

Phenotypic and genotypic evaluation of *Catha edulis* F. (Khat) effect on cytochrome P450 mediated drug metabolism

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This is to certify that the thesis prepared by Worku Bedada, entitled: Phenotypic and genotypic evaluation of *Catha edulis* F. (Khat) effect on cytochrome P450 mediated drug metabolism and submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy (Pharmacology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality

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ABSTRACT

Phenotypic and genotypic evaluation of *Catha edulis* F. (Khat) effect on cytochrome P450 mediated drug metabolism

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Khat (*Catha edulis* Forsk) is an evergreen perennial plant that belongs to *Celestraceae* family. The plant is cultivated primarily in East Africa and the Arabian Peninsula, harvested and then chewed to obtain stimulant effect. Khat is freely available in Ethiopia and has become popular among all segments of the population. Thus, it is highly likely that khat could be chewed while users are on medications. Ingestion of khat might affect bioavailability of concurrently taken medications, which in turn alter safety and/or efficacy. Bioavailability of drugs depends to a significant degree on the extent of elimination by cytochrome P450 (CYP) enzymes. CYP enzymes are influenced by genetic as well as non-genetic factors. Five major CYPs, namely CYP3A, CYP2D6, CYP2C9, CYP2C19 and CYP1A2 are responsible for the metabolism of over 99% of currently marketed drugs.

In an attempt to study the impact of khat on concurrently taken medications, the metabolic activities (phenotype) of these five CYPs were assessed in absence and presence of khat using appropriate probe drugs. The impacts of genetic variants (genotype) of the enzymes were also studied. To this effect, a comparative one-way crossover study was carried out on healthy Ethiopian adult habitual khat chewing volunteers to evaluate the effect of khat on CYP in two phases. Phase I was a pilot study involving the two major enzymes CYP2D6 and CYP3A enzymes, while in phase II five major CYPs were studied. After one week of abstinence from khat, blood samples were collected from 40 (phase I) and 60 (phase II) subjects to assess the baseline metabolic activities of the enzymes using 30 mg dextromethorphan (DM) (CYP3A and CYP2D6), 50 mg losartan (CYP2C9), 20 mg omeprazole (CYP2C19) and 100 mg caffeine (CYP1A2) as probe drugs. The procedure was repeated after one week of daily regular intake of 400 g fresh khat. DSM-V criteria for stimulant withdrawal and urinary cathinone level were employed to monitor the subjects' compliance to the study protocol. The metabolic activities were assessed by comparing the median metabolic ratio (MR) of DM/3-methoxymorphinan (3-MM), DM/ (dextromethorphan) DX, losartan/ losartan carboxylic acid, omeprazole/5-hydroxyomeprazole and caffeine/ paraxanthine for CYP3A, CYP2D6, CYP2C9, CYP2C19 and

CYP1A2, respectively. Wilcoxon Signed Ranks Test was used to compare the median MR in the absence and presence of khat.

The median DM/DX was significantly increased in the presence of khat in phase I ($P=0.02$) and in phase II ($P=0.001$) as well as when the data from both phases were merged ($P=0.001$). Moreover, the effect was particularly evident with CYP2D6 *1/*1 ($P=0.001$) compared to CYP2D6*1/*4 and CYP2D6*4/*4. Although the median DM/3-MM was only marginally increased in phase I ($P=0.09$), it was significantly increased in phase II ($P=0.045$) and when the data were merged ($P=0.001$). Even for phase I, the effect was significant ($P=0.02$) when an outlier was excluded. However, since CYP3A genotyping data were available for phase II alone and valid phenotype data in phase II were small, it was difficult to associate these data with phenotyping results. On the other hand, khat had no significant effect on the metabolic activities of CYP1A2 ($P=0.71$), CYP2C9 ($P=0.64$) and CYP2C19 ($P=0.15$). In addition, the logMR of probe drugs/metabolites in the presence of khat were correlated with logMR of cathinone/cathine using spearman correlation test for non-parametric tests. Consequently, moderately significant correlations were observed for CYP2D6, CYP2C9 and CYP3A, which indicate that khat is a substrate for these enzymes. Insignificant correlations were seen for CYP1A2 and CYP2C19. To the best of our knowledge, the present study is the first to investigate the impact of habitual khat consumptions on *in vivo* activity of human drug metabolizing enzymes. The results of the present study indicate that khat produces significant and reproducible inhibitory effects on the two major enzymes, CYP2D6 and CYP3A, which are responsible for metabolism of over 75% currently marketed drugs. The inhibitory effects of khat were consistently demonstrated by genotyping data for CYP2D6, but not for CYP3A enzyme. Moreover, the two major enzymes appeared to be involved in the metabolism of khat as seen from the correlation tests. Although the clinical significance of the current study is yet to be determined, it would be better to refrain from khat chewing while on medication to avoid potentially detrimental interactions.

Keywords: khat, Cytochrome P450 enzymes, Probe drugs, Phenotype, Genotype, Cathinone, Cathine, drug interaction

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Acronyms/Abbreviations

3-MM: 3-Methoxymorphinan

AAU: Addis Ababa University

NCBI: National center for biotechnology information

CNVs: Copy number variants

CYP: Cytochrome P450

DM: Dextromethorphan

DSM-V: Diagnostic and Statistical Manual for mental disorder, 5th edition

DX: Dextrorphan

EM: Extensive metabolizer

ERBT: Erythromycin Breath Test

IM: Intermediate metabolizer

INR: International normalized ratio

IQR: Interquartile range

IRB: Institutional review board

MDMA: 3,4-methylenedioxymethamphetamine

MR: Metabolic ratio

NREC: National Research Ethics Committee

PM: Poor metabolizer

SNPs: Single nucleotide polymorphism

TASH: Tikur Anbessa Specialized Hospital

UM: Ultra-rapid metabolizer

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1. Introduction

1.1 Overview of khat

Khat (*Catha edulis* Forsk) is an evergreen perennial plant that belongs to *Celestraceae* family. The plant is cultivated primarily in East Africa and the Arabian Peninsula, harvested and then chewed to obtain stimulant effect (Lemessa, 2001). The first scientific description of khat as *Catha edulis* was documented in *Flora-Aegyptiaco-Arabia* by the Swedish botanist Peter Forskal, who died in Arabia in 1768 (Al-Hebshi and Skaug, 2005). There are many different varieties of khat in Ethiopia, such as ‘Aweday’, ‘Gelemso’, ‘Beleche’, ‘Wondo’, ‘Gurage’, ‘Abomismar’ and ‘Bahirdar’ depending upon the area in which it is cultivated (Atlabachew et al., 2011). Khat is harvested in the early morning hours and sold at markets in late morning to preserve its freshness. It is wrapped in banana leaves, plastic bags or splashed with water and sold as bundles (30-60 selected slender twigs), stems and leaves (Lemessa, 2001). Normally, 100-400g of the khat leaves is consumed on daily basis (Toennes, 2003).

1.1.1 Socio-economic and legal aspects

The number of khat chewers has significantly increased in Ethiopia (Gebissa, 2008). An estimated prevalence of khat use in the general population is 27.3% among men and 11.0% among women of 15–49 years (CSA, 2011). The two major alkaloid in, cathine and cathinone, are placed group wise under international control since the early 1980s, as all amphetamine-like substances do. Thus, cathinone is placed under Schedule I and cathine in Schedule III (ECDD, 2006). Khat is freely available and a highly valued export commodity in Ethiopia. Its consumption has become popular in all segments of the population (Selassie and Gebre, 1996; Gebissa, 2008).

1.1.2 Chemistry of Khat

Many different compounds are found in khat including alkaloids, terpenoids, flavonoids, sterols, glycosides, tannins, amino acids, vitamins and minerals. The phenylalkylamines and the cathedulins are the major alkaloids. The main phenylalkylamines are phenylpropylamines consisting of *s* (-)-cathinone and the two diastereoisomers cathine and norephedrine. Cathinone is the main psychoactive alkaloid and mainly found in young shoots (Dhaifalaha and Šantavy, 2004). It has structural (**Fig 1**) and pharmacological similarity with amphetamine; thus called natural amphetamine (Kalix, 1992).

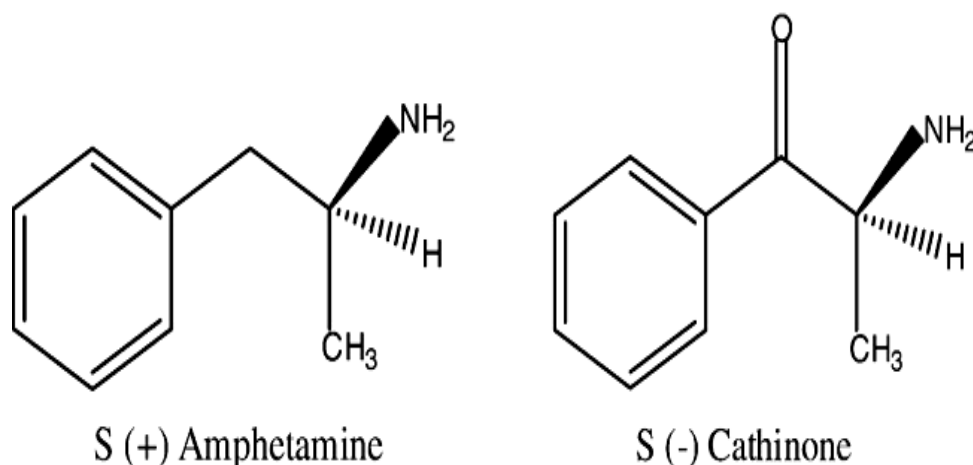


Fig.1. Chemical structures of amphetamine and *s* (-) cathinone (Kalix, 1992)

The cathedulins are based on a polyhydroxylated sesquiterpene skeleton and are basically polyesters of euonyminol. Sixty-two different cathedulins from fresh khat leaves have been characterized. There has been little investigation of the cathedulins compared to phenylalkylamines. Moreover, waves of new chemicals called substituted cathinones have emerged in response to market trends and legislative controls (Carvalho et al, 2012). Substituted cathinones are a large family of synthetic beta-keto phenethylamine (2-amino-1-phenyl-1-

propanone) derivatives chemically related to the parent compound cathinone. Examples include methcathinone, methylone and mephedrone. The general structure of cathinone derivatives is shown in **Fig 2**.

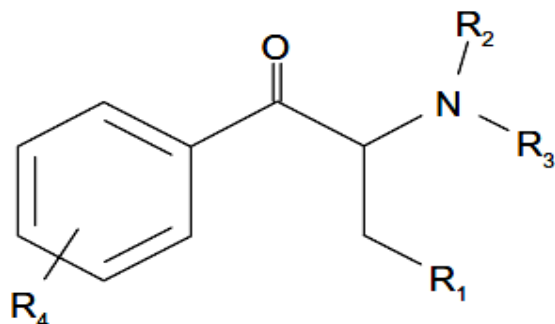


Fig. 2 General structure of substituted cathinones

1.1.3 Pharmacology of Khat

During khat chewing sessions, the leaves and the bark of the plant are chewed slowly over several hours and the juice of the masticated leaves is swallowed (Toennes et al, 2003). Cathinone has a half-life of 3h and peak plasma concentration is achieved after 1–2 h (Cox and Rampes, 2003; Toennes et al., 2003). Only less than 7% s (-) cathinone is excreted unchanged in the urine; practically all s (-) cathinone is rapidly and stereoselectively metabolized (Toennes et al., 2003) to norephedrine and cathine (**Fig 3**). The enzymes involved are not yet described. However, it is predicted from the metabolic pathways of amphetamines and synthetic cathinones that major cytochrome P450 (CYPs) are involved (Aklillu et al, 2002; Cascorbi, 2003; Toennes et al., 2003). Phase II reactions were reported to involve methylation, glucuronidation or sulfation (Paillet-Loilier et al., 2014).

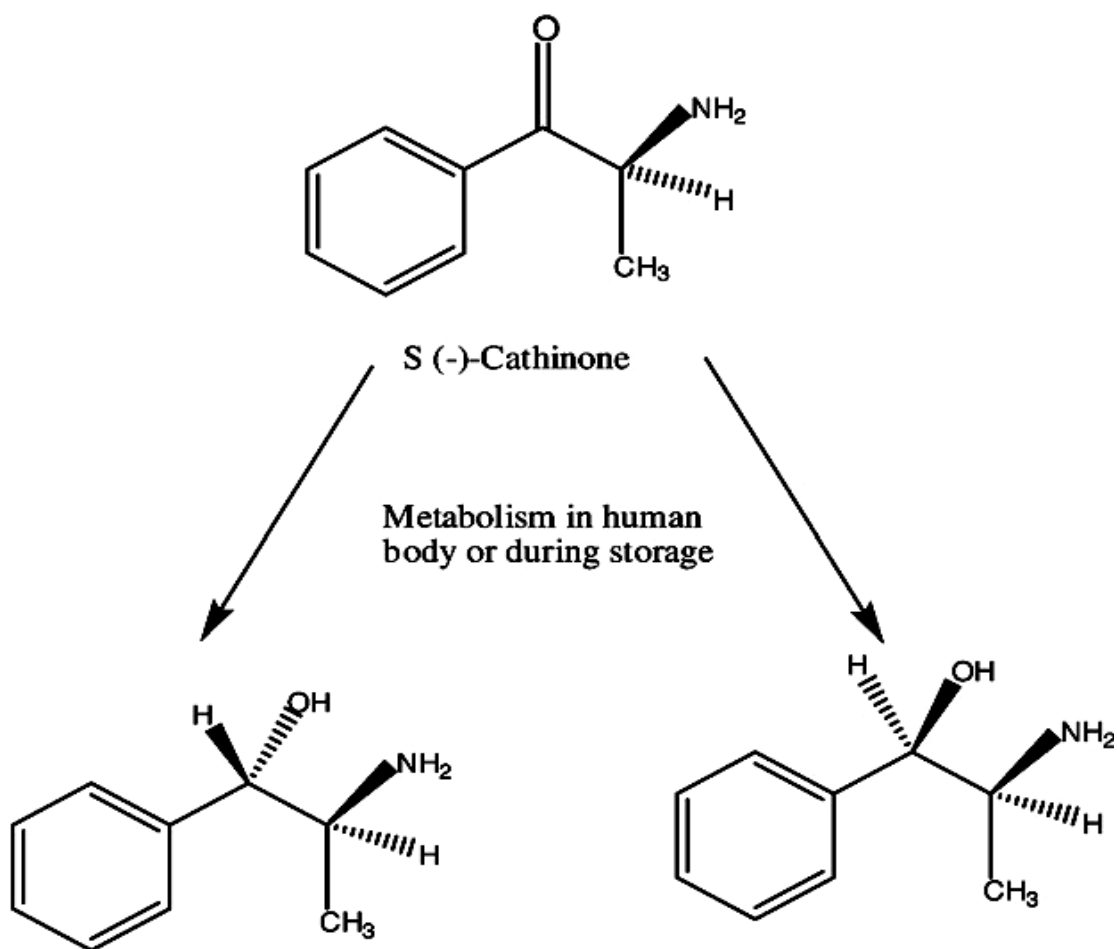


Fig 3: Stereoselective metabolism of cathinone to cathine (left) and norephedrine (right)

Cathinone is postulated to increase the levels of dopamine in the brain by acting on the catecholaminergic synapses, delaying dopamine reuptake and/or enhancing dopamine release, in particular in the striatum (Patel, 2000; Colzato et al., 2011). Moreover, serotonergic, noradrenergic and opioid systems might be involved (Feyissa and Kelly, 2008).

Khat chewing induces a state of euphoria with feelings of increased alertness. This leads to an inability to sleep at the end of chewing session (Cox and Rampes, 2003), which is often countered by drinking alcohol (Muche et al., 2006). Khat can induce psychotic symptoms (Cox and Rampes, 2003; ECDD, 2006). Self-harm and violent acts have also been reported (Alem and

Shibre, 1997). Furthermore, high frequencies of periodontal disease, gastritis, constipation, and oral keratolic lesions at the site of chewing and plasma cell gingivitis have been reported (Kassie et al., 2001). High dose of khat reduce sexual behavior in male rats (Abdulwaheb et al., 2007), affecting both motivation and performance (Mohammed and Engidawork, 2011). It has also negative effects on fetal growth and development (Jansson et al., 1988; Mwenda et al., 2003; Bedada and Engidawork, 2010). Besides, khat is associated with liver and kidney toxicities (Al-Mammary et al., 2002; ECDD, 2006) as well as cardiovascular complications (Al-Motarreb et al., 2002; Admassie and Engidawork, 2011).

Although overshadowed by detrimental features, khat has also a few actual and potential benefits. It has antimicrobial and anticancer properties (Dimba et al., 2003; Al-Hebshi and Skuag, 2005). In Ethiopia, processed leaves and roots of khat has been used traditionally to treat influenza, cough, gonorrhoea, asthma and other chest problems (Lemessa, 2001). Khat has also potential role as anti-Parkinsonian (Banjaw et al, 2005; Geresu and Engidawork, 2010) and has analgesic (Connor et al., 2000) activity.

1.2 Cytochrome P450

CYPs have evolved to enable organisms deal with lipid-soluble environmental chemicals (Martin, 2001). The human CYPs superfamily are a group of heme proteins found largely in hepatocytes, but also in intestine, lungs, kidneys, and brain (Gardiner and Begg, 2006; Zanger and Schwab, 2013). In this superfamily, more than 57 genes have been identified, which are divided into 18 families and 44 subfamilies. The families share at least 40% amino acid identity and the subfamilies share 55%. Of these, the CYP1, CYP2 and CYP3 families are particularly important in the metabolism of drugs, collectively accounting for most of Phase I

biotransformation. Recently of a member of another CYP family, CYP4 was reported to be involved in the metabolism of warfarin (Li-Wan-Po et al., 2010).

Nearly all metabolism by CYPs depends on nine subfamilies (CYP1A2, CYP2A6, CYP3A, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP2E1) (Gasche et al., 2004; Foti and Wahlstrom, 2008; Li and Bluth, 2011; Chen et al., 2012; Sherry, 2013; Al-Jenoobi et al., 2014). The relative abundance of individual CYPs in the liver has been determined as CYP1A2 (>10%), 2A6 (~10%), 2B6 (<5%), 2C8 (~5%), 2C9 (>15%), 2C19 (<5%), 2D6 (~2-5%), 2E1 (~15%), and 3A (35%) (Yang, 2009). CYP2C, CYP2D6 and CYP3A enzymes make up 60–70% of the total CYPs in the adult human liver and are responsible for the metabolism of over 85% of currently marketed drugs (Chen et al., 2012). The five major CYPs, which are responsible for 99% of currently marketed drugs are CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A (de Andreas et al., 2014).

The human *CYP* genes are highly polymorphic (de Andreas et al., 2014) mainly due to single nucleotide polymorphisms (SNPs). The major *CYP* genes and their corresponding SNPs are shown in **Table 1**. Deletion, insertion and copy number variants (CNVs) have also been observed (Ingelman-Sundberg et al., 2007). The most pharmacologically and clinically relevant CYP polymorphisms are found in CYP2D6, CYP2C9, and CYP2C19 (Li and Bluth, 2011). These polymorphisms/ mutations can result in variations in drug response among individuals. However, it is not only the individual genotype that influences the variability in responses. Factors such as sex, age, diet, disease states, organ function, concomitant drug administration and exposure to other chemicals can substantially affect CYP enzyme activity as well (Yang, 2009). Thus, since genetic and non-genetic factors interact and both contribute to the final drug-

metabolizing enzyme activity, when data on the *in vivo* enzyme activity is required, both genotype and phenotype study should be applied (Yang, 2009; de Andreas et al., 2014).

Table 1: List of *CYP* genes and their corresponding single nucleotide polymorphisms

| Gene | Chromosomal location | Number of amino acids | Number of exons | Number of SNPs |
|----------------|----------------------|-----------------------|-----------------|----------------|
| <i>CYP1A2</i> | 15q24.1 | 516 | 7 | 31 |
| <i>CYP2C9</i> | 10q24.1 | 490 | 9 | 28 |
| <i>CYP2C19</i> | 10q24.1-q24.3 | 490 | 9 | 31 |
| <i>CYP2D6</i> | 22q13.1 | 497 | 9 | 52 |
| <i>CYP3A4</i> | 7q21.1 | 503 | 13 | 32 |
| <i>CYP3A5</i> | 7q21.1 | 502 | 13 | 15 |

1.2.1 *CYP1A2*

CYP1A2 is mainly expressed in the liver and slightly in lung (Yang, 2009). It is involved in the metabolism of 8–10% of clinically important drugs such as caffeine, olanzapine and theophylline. However, it is neither the sole metabolizing enzyme, nor a rate-limiting one for most of the drugs. *CYP1A2* is also involved in the catalytic activation of several carcinogenic heterocyclic amines to reactive metabolites that are implicated in the development of various cancers (Lim et al., 2010; Thorna et al., 2012). Common substrates, inhibitors and inducers for *CYP1A2* are listed in **Table 2**. The activity of *CYP1A2* is induced by tobacco smoke or drugs such as omeprazole and is inhibited by drugs such as fluvoxamine, Cimetidine or oral hormone

replacement therapy and contraceptives (Laika et al., 2010; Thorna et al., 2012). As the N3-demethylation of caffeine to paraxanthine (1,7-dimethylxanthine) is predominantly (>95%) mediated by CYP1A2, the metabolic ratio (MR) of caffeine to paraxanthine is widely used as a main probe drug to assess CYP1A2 activity *in vivo*. Theophylline and melatonin are also used, whereas in-vitro studies often involve Phenacetin (Browninga et al., 2010; Thorna et al., 2012).

