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Research Article

In-vitro assessment of CYP3A4 and CYP2C9 inhibition potential of Lupeol using human liver microsomes

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ABSTRACT

Background: Lupeol is a dietary triterpene, possesses numerous biological activities. Lupeol is currently under development for chemotherapy and chemoprevention. The aim of present study was to determine the potential inhibitory effect of Lupeol on cytochrome P450 (CYP3A4 and CYP2C9 isozymes) activities in human liver microsomes (HLM).
Methods: The inhibition studies were conducted using testosterone 6β-hydroxylase (CYP3A4), and diclofenac 4'-hydroxylase (CYP2C9) activity assay using positive control Ketoconazole and Sulphaphenazole, respectively. Inhibition study was performed by incubating Lupeol (0 to 20 µM) with human liver microsomes, and the metabolite formation was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).
Results: Luepol did not inhibit CYP3A4 and CYP2C9 isozymes mediated activities in human liver microsomes up to a maximum tested concentration of 20µM based on solubility under tested invitro conditions.
Conclusions*:* Lupeol is not an inhibitor of the CYP3A4 and CYP2C9 isozymes. IC_{50} is greater than the highest tested concentration as well as physiological concentration, where effect was measured with confidence. Therefore, clinically relevant pharmacokinetic herb-drug interactions are unlikely to occur between Lupeol and co-administered substrates of these CYP isozymes. Looking at the spectrum of biological activities and CYP inhibition potential of Lupeol; Lupeol can be used as adjuvant/ chemotherapy agent/ chemopreventive agent in therapy.

Keywords: Lupeol, HLM, CYP3A4 and CYP2C9, Inhibition, herb–drug interactions

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1. INTRODUCTION

Lupeol [Lup-20(29)-en-3β-ol] (Figure 1) is a dietary triterpene, also known as clerodol, fagarsterol, and lupenol. Lupeol is common constituent of grape, cocoa butter, hazelnut, olive oil, white cabbage, mango pulp, and a variety of therapeutic plants. Lupeol exhibits multiple pharmacological properties that include antiarthritic, antioxidant, anti-inflammatory, antitumor, antimalarial activity, antimicrobial, antiprotozoal, cancer chemopreventive and cardio-hepato protective [1-3]. Lupeol possesses anti-cancer activity against various cancers that include melanoma, prostate, head, and neck cancer. Activity of topoisomerase II (a known target for anticancer chemotherapy) is inhibited by Lupeol. Lupeol is currently under development for chemotherapy as well as chemoprevention [1].

Herbal medicines are increasingly being used as alternative medicines worldwide over the last few decades. Most of the herbal medicines are non-toxic, safe in nature, and easier to obtain, hence preferred over allopathic medicines [4]. As a result, it is very likely that some patients will take herbal medicines concomitantly with conventional prescription medications. This may lead to unwanted adverse effects produced by herbal-drug interactions [5]. Serious questions concerning the safety, quality, and efficacy of these products has raised due to widespread use of herbal medicines. Risk for herb-drug interactions has increased as more than 80% of worldwide population makes use of traditional medicines, often in combination with prescription drugs [6-8].

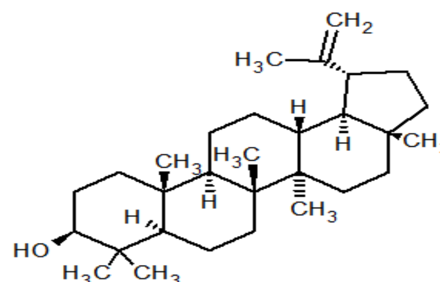

Figure 1: Structure of Lupeol

Cytochrome P450 (CYP) is a superfamily of mixed function oxidases that are responsible for the metabolism of many drugs (~ 80%) including anticancer agents. CYP3A4 and CYP2C9 isozymes are responsible for the metabolism of more than 60% of all prescribed drugs. When patients receive several medications concurrently, unwanted and life-threatening effects can result from the competition for the same drug-metabolizing enzyme affecting the blood levels of those drugs, leading to toxicity [9-12]. Numbers of new chemical entities and marketed drugs have been withdrawn from the pharmaceutical market because of toxicity issue [13]. Cancer patients would seem to be significantly at high risk, because CYP3A4 metabolizes a large number of anticancer drugs and patients are generally prescribed other medications to relieve symptoms (e.g., analgesics) and side-effects (e.g., antidiarrheals and antiemetics) and to treat comorbidities [14]. For a new molecular entity, it is mandatory to assess its likely inhibitory or inductive effects at preclinical drug discovery research level as recommended by the U.S. Food and Drug Administration and the EMEA guidelines [15-16].

