Original Article

Lack of the cytochrome P450 3A interaction of methanolic extract of *Withania somnifera*, Withaferin A, Withanolide A and Withanoside IV

Jay Savai, Alice Varghese, Nancy Pandita

Department of Pharmaceutical Analysis, Shobhaben Pratapbhai Patel, School of Pharmacy and Technology Management, SVKM's NMIMS, Mumbai, Maharashtra, India

Abstract

Aims: *Withania somnifera* is widely employed as a rejuvenator and expected to promote physical health and increase longevity. The aim of the present research work was to evaluate Cytochrome P450 3A (CYP3A) interaction of *Withania somnifera*. **Materials and Methods:** *In vitro* CYP3A interaction of methanolic extract of *Withania somnifera* (WS) and its principal phytoconstituents: Withaferin–A (WA), Withanolide–A (WL-A) and Withanoside–IV (WS-IV) were investigated in rat and human liver microsomes. *In vivo* CYP3A interaction potential was investigated by administering methanolic extract of WS orally at a dose of 500 mg/kg in female Wistar rats. Sildenafil citrate was used to index the activity of CYP3A. **Results:** IC_{50} values of methanolic extract of *Withania somnifera,* WA, WL-A, WS-IV were found to be 200 μg/ml, >20 μM, >64 μM and >64 μM for CYP3A both in rats and humans, respectively. When sildenafil citrate was orally co-administered with methanolic extract of WS and compared with orally administered sildenafil citrate alone, the area under plasma concentration time (AUC) curve and C_{max} did not significantly differ as compared to the group which received rifampicin orally (positive control). **Conclusions:** Results suggested that methanolic extract of WS, WA, WL-A, WS-IV showed no *in vitro* CYP3A inhibition in rats and humans. Methanolic extract of WS did not significantly alter the pharmacokinetics of sildenafil citrate in rats; indicating its safety when co-administered with other drugs that are substrates of CYP3A. Thus the results indicate the lesser likelihood of drug herb interactions when concomitantly administered with CYP3A substrates.

Key words: Cytochrome 3A interaction, human liver microsomes, rat liver microsomes, sildenafil citrate

INTRODUCTION

The most versatile enzyme system involved in the metabolism of xenobiotics is cytochrome P450. The cytochrome P450 enzymes (CYP) represent a large family of proteins involved in the metabolism of drugs and other xenobiotics, as well as some endogenous substrates. The major drug-metabolizing enzyme (DME), cytochrome P450 (CYP), consists of the superfamily of hemeproteins that catalyze the oxidative metabolism of a wide variety of exogenous chemicals including drugs. Several isoforms, such as CYP1A2, CYP2C9, CYP2D6 and CYP3A4 appear to be the most relevant isozymes involved in the metabolism of clinically significant drugs. Inhibition of these enzymes often results in unexpected and sometimes severe adverse drug interactions, as the metabolic clearance of co-administered drugs can get be altered dramatically.[1]

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Address for correspondence:
Asst. Prof. Alice Varghese, Department of Pharmaceutical Analysis, Shobhaben Pratapbhai Patel, School of Pharmacy and Technology Management, SVKM's NMIMS, Mumbai, Maharashtra, India. E-mail: alicevarghese02@gmail.com

Drug interactions can frequently arise when drugs are co-administered and one drug inhibits the metabolic clearance of the second drug by inhibition of a specific CYP enzyme. Inhibition of CYP enzymes can also be effected by natural products. A notable example of this is the inhibition of CYP3A by grapefruit juice, which can result in elevations of systemic exposure to CYP3A-cleared compounds.[2]

The CYP3A family of enzymes constitutes the most predominant Phase I drug-metabolizing enzymes and accounts for approximately 30% of hepatic CYP and more than 70% of intestinal CYP activity. Moreover, CYP3A is estimated to metabolize between 50-70% of currently administered drugs. The level of CYP3A activity can be decreased by inhibition of enzyme activity, or increased by induction of new protein synthesis. Changes in CYP3A activity, either through inhibition or induction, can result in potentially serious drug-drug interactions (DDIs). DDIs caused by induction can increase the clearance of many co-administered drugs, either resulting in a potential loss of treatment efficacy, or leading to severe intoxication in the case of prodrugs.[3,4]