Table 2: Selected substrates, inhibitors and inducers of CYP1A2

| Substrates | Inhibitors | Inducers |
|---------------------------|-------------------|-----------------|
| Acetaminophen, Naproxen | Amiodarone | Insulin |
| Tricyclic Antidepressants | Cimetidine | Modafinil |
| Caffeine, Theophylline | Fluvoxamine | Naficillin |
| Clozapine, Haloperidol | Interferon | Tobacco |
| Estradiol | | Omeprazole |
| Melatonin | | |
| Mexiletine | | |
| Phenacetin | | |
| R-warfarin | | |

Approximately 35 to 75% of the interindividual variability in CYP1A2 activity is reported to be due to genetic factors (Yang, 2009; Lim et al., 2010). However, *CYP1A2* polymorphism has rare functional effects (Johansson and Ingelman-Sundberg, 2011). More than 15 variant alleles and a series of subvariants (**1B* to **16*) have been identified have been found in the *CYP1A2* upstream sequence, introns and exons in NCBI dbSNP. *CYP1A2*1* (also known as **1A*) is referred to as the wild-type. The most common variants are *CYP1A2*1K* (-163C>A; -739T>G; -729C>T, all located in intron 1) and *CYP1A2*1F* (-163C>A located in intron 1) (Aklillu et al., 2003;

Johansson and Ingelman-Sundberg, 2011). The allelic variant *CYP1A2*1F* (-163C>A/ rs762551) is associated with higher clearance of the CYP1A2 substrate, caffeine in Caucasian smokers with the AA genotype compared with the CA and CC genotypes, but the effect was not seen in nonsmokers. In a study on Ethiopian smokers, the *CYP1A2*1F* haplotype was not associated with increased caffeine metabolism. Apart from genetic variation, epigenetic and environmental factors play a role in determining variability in *CYP1A2* expression and enzyme activity (Laika et al., 2010; Lim et al., 2010; Thorna et al., 2012).

1.2.2 CYP2C9

CYP2C9 is one of the most abundant CYP enzymes in the human liver and metabolizes more than 100 clinically important drugs (Yang, 2009). It is primarily involved in the metabolism of s-warfarin. Common substrates, inhibitors and inducers for CYP2C9 are listed in **Table 3**. The most commonly used probe drug for phenotype of CYP2C9 is losartan (de Andreas et al., 2014). Flurbiprofen and Tolbutamide can also be used (Yang, 2009; Bosilkovska et al., 2014).

The *CYP2C9* gene has about 28 known variant alleles. Individuals homozygous for the reference *CYP2C9* allele (*CYP2C9*1*) have the “extensive metabolizer” phenotype. Common variants with reduced enzyme activity among individuals of European ancestry are *CYP2C9*2* (rs1799853) and *CYP2C9*3* (rs1057910). *CYP2C9*2* is a missense mutation of 430T>C causing the substitution of R144C. Typically, this mutation causes a decrease in enzyme activity toward CYP2C9 substrates such as s-warfarin and Tolbutamide. *CYP2C9*3* is a missense mutation of 1075A>C on exon 7 that leads to an I359L (Yang, 2009). The frequencies of the *CYP2C9* variant alleles differ between racial/ethnic groups. *In vitro* and *ex vivo* studies suggest that *CYP2C9*2* and *3 impair metabolism of s-Warfarin by ~30–40% and ~80–90%, respectively.

Table 3: Selected substrates, inhibitors and inducers of CYP2C9

| Substrates | Inhibitors | Inducers |
|---|---|-----------------|
| Diclofenac, Flurbiprofen, Ibuprofen, Celecoxib, Proxicam | Fenofibrate, Fluvastatin, Lovastatin | Rifampin |
| Amytriptline, Fluoxetine | Amiodarone | Secobarbital |
| Fluvastatin | Fluconazole, Voriconazole | |
| Sulfonylurea, Nateglinide, Rosiglitazone, | Fluvoxamine, Sertraline | |
| Irbesartan, Losartan | Sulfamethoxazole | |
| S-Warfarin | INH | |
| Tamoxifen | Probenecid | |
| | Phenylbutazone | |
| | Zafirlukast | |

As compared with patients who are homozygous for *CYP2C9**1, individuals who inherit one or two copies of *CYP2C9**2 or *3 are at greater risk of bleeding during warfarin therapy, require lower doses to achieve similar levels of anticoagulation and require more time to achieve a stable International normalized ratio (INR) (Takahashi and Echizen, 2003; Johnson et al., 2011). Additional *CYP2C9* variant alleles with reduced activity (*CYP2C9**5, *6, *8, and *11) contribute to dose variability among African Americans. The *CYP2C9**5 allele contains the 1080C>G transversion in exon 7 causing a D360E change, which has been found almost exclusively in African-Americans (Yang, 2009). Including these additional *CYP2C9* variants in dosing algorithms for warfarin may improve predictability for African Americans (Johnson et al., 2011).

1.2.3 CYP2C19

CYP2C19, which is also called s-mephenytoin hydroxylase (Persson et al., 1996), is primarily expressed in hepatic tissue, but a significant amount is also found in the gut wall, particularly the duodenum. It is responsible for the metabolism of many drugs such as proton pump inhibitors (Omeprazole, Lansoprazole and Pantoprazole), antidepressants (Imipramine, Amitriptyline and Escitalopram), benzodiazepines (Diazepam and Flunitrazepam), anticancer drugs (Cyclophosphamide), anti-epileptics (Phenytoin, s-Mephenytoin, Phenobarbital), Clopidogrel and propranolol. The enzyme is also involved in the activation of the prodrug proguanil to cycloguanil (Persson et al., 1996; Yang, 2009). CYP2C19 also contributes to the catabolism of endogenous substrates like estradiol, progesterone and testosterone (Yang, 2009). Common substrates, inhibitors and inducers for CYP2C19 are indicated in **Table 4**. Its activity can be inhibited by Fluoxetine, Fluvoxamine, Lansoprazole, Pantoprazole and Ticlopidine, but induced by Phenobarbital and Rifampin (Lee, 2013). The phenotype of CYP2C19 is usually studied using omeprazole (de Andreas et al., 2014).

The *CYP2C19* gene is highly polymorphic and more than 30 known allelic variants and subvariants have been identified. *CYP2C19**1 is the wild-type allele encoding a fully functional enzyme (Hicks et al., 2013). The most common loss-of-function alleles are *CYP2C19**2 (rs4244285), characterized by a 681G→A substitution in exon 5 leading to a splice-defective site and *CYP2C19**3 (rs4986893), the result of the 636G>A mutation in exon4, creating a premature stop codon (Person et al., 1996). The presence of these alleles is generally considered to be sufficiently predictive for the phenotypes to be inferred from them. The phenotype of *CYP2C19* metabolic capacity can be categorized based on genotypes and includes extensive metabolizers (EM, two wild-type functional alleles), intermediate metabolizers (IM, two reduced functional

alleles or one null allele and a functional allele) and poor metabolizers (PM, two non-functional alleles) of drugs (Li-Wan-Po et al., 2010; Hicks et al., 2013; Lee, 2013; Hulot et al., 2015).

Table 4: Selected substrates, inhibitors and inducers of CYP2C19

| Substrates | Inhibitors | Inducers |
|---|-------------------------|-----------------|
| Tricyclic antidepressants, Citalopram, Moclobemide | Chloramphenicol | Carbamazepine |
| Chloramphenicol | Cimetidine | Norethindrone |
| Diazepam, Phenobarbitone | Ketoconazole | Prednisone |
| Clopidogrel | Fluoxetine, Fluvoxamine | Rifampin |
| Cyclophosphamide | Proton Pump Inhibitors | |
| Phenytoin | Modafinil | |
| Indomethacin | Indomethacin | |
| Proton pump inhibitors | Topiramate | |
| Nelfinavir | | |
| Progesterone | | |
| Propranolol | | |
| Proguanil | | |

The *CYP2C19**17 allele results in enhanced gene transcription which, purportedly leading to increased metabolic activity. However, the clinical importance of the *CYP2C19**17 allele is a matter of debate (Hicks et al., 2013). The *CYP2C19**17 allele is characterized by two SNPs in the 5'-flanking region (-3402C > T and -806C > T) of the gene. There is low frequency of the *CYP2C19**17 allele in Chinese subjects (4%) relative to Ethiopians and Swedes, both of which

have 18% distribution (Li-Wan-Po et al., 2010). The *CYP2C19*17* allele has been associated with increased bleeding among carriers treated with Clopidogrel (Hulot et al., 2015).

1.2.4 CYP2D6

The human CYP2D6 is mainly expressed in the liver (Arneth et al., 2009). Although it accounts for only 2–5% of all hepatic CYP isozymes, CYP2D6 metabolizes approximately 25% of all clinically used medications currently available in the market (Bathum et al., 1999; Dorado et al., 2005; Ingelman-Sundberg, 2005). CYP2D6 is also reported to metabolize amphetamines (Yang, 2009). CYP2D6 has a very high affinity for alkaloids and does not undergo a substantial induction by xenobiotic or drugs like Rifampicin (Cascorbi, 2003). Selected substrates, inhibitors and inducers for CYP2D6 are indicated in **Table 5**. Conflicting reports have been documented in pregnancy (Frank et al., 2007; Nylen et al., 2011).

CYP2D6 activity shows a very high degree of interindividual variability, which is primarily due to genetic polymorphism that influences both enzyme expression and function (Bertilsson et al., 2002; Chou et al., 2003). The most important variant alleles are *CYP2D6 *3/ CYP2D6A* (2549-A deletion), *CYP2D6*4/ CYP2D6B* (G1846A transition resulting in splicing defect), *CYP2D6*5/CYP2D6D* (gene deletion), *CYP2D6*10B/CYP2D6Ch1* (A proline to serine exchange in codon 34 resulting with reduced function) and *CYP2D6*41* (a variant of *CYP2D6*2* having -1584 C instead of G, but less expressed than the corresponding *CYP2D6*2*) and gene duplications of allele **1(*1×2)* and **2(*2×2)* (Masimirembwa et al., 1996, Cascorbi, 2003; Ingelman-Sundberg, 2005).

Table 5: Selected Substrates and Inhibitors of CYP2D6

| Selected Substrates | Inhibitors |
|---|--|
| Fluoxetine, Fluvoxamine, Paroxetine, Venlafaxine, Tricyclic Antidepressants | Diphenhydramine , Chlorpheniramine, Metoclopramide |
| Carvedilol, Propranolol, Metoprolol, Flecainide, Lidocaine | Bupropion ,Citalopram, Fluoxetine, Paroxetine, Sertraline ,Cloimpiramine |
| Codeine , Dextromethorphan | Celecoxib |
| Debrisoquine | Methadone |
| Amphetamine | Chlorpromazine |
| Chlorpromazine, Thioridazine, Haloperidol, Risperidone, Aripiprazole | Cimetidine , Ranitidine |
| Metoclopramide, Ondansetron, Promethazine | Amiodarone, Quinidine |
| Tamoxifen | Doxorubicin |
| | Ritonavir |
| | Halofantrine |
| | Terbinafine |

The most common *CYP2D6* variant alleles are shown in **Table 6**. The alleles *1 and *2 are functional/reference/wild type alleles; whereas the alleles *3, *4 and *5 are inactive/deficient alleles. The alleles with decreased activity are *10, *17 and *41. Gene duplications or multiplications of the active alleles (*1xN and *2xN) results in increased activity (Chou et al.,2003; Ingelman-Sundberg, 2005; Frank et al.,2007; Miranda et al., 2007; Yang, 2009).The importance of the genetic polymorphisms of *CYP2D6* for efficient drug therapy is well established. Patients who are taking drugs that are CYP2D6 substrates and who have such mutations may have elevated drug levels. Conversely, gene duplication or multiplication is

thought to lead to increased enzyme production, which results in an increased rate of drug metabolism, thereby leading to decreased drug levels (Aklillu et al., 1996, 2002; Bernard et al., 2006; Arneth et al., 2009; Roden et al., 2011).

Table 6: Most common allelic variants of CYP2D6

| Allele | Nucleotide change | Amino Acid change | Impact on enzyme |
|-------------------|---|----------------------------------|---------------------------------|
| *1 | None | None | Normal activity |
| *2 | None | None | Normal activity |
| *1xN, *2xN | Gene duplication | N-active genes | Increased activity |
| *3 | 2549A>del | 259 Frame shift | Inactive enzyme |
| *4 | 1846G>A | Splicing defect | Inactive enzyme |
| *5 | Gene deletion | CYP2D6 deleted | No enzyme |
| *10B | 100C>T, 188C>T | P34S, S486T | Unstable enzyme |
| *17 | 1023C>T, 1111C>T, 1661G>C, 2850C>T, 4180G>C | T107I, R296C, S486T | Reduced affinity for substrates |
| *41 | -1584G>C | R296C, S486T, Splicing defect | Reduced activity |

More than 150 different alleles of the *CYP2D6* gene are currently known to occur at different frequencies in different populations of the world and have been linked to four distinct phenotype ultrarapid metabolizer (UM), EM, IM and PM (Arneth et al., 2009). The likely phenotypic classification based on *CYP2D6* diplotypes are shown in **Table 7**.

Table 7: The likely CYP2D6 phenotypes based on CYP2D6 diplotypes

| Likely CYP2D6 Phenotype | Activity Score | Genotypes |
|--------------------------------|-----------------------|--|
| UM | >2 | An individual carrying duplications of functional alleles |
| EM | 1-2 | An individual carrying two functional alleles/ two reduced function alleles/ one functional and non-functional allele/one functional and one reduced function allele |
| IM | 0.5 | An individual carrying one reduced function allele and non-functional allele |
| PM | 0 | An individual carrying two non-functional alleles |

UMs carry duplicated functional alleles and show enhanced enzymatic activity compared to EMs, who carry 2 functional copies and exhibit normal enzymatic activity. Individuals containing one functional and one defective alleles are classified as IMs and display weak enzyme activity, while subjects carrying 2 deficient alleles are classified as PMs and lack total CYP2D6 activity (Trojan et al., 2012). CYP2D6 PMs have significantly higher risk of relapse after tamoxifen therapy (Ferraldeschi and Newman, 2010). UMs have greater risk of toxicity due to codeine (Gasche et al., 2004). Such codeine mediated toxicities are especially practical in Ethiopian populations (Aklillu et al., 1996). The distributions of CYP2D6 alleles exhibit notable interethnic differences (Cascorbi, 2003). Approximately, 7 to 10% of European Caucasians, 0 to 5% Africans and 1% of Chinese are PMs (Aklillu et al., 1996; Li and Bluth, 2011) mainly resulting from CYP2D6*4. However, the overall CYP2D6 activity is lower in Chinese compared to Caucasians. This is due CYP2D6*10, which occurs in 50% of Chinese, but largely absent in

Caucasians. This variant produces an unstable enzyme with reduced activity. The frequency of the IM phenotype was estimated to be around 10–15% of the European population (Raimundo et al., 2004).

At the other end of the spectrum, gene duplication occurs in 7% of Tanzanians, 20% Saudi Arabians and 29% of Ethiopians (Aklillu et al., 1996; Li and Bluth, 2011), whereas it is only less than 2% in other Africans. This frequency is generally low in Northern Europe and Asian populations compared to populations in the Mediterranean, where 10% of them have duplicated *CYP2D6* genes (Cascorbi, 2003; Raimundo et al., 2004; Carmo et al., 2007). The frequency and geographical distribution of UMs is shown in **Figure 4**.

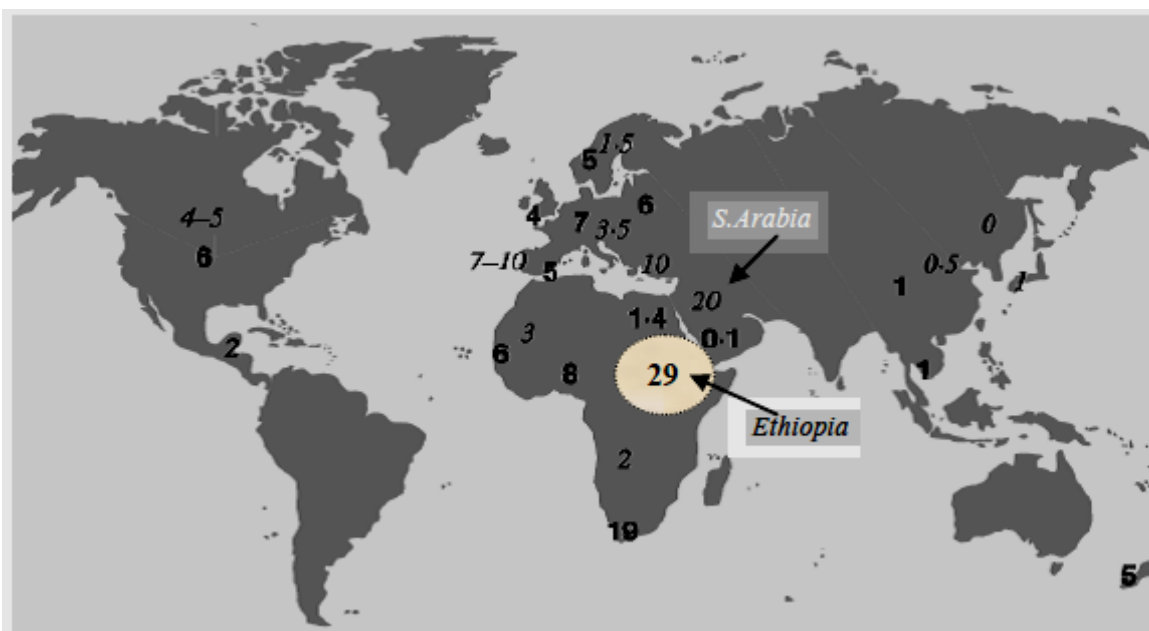


Fig 4: Frequency of UM distribution of *CYP2D6* across the globe (Ingelman-Sundberg, 2005)

The *CYP2D6* enzyme is not inducible by conventional means. The likely cause for the gain of duplicated genes in populations like Ethiopians is natural selection (Ingelman-Sundberg, 2005). Environmental factors, such as diet and herbs containing alkaloids could have exerted a selective

advantage on duplicated *CYP2D6* genes, increasing the survival rates of these individuals. The basis of this selection would be the ability of CYP2D6 to metabolize or detoxify these agents (Aklillu et al., 2002).

Generally, there is a homogeneous distribution of the CYP2D6 genetic polymorphism among native Ethiopians; Cushitic (Oromo) as well as Semitic speakers (Amhara, Tigriyans and Gurage) (Passarino et al., 1998). However, important difference in CYP2D6 enzyme activity between Ethiopians living in Ethiopia as compared to Ethiopians living in Sweden was reported. Native Ethiopians of the same genotype demonstrated reduced CYP2D6 activity compared to Ethiopians in Sweden (Aklillu et al, 2002). The same dietary and herbal agents that triggered selective genetic selection many years ago might inhibit the same enzyme (Aklillu et al, 2002; Ingelman-Sundberg, 2005; Gressier et al.,2012) when still used. In this sense, typical dietary experience of native Ethiopians and herbal agents such as khat that are freely available to native Ethiopians could make important differences. As a result, variability of responses to drugs cannot be exclusively explained by *CYP2D6* genotype (Wojtczak et al., 2007). Although *CYP2D6* genotyping is widely used to evaluate CYP2D6 enzyme activity, the CYP2D6 metabolic capacity is determined by phenotype analysis.

CYP2D6 phenotype study involves specific probe drugs, such as DM, debrisoquine or sparteine (Dorado et al., 2012). Debrisoquine and sparteine are marketed only in a limited number of countries and are also associated with serious side-effects such as orthostatic hypotension. DM is, thus a safe alternative. The average adult dose is 15 to 30 mg (Wojtczak et al., 2007). The phenotype of CYP2D6 is studied by measuring the MR of DM/DX in 3h single point plasma or 8h urine sample. DM is primarily metabolized by CYP2D6 through O-demethylation into DX.

A minor pathway is metabolism by CYP3A into 3-MM) (Wojtczak et al., 2007, Dorado et al., 2012, de Andres et al., 2014).

1.2.5 CYP3A

CYP3A is the most abundant CYP isoforms, accounting for approximately 60% of the total CYPs in the body, and exhibits broad and overlapping substrate specificity (Yang, 2009). Nearly, half of all current clinical drugs are substrates for CYP3A (Luo et al., 2009). The total CYP3A catalytic activity is largely attributable to the two most common isoforms; CYP3A4 and CYP3A5 (Gasche et al., 2004). Selected substrates, inhibitors and inducers for CYP3A are shown in **Table 8**.

CYP3A4 is the dominant CYP3A enzyme expressed in the liver and small intestine. Genetic variants of the *CYP3A4* are relatively common, but less likely to cause a phenotypic change in drug metabolism (Gasche et al., 2004; Roden et al., 2011). CYP3A4 activity is affected by sex where it can be 20–40% higher in women than in men, and also increase during pregnancy (Luo et al., 2009; Nylén et al., 2011). CYP3A5 has relatively weak catalytic capability and is consistently expressed in extrahepatic tissues, such as kidney, lung, colon, and esophagus.