Lupeol possesses several biological activities. Hence, it is likely that Lupeol will be administered with other therapeutic agents in clinical settings. This study was conducted to evaluate the potential of lupeol for significant metabolic-based interactions in human liver microsomal enzymes. Seervi et. al. [17] clearly states that further studies of lupeol on human liver microsomes will help in confirming CYP-mediated interaction potential in human beings. Peak plasma concentration of lupeol upon administration of 200mg/kg one time intra peritoneal dose is 5.22 µM at 8 hrs in mice [2]. The concentrations of Lupeol employed in this study based on solubility of Lupeol span and exceed the C_{max} reported by Siddique et. al. [2].

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Lupeol (purity 99 %) was purchased from Natural Remedies Private Limited, Bangalore, India. Ketoconazole, Sulfaphenazole, Diclofenac, and Testosterone were received as gift Samples from CIPLA (Patalganga Panvel, Maharashtra, India). Corticosterone, Hydroxy- Diclofenac D4, Hydroxy-Testosterone and Hydroxy-Diclofenac were obtained from CDRI (Luckhnow, Delhi, India). Human liver microsomes were procured from Sekisui XenoTech, LLC (Kansas City, North America). Nicotinamide adenine dinucleotide phosphate (NADPH), formic acid 98% (MS grade), Potassium phosphate monobasic and Potassium phosphate dibasic were purchased from Sigma-Aldrich; Fluka (St. Louis, MO, USA). HPLC- grade acetonitrile and dimethyl sulphoxide (DMSO) were obtained from Merck (Mumbai, India). All other chemicals and reagents were of analytical grade and procured from commercial suppliers in India.

2.2 LC-MS/MS Instrument and Conditions

Separation was performed on HPLC System (Shimadzu Corporation, Kyoto, Japan). HPLC system equipped with LC-20AD binary pump, a DGU20A degasser, an SIL-HTC auto-sampler and a CTO-20A thermo-stated column oven maintained at 40°C during analysis. Optimized Chromatographic conditions are mentioned in table 1.

Table 1: Optimized chromatographic conditions employed in analysis

<table>
<tr><th>Parameter</th><th colspan="2">CYP3A4 Inhibition Assay</th><th colspan="2">CYP 2C9 Inhibition Assay</th></tr>
<tr><td>Mobile phase A</td><td colspan="2">0.1% Formic acid</td><td colspan="2">10 mM Ammonium Formate with 0.1% formic acid</td></tr>
<tr><td>Mobile phase B</td><td colspan="2">Acetonitrile</td><td colspan="2">Acetonitrile</td></tr>
<tr><td>Flow rate</td><td colspan="2">0.8ml/min</td><td colspan="2">0.8ml/min</td></tr>
<tr><td>Run time</td><td colspan="2">5.0 min</td><td colspan="2">5.7 min</td></tr>
<tr><td>Column</td><td colspan="2">Thermo Scientific, Acclaim polar advantage, 4.6*50mm, 3.5µm</td><td colspan="2">Agilent, Zorbax SB-CN, 4.6*50mm, 3.5µm</td></tr>
<tr><td rowspan="2">Retention time</td><td colspan="2">6-β-hydroxy testosterone: 3.08 min</td><td colspan="2">4-OH-diclofenac: 3.10 min</td></tr>
<tr><td colspan="2">Corticosterone: 3.24 min</td><td colspan="2">4-OH-diclofenac-D4: 3.10 min</td></tr>
<tr><td rowspan="6">Gradient programme</td><td>Time (min)</td><td>% B</td><td>Time (min)</td><td>% B</td></tr>
<tr><td>0.5</td><td>20</td><td>0.5</td><td>20</td></tr>
<tr><td>2.0</td><td>95</td><td>2.5</td><td>90</td></tr>
<tr><td>3.5</td><td>95</td><td>3.7</td><td>90</td></tr>
<tr><td>3.6</td><td>20</td><td>3.8</td><td>20</td></tr>
<tr><td>5.0</td><td>STOP</td><td>5.7</td><td>STOP</td></tr>
</table>

Mass spectrometric detection was performed on an API-4000 triple quadrupole instrument from Sciex (Applied Biosystems, Foster City, CA, USA) using MRM (Multiple Reaction Monitoring) mode. Optimized electrospray ion source parameters are mentioned in Table 2. Optimized multiple reaction monitoring (MRM) parameters for analyte and internal standard (IS) are mentioned in Table 3. Data acquisition and analysis were performed using the analyst software version 1.6 (Applied Biosystems, Foster City, CA, USA).

