EFFECT OF *HIBISCUS SABDARIFFA* (LINN) WATER EXTRACT ON THE PHARMACOKINETICS OF METFORMIN AND LISINOPRIL IN HEALTHY HUMAN VOLUNTEERS

BY

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Declaration

I declare that the work in this thesis entitled '**EFFECT OF *HIBISCUS SABDARIFFA* (LINN) WATER EXTRACT ON THE PHARMACOKINETICS OF METFORMIN AND LISINOPRIL IN HEALTHY HUMAN VOLUNTEERS**' has been carried out by me in the Department of Pharmaceutical and Medicinal Chemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

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The thesis entitled '**EFFECT OF *HIBISCUS SABDARIFFA* (LINN) WATER EXTRACT ON THE PHARMACOKINETICS OF METFORMIN AND LISINOPRIL IN HEALTHY HUMAN VOLUNTEERS**' by Ibrahim NASIR meets the regulations governing the award of the degree of Doctor of Philosophy (Ph.D) in Pharmaceutical Chemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Abstract

The concurrent use of herbal drugs and orthodox medicines for the treatment of various health conditions is a common practice amongst patients; a practice that could result in drug-herb interaction. This study evaluates the effects of *Hibiscus sabdariffa*water extract on the*in vitro* and *in vivo* availability, as well as pharmacokinetics of metforminand lisinopril in healthy human volunteers.For the determination of metformin and lisinopril *in vitro*in simulated physiological pHs(0.1 M HCl, phosphate buffers pH of 6.8 and 7.4), three simples UV spectrophotometric methodswere developed and validated according to ICH guidelines. The *in vitro*availability of metformin and lisinopril alone and in presence of *Hibiscus sabdariffa* calyxes water extracts (1 g/L) in each of the three media(1 L and 900 mL) were determinedusing dissolution apparatus (BP, 2013) set at 100and 50 rpm at 37 °C. Samples (5 mL) withdrawn from the media at 5, 10, 15, 30, 45, and 60 min were analysed for the metformin and lisinopril content released using the appropriate developed method. For the quantification of metformin and lisinopril in the *in vivo*study, a RP-HPLC method was developed and validated for each drug using caffeine as internal standard IS.The optimized conditions comprise of a Chemsl ODS®C_{18} column (200 x 4.6 mm), binary mobile phase (Methanol: Water 80:20)containing 0.1 % orthophosphoric acid as additive, isocratic elution mode, 232 and 218 nm detection wavelengths flow rate of 1.0 mL/min and 10 µL injection volume. The*in vivo* study involved 48 apparently healthy human volunteers randomly selected and divided into four groups of 12 subjects each. The study was carried out in four phases; phase 1 (administration of the drugs alone), phase 2 (concurrent administration of drugs with *Hibiscus sabdariffa* calyxes water extracts), phase 3 (administration of drugs30 min after administration of *Hibiscus sabdariffa* water extracts) and phase 4 (administration of *Hibiscus sabdariffa*water extracts 30 minutes after administration of drugs). Saliva samples collected at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 12.0, 24.0 and 36.0 hrin each phase were analysed

for metformin and lisinopril content using the appropriate developed RP-HPLC method.The results indicated that the UV spectrophotometric methods obeyed Beer's law within the calibration range (2.5 to 15 μg/mL) with correlation coefficients of 0.999. The $\lambda_{max}$for metformin/lisinopril observed in 0.1 M HCl, phosphate buffer pH of 6.8 and 7.4 were 205/215, 235/210 and 232/210 nm respectively.The presence of *Hibiscus sabdariffa* significantly ($p < 0.05$) increased the *in vitro* availability of metformin and lisinopril in simulated gastric and intestinal pHs. In simulated blood pH however, the availability of metformin was significantlydecreased($p < 0.05$) by the presence of *Hibiscus sabdariffa*while that of lisinopril was significantly increased. *In vivo* availability of metformin and lisinopril were significantly ($p < 0.05$) increased by the presence of *Hibiscus sabdariffa*in all phases.For the RP-HPLC methods, retention times of 1.6 and 1.7 min were observed for metformin and lisinopril respectively. Pharmacokinetic parameters of the drugs administered alone and when interacted with *Hibiscus sabdariffa* calyxes water extracts showed that metformin administered alone achieved C_{max} of 892.320ng/mL at 5 hr (T_{max}), while lisinopril attained C_{max} of 95.360ng/mL at 7 hr (T_{max}). Furthermore,AUC_0^{∞} of5460216.284ng.hr/mLand736805.828ng.hr/mLwere recorded for metformin and lisinopril respectively.Small increases in $C_{max}$and AUC_0^{∞} were observed when both metformin and lisinopril were interacted with *Hibiscus sabdariffa*in all the phases.However, the increases observed in all the pharmacokinetic parameters for all phases were not statistically significant ($p < 0.05$). It can therefore be concluded that *Hibiscus sabdariffa* calyxes water extract does not significantly affect the pharmacokinetics of metformin and lisinopril in healthy humans.

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Abreviations

IDDM	Insulin-Dependent Diabetes Mellitus
NIDDM	Non-Insulin-Dependent Diabetes Mellitus
UKPDS	United Kingdom Prospective Diabetes Study
ACEI	Angiotensin Converting Enzyme Inhibitor
LDL	Low Density Lipoprotein
HDL	High Density Lipoprotein
SBP	Systolic Blood Pressure
DBP	Diastolic Blood Pressure
UV-VIS	Ultraviolet-visible
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
BMI	Body mass index
SD	Standard Deviation
ABUCUHSR	Ahmadu Bello University Committee for the use of human Subjects for Research
IDF	International Diabetes Federation
P-gp	P-glycoprotein
MDRl	Multi Drug Resistance Protein
CYPs	Cytochrome P450
QSAR	Quantitative Structure Activity Relationships
cDNA	Complementary Deoxyribonucleic Acid
Caco-2	Colon Carcinoma Cells/ Human Epithelial Cell Line
FDA	Food and Drug Administration
PXR	Pregnane X Receptor

HIV	Human Immunodeficiency Virus
BP	British Pharmacopoeia
USP	United States Pharmacopoeia
BC	Before Christ
HCA	Hydroxycitric Acid
PCA	Protocatechuic Acid
EDNO	Endothelium Derived Nitric Oxide
cGMP	Cyclic Guanosine Monophosphate
AGE	Advanced Glycation End Products
TBARs	Thiobarbituric Acid Reactive Substance
Akt	Protein Kinase B
LD	Lethal Dose
λmax	Wavelength of Maximum Absorption
LOD	Limit of Detection
LOQ	Limit of Quantification
IR	Infrared Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
IS	Internal Standard
PAS	Photoacoustic Spectroscopy
GC	Gas Chromatography
API	Active Pharmaceutical Ingredient
LC	Liquid Chromatography
rpm	Revolutions Per Minute
K_a	Absorption Rate Constant
K_E	Elimination Rate Constant

$T_{1/2}$	Half Life
C_{max}	Maximum Plasma Drug Concentration
T_{max}	Time at which C_{max} is attained
Cl	Clearance
V_d	Volume of Distribution
AUC_0^t	Last Area Under Curve
AUC_t^∞	Extrapolated Area Under Curve
AUC_0^∞	Total Area Under Curve

CHAPTER ONE

1.0 INTRODUCTION

1.1 Comorbidity of diabetes mellitus with hypertension

Diabetes mellitus and hypertension have emerged as major medical and public health issue for cardiovascular diseases (Marwa *et al.*, 2017).Hypertension occurs with twice the frequency in the diabetic compared with the nondiabetic population, and up to 50% of patients with type 2 diabetes mellitus are reported to become hypertensive.The reasons proposed for this increased prevalence are controversial, but insulin resistance has been implicated (Raeven, 1996). In addition to being a major risk factor for atherosclerosis in large blood vessels, hypertension in diabetes appears to contribute to small vessel disease and is a risk factor for diabetic nephropathy and possibly for diabetic retinopathy. The United Kingdom prospective diabetes study (UKPDS, 2000) group has reportedthat tight control of blood pressure (with a target of <150/85 mmHg) reduces the risk of diabetes-related death and diabetic complications, including diabetic retinopathy, in Type 2 diabetics.

Hypertension is said to affect about one billion people worldwide and the figure is estimated to rise to 1.56 billion by 2025 (Jakovljevic and Ostojic, 2013; Mohan *et al.,* 2013). On the other hand, the estimated number of people living with diabetes in Africa is currently 12.1 million, with a recent projection showing that, the number will reach 239 million by 2030 (Mohan *et al.,* 2013). According to International Diabetes Federation (IDF) estimates for 2012, it is approximated that 80% of those affected by diabetes are in Sub-Saharan Africa, and 81.2% of the cases are undiagnosed or are not aware they have diabetes (Marwa *et al.*, 2017).Diabetes mellitus (DM) is a group of disorders of carbohydrate metabolism in which the action of insulin is diminished or absent through altered secretion, decreased insulin activity, or a combination of both factors. It is characterised by hyperglycaemia. DM may be

categorised into several types but the two major types are Type 1 (insulin-dependent diabetes mellitus; IDDM) and Type 2 (non-insulin-dependent diabetes mellitus; NIDDM). Malnutrition-related diabetes is no longer considered a separate entity (Sweetman, 2002). Type 1 DM is present in patients who have little or no endogenous insulin secretory capacity and who therefore require exogenous insulin therapy for survival. The associated hypoinsulinaemia and hyperglucagonaemia put such patients at risk of ketosis and ketoacidosis. Obesity is present in the majority of Type 2 patients; non-obese patients tend to have low insulin secretory capacity (although not as low as in type 1 diabetes) rather than appreciable insulin resistance. It is closely associated with cardiovascular disease, and the Type 2 may be described as the metabolic syndrome.DM Type 2 is often associated with arterial hypertension (Pronina *et al*., 2010).

Hypertension is extremely widespread in patients with Type 2 diabetes mellitus and it is reported to be around 40 – 80 % (Abougalambou *et al*., 2011). Caocalvo *et al*. (2006) reported that patients with long term diabetes had associated hypertension in 50 – 75 % of the cases. This high rate is partly explained by associated obesity, older age and essential hypertension (Tank *et al*., 2002). It was also reported by Abougalambou and Ayman (2013) that hypertension is extremely common disease found in patients with DM and that 80 – 90 % of patients with Type 2 diabetes mellitus will develop hypertension and about 20 % of hypertensive patients develop diabetes.

1.1.1 Treatment of diabetes and hypertension

All the main groups of antihypertensive drugs can be used in diabetics (Kaplan, 2001) and most patients will require at least two drugs to achieve target blood pressure. ACE inhibitors have been particularly recommended, as there is evidence of benefit in preserving renal function in patients with nephropathy; they have been reported to decrease proteinuria and

preserve glomerular filtration rate in diabetic patients independently of changes in systemic blood pressure (Abougalambou *et al.*, 2011). In the United Kingdom, prospective diabetes study (UKPDS) treatment with an ACE inhibitor (e.g captopril) or a beta blocker (e.g atenolol) was equally effective in reducing the risk of diabetic complications, although the ACE inhibitor appeared to be better tolerated.Although there has been concern regarding the safety of calcium-channel blockers, studies have confirmed that long-acting calcium-channel blockers are also a suitable choice (Abougalambou *et al.*, 2011; Peters*et al.*, 2015).

Currently available therapy for diabetes includes insulin and various oral hypoglycemic agents such as sulfonylureas, metformin, glucosidase inhibitors, troglitazone. But these are reported to produce serious adverse side effects such as liver problems, lactic acidosis and diarrhea (Seidowsky*et al.*, 2009). Diabetes is currently affecting around 143 million people (Mentreddy *et al.*, 2005) and the number of those affected is increasing day by day, by 2030 it is predicted to reach 366 million population worldwide (Ponnusamy *et al.*, 2011).

1.2Role of herbs in the treatment of diabetes and hypertension

Herbal medicine is used for treatment of diabetes in developing countries where the cost of conventional medicines is a burden to the population (Saravanan and Pari, 2008). Despite the introduction of hypoglycemic agents from natural and synthetic sources, diabetes and its secondary complications continue to be a major medical problem. Many indigenous medicinal plants have been found to be useful to successfully manage diabetes. One of the great advantages of medicinal plants is that they are readily available and have very low side effects. Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them.About 800 plant species have been reported to possess antidiabetic and antihypertensive properties. Several plant species have been used for prevention or management of diabetes by the native Americans,

Chinese, South Americans and Asian Indians (Seidowsky*et al*., 2009). A study conducted showed that Asia and Africa continents have 56 % and 17 % share of the worldwide distribution of therapeutic herbal plants respectively (Chun-Hung *et al.,* 2012). Biological actions of isolates from the medicinal plants are related to their chemical composition most of which are rich in phenolic compounds, alkaloids, flavonoids, terpenoids, coumarins, and glycosides. On the other hand, many conventional drugs for treatment of hypertension (e.g reserpine) and diabetes(e.g metformin) are secretagogues which have a plant origin (Grover *et al*., 2002).*Hibiscus sabdariffa*L.is among the herbs that are reported to possess both the antihypertensive and antihyperglycaemic potential(Alarcon *et al.,* 1998).

Animal studies have consistently shown that consumption of *Hibiscus sabdariffa* extract reduces blood pressure in a dose dependent manner. They have also shown that total cholesterol, LDL-C, and triglycerides were lowered in the majority of normolipidemic, hyperlipidemic, and diabetic animal models, whereas HDL-C was generally not affected by the consumption of *Hibiscus sabdariffa*L. extract. The daily consumption of *Hibiscus sabdariffa* calyx extracts significantly lowered SBP and DBP in adults with pre-to moderate essential hypertension and type 2 diabetes. In addition, *Hibiscus sabdariffa* tea was as effective at lowering blood pressure as captopril, but less effective than lisinopril (Allison *et al*., 2013).*Hibiscus sabdariffa L.* extracts have been reported to show antibacterial, antioxidant, nephro and hepato-protective, renal or diuretic effect, effects on lipid metabolism (anti-cholesterol), antidiabetic and antihypertensive effects among others(Da-Costa-Rocha *et al*., 2014). This might be linked to strong antioxidant activities, inhibition of α-glucosidase and α-amylase, inhibition of angiotensin converting enzymes (ACE), and direct vasorelaxant effect or calcium channel modulation. Phenolic acids (especially protocatechuic acid), organic acid (hydroxycitric acid and hibiscus acid) and anthocyanins

(delphinidin-3-sambubioside and cyanidin-3-sambubioside) that were isolated from the plant are likely to contribute to the reported effects (Da-Costa-Rocha *et al*., 2014).

The reported traditional and pharmacological uses of *Hibiscus sabdariffa*water extract are likely the reason behind it been taken concurrently with metformin and lisinopril especially in people with comorbid conditions (Type 2 diabetes and hypertension) to probably have synergistic effect to alleviate their condition, a practice that may or may not be dangerous. In view of these, there is need to evaluate such practice by establishing the pharmacokinetic profile of the drugs in question and the resulting effect when interacted with *Hibiscus sabdariffa*L. waterextract.

1.3 Drug herb interaction

Drugs have a great potential for interactions with other drugs, foods,herbs and diseases. These interactions may be beneficial or harmful (Amorha *et al*., 2013). There is emerging challenge in the medical literature with respect to documentation of case reports of adverse interactions between herbal medicines and conventional drugs. Thus, it is important to anticipate when a potential drug interaction might have clinically significant consequences for the patient so that advice may be given to minimize the risk of harm. This may be achieved by avoiding the combination, making dosage adjustments, spacing dosing times or close monitoring of patient (Lee and Stockley, 2007). There is need to evaluatedrug-herb interactions especially due to an increase in the global acceptance in the use of herbal medicinescurrently (Hopkins *et al*., 2013).

1.4Statement of research problem

Some patients take their prescription drugs with herbs which can result in possible serious interactions(Chen *et al*., 2012). It was reported that 47.5% of the diabetic and hypertensive

respondents from a secondary health care facility in Maiduguri, a town in north east Nigeria, in addition to orthodox medications prescribed, used herbal remedies (Olisa and Oyelola, 2009). These patients were oblivious of the possibility of drug-herb interactions and probable complications (Olisa and Oyelola, 2009;). It has been recommended that, particularly for drug action that is intensified by similar effect of herbs addition of herbal therapy should be avoided when the drug therapy is already addressing the therapeutic goal and only a well-reasoned analysis of the potential for drug herb interaction will often permit the use of drugs and herbs together (Subhuti, 2000). For comorbid patients living with hypertension and Type 2 diabetes, the most frequently prescribed drugs are metformin and lisinopril (Jacoba*et al.*, 2004; Abougalambou *et al.*, 2011).A good number of these patients take their prescribed drugs with *Hibiscus sabdariffa*water extracta practicethatneeds to be evaluated.

1.5Justification of the study

Diabetes mellitus type 2 is often associated with arterial hypertension and they are treated concurrently (Pronina *et al.*, 2010). For comorbid Type 2 DM and hypertensive,metformin and an ACE inhibitors have been particularly recommended and are the most frequently prescribed drugs as there is evidence of benefit in preserving renal function and glomerular filtration rate (Abougalambou *et al.*, 2011). Studies both on experimental animal model and humans have reported the antidiabetic and antihypertensive potentials of *Hibiscus sabdariffa*L. water extracts (Da-Costa-Rocha *et al.*, 2014). The ethno pharmacological claims of *Hibiscus sabdariffa*L. water extract in lowering BP, antihyperglycaemic effect makes these comorbid patients to be taking these drugs concurrently with the extract justify the need to evaluate the effect of *Hibiscus sabdariffa*calyxes extract on the pharmacokinetics profile of these commonly prescribed drugs.To the best of our knowledge, there is no documented study

on the interaction between metformin, lisinopril and *Hibiscus sabdariffa*L. calyxes water extract.

1.6Aim of the study

This research is aimed at evaluating the effect of *Hibiscus sabdariffa* L. water extract on the pharmacokinetics of metformin and lisinopril in healthy human volunteers.

1.7 Objectives of the study

Thespecific objectives of this study are to:

1. collect, identify and prepare*Hibiscus sabdariffa* calyxes water extract.
2. sample and carry out quality control studies on metformin and lisinopril tablets to be used.
3. develop and validate a UV-vis spectrophotometric method for the determination of metformin/lisinopril in dissolution media pH 1.2, 6.8 and 7.4.
4. develop and validate a RP-HPLC method for the determination of metformin/lisinopril in human saliva.
5. determine the pharmacokinetic parameters of metformin and lisinopril in healthy human volunteers.
6. determine the respective pharmacokinetic parameters of metformin and lisinopril when interacted with *Hibiscus sabdariffa* L. water extract in the volunteers.

1.8Research Hypothesis

The pharmacokinetic parameters of metformin and lisinopril are not significantly affected when the drugs are interacted with *Hibiscus sabdariffa* L. water extract.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Herbal Medicines

Herbal medicines are accepted and commonly used worldwide with a hope of improving health conditions and managing various diseases such as pains, cold, inflammation, heart diseases, and diabetes. However, their mechanisms of action are generally unknown and there is a lack of clinical efficacy and safety data (Sahoo *et al.*,2010). An estimated one fourth of adults in developed countries and more than 80% of the population in most developing countries utilize herbal medicines. The 2002 National Health Interview Survey of the United States (n = 5,456) indicates that 19 % of adults used herbal supplements within the past 12 months of February, 2007 to January, 2008 (Mehta *et al.,* 2008). In a study including 61, 587 individuals aged 50 to 76 years in the United States, one third (20, 528) of the population stated that they were using herbal supplement (Gunther *et al.,* 2004). Based on the 2002 National Health Interview Survey, an estimated 38.2 million adults in the United States used herbs and supplements in 2002 (Kennedy, 2005). To date, there are more than 11,000 species of herbal plants that are in use worldwide and about 300 species are commonly used in Asian and other countries.

African traditional medicine in Nigeria has survived through the worst catastrophe of human history, the slave trade era (Spitzer, 2002). This is evident in the level of dependence of Nigerian locals in the twenty-first century on this medicinal system which has outlived several

other systems and also in thriving African communities of Nigerian roots in diaspora, particularly South American countries such as Brazil, Peru which have strong, thriving ethnomedicinal practices till date (Carney, 2003; Mitchell and Ahmad, 2006). During the slavery period, slaves from West Africa particularly Nigeria, usually predominate and emerge as the traditional healers of the African settlements (Carney, 2003). A study conducted which covered 66.6% of Edo state, central southern Nigeria, reported the interview of about 189 traditional medical practitioners using semi-structured questionnaire administered by trained interviewers. The study indicated 70 plants belonging to 67 genera in 43 families are commonly prescribed, with 39 species of these plants being cultivated, while 29 species grew in the wild, and 2 plants are both cultivated and grew in the wild. Herbal antihypertensive and antihyperglycaemic recipes were prepared as decoctions, infusions, powders and or juice. Plants which were frequently prescribed included *Allium species*, *Persea americana*, *Zingiber officinale*, *Hunteria umbellata,* and *Rauwolfia vomitoria*(Gbolade, 2012). Another study carried out in Sokoto north west Nigeria reported the interview of 40 traditional medicine practitioners and herbal sellers (65% males and 35% females) using semi-structured questionnaires and open-ended conversations. The study indicated 34 plants belonging to 30 families with reports of validation of some of the antihypertensive and antihyperglycaemic plants some of which include*Allium sativum,Commiphora kastingii,Moringa oleifera* and *Hibiscus sabdariffa* (Raji *et al.,* 2013).

2.2 Herb-drug interaction

Herbal remedies are often co-administered with orthodox drugs, raising the potential for herb-drug interactions, which may have important clinical significance based on an increasing number of clinical reports of such interactions (Chen *et al.,* 2012). The interaction of herbal medicines with prescribed drugs is a significant safety concern, especially for drugs with narrow therapeutic indices (e.g. warfarin and digoxin). Because the pharmacokinetics and/or

pharmacodynamics of the drug may be altered by combination with herbal remedies, severe and perhaps even life threatening adverse reactions may occur in clinical practice. Due to the clinical significance of herbal interactions with conventional drugs, it is important to identify herbs and drugs that may interact with each other and cause clinical consequences (Chen *et al.,* 2012).

2.2.1 Approaches to identifying herb-drug interactions

It is important to identify drugs that can interact with herbal medicines using proper *in vitro* and *in vivo* models to avoid or minimize herb-drug interactions. Such models have very different cost, reliability and possibility for high throughput studies. Thus, these models may be used in combination to obtain sufficient information that is useful for providing warning and proper professional advices to patients in clinical practice or consumers of herbal remedies (Chen *et al.,* 2012). There is an increasing use of in-silico methods to study human cytochrome P450s (CYPs), Phase Il enzymes, P-glycoprotein (P-gp/MDRl./ABCBl) and their interactions with xenobiotics including herbal medicines and conventional drugs (Jacob *et al.,* 2009). The in-silico methods mainly include simple rule-based modelling, structure activity relationships, three-dimensional quantitative structure activity relationships (QSAR) and pharmacophore modelling (Khakar, 2010). Knowledge of substrate specificity and regulation of the CYPs is important, as this will provide information on the possible drug interactions with herbal medicines. A number of *in vitro* systems are available to investigate the potential for drug interactions with herbal medicines. For metabolic drug interactions, the major models include subcellular fractions (liver microsomes, cytosols and homogenates), precision-cut liver slices, isolated and cultured hepatocytes or liver cell lines, and cDNA-expressed enzymes (Asha and Vidyavathi, 2010). For transport studies, human epithelial cell line Caco-2, MDCKII, oocytes with highly expressed drug transporters, membrane vesicles and cDNA expressed drug transporters are widely used (Giacomini *et al.*, 2010). Recent

advancement has been made in the development of *in vitro* analogues to physiologically-based pharmacokinetic models that represents the body as interconnected compartments specific for a particular organ (Maguire *et al.*, 2009). Each of these systems has advantages and limitations, and a combination of these methods will provide the most accurate information on how herbal medicines inhibit or induce CYPs and P-gp and the potential for herb-drug interactions. For example, cultured human hepatocytes provide cellular integrity with respect to enzyme architecture, and allow the study of Phase I and Il reactions and transport; however, some drug transporters and enzymes are rapidly down-regulated after isolation of hepatocytes (Gebhardt *et al.,* 2003).

The application of genomic and proteomic techniques and incorporation of systems biology approaches to the study of herb-drug interactions has the potential to lead to a more effective screening due to the high-throughput capacity and insights into the mechanisms for herb-drug interactions (Chen *et al*., 2012). The application of high throughput approaches to the study of herb-drug, herb-CYP and herb-P-gp interactions is becoming possible (Foti *et al.,* 2010; Giacomotto and Segalat, 2010). Animal models are widely used for the evaluation of new drugs, drug-drug interactions, and herb-drug interactions (Amore *et al.,* 2010). When drug interactions with an herbal remedy are suspected to be likely or significant in animal studies, they should be confirmed by well-designed clinical studies. Selective and specific probes should be chosen for *in vivo* drug interactions studies when modulation of CYP and P-gp is a possible interaction mechanism (Chen *et al.,* 2012). In some cases, pharmacogenetic studies can be incorporated to explore the interplay of genetic mutations and combined use of herbal medicines (Yin *et al.,* 2004). This is particularly important when some herbal medicines are consumed by specific ethnic groups who carry characteristic genetic mutations of genes encoding key drug metabolizing enzymes and drug transporters (Chen *et al.,* 2012).

2.2.2 Potential mechanisms for herb-drug interactions

The mechanisms for most reported drug interactions with herbal medicines have not been fully elucidated. As with drug-drug interactions, both pharmacokinetic and pharmacodynamic mechanisms are implicated in these interactions (Chen *et al.,* 2012).

2.2.2.1 Pharmacokinetic mechanisms

Alterations in absorption, metabolism, distribution or excretion of drugs are the cause for pharmacokinetic interactions. Altered drug metabolism by herbal medicines is often a result of CYP induction and/or inhibition (Zhou *et al.*, 2003). The most well-studied and understood example of this is the induction of CYP3A4 and 2B6 by St John's wort (*Hypericum perforatum*) in humans. Of the components of St. John's wort, hyperforin is reported to be the active constituent and it is the most potent agonist for pregnane X receptor PXR (Moore *et al.*, 2000). Some herbal medicines including ginkgo (Ginkgo biloba), gugulipid (*Commiphora mukul*) and licorice (*Glycyrrhiza uralensis*) have been reported to activate human PXR (Chen *et al.*, 2012). Due to the important role of P-gp in drug transport and excretion, modulation of P-gp by herbal medicines may have significant pharmacokinetic consequences (Zhou *et al.,* 2004). St John's wort induces intestinal P-gp *in vitro* and *in vivo*. Oral administration of St John's wort for 14 days in healthy volunteers resulted in a 1.4-fold increase in P-gp expression (Durr *et al*., 2000). The substrates of P-gp, fexofenadine and digoxin which are often used as probes for examining P-gp activity *in vivo*, were found to have increased clearance in healthy subjects treated with St. John's wort (Dresser *et al.*, 2003). However, there is rare clinical evidence for altered protein binding of drugs by herbal medicines. Given that many herbal components are highly bound by plasma proteins, they may displace drugs from the binding sites (Chen *et al.*, 2012). Herbal medicines are often administered orally and they can attain moderate to high concentrations in the gut lumen (the primary site of absorption for most orally-administered drugs) and liver, and may exert a

significant effect on enterocytes and hepatocytes. Both P-gp and CYP3A4 are abundantly expressed in the villus tip of enterocytes and hepatocytes (Berggren *et al.,* 2007). The interplay of both intestinal P-gp and CYP3A4 has a strong effect on the bioavailability of many orally-administered drugs including cyclosporine, midazolam, statins, HIV protease inhibitors and verapamil (Kato *et al.*, 2003). Thus, the modulation of intestinal and hepatic P-gp and CYP3A4 by herbal medicines represents a potentially important mechanism by which the bioavailability of coadministered drugs can be modulated (Chen *et al.*, 2012).

2.2.2.2 Pharmacodynamic mechanisms

Altered pharmacokinetics almost inevitably leads to a significant change in response to drugs that have narrow therapeutic indices (example warfarin and digoxin). However, given that a single herbal preparation may contain more than 100 components, all of which may have unknown biological activities, herbal medicine can potentially mimic, increase, or reduce the effects of co-administered drugs through simultaneous effects on the same drug targets (Fugh-Berman, 2000). If the effect of the orthodox drug in combination with the herbal medicine is enhanced (synergistic or additive effect), which can be unfavorable on target, this may result to toxicity. In contrast, some herbal medicines may contain compounds with antagonistic properties, which are likely to reduce drug efficacy and produce therapeutic failure. The synergistic or antagonistic effects between herbs and drugs often result from the competitive or complementary effect of the drug and the coadministered herbal compounds at the same drug targets. For example, herbal medicines such as danshen, ginkgo, ginseng, ginger and garlic may enhance the anticoagulant activity of warfarin by acting on the same drug target vitamin K epoxide reductase or other critical components in the clotting system which may increase bleeding in people on chronic warfarin therapy (Greenblatt and Von-Moltke 2005; Chen *et al.*, 2012).

2.2.3 Clinical outcomes of herb-drug interactions

When a drug's clearance is significantly altered, or its drug targets are the same as the herbal components, a clinically important herbal interaction with the drug may occur. The clinical outcome of an herb-drug interaction varies, being well tolerated, mild, or sometimes lethal (Chen *et al.,* 2012).

2.2.3.1 Altered drug plasma concentration

Herbal medicines that are able to modulate intestinal and hepatic CYPs and P-gp often alter the oral absorption, bioavailability, systemic exposure and clearance of coadministered drugs (Zhou*et al*., 2004; Izzo and Ernst, 2009). For example, long-term treatment of St John's wort reduces the plasma levels of co-administered cyclosporine, amitriptyline, imatinib, nifedipine, digoxin, indinavir, nevirapine, oral contraceptives, warfarin, phenprocoumon, theophylline, and simvastatin (Chen *et al*., 2012). Garlic preparations decrease the plasma concentrations of saquinavir, but not ritonavir (Piscitelli *et al.*, 2002). Consequently, drug efficacy may be changed and toxicity may occur. For example, decreased blood trough concentrations of cyclosporine have been observed in patients also taking St John 's wort and this was associated with transplant graft rejection (Ruschitzka *et al.,* 2000; Moschella and Jaber, 2001).

2.2.3.2 Altered drug efficacy

There are limited clinical studies addressing the effect of combined herbal medicines on drug efficacy due to pharmacokinetic and/or pharmacodynamic mechanisms. When the systemic exposure of a drug is significantly increased or reduced by herbal medicines, the clinical response to this drug may change. A direct additive, synergistic or antagonistic interaction between the drug and herbal medicines will also alter the magnitude of drug response. Many herbal medicines such as danshen (*S. miltiorrhiza*), goldenseal (*Hydrastis canadensis*),

ginseng, horse chestnut (*Aesculus hippocastanum*), red clover (*Trifolium pratense*), turmeric, passionflower (*Passiflora incarnata*), and ginkgo can enhance the anticoagulant effect of warfarin since they contain active compound (coumarin) that possess anticoagulant activity (Wittkowsky, 2008). A case was reported where combined use of *Hypericum perforatum* (St John's wort) and selective serotonin reuptake inhibitors (sertraline and nefazodone) caused symptoms characteristic of central serotonergic syndrome (characterized by symptoms such as lack of co-ordination, hyperflexia, tremor, fever, nausea, diarrhoea, coma, confusion and agitation) in the elderly patients (Chen *et al.,* 2012). It appears that CYP and P-gp induction is not the underlying mechanism for the additive effect of St John's wort on serotonin reuptake inhibitors St John's wort may inhibit serotonin reuptake in the brain and thus enhance the effect of serotonin reuptake inhibitors (Chen *et al.,* 2012).

2.2.3.3 Occurrence of adverse effects

An herb-drug interaction may lead to adverse reactions that may be mild, moderate, or even life-threatening. Mild to moderate adverse events due to herb-drug interactions are generally well tolerated, but some herb-drug interactions may cause severe adverse reactions. For example, when St John' wort was combined with oral contraceptives (ethinylestradiol) or loperamide, it caused intermenstrual bleeding or delirium in some patients (Zhou and Lai, 2008). There are case reports where ginkgo raised blood pressure when combined with a thiazide diuretic and caused coma when combined with trazodone, an atypical antidepressant (Shaw *et al*., 1997; Galluzzi *et al.,* 2000). Only few of these interactions were reported, but for most of them, pharmacodynamic mechanism may provide an explanation, although altered pharmacokinetics may also contribute to the interactions (Chen *et al*., 2012). The clinical outcome of herb-drug interactions depends on factors that are related to the coadministered drug (dose, dosing regimen, administration routes and therapeutic range), herbal medicine (species, chemical composition, dose, dosing regimen, and administration

route) and patients (age, gender, disease status, hepatic and renal function, and genetic polymorphism). Generally, a doubling or more in drug plasma concentration has the potential for enhanced drug effects and/or appearance of adverse effects (Yang *et al.*, 2010). However, less marked changes may still be clinically important for drugs with a steep concentration-response relationship or a narrow therapeutic index (Chen *et al.*, 2012).

2.2.4 Predicting pharmacokinetic herb-drug interactions

It is important to predict clinical herb-drug interactions, since toxic herb-drug interaction may be avoided and reduced by timely warning of the potential risks to patients. It is likely to predict pharmacokinetic drug-drug interactions when proper procedures are followed and there are a number of prediction successes with drugs mainly metabolized by CYPs or which are P-gp substrates (Perdaems *et al.*, 2010). The prediction is possibly successful when the following criteria are met:

1. The clearance of the drug is predominantly through hepatic metabolism (>80%).
2. The drug is not subject to substantial Phase II conjugation or other non-CYP metabolism and
3. The drug does not have absorption problems.