CYP3A5 expression is highly polymorphic, with 25 allelic variants of CYP3A5. Functional CYP3A5 is encoded by the *CYP3A5*1* allele. *CYP3A5*2* (rs28365083; g.27289C > A; T398N), considered to be not fully functional, was found at a very low frequency ($\approx 1\%$) in white population.

Table 8: Selected substrates, inhibitors and inducers of CYP3A enzyme

| Selected Substrates | Inhibitors | Inducers |
|---|---|-----------------------------|
| Fentanyl, Methadone, Codeine, Dextromethorphan | Amiodarone | Phenobarbitone |
| Alprazolam, Midazolam, Zolpidem, Zaleplon | Chloramphenicol, Erythromycin, Norfloxacin | Efavirenz, Nevirapine |
| Calcium channel blockers, Statins, Quinine, Propranolol, Lidocaine | Cimetidine | Phenytoin, Carbamazepine |
| Haloperidol, Aripiprazole, Risperidone, Quetiapine | Delavirdine, Ritonavir, Nelfinavir | Pioglitazone |
| Antihistamines, Ondansetron | Fluvoxamine | Glucocorticoids |
| Erythromycins, Dapsone | Mifepristone | |
| Protease inhibitors | Verapamil | |
| Glucocorticoids, Progesterone, Estradiol, Testosterone | | |
| Cyclosporine, Vincristine, Tamoxifen, Tacrolimus, Irinotecan | | |
| Sildenafil | | |
| Nateglinide | | |

The most common nonfunctional variant of *CYP3A5* is designated as *CYP3A5*3*. *CYP3A5*3* is the most frequent and well-studied variant allele of *CYP3A5*. It is designated as 6986A > G (*CYP3A5*1* has an A at this position). A change from A to G at this position creates a cryptic splice site in intron 3, resulting in altered mRNA splicing. The alternatively spliced isoform has an insertion from intron 3, which alters the reading frame and results in a premature termination codon and hence a nonfunctional protein. Individuals with the *CYP3A5*3/*3* genotype are considered to be *CYP3A5* nonexpressors. The likely phenotypes based on *CYP3A5* diplootypes are described in **Table 9**.

*CYP3A5**1 carriers have a higher rate of Tacrolimus clearance than those with the other genotypes, with *1/*1 individuals having a higher clearance than *1/*3 individuals, who have higher clearance than *3/*3 individuals (Yang, 2009; Lamba et al., 2012). The other two most studied *CYP3A5* alleles are *6 and *7. *CYP3A5**6 (14690G > A; rs10264272) is a nonfunctional allele, present predominantly in the African American population and occasionally found in other populations. *CYP3A5**6 causes alternative splicing of *CYP3A5* (a G-to-A transition in exon 7 results in exon 7 skipping) and protein truncation, resulting in the absence of the *CYP3A5* protein. *CYP3A5**6 alleles are relatively frequent in African individuals (7–17%), but are very rare in White and Asian populations. *CYP3A5**7 (rs76293380; 27131–27132ins T) is an insertion polymorphism that resides between codons 345 and 346 and results in a shift in reading frame. This shift introduces a premature termination codon at position 348 (D348), resulting in a truncated and nonfunctional protein. *CYP3A5**7 occurs at a frequency of about 8% in the African population but has not been found in White or Asian populations (Lamba et al., 2012; Birdwell et al., 2015). Ethiopians have lower frequency of *CYP3A5**1 and do not contain African population-specific allele, *CYP3A5**7 (Mirghania et al., 2006; Gebeyehu et al., 2011).

There are a number of clinical markers, endogenous and exogenous, used to assess the activity of CYP3A. The plasma 4 β -hydroxycholesterol/cholesterol and urinary 6 β -hydroxycortisol/cortisol ratio is a non-invasive method (Chen et al., 2012), while plasma midazolam clearance, plasma alprazolam clearance, plasma quinine clearance, and the erythromycin breath test (ERBT) (Mirghania et al., 2006; Luo et al., 2009; Allqvist, 2010; Nylen et al., 2011) are all invasive methods. The ratio of 4 β -hydroxycholesterol/cholesterol is preferable under conditions where the cholesterol level is expected to change, such as pregnancy (Nylen et al., 2011). ERBT requires

IV administration of radioactively labeled dose of ^{14}C N-methylerythromycin followed by a single breath sample collected after 20 min (Franke et al., 2008) and the rate of $^{14}\text{CO}_2$ is calculated.

Table 9: Assignment of likely phenotypes based on CYP3A5 Diplotypes

| Likely Phenotype | Genotypes | Examples of Diplotypes |
|----------------------------------|--|--|
| EM (CYP3A5 expresser) | An individual carrying two functional alleles | <i>*1/*1</i> |
| IM (CYP3A5 low expresser) | An individual carrying one functional allele and one non-functional allele | <i>*1/*3, *1/*6, *1/*7</i> |
| PM (CYP3A5 non-expresser) | An individual carrying two non-functional alleles | <i>*3/*3, 6/*6, *7/*7, *3/*6, *3/*7, *6/*7</i> |

The plasma quinine is shown to be reproducible and sensitive to reflect the activity of both CYP3A4 and CYP3A5 (Mirghania et al., 2006), but single point blood sample must be taken after 14h. Midazolam plasma clearance also reflects the activity of both CYP3A4 and CYP3A5. However, single point plasma clearance does not concur well with the area under the concentration-time curve (AUC) for Midazolam clearance (Allqvist, 2010).

Plasma alprazolam clearance reflects the activity of both CYP3A4 and CYP3A5, but a single plasma sample at 8h post dose is shown to be highly correlated with the AUC of alprazolam clearance (Wennerholm et al., 2005; Allqvist, 2010). Alprazolam, a triazolo 1, 4-

benzodiazepine analog, is extensively metabolized in humans, primarily by CYP3A4 to the primary metabolite 4-hydroxyalprazolam and in addition by CYP3A5 to α -hydroxyalprazolam (Allqvist, 2010). Recently, the use of DM for simultaneous assessment of the activities of CYP3A and CYP2D6 using single sample is well appreciated, as this reduces time and cost per analysis, and also avoids discomfort to study participants (de Andres et al., 2014).

1.3 Rationale for the study

Bioavailability of drugs depends, to a significant degree, on the extent of elimination by xenobiotic-metabolizing enzymes in the human liver. The five human CYP enzymes that are primarily responsible for the metabolism of over 99% xenobiotics are CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A (de Andreas et al., 2014). The drug-metabolizing activities of different CYP isoforms show high inter- and intra-individual variations. Genetic variation contributes most to individual differences in capacity to metabolize drugs and herbs (Gasche et al., 2004). Besides; age, sex, concomitant drug use, herbs and dietary administration can affect drug metabolizing capacity of CYPs (Tanaka et al., 2014). Co-administration of two or more drugs or herbs may give rise to drug interactions due to an alteration, inhibition/ induction, of CYPs activity (Lynch and Price, 2007) if the drugs or herbs are metabolized by the same enzyme system(s). Interaction of herbs, e.g. khat, with drugs is more troublesome, at least theoretically, because the multitude of pharmacologically active compounds in the herb can apparently intensify and complicate potential interaction (Izzo, 2004; Al-Jenoobi et al, 2014; Tanaka et al., 2014). If such interaction occurs to a significant extent, clinical efficacy of concomitantly administered drugs might be compromised. Moreover, adverse effects can increase (Yang, 2009).

The enzymes involved in the metabolism of khat are not yet known but it is anticipated that CYPs play a role (Toennes et al., 2003; Chen et al., 2006; Carmo et al., 2007; Samer et al., 2013). *In vitro* studies have shown that amphetamine is a CYP2D6 substrate and this opens the possibility that some of the active substances in khat or metabolites may act as substrate or inhibitor of CYP2D6 (Bach et al., 1999, 2000; Aklillu et al., 2002; Cascorbi, 2003; Toennes et al., 2003). Moreover, CYP3A exhibits broad substrate specificity (Luo et al., 2009, Yang, 2009) and khat constituents could possibly be substrates, inhibitors or inducers for CYP3A. The contribution of other CYPs cannot be ruled out too.

In khat belt countries (East Africa and Arabian Peninsula) where khat chewing is prevalent (Gebissa, 2008), there is a high possibility of chewing khat while on medications. Such concurrent ingestion of khat and medications may affect the therapeutic outcome of the medications. Besides reports of khat affecting the bioavailability of ampicillin and amoxicillin (Attef et al., 1997; Bathum et al., 1999), there are no data describing the effects of khat on CYPs mediated drug metabolism. In view of this, activities of the five human CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A) that are primarily responsible for the metabolism of xenobiotics were assessed using specific probe drugs administered alone and with khat. The finding of this study will increase knowledge on the impact of khat on CYPs mediated drug metabolism. It will also serve as a spring board for researchers, health care practitioners and policy makers for further study and intervention.

2. Objectives

2.1 General objective

The present study was aimed at phenotypic and genotypic evaluation of regular khat consumption on five major CYPs (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A) in healthy habitual khat chewing Ethiopian population.

2.2 Specific objectives

- To conduct genotyping of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A
- To conduct phenotyping of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A in absence and presence of khat
- To study the correlation of MRs from phenotyping outcomes with MR of cathinone/cathine
- To draw inference of interaction of khat with drugs from genotyping, phenotyping and correlation results of the five CYPs.

3. Material and Methods

3.1 Chemicals

Losartan, losartan carboxylic acid (E-3174), and 5-hydroxyomeprazole sodium salt were purchased from Santa Cruz Biotechnology (CA, USA). Dextromethorphan hydrobromide, dextrorphan tartrate, 3-methoxymorphinan hydrochloride, omeprazole, caffeine, 1, 7-dimethylxanthine (paraxanthine), the internal standard (IS) levallorphan, and b-glucuronidase were obtained from Sigma-Aldrich (MO, USA). LC–MS-grade solvents (methanol, acetonitrile and formic acid), analytical-grade sodium acetate and acetic acid used to prepare the b-glucuronidase treatment buffer, ammonium formate (analytical-grade), and SOLA® cartridges for SPE were purchased from Fisher Scientific (Geel, Belgium). Potassium dihydrogen phosphate and sodium hydroxide, both analytical grades, were used to prepare the solid phase extraction (SPE) buffer and were purchased from Merck (Darmstadt, Germany). De-ionized water was purified through a water purification system from Millipore (MA, USA). Amphetamine, 3, 4-methylenedioxymethamphetamine (MDMA), metamphetamine, cathinone-HCl, phenylpropanolamine-HCl, 0.1% formic acid (generously provided from karolinska Institutet).

3.2 Study Setting

The study was conducted in Addis Ababa, Ethiopia. Addis Ababa is the capital city of Ethiopia with a population of 3,195,000 (*male: 1, 515,000 and Female: 1,680,000* (Gebretekle and Serbessa, 2016)). The city has ten administrative sub-cities and 99 woredas. The sub-cities are Akak-kality, Nefas-Silk-Lafto, Kolfe-Keraniyo, Gulelle, Lideta, Kirkos, Arada, Addis Ketema, Yeka and Bole. All Ethiopian ethnic groups are represented in Addis Ababa, owing to the fact that it is the capital of the country. There are over 38 hospitals, 5 of them owned by Addis Ababa

Health Bureau. There are also about 27 governmental owned health centers, 19 higher and 103 medium private clinics (Asemahagn, 2014). The city has 308 pharmacies, 249 drug stores and 1 rural drug vendor. Moreover, 140 importers, 93 wholesalers and 6 pharmaceutical industries are found in the city (Gebretekle and Serbessa, 2016).

3.3 Research participants

Participants were recruited from Addis Ababa through an open advertisement. All subjects had undergone thorough history taking and physical examination. Volunteers with a previous history of adverse drug effects and those with any drug intake within 2 weeks before the study were excluded from the study. Most of the study participants were students at Addis Ababa University (AAU). A medical doctor from Tikur Anbessa Specialized Hospital (TASH), AAU examined subjects' health status prior to their participation. Subjects were not allowed to take any medication during the study period.

3.4 Study Design

A comparative one-way crossover study was carried out to evaluate the effect of khat on CYP in two phases. In phase one study (pilot study), the two major enzymes CYP2D6 and CYP3A4 enzyme activity was assessed, while in phase II the five CYPs (CYP3A4, CYP2D6, CYP2C9, CYP2C19 and CYP1A2) which are involved in the metabolism of over 99% of currently marketed drugs were assessed. CYP2B6 were excluded from phase II study due to unavailability of subtherapeutic doses of the probe drug, bupropion. Efavirenz could not be replaced due to complex ethical concerns.

In phase I study, after one week of khat abstinence, baseline CYP2D6 and CYP3A4 metabolic activities were determined in 40 Ethiopian male volunteers using 30 mg DM as a probe drug and

then repeated after one week of daily use of 400 g fresh khat leaves. In phase II study, the metabolic activities of the five CYPs were determined in 60 healthy Ethiopian male volunteers using 30mg DM (CYP3A4 and CYP2D6), 50mg Losartan (CYP2C9), 20mg omeprazole (CYP2C19) and 100mg caffeine (CYP1A2) as probe drugs. Subjects were allowed to chew khat until the last minute of blood sample collection for phenotyping. Urinary concentrations of cathinone and cathine were determined to monitor the subjects' compliance to the study protocol. Khat withdrawal symptoms were also assessed using DSM-V criteria (APA, 2013) by substance abuse specialist from St. Paul Specialized Hospital, Ministry of Health, and Ethiopia.

3.5 Assay of CYP activity

In Phase I study, the activities of CYP2D6 and CYP3A4 enzyme were assessed by comparing median MR of DM/DX and DM/3-MM respectively in the presence and absence of khat. In phase II study, the metabolic activities of the five CYPs were determined in 60 healthy Ethiopian male volunteers using 30mg DX (CYP3A4 and CYP2D6), 50mg Losartan (CYP2C9), 20mg omeprazole (CYP2C19) and 100mg caffeine (CYP1A2) as probe drugs. The metabolic activities of the enzymes were assessed similarly by comparing median MR of DM/DX, DM/3-MM, Losartan/ losartan carboxylic acid, omeprazole /5-hydroxyomeprazole and caffeine/ paraxanthine for CYP2D6, CYP3A4, CYP2C9, CYP2C19 and CYP1A2, respectively. The metabolic pathways of probe drugs are indicated in **Fig 5**.

3.6 Sample collection

Three milliliters of blood sample was centrifuged for 10 min at $3500 \times g$ to obtain plasma and aliquots were stored at -80°C until LC–MS/MS analysis. Two milliliters of blood sample was also collected from each subject in EDTA containing vacutainer tube for genotyping. Two

milliliters of urine aliquots were also kept at -80°C until analysis. No significant treatment associated adverse events had been noticed that were attributable to probe drugs.

3.7 Phenotyping

3.7.1 Quantification of plasma probe drugs and metabolites

Frozen plasma was transported in dry ice to Extremadura University Hospital & Medical School, E-06071, Badajoz, Spain for quantification and analysis of plasma probe drugs and metabolites using procedures described elsewhere (de Andreas et al., 2014). The stock solutions of all probes, their metabolites and levallorphan (IS) were prepared at 1 mg/ml in methanol and stored in darkness at -20°C , except for caffeine and paraxanthine that were prepared in milliQ purified water and stored in darkness at -20°C . Working standard solutions were prepared at 10 $\mu\text{g}/\text{ml}$ by appropriate dilution in methanol/water (50:50%, v: v) and stored at -20°C . Standard calibration samples (consisting of at least seven different concentrations covering a sufficient range in each case) were prepared by spiking the blank human plasma with the working solution of each analyte. QC samples were similarly prepared from the stock solutions at three different concentrations within the designated low-, medium- and high-concentration ranges.

The samples were analyzed on an Agilent 1200 Series HPLC system (Agilent, CA, USA) equipped with a G1311A binary pump, a G1329A autosampler, a G1322A degasser and a G1316A column oven. The chromatographic column was a Poroshell SB-C18 column (75mm \times 3mm internal diameter; 2.7 μm) from Agilent that was kept at a constant temperature of 30°C . An API2000 triple quadrupole mass spectrometer from AB Sciex (MA, USA) equipped with an atmospheric pressure electrospray ionization (ESI) interface was used for the mass analysis and detection, and operated with Analyst software (version 1.5.1). Samples were incubated with b-

glucuronidase in a water bath from GFL (Burgwedel, Germany), and centrifuged in a Spectrafuge 24D microcentrifuge from Labnet International (NJ, USA). For the SPE, 100 μ l of human plasma was mixed with 1 μ l of IS (10 μ g/ml). Then, 400 μ l of extraction buffer (potassium dihydrogen phosphate 0.1 M at pH 7.5) was added, and the resulting mixture was vortexed. The sample was then loaded into a SOLA cartridge previously conditioned with 500 μ l of methanol followed by 500 μ l of extraction buffer. The extraction cartridge was washed with 500 μ l of extraction buffer, and all the analytes to be determined and the IS were then eluted with 500 μ l of methanol. The elute was evaporated to dryness at 40°C under a stream of nitrogen, and the dried extract then reconstituted in 100 μ l of mobile phase. Aliquots of 10 μ l of the extract were injected into the chromatographic system.

To determine total concentrations of dextropran, 3-methoxymorphinan and losartan carboxylic acid, a prior deglucuronidation process is required to determine the actual plasma levels of these analytes. To this end, an aliquot of 100 μ l of plasma was mixed with 100 μ l of β -glucuronidase solution containing 1300 U/ml in 0.1 M acetate buffer, pH 5.0. The solution was kept at 37°C for 18 h and then 200 μ l of cold methanol and 200 μ l of acetonitrile were added to precipitate plasma proteins. The sample was centrifuged for 5 min at 16300 \times g in an Eppendorf centrifuge; the supernatant was transferred to a clean tube and evaporated at 40°C under a stream of nitrogen. The dried extract was reconstituted in 500 μ l of extraction buffer pH 7.5, and the analytes were then extracted by SPE. The compounds were separated using 0.1% formic acid in water and 0.1% formic acid in acetonitrile as mobile phase. The flow rate was 0.4 ml/min. Elution was in a linear gradient, with acetonitrile content changing from 17 to 50% between 0.1 and 4.5 min. Acetonitrile content was maintained at 50% from 4.5 to 8 min, and then decreased

to 17% over 1 min, followed by column equilibration for 4 min. The total run time was, thus, 13 min per sample.

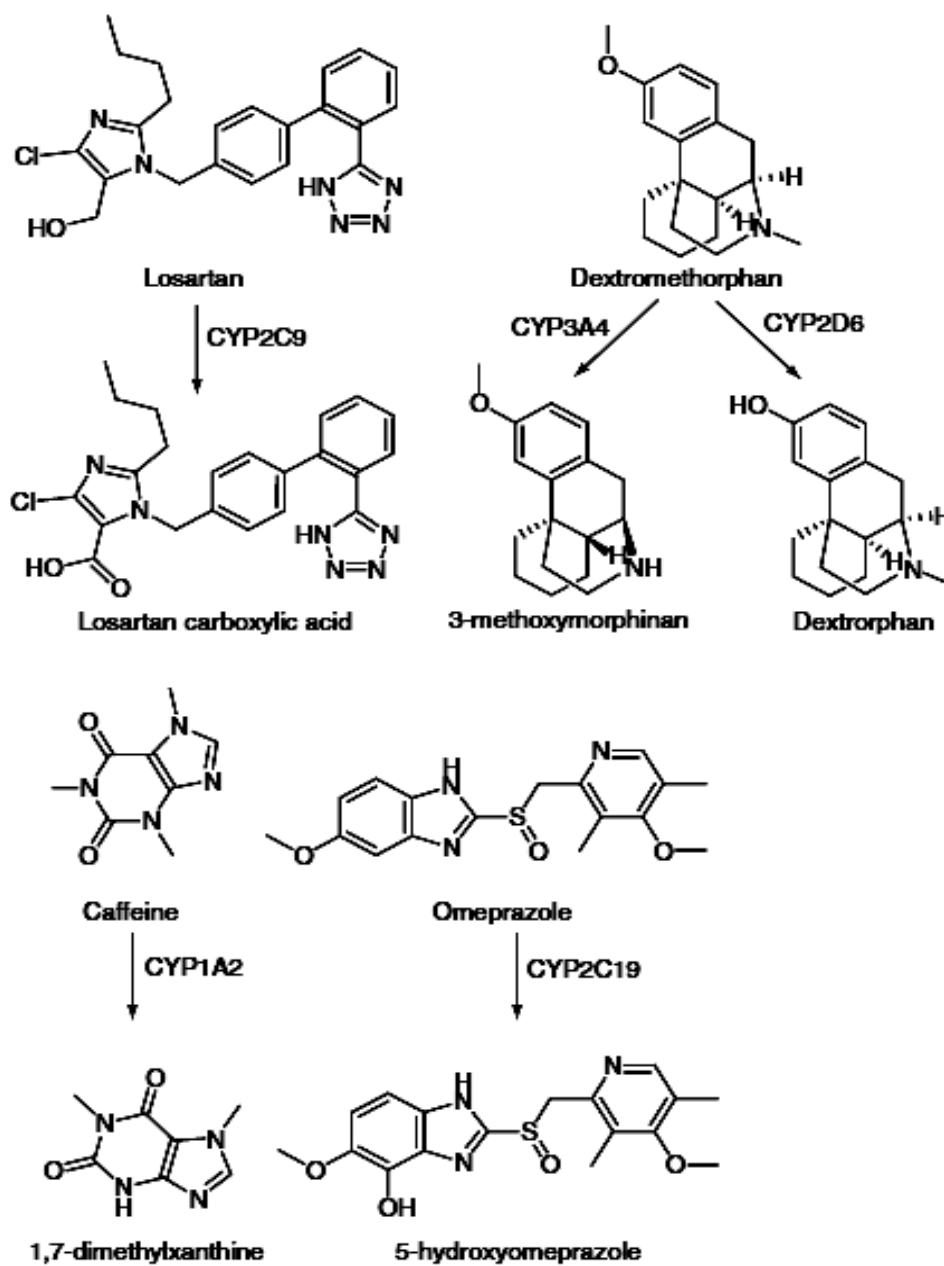


Fig 5. Probe drugs used and *in vivo* metabolic conversion products and pathways (de Andreas et al., 2014).