Table 2: Optimized source parameters employed in analysis

Source Parameter	CYP3A4 Inhibition Assay	CYP 2C9 Inhibition Assay
Ionization mode	ESI Positive	ESI Negative
Curtain gas (psi)	20	20
Collision-activated dissociation (CAD) gas (units)	6	**6**
Gas-1(psi)	50	50
Gas-2 (psi)	70	70
Ion Spray Voltage (V)	5500	-4500
Ion source temperature (°C)	500	500

Table 3: Optimized Multiple Reaction Monitoring (MRM) parameters employed in analysis

Name of Metabolite/ Internal Standard	Parent ion (m/z) (Q1)	Daughter ion (m/z) (Q3)	DP	EP	CE	CXP
CYP3A4 Inhibition Assay - Positive polarity						
6-β-hydroxy testosterone	305.20	287.5, 269.70	61	10	19,18	5
Corticosterone*	347.18	121.20, 329.30	45	10	33,21	4
CYP 2C9 Inhibition Assay - Negative polarity						
4-OH-diclofenac	310.10	265.90	-45	-10	-20	-15
4-OH-diclofenac-D4*	314.03	269.90, 234.10	-50	-10	-18,-14	-19,-5

*used as internal Standard; DP: declustering potential (V); EP: entrance potential (V); CE: collision energy (V) and CXP: collision cell exit potential (V)

2.3 Microsomal incubation

Stock solution of Lupeol and all other substrates were prepared in DMSO. Stock solutions of positive controls were prepared in acetonitrile; further dilutions were prepared in acetonitrile: DMSO (80:20 v/v). Testosterone and Diclofenac were used as selective probe substrates for CYP3A4 and CYP2C9, respectively. Testosterone 6β-hydroxylase and diclofenac 4'-hydroxylase assays were standardized with modification of reported methods [18-24].

Standardization of incubation conditions

Incubation time and protein concentration were standardized in the human liver microsomes. The Km values for each of the respective marker substrates were determined using a range of substrate concentrations with optimized protein concentration and time.

2.4 Data analysis

The standard selective inhibitors, namely, sulfaphenazole, and ketoconazole were used for CYP2C9, and CYP3A4 inhibition, respectively. The IC_{50} values of the inhibitors were determined graphically (plot of the logarithm of inhibitor concentration versus the percentage of enzyme activity) by fitting a non-linear regression constrain (2PL) of Bottom constant equal to ''0'' and Top constant equal to ''100'' by Graph Pad Prism 6.0 (GraphPad software Inc., CA, USA). Area ratio (Metabolite/IS) and inhibitor concentration in µM is used to calculate the IC_{50} by Graph Pad Prism 6.0.

3. RESULTS

3.1 Standardization of incubation conditions

Incubation conditions were standardized by incubating the respective probe substrates with different protein concentrations (0.10–1.0 mg/mL) of microsomes and by terminating the reactions at different time intervals (0–60 min). The optimum incubation time and protein concentration was selected on the basis of linear metabolite formation. For the determination of Km, different concentrations of CYP450 substrates were incubated with the standardized protein concentrations. Km values were determined by the Eadie-Hofstee plot and Lineweaver-Burk plot and final optimized incubation conditions are mentioned in Table 4.

Table 4: Standardized Incubation conditions

Parameter	CYP 3A4 Inhibition assay reaction conditions	CYP 2C9 Inhibition assay reaction conditions
Buffer	50mM Potassium Phosphate Buffer (pH 7.4)	50mM Potassium Phosphate Buffer (pH 7.4)
Substrate conc. Km	Testosterone, 65 µM	Diclofenac, 10 µM
Positive control conc. used (µM)	Ketoconazole: 1,0.5,0.25,0.062,0.031,0.016,0.008,0.004,0.002,0.001,0	Sulfaphenazole: 20,10,5,2.5,1.25,0.625,0.312,0.156, 0.078,0.039,0.019,0
Lupeol conc. used (µM)	20,10,5,2.5,1.25,0.625,0.312,0.156,0.078,0.039,0.019,0	20,10,5,2.5,1.25,0.625,0.312,0.156, 0.078,0.039,0.019,0
Protein (HLM)	0.15 mg/mL	0.15 mg/mL
Pre-incubation time and temp	5 min and 37 degree	5 min and 37 degree
NADPH	2 mM	2 mM
Final reaction Volume	200 µL	200 µL
Final Organic content (% v/v)	0.5% (0.4% Acetonitrile + 0.1% DMSO)	0.5% (0.4% Acetonitrile + 0.1% DMSO)
Incubation time and temp	5 min and 37 degree	5 min and 37 degree
Metabolite	6-β-hydroxytestosterone	4'-Hydroxydiclofenac
IS conc.	Cotricosterone (1 µM)	4-OH Diclofenac-D4 (1µM)
After incubation	Reaction was terminated by addition of 200 µL of ice cold acetonitrile containing IS, then samples were allowed for protein precipitation on ice for 5 min, then vortexed and centrifuged at 3000g for 10 min. Supernatants were transferred into 96-well plates for LC–MS/MS analysis.	