However, the prediction of pharmacokinetic herb-drug interactions appears to be difficult given that herbal medicines comprise a number of active components that may contribute to herb-drug interactions. These active constituents may inhibit and/or induce CYPs and P-gp, and the inhibition/induction of CYPs and P-gp may by temporally distinguishable. Many herbal medicines are administered orally with a chronic regimen and thus they may interact with drugs at the intestine, liver, kidney and targets of action. On the other hand, a number of drug and patient-related factors such as extra-hepatic metabolism, auto-induction, active

transport in the liver, enterohepatic circulation, disease status, renal and hepatic functions, and genetic polymorphisms can affect herb-drug interactions (Izzo and Ernst, 2009; Williamson, 2005; Kennedy and Seely, 2010).

Although it is difficult to precisely predict the potential/extent of pharmacokinetic herb-drug interactions, *in vitro* models such as hepatic microsomes and cultured hepatocytes can provide useful data on how herbal compounds affect the expression and activity of CYPs and P-gp and consequently alter drug metabolism (Zhou *et al.,* 2005). The data from *in vitro* studies can be used to extrapolate *in vivo* situations. An initial qualitative prediction of the potential for herb-drug interactions can be made based on the pharmacological and medicinal properties of the drugs/herbs. If a drug is a high-affinity substrate for CYP3A4 and/or P-gp, the potential for herbal interaction is likely to be high when the herbal medicines contain ingredients that can cause potent inhibition and/or induction of CYPs and P-gp. On the other hand, St John's wort contains potent CYP3A4 and P-gp inducer (hyperforin) and thus it would decrease the oral bioavailability and increase the clearance of coadministered drugs that are good substrates of CYP3A4 and P-gp (Chen *et al*., 2012).

2.2 Metformin

Metformin (I) is chemically 1,1-dimethylbiguanide hydrochloride, a biguanide antidiabetic appearing as white or almost white crystals with melting point of 222 – 226ºC and content range 98.5 – 101.0 % dried substance, freely soluble in water, slightly soluble in alcohol, practically insoluble in acetone and in methylene chloride (BP, 2013). Physicochemical features

H_3C

NH NH_2

H_3C

HN HN

I

of metformin showed that the drug has pKa values of 2.8 and 11.5, so exists very largely as the hydrophilic cationic species at physiological pH values. The metformin pKa values make metformin a stronger base than most other basic drugs with less than 0.01% unionized in blood. The lipid solubility of the unionized species is slight as shown by its low logP value [log (10) of the distribution coefficient of the unionized form between octanol and water] of -1.43. These chemical parameters indicate low lipophilicity and, consequently, rapid passive diffusion of metformin through cell membranes is unlikely. The logP of metformin is less than that of phenformin (-0.84) because two methyl substituents on metformin impart lesser lipophilicity than the larger phenyl ethyl side chain in phenformin. More lipophilic derivatives of metformin are presently being investigated with the aim of producing prodrugs with better oral absorption (Garry *et al.*, 2011; Stella *et al.,* 2015).

2.2.1 Pharmacokinetics of metformin

Metformin hydrochloride is slowly and incompletely absorbed from the gastrointestinal tract.Peak plasma concentrations (C_{max}) are reached within one to three hours of taking immediate-release metformin and four to eight hours with extended-release formulations.the absolute bioavailability of a single 500-mg dose is reported to be about 50 to 60% under fasting conditions, and is absorbed slowly, although this is reduced somewhat if taken with food. Once absorbed, plasma protein binding of metformin is negligible, as reflected by its very high apparent volume of distribution(300–1000 L after a single dose)the drug is excreted unchanged in the urine. The average elimination half-lifein plasma is 6.2 hr.

Metformin is well distributed and appears to accumulate in red blood cells, with a much longer elimination half-life of 17.6 hr. The range is from 18.5 to 31.5 hr in a single-dose study of non-diabetic people.Metformin is distributed into breast milk in small amounts (Sweetman, 2002; Robert *et al*., 2003).

2.2.2 Interactions of metformin with other drugs and herbs

Use of a biguanide with other drugs that lower blood-glucose concentrations increase the risk of hypoglycaemia, while drugs that increase blood glucose may reduce the effect of biguanide therapy. In general, fewer drug interactions have been reported with biguanides than with sulfonylureas. Alcohol may increase the risk of lactic acidosis as well as of hypoglycaemia. Care should be taken if biguanides are given with drugs that may impair renal function (Sweetman, 2002; Garry *et al*., 2011). Metformin has been reported to diminish the activity of anticoagulant phenprocoumon. Fatal lactic acidosis has been reported in a patient given metformin with antivirals *Didanosine*, *Stavudine*, and *Tenofovir.*Cimetidine increased plasma-metformin concentrations in 7 healthy subjects. The renal clearance of metformin was reduced; competition for proximal tubular secretion was considered responsible. A reduction in metformin dosage may be required in patients taking metformin and cimetidine, in order to reduce the risk of lactic acidosis. Platelet counts in 10 diabetic patients receiving biguanides fell (markedly in 3 patients) when they were also given ketotifen.Counts returned to normal a few days after the end of ketotifen therapy. However, the investigators did not consider the effect clinically significant (Sweetman, 2002).

Study conducted showed significant increase in the pharmacokinetics parameters C_{max}, and AUC_0^∞ of metformin interacted with *Allium sativum* in rat plasma with repeated dose administration. This study was reported by Kumar *et al.* (2011). A slight increase in $T_{1/2}$ was also observed. They however, suggest that since the study was carried out in small animals an

extensive clinical pharmacokinetic study is necessary to establish such drug-drug interactions in humans to ascertain the true degree of interactions.

An interaction study between metformin and lisinopril *in vitro*was reported by Amorha *et al.* (2013). The degree of interaction was established after analysing the dissolution samples with validated HPLC methods. The study indicated a statistically significant difference ($p < 0.05$) in the effect of lisinopril on dissolution profile of metformin at pH 1.2, 4.5 and 6.8 with decrease in the percentage of metformin release. The effect of metformin on dissolution profile of lisinopril was only significant ($p < 0.05$) at pH 1.2 and 6.8 with an increase in percentage lisinopril release. They recommend the study to be conducted in vivo to further ascertain the level of interactions using animal model or in humans.

2.2.3 Analytical methods for determination of metformin in biological fluids

An HPLC method for quantification of metformin in brain regions, cerebrospinal fluid and plasma of rats treated with polysaccharides was reported to be developed and validated. They use ranitidine HCl as internal standard(IS) and pentafluorophenylpropyl column (HS F5, 150 × 4.6 mm id), binary mobile phase consisting of ammonium acetate-acetonitrile in ratio (47.8:52.2), flow rate of 1.3 mL/min, injection volume 20 µL and detection λ 233 nm. The method successfully resolve metformin from IS with retention time (9.56 and 4.91 mins) and (12 and 6 mins) for metformin and IS in plasma and brain samples respectively (Krzystof *et al.,* 2010).

Metformin was also reported to be quantified in HPL-MS/MS method for comparative pharmacokinetic study among 3 metformin formulations in healthy Mexican volunteers in a single-dose, randomized, open-label, 3-period crossover study. The method utilized C18 (4.6 × 100 mm id) column, binary mobile phase of acetonitrile-formic acid (40:60), column temperature of 21°C, flow rate 0.8 mL/min and retention time of metformin 1.8 min (Montoya-Eguía *et al.,* 2015).

The plasma metformin concentrations were also determined using HPLC method in a pharmacokinetic interaction study between metformin and teneligliptin in healthy adults. The column used was (µBondapak Phenyl, 300 ×3.9 mm, 10 mm; Waters) isocratic elution. The mobile phase is ternary consisted of acetonitrile-6.7 mM potassium dihydrogen phosphate-triethylamine (500:1500:1) adjusted with phosphoric acid to pH 7.6. The analyte was detected using (SPD-6A; Shimadzu Corporation, Kyoto, Japan) at a wavelength of 234 nm (Nakamaru *et al.,* 2015).

A RP-HPLC method for determination of metformin in human plasma was reported to be developed and validated. The method utilized propranolol as IS, C18 (150 × 4.6 mm id) column at 40 °C, flow rate 1 mL/min, binary mobile phase consisted of phosphate buffer pH 7.0-acetonitrile in ratio (50:50) and retention time of 7.5 and 9.5 for metformin and IS respectively. The method was applied only for bioequivalence studies using 2 metformin products and suggests that, it should be used for pharmacokinetic study of metformin (Valentina *et al.,* 2008).

2.3 Lisinopril

Lisinopril (II) is a metallopeptidase antihypertensive drug defined as a (2*S*)-1-[(2*S*)-6-Amino-2-[[(1*S*)-1-carboxy-3-phenylpropyl]amino]hexanoyl]pyrrolidine-2- carboxylic acid dihydrate, an angiotensin converting enzyme inhibitor also white to almost white crystalline powder in appearance with content range 98.5 – 101.5 % anhydrous substance, soluble in water, sparingly soluble in methanol, practically insoluble in acetone, chloroform, acetonitrile and in anhydrous ethanol (BP, 2013). Lisinopril has pKa value of 3.85 which makes it a stronger basicdrug. The lipid solubility of the unionized species is slight as shown by its low logP value [log (10) of the distribution coefficient of the unionized form between octanol and water] of -0.9. These parameters indicate low lipophilicity and low rapid passive diffusion as

it exists largely as the hydrophilic cationic species at physiological pHs (Krisztina *et al.*, 2013).

II

2.3.1 Pharmacokinetics of lisinopril

Lisinopril is slowly and incompletely absorbed after oral doses. About 25% of a dose is absorbed on average, but the absorption varies considerably between individuals, ranging from about 6 to 60%. It is already an active diacid and does not need to be metabolised *in vivo*. Peak concentrations in plasma are reported to occur after about 7 hr. Lisinopril is reported not to be significantly bound to plasma proteins, this is reflected by its high volume of distribution. It is excreted unchanged in the urine. The effective half-life for accumulation after multiple doses is 12 hours in patients with normal renal function. Lisinopril is removed by haemodialysis (Sweetman, 2002).

2.3.2 Interactions of lisinopril with other drugsand herbs

Excessive hypotension may occur when lisinopril is used with diuretics, other antihypertensives, or other agents, including alcohol, that lower blood pressure. An additive hyperkalaemic effect is possible in patients receiving ACE inhibitors with potassium-sparing diuretics, potassium supplements (including potassium-containing salt substitutes), or other drugs that can cause hyperkalaemia (such as ciclosporin or indomethacin), and serum-potassium concentrations should be monitored. Potassium-sparing diuretics and potassium supplements should generally be stopped before starting ACE inhibitors in patients with heart

failure.

However, ACE inhibitor therapy does not obviate the possible need for potassium supplementation in patients receiving potassium-wasting diuretics and potassium concentrations should also be monitored in these patients. The adverse effects of ACE inhibitors on the kidneys may be potentiated by other drugs, such as NSAIDs, that can affect renal function. It has also been suggested that part of the hypotensive effect of ACE inhibitors is prostaglandin-dependent, which might explain this interaction with drugs such as NSAIDs that block prostaglandin synthesis (Sweetman, 2002). Antacids are not reported to affect bioavailability of lisinopril but use of captopril with antacids reduced the bioavailability of the drug although this did not significantly alter the effects on blood pressure and heart rate.Study have investigated the effects of concomitant aspirin and lisinopril on haemodynamic parameters, but results have been conflicting and the clinical relevance of these findings is not clear. Given the well-established benefits of both lisinopril and aspirin in patients with heart failure associated with ischaemic heart disease, it is generally recommended that patients should continue to receive treatment with both (Sweetman, 2002).

An *in vitro* interaction study between lisinopril and Yoyo Bitters conducted by Olubunmi *et al.* (2015) also reported that Yoyo Bitters significantly increased the dissolution of lisinopril at pH 1.2 and that there was no statistically significant difference between the dissolution of lisinopril with and without Yoyo Bitters at pH 4.5. The dissolution of lisinopril decreased significantly in the presence of Yoyo Bitters at pH 6.8. They also recommend conducting same study in humans to ascertain the interaction *in vivo*.

Obamiro *et al*. (2013) in an *in vitro* interaction study between lisinopril and black tea reported a significant increase in the dissolution profile of lisinopril in simulated pH 4.5 and 6.8 when coadministered with black tea while the release profile of lisinopril at pH 1.2 shows the effect of black tea in decreasing the dissolution profile of lisinopril which is found to be statistically

significant. Despite the fact that available literature show that black tea may have some cardio friendly properties they recommend that coadministration of black tea withlisinopril should be discouraged as it can impact on tablet dissolution and may result in unpredictable effect.

2.3.3 Analytical methods for determination of lisinopril in biological fluids

An LC-MS/MS method was developed and validated for pharmacokinetic evaluation of lisinopril in Wister rat's plasma. The observed C_{max} and $t_{1/2}$ were 0.082 µM and 3.1 hr respectively, the data indicate that bioavailability of lisinopril was approximately 5.4 % .They attribute the poor bioavailability to low permeability and thus poor absorption in the experimental animalsused (Denti *et al.,* 2014).

El-Emam*et al.*(2004) reported HPLC method for the determination of lisinopril in dosage forms and spiked human plasma through derivatization with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole with fluorimetric detection at 540 nm. The method utilized bumetanide as IS with mobile phase consisting of methanol-0.02M disodium hydrogen phosphate pH 3.0 in ratio (55:45) and flow rate of 1.0 mL/min. However, their method was only applied for the *in vitro* determination of the drug in spiked human plasma thus, its application in biological fluids is required since the method have a good LOD of0.008 µg/mL.

A sensitive HPLC method for the determination of lisinopril in human plasma and urine with fluorescence detection at 477 nm was reported by Olcay and Lale (2004). NoIS to overcome sample-to-sample variation was used in this method which they conclude was unnecessary since good reproducibility was obtained. Mobile phase in this method consisted of methanol-0.02M phosphate buffer pH 3.2 in ratio (50:50), column temperature 55 °C with lisinopril retention time of 11.5 mins and LOQ of 0.2 – 0.7 ng/mL.

Lisinopril was reported to be quantified in liquid chromatography–mass spectrometry method in serum. The method successfully resolved lisinopril from hyoscyamine IS with retention

time 4.33 and 5.29 minutes respectively. The mobile phase is ternary consisted of 50 mM ammonium formate buffer (pH 3)-acetonitrile-methanol in ratio (72:7:21). The column is C18 (250 × 3.2 mm id) which was flushed throughout the experiment with acetonitrile-water (60:40) to maintain its efficiency due to utilization of buffer. The method LOD and LOQ were 0.5 and 6.0 ng/mL respectively reflecting good sensitivity but it was only applied in bioequivalence studies of lisinopril hence it was suggested to be applied in pharmacokinetic and bioavailability studies (Tsakalof *et al.,* 2003).

Siddiqui *et al.*(2014) reported HPLC method for concurrent determination of metformin and three ACE inhibitors (lisinopril, captopril and enalapril). The method utilized caffeine as IS and C18 (250 × 4.6 mm id) column at temperature 25°C, pH adjusted to 3.0 with phosphoric acid and mobile phase composition of acetonitrile-water in ratio (50:50). Flow rate was 1 mL/min and detection λ 218 nm.The method LODs were 3.26 and 0.98ng/mLand LOQs 9.26 and 2.22 ng $mL^{-1}$for lisinopril and metformin respectively. They failed to mention the retention time of the analysed drugs and the displayed chromatogram makes it difficult to ascertain the actual retention time as the chromatogram is not well labelled. The method was successfully applied in the determination of the drugs in their finished product and suggested its application for pharmacokinetic studies of the drugs. The question here will be whether there are patients taking these analysed drugs at the same time or not.

2.4 *Hibiscus sabdariffa*

The genus Hibiscus (Malvaceae) includes more than 300 species of annual or perennial herbs, shrubs or trees (Wang *et al*., 2012). *H. sabdariffa* is commonly known as roselle, hibiscus, Jamaica sorrel or red sorrel (English) and in Arabic, karkadeh (Ali *et al.,* 2005; Tasamaporn, 2008). Its native distribution is uncertain, some believe that is from India or Saudi Arabia (Ismail *et al*., 2008), while Murdock showed evidence that *H. sabdariffa* was domesticated by

the black populations of western Sudan (Africa) sometime before 4000 BC(Murdock, 1959; Da-Costa-Rocha *et al.,* 2014). Nowadays, it is widely cultivated in both tropical and subtropical regions including India, Saudi Arabia, China, Malaysia, Indonesia, The Philippines, Mexico, Vietnam, Sudan, Egypt, and Nigeria (Ismail *et al*., 2008; Eslaminejad and Zakaria, 2011 and Mohamed *et al*., 2012). The species *H. sabdariffa* comprises a large number of cultivated types which, on the basis of their growth habit or end use, are classified broadly under two varieties, *H. sabdariffa* var. sabdariffa ruber and *H. sabdariffa* var. altissima Wester. Former is generally bushy and pigmented and cultivated for the edible calyces; the latter includes tall growing, unbranched types bearing inedible calyces and mainly cultivated for its jute-like fibre (Gautam, 2004).

Figure 2.1: Photograph of *Hibiscus sabdariffa*plant
Source: Adopted from Tasamaporn, 2008

2.4.1 Uses of *H. sabdariffa*

In China, the seeds are used for their oil and the plant is used for its antihypertensive, antihyperglycaemic and antimicrobial properties, while in West Africa the leaves and

powdered seeds are in widespread use as a beverage and in meals. Additionally, it is used in the pharmaceutical and food industries (Da-Costa-Rocha *et al.,* 2014). *H. sabdariffa* is used in various ways some of which are described under the following headings:

2.4.1.1 Traditional culinary use

Fresh or dried calyces of *H. sabdariffa* are used in the preparation of herbal drinks, hot and cold beverages, fermented drinks, wine, jam, jellied confectionaries, ice cream, chocolates, flavouring agents, puddings and cakes (Ismail *et al.,* 2008; Bako *et al.,* 2009; Bolade *et al*., 2009). In Egypt, the flesh calyces are used in making "cacody tea" and fermented drinks, while in Sudan and Nigeria, the calyces are boiled with sugar to produce a drink known as "Karkade" or "Zoborodo". In Mexico, this drink is called Jamaica or "agua de Jamaica" or "te de Jamaica". In the West Indies, the calyces can also be used as colouring and flavouring ingredient in rum (Ismail *et al*., 2008; Da-Costa-Rocha *et al*., 2014). The seeds are eaten roasted or ground in meals, while the leaves and shoots are eaten raw or cooked, or as a sour-flavoured vegetable or condiment. In Sudan, the leaves are eaten green or dried, cooked with onions and groundnuts, while in Malaysia the cooked leaves are eaten as vegetables (Ismail *et al.,* 2008). In Africa, the seeds are roasted or ground into powder and used in meals, such as oily soups and sauces. In China and West Africa, the seeds are also used for their oil (Atta and Imaizumi, 2002).

2.4.1.2 Use in local traditional food and medicine

H. sabdariffa has been widely used in local medicines. In India, Africa and Mexico, infusions of the leaves or calyces are traditionally used for their diuretic, febrifugal and hypotensive effects, decreasing the viscosity of the blood and stimulating intestinal peristalsis. It is also recommended as a hypotensive in Senegal (Da-Costa-Rocha *et al.*, 2014). In Egypt, preparations from the calyces have been used to treat cardiac and nerve

diseases and also to increase the production of urine (diuresis). In Egypt and Sudan, an infusion of “Karkade” calyces are also used to help lower body temperature (Leung and Foster 1996). In north Africa, the calyces preparations are used to treat sore throats and coughs, as well as genital problems, while the emollient leaf pulp is used for treating external wounds and abscesses (Tasamaporn, 2008). In india, a decoction from the seeds is used to relieve pain in urination and indigestion. In brazil, the roots are believed to have stomachic and emollient properties. In chinese folk medicine, it is used to treat liver disorders and high blood pressure (Da-Costa-Rocha *et al.*, 2014). In iran, sour hibiscus tea is reportedly a traditional treatment for hypertension (Burnham *et al.*, 2002), while in Nigeria the decoction of the seeds is traditionally used to enhance or induce lactation in cases of poor milk production, poor letdown and maternal mortality (Gibbon and Pain, 1985; Gaya *et al.,* 2009).

2.4.1.3 Use as source of fibre and animal feed

H. sabdariffa var. altissima Wester is one of the most important species grown commercially as a fibre plant. However, *H. sabdariffa* fibres are subject to ongoing research showing promising technical properties when used as a substitute for synthetic or mineral fibres in composite materials, as well as a source material for high quality paper production (Dutt *et al.,* 2010; Kumar *et al.,* 2013). The leaves are used for animal fodder and fibre (Plotto, 2004). The seeds can be used to feed poultry as well as sheep and the residue from the seeds oil extraction can also be used to feed cattle and chicks (Elamin *et al.*, 2012).

2.4.2 Bioactive constituents of *H. sabdariffa*

The main constituents of *H. sabdariffa* relevant in the context of its pharmacological effects are organic acids, anthocyanins, polysaccharides and flavonoids (Eggensperger and Wilker, 1996).

2.4.2.1 Hydroxycitric acid

Hydroxycitric acid (III) has four stereoisomers, (2S, 3S), (2R, 3R), (2S, 3R) and (2R, 3S), of these isomers(2S, 3R)-hydroxycitric acid was the principal organic acid isolatedfrom the calyces of *H. sabdariffa* (Hida *et al.,* 2007).

III

2.4.2.2 Hibiscus acid

Hibiscus acid (IV) is the lactone form of (+)-allo-hydroxycitric acid. It compromises a citric acid moiety with an additional hydroxyl group at the second carbon and has two diastereomers due to the existence of two chiral centers in the molecule (Eggensperger and Wilker, 1996). Hydroxycitric acid, hibiscus acid and it derivatives as the major organic acids in the leaves and calyces extracts of *H. sabdariffa* (Beltran-Debon *et al.,* 2010; Peng *et al.,* 2011; Herranz-Lopez *et al.,* 2012).

IV

2.4.2.3 Anthocyanins

The anthocyanins are water soluble natural pigment present as flavonoid derivatives in the calyx or flowers of *H. sabdariffa* and their colour varies with pH. They are glycosides containing various classes such as delphinidin, cyanidin, malvidin, petunidine and peonidine together with different aglycones type depending on their hydroxylation (Williamson *et al.*, 2013). The first anthocyanin isolated from the calyx of *H. sabdariffa* was “hiviscin”, also known as “hibiscin”, later named delphinidin-3-sambubioside (V) and later renamed as delphinidin-pentoside-glucoside. Several studies have identified cyanidin-3-sambubioside (VI), cyanidin-3-glucoside (VII) and delphinidin-3-glucoside (VIII) as the major anthocyanins present in the extract of *H. sabdariffa* (Da-Costa-Rocha *et al.,* 2014).

General structure of anthocyanins

From the main structure above several derivatives can be obtained by substituting the respective R-groups as follows:

R_1=OH, R_2=OH, R_3=Sambubioside (V)

R_1=OH, R_2=H, R_3=Sambubioside: (VI)

R_1=OH, R_2=H, R_3=Glucose: (VII)

R_1=OH, R_2=OH, R_3=Glucose: (VIII).

2.4.2.4 Flavonoids

H. sabdariffa contain polyphenols of the flavonol and flavanol type in simple or polymerised form. The following flavonoids have been described in *H. sabdariffa* extracts: hibiscitrin (hibiscetin-3-glucoside), sabdaritrin, gossypitrin, gossytrin and other gossypetin glucosides, quercetin and luteolin (McKay, 2009); as well as chlorogenic acid, protocatechuic acid, pelargonidic acid, eugenol, quercetin, luteolin and the sterols β-sitosterol and ergosterol (McKay, 2009). The amount of quercetin (quercetin-3-glucoside) and rutin (quercetin-3-rutinoside IX) present in calyces of *H. sabdariffa* water extracts were reported to be 3.2 mg/g and 2.1 mg/g respectively (Alarcon-Alonso *et al*., 2012). In addition to quercetin and rutin kaempferol was also identified in calyces of *H. sabdariffa* water extracts(Beltran-Debon *et al*., 2010; Peng *et al.,* 2011; Herranz-Lopez *et al*., 2012). Phenolic acid such asprotocatechuic acid (PCA) is an important phenolic acid present in *H. sabdariffa* extract (Lee *et al.*, 2003; McKay, 2009). It was isolated from the dried flowers of *H. sabdariffa* and assigned the structure of 3,4-dihydrobenzoic acid) (Da-Costa-Rocha *et al.,* 2014). Chlorogenic acid is another phenolic acid present in both leaf and calyces of *H. sabdariffa* extracts and belongs to a family of esters formed between certain trans-cinnamic acids (caffeic acid, ferulic acid and p-coumaric acid) and quinic acid (Clifford *et al*., 2003). Several studies reported the presence of this acid and its derivatives in extracts of calyces and leaves of *H. sabdariffa* (Beltran-Debon *et al*., 2010; Peng *et al.,* 2011 and Herranz-Lopez *et al*., 2012). The amount of chlorogenic acid in the extract of *H. sabdariffa* calyxwas reported to be 2.7 mg/g (Alarcon-Alonso *et al.,* 2012).

IX

2.4.2.5 Pectin, mucilage and carbohydrates (polysaccharides)

Polysaccharides are another key group of compounds present in large quantities in the calyces of *H. sabdariffa.* In one study, the ethanol precipitated water extract yielded 10% of reddish polysaccharides. The following compounds were identified in two different fractions, arabinose, galactose, glucose, rhamnose and smaller amounts of galacturonic acid, glucuronic acid, manose and xylose (Muller *et al.,* 1989). Similar results were obtained in two other studies (Muller and Franz, 1992; Brunold *et al.,* 2004). The mucilage content was determined in the calyces of five strains of *H. sabdariffa* var. sabdariffa, reaching 24–28% in strains from Central America and Egypt but only 15% in an Indian strain. The pectin content only accounted for 2–4% while the sugars reached a maximum of 3–5% in these five strains. Mucilage and pectin consisted of 60–80% anhydrouronic acid (Da-Costa-Rocha *et al.,* 2014). The petals of *H. sabdariffa* yielded 65% of dry weight of mucilage, which on hydrolysis produced galactose, galacturonic acid and rhamnose, while the leaves only yield 10% (Sengupta and Banik, 2011).

2.4.2.6 Volatile compounds

Volatile compounds are responsible for the aroma of *H. sabdariffa.* In a study conducted in 1992, more than twenty-five volatile compounds (accounting for less than 8% of total *H. sabdariffa* seeds composition were reported in seed oil of *H. sabdariffa.* They were mainly unsaturated hydrocarbons, alcohols and aldehydes from C_8 to C_{13} (Jirovetz *et al.,* 1992).

Subsequently, thirty-seven volatile compounds from five different groups from the calyces of *H. sabdariffa* water extracts were characterised. These compounds included fatty acid derivatives (such as 2- ethylfuran and hexanal), sugar derivatives (furfural and 5methyl-2-furaldehyde), phenolic derivatives (eugenol), terpenes (such as 1,4-cineole, limonene) and miscellaneous compounds (acetic acid) (Chen *et al.*, 1998). In another study, the volatile component profile were examined from fresh and dried calyx (two samples each) using different time and temperature extraction conditions by GC–MS. A total of 32 compounds were identified and were divided into five chemical groups: aldehydes (fourteen compounds), alcohols (ten compounds), ketones (five compounds), terpenes (two compounds) and acids (one compound identified as protocatechuic acid X)(Ramirez-Rodrigues *et al.,* 2011). A total of seven aromatic volatiles were common to all the four samples tested, they are listed here (hexanal, 3-octanone, octanal, 1-octen-3-one, nonanal, 2 ,4-nonadienal, and geranyl acetone) (Da-Costa-Rocha *et al.*, 2014).

COOH
OH
OH

X

2.4.3 Pharmacological activities of *H. sabdariffa*

Below are some of the pharmacological effects of *H. sabdariffa* extracts:

2.4.3.1 Anti-hypertensive activity of H. sabdariffa

Decoctions of *H. sabdariffa* have been used traditionally in West Africa and Mexico as an anti-hypertensive remedy. Several *in vitro* (Obiefuna *et al.*, 1994) and *in vivo* studies have shown that the extract of the calyces (ranging from 125 to 500 mg/kg) indeed reduce both the

systolic and diastolic pressures, lowering heart rate and working as a vasodilator (Inuwa *et al.*, 2012). The anti-hypertensive activity might be through inhibition of angiotensin-converting enzymes (ACE) (Ojeda *et al.*, 2010), acetylcholine-like and histamine-like mechanisms (Adegunloye *et al.*, 1996), diuretic effect (Mojiminiyi *et al.*, 2000), reduction in the diffusion distance between capillaries and myocytes, as well as new vessel formation (Inuwa *et al.*, 2012) and direct vaso-relaxant effects (Adegunloye *et al.*, 1996 and Ajay *et al.*, 2007). The relaxant effect might be partially endothelium independent and possibly mediated by endothelium-derived nitric oxide (EDNO) dependent action. Endothelium-dependent vasodilator component results through activation of the endothelium-derived nitric oxide-cyclic GMP-relaxant pathway, whereas the endothelium-independent component could be due to inhibition of Ca^{2+} influx (Ajay *et al.*, 2007). Additionally, *H. sabdariffa* showed antiplatelet but no thrombolytic activity in vitro (Yamamoto *et al*., 2005). Despite the beneficial effect of *H. sabdariffa*as an anti-hypertensive, it was reported to produce an undesirable effect on gonadal activity (Da-Costa-Rocha *et al.*, 2014).

*Clinical studies on anti-hypertensive activity of H. sabdariffa:*Several clinical trials were carried out to determine the antihypertensive effect of *H. sabdariffa* (Herrera-Arellano *et al.*, 2004; Herrera-Arellano *et al.*, 2007; Mozaffari-Khosravi *et al.*, 2009). Both a cochrane review and a systematic review carried out in 2010 concluded that the studies did not provided reliable evidence to support recommendation of this plant to control or lower blood pressure in hypertensive patients, when compared to placebo or no treatment (Ngamjarus *et al.*, 2010; Wahabi *et al.,* 2010). However, a recent randomised, double-blind, placebo-controlled clinical trial showed that *H. sabdariffa* tea (1.25 g of *H. sabdariffa* per 240 mL boiled water; 3 servings a day for 6 weeks) effectively reduced blood pressure in pre-and mildly hypertensive adults (McKay *et al.,* 2010). Similar effects on decreasing systolic and diastolic blood

pressures were observed in mildly hypertensive Type 2 diabetic individuals when taking green or hibiscus (sour) tea for 4 weeks (three times a day, 2 hr after each meal) (Mozaffari-Khosravi *et al.*, 2013). The authors also concluded that this might be useful in preventing the progression to moderate or more severe hypertension, potentially decreasing cases of cardiovascular disease. Furthermore, a recent comprehensive review on animal and human studies on the effect of *H. sabdariffa* in the treatment of hypertension and hyperlipidemia concluded that *H. sabdariffa* has great potential to reduce risk factors associated with cardiovascular diseases and warrants further studies (Hopkins *et al.*, 2013). Anthocyanins, including delphinidin-3-*O*-sambubioside (hibiscin) and cyanidin-3-*O*-sambubioside (gossypicyanin), have been identified as being responsible for ACE inhibition (Herrera-Arellano *et al.*, 2007; Ojeda *et al.*, 2010).

2.4.3.2 Anti-diabetic activity of H. sabdariffa

Diabetes mellitus (DM) can be defined as an endocrine and metabolic disorder characterised by chronic hyperglycaemia, dyslipidemia, and protein metabolism that results from defects in both regulations of insulin secretion and/or insulin action (Sweetman, 2002). The protective effect of a polyphenol extract of *H. sabdariffa* was studied in a Type 2 diabetic rat model (high fat diet model). At a dose of 200 mg/kg, the extract demonstrated anti-insulin resistance properties as it reduced hyperglycaemia and hyperinsulinemia. It decreased serum triacylglycerol, cholesterol and the ratio of low density lipoprotein/high-density protein (LDL/HDL), as well as reduced the plasma advanced glycation end products (AGE) formation and lipid peroxidation (Peng *et al.*, 2011). The currently accepted therapeutic strategy for the control of postprandial hyperglycaemia is based on the inhibition of α-glucosidase and α-amylase. This results in an aggressive delay of carbohydrate digestion to absorbable monosaccharide. With this in mind, a study was conducted to determine the effect of *H. sabdariffa* extract on intestinal α-glucosidase and pancreatic α-amylase activity *in vitro*.

As a result, *H. sabdariffa* extract was shown to be a potent pancreatic α-amylase inhibitor (Adisakwattana *et al.*, 2012). Similar results were found for hibiscus acid (hibiscus-type (2S,3R)-hydroxycitric acid lactone) (Yamada *et al.*, 2007), which inhibited pancreatic α-amylase and intestinal α-glucosidase enzyme (Hansawasdi *et al.*, 2001). DM is a risk factor for coronary heart diseases as well as atherosclerosis. An ethnobotanical study conducted in the Caribbean for urinary problems and DM revealed that *H. sabdariffa* is traditionally used to clean the liver and blood within a group of plants used for cooling, high cholesterol and urinary problems. When the respondents were asked which medicinal plants were used for high blood pressure, diabetes and jaundice, *H. sabdariffa* was referred to hypertension (Lans, 2006). A study in alloxan-induced diabetic rats showed that an ethanolic extract of *H. sabdariffa* flowers (200 mg/kg) had a strong hypolipidemic as well as antioxidant effect. Thus, *H. sabdariffa* extract showed therapeutic promise in decreasing and preventing the development of atherosclerosis and possible related cardiovascular pathologies linked with diabetes. The authors suggest that this activity might be linked to polyphenolic compounds and dihydrobenzoic acids, like protocatechuic acids, but further identification of the active compounds is warranted (Farombi and Ige, 2007; Da-Costa-Rocha *et al.*, 2014). A similar effect was reported by (Huang *et al.*, 2009) with the extract suppressing the high-glucose-induced migration in a vascular smooth muscle cell model.