The injection volume was 10 μ l. Data acquisition was performed in single reaction monitoring (SRM) mode with positive ESI for all analytes except E-3174 whose data were acquired with negative ESI from 8.5 to 13 min. The ESI inlet conditions were as follows: curtain gas, nitrogen (20 psi); collision gas, nitrogen (6 psi); ion spray voltage, 5500 V in positive mode and 4500 V in negative mode; ion source temperature, 500°C; ion source gas 1, nitrogen (50 psi); and ion source gas 2, nitrogen (70 psi). SRM was optimized for each analyte/ standard by direct infusion using the ESI source. Dwell times of 50 ms were used for each SRM reaction and mass peak widths were 0.7 Da at 50% of peak height for both the first and third quadrupoles, which is selective enough to avoid potential interferences from other compounds.

The five CYP enzymes activities were calculated as follows: CYP1A2 activity as the concentration of caffeine divided by the concentration of paraxanthine in the 4h plasma sample; CYP2C19 activity as the concentration of omeprazole divided by the concentration of 5-hydroxyomeprazole in the plasma samples at 4h; CYP2C9 activity as the losartan to losartan carboxylic acid plasma ratio at 4h; CYP2D6 activity as the dextromethorphan to dextrorphan plasma ratio at 3h; and CYP3A4 activity as the 3h metabolic plasma ratio given by the ratio of the concentration of dextromethorphan to the concentration of 3-methoxymorphinan (de Andreas et al., 2014).

3.7.2 Quantification of urinary cathinone and cathine concentration

Frozen urine samples were sent in dry ice to Karolinska Institutet, Laboratory, and Stockholm, Sweden, where routine testing for substance of abuse is performed. Urinary cathinone and cathine concentration was determined as described previously (Al-Saffar et al., 2013) using LC–MS/MS system consisted of a Waters Acquity UPLC (ultra-performance liquid chromatography)

with a vacuum degasser, binary pumps, autosampler (12 °C) and sample manager connected to a Xevo TQ tandem mass spectrometer with MassLynx™/Target Lynx™ Software version 4.1 (Waters Co., Milford, MA, USA). In brief, 50-µL aliquot of urine was added to an autosampler vial with 200 µL of IS working solution (40 ng). Thereafter, the vials were capped, vortexed for ~10 sec, and were loaded onto the sample manager kept at +4 °C. Amphetamine, 3-4 methylenedioxymethamphetamine (MDMA) and metamphetamine (LGC Standards) in 100 µg/mL concentration were diluted to 500 ng/mL in ddH₂O and used as internal standards. The reference standards, cathinone-HCL and Phenylpropanolamine-HCL in 1mg/mL ampoule (LGC Standards) were diluted in 0.1% formic acid to give 100mg/mL. Quality control (QC, 100 ng/mL) and standards for calibration curves covering a 0–10,000 ng/mL concentration range were prepared by dilution of blank urine with working solutions of the analytes with blank urine (Al-Saffar et al., 2013).

3.8 Genotyping

Blood samples were transported in dry ice to Division of Clinical Pharmacology, Department of Laboratory Medicine, Karolinska Institutet, and Stockholm, Sweden for genotyping. Genomic DNA was isolated from peripheral blood leukocytes using QIAamp DNA Maxi Kit (QIAGEN GmbH, Hilden, Germany) and analyzed at Division of Clinical Chemistry, Karolinska Institutet. Common variant alleles of CYP2D6 (*4), CYP3A5 (*3,*6), CYP2C19 (*2,*3), CYP2C9 (*2,*3) and CYP1A2 (*1F) were determined using TaqMan® SNP genotyping assay (Applied Biosystems, CA, USA). Genotyping variants assessed were -163C>A (CYP1A2*1F), 430T>C (CYP2C9*2), 1075A>C (CYP2C9*3), 681G>A (CYP2C19*2), 636G>A (CYP2C19*3), 1846G>A (CYP2D6*4), 6986A>G (CYP3A5*3) and 14690G>A (CYP3A5*6) on ABI 7500 FAST (Applied Biosystems, Foster City, CA). The final volume for each reaction was 10µl,

consisting of 2x TaqMan® Fast Universal Master Mix(Applied Biosystems), 20 X drug metabolizing genotype assay mix and 10ng genomic DNA. The polymerase chain reaction (PCR) profile consisted of an initial step at 60°C for 30 sec, hold stage at 95°C for 10 min and PCR stage for 40 cycles step 1 with 95°C for 15 and step 2 with 60°C for 1 min and post read stage with 60°C for 30 sec.

3.9 Statistical Analysis

Median (interquartile range) and proportions in percentage were used to describe baseline characteristics of the study participants. Median (interquartile range) was used to describe plasma MR of caffeine/ paraxanthine (CYP1A2 MR), losartan/ losartan carboxylic acid (CYP2C9 MR) omeprazole/ 5-hydroxyomeprazole (CYP2C19 MR), dextromethorphan/dextrophan (CYP2D6 MR) and dextromethorphan/3-methoxy morphinan(CYP3A MR). The change in median caffeine/ paraxanthine, losartan/ losartan carboxylic acid, omeprazole/ 5-hydroxyomeprazole, DM/DX and DM/3-MM ratios before and after khat was analyzed using Wilcoxon matched-pairs signed rank test. Spearman coefficient was used to assess correlations. Percent change in these MRs in the presence of khat from baseline was calculated using the following formula:

$$\% \text{ change in MR} = \left[\frac{\text{MR in the presence of khat} - \text{MR in the absence of khat}}{\text{MR in the absence of khat}} \right] \times 100$$

Log transformed values were used for graphical presentations. SPSS Statistics (IBM Corporation, Somers, NY) software, version 22.0 was used for statistical analysis. P values <0.05 were considered to be statistically significant.

3.10 Ethical Considerations

Ethical approval was obtained from the institutional review board (IRB) of College of Health Sciences, Addis Ababa University and from national research ethics committee (NREC), Federal Ministry of Science and Technology, Ethiopia. Participants were provided with information sheet that explains the purpose, nature and the risk of the study. Participants were informed that their participation would be voluntarily and they could withdraw from the study at any time. The research participants finally signed consent form before taking part in the study. The confidentiality of the information was assured through anonymity of the data and assignment of codes. The study was performed in accordance with the principles outlined in the Declaration of Helsinki for human experimental research.

4. Results

4.1 Sociodemographic characteristics

In Phase I study, 40 healthy Ethiopian male habitual khat-chewing volunteers (median age, 23 years; median weight, 58kg; median height, 172cm) residing in Addis Ababa participated in the study. In phase II, 70 healthy Ethiopian male participants have started the study. Blood sample was collected from all for genotyping study. However, only 60 have completed the phenotyping study (median age, 25 years; median weight, 59.5 kg; median height, 170 cm) participated in the study (**Table 10**). The study participants were mainly from Amhara and Oromo ethnic background and most of them were orthodox by religion. Females were not included in the study; first and foremost it was difficult to find a female volunteer participating publicly in the study setting. Secondly, pregnancy test must be done before approving potential female candidate as study participant. This is because, khat associated fetal damage is not well documented and even the safety of probe drugs used in the study cannot warrant zero harm to the fetus (e.g. Losartan).

Table 10: Sociodemographic characteristics of volunteers for both phase I and Phase II studies

| Sociodemographic Characteristics | | Frequency | |
|---|-------------------|------------------|-----------------|
| | | Phase I | Phase II |
| Sex | Male | 40 | 60 |
| | Female | - | - |
| Age | 18-20 | 2 | 3 |
| | 21-29 | 30 | 48 |
| | 31-39 | 7 | 7 |
| | 40-50 | 1 | 2 |
| Weight | 40-49 | 1 | 2 |
| | 50-59 | 20 | 27 |
| | 60-69 | 11 | 21 |
| | 70-80 | 8 | 7 |
| Religion | Orthodox | 27 | 44 |
| | Protestant | 2 | 11 |
| | Muslim | 5 | 1 |
| | Catholic | 4 | 1 |
| | Others | 2 | 3 |
| Ethnicity | Amhara | 17 | 30 |
| | Oromo | 10 | 16 |
| | Tigre | 5 | 4 |
| | Others | 8 | 10 |

In phase I, 95% of participants were alcohol users, 55% were cigarette smokers and 8 of the participants were reported to sniff shisha. Whereas, in phase II, more than 70% were alcohol users, more than 75% cigarette smokers and 3 participants were reported to sniff shisha. The most favorite beverages accompanying khat were tea and coffee, followed by soft drinks and water. Only two of the participants had experience of using peanut with khat (**Table 11**).

Table 11: Drug use and Medical History of Volunteers for both phase I and phase II studies.

| History | | Frequency | |
|-----------------------------------|-------------|------------------|-----------------|
| | | Phase I | Phase II |
| Khat use experience | <5 yrs | 5 | 29 |
| | 5-10yrs | 17 | 22 |
| | >10yrs | 8 | 9 |
| Beverages/ Flavor used | Water | 10 | 58 |
| | Soft Drinks | 14 | 27 |
| | Tea/Coffee | 27 | 53 |
| | Peanut | 2 | - |
| Alcohol Use | | 38 | 42 |
| Cigarette Smoking | | 22 | 47 |
| Glue-Sniffing | | 8 | 3 |

4.3 Monitoring of khat abstinence

4.3.1 Assessment of withdrawal symptoms

Khat withdrawal symptoms were observed in all participants after one week of khat abstinence according to the DSM-V criteria for stimulant withdrawal effect. As a result, nearly all participants experienced dysphoria, fatigue, and increased appetite. Agitation was also observed in over two-third of the subjects. Moreover, majority of the participants reported hypersomnia, vivid and unpleasant dreams. Fatigue and increased appetite was the most common complaints of study participants during random visits. In particular, the subjects were sluggish while blood sample were collected during khat abstinence periods. When khat chewing was allowed at the

end of sample collection during the same day, most of the study participants had thrilling expression, but a sense of hurry was also observed.

4.3.2 Determination of urinary cathinone levels

Study participants were instructed to abstain from khat for one week for baseline assessment of CYP2D6 phenotype on day 8. Based on the reported short half-life of cathinone (1.5 ± 0.8 h) and cathine (5.2 ± 3.4 h) (Toennes et al, 2003), a one week wash out period was considered to be sufficient to eliminate cathinone and cathine, resulting from khat ingestion prior to enrolment in the study. Furthermore, cathinone and cathine are detectable in urine only up to 22–26 h and 50–70 h, respectively after ingestion (Toennes and Kauert, 2002). Accordingly, no cathinone or cathine was detected in urine samples collected from 35 subjects in phase I and 52 subjects in phase II study. However, traces of urinary cathinone and cathine were detected in others, perhaps indicating some levels of non-compliance to the study protocol. Thus, only data for compliant subjects were analyzed and interpreted for phenotyping study. The aggregated phenotype data are shown in **Table 13**.

4.4 Effect of khat on CYP2D6 enzyme activity

4.4.1 Phenotyping

Phase I study

Considering only those subjects who were fully compliant to khat abstinence, the median CYP2D6 MR was strongly increased by khat use compared to the baseline (Wilcoxon matched-pair signed-rank test, $P=0.02$). A paired t-test performed on log DM/DX ratio demonstrated that concurrent khat use significantly inhibited CYP2D6 activity ($p=0.003$, mean difference 0.014, 95% CI: 0.042-0.186; **Figure 6a**). The median percent change in CYP2D6 MR by concomitant

khat consumption was 45%. CYP2D6 enzyme activity was determined using DM/DX MR_{0-3h} as an index. One subject (2.5%) was assigned as CYP2D6 PM, using the log DM/DX ratio of 0.8 as a cut-off for PM phenotype (Trojan et al, 2012). The DM/DX ratio of this subject was 12.3 in the absence of khat that increased to 19.2 in the presence of khat.

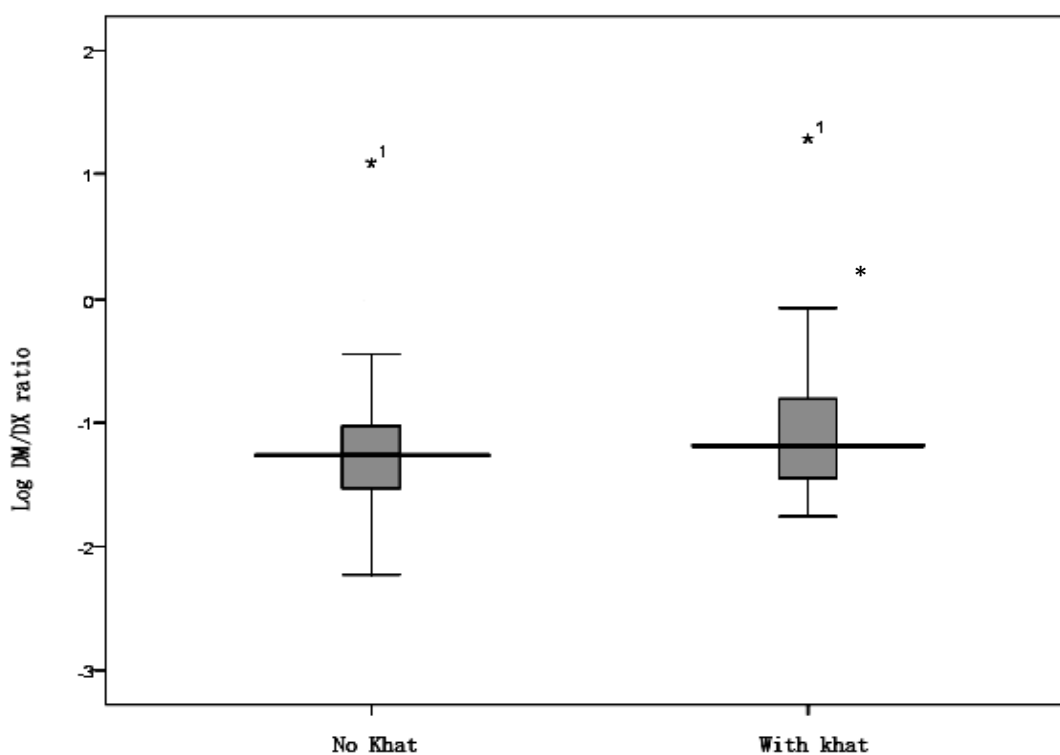


Fig 6a: Box-and-Whisker plots for metabolic activity of CYP2D6 in the absence and presence of khat for phase I study (N=35) values are described as log MR of DM/DX. Each box displays the median (black bar), and the 25th and the 75th quartiles (the lower and the upper edges of the box, respectively). The box length is the IQR. Khat significantly affected (P=0.02) the metabolic activity of CYP2D6 enzyme. Extreme values are indicated by asterisk.

Phase II Study

The median CYP2D6 MR was very strongly increased by khat use compared to the baseline (Wilcoxon matched-pair signed-rank test, p=0.001). A paired t-test performed on log DM/DX ratio demonstrated that concurrent khat use significantly inhibited CYP2D6 activity (p=0.001,

mean difference 0.1185, 95% CI: -0.182- -0.055; **Figure 6b**). The median MR percent change in CYP2D6 by concomitant khat consumption was 56.9%. In phase II study, two subjects (3.2%), having heterozygous (*1/*4) and homozygous (*4/*4) genotypes were assigned as PM, using the log DM/DX ratio of 0.8 as a cut-off for poor-metabolizer phenotype (Trojan et.al, 2012). The one with heterozygous alleles had DM/DX ratio of 0.44 in the absence of khat that increased to 0.96 in the presence of khat. The second subject had a DM/DX ratio of 3.44 in the absence of khat and 3.75 in the presence of khat.

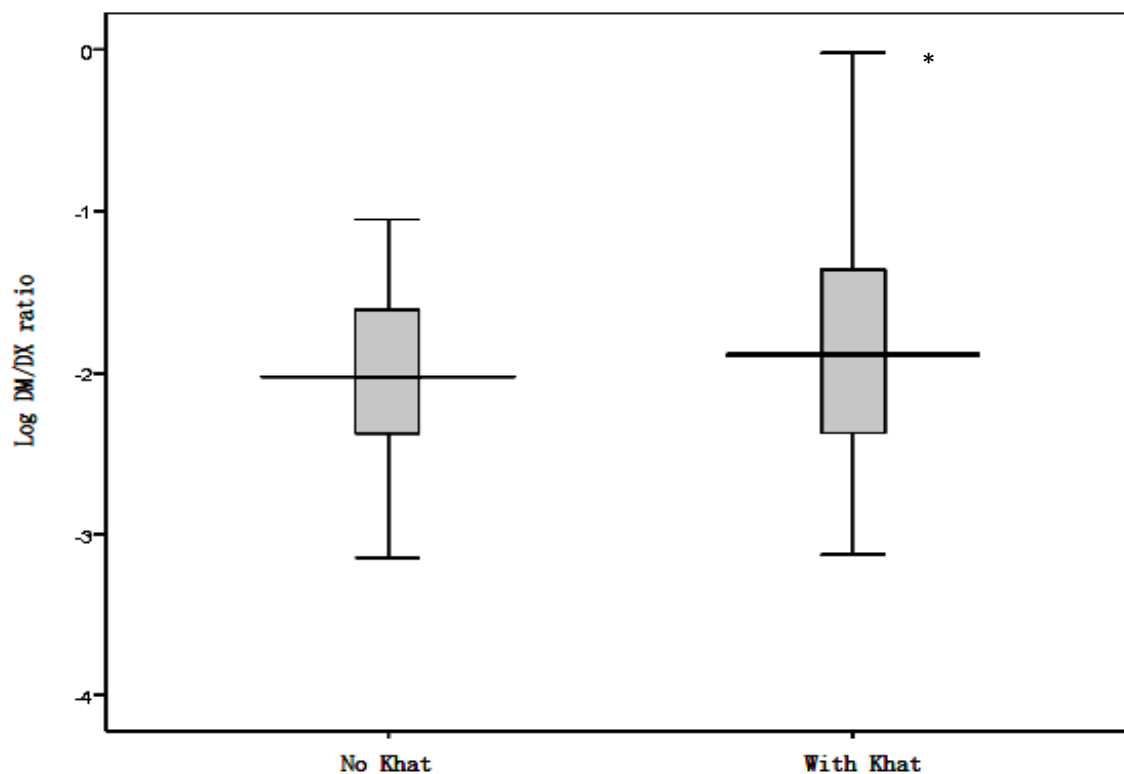


Fig 6b: Box-and-Whisker plots for metabolic activity of CYP2D6 in the absence and presence of khat for phase II study (N=52): values are described as log MR of DM/DX. Each box displays the median (black bar), and the 25th and the 75th quartiles (the lower and the upper edges of the box, respectively). The box length is the IQR. Khat significantly affected (P=0.001) the metabolic activity of CYP2D6 enzyme.

Phase I & II studies

The phenotyping data from both phase I and phase II were merged and analyzed accordingly. Khat had a very significant inhibitory effect on the metabolic activity of CYP2D6 enzyme ($p=0.001$) (**Fig 6c**).