3.2 Inhibitory effect on CYP3A4 and CYP2C9 isozymes/ Determination of IC_{50}

Both positive control inhibitors produced inhibitory effects on the respective catalytic activities with IC_{50} value of 0.029 µM of Ketoconazole for CYP3A4 and 0.150 µM of sulphaphenazole for CYP2C9, observed IC_{50} values are by previous reports [18-19]. The compounds were classified as potent ($IC_{50} \leq 10$ µM), moderate (IC_{50} from 10 to 50 µM), or weak ($IC_{50} \geq 50$ µM) CYP inhibitors [25]. Lupeol did not inhibit

CYP2C9 and CYP3A4-mediated activities up to a 20µM concentration under the tested in vitro conditions. Figure 2-5 shows the relationship between inhibitor concentration and enzyme activity (enzyme activity was calculated by amount of formation of metabolite in presence of different concentration of inhibitor).

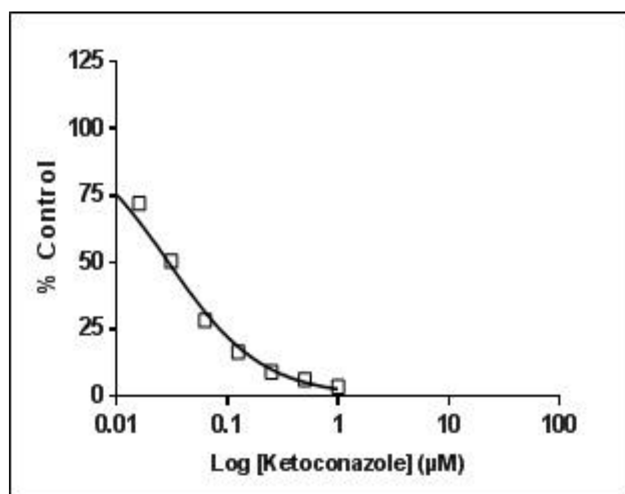

Figure 2: Log of Ketoconazole concentration (µM) Vs % Control (for Isozyme CYP3A4)

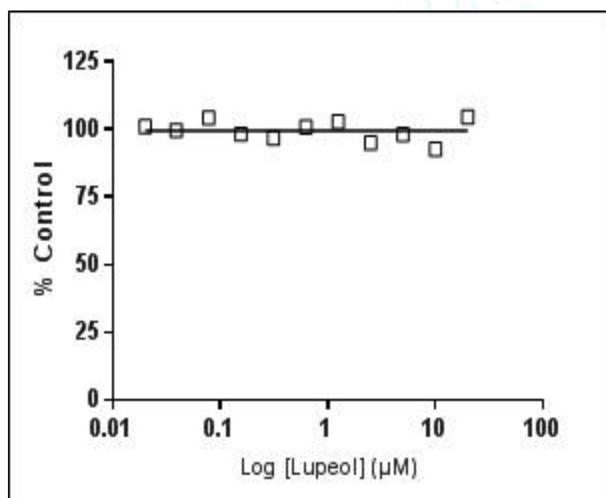

Figure 3: Log of Lupeol concentration (µM) Vs % Control (for Isozyme CYP3A4)

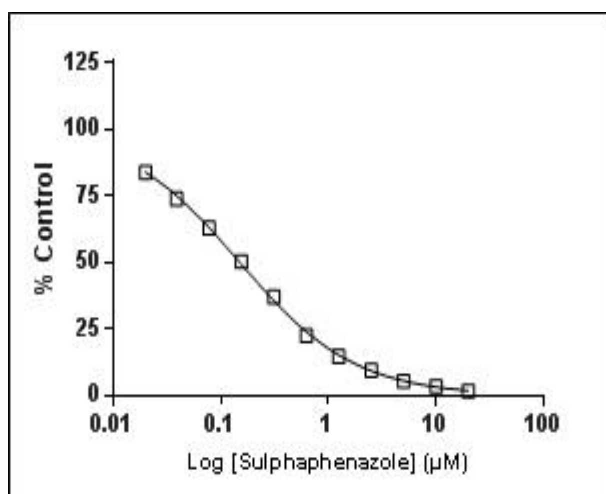

Figure 4: Log of Sulphaphenazole concentration (µM) Vs % Control (for Isozyme CYP2C9)

