

***In vitro* Effect of *Withania somnifera*, *Mucuna pruriens* and *Pausinystalia johimbe* on Hepatic Cytochrome P450 in Rat**

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Abstract

The effect of *Withania somnifera*, *Mucuna pruriens* and *Pausinystalia johimbe* extracts on hepatic cytochrome P450 enzyme CYP3A4 activities was studied using rat liver microsomes. CYP3A4-dependent testosterone 6 β -hydroxylation activities were determined by ELISA. In the study, rats were treated with *W. somnifera* (0.5 g/kg/day), *M. pruriens* (0.5 g/kg/day) and *P. johimbe* (0.25 g/kg/day) extracts for 20 days. It was found that *W. somnifera*, *M. pruriens* and *P. johimbe* extracts showed potent to moderate inhibitory effect on CYP3A4 activities in rat liver microsomes, with IC₅₀ values of 18.01 ng/mL, 14.93 ng/mL and 21.03 ng/mL, respectively.

Key words: *Withania somnifera*, *Mucuna pruriens*, *Pausinystalia johimbe*, cytochrome P450, inhibition.

Introduction

Herbal medicines are widely used around the world with the aim to obtain synergistic effects or decrease the side effects or toxicity (Pekthong *et al.*, 2008; Nature, 2007). As alternative or complementary therapy in this world, it is gaining increasing popularity. It is used in many countries, including China, India, Bangladesh, Korea, Canada, Norway, the United Kingdom and other countries in Europe and South America, South Africa and the United States. The World Health Organization (WHO) estimates that 80 percent of the population of some Asian and African countries presently uses herbal medicine for some aspect of primary health care.

Withania somnifera, known as Ashwagandha, is a plant in the Solanaceae family. The roots, leaves and berries of the plant are most useful. The climates of India, Pakistan, Srilanka and Bangladesh are good for Ashwagandha cultivation. The main chemical constituents are alkaloids and steroidal lactones, tropine containing a hydroxyl group. The leaves contain the steroidal lactones. It is used as a

medicinal herb in Ayurvedic medicine (MedlinePlus, 2017; Memorial, 2018).

Mucuna pruriens Linn. (Family: Fabaceae), known as Alkushi, is one of the popular medicinal plant in Africa and tropical Asia. It silage contains 11-23% crude protein, 35-40% crude fiber, and the dried beans contain 20-35% crude protein. The seeds of the plant contain about 3.1-6.1% L-DOPA (L-3,4-dihydroxyphenylalanine) with trace amount of alkaloids, serotonin, nicotine, mucunine, mucunadine, pruriendine, prurieninine, lecithin, steroids, and bufotenine (Rastogi *et al.*, 1991a; Singh *et al.*, 1995). *M. pruriens* was able to rescue sperm motility and count as well as possibly fertility in men suffering from infertility (Shukla *et al.*, 2010). It is also used in Ayurvedic medicine in an attempt to treat diseases including Parkinson's disease (Katzenschlager *et al.*, 2004).

Pausinystalia johimbe (Family: Rubiaceae), common name Yohimbe, is a plant species native to western and central Africa (Nigeria, Cabinda, Cameroon, Congo, Gabon, and Equatorial Guinea). Bark is used in extractions to make tinctures for

traditional medicine and dietary supplements. The main active ingredient in the extract is yohimbine. It also contains other alkaloids with undefined properties as corynanthine, an alpha-1 adrenergic receptor blocker and raubasine (Beille, 2013; Doxey *et al.*, 1984). Extracts from the bark of yohimbe are used as a general tonic and as an aphrodisiac (Beille, 2013). Side effects of using yohimbe may include high blood pressure, increased heart rate, headache, nausea, tremors and sleeplessness.

Cytochromes P450 (CYPs) are hemoproteins which use a variety of small and large molecules as substrates in enzymatic reactions (Gonzalez and Gelboin, 1992). CYPs enzymes are present in most tissues of the body, and play important roles to metabolize thousands of endogenous and exogenous chemicals including drugs. It is reported that CYPs involve about 75% of the total metabolism of drugs (Guengerich, 2008). Many drugs may increase or decrease the activity of CYPs by induction, or direct inhibition of the enzymes. This is a major source of adverse drug interactions, since changes in CYP enzyme activity may affect the metabolism and clearance of various drugs (Guengerich, 2008).

Humans have 57 genes and more than 59 pseudogenes divided among 18 families of cytochrome P450 genes and 43 subfamilies (Nelson *et al.*, 2004). Cytochrome P450 3A4 (CYP3A4) is an important gene of the enzyme cytochrome P450, mainly found in the liver and in the intestine, which is responsible to oxidize small organic foreign molecules e.g. drugs. The main aim of the present study was to assess the effects of *W. somnifera*, *M. pruriens* and *P. johimbe* extract on microsomal hepatic P450 (CYP3A4) activities *in vitro* in rat liver microsomes.