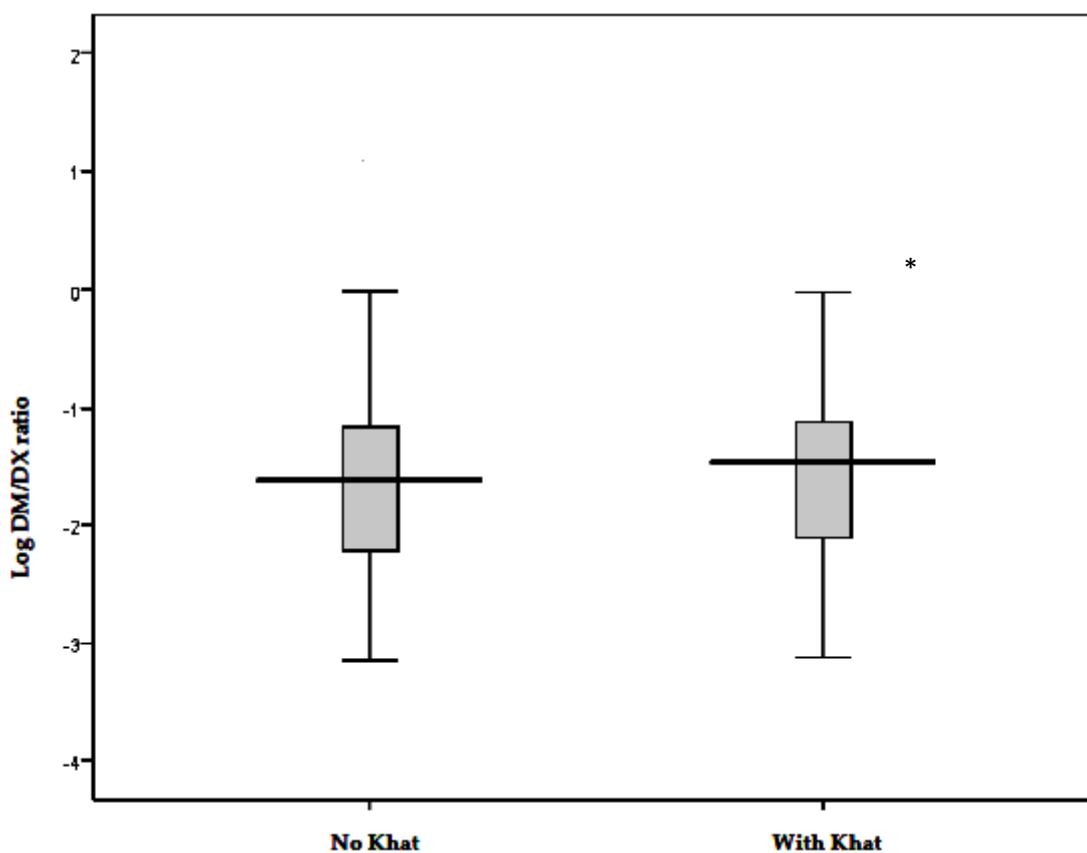


Fig 6c: Box-and-Whisker plots for metabolic activity of CYP2D6 in the absence and presence of khat for merged phase I & II data (N=87): values are described as log MR of DM/DX. Each box displays the median (black bar), and the 25th and the 75th quartiles (the lower and the upper edges of the box, respectively). The box length is the IQR. Khat significantly affected ($P=0.001$) the metabolic activity of CYP2D6 enzyme.

4.4.2 Genotyping

Phase I study

Ten subjects were heterozygous for *CYP2D6*4*. There were no significant differences between observed and expected genotype frequencies according to the Hardy–Weinberg equilibrium ($p=0.36$). *CYP2D6*3* was not detected. None of the study participants were homozygous for *CYP2D6*4*. In *CYP2D6*1/*1* genotypes, the mean log DM/DX MR was significantly increased in the presence of concomitant khat use (paired t-test $p= 0.01$, paired mean differences in log DM/DX ratio = -0.12 with 95% Confidence Interval (CI) of - 0.202 to -0.028). A similar but non-significant increase was observed in *CYP2D6*1/*4* genotypes (paired t-test $p= 0.15$, paired mean differences in log DM/DX MR = -0.11, with 95% CI of - 0.269 to 0.049).

Phase II study

Nine subjects were heterozygous for *CYP2D6*1/*4* (14.3%) and one subject (1.6%) was homozygous for *CYP2D6*4/*4* (**Table 12**). There were no significant differences between observed and expected genotype frequencies according to the Hardy–Weinberg equilibrium ($p=0.41$). Univariate ANOVA indicated significant influence of *CYP2D6* genotype on log DM/DX MR before ($p < 0.0001$) and during Khat ($p < 0.0001$). Repeated measure ANOVA indicated significant effect of *CYP2D6* genotype on log DM/DX MR in the absence or presence of Khat ($p < 0.0001$).

In *CYP2D6*1/*1* genotypes, the mean log DM/DX MR was significantly increased in the presence of concomitant khat use (paired t-test $p= 0.001$, paired mean differences in log DM/DX = 0.122 with 95% Confidence Interval (CI) of 0.054 to 0.190). A similar but non-significant

increase was observed in *CYP2D6**1/*4 genotypes (paired t-test $p=0.52$, paired mean differences in log DM/DX MR = -0.077, with 95% CI of -0.385 to 0.230).

Table 12: Genotype frequency distribution among phase II study populations

| Gene | Genotype | Genotype frequency n (%) |
|------------------------------------|-----------------|---------------------------------|
| <i>CYP1A2</i> | *1/*1 | 20 (20.6 %) |
| | *1/*F | 27 (42.9 %) |
| | *1F/*1F | 23 (36.5 %) |
| <i>CYP2C9</i> | *1/*1 | 49 (77.8%) |
| | *1/*2 | 9 (14.3%) |
| | *1/*3 | 4 (6.3%) |
| | *2/*2 | 1 (1.6%) |
| <i>CYP2C19</i> | *1/*1 | 50 (79.4%) |
| | *1/*2 | 12 (19.0%) |
| | *1/*3 | 1 (1.6%) |
| <i>CYP2D6</i> | *1/*1 | 53 (84.1%) |
| | *1/*4 | 9 (14.3%) |
| | *4/*4 | 1 (1.6%) |
| <i>CYP3A5 c.6986A>G</i> (*3) | *1/*1 | 15 (23.8%) |
| | *1/*3 | 31 (49.2%) |
| | *3/*3 | 17 (27.0%) |
| <i>CYP3A5 c.14690G>A</i> (*6) | *1/*1 | 43 (68.3%) |
| | *1/*6 | 18 (28.6%) |
| | *6/*6 | 2 (3.2%) |
| Number of <i>CYP3A5</i> *1 alleles | Zero | 19 (30.2%) |
| | One | 37 (58.7%) |
| | Two | 7 (11.1%) |

Phase I & II studies

Similar to the phenotyping data, genotyping data were also merged and evaluated. The impact of khat was more evident with *1/*1 diplotypes ($p=0.001$) compared to *1/*4 diplotypes ($p=0.46$) as presented in **Fig 7**.

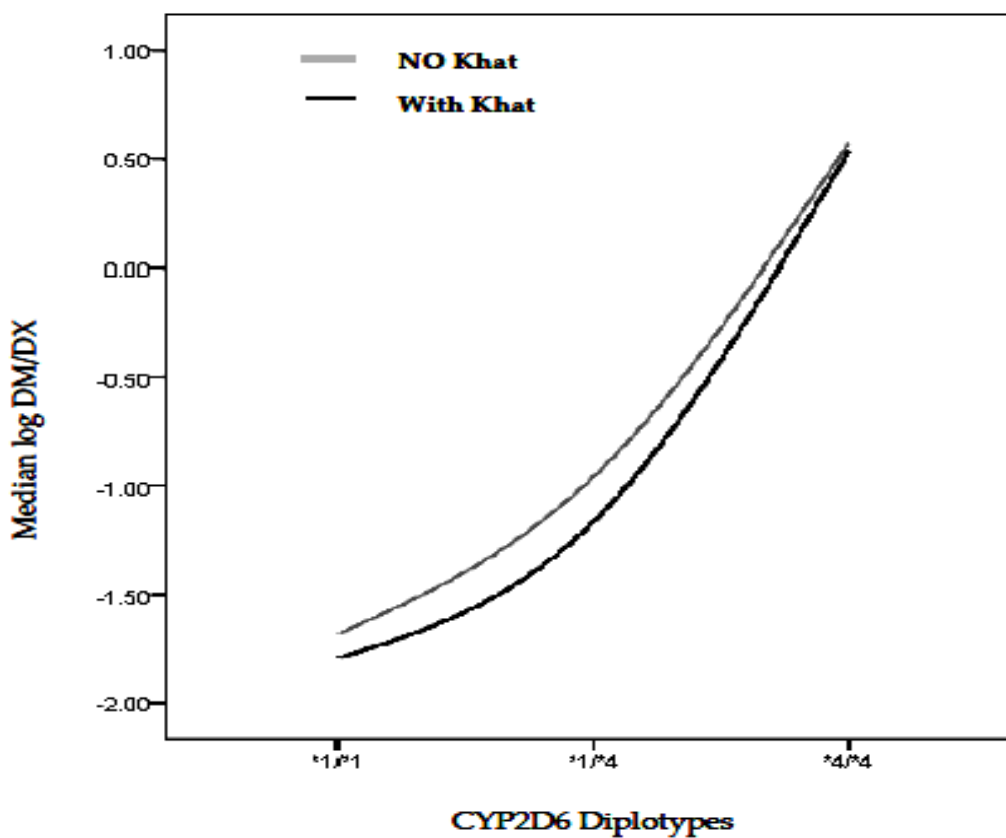


Fig 7: Line chart showing the metabolic activity of CYP2D6 for all data, described as log median MR of DM/DX, in absence and presence of khat against common CYP2D6 allelic variants (N=110). The impact of khat was significant with *1/*1 diplotypes ($p=0.001$), but not in *1/*4 diplotypes ($p=0.46$).

4.5 Effect of Khat on CYP3A enzyme activity

4.5.1 Phenotyping

Phase I study

The goal was to compare the metabolic activity of CYP3A4 from MRs of alprazolam/4-hydroxyalprazolam and DM/3-MM. However, due to an unanticipated slip that resulted in the omission of the metabolite of alprazolam, 5-hydroxyalprazolam, it was not possible to measure the metabolite. As a result, CYP3A enzyme activity was determined using DM/3-MM ratio (de Andreas et al, 2014) in the presence and absence of khat. Compared to baseline value, Wilcoxon matched-pair signed-rank test indicated that median DM/3-MM ratio ($p=0.09$) was non-significantly increased in the presence of khat. Similarly, paired t-test on log DM/3-MM ratio (paired mean differences = 0.054, 95% CI -0.039 to 0.147, $p=0.24$) demonstrated a non-significant inhibition of CYP3A4 by khat (**Figure 8a**).

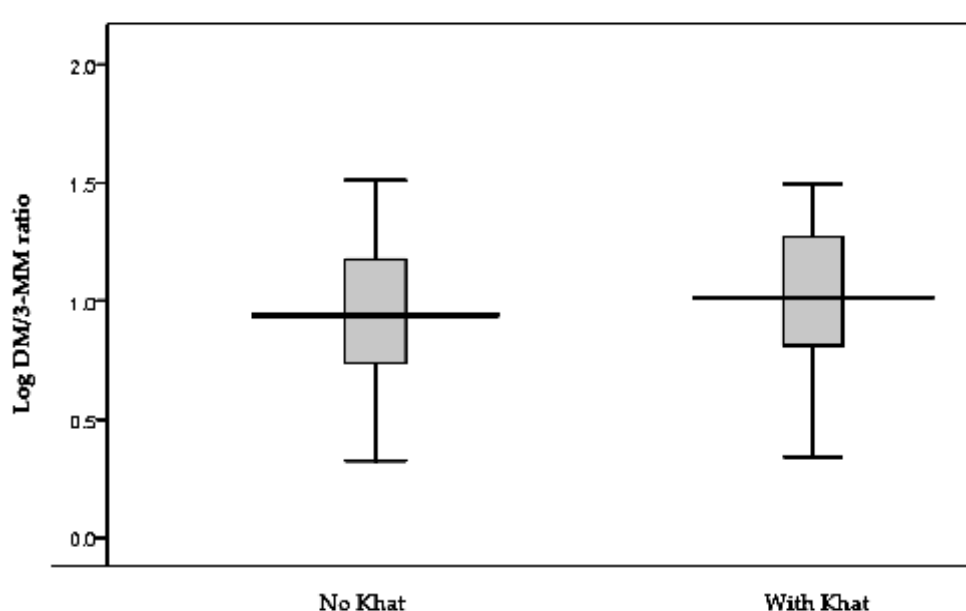


Fig 8a: Box-and-Whisker plots for metabolic activity of CYP3A4 in the absence and presence of khat for phase I study (N=35): values are described as log MR of DM/3-MM. Each box displays the median (black bar), and the 25th and the 75th quartiles (the lower and the upper edges of the box, respectively). The box length is the IQR. Khat had no significant effects ($P=0.09$) on the metabolic activity of CYP3A4 enzyme.

The median percent change during concomitant khat consumption for DM/3-MM ratio was 14.2. One subject was an outlier in CYP3A mediated metabolism of DM. When this outlier is excluded from the analysis of plasma DM/3-MM, there was a significant inhibition ($p=0.02$) of khat on CYP3A as indicated in **Fig 8b**.

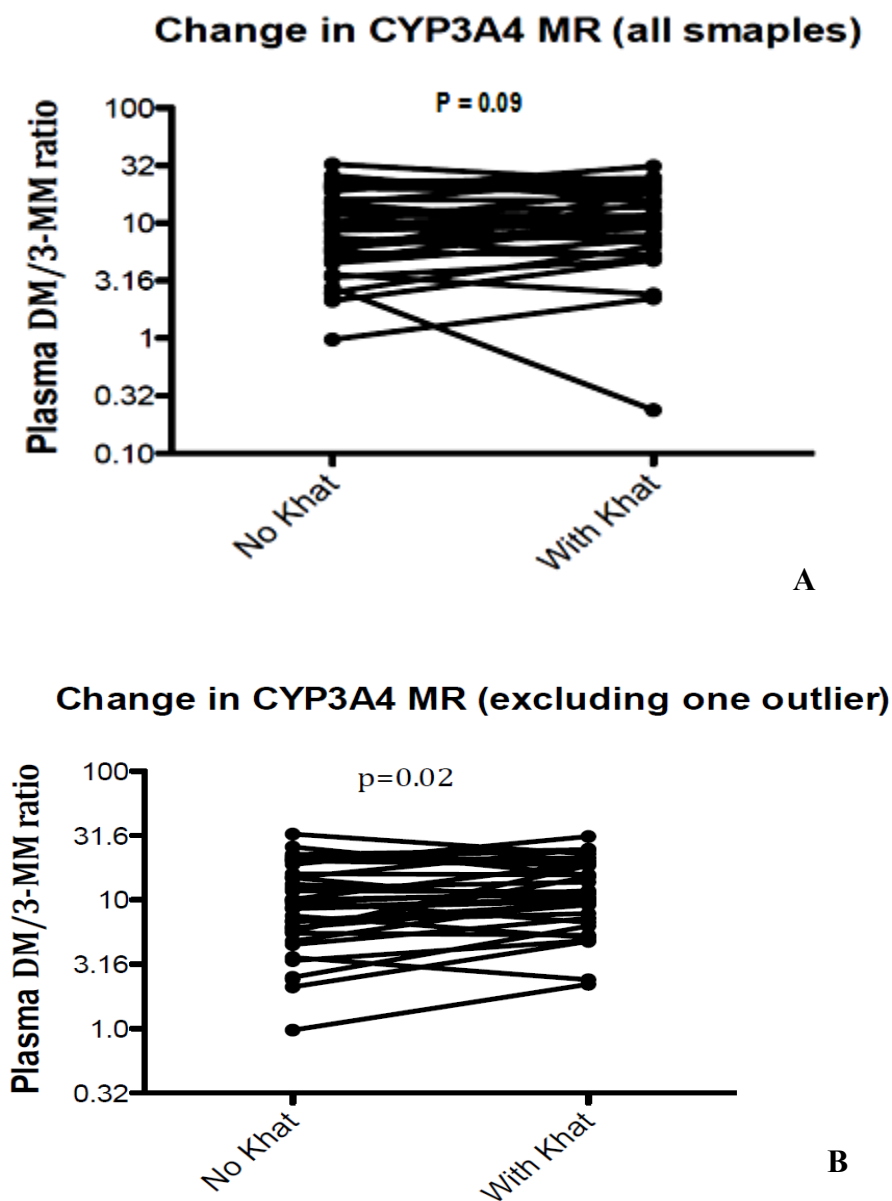


Fig 8b. Comparison of plasma DM/3-MM analysis of the whole sample population (A) and after excluding one outlier (B).

Phase II Study

The effects of khat on CYP3A enzyme activity was significant in phase II study. Compared to baseline value, Wilcoxon matched-pair signed-rank test indicated that median DM/3-MM ratio ($p=0.045$) was significantly increased in the presence of khat. However, Paired t-test on log DM/3-MM ratio (paired mean differences = -0.151 , 95% CI -0.355 to 0.053 , $p=0.14$) demonstrated only a marginal effect (**Figure 8c**). The percent change in median MR DM/3-MM was 27.4%.

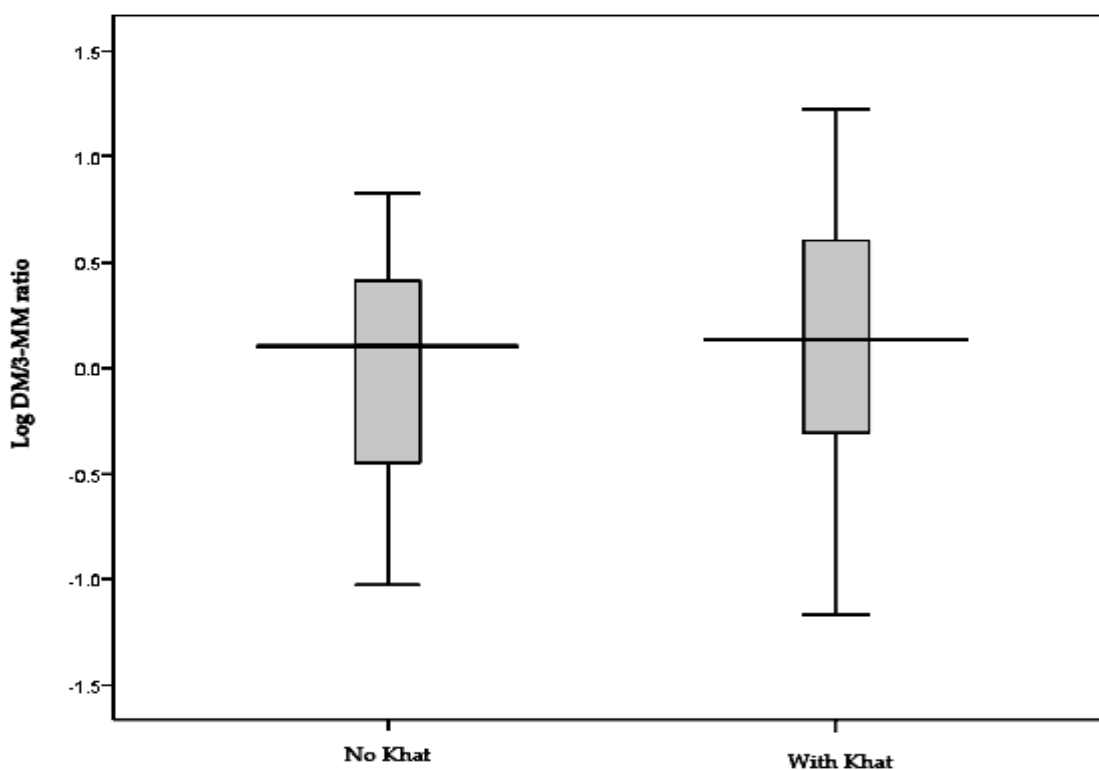


Fig 8c: Box-and-Whisker plots for metabolic activity of CYP3A4 in the absence and presence of khat for phase II study (N=52): values are described as log MR of DM/3-MM. Each box displays the median (black bar), and the 25th and the 75th quartiles (the lower and the upper edges of the box, respectively). The box length is the IQR. Khat had significant effects ($P=0.045$) on the metabolic activity of CYP3A4 enzyme.

Phase I & II studies

The phenotyping data for phase I & II were merged and analyzed as in indicated in **Fig 8d**. There was a very significant inhibitory effect ($p=0.001$) of khat on the metabolic activity of CYP3A4.

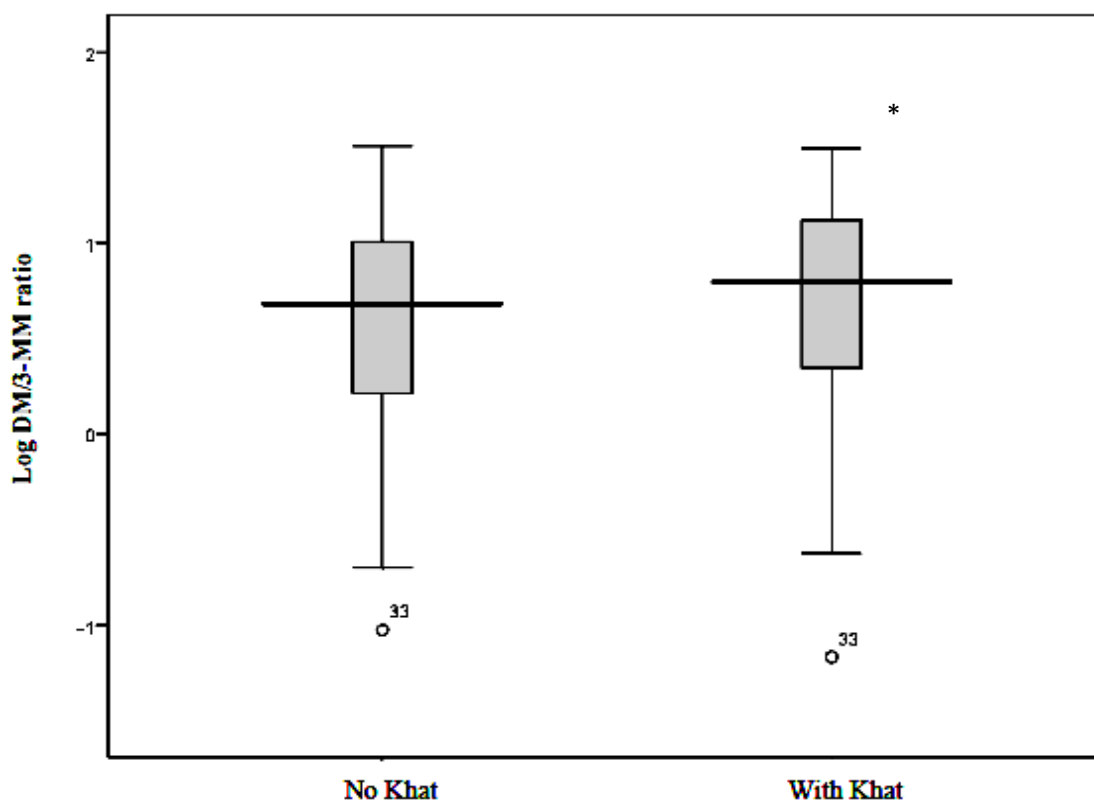


Fig 8d: Box-and-Whisker plots for metabolic activity of CYP3A4 in the absence and presence of khat for phase I & II studies (N=87): merged values are described as log MR of DM/3-MM. Each box displays the median (black bar), and the 25th and the 75th quartiles (the lower and the upper edges of the box, respectively). The box length is the IQR. The circle shows an outlier. Khat had a very significant effects ($P=0.001$) on the metabolic activity of CYP3A4 enzyme.