Clinical studies anti-diabetic activity of H. sabdariffa: Recently a double-blind, randomised, controlled trial was carried out to compare the anti-hypertensive efficacy of *H. sabdariffa* (tea) in diabetic patients. The results demonstrated that the consumption of the sour tea had positive effects on blood pressure in Type 2 diabetic patients with mild hypertension (Mozaffari-Khosravi *et al.,* 2009). Following this study another randomised clinical trial (no control group or double-blinding) in identical patients (100 individuals) showed that

consuming three glasses of green or hibiscus (sour) tea daily for a period of 4 weeks significantly decreased systolic and diastolic blood pressures in those patients (Mozaffari-Khosravi *et al.*, 2013). However, sodium, potassium or calcium concentrations were neither qualified nor specific amounts of the other active constituents of tea, such as caffeine, were taken into consideration. Other clinical trial was conducted to investigate the hypolipidemic effects of sour tea in patients with diabetes. Again, the beneficial effect of the sour tea in diabetic patients was found. The sour tea was able to significantly affect the blood lipid profile by increasing high-density lipoprotein-cholesterol, decreasing total cholesterol, low density lipoprotein-cholesterol, triglycerides with no effect on apolipoprotein-A1 (Mozaffari-Khosravi *et al.,* 2009).

2.4.3.3 Antioxidant activity of H. sabdariffa

Studies both *in vitro* (Steenkamp *et al.,* 2004; Mohd-Esa *et al*., 2010) and *in vivo* (Farombi and Fakoya, 2005; Mossalam *et al.*, 2011) have shown that extracts of *H. sabdariffa* have a potent antioxidant effect as demonstrated by strong antioxidant activity of the extract is due to its strong scavenging effect on reactive oxygen and free radicals (Farombi and Fakoya, 2005; Usoh *et al*., 2005; Mohd-Esa *et al*., 2010). The extract was also reported to act via inhibition of xanthine oxidase activity, protective action against tert-butyl hydroperoxide (t-BHP)-induced oxidative damage (Tseng *et al.,* 1997).*H. sabdariffa*extract also inhibit the formation of malondialdehyde content (100–300 mg/kg), reduction of glutathione depletion, increase of the liver and decrease blood activity of superoxide dismutase and catalase (Farombi and Fakoya, 2005; Usoh *et al.,* 2005), while in the liver it increased superoxide dismutase, catalase and glutathione and decreased malondialdehyde (Mossalam *et al.,* 2011). The effects were observed for both water and ethanolic extracts from flowers of *H. sabdariffa*, as well as from the seeds and leaves (Mohd-Esa *et al*., 2010). One single randomised, open-label, two-way cross-over study was conducted with 8 healthy volunteers.

One single dose (0.05 g/mL) of a *H. sabdariffa* water extract significantly increased the systemic antioxidant potential in plasma and urine, increasing the hippuric acid excretion with decreased malondialdehyde concentration in urine (biomarker for oxidative stress) (Frank *et al.*, 2012).

2.4.3.4 Nephroprotective activity of H. sabdariffa

Two studies were reported on the nephroprotective activity of *H. sabdariffa* extracts on diabetic nephropathy in streptozotocin-induced Type-1 diabetic rats (Lee *et al*., 2009; Wang *et al*., 2011). Nephropathy may progress to end-stage renal disease. A study was conducted to investigate the effect of the polyphenol extract of *H. sabdariffa* (100 and 200 mg/kg/day) in streptozotocin-induced diabetic nephropathy in rats. The extract revealed beneficial effects as the kidney mass was reduced and the hydropic change of renal proximal convoluted tubules was improved, it reduced serum triglyceride, total cholesterol and LDL as well as increased the activity of catalase and glutathione and reduced lipid peroxidation in the kidney (Lee *et al*., 2009). It was found that the extracts reduced kidney mass and improved hydropic change of renal proximal convoluted tubules in this rat model. The positive effect shown by the extracts might be via improving oxidative status and regulating Akt/Bad/14-3-3γ signaling (anti-apoptotic mechanisms). Another *in vivo* study also revealed that its nephroprotective effect is a result of the protection of the kidney from the oxidative stressed (Mossalam *et al.,* 2011).

2.4.3.5 Anti-anaemic activity of H. sabdariffa

A preliminary study on the use of *H. sabdariffa* decoctions as an alternative source of iron for the treatment of anaemia and some other mineral deficiency diseases was conducted and showed that dry fermented calyces of hibiscus exhibited a very low pH value which enhanced mineral availability. Another reason for enhancing mineral (iron, zinc, calcium and magnesium) bioavailability is the high concentration of ascorbic acid (Falade *et al.*, 2005). The effect of calyces of *H. sabdariffa* extract (200 to 1000 mg/kg body weight) on some haematological parameters in rats was studied to determine its medicinal usefulness in the treatment of anaemia. The study suggested that at a comparatively high dose range of 200 to 400 mg/kg, the extract had a beneficial effect on the red cells, but this was not sustained at higher doses (Adigun *et al.,* 2006). Another study using a rat model of infection with *Trypanosoma congolense* showed that the use of *H. sabdariffa* water extracts (equivalent to 9.61 mg/100g/day of ascorbic acid for 3 weeks) prevented the disease-induced anomalies with increase of serum creatinine and urea levels. It was concluded that consumption of the extract ameliorated the pathological changes in blood as well as hepatic and renal structures of *T. congolense*-infected rats. The observed effects might be due to the ascorbic acid component or other antioxidants present, which presumably kept the free radical load in infected rats (Umar *et al.,* 2009).

Other effects of *H. sabdariffa* reported in the literature include: hepatoprotective (Lee *et al.,* 2012), inhibition of vascular smooth muscle cell proliferation (Fouda *et al.,* 2007), antibacterial, antifungal and anti-parasitic (Da-Costa-Rocha *et al.,* 2014), antipyretic, antinociceptic and anti-inflammatory (Ali *et al.,* 2011), cancer preventive (Lin*et al.,* 2005), anticholesterol (Lee *et al.,* 2012), anti-obesity (Perez-Torres *et al.*, 2012) and delayed puberty (Iyare *et al.*, 2010) activities.

2.4.4 Safety (toxicology) of *H. sabdariffa*

H. sabdariffa preparations, predominantly the infusion and aqueous extracts, have a long standing traditional use both in food and in medicine, and in general are considered to be safe. The available toxicological data, however limited, are in support of this assessment. There are no any case reports of adverse reactions following oral consumption of *H. sabdariffa* preparations (Da-Costa-Rocha *et al.,* 2014). According to a study conducted, no deaths were observed in albino mice after fourteen day's administration (*ip*) at doses of 1000–5000 mg/kg body weight/day, thus the calculated LD_{50} of calyces of *H. sabdariffa* aqueous extract was >5000 mg/kg body weight. The same authors assessed the effect of the extract on blood pressure in spontaneously hypertensive and normotensive Wistar-Kyoto rats. As part of this study it was observed that between the seventh and the twenty-first day after extract administration, the highest dose of 1000 mg/kg resulted in spontaneous deaths in hypertensive but not in normotensive rats. With reference to the well-known increased risk of sudden cardiac death in patients receiving non-potassium sparing diuretics, the authors speculate that the death of the animals may have been due to a diuretic effect of the extract (Onyenekwe *et al.*, 1999). Lack of acute toxicity with calculated LD_{50} values >5000 mg/kg body weight was reported for a methanolic dried flower extract in adult albino mice on a herb-drug interaction study after 24 h administration (*ip*) (Ndu *et al.*, 2011) and for an ethanolic extract of *H. sabdariffa* seed in albino Wistar rats while studying the effect of the extract on lactogenic activity (Gaya *et al.,* 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

HPLC grademethanol and water were obtained from Sigma-Aldrich (Germany). Potassium dihydrogen orthophosphate and disodium hydrogen orthophosphate were BDH-Laboratory reagents. Distilled deionized water was used in preparing some solutions in this study. Other chemical reagents used include:

1. Sodium hydroxide pellets
2. 0.1M perchloric acid
3. Concentrated hydrochloric acid
4. Absolute ethanol (99.5 % v/v)
5. Anhydrous formic acid
6. Acetonitrile
7. Anhydrous acetic acid
8. Acetic anhydride
9. Toluene
10. Phosphoric acid

3.1.2 Drug and drug reference standard

Reference standard of metformin, lisinopril and caffeine (Sigma-AldrichGermany). Tablet brands of metformin (500 mg) and lisinopril(10 mg).

3.1.3 Equipment and glasswares

1. Dissolution machine 3 in 1 78×2Btype; Shanghai Huang Hai Medicament Testing Instrument Company Limited.
2. Bibby Stuart Scientific Melting Point Apparatus, SMP1 07030010 Ruilian Science and Technology company Limited.
3. HPLC column:Chemsl ODS C18 (200 mm×4.6 mm i.d., 5µ particle size)
4. ShimadzuD439300179 digital analytical weighing balance.
5. Thermo Electron Corporation Centra CL2 centrifuge
6. McDonald HH-S digital thermostatic water bath.
7. HPLC sample bottles 1.5 mL
8. Capillary tubes
9. Beakers, 50, 100, 250 mL
10. Measuring cylinders, 10, 50, 100 mL
11. Pipettes, 1, 5, 10 mL
12. Volumetric flasks, 5, 10, 100, 500 mL
13. Whatman filter paper no. 41
14. Test tubes and test tube holders
15. Spatula
16. Hand gloves

3.1.4 Instrumentations

HPLC machine used was Agilent technologies (Model 1200 Infinity Series). FTIR machine (Agilent technologies model 1260 Infinity Series). A double scanning UV/vis spectrophotometer (Model SP 3000) was used to monitor the drug content throughout the UV-visanalysis.

3.2 Methods

3.2.1 Collection of sample

3.2.1.1 Collection of Hibiscus sabdariffa calyxes

The plant *Hibiscus sabdariffa*was collected in Samaru, Zariaduring the month of september, 2016. It was identified at the Department of Biological Sciences, Ahmadu Bello University, Zaria by Malam Namadi Sanusi and was assigned a voucher number of 1056. The calyxes were removed, shed dried and size reduced and kept in air tight container for subsequent use.

3.2.1.2 Collection of drug samples

Tablet brands of metformin (500 mg) and lisinopril (10 mg) were purchased from a reputable pharmacy in Samaru, Zaria.

3.2.2 Identification and assay of metformin, lisinopril and caffeine standard powders

3.2.2.1 Identification and assay of metformin standard powder

Metformin standard powder was identified by taking its melting point using sealed capillary tube and also by subjecting it to FTIR analysis and comparing the spectrum obtained with a reference spectrum.

The assay was carried out by dissolving the powder (0.1 g) in anhydrous formic acid (4 mL), and acetonitrile (80 mL) was then added followed by immediate titration with 0.1M perchloric acid. Metformin content was determined using the relation 1 mL 0.1M perchloric acidis equivalent to 16.56 mg of metformin (BP, 2013).

3.2.2.2 Identification and assay of lisinopril standard powder

Lisinoprilstandard powder was subjected to FTIR analysis for identification and the obtained spectrum was compared with the reference spectrum.

The assay was carried out by dissolving 0.175 g of the powder in 25 mL of distilled water followed by titration immediately with 0.1M sodium hydroxide. Lisinopril content was

calculated using the relation 1 mL 0.1M sodium hydroxide is equivalent to 40.55 mg of lisinopril (BP, 2013).

3.2.2.3 Identification and assay of caffeine standard powder

Caffeinestandard powder was identified bytaking its melting point using sealed capillary tube and also by subjecting it to FTIR analysis.

The assay was carried out by dissolving the powder (0.170 g) in anhydrous acetic acid (5 mL). It was heated and allowed to cool, after cooling acetic anhydride (10 mL)and toluene (20 mL) were added. The mixture was titrated with 0.1M perchloric acid (BP, 2013).

3.2.3Quality control studies on metformin and lisinopril tablets to be used

3.2.3.1 Identification and assay of metformin tablets

Identification of the tablets was carried out by shaking a quantity of powdered tablets containing 20 mg metformin hydrochloride with 20 mL absolute ethanol and filtered using Whatman filer paper number 41 and the filtrate was evaporated to dryness for 1 hr. A portion was subjected to melting point determination and also to infrared absorption spectrophotometry. The spectrum obtained was compared with the reference spectrum.

The assay was carried by weighing and powdering 20 tablets. A quantity of the powder containing equivalent of 0.1 g metformin hydrochloride was shaken with water(70 mL) for 15 min, it was diluted to 100 mL with water and filtered, the first 20 mL was discarded.Ten (10 mL) of the filtrate was diluted to 100 mL withwater. Ten (10 mL) of the resulting solution was diluted to 100 mL with water and theabsorbance was measured at 232 nm. Metformin content was determined using A(1%, 1 cm) value of 798 (BP, 2013).

3.2.3.2 Identification and assay of lisinopril tablets

Identification of the tablets was carried out by shaking a quantity of powdered tablets containing 15 mg lisinopril with 20 mL absolute ethanol and filtered using Whatman filer paper number 41 and the filtrate was evaporated to dryness for 1 hr. A portion was subjected to melting point determination and also to infrared absorption spectrophotometry. The spectrum obtained was compared with the reference spectrum.

The assay was carried by weighing and powdering 20 tablets. A quantity equivalent to 4 mg of lisinopril was dissolved in 20 mL of HPLC grade water and filtered using a Whatman filter paper number 41. Also,lisinopril standard powder (4 mg) was weighed and dissolved in HPLC grade water (20 mL). Equal volumes (20 μL) of the solutions were injected into the chromatograph (flow rate 1 mL/min, detection λ 210 nm, mobile phase methanol:acetonitrile ratio 96:4 and column temperature 50 °C) and the chromatograms were recorded. Lisinopril content in the tablet was calculated using the formula (TC/D)(ru/rs) where T is the label quantity, in mg, of lisinopril in the tablet; C is the concentration, in mg, of lisinopril standard powder; D is the concentration, in mg, of lisinopril in the tablet preparation based upon the labelled quantity and extent of dilution; *ru* and *rs* are lisinopril peak heights of tablet and standard powder solutions respectively (USP, 2012).

3.2.3.3 Dissolution rate

Dissolution test for metformin tablet was carried out in the medium phosphate buffer pH 6.8 (900 mL), basket rotation of 100 rpm at 37 °C. Sample(10 mL) was withdrawn at 30 min and filtered. Filtrate (10 mL) was diluted to 100 mL with water and 10 mL of the resulting solution was further diluted to 100 mL with water. The absorbanceof the final solution was measured at 233 nm and the content of metformin hydrochloride was calculated in the medium taking A (1%, 1 cm) value of 806 at 233 nm (BP, 2013).

For lisinopril, dissolution was carried out in the medium 0.1M hydrochloric acid (900 mL), basket rotation of 50 rpm at 37 °C. Sample(10 mL) was withdrawn at 30 min and filtered. Filtrate (10 mL) was diluted to 100 mL with *water* and 10 mL of the resulting solution was further diluted to 100 mL with *water*. A solution of same concentration of lisinopril standard powder was also prepared in the medium. Equal volumes (20 μL) of the solutions were injected into the chromatograph (flow rate 1 mL/min, detection λ 215 nm, mobile phase methanol:water ratio 1:4 and temperature 50 °C) and the chromatograms were recorded. Lisinopril content in the tablet was calculated using the formula described in section 3.2.3.2 (USP, 2012).

3.2.3.4Disintegration test

Disintegration test for metformin tablets was conducted by taking six tablets and 1 dosage unit was placed in each of the 6 tubes of the basket and the apparatus was operated using 0.1M HClas the medium which was maintained at 37 ± 2 °C, as the disintegration test fluid. At time interval, the basket was lifted from the fluid and observe the dosage units, the time at which each dosage unit disintegrated wasrecorded until all of the dosage units have disintegrated completely. The same was done for lisinopril tablets (BP, 2013).

3.2.3.5Uniformity of weight test

Uniformity of weight tests were conducted for metformin and lisinopril by randomly selecting and weighing 20 tablets each and the mean weights were determined. Percentage deviation of each of the tablets were also determined (BP, 2013).

3.2.4 UV-visspectrophotometric methods foranalysis of metformin

UV-vis methods for determination of metformin in dissolution media (pH 1.2, 6.8 and 7.4) respectively were developed and validated as follows:

3.2.4.1 Preparation of solutions and reagents

Preparation of dissolution media

The media used for dissolution were 0.1 M hydrochloric acid (simulated gastric pH), phosphate buffers pH 6.8 (simulatedintestinal pH) and phosphate buffer pH 7.4 (simulated blood pH). All were prepared using official methods (BP 2013).

Preparation of stock solutions of metformin in the media

Stock solutions of metformin were prepared by accurately weighing and dissolving 5 mg of pure metformin powder in 50 mL of each of the media to obtain concentrations of 100 μg/mL.

3.2.4.2 Determination of λ_{max} for metformin

A portion (1 mL) of each stock solution of metformin was taken and diluted to 10 mL with the same medium. The resulting solution in each case was scanned at a wavelength of range 200 to 600 nm on the UV-spectrophotometer and the wavelength of maximum absorption determined.

3.2.4.3 Preparation of calibration curves for metformin

From each stock solution, different aliquots in the range 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mL were transferred into series or 10 mL volumetric flask and the volume made up to the mark with the same medium to obtain solutions with concentrations 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 μg/mL respectively. The absorbance of each solution was then determined at the respective λ_{max} of metformin in each of the three media. The absorbances obtained were then plotted against their corresponding concentrations.

3.2.4.4 Validation of UV spectrophotometric methods for metformin

The methods were validated with respect to their precisions, accuracies, percentage recoveries, limits of detection and limits of quantitation (ICH, 2006).

Precision

The precision of this method was checked by replicate analysis of the calibration curve responses determined. This was done by taking five replicates of five different determinations for the analysis. The percentage coefficient of variation (% CV) for the replicate analysis of each of the determinations were taken as a measure of precision.

$$\% \ CV = \frac{S}{X} \times 100 \text{..........Formula 1}$$

Where S is the standard deviation and X is the mean.

Accuracy

The accuracy of these methods was checked by standard addition methods. This was done by preparing 10 μg/mL solutions in five 5 mL volumetric flasks for each of the medium containing 4 ml of 5 μg/mL metformin concentrations, 0.3 mL of stock solutions were added to each of the flasks and made up to the mark with each of the medium respectively. Five replicate analysis of each of the resulting solutions were done for the three media. Accuracy wasexpressed as percentage relative error (%Er).

$$\% \text{ Er} = \frac{x - \mu}{\mu} \times 100 \ldots\ldots\ldots \text{Formula 2}$$

Where X is the mean and μ is the expected value.

Recovery

The recovery of this method was checked by having six 5 mL volumetric flasks for the three media each containing 4 mL of 1 μg/mL initial concentration of metformin. Flask one was left unspiked but 0.46, 0.56, 0.66, 0.76 and 0.86 mL of the stock solutions of metformin were added to flasks 2, 3, 4, 5 and 6 of each of the medium respectively and made up to the volume with the same medium to obtain concentrations 10, 12, 14, 16 and 18 μg/mL respectively. Thereafter, the absorbance of each solution was measured and the drug content was determined by subtracting the absorbance of the unspiked solution from that found in each of the spiked solutions and interpolating the final concentration from the calibration curve, thus the percentage recovery was computed using the formula:

$$\% \text{ Recovery} = \frac{\text{measured concentration}}{\text{added concentration}} \times 100 \ldots\ldots\text{Formula 3}$$

Limit of detection

The limit of detection (LOD) was determined by studying the calibration curve using samples containing the drug in the range of LOD. The standard deviation of y-intercepts of the regression lines was used as standard deviation. LOD is expressed as:

$$\text{LOD} = \frac{3.3\,\sigma}{S} \ldots\ldots\ldots\ldots\ldots \text{ Formula 4}$$

Where σ is the standard deviation of y-intercepts of the regression lines and S is the slope of the calibration curve.

Limit of quantification

The limit of quantitation (LOQ) was determined using the expression:

$$LOQ = \frac{10\,\sigma}{S} \text{………. …..Formula 5}$$

Where σ is the standard deviation of y-intercepts of the regression lines and S is the slope of the calibration curve.

3.2.4.5 Application of the developedUV spectrophotometric methods for metformin

Twenty (20)tabletswere randomly selected from the metformin tablet brand, ground into a fine powder using mortar and the resulting powder was weighed. A quantity of the powder equivalent to 50 mg metformin was weighed and transferred separately into three different volumetric flasks (100 mL) labelled A, B and C containing calibrated volumetric flasks containing about half their volumes with 0.1M hydrochloric acid, phosphate buffer pH 6.8 and phosphate buffer pH 7.4 respectively. The volume of each flask was made up to the mark with the medium and shaken for 10 minutes. The solutions were filtered through a Whatman Filter paper no. 41. A portion (1 mL) of each filtrate was transferred into a volumetric flask (10 mL) and made up to the mark with the medium to obtain a solution of concentration 50 µg/mL in each case. The absorbance of each of the resulting solutions was determined at the respective λ_{max} of each medium.

The proposed methods were compared with an official method for assay of metformin by assaying the same tablet brand using (BP, 2013) method.

3.2.5*In vitro* availability of metformin

The *in vitro* availability of metformin in simulated pHs (gastric, intestinal and blood), at 37ºC was determined using dissolution apparatus as described in BP (2013) with slight modification to the top of the basket in order to prevent air entrapment during dissolution. A tablet of metformin (500 mg) was placed in the dissolution basket and allowed into the dissolution medium (1 L) set at 100 rpm. The dissolution process was monitored over a period of 60 min, with aliquots (5 mL) being withdrawn at 5, 10, 15, 30, 45 and 60 min intervals. The volume of the dissolution medium was maintained after each withdrawal by an immediate replacement with 5 mL of the dissolution medium maintained at the same temperature in the same bath. The drug content available was determined using the UV methods developed by diluting 1 mL of the sample to 10 mL and determining the absorbance at the respective λmax.

For the *in vitro* availability of metformin in the presence of *Hibiscus sabdariffa*, the dissolution media were prepared by weighing a quantity(5) g of dried size reduced*Hibiscus sabdariffa*calyxes was cold macerated in 1 L of each of the media for 24 hr. It was filtered and a portion of the filtrate (100 mL) was evaporated to dryness using water bath. The extract was weighed and the concentration was found to be 0.25 g/100mL of the extract. From the determined concentration (0.25 g/100 mL), a portion (400 mL) was diluted to 1 L to obtain solutions containing 1 g/L for each medium. The process described above was then repeated to determine the drug content after each sampling.

3.2.6 UV-visspectrophotometric methods for lisinopril

UV-vis methods for determination of lisinopril in dissolution media (pH 1.2, 6.8 and 7.4) respectively were developed and validated as follows:

3.2.6.1 Preparation of solutions and reagents

Preparation of stock solutions of lisinopril in the media

Stock solution of lisinopril were prepared by accurately weighing and dissolving 5 mg of pure lisinopril powder in 50 mL of each of the media to obtain concentrations of 100 µg/mL.

3.2.6.2 Determination of λ_{max} for lisinopril

A portion (1 mL) of each stock solution of lisinopril, was taken and diluted to 10 mL with the same medium. The resulting solution in each case was scanned at a wavelengths of range 200 to 600 nm on the UV-spectrophotometer and the wavelengths of maximum absorption determined.

3.2.6.3 Preparation of calibration curves for lisinopril

From each stock, different aliquots in the range 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mL were transferred into series of 10 mL volumetric flask and the volume made up to the mark with the same medium to obtain solutions with concentrations 2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 µg/mL respectively.The absorbance of each solution was then determined at the respective λ_{max} of lisinopril in each of the three media. The absorbances obtained were then plotted against their corresponding concentrations.

3.2.6.4 Validation of UV spectrophotometric methods for lisinopril

The methods were validated with respect to their precisions, accuracies, percentage recoveries, limits of detection and limits of quantitation (ICH, 2006).

Precision

The precision of this method was checked by replicate analysis of the calibration curve responses determined. This was done by taking five replicates of five different determinations for the analysis. The percentage coefficient of variation (% CV) for the replicate analysis of each of the determinations were taken as a measure of precision.The % CV was calculated using formula 1.

Accuracy

The accuracy of these methods was checked by standard addition methods. This was done by preparing 10 µg/mL solutions in five 5 mL volumetric flasks for each of the medium containing 4 ml of 5 µg/mL lisinopril concentrations, 0.3 mL of stock solutions were added to each of the flasks and made up to the mark with each of the medium respectively. Five replicate analysis of each of the resulting solutions were done for the three media.The accuracy was calculated using formula 2.

Recovery

The recovery of this method was checked by having six 5 mL volumetric flasks for the three media each containing 4 mL of 1 µg/mL initial concentration of lisinopril. Flask one was left unspiked but 0.46, 0.56, 0.66, 0.76 and 0.86 mL of the stock solutions of lisinopril were added to flasks 2, 3, 4, 5 and 6 of each of the medium respectively and made up to the volume with the same medium to obtain concentrations 10, 12, 14, 16 and 18 µg/mL respectively. Thereafter, the absorbance of each solution was measured and the drug content was determined by subtracting the absorbance of the unspiked solution from that found in each of the spiked solutions and interpolating the final concentration from the calibration curves, thus the percentage recoveries were computed using formula 3.

Limit of detection and limit ofquantification

The limit of detection (LOD) and limit of quantification (LOQ) were determined in a similar manner as described in method using formulae 5 and 6 respectively.

3.2.6.5 Application of the developedUV spectrophotometric methods for lisinopril

Tablets (20) were randomly selected from the lisinopril tablet brand, ground into a fine powder using a mortar until and the resulting powder was weighed. A quantity of the powder equivalent to 20 mg lisinopril was weighed and transferred separately into three different volumetric flasks (100 mL) labelled A, B and C containing about half their volumes with 0.1M hydrochloric acid, phosphate buffer pH 6.8 and phosphate buffer pH 7.4 respectively. The volume of each flask was made up to the mark with the same medium and shaken for 10 min. The solutions were filtered through a Whatman filter paper no. 41. A portion (1 mL) of each filtrate was transferred into a volumetric flask (10 mL) and made up to the mark with the same medium to obtain a solution of concentration 20 µg/mL in each case. The absorbance of each of the resulting solutions was determined at the respective λ_{max} of each medium.

The proposed methods were compared with an official method for assay of lisinopril by assaying the same tablet brand using (USP, 2012) method.

3.2.7*In vitro* availability of lisinopril

The *in vitro* availability of lisinopril in simulated pHs (gastric, intestinal and blood), at 37ºC was determined using dissolution apparatus as outlined in BP (2013) with slight modification to the top of the basket in order to prevent air entrapment during dissolution. A tablet of lisinopril (10 mg) was placed in the dissolution basket and allowed into the dissolution medium (900 mL) set at 50 rpm. The dissolution process was monitored over a period of 60 minutes, with aliquots (5 mL) being withdrawn at 5, 10,15, 30, 45 and 60 min intervals.The volume of the dissolution medium was maintained after each withdrawal by an immediate

replacement with 5 mL of the dissolution medium maintained at the same temperature in the same bath. Thedrug content available was determined using the UV methods developed by taking the absorbances of the withdrawn samples directly without dilution at the respective λ_{max}.

For the *in vitro* availability of lisinopril in the presence of *Hibiscus sabdariffa*, the dissolution media were prepared by weighing a quantity(5 g) of *Hibiscus sabdariffa*calyxes was cold macerated in 1 L of each of the media for 24 hr. It was filtered and a portion of the filtrate (100 mL) was evaporated to dryness using water bath. The extract was weighed and the concentration was found to be 0.25 g/100mL of the extract. From the determined concentration (0.25 g/100 mL), a portion (400 mL) was diluted to 1 L to obtain solutions containing 1 g/L for each medium. The process described above was then repeated to determine the drug content after each sampling.

3.2.8 HPLC method development for metformin

High performance liquid chromatographic method for the analysis of metformin hydrochloride extracted from saliva samples was developed and validated as follows:

3.2.8.1 Preparation of suitable solvent (diluent) for dissolution and extraction of metformin hydrochloride standard powder

Although metformin is highly soluble in water, it was observed that the solvent that gives better resolution both for the drug and internal standard (caffeine) is methanol:water (50:50). This solvent was used in dissolving metformin and internal standard throughout the analysis.

3.2.8.2 Preparation of stock solution of metformin hydrochloride

Stocksolution of metformin hydrochloride was prepared by accurately weighing and dissolving 2mg of pure metformin powder in 20 mL of M:W to obtain a concentration of 100 µg/mL.

3.2.8.3 Preparation of internal standard (caffeine)

A stocksolution of caffeine was prepared by accurately weighing and dissolving 2 mg of pure caffeine powder in 20 mL of M:W to obtain a concentration of 100 µg/mL. Further dilutions were appropriately made where necessary.

3.2.8.4Chromatographic conditions

Chromatographic separation was achieved using Chemsl ODS® C18 (200 mm × 4.6 id). The mobile phase consists of methanol-water containing 0.1 % orthophosphoric acid as additive. Isocratic separation conditions were achieved through varying and optimization of mobile phase ratios, injection volume, temperature, detection wavelength and flow rate after several trials. These stated conditions allowed the detection of metformin and caffeine (internal standard) with ample sensitivity.

3.2.8.5Preparation of metformin-saliva sample

A solution of metformin(12.5µg/mL) was prepared from the stock solution and a portion (2 mL) was added to blank saliva (2 mL) followed by 1 mL of a solution (0.5µg/mL) of the internal standard to obtain a mixture containing 5.0 µg/mLof metformin. The mixture was vortex mixed and centrifuged at 3000 rpm for 10 min which gives a relatively clear solution.A quantity (0.5 mL) of the clear solution was injected into the HPLC machine and the chromatogram was obtained which resolved metformin from the internal standard.

3.2.8.6Preparation of calibration curve of metformin

Various concentrations (3.125, 6.25, 12.5, 25.0, 50.0 and 62.5 µg/mL) were prepared from the stock solution and portions(2 mL each)were transferred into series of 5 mLsamplebottles each containing blank saliva(2 mL) followed by 1 mL of a solution (0.5 µg/mL) of the internal standardto obtain mixtures containing of 1.25, 2.5, 5.0, 10.0, 20.0 and 25.0 µg/mLrespectively of metformin and 100 µg/mL of the internal standard.The mixtures were vortex mixed and centrifuged at 3000 rpm for 10 min resulting in clear solutions. A quantity(0.5 mL) of each clear solution was injected into the HPLC machine and the respective chromatograms wereobtained. The peak height ratios of metformin/internal standardobtained were then plotted against the corresponding concentrations.

3.2.8.7Validation of HPLC method for metformin

The method was validated with respect to precision, accuracy, percentage recovery, limit of detection and limit of quantitation (ICH, 2006).

Intraday precision

The intraday precision of this method was determined by taking five replicates analysis of a 20.0 µg/mLsolution of metformin using the method at 1 hr intervals within the same day. The percentage coefficient of variation (% CV) was taken as a measure of precision.

Interday precision

The interday precision of this method was determined by taking five replicates analysis of the 20.0 µg/mLsolution of metformin using the methodfor three consecutive days.The percentage coefficient of variation (% CV) was taken as a measure of precision.The % CV was calculated using formula 1.

Accuracy and recovery

The accuracy of this method was checked by standard addition methods, where 80, 100 and 120 % of a 10 μg/mLsolution of metformin were added to same and treated with saliva and the IS as described in the methodology to obtain final concentrations 18, 20 and 22 μg/mL of metformin. The mixtures were centrifuged as described in section 3.2.8.6 before finally injecting into the HPLC machine. After obtaining the chromatograms, the metformin content was determined by subtracting the peak height ratio of metformin/internal standard of the unspiked solution (10 μg/mL) from that found in each of the spiked solutions and interpolating the final concentrations from the calibration curve.The accuracy was calculated using formula 2 while the percentage recoveries were computed using formula 3.

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were determined in a similar manner as described using formulae 5 and 6 respectively.Where σin this case is the standard deviation of y-intercepts of the regression lines determined through LINEST function in Microsoft Office Excel® 2016.

3.2.9 HPLC method development for lisinopril

High performance liquid chromatographic method for the analysis of lisinopril extracted from saliva samples was developed and validated as follows:

3.2.9.1 Preparation of suitable solvent (diluent) for dissolution and extraction of lisinopril standard powder

Although lisinopril is highly soluble in water, it was observed that the solvent that gives better resolution both for the drug and internal standard (caffeine) is methanol:water(50:50). This solvent was used in dissolving the lisinopriland ISthroughout the analysis.

3.2.9.2 Preparation of stock solution of lisinopril

A stocksolution of lisinopril was prepared by accurately weighing and dissolving 2mg of pure lisinopril powder in 20 mL of M:W to obtain a concentration of 100 µg/mL.