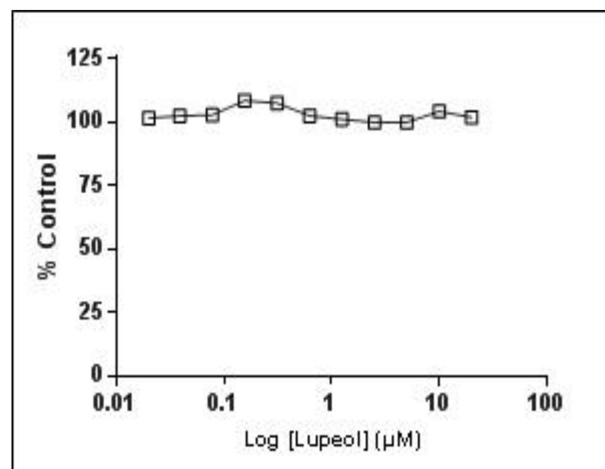

Figure 5: Log of Lupeol concentration (µM) Vs % Control (for Isozyme CYP2C9)

4. DISCUSSION

Currently, there has been an increase in the use of herbal remedies as complementary and alternative medicines along with the therapeutics drugs. More than 80% of the prescribed drugs are metabolized by CYP enzymes, therefore concomitant administrations of herbal and allopathic medicines are liable to serious herb-drug interactions [12]. The major isozymes present in human liver microsomes are CYP3A4/5, CYP2C9/19, and CYP1A2 [26]. There are several reported herb-drug interactions involving CYP inhibition, such as the components of grapefruit juice interfering with the metabolism of terfenadine and nifedipine, garlic with saquinavir, etc. [27-28]. Upon consideration of above-mentioned facts, it becomes imperative to predict herb-drug interactions with respect to CYP enzymes in the early drug discovery and development era.

Lupeol is widely distributed triterpenoid present in significant amounts in dietary substances and may modulate the CYP enzymatic activity. Lupeol exhibits several biological activities. Recently, Lupeol was studied in clinical settings for its anti-acne effect [29] and non-inflammatory acne lesions [30]. Lupeol is currently under development for chemoprevention, chemotherapy, and also called as 'magical drug' [31]. Therefore, it is likely that Lupeol will be administered with other therapeutic agents in clinical settings some of which are likely to be CYP3A4 and CYP2C9 substrates. It is important to understand any potential herb–drug interactions due to inhibition of these CYP enzymes by Lupeol. The results of this study demonstrated that Lupeol has very low propensity to interact with CYP isozymes and did not inhibit CYP3A4 and CYP2C9 enzymes in human liver microsomes.

Additional experiment of in-vitro metabolic stability study (mice, rat and human liver microsomes) and hepatocyte stability (mice, rat and human hepatocyte) of lupeol shows that lupeol did not undergo phase I or phase II metabolism (Data not shown). Hence, CYP enzymes did not play a role in metabolic clearance of Lupeol. Therefore, co-administration of any potent CYP inhibitors (e.g., ketoconazole, fluconazole, fluoxamine, and quinidine)) or inducers are not likely to result in clinically relevant effects on the exposure of Lupeol.

Peak plasma concentrations of Lupeol reported by Siddique et. al. [2] upon administration of 200mg/kg one time intra peritoneal dose is 5.22µM in mice, which is considerably lower than the highest concentration (20µM) evaluated in

this in vitro study. Also, CYP induction study conducted by Seervi et al. [17] demonstrated that lupeol did not have any induction potential. Therefore, Lupeol is not anticipated to precipitate clinically relevant pharmacokinetic herb–drug interaction when co-administered with CYP substrates due to inhibition or induction.

5. CONCLUSION

Lupeol is not an inhibitor of CYP3A4 and CYP2C9 enzymes. IC_{50} is greater than the highest tested concentration as well as physiological concentration. Therefore, clinically relevant pharmacokinetic herb–drug interactions are unlikely to occur between Lupeol and co-administered substrates of these CYP isozymes. Looking at the spectrum of biological activities and CYP inhibition potential of Lupeol; Lupeol can be used as adjuvant/ chemotherapy agent/ chemopreventive agent in therapy.

Author Contributions

The manuscript was written through the contributions of all authors. All authors have approved the final version of the manuscript.

Conflict of Interest Disclosure

The authors declare no competing financial interest.

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