4.5.2 Genotyping

Genotyping were conducted for CYP3A5 allelic variants *3 and *6. There were no significant differences between observed and expected genotype frequencies according to the Hardy–Weinberg equilibrium (CYP3A5*3, $p= 0.91$) and (CYP3A5*6, $p=0.94$). The outcome of genotyping studies revealed that there was low homozygous wild type genotype (*1/*1), i.e.

11.1%. Homozygous defective alleles (*3/*3, *6/*6) predict PM/non-expressor status and had 30.2 frequency. Whereas, heterozygous variants (*1/*3, *1/*6) which are responsible for IM (low expressor) had higher frequency, 58.7% (Table 12).

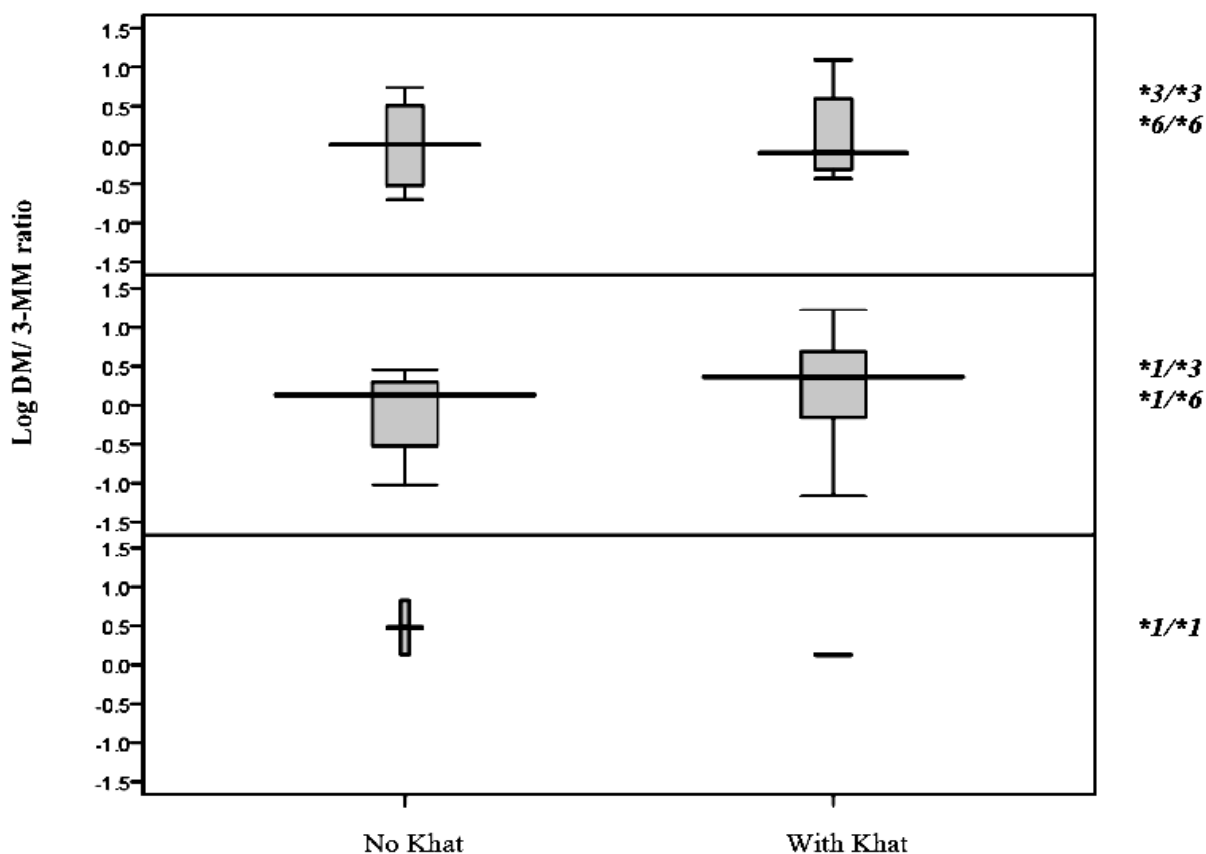


Fig 9: Box-and-Whisker plots for log MR of DM/3-MM in absence and presence of khat against common CYP3A5 allelic variants (N=63). Each box displays the median (black bar), and the 25th and the 75th quartiles (the lower and the upper edges of the box, respectively). The box length is the IQR. The effects of khat was significant ($p=0.009$) for *1/*3 and *1/*6 diplotypes that determine IM status.

4.6. Effects of khat on CYP1A2, CYP2C9 and CYP2C19

The study of the effects of khat on the metabolic activities of CYP1A2, CYP2C9 and CYP2C19 were not included in phase I study. Thus, only the results of phase II study are presented here.

4.6.1 Phenotyping

CYP1A2 activity was determined using caffeine/ paraxanthine ratio (de Andreas et.al, 2014) in the presence and absence of khat. Compared to the baseline value, Wilcoxon matched-pair signed-rank test indicated that log median caffeine/ paraxanthine ratio ($p=0.71$) was non-significantly increased in the presence of khat. Similarly, Paired t-test on log caffeine/ paraxanthine ratio (paired mean differences = 0.004, 95% CI -0.063 to 0.055, $p=0.90$) demonstrated a non- significant inhibition of CYP1A2 by khat (**Figure 10**).

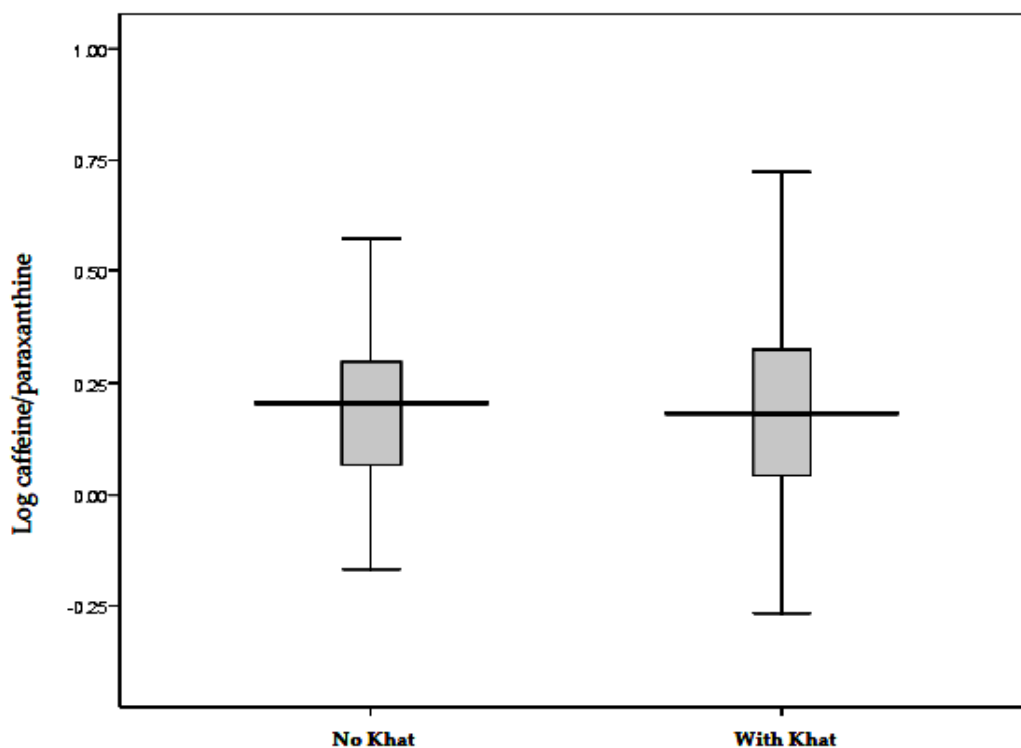


Fig 10 Box-and-Whisker plots for metabolic activity of CYP1A2 in the absence and presence of khat (N=52): values are described as log MR of caffeine/paraxanthine. Each box displays the median (black bar), and the 25th and the 75th quartiles (the lower and the upper edges of the box, respectively). The box length is the IQR. Khat had no significant effects ($P=0.71$) on the metabolic activity of CYP1A2 enzyme.

The percent change in median MR during concomitant khat consumption for caffeine/ paraxanthine was 5.6%. CYP2C9 activity was determined using Losartan/ losartan carboxylic

acid ratio (de Andreas et al., 2014) in the presence and absence of khat. Compared to the baseline value, Wilcoxon matched-pair signed-rank test indicated that log median Losartan/ losartan carboxylic acid ratio ($p=0.64$) was non-significantly increased in the presence of khat. Similarly, Paired t-test on log Losartan/ losartan carboxylic acid ratio (paired mean differences = -0.003 , 95% CI -0.113 to 0.11 , $p=0.96$) demonstrated a non- significant induction of CYP2C9 by khat (**Figure 11**). The percent change in median MR during concomitant khat consumption for Losartan/ losartan carboxylic acid was 20%.

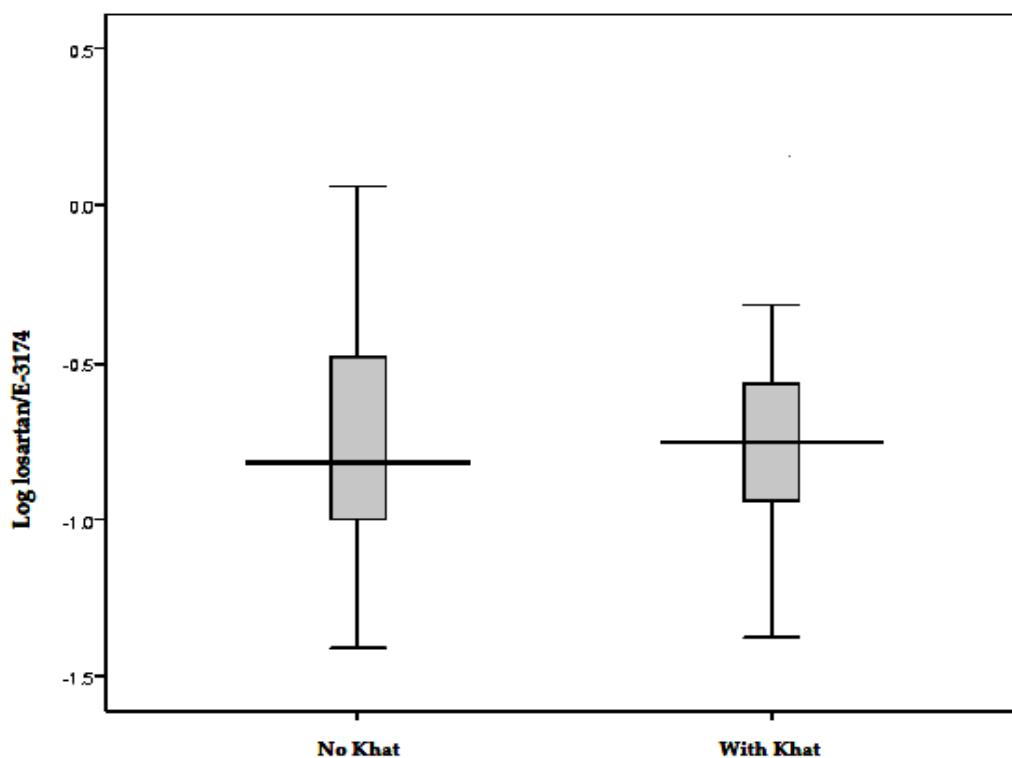


Fig 11 Box-and-Whisker plots for metabolic activity of CYP2C9 in the absence and presence of khat (N=52): values are described as log MR of losartan/E-3174. Each box displays the median (black bar), and the 25th and the 75th quartiles (the lower and the upper edges of the box, respectively). The box length is the IQR. Khat had no significant effects ($P=0.64$) on the metabolic activity of CYP2C9 enzyme.

CYP2C19 activity was determined using omeprazole/ 5-hydroxyomeprazole ratio (de Andreas et.al, 2014) in the presence and absence of khat. Compared to the baseline value, Wilcoxon

matched-pair signed-rank test indicated that log median omeprazole/ 5-hydroxyomeprazole ratio ($p=0.15$) was non-significantly increased in the presence of khat. Similarly, Paired t-test on log omeprazole/ 5-hydroxyomeprazole ratio (paired mean differences = -0.030 , 95% CI -0.118 to 0.293 , $p=0.50$) demonstrated a non- significant inhibition of CYP2C19 by khat (**Figure 12**). The percent change in median MR during concomitant khat consumption for omeprazole/ 5-hydroxyomeprazole was 59%.

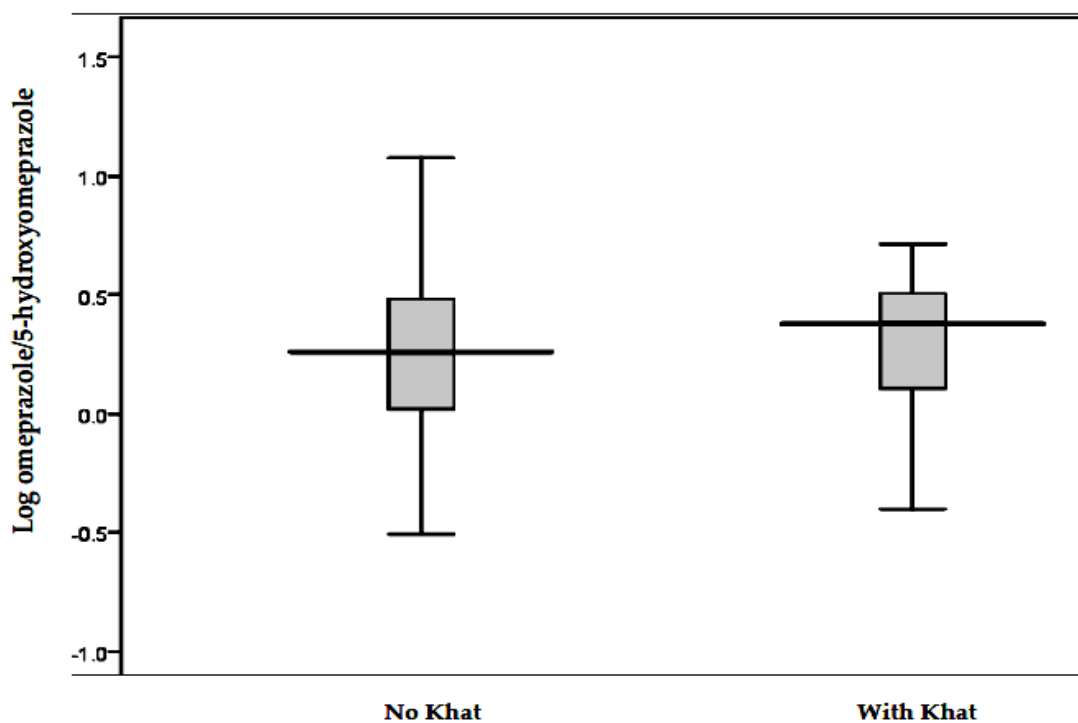


Fig 12: Box-and-Whisker plots for metabolic activity of CYP2C19 in the absence and presence of khat (N=52): values are described as log MR of omeprazole/5-hydroxyomeprazole. Each box displays the median (black bar), and the 25th and the 75th quartiles (the lower and the upper edges of the box, respectively). The box length is the IQR. Khat had no significant effects ($P=0.29$) on the metabolic activity of CYP2C19 enzyme.

4.6.2 Genotyping

The genotype frequencies of CYP1A2*1F, CYP2C9 (*2 and *3) and CYP2C19 (*2 and *3) is presented in **Table 12**. There were no significant differences between observed and expected

CYP1A2*1F genotype frequencies according to the Hardy–Weinberg equilibrium ($p=0.36$). Univariate ANOVA indicated no significant influence of CYP1A2*1F genotype on log CYP1A2 MR before ($p=0.73$) and during Khat ($p=0.12$). Repeated measure ANOVA indicated no significant effect of CYP1A2*1F genotype on log CYP1A2 MR in the absence or presence of Khat ($p=0.30$).

4.7 Correlation of cathinone/cathine ratio with MR of CYPs

Correlation of log CYP2D6 MR in the presence of khat and urinary log cathinone/cathine ratio for both phase I and phase II data is presented in **Figure 13**.

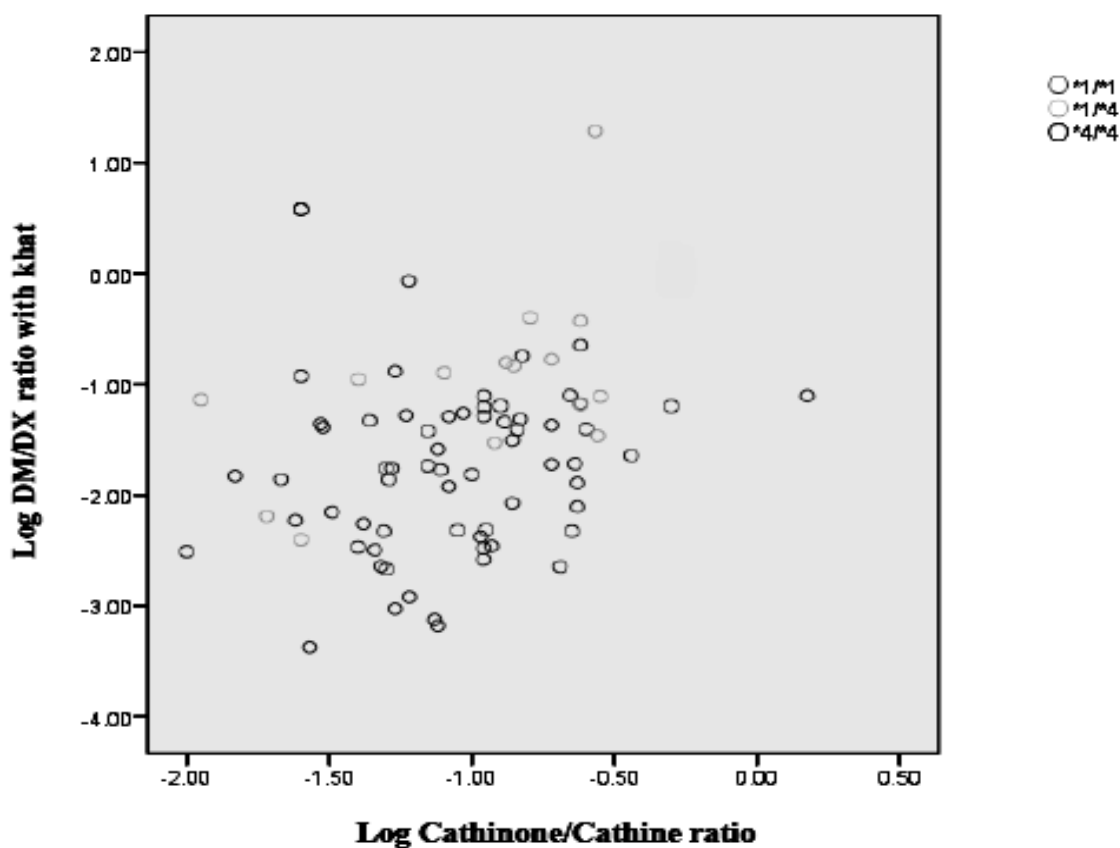


Fig 13: Scatter plot showing correlation between log CYP2D6 MR (DM/DX) and log urinary cathinone/cathine ratio stratified by CYP2D6 genotypes (N=87) for both phase I and phase II (spearman correlation $p=0.002$) data.