There is increasing consumption of medicinal herbs and herbal products globally, cutting across social and racial classes as it has been observed it is observed both in developing and developed countries. According to the World Health Organization (WHO), about 70% of the world population currently uses medicinal herbs as complementary or alternative medicine. Herb-drug interactions (HDIs) are one of the most important clinical concerns in the concomitant consumption of herbs and prescription drugs. The necessity of polypharmacy in the management of most diseases further increases the risk of HDI in patients. The ability of intestinal and hepatic CYP to metabolize numerous structurally unrelated compounds, The ability of intestinal and hepatic CYP to metabolize numerous structurally unrelated compounds is responsible for the large number of documented drug-drug and drug-food interactions. for the large number of documented drug-drug and drug-food interactions.[5]

Withania somnifera Dunal (WS), commonly known as ashwagandha, Indian ginseng and Winter cherry has been used for centuries in Ayurvedic medicine to increase longevity and vitality.[6,7] Western research supports its polypharmaceutical use, confirming antioxidant, anti-inflammatory, immune-modulating, and anti-stress properties in the whole plant extract and several separate constituents.[8] As an antioxidant, WS and active constituents, sitoindosides VII-X and Withaferin A (WA), Withanolide-A (WL-A) and Withanoside I-IV have been proven to increase levels of endogenous superoxide dismutase, catalase, and ascorbic acid, while decreasing lipid peroxidation.[9-12] It also shows certain potential therapeutic activities like anti-tumor and antibacterial.[13-16]

In the present research work, we have evaluated the *in vitro* and *in vivo* interaction potential of *Withania somnifera* with CYP3A in rats and humans to indicate the possibility of herb-drug interactions.

MATERIALS AND METHODS

Chemicals and equipment

All the solvents, chemicals and reagents used were of analytical grade and purchased locally. Testosterone and 6-hydroxy Testosterone were gift samples from AvikPharma, Vapi, Gujarat and Piramal Life Sciences Ltd, Mumbai, respectively. Phenacetin and Caffeine were purchased from Sigma-Aldrich Ltd. Nicotinamide adenine dinucleotide phosphate-reduced tetrasodium salt (NADPH) was purchased from SRL Labs Pvt. Ltd. HPLC grade acetonitrile was purchased from Thermo Fischer Scientific India Pvt. Ltd. Withaferin-A, Withanolide-A and Withanoside-IV were purchased from Natural Remedies, Bangalore, India and human liver microsomes (HLM) from Invitrogen Services.

HPLC system consisted of a Shimadzu LC 2010, with an autosampler, Photo Diode Array (PDA) detector using LC Solutions® software.

Preparation of test samples

Fresh roots of *Withania somnifera* (WS) were purchased from Zandu Foundation, Gujarat, India. Roots were identified by Dr. Naik, Senior Research Scientist, Piramal Life Sciences Ltd. and aunthenticated by Agharkar Research Institute, Pune with the voucher specimen (R-128) deposited for further reference. Fresh roots were dried, powdered and extraction was carried out by cold maceration with methanol following defatting with Petroleum Ether (60-80°C). The methanolic extract was evaporated to dryness by rotary vacuum evaporator, yielding a semi-solid residue. The yield of the methanolic extract residue was about 2.5% (w/w). This methanolic extract residue was stored in vacuum desiccator until further use. Solutions of methanolic extract, Withaferin-A (WA), Withanoside-IV (WS-IV) were prepared in methanol and Withanolide-A (WL-A) was dissolved in DMSO and used for the studies The methanolic extract of *Withania somnifera*,Withaferin A (WA) and Withanoside IV (WS

-IV) were solubilized in methanol and Withanolide A (WL-A)was solubilized in DMSO and used further in the study

Preparation of rat liver microsomes

Rat liver microsomes were isolated from male Swiss Wistar (150-200 g) strain rats based on the methods described by the calcium aggregation method.[17] The experiment was performed according to the guidelines of Institutional Animal Care Committee constituted as per the guidelines of the Committee for the purpose of control and supervision of experimental animals (CPCSEA) and the protocol [Protocol no. CPCSEA/IAEC/SPTM/P-59/211] was duly approved by the Institutional Animal Ethics Committee. Briefly, the rats were euthanized by cervical dislocation and the livers (20 g) were quickly removed, perfused with 1.15% potassium chloride (KCl) solution and homogenized with four volumes (80 ml) of ice cold 10 mMTris-HCl buffer containing 0.25 M Sucrose, pH 7.4, in a Potter glass homogenizer equipped with a Teflon pestle. The homogenate was centrifuged at 13,000 × g for 10 min at 4°C in a refrigerated centrifuge (Eppendorff) and the precipitate was discarded. To the supernatant, calcium chloride was added to yield a final concentration of 10 mM. The solution was stirred for 15-20 min and then centrifuged at 25,000 × g for 10 min at 4°C. The firmly packed pellets of microsomes were resuspended by homogenization in 100 mMTris-HCl buffer containing 20% w/v glycerol and 10 mM EDTA, pH 7.4. The microsomes were stored at −80°C until use. Protein concentrations were determined by Folin Lowry method[18] using bovine serum albumin as standard. The optical density was recorded on a Perkin Elmer UV/vis spectrophotometer at 625 nm.

