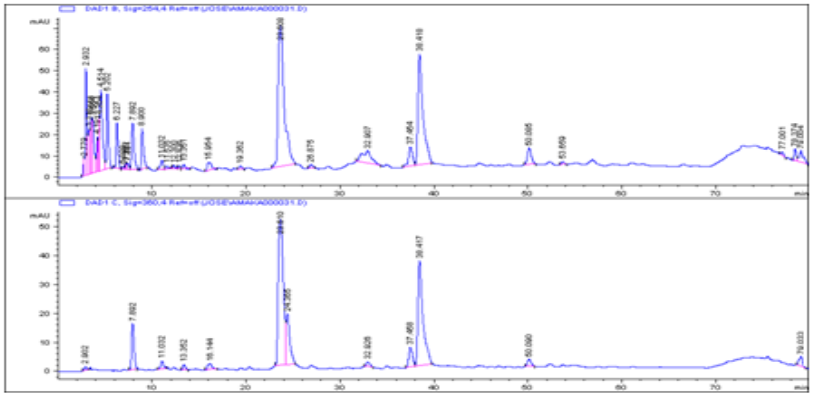
Mondia whitei stem (MW1)

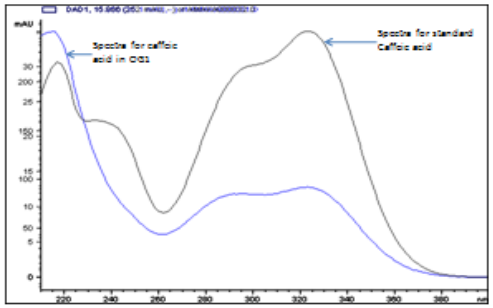


Picralima nitida seeds (PN1)

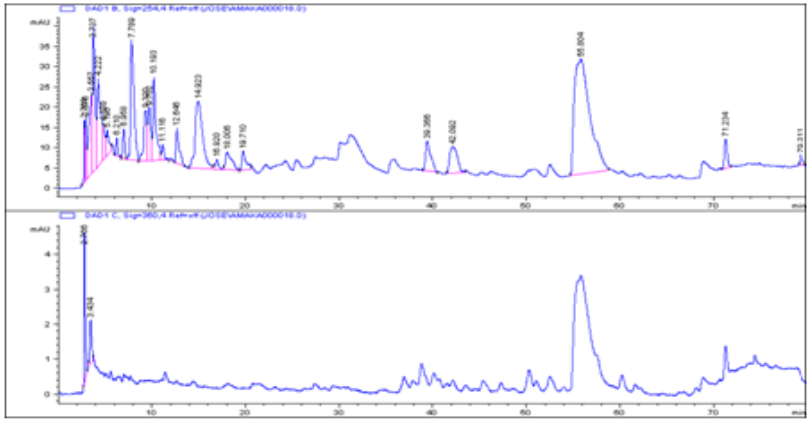


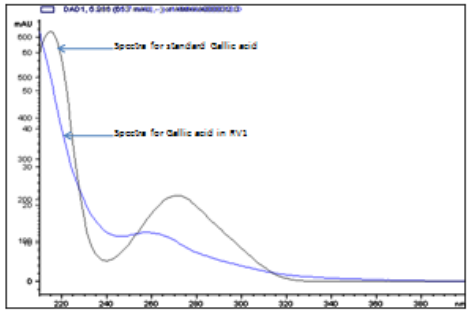
Ocimum gratissimum leaves (OG1)



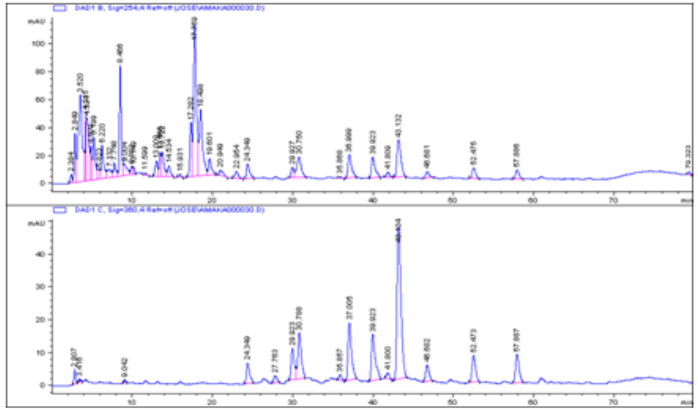


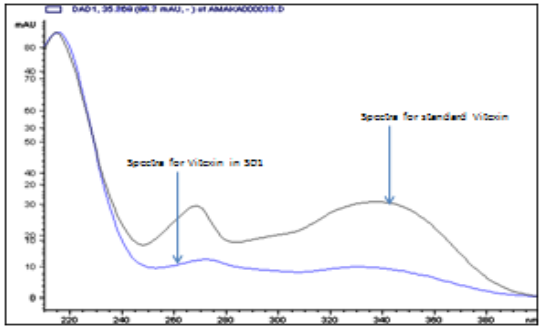
Rauwolfia vomitoria (RV1)