There was a significant positive correlation between log DM/DX ratio during khat consumption and log cathinone/cathine concentrations ($p=0.002$) based on spearman correlation test for non-parametric tests. The magnitude of correlation was moderate (coefficient=0.35). Similarly, significant but moderate negative correlation for CYP2C9 ($P=0.015$, coefficient= -0.33) and positive correlation for CYP3A ($P=0.001$, coefficient=0.39) was observed. This might indicate the involvement of these enzymes in the metabolic pathways of khat in some ways. Particularly for CYP2D6, khat can be considered both as substrate and inhibitor for khat. However, no significant correlation was seen for CYP1A2 ($P=0.75$, coefficient=-0.043) and CYP2C19 ($P=0.18$, coefficient =-0.19).

Table 13: Aggregated Median MR and IQR of CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 in the absence and presence of khat and P values using Wilcoxon Matched-Pair Signed Rank Test(N=35, Phase I; N=52, phase II).

| CYP Subtypes | Phase Category | No Khat | With Khat | P-value (Wilcoxon) |
|----------------|----------------|--------------|--------------|--------------------|
| | | Median(IQR) | Median(IQR) | |
| <i>CYP1A2</i> | Phase II | 1.61(0.84) | 1.52(1.02) | 0.71 |
| <i>CYP2C9</i> | Phase II | 0.15(0.24) | 0.18(0.16) | 0.64 |
| <i>CYP2C19</i> | Phase II | 1.82 (2.13) | 2.41 (1.94) | 0.15 |
| <i>CYP2D6</i> | Phase I | 0.05(0.07) | 0.07(0.13) | 0.02 |
| | Phase II | 0.007(0.02) | 0.012(0.04) | 0.001 |
| | Phase I & II | 0.019(0.061) | 0.033(0.061) | 0.001 |
| <i>CYP3A4</i> | Phase I | 9.43(10.43) | 10.45(11.98) | 0.09 |
| | Phase II | 1.06 (2.05) | 1.35 (3.72) | 0.045 |
| | Phase I & II | 0.10(0.98) | 5.31(10.7) | 0.001 |

5. Discussion

To the best of our knowledge, the present study is the first to investigate the impact of habitual khat consumptions on *in vivo* activity of human drug metabolizing enzymes. This dissertation discusses two phases of a study describing the effects of khat on CYP mediated drug metabolism. Phase I study was a pilot study focusing on the two major CYPs, CYP3A and CYP2D6. The effect of concomitant khat use on CYP3A and CYP2D6 enzyme activities and any implication of *CYP2D6* genotype on khat-CYP2D6 interaction were evaluated using established techniques (Ducharme et al,1996; Allqvist, 2010; de Andreas et al.,2014). In Phase II five major CYPs namely, CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A were examined.

The study was controlled by monitoring subjects' compliance to khat abstinence and chewing. Khat withdrawal symptoms were assessed using DSM-V criteria (APA, 2013). With this scale, stimulant withdrawal symptoms were observed in all subjects during abstinence periods, which seemingly substantiate total adherence to study protocol by subjects. However, a DSM-V criterion for amphetamine withdrawal is somehow subjective and might not be a sensitive indicator of khat abstinence. Moreover, the DSM-V criteria set for amphetamine might not fully stand for khat. This is because the urinary cathinone-cathine test result was positive for some volunteers and this might possibly imply some non-conformity to the study protocol. As a result, the statistical analysis and outcome interpretation took into account all these irregularities.

Khat had a significant and reproducible (Bedada et al., 2015) inhibitory effect on human CYP2D6 as demonstrated by median MR of DM/DX in absence and presence of khat in both phase I (P=0.02) and phase II (P=0.001). Merger of the two data also demonstrated significant (P=0.001) effect. When sample size was increased (e.g. phase II), statistical significance was

observed. Moreover, it was possible to see rare defective diplotypes with better sample size. As there were no similar previous studies, the results of our findings were compared with studies on amphetamines and synthetic cathinones (Kalix, 1992; Paillet-Loilier et.al, 2014).

Khat is considered natural amphetamine due to structural and pharmacological similarity between the two chemicals (Kalix, 1992; Carvalho et al., 2012). Amphetamines have been reported to be inhibitors of CYP2D6 (Wu et al.,1997; Kotlyar et al., 2005; Carvalho et al., 2012; delaTorre et al., 2012) and these findings could support our observations. Moreover, *in vitro* studies have indicated that synthetic cathinones are inhibitors of CYP2D6 enzymes (Pedersen et al., 2013; Paillet-Loilier et al., 2014), which further supports our findings.

The inhibition of CYP2D6 by khat was more pronounced in *CYP2D6*1/*1* than *CYP2D6*1/*4* and *CYP2D6*4/*4* genotypes. This finding is consistent with previous reports, which demonstrated that the extent of inhibition of CYP2D6 is genotype-dependent and those with greater baseline enzyme activity show greater magnitude of inhibition (Ozdemir et al., 1998). This means that the inhibition is more pronounced in *CYP2D6* duplicated and wild type genotype than those heterozygous or homozygous defective genotypes. Given the fact that majority of the Ethiopian populations are carriers of active CYP2D6 genes and also chew fresh khat leaves on daily basis (Selassie and Gebre, 1996; Aklillu et al., 1996, 2002), the magnitude of inhibition by khat on CYP2D6 enzymes is anticipated to be stronger. It would be even higher for population who are carriers of CYP2D6 gene duplication coding for UM phenotype (Ozdemir et al., 1998).

The prevalence of CYP2D6 PM Phenotype in the present study was 2.5% and 3.2 %, for phases I and II respectively. This is not far from previous reports (2%) for Ethiopians (Aklillu et al., 1996, 2002). Compared to other African populations, Ethiopians rather display unique characteristics

with respect to CYP2D6 genetic polymorphisms (Aklillu et al., 1996). As much as 29% of the Ethiopians carry functionally active duplicated or multi-duplicated CYP2D6 genes (Aklillu et al., 1996), contrary to other Africans who have only 2% duplicated but non-functional alleles (Masimirembwa et al., 1996). Since the enzyme is not inducible by conventional means, such large differences in the frequency of CYP2D6 duplication can only be explained by selection pressure from environmental factors mainly of dietary or non-dietary sources (Ingelman-Sundberg, 2005). This later view was further corroborated by the observed differences in the activity of CYP2D6 between Ethiopians living in Ethiopia and Ethiopians living in Sweden (Aklillu et al., 2002). The study hypothesized that inhibitory dietary or non-dietary factors such as khat use present in Ethiopia but nearly absent in Sweden may explain the observed low CYP2D6 enzyme activity in Ethiopians living in Ethiopia compared to those living in Sweden.

CYP2D6 is constitutively expressed in all regions of human brain, where it is involved in endogenous metabolism including in the conversion of tyramine to dopamine and regeneration of serotonin from 5-methoxytryptamine (Haduch et al., 2013; Miksys and Tyndale, 2013). Cathinone similar to amphetamines increases the levels of dopamine in the brain possibly by acting on the catecholaminergic synapses (Patel, 2000; Colzato et al., 2011). Previously, it was suggested that CYP2D6 slow metabolizers might have a higher dopamine tone in the pituitary (Aklillu et al., 2007), probably due to their reduced capacity to convert tyramine to dopamine. Whether similar effects happen in rewarding center is not currently known. However, it is clear that inhibition of CYP2D6 throughout the body system results in variability of responses to endogenous chemicals and xenobiotics; which results in changes in physiological or pharmacological effects.

Several studies suggest that polymorphic CYP2D6 enzyme activity may cause inter-individual variability in personality, cognition, risk for depression and suicide (Llerena et al., 1993; Ingelman-Sundberg et al., 2014; Penas-Lledo and Llerena, 2014). The CYP2D6 genotype may thus play a role in inter-individual variation in khat-induced CNS stimulation, psychic dependence, personality disorder or risk of suicide. Furthermore, CYP2D6 is the major enzyme responsible for metabolism of several neuroactive drugs including psychotropic, anti-depressant and antipsychotic drugs (Bertilsson et al., 2002; Ravindranath and Strobel, 2013). Chewing large amounts of khat is associated with manic illness, psychosis, violent reactions, state of euphoria and elation with feelings of increased alertness and arousal, increase in suicidal depression and an increased relative risk of hallucinations. Many khat addicts tend to develop aggressive behavior, paranoia and ultimately personality disorder and psychic dependence (Alem and Shibre, 1997; Cox and Rampes, 2003; Bhui et al., 2006; Hoffman and Al'Absi, 2010; Kroll et al., 2011; Widmann et al., 2014). Added to these complications, it is highly likely that addicted psychiatric patients in Ethiopia use khat while on medication. Khat-drug interaction can potentially affect the treatment outcomes to cause unanticipated toxicity or lack of treatment response, in addition to aggravation of the diseases through its negative neuropsychiatric effects. This is especially highly likely for psychotropic medications where there is low therapeutic index, low response rate, therapeutic lag and early emergence of adverse effects that affect compliance to therapeutic regimen.

Beside neuropharmacological effects described above, khat can also affect the treatment outcome of other drugs that are substrates of CYP2D6. Like most sub-Saharan Africa countries, Ethiopia is affected by high burden of infectious diseases such as HIV/AIDS, tuberculosis and malaria. Likewise, opportunistic infections like oral Candidiasis and diarrhea continue to cause

substantial morbidity and mortality due to poor adherence of patients to multiple drug therapy where drug-drug and drug-khat interaction also plays a role. High prevalence of HIV in khat users is documented and HIV patients on antiretroviral therapy also chew khat concomitantly (Berhanu et al., 2012; Malaju and Asale, 2013; Tadesse et al., 2013). Ethiopia is a malaria endemic country with about 68% of the total population living in areas at risk of malaria infection. *P. vivax* malaria is responsible for 40% of all malaria cases in Ethiopia and chloroquine and primaquine (both substrates of CYP2D6) (Projean et al., 2003; Pybus et al., 2012) are the only remedies. Primaquine metabolite that is responsible for hypnozoite killing is generated by CYP2D6-dependent pathway and concomitant khat use may hamper this process. Indeed, a recent clinical study by Bennett et al (2013) reported significant associations between low-activity CYP2D6 phenotypes and the initial relapse and number of malaria relapses.

Overall the present study provides evidence that simultaneous ingestion of khat and drugs metabolized by CYP2D6 may result in significant unanticipated pharmacological consequences.

Khat had only marginal inhibitory effects ($P=0.09$) on CYP3A4 activity, in phase I, as revealed from median MR of DM/3-MM. Nevertheless, when the only outlier was excluded, significant inhibitory effects were demonstrated ($P=0.02$). The latter statement is further reinforced by significant ($P=0.045$) inhibitory effect of khat observed on CYP3A in phase II study. Such significant effects of khat were furthermore corroborated by analysis of merged data from both phases. Merger of data showed strong inhibitory effects ($P=0.001$) of khat on the same enzyme. The overall result indicates that relatively larger sample size demonstrated better statistical outcomes. Given that CYP3A is the most important liver enzymes metabolizing approximately over 50% of all medications and has broader and overlapping substrate specificity (Luo et.al,

2009; Yang, 2009), such magnitude of inhibition by khat has vital pharmacological implication. Similar to CYP2D6, findings for CYP3A were compared with studies on amphetamines and synthetic chemicals (Kalix, 1992; Paillet-Loilier et.al, 2014).

In vitro study with MDMA has demonstrated that amphetamines have only little potential interaction with CYP3A (Paillet-Loilier et.al, 2014). Although this finding must be substantiated by *in vivo* studies, the report did not rule out the effects of cathinone on CYP3A enzymes. Moreover, the pharmacological differences between khat and amphetamine may play a role. Many different compounds are found in khat including alkaloids, terpenoids, flavonoids, sterols, glycosides, tannins, amino acids, vitamins and minerals. Therefore, amphetamine could only be compared with the main alkaloids-(–)-cathinone (Dhaifalaha and Šantavy, 2004), as these compounds in khat are likely to contribute to its overall effects.

The total CYP3A catalytic activity is mainly due to CYP3A4 and CYP3A5. Although CYP3A5 may not appear as important as CYP3A4 in drug metabolism, its expression and genotype may have clinical importance in African populations, where the enzyme is expressed to a larger extent. However, there is a unique distribution of *CYP3A5* genetic variants among Ethiopians, i.e. they have lower frequency of *CYP3A5*1* and do not contain African population-specific allele, *CYP3A5*7* (Gebeyehu et al., 2011). *CYP3A5*1* (wild type allele), *CYP3A5*3* and *CYP3A5*6* were evaluated in the current study.

Homozygous wild type genotype was very low (7.1%), whereas the heterozygous genotypes (**1/*3*, **1/*6*) responsible for low expressor/IM status had high frequency (67.9%). Homozygous defective genotypes, considered *CYP3A5*0/*0* (**3/*3*, **6/*6*) responsible for PM status accounted for 25%. Although it is not rational to draw comparisons due to important

differences in sample size and nature of participants, similar patterns were observed in previous reports. Ethiopians have low frequency wild type alleles and high frequency of CYP3A5*5 allelic variant which is most responsible for low-/non-expressor status (Gebeyehu et al., 2011). However, it is still difficult to justify the impact of CYP3A5 polymorphism on CYP3A4 phenotype due to many factors. First of all, genotyping data were lacking in phase I. The other problem was that, significant amount of the corresponding phenotyping data were missed in phase II study. For example, from 8 subjects containing homozygous for wild type alleles, only one has complete phenotyping data. Thirdly, CYP3A5 enzyme is affected by sex. Its sensitivity is high in females compared to males (Gebeyehu et.al, 2011). The current research involved only male volunteers. Fourthly and most importantly using DM as probe drugs for phenotyping of CYP3A might not be appropriate (Allqvist, 2010). Additionally, since CYP3A is also involved in the metabolism of omeprazole, weak probes like DM might not clearly demonstrate phenotypic characteristics of CYP3A. The substrates of CYP3A are structurally diverse and exhibit wide range of size and affinities. Moreover, the interactions between CYP3A4 and its substrates and inhibitors are thought to be complex (Kenworthy et al., 1999). Lastly, the functional relevance of CYP3A5 polymorphism is also questionable (Johansson and Ingelman-Sundberg, 2011). Nevertheless, these aforementioned possible factors could only point to the gaps that preclude genotype-phenotype association. Otherwise, they cannot nullify the significant phenotyping outcome. Further study, with sensitive probe drug and larger sample size is recommended to identify those gaps.

Khat had no significant effects on CYP1A2 ($p=0.71$), CYP2C9 ($p=0.64$) and CYP2C19 ($p=0.15$). The metabolic activities of most common allelic variants for the enzymes were

analyzed separately and again no statistically significant effects of khat were observed for each variant group.

The percentage of homozygous wild type genotype of CYP1A2 *1/*1 (also known as *1A/*1A) was 20.6%. CYP1A2*1/*F accounts for 42.9 and homozygous CYP1A2*F/*F accounts for remaining 36.5%. Significant association was not observed between these genotypes and their corresponding phenotypes. Whatever the results might be, the functional significance of CYP1A2 polymorphism is questionable (Johansson and Ingelman-Sundberg, 2011). To settle these disputes, there is a need for well-defined phenotype groups so that studies have adequate power to discern effects of genomic variants against the background of potential inducers (caffeine, smoking, diet, etc.) (Thorn et al., 2012). In the current study, more than 75% of the study participants were smokers. However, significant differences were not observed between smokers and non-smokers. This finding is in line with previous reports by Aklillu et al (2003).

The frequency of CYP2C9*1/*1,*1/*2,*1/*3, ,*2/*2 and genotypes was 77.8%, 14.3%, 6.3% and 1.6% , respectively. The homozygous genotype (*2/*2) is responsible for PM status. Higher values of *1/*1(86.7%) and lower values of *1/*2 (8.7%) were reported in previous studies with Ethiopians (Scordo et al., 2001). There was no significant difference for *1/*3 (5.8% vs. 4.6%). Homozygous defective genotype (*2/*2) was not reported in the study by Scordo et al (2001) for Ethiopians.

Regarding CYP2C19, there was 79.4% (*1/*1), 19% (*1/*2) and 1.6% (*1/*3) genotype frequency. Previous studies reported 74.6% (*1/*1), 19.3% (*1/*2) and 0.9% (*1/*3) for Ethiopians (Persson et al., 1996). The study also reported overall 5.2% homozygous and heterozygous defective genotypes (*2/*2 and *2/*3) that are responsible for PM status. The

genotypes for PM were absent in our findings. The discrepancy could be due to sample size difference, 60 in our case 114 in previous reports. Although the effects of sex is not clearly indicated, it might play some role, as no females in the present study and 54 females were involved in the other study.

Beside khat having significant inhibitory effect on CYP2D6, khat is also a possible substrate for the enzyme CYP2D6. The main psychoactive chemical in khat, cathinone, readily undergoes stereospecific metabolism by liver microsomal enzymes (Brenneisen et al.,1986; Toennes et al.,2003) to produce the phenylpropanolamine stereoisomers cathine and norephedrine (Mathys and Brenneisen,1992; Toennes et al.,2003). Cathine is the major metabolite and is a less active as a stimulant, whereas norephedrine is the minor metabolite and has no psychostimulant effect. There was significant and moderate ($p=0.002$; coefficient=0.35) correlation between the metabolic pathways of DM and cathinone. CYP2D6 was reported to be involved in the metabolic pathway of methamphetamines, such as 4-fluoromethamphetamine, 4-chloromethamphetamine, 4-bromomethamphetamine, 4-iodomethamphetamine, 4-nitromethamphetamine, and 4-methoxymethamphetamine (Taniguchi et al., 2013). Similarly, CYP2D6 was reported to be involved in the metabolism of related chemical, 4-Methylthioamphetamine (Carmo et al., 2007). Furthermore, *in vitro* studies have shown that amphetamines and cathinones are substrates for CYP2D6 enzymes (Kotlyar et al, 2005; delaTorre et al., 2012; Pedersen et al., 2013; Tyrkkö et al., 2015). Finally, the study conducted by Wu et al (1997) on interaction of amphetamines with human liver CYP2D6 supports our findings.

Khat is also a possible substrate for other enzymes as hinted by Tyrkkö et al (2015). The current study has demonstrated similarly that khat is a possible substrate for CYP3A ($P=0.001$, coefficient=0.39) and CYP2C9 ($P=0.015$, coefficient= -0.33). This finding is further supported

by previous reports indicating that CYP3A4 is involved in the *in vitro* N-demethylation of benzphetamine (Bach et.al, 2000). There was similar report by Paillet-Loilier et al (2014) for both CYP3A and CYP2C9.

Although Paillet-Loilier et al (2014) and Pedersen et al(2013) reported that amphetamines and synthetic cathinones are also substrates for CYP1A2 and CYP2C19, this was not demonstrated in the current study.

In general, results from the present study may serve as a basis for future clinical studies for investigating the impact of concurrent habitual khat consumption on treatment outcome of most important diseases in Ethiopia such as HIV, TB, Malaria, Neuropsychiatric and Cardiovascular disorders.

Conclusions

The results of the present study indicates that khat produces significant and reproducible inhibitory effects on the two major enzymes, CYP2D6 and CYP3A, which are responsible for metabolism of over 75% of currently marketed drugs. The inhibitory effects of khat were consistently demonstrated by genotyping data for CYP2D6, but not for CYP3A enzyme. To avoid potentially detrimental outcomes of khat-drug interactions, it would be better to refrain from khat chewing while pharmacotherapeutic disease management is underway. Our study outcomes has clear relevance in khat belt countries where several million people living in East Africa and Arabian peninsula chew khat almost on a daily basis.

Recommendations

1. The clinical significance of CYP2D6 and CYP3A inhibition must be studied.
2. As this study is the first of its kind, it must be studied with other CYPs such as CYP2A6, CYP2B6 and CYP2C8.
3. Multiple plasma samples are also important for future studies, for better correlation with AUC.
4. Study of the same procedure using non-chewing volunteers as control might be important
5. Future incorporation into pharmacotherapeutic care plan should be devised. Policy makers should weigh research outcomes from scientific point of view and use it for betterment of patient's health. The case of codeine should be taken as a good lesson in this respect.

6. References

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Appendices

1. Subject information sheet

Background Information

Cytochrome P450 (CYP) enzymes comprise the major drug metabolizing enzyme system in humans. Genetic polymorphisms or environmental factors such as dietary components, toxins, or drugs can affect the activity of these enzymes and result in interindividual variations in drug concentrations. The five commonest human CYP enzymes that are primarily responsible for the metabolism of drugs are CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A. The enzymes involved in the metabolism of khat are not yet known but it is anticipated that the CYP play a critical role. The current study attempts to sort out the effect of khat on these five major enzymes. The finding of this study will boost our understanding of the interaction between khat and clinically used medications.

How many people will take part in this study?

100 healthy Ethiopian male volunteers are recruited from Addis Ababa for the study at the Black Lion Hospital.

What is involved in the study?

All subjects will refrain from chewing khat for 1 week (day 1-7). On day 8, the subjects will be administered subtherapeutic doses of the following cocktail drugs: 100mg caffeine (CYP1A2), 50mg losartan (CYP2C9), 20 mg omeprazole (CYP2C19) and 30mg Dextromethorphan (CYP2D6, CYP3A4). Blood samples (5ml) will be collected 3h after administering dextromethorphan and 4h for other probe drugs. The blood collected will be centrifuged for 11 min at $3500 \times g$ to obtain plasma aliquots that is stored at -80°C until LC-MS/MS analysis. On same day, day 8 after blood sample collection is over, the subjects will start to chew 400g of khat and this will continue daily at similar time until the last blood sample is taken, i.e. day 15. All phenotyping procedures performed before khat is repeated after participants are allowed to chew khat. Genomic DNA will be isolated from peripheral blood leukocytes using QIAamp DNA Maxi Kit (QIAGEN GmbH, Hilden, Germany) and will be sent to Division of Clinical Chemistry, Karolinska Institutet, and Stockholm, Sweden for analysis. CYP1A2(*1A, *1F), CYP2C9(*2, *3), CYP2C19 (*2, *3), CYP2D6(3*, 4*) and CYP3A5 (*3, *6) genotypes will be determined. Urine sample will be collected too.