CYP3A inhibition assay in rat and human liver microsomes

Rat and human liver microsomes were used for assessing the inhibition potential of methanolic extract of *Withania somnifera* (10-640 µg/ml), Withaferin-A (1-20 µM), Withanolide-A (1-64 µM) and Withanoside-IV (1-64 µM) by estimating testosterone hydroxylation activity, and the inhibition potential was compared with the positive control Ketoconazole (known CYP3A4 inhibitor in rats and humans). Briefly, a standard 100 µL incubation mixture contained liver microsomes (0.5 mg/ml and 0.25 mg/ml protein concentration in RLM and HLM, respectively), Testosterone [40 µM in RLM and 100 µM in HLM[19]] in 0.1 M sodium phosphate buffer pH 7.4 at 37°C was incubated for 30 min for WS and WS-IV, and 15 min for WA and WL-A (WA and WL-A are unstable in RLM and HLM), in duplicate. The reactions were initiated with NADPH (final concentration 1 mM) and then terminated with 50 µl of internal standard Caffeine (50 µg/mL) in acetonitrile. The samples were centrifuged at 4000 rpm for 10 min at 4°C and the supernatants were subjected to Reverse phase high performance liquid chromatography (RP-HPLC) analysis. Samples were run on a Shiseide Cyano column 5 µ (4.6 mm × 150 mm) and mobile phase used was (A) HPLC-grade Water and (B) HPLC-grade Acetonitrile and was pumped at a flow rate of 1 ml/min. The gradient program used was Time: %B – 0/20; 8/50; 12/60; 15/20; 16/20. Detection of testosterone and its metabolite 6-hydroxy testosterone was accomplished by Ultra violet (UV) absorbance at a wavelength of 245 nm. Modulatory effects of methanolic extract of WS, WA, WL-A, WS-IV were evaluated by incubation of rat and human liver microsomes, testosterone with or without crude extract/marker compounds. Solutions of different concentration of the methanolic extract of WS, WA and WS-IV were prepared in methanol and WL-A in dimethyl sulfoxide (DMSO). Negative control incubations with methanol and DMSO and positive control incubations with ketoconazole were run simultaneously. Each concentration was run in duplicate. In all the incubations organic content was not more than 1%v/v. The formation of 6-OH Testosterone was subsequently quantified using RP-HPLC. Retention times for Caffeine and 6-OH Testosterone were 2.5 and 5.05 min, respectively. IC_{50} values were calculated using Graph Pad Prism®.

In vivo pharmacokinetic study design in rats

Male Swiss Wistar albino rats were selected for the *in vivo* pharmacokinetic (PK) study. They were housed under standard conditions for a week. The experiment was performed according to the guidelines of the Institutional Animal Care Committee constituted as per the guidelines of the CPCSEA and the protocol [Protocol no. CPCSEA/IAEC/SPTM/P-60/211] was duly approved by the Institutional Animal Ethics Committee. Wistar rats were randomly divided into three groups (n = 6). Group I was orally administered 0.5% sodium carboxymethyl cellulose for 10 days. Group II was orally administered methanolic extract of *Withania somnifera* (500 mg/kg suspended in 0.5% sodium carboxymethyl cellulose) for 10 days. Group III was orally administered rifampicin (50 mg/kg suspended in 0.5% sodium carboxymethyl cellulose) for 10 days. Twenty-four hours after the last dose, animals of Groups I-III were administered with a single oral dose of sildenafil citrate (200 mg/kg suspended in 0.5% sodium carboxymethyl cellulose). Whole blood samples (500 µL) were withdrawn from the retro-orbital sinus at 5, 15, 30, 60, 120, 240 and 360 min

after sildenafil citrate administration. Disodium edetate was used as the anticoagulant. The blood samples were centrifuged at 4000 rpm for 10 min at 4°C and plasma was separated and stored at – 30°C until RP-HPLC analysis was carried out.