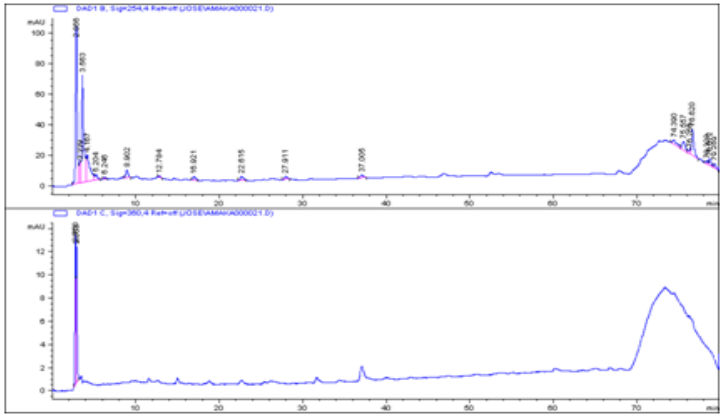


Scoparia dulcis whole plant (SD1)

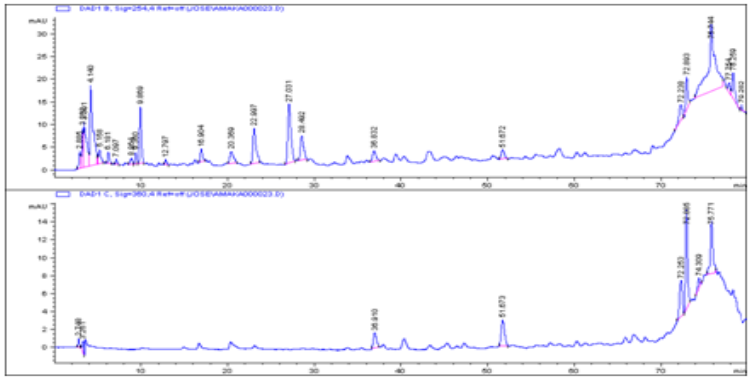




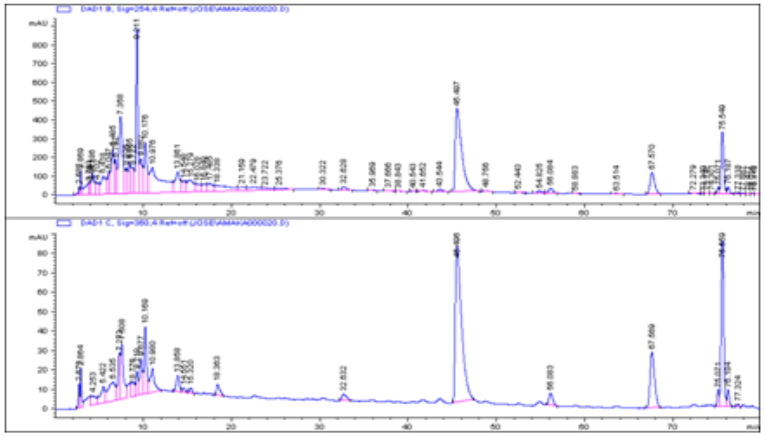
Strophantus hispidus stem (SH1)

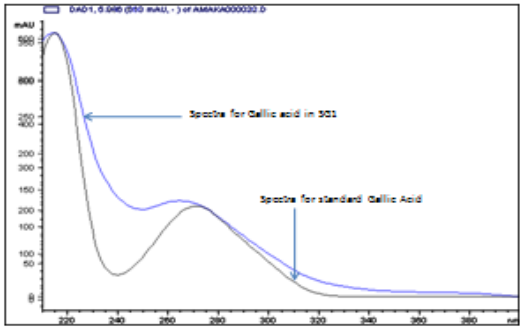


Securidaca longepedunculata (SL1)