Materials and Methods

Chemicals: Ethanol, CHCl₃, KCl were purchased from Merck, Germany. EDTA and acetonitrile were purchased from Sigma-Aldrich, Germany. Tris-HCl (LOBA CHEMIE, India), testosterone (Atlas Medical, UK) and reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (Origin- Sisco

Research Lab.) were also purchased. All other laboratory chemicals were used as the highest purity and from commercial suppliers.

Extraction of *W. somnifera*, *M. pruriens* and *P. johimbe*: *W. somnifera* and *M. pruriens* (seed) were purchased from the local market of Dhaka, Bangladesh and identified by a botanist. *P. johimbe* was a kind gift of S.B. Herbal & Nutraceuticals, Rajshahi, Bangladesh. Dried raw materials of *W. somnifera*, *M. pruriens* were ground, soaked and stirred intermittently for 3-4 days in ethanol. The extraction of *P. johimbe* was carried out by dissolving powder of the plant in ethanol for 3 days. The extracts were filtered followed by drying using a rotary evaporator under reduced pressure at low temperature (40°C) and yielded semisolid residues which were kept at 4°C until use.

Preparation of microsomes: In this study 20 female Wistar rats (8 weeks old) weighing about 200 g were purchased from Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, Bangladesh. Prior to commencement of the experiment, all the rats were acclimatized to the new environment for a period of one week. During the experiment period the rats were kept in a well ventilated animal house at room temperature of 25°C. They were supplied with standard pellets and fresh drinking water. All the rats were kept in cage and maintained with natural 12 h light and dark cycle in the Animal House of the Institute of Food and Nutrition Science, University of Dhaka, Bangladesh. This study was conducted according to the Declaration of Helsinki. The rats were randomly assigned into four groups and 5 rats were taken in each group.

Group-I was treated with *W. somnifera* (0.5 g/kg/day), Group-II was *M. pruriens* (0.5 g/kg/day), Group-III was *P. johimbe* (0.25 g/kg/day) extracts for 20 days and Group-IV was control group. The rats were sacrificed 24 h after the last treatment. The liver was immediately removed, weighed and washed in cold homogenization buffer (50 mM Tris-HCl, 150 mM KCl, 2 mM EDTA, pH 7.4). Then the liver was homogenized in 4 ml buffer per 1.0 g of liver. The

homogenates were submitted for centrifugation at 4000 rpm for 30 min (Pekthong *et al.*, 2008; Pekthong *et al.*, 2009; Richert *et al.*, 2002). Finally, microsomal samples containing supernatant collected and frozen at -80°C until analysis.

CYP3A4 testosterone 6 β -hydroxylase assay: Testosterone 6 β -hydroxylation was measured by slightly modified method described by Pearce *et al.* (1996). According to this method, hepatic microsomes (100 μl) were incubated at 37°C with testosterone (50.88 nM) and NADPH (2 mM) for 1 h. The reaction was stopped by adding 300 μl ice-cold acetonitrile and the samples were kept at ice for 30 min. Then the samples were centrifuged at 4000 rpm for 20 min and the supernatants were collected for analysis.

ELISA assay: For this assay 96 well plate for ELISA (LisaScan EM, Erba Mannheim, Germany) was used. According to Chen *et al.* (1991), 50 μl of standards, samples and control microsomes were dispensed into appropriate wells. 100 μl of TMB (tetramethyl benzidine) substrate was dispensed to each well and gently mix for 10 sec. After incubating at room temperature ($\sim 22^{\circ}\text{C}$) for 20 min, the reaction was stopped by adding 100 μL of stop solution (1 M sulfuric acid) to each well. After gently mixing 30 sec, all the blue color was changed to yellowish color and the absorbance were measured at 450 nm immediately with ELISA reader.

Determination of IC₅₀: IC₅₀ is a measurement of the effectiveness of a compound in inhibiting biochemical processes and biological functions. According to the FDA (<http://www.fda.gov>), IC₅₀ is the concentration of a particular drug or other substance/or inhibitor that is required to inhibit the biological process for 50% inhibition *in vitro*.