Plasma sample preparation and analysis

To a 100-µL plasma sample, 10 µL of Caffeine (internal standard) was spiked. Samples were then vortex-mixed for 1-2 min and extracted with 1 mL Acetonitrile by vortex-mixing for 5 min. After centrifugation at 4000 rpm, 4°C for 10 min, the upper organic layer was separated and evaporated to dryness at 30°C in a nitrogen evaporator under a gentle stream of nitrogen. The residue was reconstituted with 100 µL mobile phase (A-Water: B-Acetonitrile in the ratio 1:1), centrifuged and the supernatant was subjected to RP-HPLC analysis.

Pharmacokinetic and statistical analysis

All pharmacokinetic parameters were determined by non-compartmental analysis. Plasma concentration against time curves was constructed. The peak plasma concentration (Cmax) was directly obtained from the graph and the area under the plasma concentrations against time curve (AUC) was obtained by the trapezoidal rule.

Comparisons between the control and the treated groups were performed by analysis of variance followed by one-way ANOVA by Dunnett's test using GraphPad Prism.

RESULTS

Cytochrome P450 inhibition assay

The concentration of protein present in isolated rat liver microsomes (RLM) was estimated by the Lowry *et al*., method and it was found to be 33 mg/ml.

In vitro CYP3A inhibition assay

Methanolic extract of *Withania somnifera* (10-640 µg/ml), WA (1-20 µM), WL-A (1-64 µM) and WS-IV (1-64 µM) were evaluated for the CYP3A inhibitory activity in both rat and human liver microsomes ($n = 2$). Figure 1 shows a representative chromatogram of Caffeine (IS), 6-OH Testosterone and Testosterone in microsomes. The assay was performed as mentioned earlier and IC_{50} values of methanolic extract of WS [Figure 2], WA [Figure 3], WL-A [Figure 4] and WS-IV [Figure 5] was found to be 200 µg/ml, >20 µM, >64 µM and > 64 µM respectively for CYP3A, both in rats and humans.

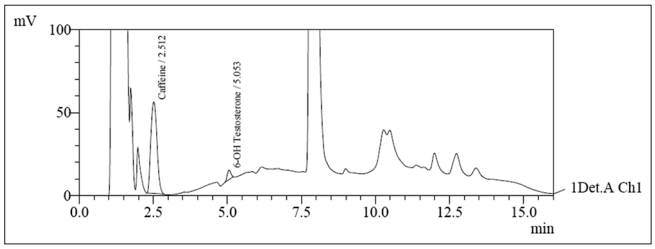

Figure 1: Representative chromatogram of Caffeine, 6-OH Testosterone and Testosterone

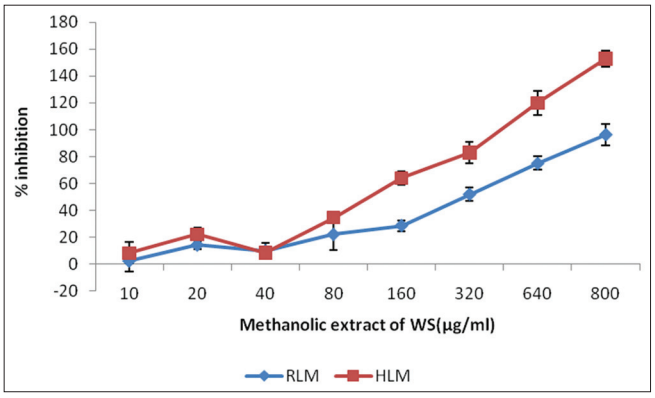

Figure 2: *In vitro* inhibitory effect of methanolic extract of *Withania somnifera* (10-800 µg/ml) on CYP3A4 in RLM and HLM. Values expressed as Mean ± SD ($n = 2$)