3.2.9.3 Preparation of internal standard (caffeine)

A stocksolution of caffeine was prepared by accurately weighing and dissolving 2 mg of pure caffeine powder in 20 mL of M:W to obtain a concentration of 100 µg/mL. Further dilutions were appropriately made where necessary.

3.2.9.4Chromatographic conditions

Chromatographic separation was achieved using Chemsl ODS® C18 (200 mm × 4.6 id). The mobile phase consists of methanol-water containing 0.1 % orthophosphoric acid as additive. Isocratic separation conditions were achieved through varying and optimization of mobile phase ratios, injection volume, temperature, detection wavelength and flow rate after several trials. These stated conditions allowed the detection of lisinopril and caffeine (internal standard) with ample sensitivity.

3.2.9.5Preparation of lisinopril-saliva sample

A solution of lisinopril (12.5 µg/mL) was prepared from the stock solution and a portion (2 mL) was added to blank saliva (2 mL) followed by 1 mL of a solution (0.05 µg/mL) of the internal standard to obtain a mixture containing (5.0 µg/mL) of lisinopril. The mixture was vortex mixed and centrifuged at 3000 rpm for 10 min which gives a relatively clear solution. A quantity (0.5 mL) of the clear solution was injected into the HPLC machine and the chromatogram was obtained which resolved lisinopril from the internal standard.

3.2.9.6Preparation of calibration curve of lisinopril

Various concentrations (2.5, 6.25, 12.5, 25.0, 50.0, 62.5 and 125.0 µg/mL) were prepared from the stock solution and portions (2 mL each) were transferred into series of 5 mL sample bottles each containing blank saliva (2 mL) followed by 1 mL of a solution (0.05 µg/mL) of theinternal standard to obtain mixtures containing of 1.0, 2.5, 5.0, 10.0, 20.0, 25.0 and 50.0 µg/mL respectively of lisinopril and 50 µg/mL of the internal standard.The mixtures were vortex mixed and centrifuged at 3000 rpm for 10 minresulting in clear solutions. A quantity(0.5 mL) of each clear solution was injected into the HPLC machine and the respective chromatograms were obtained. The peak height ratios of lisinopril/internal standard obtained were then plotted against the corresponding concentrations.

3.2.9.7Validation of HPLC method for lisinopril

The method was validated with respect to precision, accuracy, percentage recovery, limit of detection and limit of quantitation (ICH, 2006).

Intraday precision

The intraday precision of this method was determined by taking five replicates analysis of a 10.0 µg/mLsolution of lisinopril using the method at 1 hr intervals within the same day. The percentage coefficient of variation (% CV) in this case was taken as a measure of precision.

Interday precision

The interday precision of this method was determined by taking five replicates analysis of the 10.0 µg/mLsolution of lisinopril using the method for three consecutive days. The % CV was calculated using formula 1.

Accuracy and recovery

The accuracy of this method was checked by standard addition methods, where 80, 100 and 120 % of a 10 µg/mL solution of lisinopril were added to same and treated with saliva and the IS as described in the methodology to obtain final concentrations 18, 20 and 22 µg/mL of lisinopril. The mixtures were centrifuged as described in section 3.2.9.6 before finally injecting into the HPLC machine. After obtaining the chromatograms, the lisinopril content was determined by subtracting the peak height ratio of lisinopril/internal standard of the unspiked solution (10 µg/mL) from that found in each of the spiked solutions and interpolating the final concentrations from the calibration curve.The accuracy was calculated using formula 2 while the percentage recoveries were computed using formula 3.

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were determined in a similar manner as described using formulae 5 and 6 respectively.Where σin this case is the standard deviation of y-intercepts of the regression lines determined through LINEST function in Microsoft Office Excel® 2016.

3.2.10 *In vivo* interaction studies of metformin and lisinopril with *Hibiscus sabdariffa* water extract

This study was conducted using the volunteers who satisfy the following inclusion and exclusion criteria:

Inclusion criteria

1. Healthy male volunteers
2. Age between 18 and 40 years
3. Body mass index (BMI) in the range of 18 – 25 kg/m^3

Exclusion criteria

1. Smoking (more than 10 cigarettes per day)
2. History of regular alcohol consumption
3. Taking other medications within two (2) weeks before entering the study
4. Allergic reactions and hereditary disorder (angioedema) in which lisinopril is contraindicated
5. Subjects with history of liver, kidney and cardiovascular disease that could affect bioavailability of metformin or lisinopril
6. Participated in other clinical experiments within 1 month prior to this study
7. Subjects that took either metformin or lisinopril within the previous two weeks.

3.2.10.1 Ethical approval

Ethical clearance was obtained from the Ahmadu Bello University, Zaria ethical Committee for the Use of Human subjects for Research (ABUCUHSR). An informed written consent was also obtained from the volunteers (see appendix 1 and 2).

The total number of subjects that participated were 48 who were randomly selected and divided into four (4) groups of 12 subjects each.Study was carried out in four (4) phases as

double blind randomized crossover studies with a washout period of two (2) weeks in between the phases.

3.2.10.2 Preparation of Hibiscus sabdariffa water extract

The *Hibiscus sabdariffa* solution in water was prepared by weighing a quantity (5) g of *Hibiscus sabdariffa*calyxes and cold macerated in 1 L of water for 24 hr. It was filtered and a portion of the filtrate (100 mL) was evaporated to dryness using water bath. The extract was weighed and the concentration was found to be 0.25 g/100mL of the extract. By diluting 100 mL of the filtrate containing 0.25 g/100 mL to 1 L a solution with strength of 25 mg/mL was obtained.

3.2.10.3Phase 1 (Administration of drugs alone)

Blank saliva was collected from each subject in group 1 before administration of metformin tablet (500 mg)with 200 mL water.Thereafter, saliva samples were collected at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 12.0, 24.0 and 36.0 hr.A quantity (2 mL)of saliva samples for each volunteer was treated as described in the method.

For lisinopril blank saliva was collected from each subject in group 3 before administration of lisinopril tablet (10 mg)with 200 mL water. Thereafter, saliva samples were collected at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 12.0, 24.0 and 36.0 hr. A quantity (2 mL) of saliva samples for each volunteer was treated as described in the method.

3.2.10.4 Phase 2(Concurrent administration of drugs with Hibiscus sabdariffa water extracts)

In this phase blank saliva was collected from each subject in group 2 before administration of metformin tablet (500 mg)concurrently with 200 mL of *Hibiscus sabdariffa* water extracts (25 mg/mL). Thereafter, saliva samples were collected at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0,

9.0, 12.0, 24.0 and 36.0 hr. A quantity (2 mL) of saliva samples for each volunteer was treated as described in the methodology for metformin.

For lisinoprilblank saliva was collected from each subject in group 4 before administration of lisinopril tablet (10 mg) concurrently with 200 mL of *Hibiscus sabdariffa* water extracts (25 mg/mL). Thereafter, saliva samples were collected at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 12.0, 24.0 and 36.0 hr. A quantity (2 mL) of saliva samples for each volunteer was treated as described in the methodology for lisinopril.

The next two phases (phase 3 and 4) were carried out after the washout period of two (2) weeks.

3.2.10.5 Phase 3(Administration of drugs 30 min after administration of Hibiscus sabdariffa water extracts)

In this phase,blank saliva was collected from each subject in group 3 before administration of 200 mL of *Hibiscus sabdariffa* water extracts (25 mg/mL). After 30 min metformin tablet (500 mg) was administered to the volunteers and saliva samples were collected at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 12.0, 24.0 and 36.0 hr. A quantity (2 mL) of saliva samples for each volunteer was treated as described in the methodology for metformin.

For lisinoprilblank saliva was collected from each subject in group 1 before administration of 200 mL of *Hibiscus sabdariffa* water extracts (25 mg/mL). After 30 min lisinopril tablet (10 mg) was administered to the volunteers and saliva samples were collected at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 12.0, 24.0 and 36.0 hr.A quantity (2 mL) of saliva samples for each volunteer was treated as described in the methodology for lisinopril.

3.2.10.6 Phase 4(Administration of Hibiscus sabdariffa water extracts 30 minafter administration of drugs)

In this phase, blank saliva was collected from each subject in group 4 before administration of metformin tablet (500 mg). After 30 min 200 mL of *Hibiscus sabdariffa* water extracts (25 mg/mL) was administered to the volunteers and saliva samples were collected at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 12.0, 24.0 and 36.0 hr.A quantity (2 mL) of saliva samples for each volunteer was treated as described in the methodology for metformin.

For lisinopril blank saliva was collected from each subject in group 2 before administration of lisinopril tablet (10 mg). After 30 min 200 mL of *Hibiscus sabdariffa* water extracts (25 mg/mL) was administered the volunteers and saliva samples were collected at 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 12.0, 24.0 and 36.0 hr. A quantity (2 mL) of saliva samples for each volunteer was treated as described in the methodology for lisinopril.

3.2.11Evaluation ofpharmacokinetic parameters

The drug-internal standard peak height ratio obtained from the chromatograms of each volunteerwere used to determine the drug concentrations by interpolating from the calibration curves of each drug.Pharmacokinetic parameters like elimination rate constant (K_E), biological half-life ($t_{1/2}$), absorption rate constant (Ka), absorption half-life ($t_{1/2}$), volume of distribution (V_d),clearance (Cl), maximum plasma drug concentration (C_{max}), time at which C_{max} is observed (T_{max}) and total area under the curve (AUC_0^∞)which is the sum of the last and extrapolated areas under concentration time curve ($AUC_0^t + AUC_t^\infty$)were all computed using Kinetica software version 5.0 from the mean drug plasma concentrations.

3.2.12 Statistical analysis

Results obtained were analyzed with Graph Pad Prism 6 software using Students *t-test* and one-way ANOVA then followed by a Post hoc test where applicable and it was presented as percentages, means ± SD.

CHAPTER FOUR

4.0 RESULTS

4.1Quality control of metformin, lisinopril and caffeine (standard powders and tablets)

The labeled information of the tablet brands used for this study are shown in Table 4.1. The melting point and assay results of both standard powders and tablet samples used in this study are shown in Table 4.2. Other parameters including dissolution and uniformity of weight are shown in Table 4.3, while disintegration test results are shown in Table 4.4. FT-IR spectra of the standard and tablet samples superimposed with reference spectra are shown in Figure 4.1, 4.2, 4.3, 4.4 and 4.5 respectively.

Table 4.1: Labeled information of tablet brands used

Drug	Brand	Batch Number	Expiry Date	NAFDAC NO
Metformin	Glucophage® (500 mg)	Y00309	03/2020	04-6233

Lisinopril	Zestril® (10 mg)	LT626	01/2019	04-9760

Table 4.2: Assay and melting point of metformin, lisinopril and caffeine standard powders and tablets

Samples	Drug content (BP range) (%)	Melting point (°C)
Metformin standard powder	99.36 (98.50 – 101.00)	223 - 225
Metformin tablets	102.00 (95.00 – 105.00)	224 - 226
Lisinopril standard powder	100.40 (98.50 – 101.50)	157 – 160
Lisinopril tablets	103.50 (98.50 – 105.00)	159 - 161
Caffeine standard powder	100.26 (98.50 – 101.50)	235 - 237

Melting point range:

Metformin standard powder (222 – 226)
Metformin tablets (222 – 226)
Lisinopril standard powder (158 – 162)
Lisinopril tablets (158 – 162)
Caffeine standard powder (234 – 239)

Table 4.3: Uniformity of weight and dissolution of metformin and lisinopril tablets

Samples	Percentage deviation (%) n=20	Dissolution testdrug release (BP range) (%)
Metformin tablets	≤ 3.5	89.0 (≥ 85)
Lisinopril tablets	≤ 2.3	87.0 (≥ 85)

BP, 2013 range for uniformity of weight
For tablet 80 mg or less = 10 % deviation allowed
For tablet 250 mg or more = 5 % deviation allowed

Table 4.4: Disintegration test of metformin and lisinopril tablets

Samples S/No	Metformin disintegration time (min)	Lisinopril disintegration time (min)
1	6.05	5.20
2	6.10	5.20
3	6.15	5.25
4	6.20	5.30
5	6.20	5.35
6	6.21	5.40

BP specification: All 6 tablets should dissolve completely in < 15 min

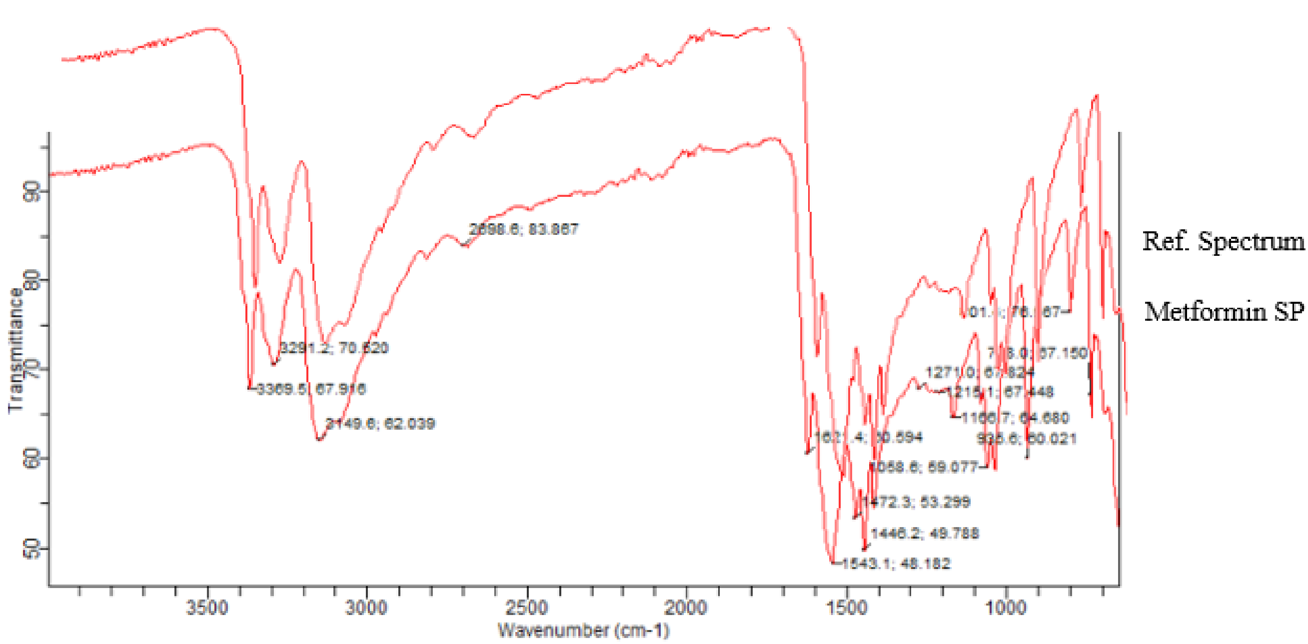


Figure 4.1: Superimposed FT-IR spectra of metformin standard powder and reference

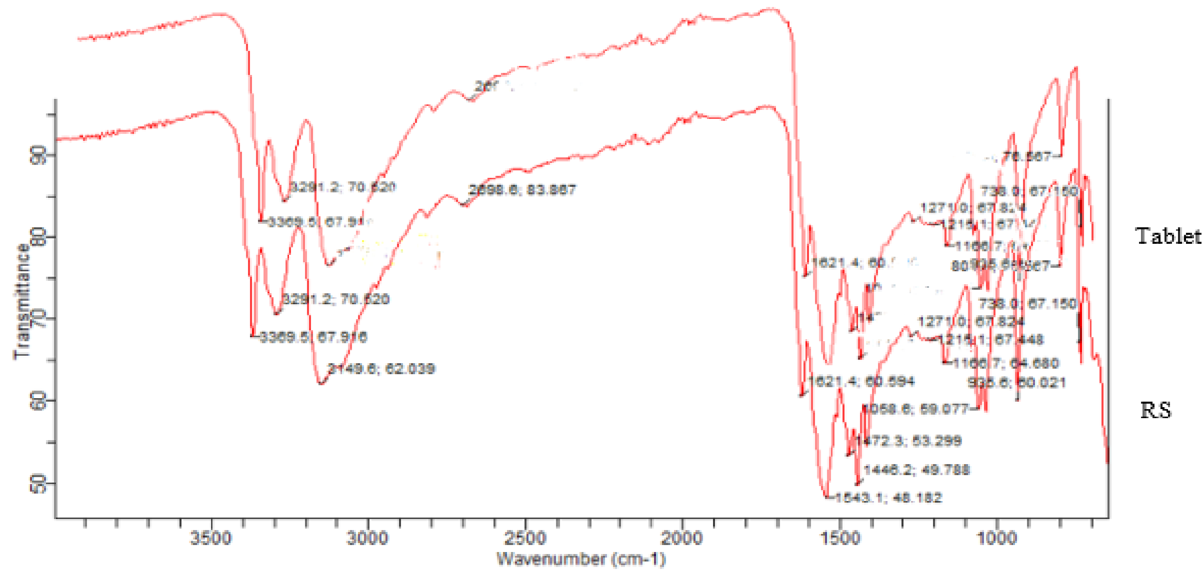


Figure 4.2: Superimposed FT-IR spectra of metformin standard powder and tablet

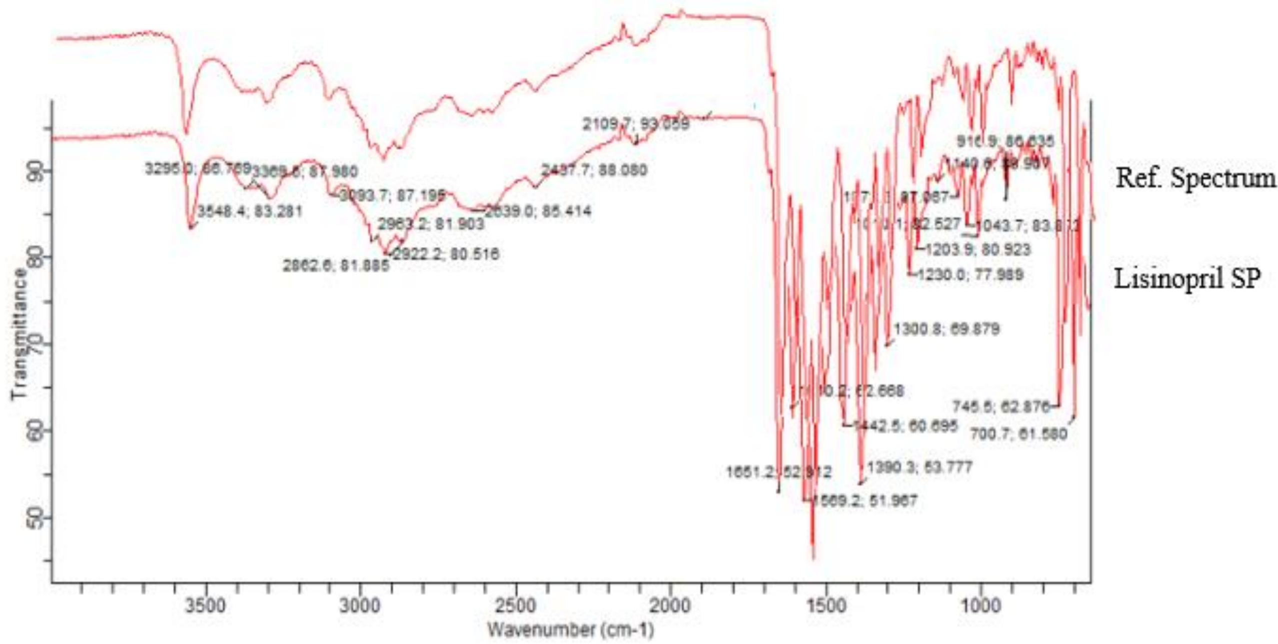
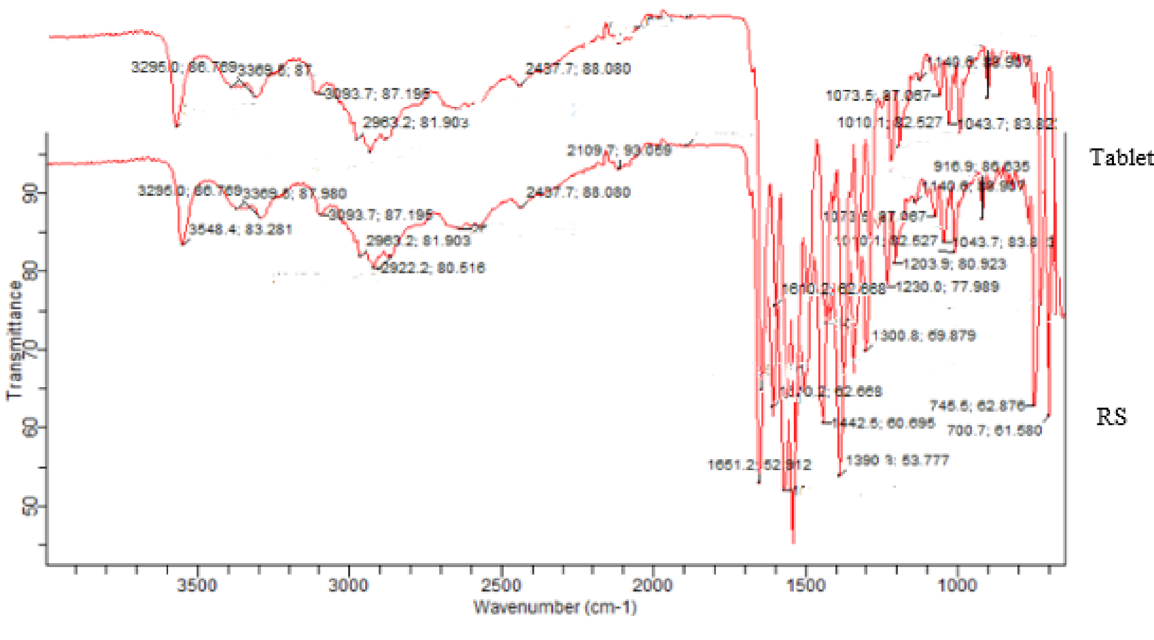


Figure 4.3: Superimposed FT-IR spectra of lisinopril standard powder and reference



Tablet
RS
Transmittance
Wavenumber (cm-1)
3500
3000
2500
2000
1500
1000
90
80
70
60
50
3548.4; 83.281
2922.2; 80.516
2437.7; 88.080
1300.8; 69.879
1230.0; 77.989
1203.9; 80.923
1442.5; 60.695
745.6; 62.876
700.7; 61.580
1073.5; 87.067

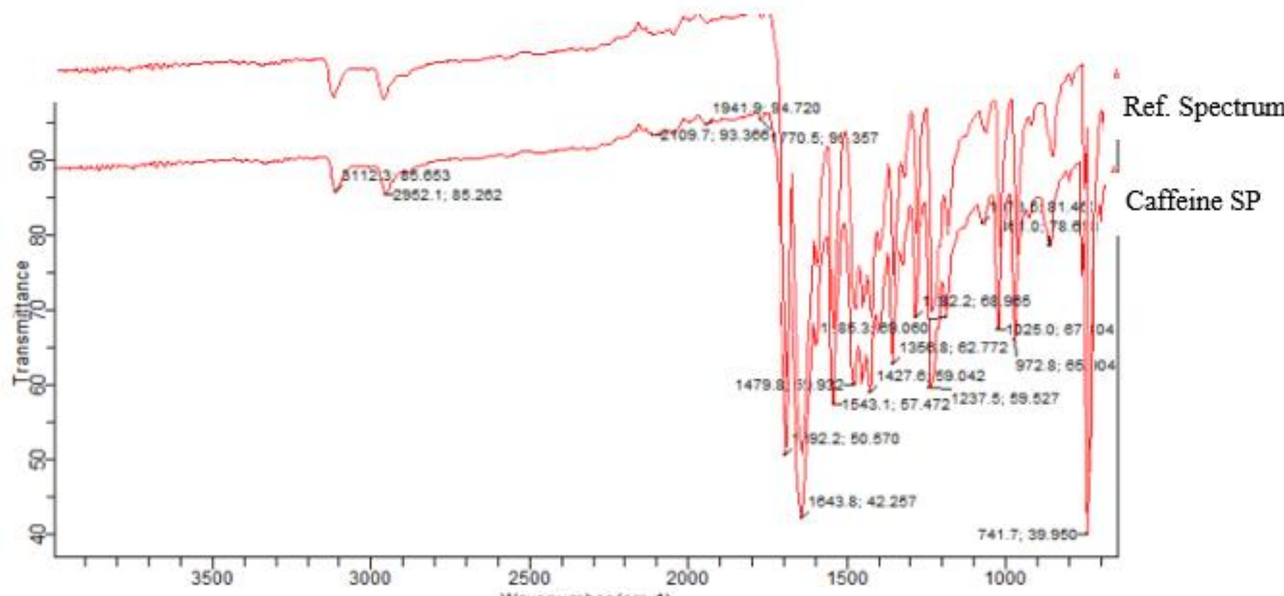


Figure 4.5: Superimposed FT-IR spectra of caffeine standard powder and reference

4.2 UV Developed and validated UV spectrophotometric methods

The calibration curves for the determination of metformin and lisinopril in all the methods obeyed the Beer-Lambert's law. The linear relationship between absorbance (A) and concentration (C in µg/mL) gives the regression equations A = Cy ± x, and the coefficients of correlation (r) and wavelengths of maximum absorptions are shown in Table 4.5 and the calibration curves are presented in Figures 4.6, 4.7, 4.8, 4.9, 4.10 and 4.11.

The results of the validation parameters for the methods are shown in Table 4.6 while the results of the drugs content assayed using the developed methods and comparison with USP, 2012 and BP, 2013 methods are shown in Table 4.7.

Table 4.5: Calibration curve parameters of developed UV spectrophotometric methods

	0.1M HCl (pH 1.2)		pH 6.8		pH 7.4	
Parameter	Metformin	Lisinopril	Metformin	Lisinopril	Metformin	Lisinopril
λ_{max} (nm)	205	215	235	210	232	215
Linearity range (µg/mL)	2.50 – 15.0	2.50 – 15.0	2.50 – 12.5	2.50 – 12.5	2.50 – 10.0	2.50 – 10.0
Correlation coefficient (r)	0.9992	0.9974	0.9994	0.9918	0.9990	0.9987
Regression equation	A = Cy + x	A = Cy + x	A = Cy - x	A = Cy - x	A = Cy + x	A = Cy - x
Slope (y)	0.0524	0.0328	0.1415	0.0534	0.1561	0.04
Intercept (x)	0.012	0.0202	0.02	0.122	0.0465	0.0148