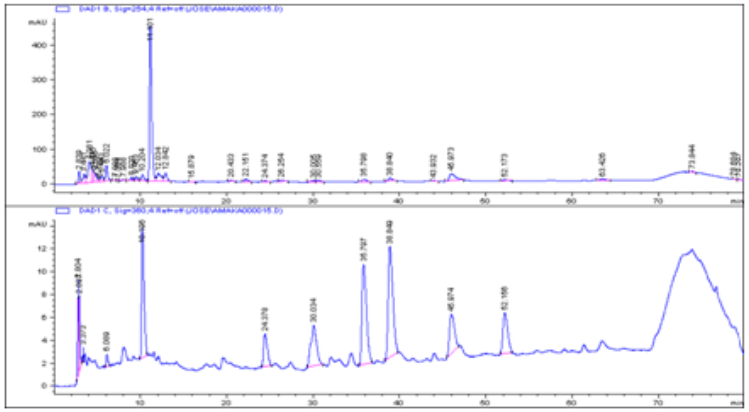


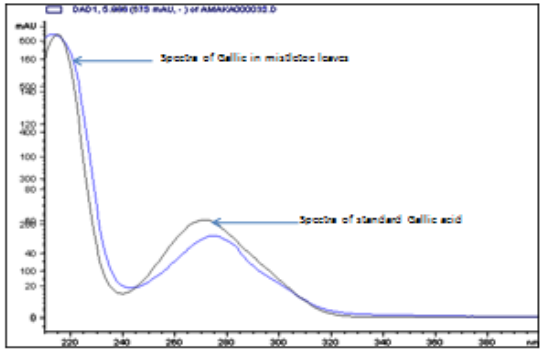
Syzygium guineense stem (SG1)



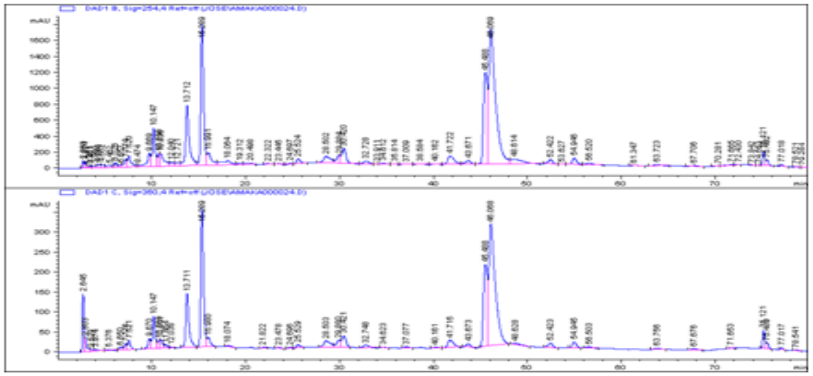


Tapinanthus bangwensis leaves (ML1)

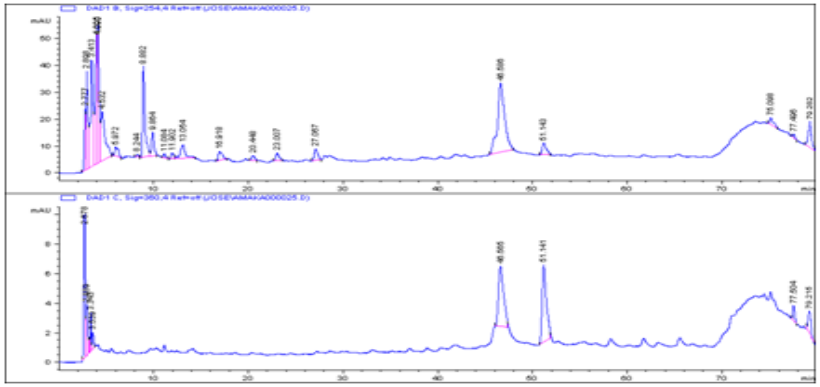




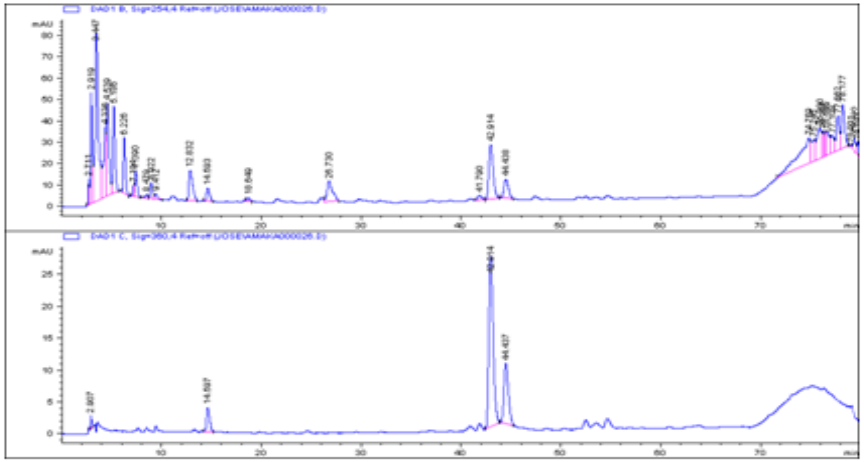
Terminalia aviceenoides stem (TA1)



Tamarindus indica stem (TI1)



Vernonia amygdalina leaves (VA1)



Ximenia americana stem (XA1)