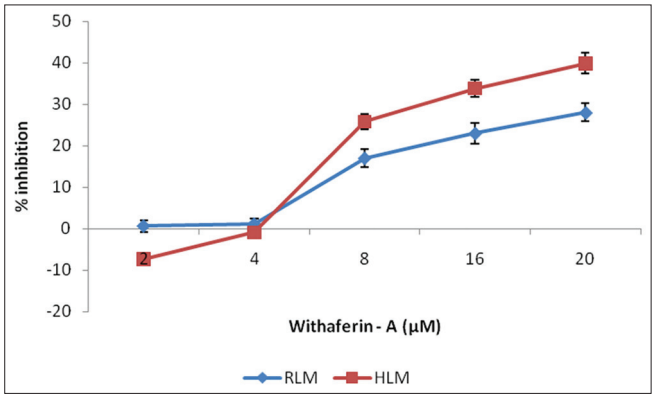

Figure 3: *In vitro* inhibitory effect of Withaferin-A (1-20 µM) on CYP3A4 in RLM and HLM. Values expressed as Mean ± SD ($n = 2$)

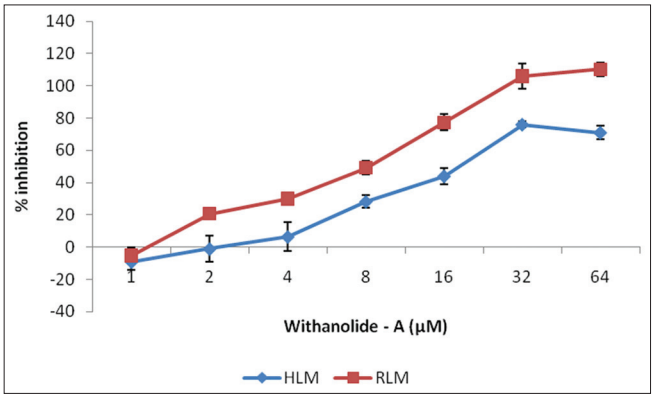

Figure 4: *In vitro* inhibitory effect of Withanolide–A (1-64 µM) on CYP3A4 in RLM and HLM. Values expressed as Mean ± SD ($n = 2$)

Table 1: Pharmacokinetic parameters of sildenafil citrate in rats after oral administration of methanolic extract of *Withania somnifera* and rifampicin

Groups	Pharmacokinetic parameters (mean±SEM)	
	AUC (µg/ml)	C_{max} (µg/ml)
I (Positive control)	275±52.83	27±8.77
II (Test group)	244±33.93	26.50±3.87
III (Standard group)	51±16.46*	10.41±2.55*

Values are expressed as mean±SD, *n*=6 in each group, *P<0.05 when standard group (Group III) compared with positive control (Group I) using One-way ANOVA by Dunnett's test; AUC: Area under curve

IC_{50} values <100 µg/ml for herbal extracts and <10 µM for active constituents are considered to be potent inhibitors of CYP450 enzymes.[19] Hence, there is no significant inhibition of CYP3A by WS, WA, WL-A and WS-IV in rats and humans.

In vivo pharmacokinetic study

Interaction potential with CYP3A in rats was determined by comparing the pharmacokinetic parameters [Area under Curve (AUC) and C_{max}] of CYP3A specific probe substrate sildenafil citrate at a dose of 200 mg/kg, p.o.[20] when administered alone and after oral administration of methanolic extract of *Withania somnifera* at dose of 500 mg/kg, p.o[21] for 10 days. Group II showed no significant change in AUC and C_{max} values in comparison with Group I indicating no significant interaction with CYP3A whereas Group III showed significant reduction in AUC and C_{max} values indicating specific CYP3A induction [Table 1]. Hence the study shows that the pharmacokinetic parameters of sildenafil citrate remained unaffected in rats after administration of methanolic extract of *Withania somnifera* which indicates no significant drug-herb interaction [Figure 6].

DISCUSSION

Trends in the usage of complementary and alternative medicine, and the use of herbal supplements was reported to have increased from 2.5% in 1990 to 12.1%, and was the second most common form of complementary and alternative medicine used in 1997.[22]

In Ayurveda, *Withania somnifera* is widely claimed to have potent aphrodisiac, sedative, rejuvenative and life-prolonging properties. It is also used as a general energy-enhancing tonic known as Medharasayana, which means 'that which promotes learning and a good memory' and in geriatric problems. The plant