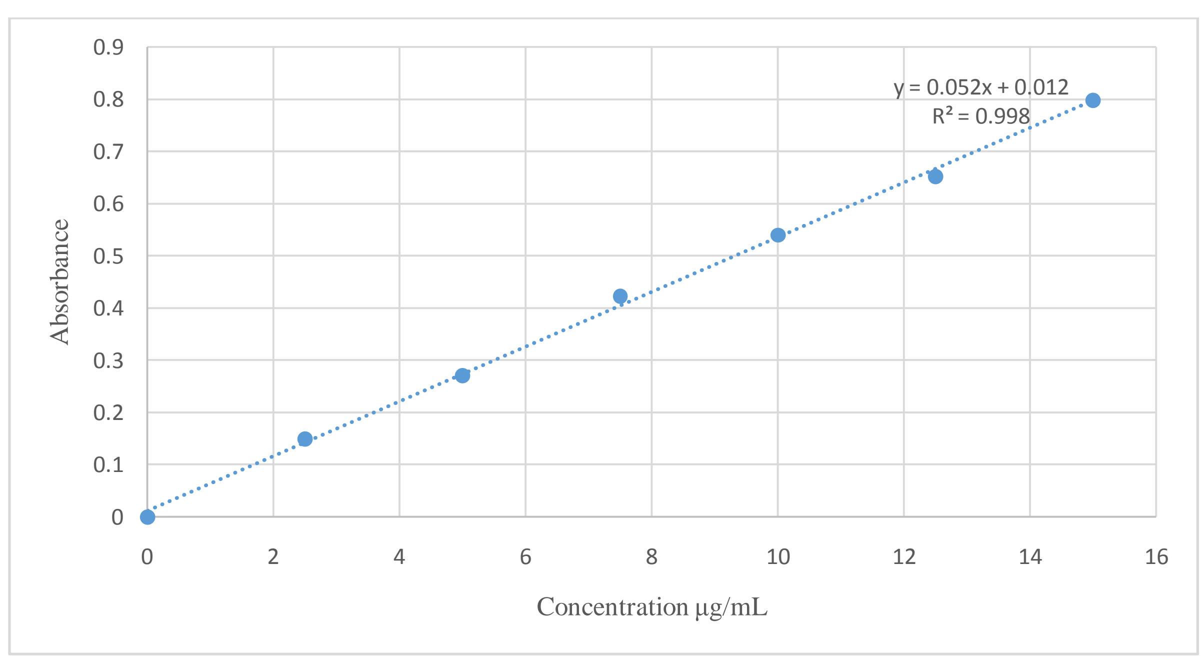


Figure 4.6: Calibration curve of metformin in 0.1 M HCl

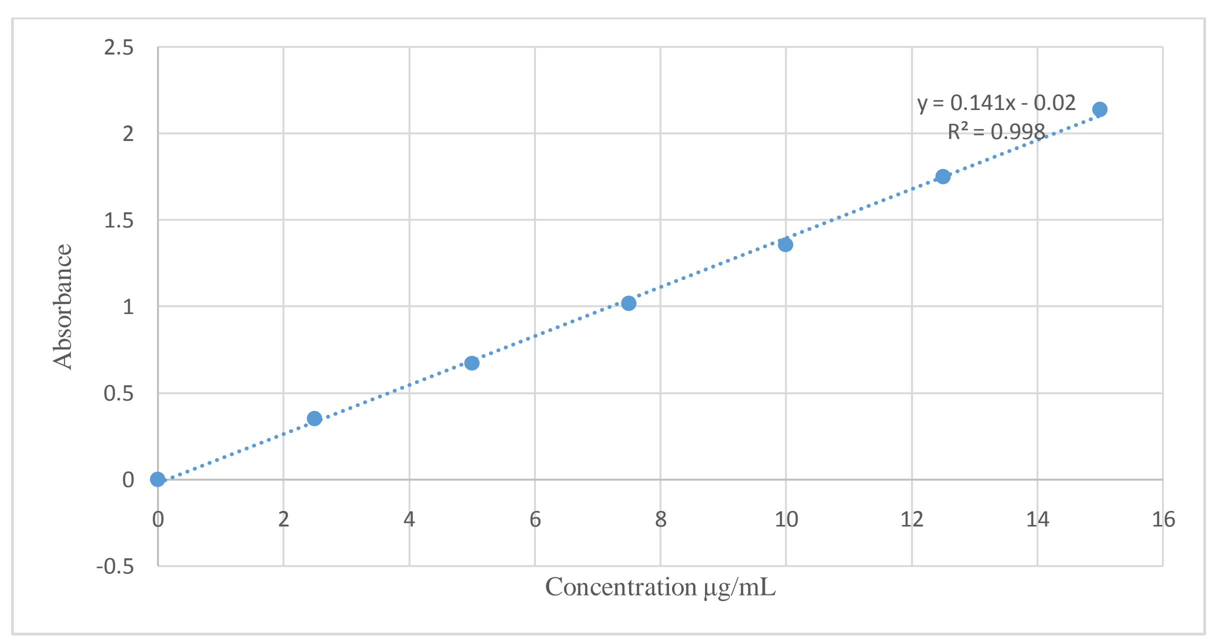


Figure 4.7: Calibration curve of metformin in phosphate buffer pH 6.8

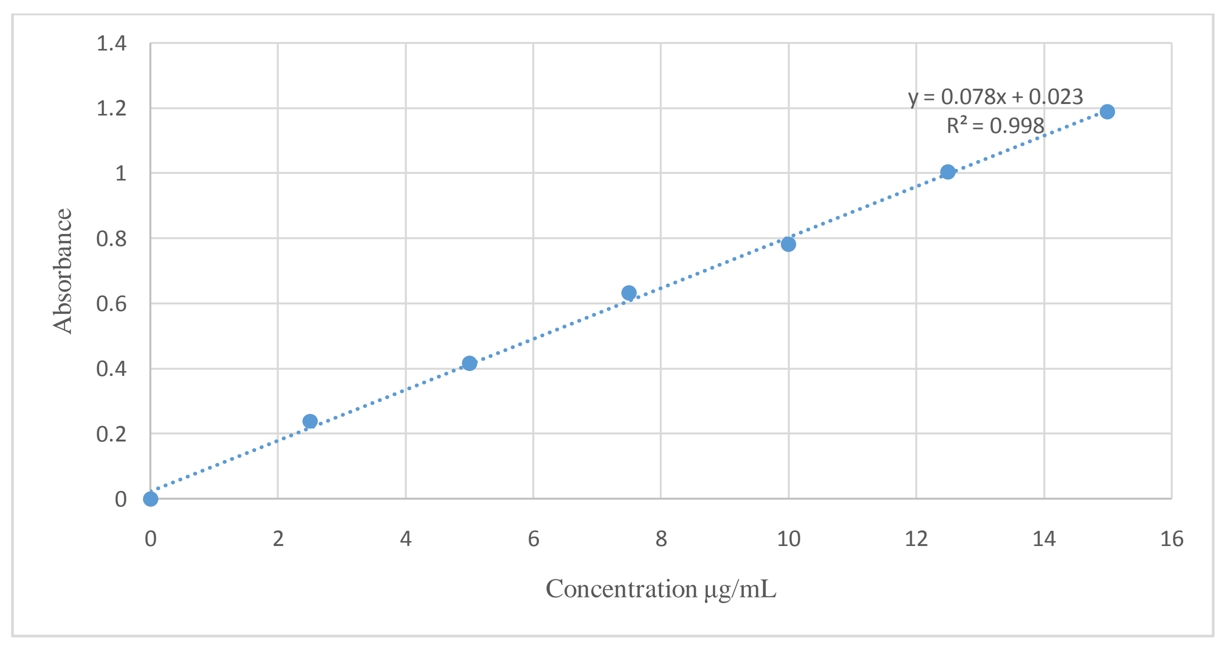


Figure 4.8: Calibration curve of metformin in phosphate buffer pH 7.4

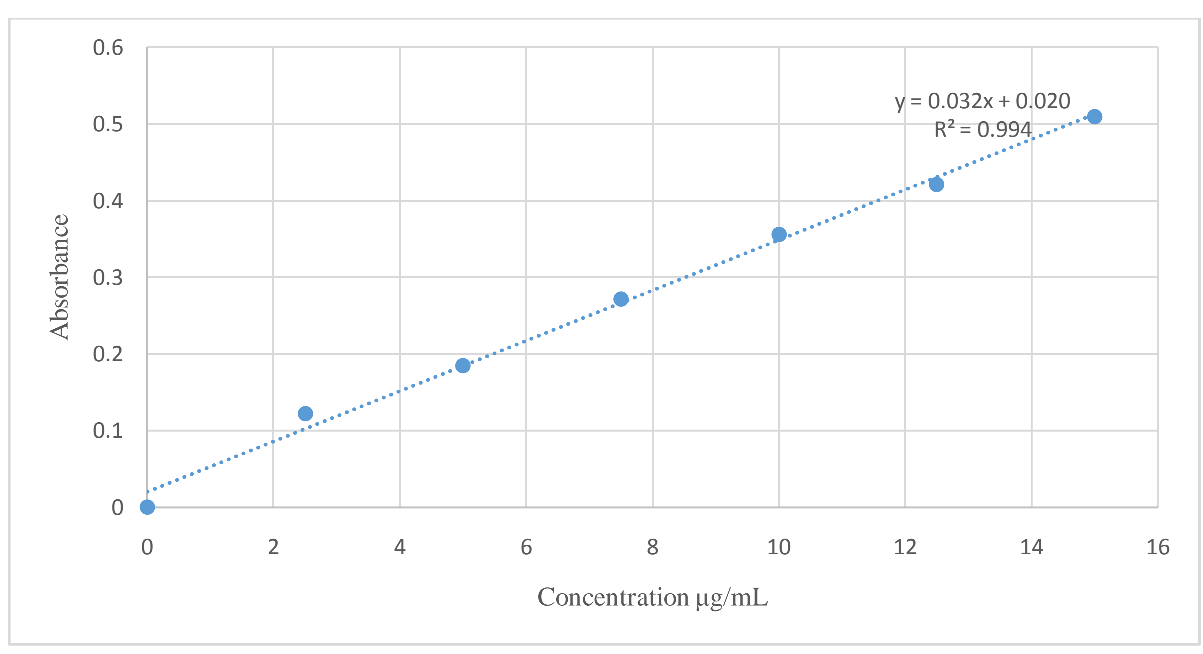


Figure 4.9: Calibration curve of lisinopril in 0.1 M HCl

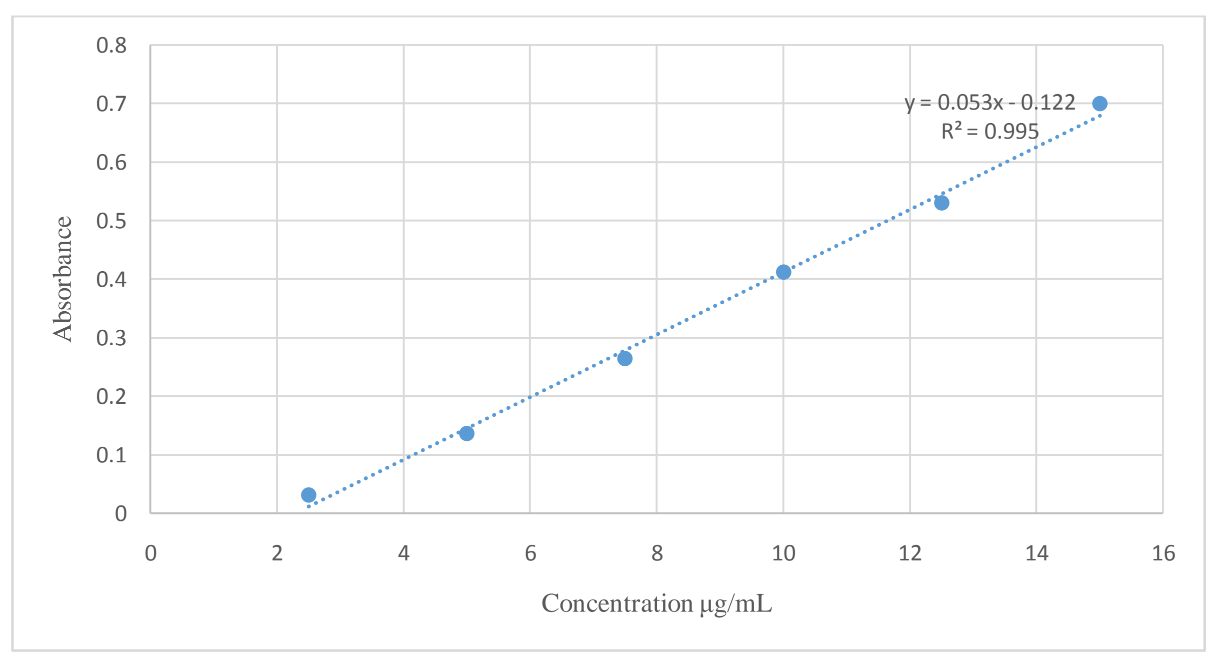


Figure 4.10: Calibration curve of lisinopril in phosphate buffer pH 6.8

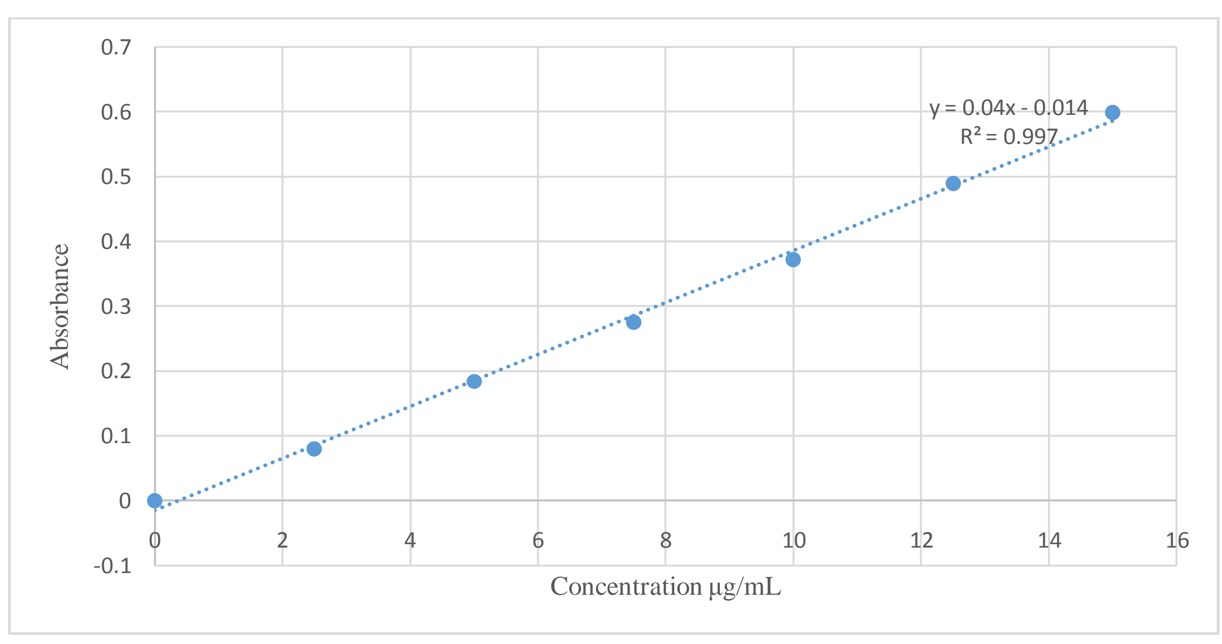


Figure 4.11: Calibration curve of lisinopril in phosphate buffer pH 7.4

Table 4.6: Validation parameters of developed UV spectrophotometric methods

	0.1M HCl (pH 1.2)		pH 6.8		pH 7.4	
Parameter	Metformin	Lisinopril	Metformin	Lisinopril	Metformin	Lisinopril
Precision (% RSD)	1.8	1.7	1.6	1.9	1.9	1.7
Accuracy ± SD (% Er)	2.3 ± 0.10	2.0 ± 0.16	2.9 ± 0.16	2.5 ± 0.14	2.0 ± 0.32	2.1 ± 0.07
Recovery ± SD (%)	99.5 ± 0.50	99.7 ± 0.11	98.8 ± 0.35	98.9 ± 0.10	99.3 ± 0.18	99.6 ± 0.20
Limit of detection (µg/mL)	0.53	0.96	0.47	1.05	0.58	0.67
Limit of quantification (µg/mL)	1.61	2.90	1.43	3.20	1.76	2.06

RSD = relative standard deviation; Er = relative error

Table 4.7: Content of metformin and lisinopril assayed using the developed methods and official method

Dissolution Media	% Content of metformin ± SD		% Content of lisinopril ± SD	
	Developed methods	BP method	Developed methods	USP method
0.1M HCl (pH 1.2)	103.00 ± 0.12	102.60 ± 0.10	102.00 ± 0.72	101.70 ± 0.20
Phosphate buffer (pH 6.8)	103.00 ± 0.07	102.60 ± 0.10	102.00 ± 0.42	101.70 ± 0.20
Phosphate buffer (pH 7.4)	103.00 ± 0.21	102.60 ± 0.10	102.00 ± 0.15	101.70 ± 0.20

BP range (95 – 105 %), n = 3; USP range (90.0 – 110.0 %), n = 3

4.3 *In vitro* availability studies

The results of the *in vitro* availability of metformin and lisinopril at different time intervals in simulated gastric pH, intestinal pH and blood pH at 37 ^{o}C alone and in the presence of *Hibiscus sabdariffa* L. water extract (1 g/L) are shown in Figures 4.12, 4.13, 4.14 and 4.15 respectively.

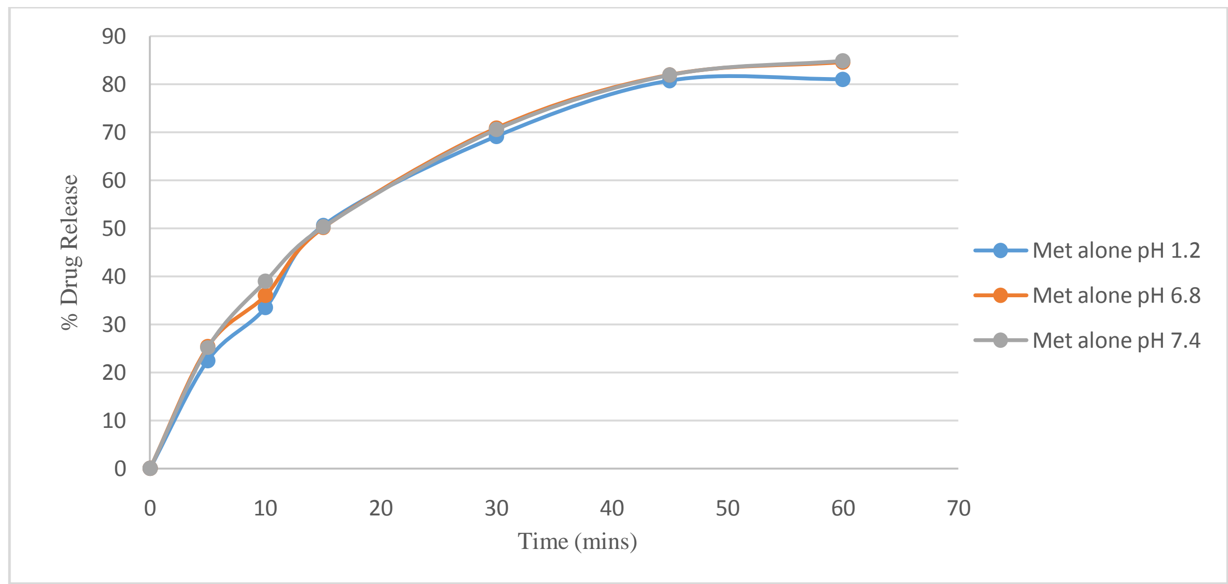


Figure 4.12: Percentage of metformin releasedover a period ofan hourin the dissolution media.

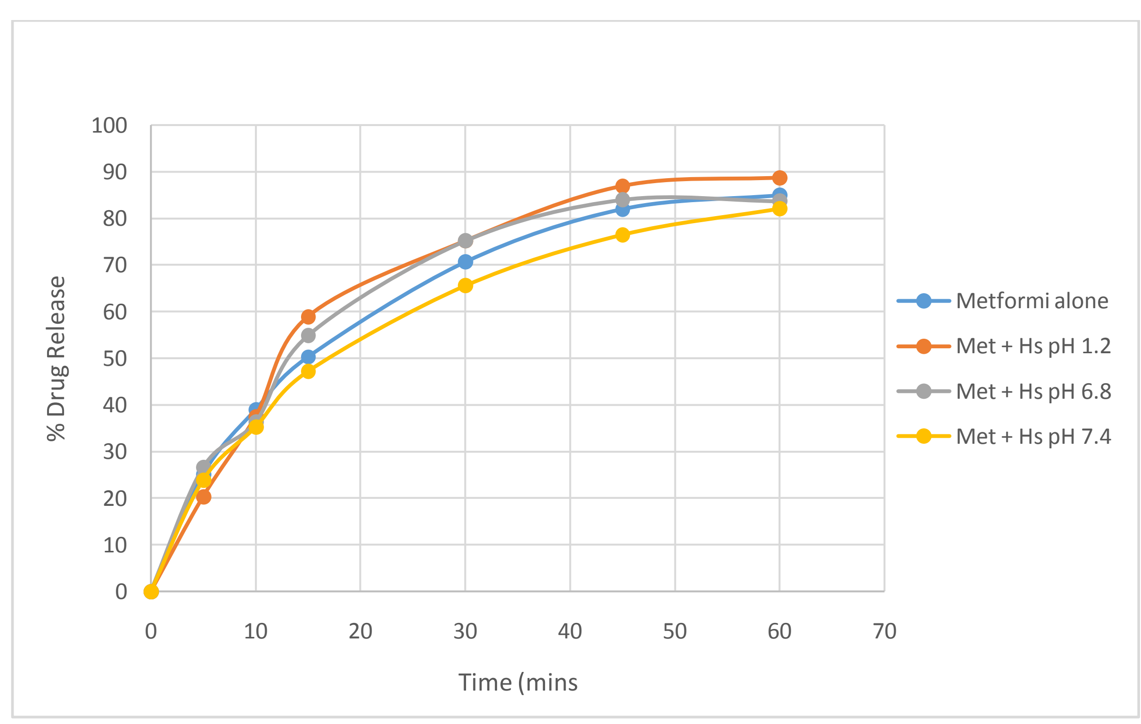


Figure 4.13: Percentage of metforminreleasedalone and when interacted with *Hibiscus sabdariffa* calyxes water extract (1 g/L) in the dissolution media.

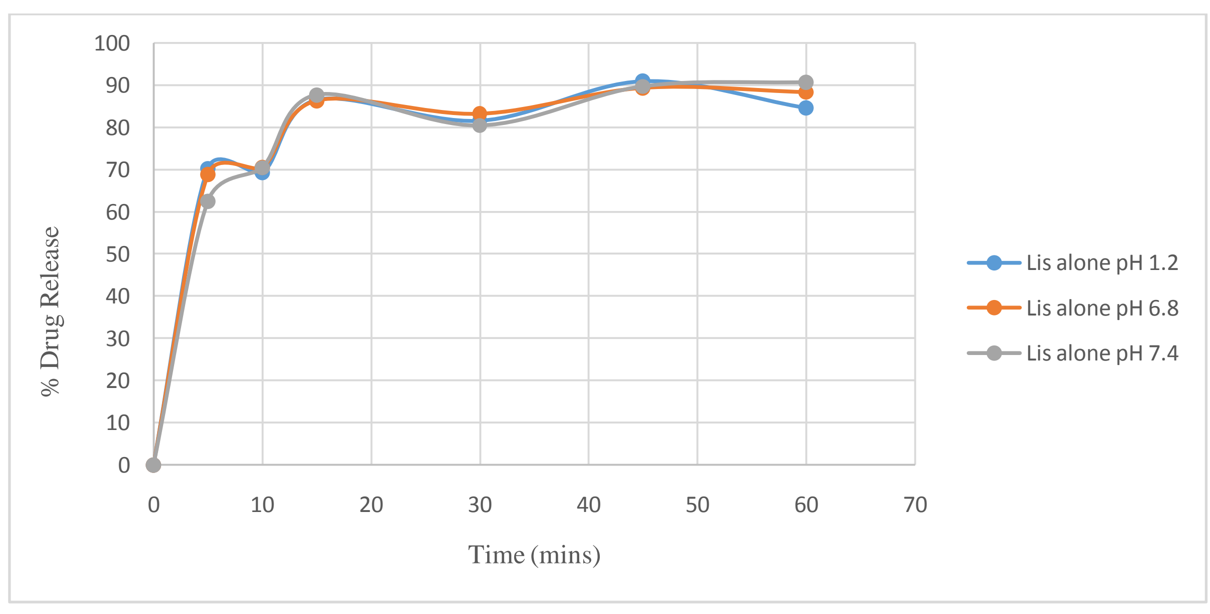


Figure 4.14: Percentage of lisinopril releasedover a period of an hour in thedissolution media.

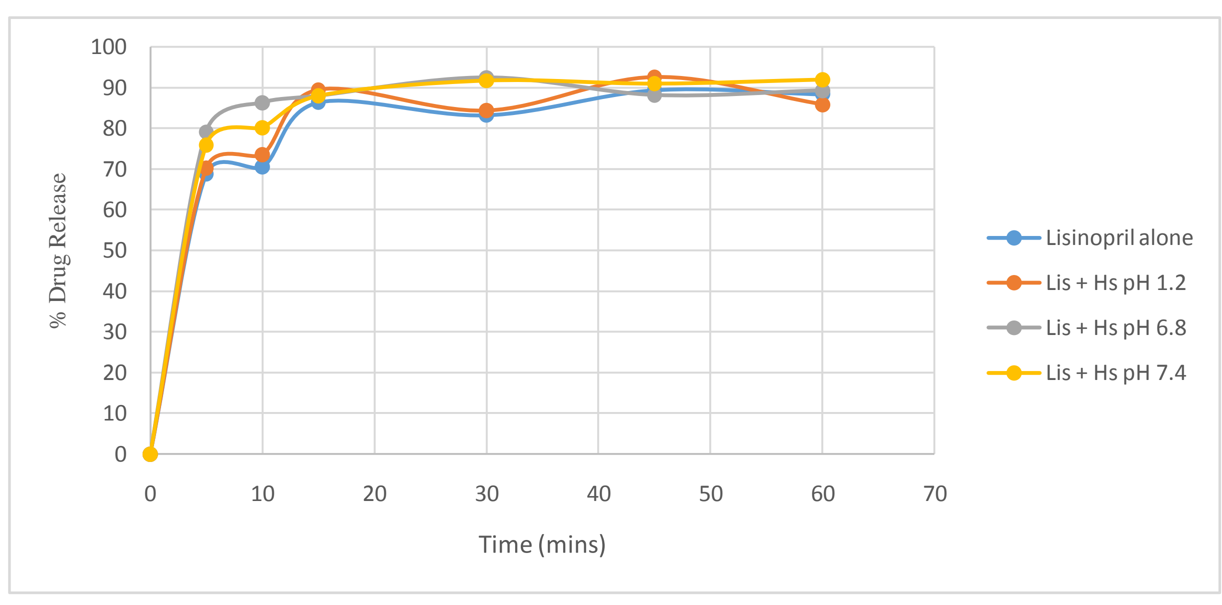


Figure 4.15: Percentage of lisinoprilreleasedalone and when interacted with *Hibiscus sabdariffa* calyxes water extract(1 g/L) in the dissolution media.

4.4Developed and validated RP-HPLC methods for metformin and lisinopril

The optimized chromatographic conditionsof the RP-HPLC methods for metformin and lisinopril are shown in Table 4.8 while the chromatograms obtained are presented in Figures 4.16, 4.17, 4.18 and 4.19.

Calibration parameters for RP-HPLC methods of metformin and lisinopril are shown in table 4.9. The linear relationship between the peak height ratios of the drugs-internal standard (A) and concentrations (C in ng/mL) gives the regression equations $A = Cy \pm x$. Calibration curves for metformin and lisinopril are shown in figures 4.20 and 4.21 respectively.

The results of the validation parameters for the RP-HPLC methods for metformin and lisinopril are shown in Table 4.10.

Table 4.8: Optimized chromatographic conditions of HPLC methods

	Descriptions	
Parameters	Metformin	Lisinopril
Mobile phase	Methanol:Water (80:20)	Methanol:Water (80:20)
Column	Chemsl ODS® C18	Chemsl ODS® C18
Column size	200 mm×4.6 mm i.d., 5µ particle size	200 mm×4.6 mm i.d., 5µ particle size
Additive	0.1% orthophosphuric acid	0.1% orthophosphuric acid
Detection wavelengthλ	232 nm	218 nm
Column temperature	35 °C	35 °C
Flow rate	10 µL	10 µL
Injection volume	1 mL/min	1 mL/min
Runtime	6 min	4 min
Retention time	1.6 min	1.7 min

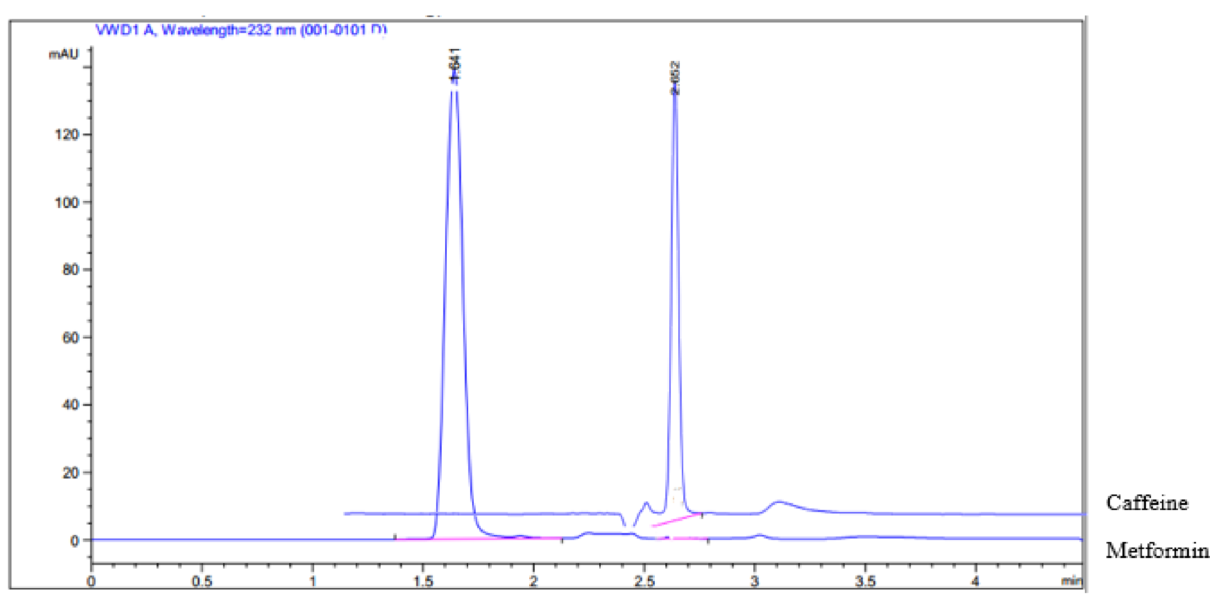


Figure 4.16: Superimposed RP-HPLC chromatogram of metformin and internal standard

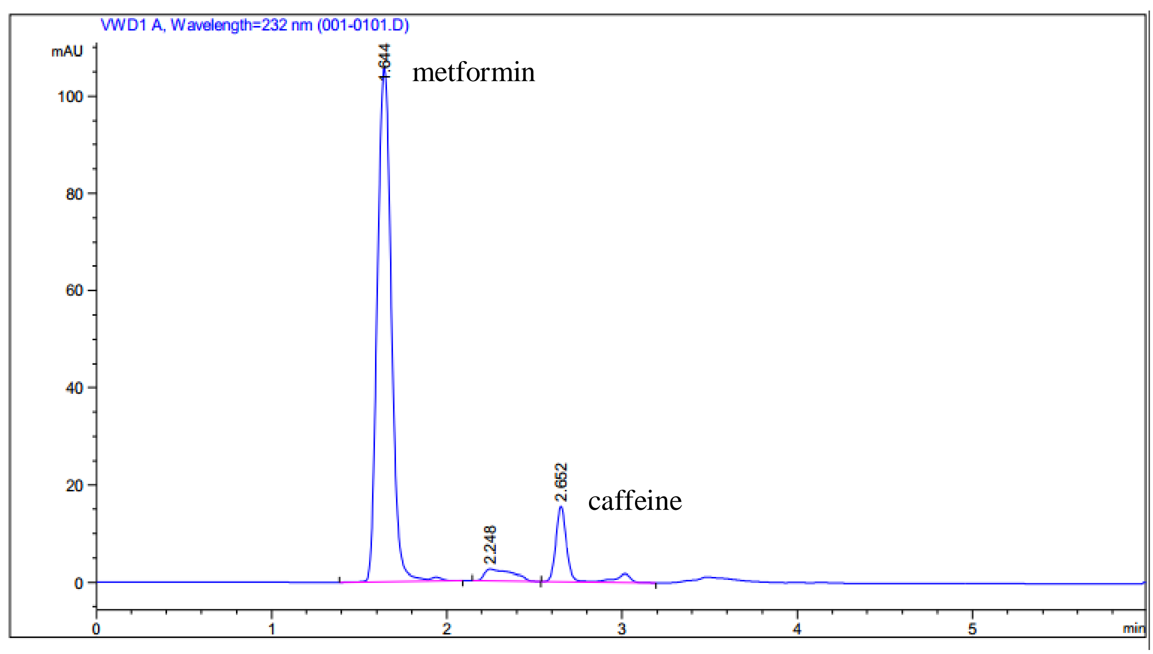


Figure 4.17: RP-HPLC chromatogram of metformin and internal standard spiked with saliva

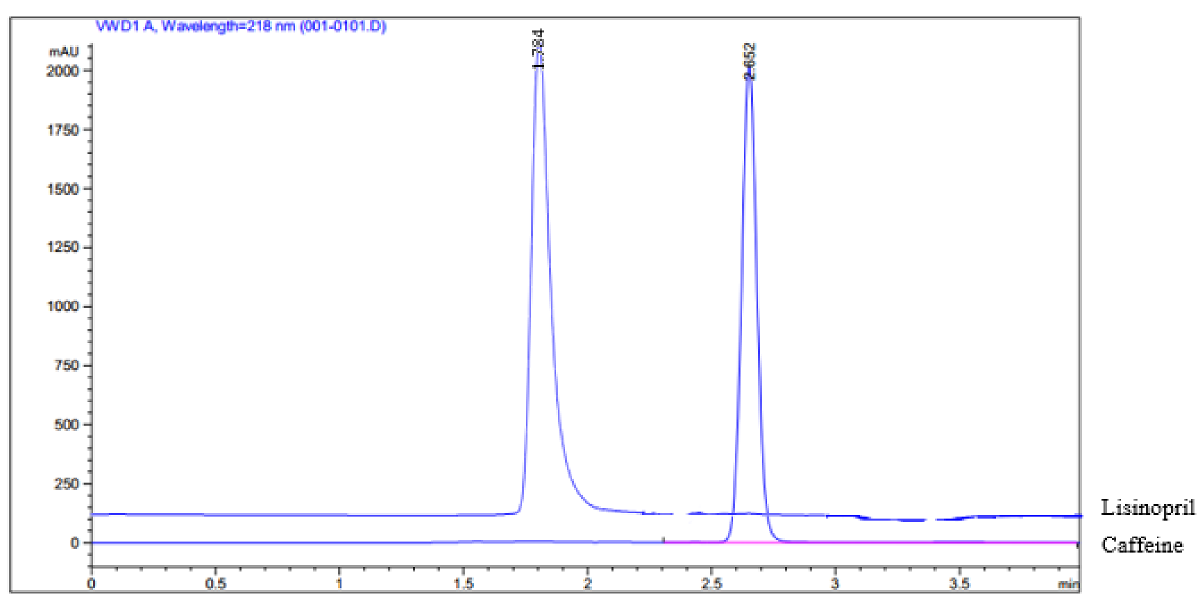


Figure 4.18: Superimposed RP-HPLC chromatogram of lisinopril and internal standard

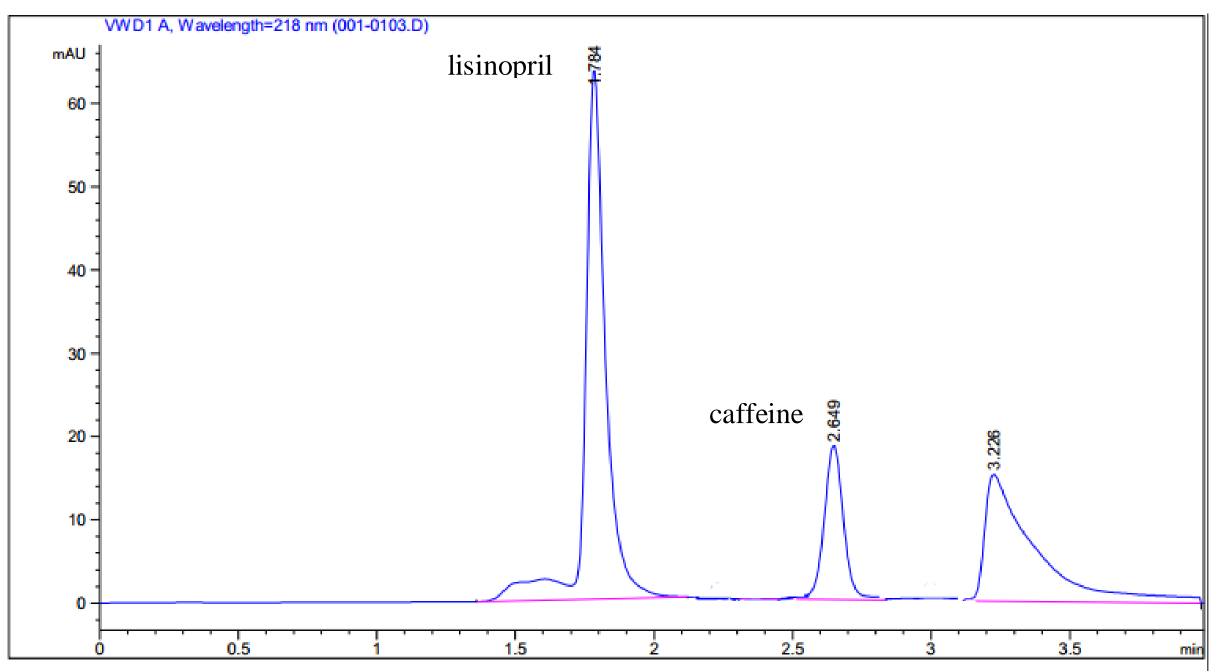


Figure 4.19: RP-HPLC chromatogram of lisinopril and internal standard spiked with saliva

Table 4.9: Calibration curve parameters of the developed RP-HPLC methods

Parameter	Value obtained	
	Metformin	Lisinopril
Linearity range (µg/mL)	1.25 – 25.0	1.0 – 50.0
Correlation coefficient (r)	0.9987	0.9981
Regression equation	A = Cy + x	A = Cy + x
Slope (y)	1.7997	0.147
Intercept (x)	0.5297	0.0984

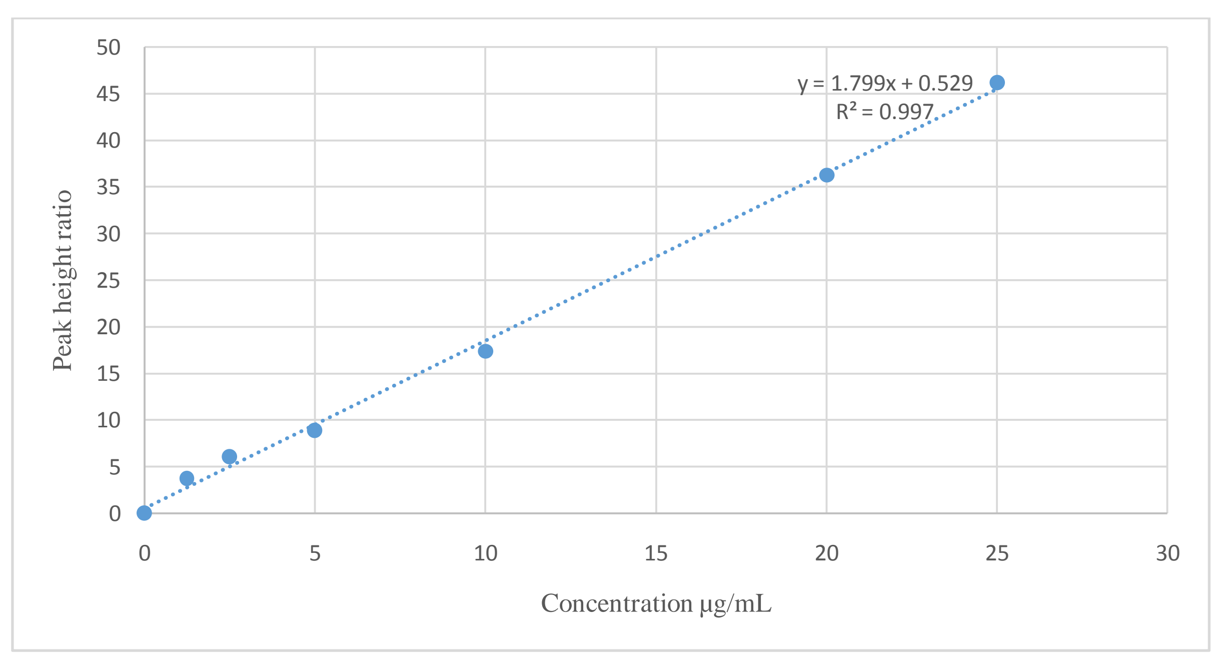


Figure 4.20: RP-HPLC calibration curve of developed method for metformin

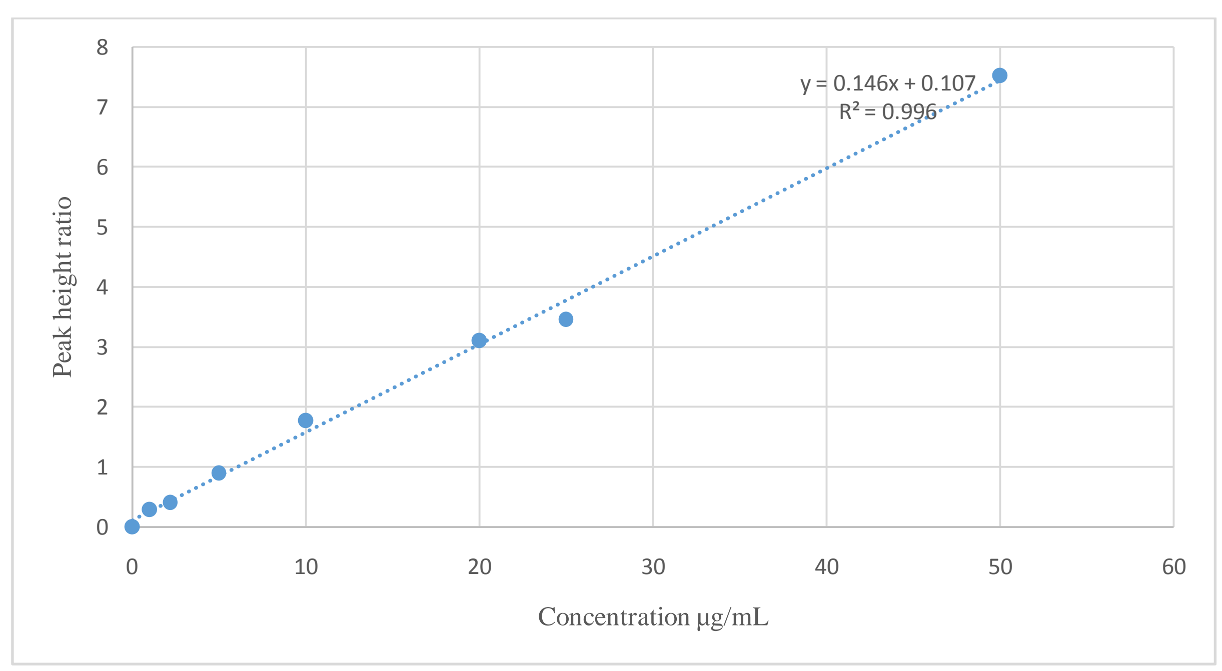


Figure 4.21: RP-HPLC calibration curve of developed method for lisinopril

Table 4.10: Validation parameters of RP-HPLC methods developed

Parameter	Value obtained	
	Metformin	Lisinopril
Precision Intraday (% RSD)± SD	0.5 ± 0.12	0.6 ± 0.16
Interday (% RSD)± SD	0.7 ± 0.11	0.8 ± 0.12
Accuracy ± SD (% Er)	1.00 ± 0.12	1.20 ± 0.30
Recovery ± SD (%)	99.98 ± 0.10	99.89 ± 0.20
Limit of detection (ng/mL)	0.22	0.15
Limit of quantification (ng/mL)	0.67	0.46

4.5 *In vivo* interaction study of metformin and lisinopril with *Hibiscus sabdariffa* L.