How long will I be in the study?

The study will take about 2 weeks

What are the risks of the study?

The drugs are safe and also taken at subtherapeutic concentration. Thus, there are no significant adverse effects associated at specified doses.

Are there any benefits for taking part in this study?

There are no direct benefits for you because you participated in this study. But, we hope the information learned and outcome obtained from this study will allow us to better understand the pharmacological and toxicological impact of khat on concomitantly taken drugs.

What are my rights as a participant?

Participation in this study is voluntary and you can discontinue taking part in the study without any explanation. Moreover, if you suffer an unanticipated injury as a direct result of this research and require emergency medical treatment, Black Lion Hospital will provide you treatment at no cost to you.

Will I be paid for my participation?

You will not be paid for participation in this study. However, all costs associated with the study will be covered by the project.

What about confidentiality?

Study records that identify you will be kept confidential. All information obtained or data generated will be strictly confidential and stored in a password protected computer file and will only be accessed by the research team. The blood sample collected will by no means be used for other test purposes than clearly indicated in the research. The data collected in this study will be used for the purpose described in the consent form. This consent form will be kept by the research team for at least six years. The study results will be kept in your research record and be used by the research team indefinitely.

Data from this study may be used in medical publications or presentations. Your name and other identifying information will be removed before this data is used.

2. Consent Form

You are being asked to participate in a research study. A member of the research team will explain what is involved in this study and how it will affect you. Your confidentiality will be maintained. Please take your time to ask questions and feel comfortable making a decision whether to participate or not. This process is called informed consent. If you decide to participate in this study, you will be asked to sign this form. You will be given a signed copy of this document. This consent form document does not have an expiration date.

The research project and the procedures associated with it have been explained to me and I have also read information for participants and I have agreed to participate in this study. My participation is voluntary and I do not have to sign this form if I do not want to be part of this research study.

Signature of Subject: _____

Date: _____ Time: _____ AM/PM (Circle)

Investigator obtaining the consent: _____

I have explained to _____ the nature and purpose of the study and the risks involved. I have answered and will answer all questions to the best of my ability. I will give a signed copy of the consent form to the subject.

Signature of Person Obtaining Consent: _____

Date: _____ Time: _____ AM/PM (Circle)

Amharic version

መግቢያ

ክፍል 1፡ ለተሳታፊዎች የሚሰጥ መረጃ

ሳይቶክሮም T450 ለሰው ልጅ ህክምና አስፈላጊ የሆኑ መድኃኒቶችን ወደ ጠቃሚ ፍቱን ንጥረ ነገር ለመቀየርም ሆነ ወደ ጎጂ አላስፈላጊ ንጥረ ነገር ለመቀየር ያገለግላል። ከሳይቶክሮም T450 ዘሮች ውስጥ አብዛኛውን ስራ የሚሰሩት አምስት ሲሆኑ እነርሱም ሳይቶክሮም T450-1ኤ2፣ሳይቶክሮም T450-2ሲ9፣ሳይቶክሮም T450-2ሲ19፣ሳይቶክሮም፣ T450-2 ዲ6 እና ሳይቶክሮም T450-3 ኤ ናቸው። ጫት ከነዚህ ኢንዱስትሪ ጋር ያለው መስተጋብር ከፈዋሽ መድኃኒቶች የሚገኘውን ጥቅም ሊቀንስ አሊያም ደግሞ በመድኃኒቱ የሚመጣውን የጎንጎሽ ችግር ሊያባብስ ይችላል ተብሎ ይገመታል። በዚህም ምርምር በጫትና በሳይቶክሮም T450 ኢንዱስትሪ መካከል ያለውን መስተጋብራዊ ሂደት ለማጥናት ታቅዷል።

ስንት ሰዎች በዚህ ጥናት ይሳተፋሉ?

100 ያህል የሚሆኑ ሰዎች በጥናቱ ይሳተፋሉ

የምርምሩ ሂደት ምን ይመስላል?

በምርምሩ ለመሳተፍ ፈቃደኛ የሆኑ ሰዎች ለአንድ ሳምንት ጫት ከመቃም ይቆጠባሉ። በ8ኛው ቀን ቀላል ቁርስ ከተመገቡ በኋላ 100ሚ.ግ ካፌይን፣50ሚ.ግ ሎሳርታን፣ 20ሚ.ግ አሜፕራዛል በአንድ ጊዜ ይወስዱና ከ1 ሰዓት በኋላ 30ሚ.ግ ዴክስትሮሜቶርፋን ይወስዳሉ።ዴክስትሮሜቶርፋንን ከወሰዱ ከ3 ሰዓት በኋላ 5ሚሊ ሌትር ደም ይሰጣሉ። ይህ ደም ግማሹ ወደ ፕላዝማ ሲቀየር፣ቀሪው ምንም ሳይደረግ ለዘረመል ጥናት ጥቅም ላይ ይውላል። በዚህ ቀን ከሰዓት በኋላ ጫት መቃም ጀምረው እስከ 15ኛው ቀን ድረስ በየቀኑ በተመሳሳይ ጊዜ 400 ግራም ጫት ይቅማሉ። በ15ኛው ቀን ጧት ልክ ከጫት በፊት እንደተወሰደው አኳኋን መድኃኒቶቹን ወስደው በተመሳሳይ መልኩ 5ሚሊ ሌትር ደም ሰጥተው ጥናቱን ያጠቃልላሉ። ሽንትም በመጨረሻው ይወሰዳል። ሽንቱም፣ ፕላዝማውም፣ደሙም በጥንቃቄ ከተያዘ በኋላ ወደ ስዊድን ሀገር ተልኮ በዘመናዊ መሳሪያ በመድኃኒቶቹና በጫት መካከል ያለው መስተጋብር ይጠናል።

ጥናቱ ስንት ቀን ይፈጃል

ምርምሩ ሁለት ሳምንታትን ይፈጃል ።

ጥናቱ እኔ ላይ ምን ጉዳት ያመጣል?

መድኃኒቶቹ በአሁኑ ወቅት ለተለያዩ ህመሞች አገልግሎት እየሰጡ ያሉ ሲሆን፣ ለዚህ ጥናት ደግሞ በጣም ትንሹን መጠን ስለምንጠቀም የጎላ ችግር አያመጡም።

በዚህ ጥናት ተሳታፊ በመሆን ምን የተለየ አገኛለሁ?

በዚህ ጥናት ውስጥ በማለፍህ ቀጥተኛ የሆነ ጥቅም አታገኝም። ነገር ግን ጥናቱ በሀገራችን በየትኛውም አካባቢ ዘውትር እየተቃመ የሚገኘው ጫት ለሀብረተሰቡ አስፈላጊ በሆኑ ፍቱን መድኃኒቶች ላይ የሚያመጣውን የጎላ ተፅዕኖ ይፋ ስለሚያደርግ ሁሉም ዜጋ ተጠቃሚ ይሆናሉ። አንተም በዚህ ጥናት ተሳታፊ በመሆንህ ሀብረተሰቡን ትጠቅማለህ ማለት ነው።

በዚህ ጥናት ውስጥ ሲሳተፍ መብቶቼ ምንድናቸው?

በዚህ ጥናት ለመሳተፍ የማንም ሰነድ ወገን ተፅዕኖ የሌለበት ፈቃደኝነት ያስፈልጋል። ከጥናቱ በፊትም ሆነ በጥናቱ ሂደት መሃል በየትኛውም ጊዜ ከጥናቱ ራስህን የማግለል መብትህ የተጠበቀ ነው። እንዲሁም ከጥናቱ ጋር በተገናኘ መልኩ ያልታሰበ እክል ብያጋጥምህ በጥቁር አንበሳ ሆስፒታል በነጻ የመታከም መብት አለህ።

በጥናቱ ተሳታፊ በመሆን የሚከፈለኝ ገንዘብ አለ?

በጥናቱ ተሳታፊ በመሆንህ ብቻ የሚከፈልህ ጥሬ ገንዘብ የለም። ነገር ግን ከጥናቱ ጋር የሚገናኙ ወጪዎችህ ሁሉ ይሸፈናሉ።

የጥናቱ ውጤት ሚስጢራዊነት እስከ ምን ድረስ ነው?

በጥናቱ ውጤት ማንነትህን የሚገልፅ ማንኛውም መረጃ በሚሰጥር ይያዛል። የሚወሰዱት የደም ፍሙናዎችም በጥናቱ ሂደት ከተገለፀው ዓለማዊ ውጪ በምንም መልኩ ለሌላ ዓለማዊ አይውልም። ተመራማሪዎቹ የጥናቱን ውጤት ያለገደብ መጠቀም ይችላሉ። በጥናቱ ሂደት የሚገኝ መረጃም በስምምነታችን መሰረት ብቻ ጥቅም ላይ ይውላል። የስምምነታችንም ሰነድ ብያንስ ለ6 ዓመታት በጥንቃቄ ይያዛል። በዚህም መሰረት መረጃው አንተን የሚገልፅ ማንኛውም አሻራ ከተደመሰሰለት በኋላ በህጉ መታወቅ መልክ ሊሰራጭ ወይም በተለያዩ መድረኮች ላይ ሊቀርብ ይችላል።

ክፍል 2: የስምምነት መግለጫ

በዚህ ጥናት በፈቃደኝነት እንድትሳተፍህን በትህትና እንጠይቅሃለን። ተመራማሪዎቹ ስለ ጥናቱ ዓላማ፤ ሂደትና ባንተ ላይ ሊመጣ የሚችለውንም መጠነኛ ጉዳት በጥልቅ ያስረዱሃል። ማንነትህን በተመለከተ ሚስጥራዊነቱ በሚገባ ይጠበቃል። ጥናቱን በተመለከተ ያለህን ማንኛውንም ጥያቄ የመጠየቅ ፤ እንዲሁም ስጋት የመግለፅ መብት አለህ። በጥናቱ ለመሳተፍ ከወሰንክ የስምምነት መግለጫውን በፈርማህ ታፀድቃለህ። የፈረምከው የስምምነት መግለጫ ቅጂ ላንተም ይደርስሃል። ይህ ሰነድ ጊዜ አያልፈበትም።

የምርምሩ ፕሮጀክትና የጥናቱ ዝርዝር ዓላማና ሂደት በሚገባ የተበራራልኝ ሲሆን በተጨማሪም ለተሳታፊዎች የተዘጋጀውን የጥናቱን አጠቃላይ ይዘት አንብቤያለሁ። በዚህም መሰረት በጥናቱ ለመሳተፍ ተስማምቼያለሁ። የተስማማሁትም የማንም ግፊት ሳይኖርብኝ በራሴ ሙሉ ፈቃደኝነት ነው። ፈቃደኛ ባልሆን ኖሮ ይህንን የስምምነት ማረጋገጫ አልፈርምም ነበር።

የተሳታፊ ፊርማ: _____

ቀን: _____ ሰዓት: _____ ጧት ማታ

የስምምነት ማረጋገጫውን ያስፈረመ ተመራማሪ: _____

ለ _____ የምርምሩን ይዘት፤ የጥናቱን ዝርዝር ዓላማና ሂደት፤ እንዲሁም ሊመጣበት የሚችለውን መጠነኛ እክል በሚገባ ገልጭለታለሁ። ከዚህም ውጪ ለተጠየቁት ጥያቄ አጥጋቢ ምላሽ ሰጥቻለሁ። ከዚህም በኋላ ለሚጠየቀው ጥያቄ በሚችለው መልኩ አጥጋቢ ምላሽ ለመስጠት ተዘጋጅቼያለሁ። የስምምነት ማረጋገጫውንም አንድ ቅጂ ለተሳታፊው እሰጣለሁ።

የስምምነት ማረጋገጫውን ያስፈረመ ተመራማሪ ፊርማ _____

ቀን _____ ሰዓት: _____

3. Stimulant Withdrawal Diagnostic criteria (DSM-5)


- A. The cessation of(or reduction in) , prolonged amphetamine-type substance, cocaine or other stimulant use
- B. Dysphoric mood and plus two (or more) of the following physiological changes developing within a few hours or several days after criterion A: Yes [] No [], if yes proceed
- | | | |
|---|---------|--------|
| 1. Fatigue | Yes [] | No [] |
| 2. Insomnia or hypersomnia | Yes [] | No [] |
| 3. Psychomotor agitation or retardation | Yes [] | No [] |
| 4. Increased appetite | Yes [] | No [] |
| 5. Vivid, unpleasant dreams | Yes [] | No [] |
- C. The criterion symptoms in B are clinically significant or cause distress in social, occupational or other important areas of functioning.
- D. The signs or symptoms are not attributable to another medical condition and are not better explained by another mental disorder, including intoxication or withdrawal fro another substance

Withdrawal symptoms: Yes No

Substance abuse specialist: _____

Signature: _____

Date: _____

| | | |
|---|--|--|
|  | Addis Ababa University Medical Faculty Institutional Review Board | SOP# AAUMF 008 Version 2.0 Effective date: 1 Feb. 2009 Page 13 of 13 |
| | Title: 3.2. Use of Study Assessment Form | |

ANNEX 3

Form AAUMF 03-008

IRB's Decision

Meeting No: 051/2013

Date (D/M/Y): April 10/2013

Protocol number: 012/13/Pharma

Assigned No.....

| | |
|---|---|
| Protocol Title: The effect of Khat on CYP2D6 & CYP3A mediated drug metabolism | |
| Principal Investigators: | Worku Bedada |
| Institute: | AAU- CHS Department of Pharmacology |
| Elements Reviewed (AAUMF 01-008) | <input checked="" type="checkbox"/> Attached <input type="checkbox"/> Not attached |
| Review of Revised Application <input type="checkbox"/> Yes <input type="checkbox"/> No | Date of Previous review: |
| Decision of the meeting: | <input checked="" type="checkbox"/> Approved <input type="checkbox"/> Approved with Recommendation <input type="checkbox"/> Resubmission <input type="checkbox"/> Disapproved |

- I. Elements approved-
1. Protocol Version No.
 2. Protocol Version Date.....
 3. Informed consent Version No.
 4. Informed Consent Version Date

II. Obligations of the PI-

1. Should comply with the standard international & national scientific and ethical guidelines
2. All amendments and changes made in protocol and consent form needs IRB approval
3. The PI should report SAE within 10 days of the event
4. End of the study, including manuscripts and thesis works should be reported to the IRB

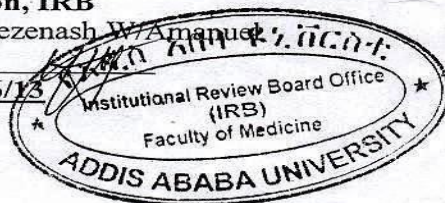
III. TO ESTM Institution Review Board (IRB) Approval: Period from 20/06/13 to 20/06/15

Follow up report expected in

3 Months _____ 6 months _____ 9 months one year _____**Chairperson, IRB**

Dr. Yimtubezzenash W. Amanuel

Signature _____

Date: 20/06/13**Associate Director of****Research and Technology Transfer**

Signature _____

Date _____



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የሳይንስና ቴክኖሎጂ ሚኒስቴር
The Federal Democratic Republic of Ethiopia
Ministry of Science and Technology

ቁጥር 3.10/586/06
Ref. No.
ቀን 28/05/06
Date

To: Addis Ababa University, College of health Sciences
Addis Ababa

Re: The effect of Khat and CYP2D6 and CYP3A4 mediated drug metabolism

Dear sir/Mr./s/Dr.

The National Research Ethics Review committee (NRERC) has reviewed the aforementioned project protocol in an expedited manner. We are writing to advise you that NRERC has granted

Full Approval

To the above named project, for a period of **one year (February 7, 2014- February 6, 2015)**. All your most recently submitted documents have been approved for use in this study. The study should comply with the standard international and national scientific and ethical guidelines. Any change to the approved protocol or consent material must be reviewed and approved through the amendment process prior to its implementation. In addition, any adverse or unanticipated events should be reported within 24-48 hours to the NRERC. Please ensure that you submit progress report once in a four month and annual renewal application 30 days prior to the expiry date.

We, therefore, request your esteemed organization to ensure the commencement and conduct of the study accordingly and wish for the successful completion of the project.



With regards,

Yohannes Sitotaw
Secretary of NRERC

Cc Mr Worku Bedada (PI)
Addis Ababa


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You may Contact

ፖ.ሳ.ቁ.
P.O.Box 2490

አዲስ አበባ ኢትዮጵያ
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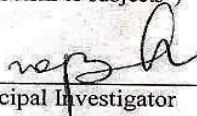


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Web site: <http://www.most.gov.et>

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Fax +251-011-4-66 02 41

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|---|--|--|
|  | Addis Ababa University Medical Faculty Institutional Review Board | SOP# AAUMF 012 Version 1.0 Effective date: 1 Jan. 2007 Page 8 of 9 |
| | Title: Review of Protocol Amendment | |

ANNEX 1
Form AAUMF 01-012

Protocol Amendment Submission Form

| | |
|--|--------------------------|
| PROTOCOL NUMBER: 012/13/Pharme | SUBMITTED DATE: 18/11/14 |
| PROTOCOL TITLE: The effects of khat on CYP mediated drug metabolism | |
| PRINCIPAL INVESTIGATOR: Worku Bedada | |
| INSTITUTE: Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, CHS, AAU | Telephone: 0911341140 |
| APPROVED DATE: 10 April 2013 | NO. OF AMENDMENT: 02 |
| REASON FOR AMENDMENT: analyzing the effects of khat on CYP3A enzyme mediated drug metabolism with better probe drug and larger sample size; and also adding other CYPs such as CYP2C9/19, CYP1A2 and CYP2B6 that are also important in the metabolism of clinically important drugs. | |
| TYPE OF AMENDMENT REQUESTED: <input checked="" type="checkbox"/> EXPEDITED (Minor changes) <input checked="" type="checkbox"/> FULL REVIEW BY IRB (More than minor changes or that amendment "materially affects risks to subjects") | |
| SIGNATURES:  Date: 18/11/14 Principal Investigator | |
| COMMENTS: <input checked="" type="checkbox"/> EXPEDITED (Minor changes) <input checked="" type="checkbox"/> FULL REVIEWED | |
| APPROVALS  Date: 22/01/15 Chairperson, IRB | |
| COMPLETION  Date: 22/01/15 Secretary, IRB | |
| ADDIS ABABA UNIVERSITY Institutional Review Board Office Faculty of Medicine (IRB) | |
| PROTOCOL NUMBER: AAUMF □□□/□□-□□ | |

File name: c:\AAUMF\IRB\SOP12\Amend\Protocol.doc

በጫት ምርምር ለመሳተፍ ፈቃደኛ ለሆኑ ወንዶች የተደረገ ጥሪ

የጥናቱ ርዕስ: Phenotypic and genotypic evaluation of *Catha edulis* F. (Khat) effect on cytochrome P450 mediated drug metabolism

ርዕሰ ጉዳይ: በሀገራችን ጫት ዕድሜንና የታን ሳይሊይ፣ በየትኛውም የሀገሪቱ ቦታዎች እንደሚታዩ ጥናቶች ያሳያሉ። በመሆኑም ታካሚዎች መድኃኒት እየወሰዱ የሚቅሙበት ዕድል ሰፊ ነው። ሆኖም በጫትና በፈዋሽ መድኃኒቶች መካከል ሊኖር የሚችለው መስተጋብር በሳይንሳዊ መንገድ አልተጠናም። በዚህ መሠረት የዚህ ምርምር ዋና ዓላማ በጫትና በፈዋሽ መድኃኒቶች መካከል የሚኖረውን መስተጋብር በሳይንሳዊ መንገድ ማጥናት ይሆናል።

- ጥናቱ የሚካሄድበት ቦታ: ጥቁር አንበሳ ስፔሻላይዜድ ሆስፒታል
- ለጥናቱ 100 የሚሆኑ ከ18-50 ዕድሜ ክልል የሚገኙ ጤናማ ወንዶች ይፈለጋሉ።
- በጥናቱ ለመሳተፍ የማንም ለስተኛ ወገን ተፅዕኖ የሌለበት ፈቃደኝነት ይፈለጋል።
- በጥናቱ ውጤት ማክትን የሚገልፁ ማንኛውም መረጃዎች በሚስጥር ይያዛሉ።
- ከጥናቱ ጋር የሚያያዙ ማንኛውም ወጪዎች በጥናቱ ፈንድ ይሸፈናሉ።

ተመራማሪዎች:

1. አቶ ወርቁ በዳዳ
2. ዶ/ር ኤፍሬም እንግዳወርቅ
3. ፕሮፌሰር አሌኔ አክሊሉ

ለበለጠ መረጃ አቶ ወርቁ በዳዳን ያነጋግሩ (ስልክ: 0911341140)