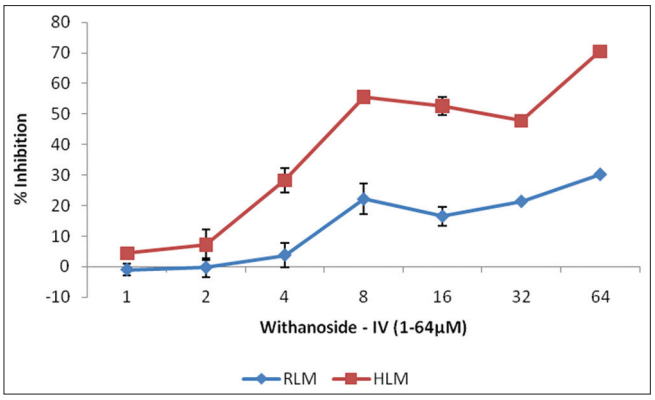

Figure 5: *In vitro* inhibitory effect of Withanoside–IV (1-64 µM) on CYP3A4 in RLM and HLM. Values expressed as Mean ± SD (n = 2)

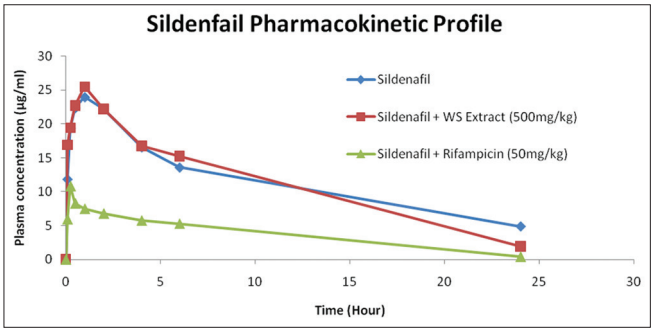

Figure 6: Plasma concentration–time curve of sildenafil citrate; 200 mg/kg in rats after oral administration of methanolic extract of *Withania somnifera* (WS); 500 mg/kg and rifampicin (50 mg/kg). Values for each time point expressed as Mean ± SD (n = 6)

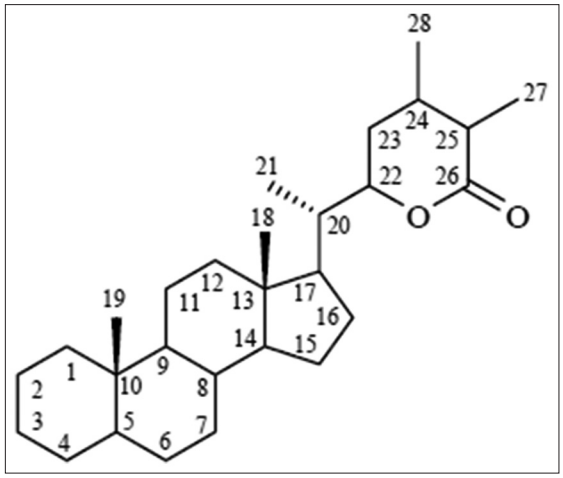

Figure 7: Basic structure of withanolides

was traditionally used to promote youthful vigor, endurance, strength, and health, nurturing the time elements of the body and increasing the production of vital fluids, muscle fat, blood, lymph, semen and cells. The similarity between these restorative properties and those of ginseng roots has led to Ashwagandha roots being called Indian ginseng.[23] The chemistry of *Withania* species has been extensively studied and

several groups of chemical constituents such as steroidal lactones, alkaloids, flavonoids, tannin, etc., have been identified, extracted, and isolated. The major chemical constituents of these plants, withanolides, are mainly localized in leaves, and their concentration usually ranges from 0.001 to 0.5% dry weight (DW) [Figure 7].[24] These compounds have been reported as the major constituents responsible for the various biological activities associated with the herbs and they were commercially available as pure compounds.

Withania somnifera is traditionally known as a tonic or adaptogenic, hence a widely used herb.

The inhibition of CYP enzymes can result in clinical drug interactions whereby the systemic exposure to one drug that is cleared primarily via CYP-mediated biotransformation is elevated when co-administered with a second drug that inhibits this activity. Such data can be used to predict whether the potential exists for a drug interaction *in vivo*.

To address whether *Withania somnifera* and its phytoconstituents inhibit or induce CYP3A in rats these parameters were evaluated.

The findings from this study suggested that the various concentrations of the methanolic extract of *Withania somnifera*, Withaferin-A, Withanolide-A and Withanoside-IV showed no significant *in vitro* CYP3A inhibition in rats and humans. Further methanolic extract of *Withania somnifera* caused no significant changes in the pharmacokinetic parameters of sildenafil citrate when administered orally in rats. Thus the data suggests that the methanolic extract of *Withania somnifera*, Withaferin-A, Withanolide-A and Withanoside-IV may not have any potential to lead to potential herb-drug interactions involved with CYP3A in rats and humans.

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