Mean saliva metformin concentrations versus time obtained from the volunteers in all the four phases of the study are displayed in Figure 4.22 and Appendix 28. The mean pharmacokinetic parameters generated for metformin administered alone and when interacted with *Hibiscus sabdariffa* calyxes water extractin all the other phases of the study aredisplayed in Table 4.11. On the other hand, mean saliva lisinopril concentrations versus time obtained from the volunteers in all the four phasesof the interaction study are displayed in Figure 4.23 and Appendix 29. The meanpharmacokinetic parameters generated for lisinopril administered alone and when interacted with *Hibiscus sabdariffa* calyxes water extracts in phase 2, 3, and 4 are also displayed in Table 4.12.

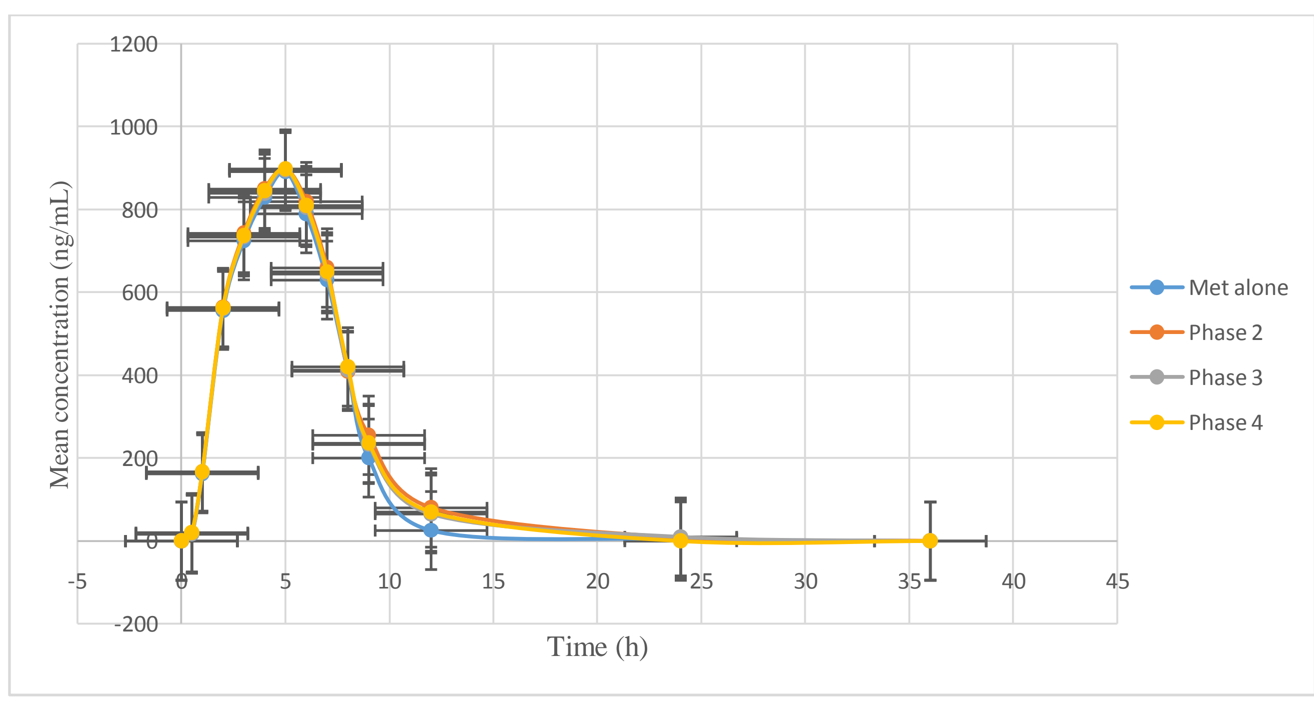


Figure 4.22: Mean saliva concentrations of metformin against time for all the phases of the study

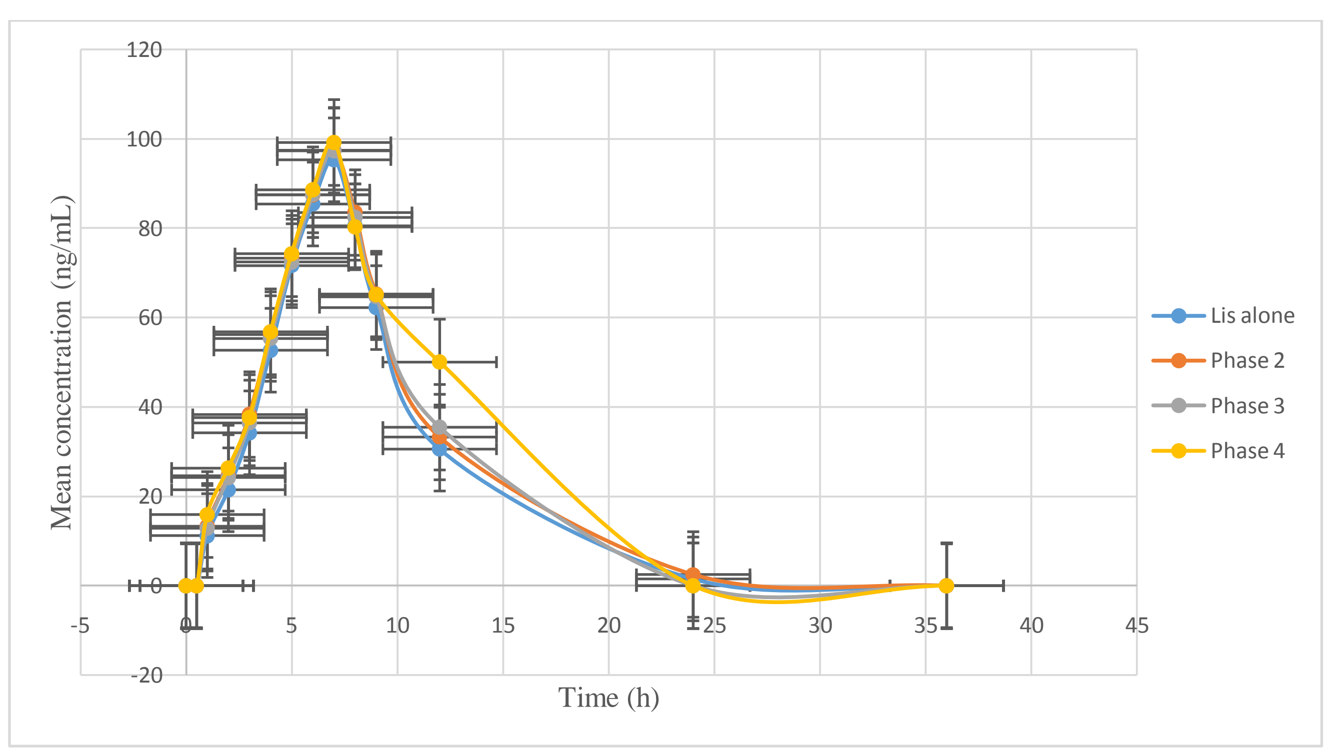


Figure 4.23: Mean saliva concentrations of lisinopril against time for all the phases of the study

Table 4.11: Mean pharmacokinetic parameters of metformin (500 mg) alone and when interacted with 200 mL of *Hibiscus sabdariffa* calyxes water extract (25 mg/mL)

PK Parameter	Phase 1	Phase 2	Phase 3	Phase 4
Tlag (hr)	0.000	0.000	0.000	0.000
K_a (ng hr^{-1})	0.823	0.753	0.929	0.409
K_E (ng hr^{-1})	0.287	0.206	0.244	0.446
$T_{1/2}$ (hr)	2.412	2.651	2.838	1.555
C_{max} (ng mL^{-1})	892.320	898.280	895.300	898.520
T_{max} (hr)	5.000	5.000	5.000	5.000
AUC_0^t (ng.hr mL^{-1})	5448963.064	6008468.234	5873608.767	5569117.265
AUC_t^∞ (ng.hr mL^{-1})	11253.220	23068.785	31360.984	152838.308
AUC_0^∞ (ng.hr mL^{-1})	5460216.284	6031537.018	5904969.750	5721955.572
Cl (Lhr^{-1})	91.572	82.898	84.674	87.383
Vd (L)	318.585	317.066	346.793	195.990

Table 4.12: Mean pharmacokinetic parameters of lisinopril (10 mg) alone and when interacted with 200 mL of *Hibiscus sabdariffa* calyxes water extract (25 mg/mL)

PK Parameter	Phase 1	Phase 2	Phase 3	Phase 4
Tlag (hr)	0.500	0.500	0.500	0.500
K_a (ng hr^{-1})	0.557	0.601	0.633	0.915
K_E (ng hr^{-1})	0.248	0.216	0.204	0.132
$T_{1/2}$ (hr)	2.799	3.202	3.391	5.270
C_{max} (ng mL^{-1})	95.360	97.500	97.370	99.240
T_{max} (hr)	7.000	7.000	7.000	7.000
AUC_0^t (ng.hr mL^{-1})	730602.846	788798.425	643247.706	679.104
AUC_t^∞ (ng.hr mL^{-1})	6202.982	11610.866	173618.763	365.923
AUC_0^∞ (ng.hr mL^{-1})	736805.828	800409.291	816866.469	1045.027
Cl (Lhr^{-1})	13.572	12.494	12.242	9.569
Vd (L)	54.802	57.721	59.892	72.756

CHAPTER FIVE

5.0 DISCUSSION

5.1 Quality control of metformin, lisinopril and caffeine (standard powders and tablets)

The FT-IR spectra of metformin and lisinopril standard powders and tablets (Figures 4.1, 4.2, 4.3 and 4.4)were seen to be completely superimposed in both the functional groups and fingerprint regions to the respective spectra of the reference standards. FT-IR spectrum of caffeine (Figure 4.5)also conformed with the official specifications (BP, 2013). Thus, the standard powders and tabletsused for the study contain the appropriate active ingredients.

The assay results (Table 4.2) revealed the content of metformin and lisinopril in both the pure powders and tablet samples and were within the officialranges. Also assay result of caffeine is in conformity with official range (BP, 2013). These indicate that the standard powders and tablets used for this study are genuine and contained the active pharmaceutical ingredients (APIs) in the labelled and stated amounts.

Weight variation and dissolution tests results (Table 4.3) indicates assurance in consistency of dosage units during compression with respect to flow properties of the powders, granule density and particle size distribution while the content of the drugs obtained during dissolution implies the*in vitro*consistency in batch-to-batch solid oral dosage forms which gives good prediction of *in vivo* drugs release profiles. Both tests fall within the stated limit (BP, 2013).

It was observed that all metformin and lisinopril tablets (6 tablets each) disintegrates in less than 10 min(Table 4.3) and therefore, are said to pass the test as prescribed by BP, (2013). This provides a hint that the tablet brands used for this study are not only of good quality but also serves as good predictors of the APIs*in vivo*.

5.2 UV spectrophotometric methods development for metformin and lisinopril

For the quantitative determination of drugs content, it was observed that the calibration curves of metformin in simulated gastric juice (0.1M hydrochloric acid), intestinal pH (Phosphate buffer pH 6.8) and blood pH (Phosphate buffer pH 7.4) at their respective λ_{max}obeyed the Beer-Lambert's Law. The linear relationship between absorbance (A) and concentration (C in % w/v) was given by the regression equationsof the type A=Cy ± x (table 4.5). The coefficient of correlation (r) in each case is approaching unity(0.999). For lisinopril, the linear relationship between absorbance (A) and concentration (C in % w/v) was also given by the regression equations of the type A = Cy ± x (table 4.5). The coefficient of correlation (r) in each case is approaching unity(0.998). This clearly shows the direct proportional relationship and high correlation between the absorbance (A) and the concentrations used for these determinations in both cases.

The low % RSD (Table 4.6) for all the methods shows that the precisions of the methods were satisfactory. With good technique and reliable methodology the precision should be < 15 % CV (Harvey, 2000). The accuracy (Table 4.6) of all the methods expressed as the measure of percentage relative error are within the range (1 – 5 %) for moderately accurately procedure (Harvey, 2000). The average percentage recovery for all the methods were found to range between 99.0 and 100.0 %, showing that they have good recoveries especially when compared with the 99.44, 99.5 and 99.09 % reported by Sulakshana*et al.*(2015) UV methods for estimation of metformin in 0.1 N HCl, 0.01 N HCl and NaOH respectively. Recoveries for lisinopril are comparable to 99.78 % reported by Digambar*et al.* (2015) and 98.0 – 102 % reported by Chavan and Poonam (2015) UV methods for estimation of lisinopril in bulk and tablet dosage form.

The LODand LOQ(Table 4.6)for both metformin and lisinopril in all the methods were satisfactory. This shows the sensitivityofthe methods for the analysis of the drugs. No statistically significant difference ($p < 0.05$) was observed between the percentage content of metformin and lisinopril quantified using the developed methods and that by official method. Thus, the developed methods can be used in place of the official method.

5.3*In vitro* availability of metformin and lisinopril

Results of the *in vitro* dissolution test (Figure 4.12) showed thatmetformin achieved a maximum availability of 84.94 and 88.74 % in simulated gastric pH for metformin alone and in the presence of *Hibiscus sabdariffa* respectively. The corresponding contents of metformin observed in simulated intestinal pH were 84.94 and 86.95 %, while in simulated blood pH 84.94 and 82.04 % of metformin was released. No significant difference ($p < 0.05$) was observed among the availabilities of metformin in all the three media.

The presence of *Hibiscus sabdariffa* significantly ($p < 0.05$) increased the *in vitro* availability of metformin in simulated gastric and intestinal pH. This increase in the availabilities of metformin in these two media therefore, may be due to *Hibiscus sabdariffa* influencing the dissolution of the drug and its subsequent release which was not observed in the dissolution of the drug alone in all the media (Figure 4.13). However, in simulated blood pH, the *in vitro* availability of metformin was significantly ($p < 0.05$) decreased by the presence of *Hibiscus sabdariffa.* This may be explained by the fact that metformin have pKa values of 2.8 and 11.5, so exists very largely as the hydrophilic cationic species at physiological pH values. The metformin pKa values make metformin a stronger base than most other basic drugs with less than 0.01% nonionized in blood (Garry *et al.,* 2011). The observed significant increase in the availability of metformin interacted with *Hibiscus sabdariffa* ($p < 0.05$) in simulated gastric and intestinal pH could serve as an advantage with respect to metformin dissolution, release

and subsequent bioavailability especially when metformin is taken with *Hibiscus sabdariffa* calyxes water extracts at the recommended therapeutic doses.

No significant difference ($p < 0.05$) was observed among the availabilities of lisinopril alone in all the three media (Figure 4.14). Results of the *in vitro* dissolution test (Figure 4.14) showed thatlisinopril achieved a maximum availability of 89.40 and 92.62 % in simulated gastric pH for lisinopril alone and in the presence of *Hibiscus sabdariffa* respectively. The corresponding contents of lisinopril observed when interacted with *Hibiscus sabdariffa*in simulated intestinal pH and simulated blood pH were 92.50 and 91.95 % respectively.

The presence of *Hibiscus sabdariffa* significantly ($p < 0.05$) increased the *in vitro* availability of lisinopril in simulated gastric, intestinal and blood pHs. This increase in the availabilities of lisinopril in the three media therefore, is due to *Hibiscus sabdariffa* which influence the dissolution of the drug and its subsequent release which was not observed in the dissolution of lisinopril alone in all the media used for this study (Figure 4.15). Obamiro *et al.* (2013) reported a significant increase in the dissolution profile of lisinopril in simulated pH 4.5 and 6.8 when coadministered with black tea while the release profile of lisinopril at pH 1.2 shows the effect of black tea in decreasing the dissolution profile of lisinopril which is found to be statistically significant. Despite the fact that available literature show that black tea may have some cardio friendly properties they recommend that coadministration of black tea withlisinopril should be discouraged as it can impact on tablet dissolution and may result in unpredictable effect. Olubunmi *et al.* (2015) also reported that Yoyo Bitters significantly increased the dissolution of lisinopril at pH 1.2 and that there was no statistically significant difference between the dissolution of lisinopril with and without Yoyo Bitters at pH 4.5. The dissolution of lisinopril decreased significantly in the presence of Yoyo Bitters at pH 6.8. In this study, the observed significant increase in the availability of lisinopril interacted with *Hibiscus sabdariffa* ($p < 0.05$) in simulated gastric, intestinal pH and blood pH could serve as

an advantage with respect to lisinopril dissolution, release and subsequent bioavailability especially when lisinopril is taken with *Hibiscus sabdariffa* calyxes water extract at the recommended therapeutic doses otherwise it could lead to an unpredictable effect.

5.4 RP-HPLC method for metformin and lisinopril

The peak height ratios of metformin-lisinopril and IS against their corresponding concentrations were determined. The linear relationship between the peak height signals (A) and their corresponding concentrations (C in % w/v) is given by the regression equations of the type A=Cy + x for metformin and lisinopril respectively (Table 4.9). The coefficient of correlation (r) in each case is approaching unity(0.998). This shows a direct proportionality relationship between peak height signals and concentrations which give the good correlations.

The low % RSD (≤ 0.80) for both methods shows the precision of the methods. With good technique and reliable methodology the precision should be < 15 % CV (Harvey, 2000). Krzysztof *et al.* (2010) reported % CV of 9.8 and 8.11 in HPLC quantification of metformin in brain regions, cerebrospinal fluid and plasma of rats. Olcay and Lale (2004) reported % RSD of 3.81 and 4.14 in HPLC method for determination of lisinopril in human plasma and urine.This shows that the precision of each of the developed methods is satisfactory. The accuracy (≤ 1.2) of the methods expressed as the measure of percentage relative errorare within the range (1 – 5 %) for moderately accurately procedure (Harvey, 2000). The average percentage recoveries for the methods were found to be 99.98 and 99.89 % for metformin and lisinopril respectively showing that methods have good recoveries especially when compared with the 98.20 % reported by Valentina*et al.*(2008) in a HPLC method for determination of metformin in human plasma and 78.25 % reported by Olcay and Lale (2004) in a HPLC method for estimation of lisinopril. The LOD (≤ 0.22ng/mL) and LOQ (≤ 0.67ng/mL) for the

developed methods are satisfactory. This shows that the methods are sensitive for the analysis of the drugs.

5.5*In vivo* interaction of metformin and lisinopril with *Hibiscus sabdariffa* calyxes water extract

The results of the *in vivo* availability study (Figure 4.22 and Appendix 28) showed that metformin administered alone (phase 1) achieved average maximum availability of 892.32 ng/mL at 5 hours. The corresponding observed maximum availabilities of metformin for phases 2, 3 and 4 were 898.28, 895.30 and 898.52 ng/mL respectively.On the other hand, *in vivo* availability study (Figure 4.23 and Appendix 29) showed that lisinopril administered alone (phase 1) achieved average maximum availability of 95.36 ng/mL at 7 hr. The corresponding observed maximum availabilities of lisinopril for phase 2 (97.50ng/mL), 3 (97.37ng/mL) and 4 (99.24ng/mL) respectively.This study showed significant increase ($p < 0.05$) in the availabilities of metformin and lisinoprilwhen interacted with *Hibiscus sabdariffa*in all the phases of interaction study which is inagreement with the *in vitro*availability study findings for both drugs. Though, an exception was observed with *in vitro* availability of metformin in the presence of *Hibiscus sabdariffa* in simulated blood pH; where significant decrease ($p < 0.05$) was observed.

The results showed that metformin administered alone achieved C_{max} of 892.320 ng/mL at 5 hr (T_{max}), a slight increase in the corresponding $C_{max}$of metformin was observed for phase 2 (898.280 ng/mL), phase 3 (895.300 ng/mL) and phase 4 (898.520 ng/mL). However, these increases were not significant ($p < 0.05$). Total area under the concentration time curve(AUC_0^∞)for metformin administered alonewas 5460216.284ng.hr/mL. The corresponding metformin$AUC_0^\infty$for phases,2, 3 and4 were6031537.018, 5904969.750 and

5721955.572ng.hr/mL respectively.Although slightly higher, they were however not significant (*p* < *0.05*).

The observed T_{max} of metformin in all the phases of interaction in this study agrees with those reported in the literature (Sweetman, 2002; Krzstof *et al.,* 2010).The observed slight increasesin the pharmacokinetic parameters are not too different from the slight increases in the parameters reported by Nakamaru *et al.* (2015) in a pharmacokinetic interaction study between metformin and teneligliptin where C_{max} increase from 1.56 to 1.75 µg/mL and AUC_0^∞ from 10.2 to 12.3 µg.hr/mL while $t_{1/2}$ remained 3.0 hr. No significant difference ($p < 0.05$) was observed in the pharmacokinetic parameters generated hence despite the observed increase they suggest coadministration of the drugs and that the increase is unlikely to be clinically significant.A pharmacokinetic study among 3 metformin formulations in healthy Mexican volunteers in a single-dose, randomized, open-label, 3-period crossover study by Montoya-Eguía *et al.* (2015) reported an increase in the C_{max} and AUC_0^∞ of metformin among the volunteers. However, the parameters were not significantly different ($p < 0.05$) with respect to the observed variation.On the other hand, Kumar *et al.* (2011) reported an interaction study between metformin and *Allium sativum* in rat plasma with repeated dose administration. A significant increase in C_{max} (from 19.54 to 31.49 µg/mL) and AUC_0^∞ (from 79.33 to 117.87 µg.hr/mL) of metformin when interacted with the drug with a slight increase in $t_{1/2}$. They however, suggest that extensive clinical pharmacokinetic studies are necessary to establish such drug-drug interactions in higher animals.

It was also observed that, lisinopril administered alone achieved C_{max} of 95.360ng/mL at 7 hr (T_{max}). Slight increases in C_{max} of lisinopril were observed when the drug was interacted with *Hibiscus sabdariffa* in phase 2 (97.500 ng/mL), phase 3 (97.370 ng/mL) and phase 4 (99.240 ng/mL). However, these increases were not statistically significant (*p* < *0.05*). Peak

concentrations in plasma are reported to occur after about 7 hr (Sweetman, 2002). Olcay and Lale (2004) reported that lisinopril administered alone in healthy volunteers attained C_{max} of 87.4 ng/mL at 7 hr. This iscomparable to the observed C_{max} and $T_{max}$of lisinopril in phase 1 of this study.

Total area under the concentration time curve (AUC_0^∞) of lisinopril administered alone was736805.828ng.hr/mL. The corresponding$AUC_0^\infty$for phase 2, 3 and 4 are 800409.291, 816866.469, and 1045.027ng.hr/mL respectively.However, these increases in $C_{max}$and AUC_0^∞also seen with lisinopril were not statistically significant ($p < 0.05$). On the other hand, a decrease in clearance (Cl) of lisinopril from 13.57 L/hr in phase 1 to 9.57 L/hr in phase 4 was observed, this indicate an increase in the mean residence time of the drug. Lisinopril is reported not to be significantly bound to plasma proteins this is reflected by its high volume of distribution (Sweetman, 2002).In this study also, a slight increase in volume of distribution of lisinopril was observed in phase 4 (Table 4.12).

A similar pattern was observed with the clearance of metformin which decrease slightly from 91.57 L/hr in phase 1 to 82.89 L/hr in phase 2. The corresponding metformin Cl for phases 3 and 4 were 84.67 and 87.38 L/hr respectively. However, these decreases were also not statistically significant ($p < 0.05$). These observations, may thus not be clinically significant as metformin and lisinopril do not fall among the class of drugs with narrow therapeutic index except in subjects with renal impairment because the excretion of both drugs is renal and thus, both drugs are contraindicated in them.

Although statistical analysis conducted for all the pharmacokinetic parameters of both metformin and lisinopril revealed no statistically significant difference ($p < 0.05$) in their respective pharmacokinetic parameters. However, the clinical relevance of these findings will

have to be validated through further pharmacodynamic interactions of *Hibiscus sabdariffa*with these drugs.

CHAPTER SIX

6.0 SUMMARY AND CONCLUSION

6.1 Summary

In this study, quality control of both standard powders and tablet samples used were conducted. Three new UV spectrophotometric methods each for the determination of metformin and lisinopril in 0.1 M HCl, phosphate buffer pH of 6.8 and 7.4 were developed, validated and used for the *in vitro* availability studies of the drugs interacted with *Hibiscus sabdariffa*water extract. A RP-HPLC methodwas developed and validated for the quantification of each of the drugsin saliva.For*in vivo*study; 48 randomly selected healthy human volunteers who were divided into 4 groups of 12 subject each were recruited. The *in vivo* interactions were carried out in four phases; phase 1(administration of drugs alone), phase 2 (administration of drugs with *Hibiscus sabdariffa* calyxes water extracts concurrently), phase 3 (administration of drugs 30 min after administration of *Hibiscus sabdariffa*) and phase 4 (administration of *Hibiscus sabdariffa*30 min after administration of drugs).

The results showed that quality control studies of both standard powders and tablet samples used revealed the content of the labelled APIs in their appropriate amount.*In vitro* study revealed a significant ($p < 0.05$) increasein the availability of the drugsin all the media; the only exception been metformin in pH 7.4 where a significant decrease ($p < 0.05$) was observed.*In vivo* study revealed that the drugs contentin the collected saliva samples of the

volunteers were significantly ($p < 0.05$) increased by the presence of *Hibiscus sabdariffa*water extracts in all phases. Pharmacokinetic parameters of the drugs administered alone and when interacted with *Hibiscus sabdariffa* calyxes water extracts showed that small increases in $C_{max}$and AUC_0^∞were observed in all the phases. However, the increases observed in all the pharmacokinetic parameters in all phases were not statistically significant ($p < 0.05$). This study therefore, suggests that *Hibiscus sabdariffa* calyxes water extract does not significantly affect the pharmacokinetics of metformin and lisinopril.

6.2 Conclusion

It could be concluded that *Hibiscus sabdariffa* water extract does not significantly affect ($p < 0.05$) the pharmacokinetic of both metformin and lisinopril. However, small increases were observed in the pharmacokinetic parameters; the clinical relevance of which will have to be validated.

6.3 Recommendations

1. Metformin and lisinopril can be taken with *Hibiscus sabdariffa* L. water extract without any fear of their pharmacokinetics been affected.
2. The interactions between *Hibiscus sabdariffa* and the drugs should be pharmacodynamically evaluated to establish benefit of concurrent use.

6.4 Contributions to Knowledge

This study established that:

1. metformin and lisinopril can be quantitatively determined at λ_{max} ranging from 210 to 232 nm in dissolution media (pH 1.2, 6.8 and 7.4) using UV-vis spectroscopy.

2. the *in vitro* availability of metformin and lisinopril were significantly ($p < 0.05$) increased when the drugs were interacted with *Hibiscus sabdariffa* L. in media of pH 1.2, 6.8 and 7.4; the only exception been metformin in pH 7.4 where a significant decrease ($p < 0.05$) was observed.

3. RP-HPLC methods for the analysis of metformin and lisinopril in human saliva with respective retention times of 1.6 and 1.7 mins in a methanol:water (80:20) solvent system were developed and validated. To the best of our literature search, this is the first report of its kind.

4. the pharmacokinetics of metformin (C_{max} 892.32 vs 898.28 and AUC_0^∞ 5460216.28 vs 6031537.02) and lisinopril (C_{max} 95.36 vs 97.50 and AUC_0^∞ 736805.83 vs 800409.29) were not significantly ($p > 0.05$) affected when interacted with *Hibiscus sabdariffa* L. water extract.

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APPENDICES

Appendix 1: Ethical approval obtained from ABUCUHSR

Committee on Use of Human Subjects for Research

Directorate of Academic Planning & Monitoring

Ahmadu Bello University, Zaria

Director: Professor M. I. Sule *B Pharm, MSc, PhD, (ABU), MPSN* Tel: 08036184581

Chairman: Prof I. H. Nock, B.Sc,M.Sc (ABU), PhD (NewDelhi) 08065425450 | Secretary: U. D. Abdullahi, 08034619772

Appl No.: ABUCUHSR/2017/Pharm, & Med. Chemistry/003 — 4th August, 2017

Approval No: ABUCUHSR/2017/003

Dr. Aminu Musa
Department of Pharm. and Medicinal Chemistry,
Faculty of Pharmaceutical Sciences,
Ahmadu Bello University,
Zaria.

Sir,

PROVISIONAL APPROVAL OF RESEARCH TITLED "EFFECT OF HISBISCUS SABDARIFFA (LINN) ON THE PHARMACOKINETICS OF METFORMIN AND LISINOPRIL IN HEALTHY HUMAN VOLUNTEERS"

This is to convey the provisional approval of the ABUCUHSR to you for the aforestated study domiciled in Department of Pharmaceutical and Medicinal Chemistry of the Ahmadu Bello University. The approval is predicated on the assumption that you shall maintain and cater for the study subjects as indicated in your application.