Inhibition of a drug in percent (I %) were calculated as follows:

$$I \% = (1 - A_{\text{Sample}}/A_{\text{Blank}}) \times 100$$

Here, A_{blank} = the absorbance of the blank (control)

A_{Sample} = the absorbance of the sample

Concentration of *W. somnifera*, *M. pruriens* and *P. johimbe* extracts providing 50% inhibition (IC₅₀) was calculated from the graph plotted inhibition

percentage against extract concentration. The equation of the IC₅₀ is given below:

$$IC_{50} = [(50-A)/(B-A)] \times (D-C) + C$$

Where, A = the first point on the curve, expressed in percent inhibition, that is less than 50%

B = the first point on the curve, expressed in percent inhibition, that is greater than 50% or equal to 50%

C = the concentration of inhibitor that gives A% inhibition

D = the concentration of inhibitor that gives B% inhibition.

Statistical analysis: Statistical analysis was conducted by the general procedures of IBM SPSS Statistics 20, IBM Corporation. The significance of the difference between the groups was assessed by one-way ANOVA. The level of significance was evaluated at p-value < 0.05.

Results and Discussion

Effect of *W. somnifera*, *M. pruriens* and *P. johimbe* on CYP3A4 activities: The absorbencies of standard testosterone obtained from ELISA were plotted in a calibration curve to get the concentration of inhibition of samples (Figure 1). *W. somnifera*, *M. pruriens* and *P. johimbe* extracts were found to be inhibitory activity of CYP3A4. The IC₅₀ values of *W. somnifera*, *M. pruriens* and *P. johimbe* extracts were found as 18.01 ng/mL, 14.93 ng/ml and 21.03 ng/mL, respectively (Figure 2). These values exhibited higher degrees of inhibition that indicating the binding of the inhibitor (drugs) to the enzyme (CYP3A4).

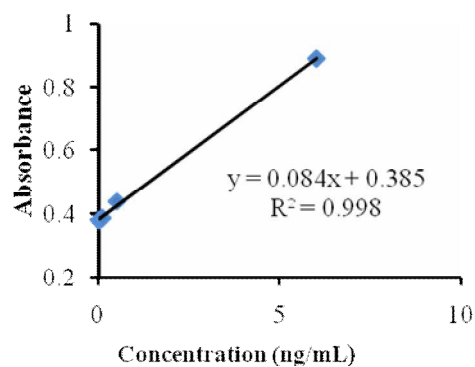


Figure 1. Standard calibration curve. Here, n=3

Cytochrome P450 (CYP) 3A4 is the most abundant human cytochrome enzyme. It plays central roles in the metabolism of xenobiotics including drugs, endogenous compounds and herbal component as effective substrates (Coon, 2005). The expression of individual CYPs is regulated by both endogenous factors and foreign compounds including drugs and natural compounds. The CYP substrates like both endogenous and exogenous compound can lead to pharmacokinetic interactions. When two or more drugs are administered at the same time, CYPs enzyme inhibition may contribute to decrease in one of the drug clearances due to drug-drug interaction and decrease in the formation of pro-drugs reactive metabolites, which can lead to decrease in the pharmacological effect of drug metabolism. However, the decrease in drug metabolism rate will increase drug toxicity (Jamal *et al.*, 2010; Wienkers *et al.*, 2005).

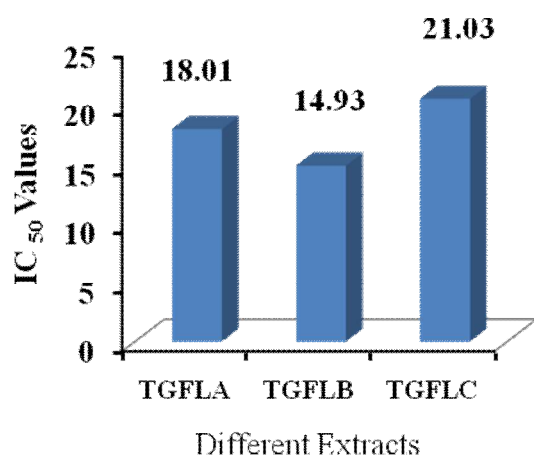


Figure 2. IC₅₀ values of Different Drug on CYP3A4-mediated activities in rat liver microsomes (TGFLA= treated with *W. somnifera*, TGFLB= treated with *M. pruriens* and TGFLC= treated with *P. johimbe*).

Conclusion

CYP3A4 is the largest single portion of the CYP450 protein and responsible for the metabolism of a wide variety of drugs. From the present *in vitro* study on rat liver microsomes, it was found that *W. somnifera*, *M. pruriens* and *P. johimbe* extracts showed moderate inhibition with IC₅₀ values of 18.01 ng/mL, 14.93 ng/mL and 21.03 ng/mL, respectively

of CYP3A4 of hepatic microsomes of rats. Based on the findings it was concluded that *W. somnifera*, *M. pruriens* and *P. johimbe* extracts were found to inhibit the catalytic activities of CYP3A4 in human also.

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