Monitoring of the Research by spot checks, invitations, interactions with the subjects any other means the Committee deems fit shall be undertaken at the convenience of the Committee.

This approval can and shall be revoked should a significant breach in the terms and condition of the approval occur. It is hence your responsibility to ensure that the agreed terms are maintained to the end of the Study.

The said approval shall be posted on the ABUCUHSR Page on the University's website.

Note upon completion of the research, ethical clearance certificate will be issued,

Prof. I.H. Nock
ABUCUHSR Chairman

Cc. Director, DAPM
Director, IC &ICT
Dean, Pharmaceutical Sciences
HOD, Pharm. & Med. Chemistry
Dr. Aminu Musa, Dept. of Pharm. & Med. Chemistry

RESEARCH CONSENT FORM

Name of Reseacher(s) *(to be completed by the researcher)*
NASIR IBRAHIM (P14PHMC9006)
Title of study *(to be completed by the researcher)*
EFFECT OF *HIBISCUS SABDARIFFA* (LINN) ON THE PHARMACOKINETICS OF METFORMIN AND LISINOPRIL IN HEALTHY HUMAN VOLUNTEERS

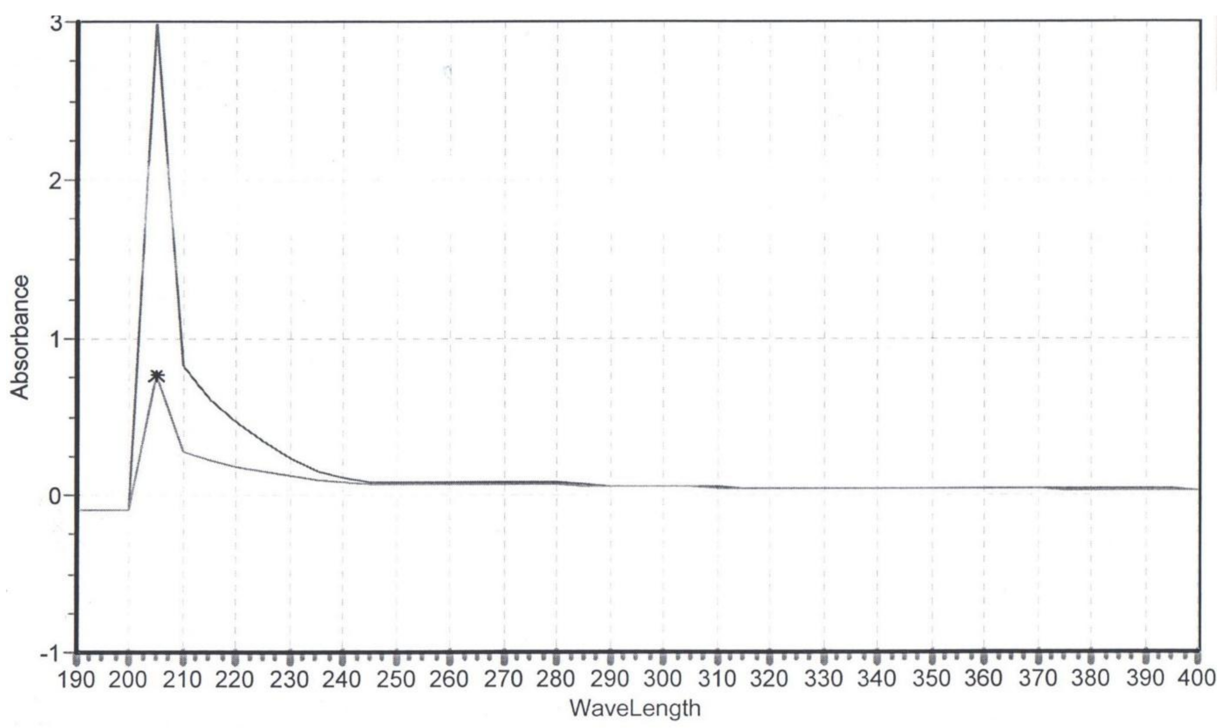
Please read and complete this form carefully. If you are willing to participate in this study, ring the appropriate responses and sign and date the declaration at the end. If you do not understand anything and would like more information, please ask.

I have had the research satisfactorily explained to me in verbal and / or written form by the researcher	**YES / NO**
I understand that the research will involve administration of single dose of metformin (500 mg) and/ or lisinopril (10 mg) with *Hibiscus sabdariffa* L. calyces water extracts (25 mg/mL) and saliva samples will be collected from me for a period of 36 hours	**YES / NO**
I understand that I may withdraw from this study at any time without having to give an explanation. This will not affect my future care or treatment.	**YES / NO**
I understand that all information about me will be treated in strict confidence and that I will not be named in any written work arising from this study	**YES / NO**
I understand that any saliva sample of me collected will be treated as described to me and will be used solely for research purposes and shall be discarded on completion of the research analysis	**YES / NO**
I understand that you will be discussing the progress of this research with the supervisory team and professional colleagues at Ahmadu Bello University, Zaria and Usmanu Danfodiyo University, Sokoto, Nigeria.	**YES / NO**

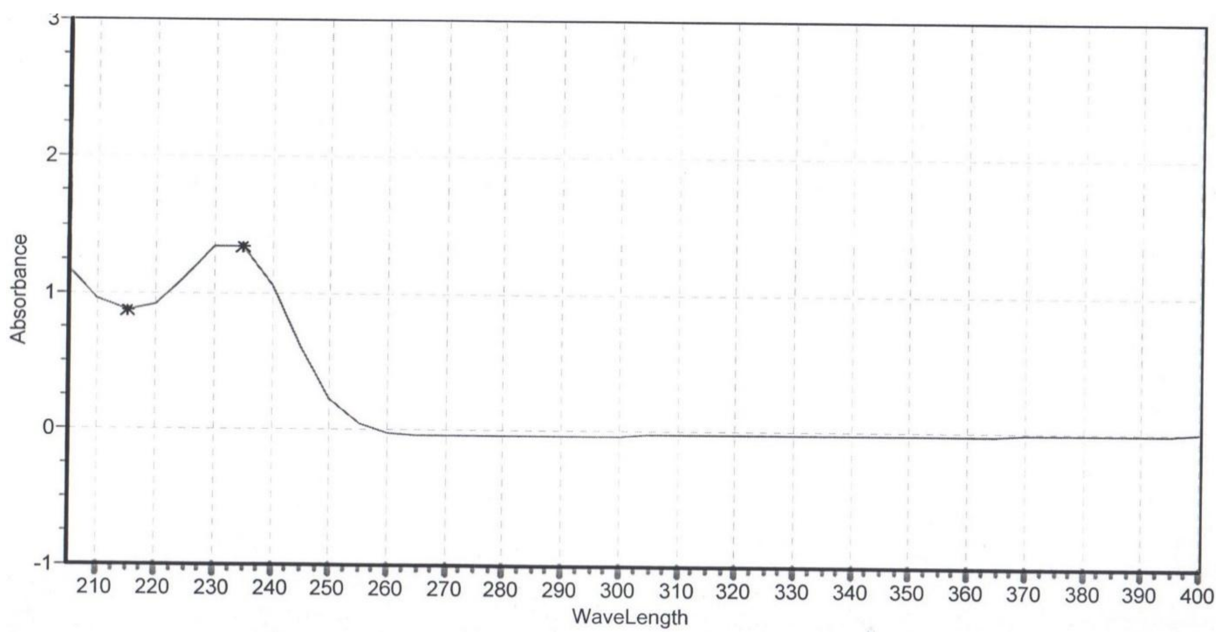
I freely give my consent to participate in this research study and have been given a copy of this form for my own information.

Signature: 17/10/17

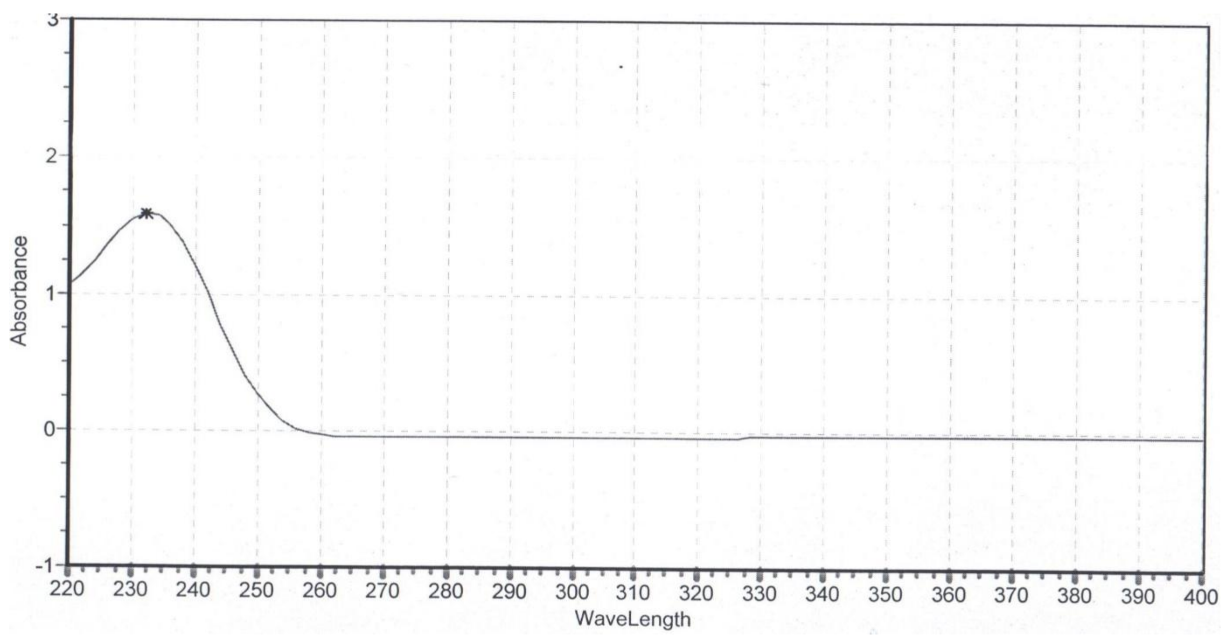
Appendix 2: Sample consent form from one of the volunteers



Appendix 3: Spectrum of metformin in 0.1 N HCl showing λ_{max} at 205 nm



Appendix 4: Spectrum of metformin in medium pH 6.8 showing λ_{max} at 235 nm



Appendix 5: Spectrum of metformin in medium pH 6.8 showing λ_{max} at 232 nm

Appendix 6: Dissolution profile of metformin tablet (500 mg) alone in 0.1M HCI

Time (min)	Absorbance	Concentration (µg/mL)	Actual Concentration (µg/mL)	Concentration (mg/mL)	Concentration (mg/L)	% Drug release
5	0.496	9.2366	92.366	0.092366	92.366	18.473
10	0.889	16.737	167.366	0.167366	167.366	33.473
15	1.337	25.286	252.863	0.252863	252.863	50.573
30	1.771	33.569	335.687	0.335687	335.687	67.137
45	1.996	37.863	378.626	0.378626	378.626	75.725
60	2.135	40.515	405.153	0.405153	405.153	81.031

$Y = 0.0524x + 0.012$ $R^2 = 0.9984$; $r = 0.9992$; $\lambda_{max} = 205$ nm

Appendix 7: Dissolution profile of metformin tablet (500 mg) + *Hibiscus sabdariffa* 1g/L in 0.1M HCI

Time (min)	Absorbance	Concentration (µg/mL)	Actual Concentration (µg/mL)	Concentration (mg/mL)	Concentration (mg/L)	% Drug release
5	0.546	10.191	101.908	0.101908	101.908	20.382
10	0.995	18.759	187.595	0.187595	187.595	37.519
15	1.557	29.484	294.847	0.294847	294.847	58.969
30	1.982	37.595	375.954	0.375954	375.954	75.191
45	2.290	43.473	434.733	0.434733	434.733	86.947
60	2.337	44.370	443.702	0.443702	443.702	88.740

$Y = 0.0524x + 0.012$ $R^2 = 0.9984$; $r = 0.9992$; $\lambda_{max} = 205$ nm

Appendix 8: Dissolution profile of metformin tablet (500 mg) alone in dissolution medium pH 6.8

Time (min)	Absorbance	Concentration (µg/mL)	Actual Concentration (µg/mL)	Concentration (mg/mL)	Concentration (mg/L)	% Drug release
5	1.771	12.657	126.572	0.126572	126.572	25.314
10	2.317	16.516	165.159	0.165159	165.159	33.032
15	3.525	25.053	250.530	0.250530	250.530	50.106
30	4.994	35.435	354.346	0.354346	354.346	70.869
45	5.778	40.975	409.753	0.409753	409.753	81.951
60	5.965	42.297	422.968	0.422968	422.968	84.594

$Y = 0.1415x - 0.02$ $R^2 = 0.9988$; $r = 0.9994$; $\lambda_{max} = 235$ nm

Appendix 9: Dissolution profile of metformin tablet (500 mg) + *Hibiscus sabdariffa* 1g/L in dissolution medium pH 6.8

Time (min)	Absorbance	Concentration (µg/mL)	Actual Concentration (µg/mL)	Concentration (mg/mL)	Concentration (mg/L)	% Drug release
5	1.862	13.300	133.004	0.133004	133.004	26.601
10	2.552	18.177	181.767	0.181767	181.767	36.353
15	3.862	27.435	274.346	0.274346	274.346	54.869
30	5.296	37.569	375.689	0.375689	375.689	75.138
45	5.920	41.979	419.788	0.419788	419.788	83.958
60	5.899	41.830	418.304	0.418304	418.304	83.661

$Y = 0.1415x - 0.02$ $R^2 = 0.9988$; $r = 0.9994$; $\lambda_{max} = 235$ nm

Appendix 10: Dissolution profile of metformin tablet (500 mg) alone in dissolution medium pH 7.4

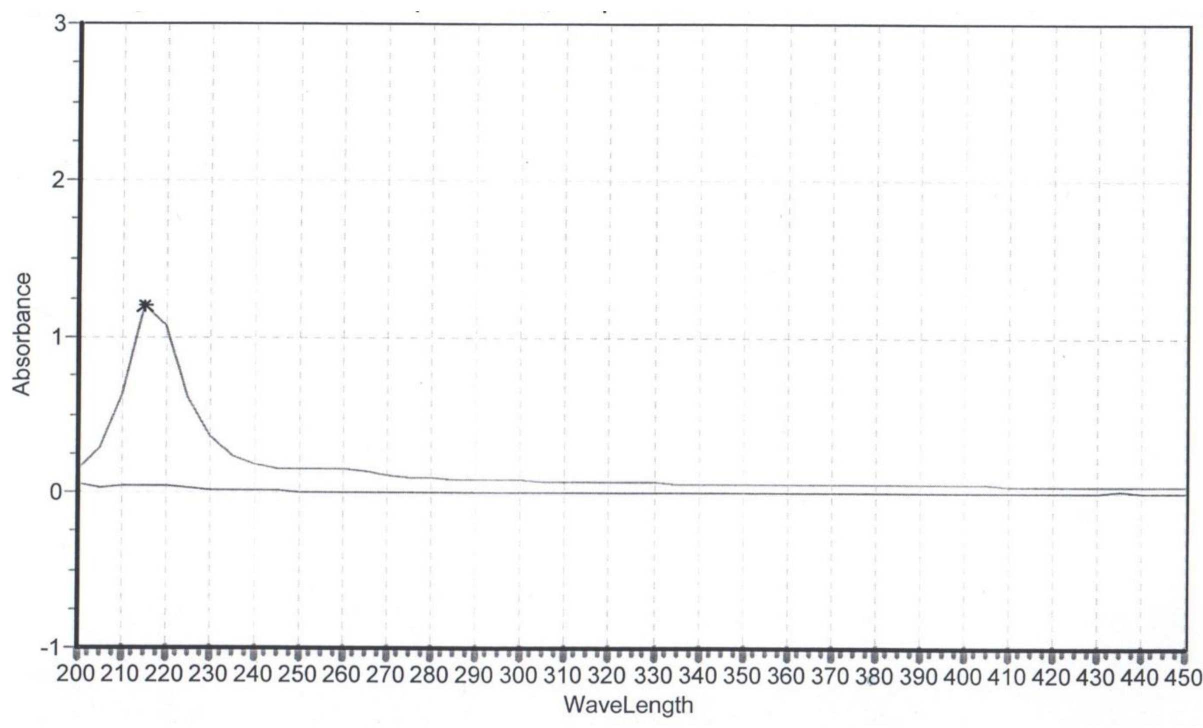
Time (min)	Absorbance	Concentration (µg/mL)	Actual Concentration (µg/mL)	Concentration (mg/mL)	Concentration (mg/L)	% Drug release
5	1.005	12.583	125.833	0.125833	125.833	25.167
10	1.544	19.494	194.936	0.194936	194.936	38.987
15	1.985	25.147	251.474	0.251474	251.474	50.294
30	2.778	35.314	353.141	0.353141	353.141	70.628
45	3.220	40.980	409.808	0.409808	409.808	81.962
60	3.336	42.468	424.679	0.424679	424.679	84.936

$Y = 0.078x + 0.0235$ $R^2 = 0.9981$; $r = 0.9990$; $\lambda_{max} = 232$ nm

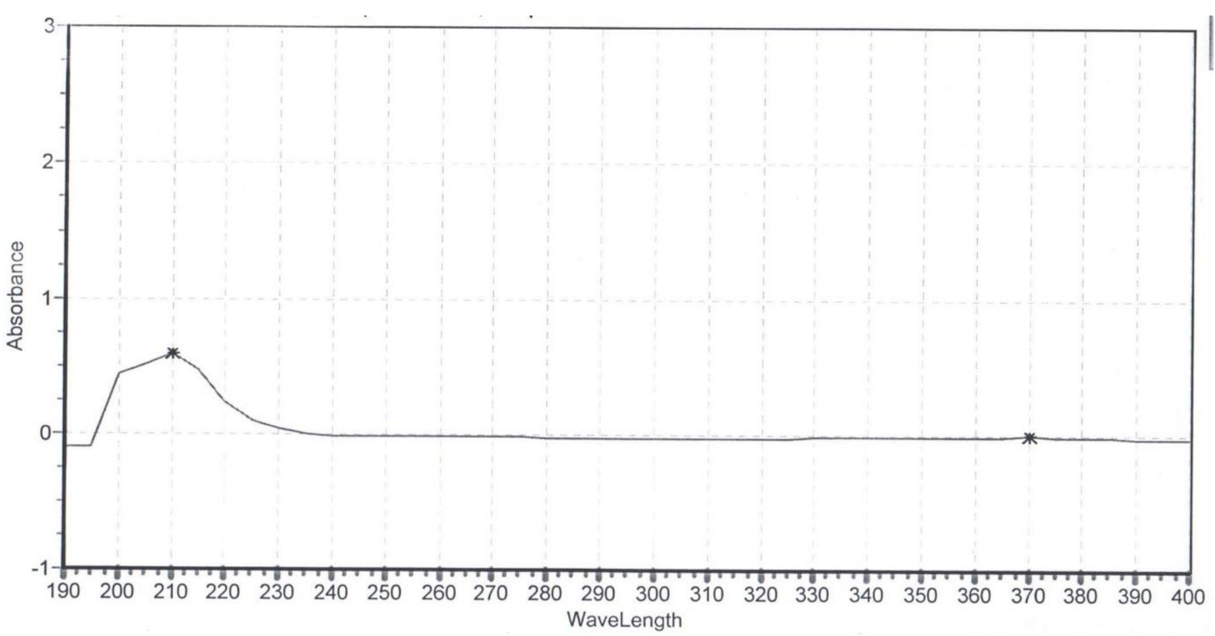
Appendix 11: Dissolution profile of metformin tablet (500 mg) + *Hibiscus sabdariffa* 1g/L in dissolution medium pH 7.4

Time (min)	Absorbance	Concentration (µg/mL)	Actual Concentration (µg/mL)	Concentration (mg/mL)	Concentration (mg/L)	% Drug release
5	0.956	11.955	119.551	0.119551	119.551	23.910
10	1.399	17.635	176.346	0.176346	176.346	35.269
15	1.864	23.596	235.962	0.235962	235.962	47.192
30	2.579	32.763	237.628	0.237628	327.628	65.526
45	3.005	38.224	382.244	0.382244	382.244	76.449
60	3.223	41.019	410.192	0.410192	410.192	82.038

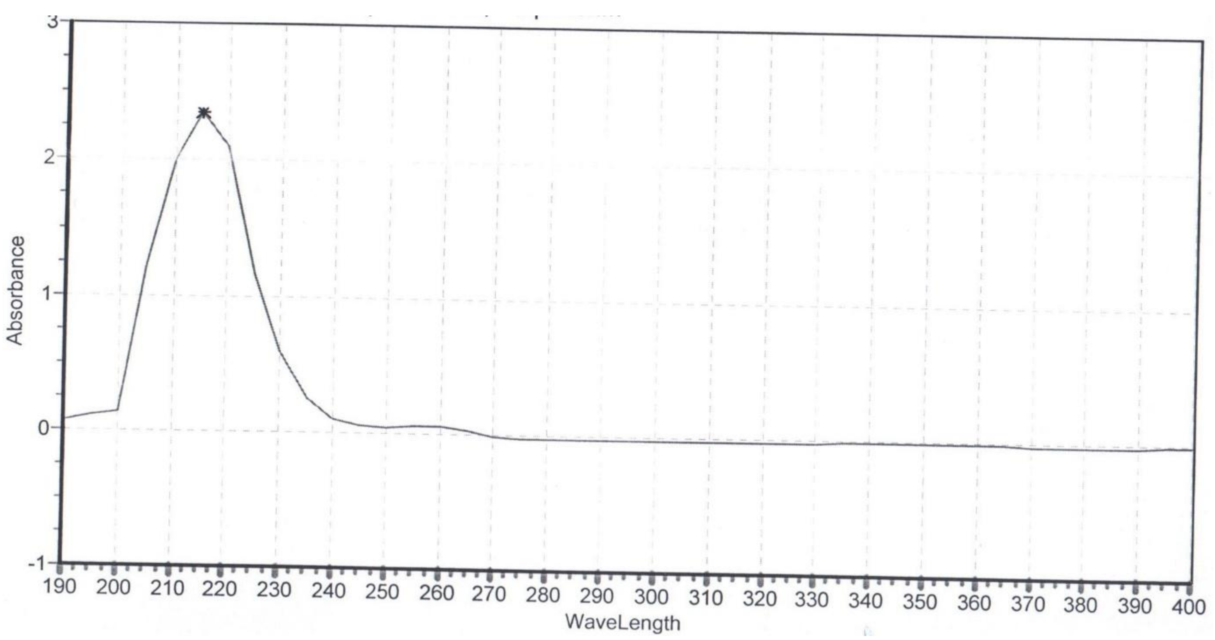
$Y = 0.078x + 0.0235$ $R^2 = 0.9981$; $r = 0.9990$; $\lambda_{max} = 232$ nm



Appendix 12: Spectrum of lisinopril in 0.1 N HCl showing λ_{max} at 215 nm



Appendix 13: Spectrum of lisinopril in medium pH 6.8 showing λ_{max} at 210 nm



Appendix 14: Spectrum of lisinopril in medium pH 7.4 showing λ_{max} at 215 nm

Appendix 15: Dissolution profile of lisinopril tablet (10 mg) alone in 0.1M HCI

Time (min)	Absorbance	Concentration (µg/mL)	Concentration (mg/900mL)	% Drug release
5	0.136	3.5305	3.5305	35.30
10	0.146	3.8354	3.8354	38.35
15	0.173	4.6585	4.6585	46.59
30	0.288	8.1646	8.1646	81.65
45	0.322	9.2012	9.2012	92.01
60	0.298	8.4695	8.4695	84.69

$Y = 0.0328x + 0.0202$ $R^2 = 0.9948$; $r = 0.9974$; $\lambda_{max} = 215$ nm

Appendix 16: Dissolution profile of lisinopril tablet (10 mg) + *Hibiscus sabdariffa* 1g/L in 0.1M HCI

Time (min)	Absorbance	Concentration (µg/mL)	Concentration (mg/900mL)	% Drug release
5	0.139	3.6219	3.6219	36.22
10	0.150	3.9573	3.9573	39.57
15	0.176	4.7500	4.7500	47.50
30	0.297	8.4390	8.4390	84.39
45	0.324	9.2621	9.2621	92.62
60	0.302	8.5914	8.5914	85.91

$Y = 0.0328x + 0.0202$ $R^2 = 0.9948$; $r = 0.9974$; $\lambda_{max} = 215$ nm

Appendix 17: Dissolution profile of lisinopril tablet (10 mg) alone in dissolution medium pH 6.8

Time (min)	Absorbance	Concentration (µg/mL)	Concentration (mg/900mL)	% Drug release
5	0.299	7.8839	7.8839	78.84
10	0.340	8.6517	8.6517	86.52
15	0.350	8.8389	8.8389	88.39
30	0.376	9.3258	9.3258	93.26
45	0.302	7.9401	7.9401	79.40
60	0.350	8.8389	8.8389	88.39

$Y = 0.0534x - 0.122$ $R^2 = 0.9957$; $r = 0.9978$; $\lambda_{max} = 210$ nm

Appendix 18: Dissolution profile of lisinopril tablet (10 mg) + *Hibiscus sabdariffa* 1g/L in dissolution medium pH 6.8

Time (min)	Absorbance	Concentration (µg/mL)	Concentration (mg/900mL)	% Drug release
5	0.301	7.9213	7.9213	79.21
10	0.339	8.632	8.632	86.33
15	0.348	8.8015	8.8015	88.02
30	0.372	9.2509	9.2509	92.51
45	0.349	8.8202	8.8202	88.20
60	0.355	8.9326	8.9326	89.33

$Y = 0.0534x - 0.122$ $R^2 = 0.9957$; $r = 0.9978$; $\lambda_{max} = 210$ nm

Appendix 19: Dissolution profile of lisinopril tablet (10 mg) alone in dissolution medium pH 7.4

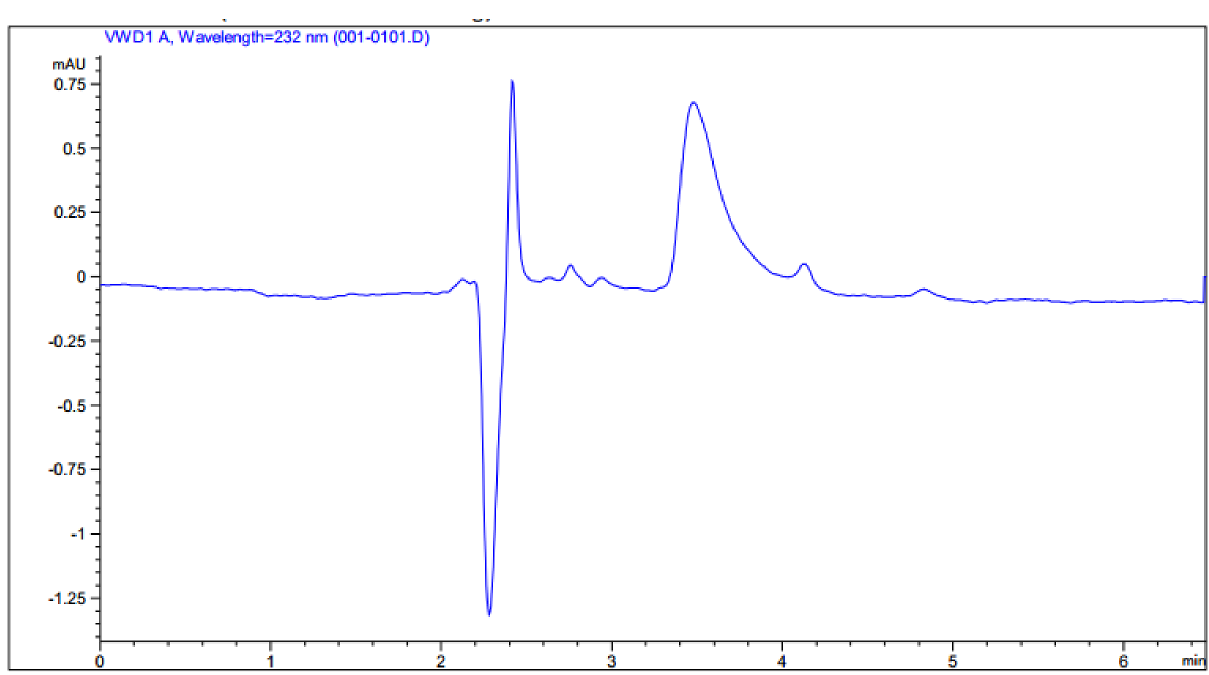
Time (min)	Absorbance	Concentration (µg/mL)	Concentration (mg/900mL)	% Drug release
5	0.223	5.9450	5.9450	59.45
10	0.259	6.8450	6.8450	68.45
15	0.272	7.1700	7.1700	71.70
30	0.299	7.8450	7.8450	78.45
45	0.344	8.9700	8.9700	89.70
60	0.352	9.1700	9.1700	91.70

$Y = 0.04x - 0.0148$ $R^2 = 0.9974$; $r = 0.9987$; $\lambda_{max} = 215$ nm

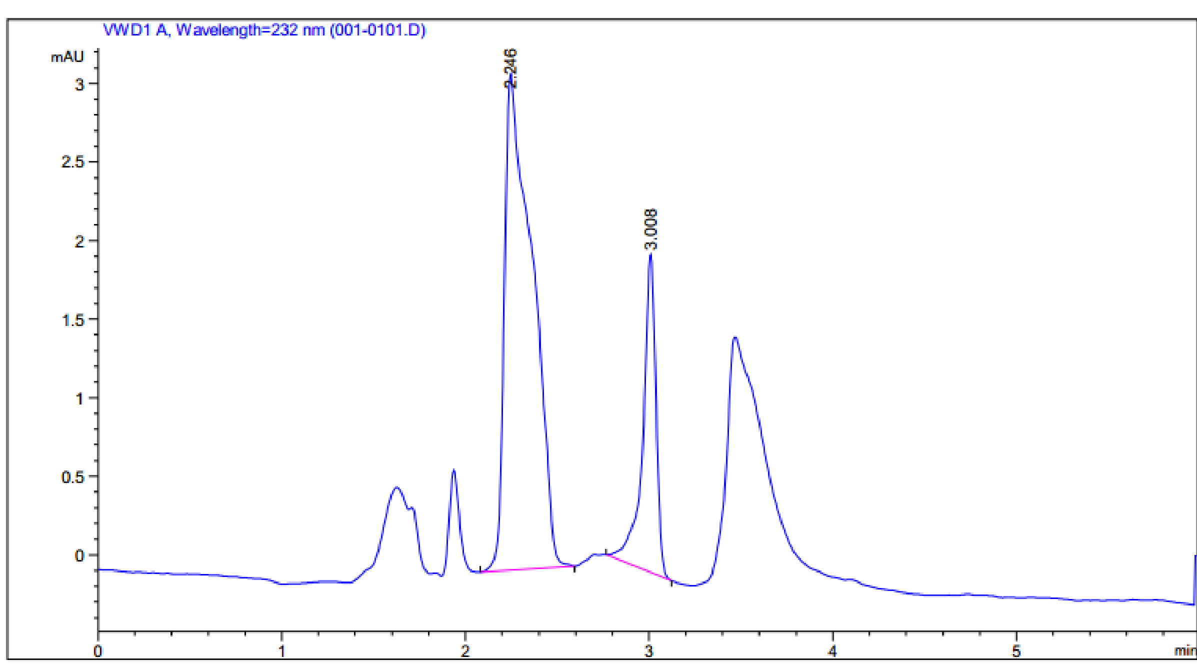
Appendix 20: Dissolution profile of lisinopril tablet (10 mg) + *Hibiscus sabdariffa* 1g/L in dissolution medium pH 7.4

Time (min)	Absorbance	Concentration (µg/mL)	Concentration (mg/900mL)	% Drug release
5	0.225	5.9950	5.9950	59.95
10	0.262	6.9200	6.9200	69.20
15	0.277	7.2950	7.2950	72.95
30	0.312	8.1700	8.1700	81.70
45	0.349	9.0950	9.0950	90.95
60	0.353	9.1950	9.1950	91.95

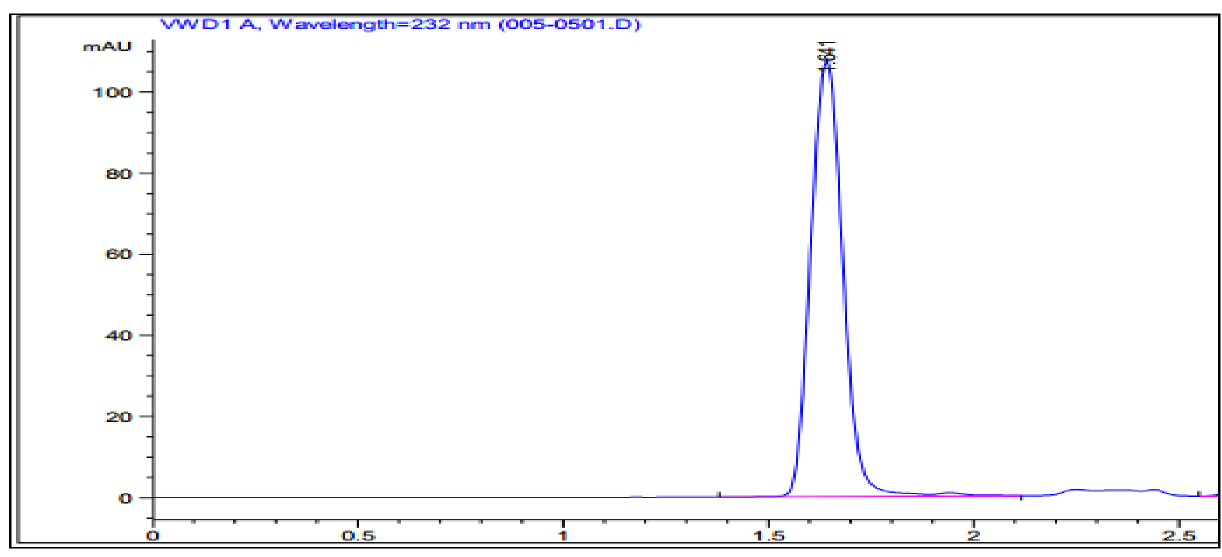
$Y = 0.04x - 0.0148$ $R^2 = 0.9974$; $r = 0.9987$; $\lambda_{max} = 215$ nm



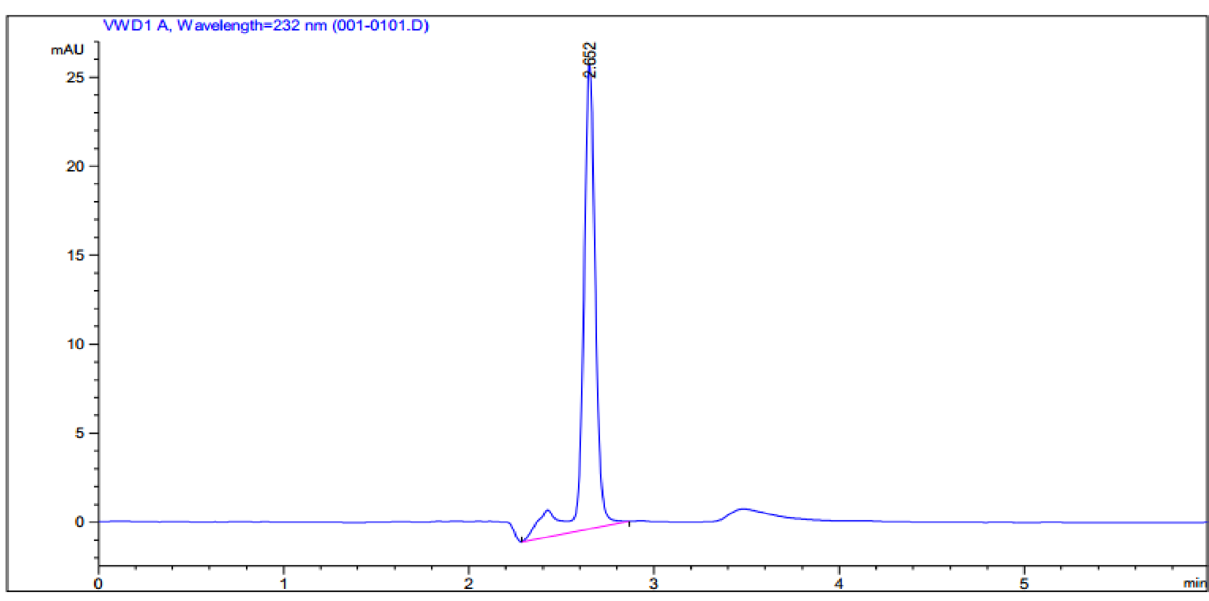
Appendix 21: RP-HPLC chromatogram of blank extracting solvent methanol:water (50:50) at 232 nm



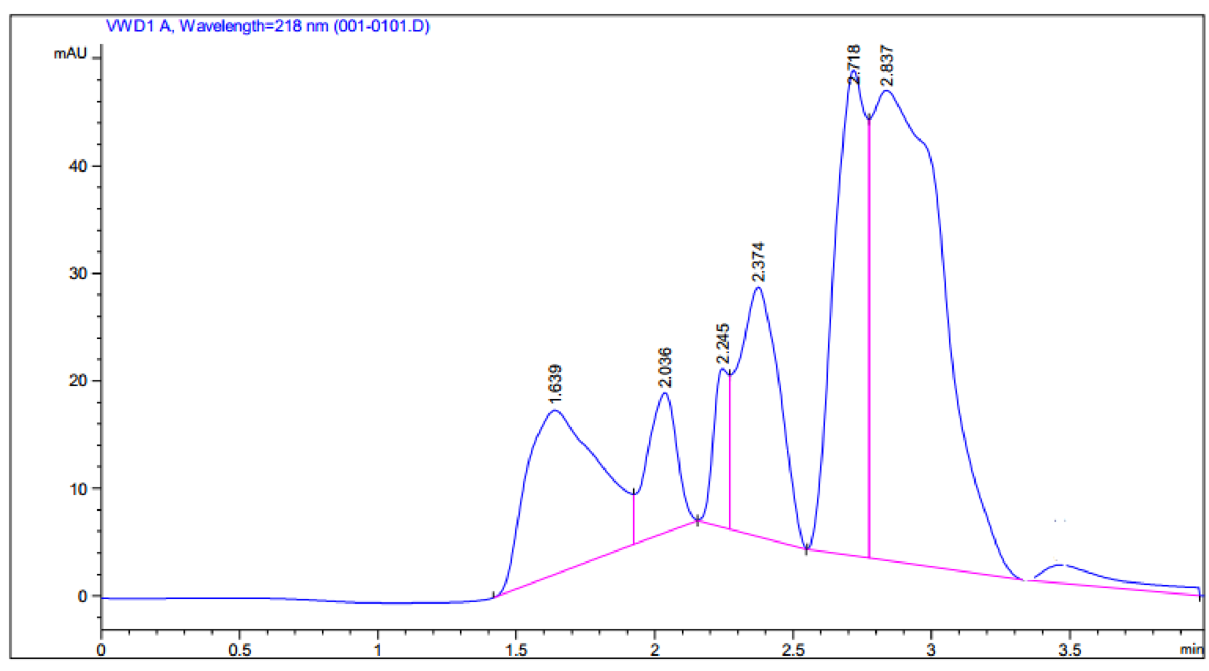
Appendix 22: RP-HPLC chromatogram of blank saliva from one of the volunteers for metformin



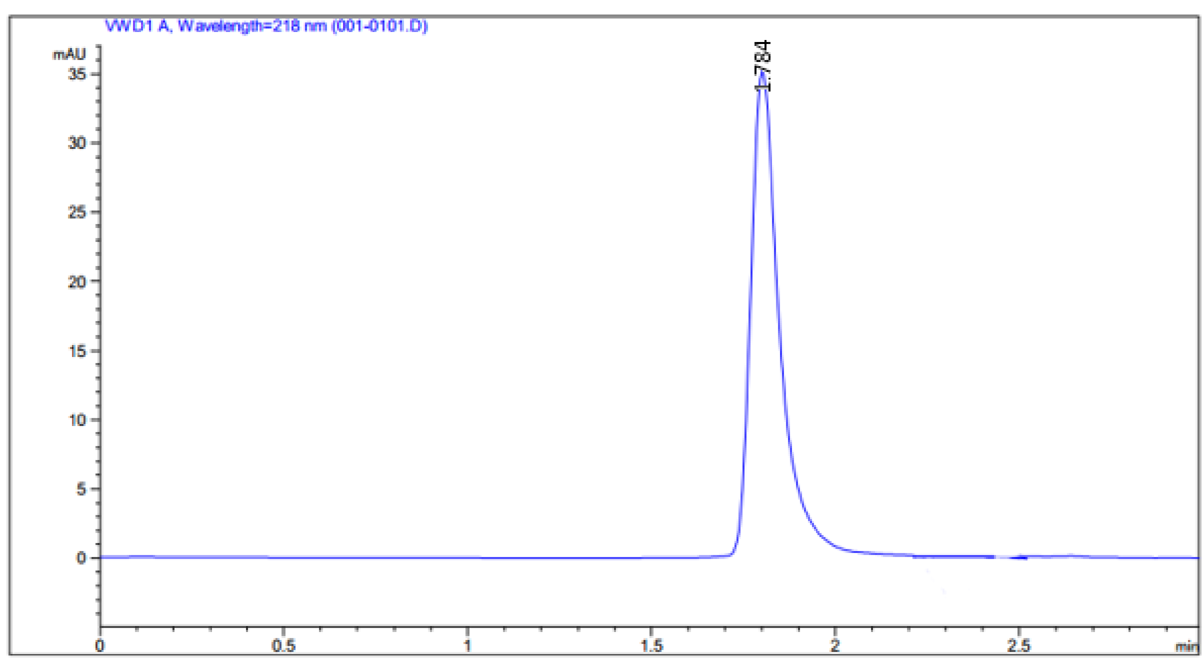
Appendix 23: RP-HPLC chromatogram of metformin alone at 232 nm



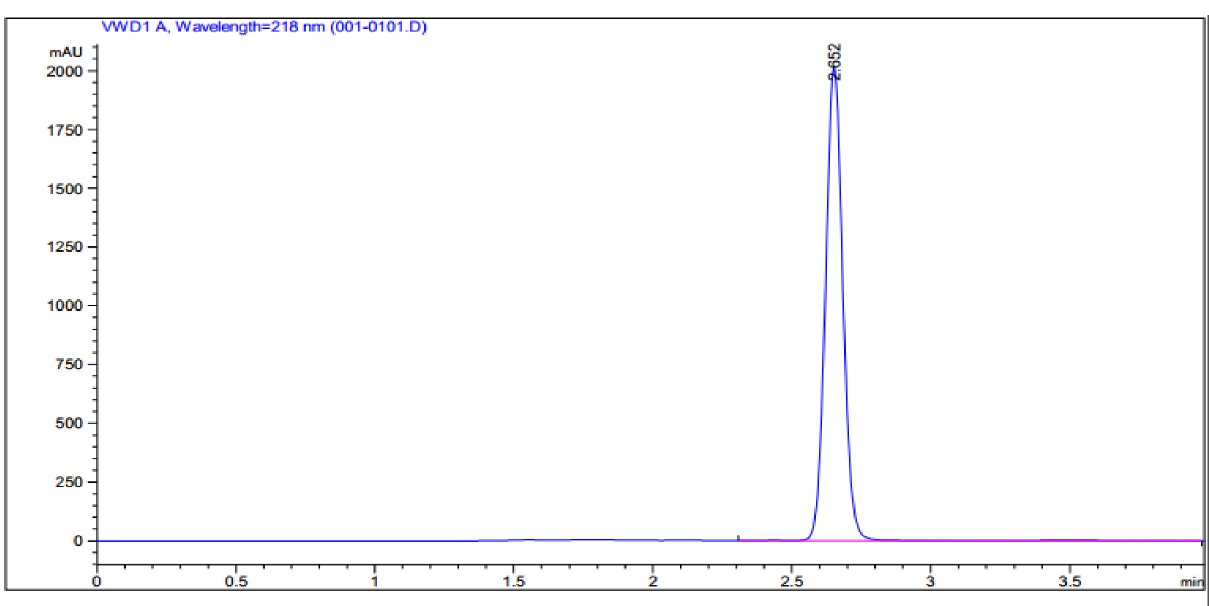
Appendix 24: RP-HPLC chromatogram of internal standard alone at 232 nm



Appendix 25: RP-HPLC chromatogram of blank saliva from one of the volunteers for lisinopril



Appendix 26: RP-HPLC chromatogram of lisinopril alone



Appendix 27: RP-HPLC chromatogram of internal standard alone at 218 nm

Appendix 28: Mean saliva concentrations versus time for metformin (500 mg) alone and when interacted with 200 mL of *Hibiscus sabdariffa* calyxes water extract (25 mg/mL)

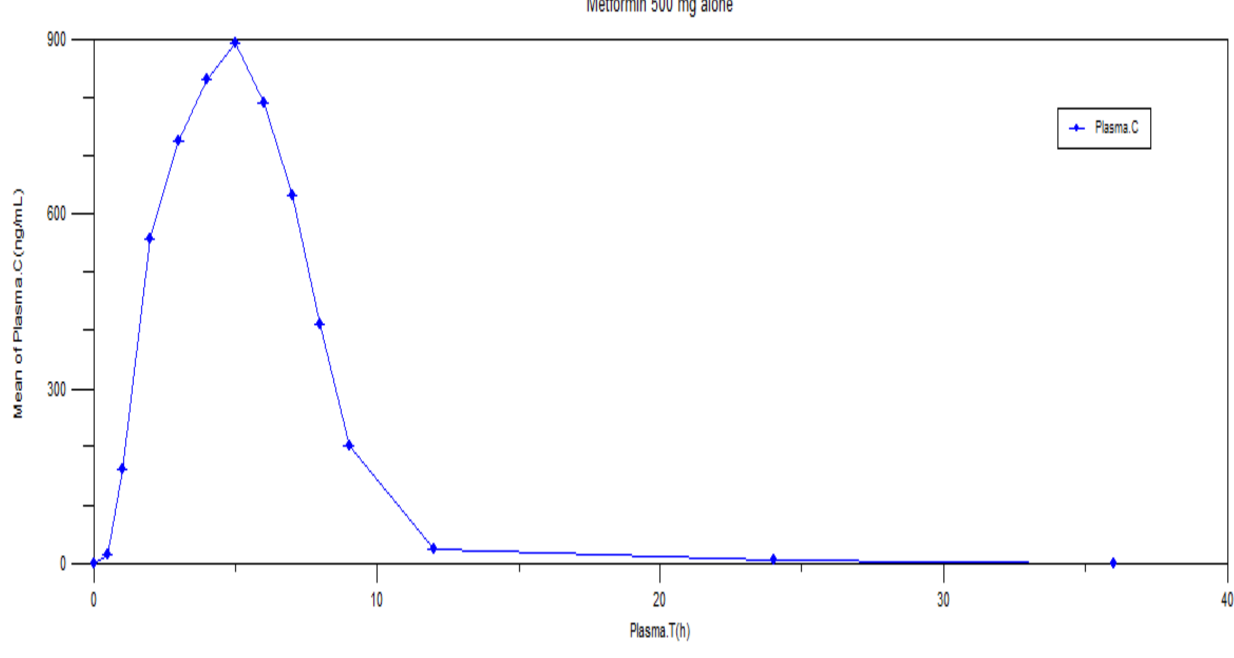
Time (hrs)	Phase 1 mean values ± SD (ng/mL)	Phase 2 mean values ± SD (ng/mL)	Phase 3 mean values ± SD (ng/mL)	Phase 4 mean values ± SD (ng/mL)
0	nd	nd	nd	nd
0.5	16.48 ± 2.64	18.36 ± 0.49	17.42 ± 1.56	20.46 ± 1.41
1	162.43 ± 1.18	167.37 ± 0.55	164.90 ± 0.87	166.47 ± 1.02
2	557.05 ± 0.28	564.22 ± 0.63	560.64 ± 0.74	562.79 ± 0.71
3	725.20 ± 0.55	743.26 ± 0.33	734.23 ± 0.44	737.65 ± 0.43
4	830.02 ± 0.94	850.12 ± 0.46	840.07 ± 0.70	845.42 ± 0.58
5	892.32 ± 0.70	898.28 ± 0.57	895.30 ± 0.64	898.52 ± 0.56
6	790.40 ± 1.52	819.89 ± 0.72	805.15 ± 1.12	810.50 ± 0.81
7	630.30 ± 1.09	659.67 ± 0.74	644.98 ± 0.92	650.34 ± 0.96
8	410.63 ± 1.36	410.07 ± 0.45	412.89 ± 0.29	420.85 ± 0.48
9	200.55 ± 1.67	255.55 ± 0.55	233.15 ± 0.31	236.31 ± 0.38
12	25.57 ± 1.29	80.26 ± 0.39	65.42 ± 0.49	70.23 ± 0.44
24	5.08 ± 1.50	7.34 ± 0.38	10.18 ± 0.52	nd
36	nd	nd	nd	nd

Number of participants = 12 for each phase
nd = not detected

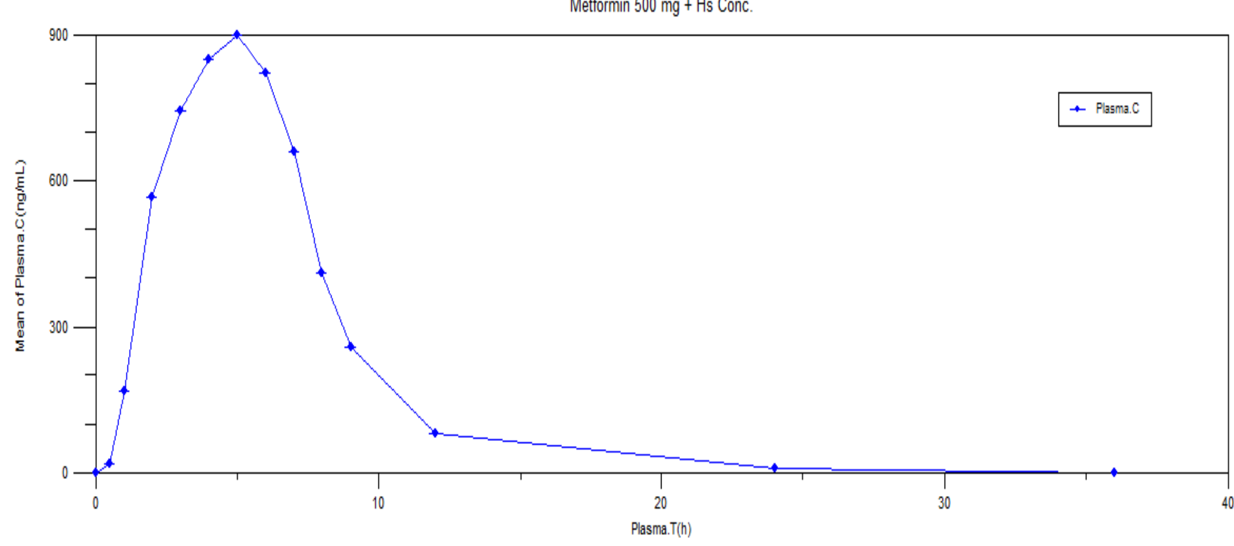
Appendix 29: Mean saliva concentrations versus time for lisinopril (10 mg) alone and when interacted with 200 mL of *Hibiscus sabdariffa* calyxes water extract (25 mg/mL)

Time (hrs)	Phase 1 mean values ± SD (ng/mL)	Phase 2 mean values ± SD (ng/mL)	Phase 3 mean values ± SD (ng/mL)	Phase 4 mean values ± SD (ng/mL)
0	nd	nd	nd	nd
0.5	nd	nd	nd	nd
1	11.23 ± 0.81	13.52 ± 0.17	12.87 ± 0.65	15.94 ± 0.38
2	21.49 ± 0.49	24.63 ± 0.38	24.24 ± 0.52	26.32 ± 0.49
3	34.24 ± 0.31	38.33 ± 0.31	36.45 ± 0.56	37.64 ± 0.45
4	52.73 ± 0.37	56.23 ± 0.21	55.35 ± 0.42	56.85 ± 0.85
5	71.66 ± 0.90	73.34 ± 0.43	72.50 ± 0.74	74.34 ± 0.56
6	85.46 ± 0.46	87.52 ± 0.23	87.55 ± 0.79	88.64 ± 0.73
7	95.36 ± 0.39	97.50 ± 0.09	97.37 ± 0.50	99.24 ± 0.86
8	80.58 ± 0.46	83.55 ± 0.57	82.46 ± 0.47	80.36 ± 0.37
9	62.27 ± 0.25	65.31 ± 0.37	64.68 ± 0.79	65.12 ± 0.57
12	30.59 ± 0.65	33.29 ± 0.39	35.49 ± 1.46	50.07 ± 0.63
24	1.53 ± 0.07	2.52 ± 0.07	nd	nd
36	nd	nd	nd	nd

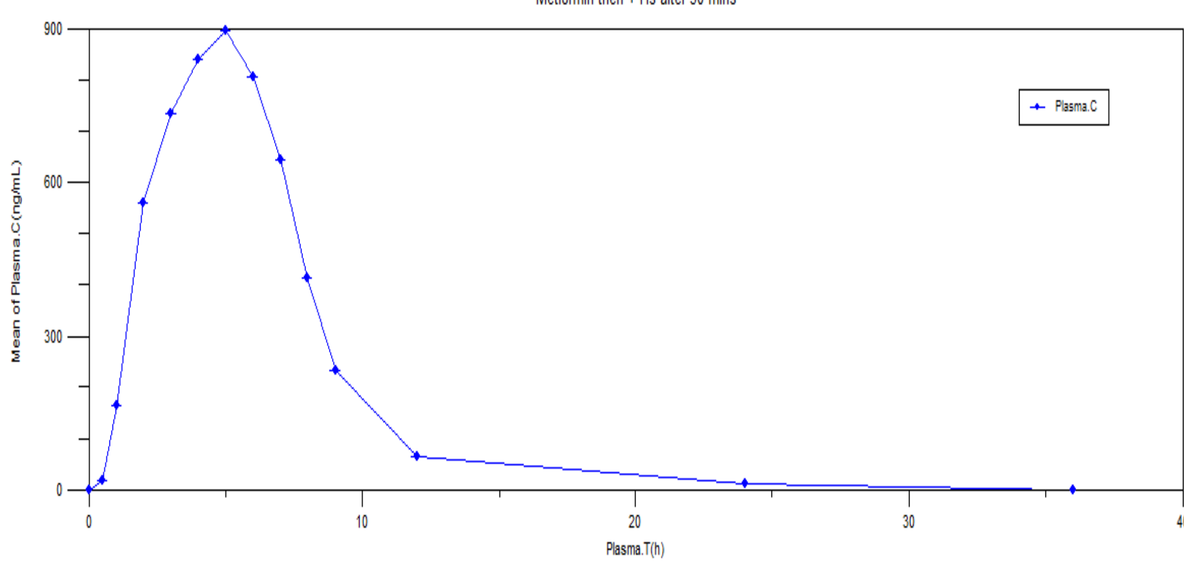
Number of participants = 12 for each phase
nd = not detected



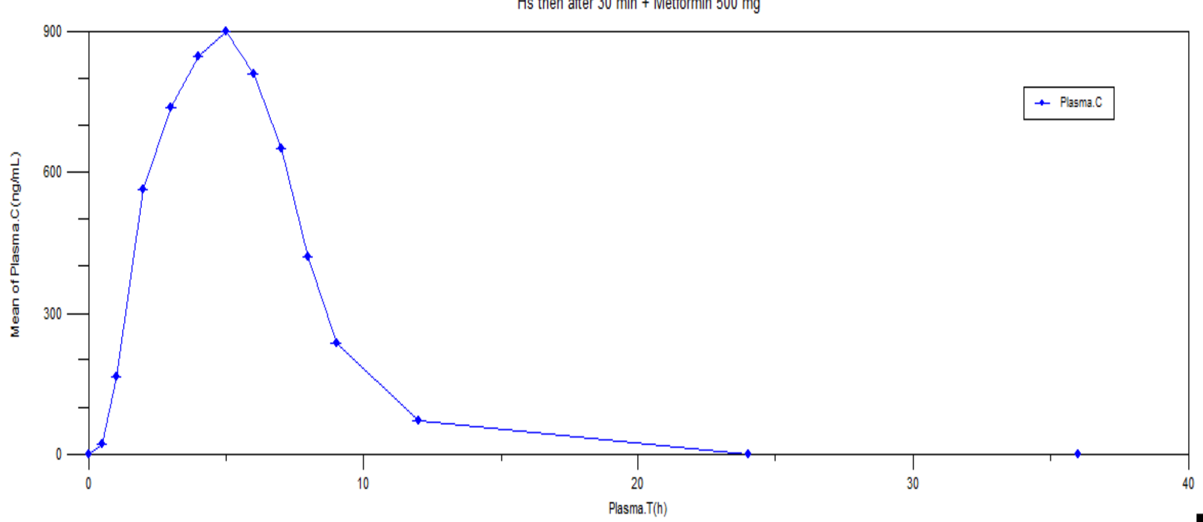
Appendix 30: Mean salivaconcentrations of metformin 500 mg administered alone



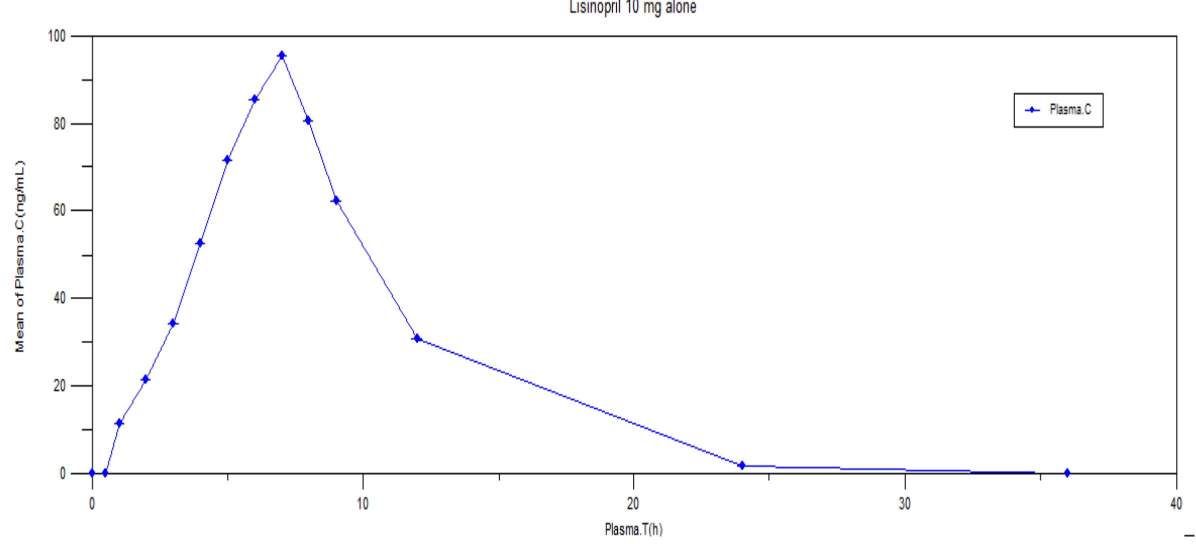
Appendix 31: Mean salivaconcentrations of metformin (500 mg) and 200 mL of *Hibiscus sabdariffa* calyxes water extract (25 mg/mL) administered concurrently



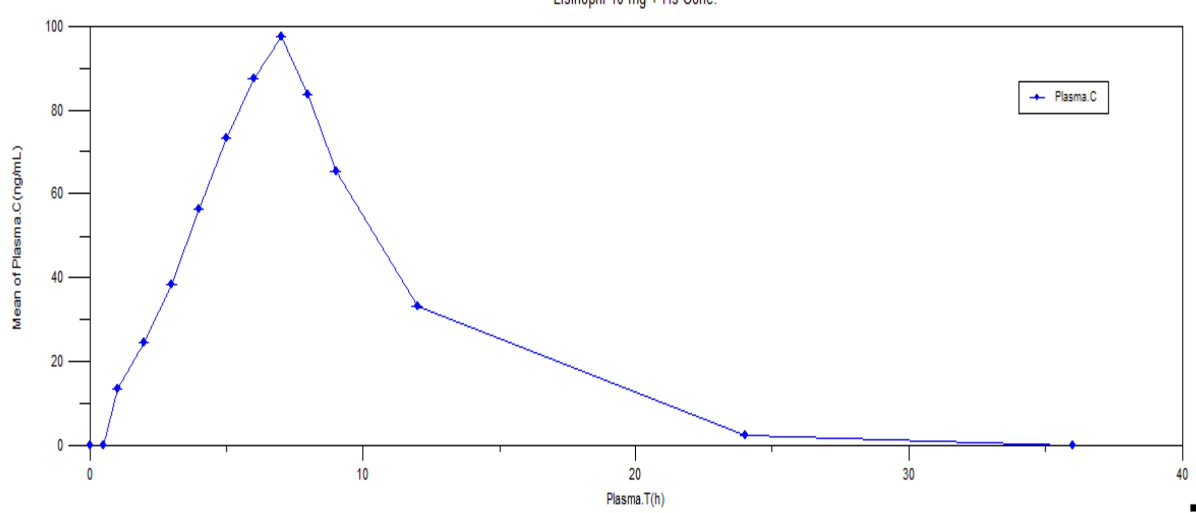
Appendix 32: Mean salivaconcentrations of metformin (500 mg) plus 200 mL of *Hibiscus sabdariffa* calyxes water extract 25 mg/mL after 30 minutes.



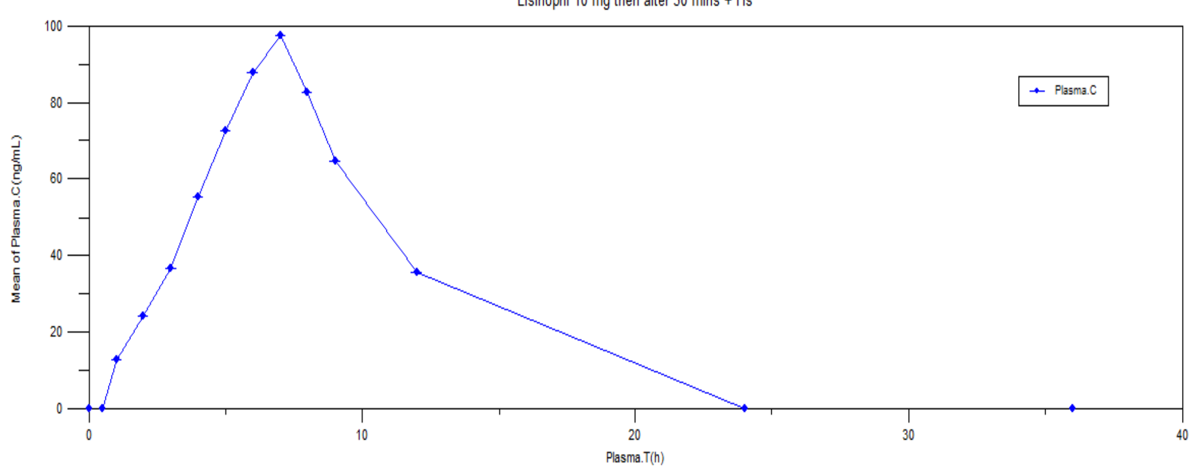
Appendix 33: Mean saliva concentrations for administration of 200 mL of *Hibiscus sabdariffa* calyxes water extract 25 mg/mL then metformin (500 mg) after 30 minutes.



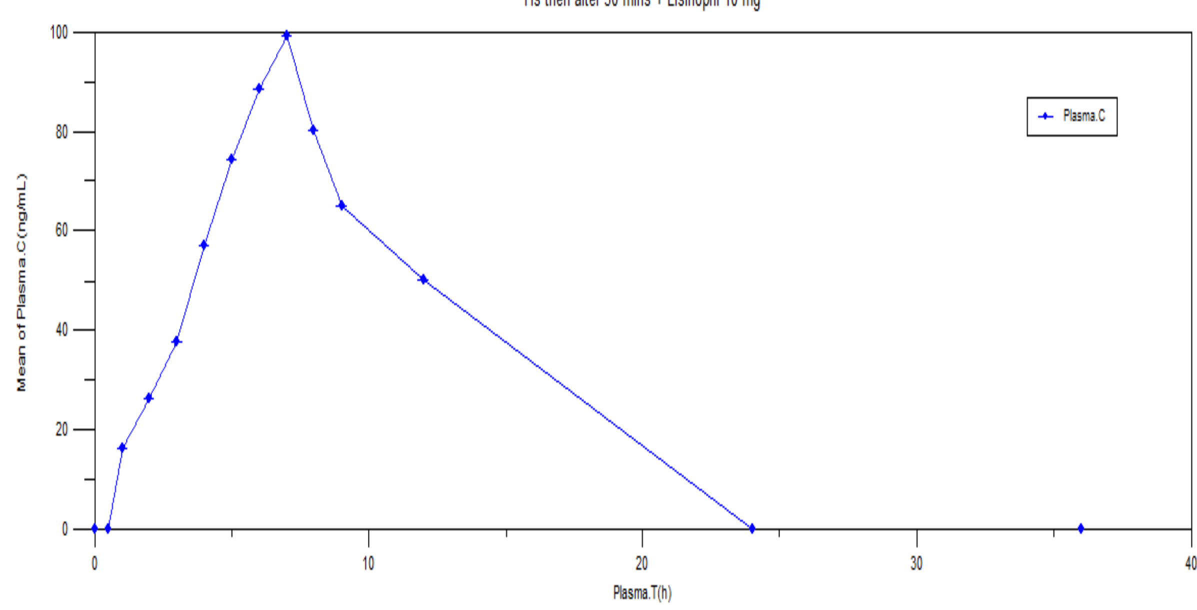
Appendix 34: Mean salivaconcentrations of lisinopril (10 mg)administered alone.



Appendix 35: Mean saliva concentrations of concurrent administration of lisinopril (10 mg) and 200 mL of *Hibiscus sabdariffa* calyxes water extract 25 mg/mL



Appendix 36: Mean saliva concentrations for administration of lisinopril (10 mg) then 200 mL of *Hibiscus sabdariffa* calyxes water extract (25 mg/mL) after 30 minutes.



Appendix 37: Mean saliva concentrations for administration of 200 mL of *Hibiscus sabdariffa* calyxes water extract (25 mg/mL) then lisinopril (10 mg) after 30 minutes.