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In vitro and ex vivo study on modulatory effect of Berberine induced CYP3A4 mediated drug metabolism

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Abstract
Berberine which is a natural phytochemical abound in traditional medicine has been reported to affect drug metabolism by inhibiting the cytochrome P450 enzyme especially the CYP3A4. The objective of this study was to determine the impact of berberine on CYP 3A4 activity in in vitro human liver microsome and ex vivo rat intestinal perfused model. With regards to microsomal assays berberine showed dose-dependent inhibition of midazolam hydroxylation with IC 50 of 12.3 µM. Non-competitive inhibition was also confirmed in the kinetic studies which implied that berberine could bind to CYP3A4 at another location rather than the substrate binding site. Midazolam intestinal perfusion presents a considerable reduction in absorption after perfusion with berberine cocktail in co-perfusion (p < 0.01) in ex vivo intestinal perfusion, which signifies the presystemic blockage of CYP3A4. Also the RT-PCR analysis of the rat intestinal mucosa demonstrated that the CYP3A4 mRNA expression was down regulated after a 7- day pretreatment with berberine. These findings stress the possibility of herb-drug interactions with berberine, especially those drugs metabolized by CYP3A4, and warrant caution that need to be clinically monitored and regulated.

Keywords: ***Berberine, CYP3A4, drug metabolism, and microsomal assay, intestinal perfusion, and herb-drug interactions.***

1. Introduction

1.1 The value of CYP3A4 with regards to Drug Metabolism and Disposition

The cytochrome P450 enzyme (CYPs) is family of enzymes that are essential in the breakdown of a large number of endogenous and exogenous chemicals, like drugs, toxics and hormones. CYP3A4 is the most common of the 57 known human CYP isoforms, with a major role in the oxidative metabolism of drugs, and is responsible of some 50% of phase I drug metabolism in liver. The enzyme plays a role in metabolism of diverse amount of pharmacologically active substances, which are antihypertensives, antifungal, immunosuppressants, statins, and anti-retrovirals.

CYP3A4 activity influences the absorption, distribution, metabolism and excretion of these drugs. Alternations in the activity of CYP3A4 that may be caused by genetic polymorphism or by variations in environmental conditions can cause major changes in both drug levels and efficacy. Blocking or stimulation of CYP3A4 may also affect the therapeutic index of drugs, which have a narrow therapeutic index as is the case with warfarin, theophylline and cyclosporine. Consequently, regulating and modulating of CYP3A4 is valuable since gaining the knowledge is necessary to improve drug therapy and reduce side effects of drugs.

1.2 Herbal Compounds Herbal Compounds such as berberine are widely used in folk medicine.

Traditional healing systems have had herbal medicine as one of their foundational lines, and many plant-based compounds have been used because of their remedial impact. Berberine is one example; it is a plant alkaloid made by many herbs like Berberis genus (e.g. Berberis vulgaris, Berberis aristata), coptis chinensis and phellodendron amurense. In traditional medicine, berberine has long been used in many different countries e.g. China, India, and South Korea to treat various diseases, e.g. diabetes, hyperlipidemia, and infections, as well as gastrointestinal disorders.(1)

New discoveries have resulted to more upsurge in the interest of berberine because of its wide-range pharmacological properties such as antidiabetic, anti-inflammatory, antioxidant, antibacterial, and cardioprotective properties. In addition, berberine is proven to affect multiple key processes in the body, including AMP-activated protein kinase (AMPK), NF-kappa B and PPAR-gamma, which have been attributed to its broad therapeutic effects. Although these are some of the positive implications, interaction between berberine and

conventional drugs is increasing especially since it is our common practice to co-administer berberine with medications of chronic illnesses.

1.3 Possible Hazards of Herb-Drug Interactions, in Specific by Changing CYP450

Although with a demonstrated therapeutic value, berberine has also been linked to herb-drug interactions, which is mostly caused when it influences cytochrome P450 enzymes. Because of being a strong CYP3A4 inhibitor, berberine may profoundly influence the metabolism of pharmacological substances that are CYP3A4 substrates. This interaction may result in rise in drug levels, toxicity and negative effects of the drug especially when the drug may have a narrow therapeutic index.(2)

As an example, statins, a type of drug that are frequently used to achieve hypercholesterolemia, get processed mostly by CYP3A4. When berberine is co-administered with statins, chances are high that the concentration of the statins in the body would be augmented and as a result, there would be an amplification of myopathy and rhabdomyolysis. In the same way, there are other classes of drugs that can be metabolized by CYP3A4 such as benzodiazepines, calcium channel blockers, immunosuppressants, and the enzyme inhibitory action of berberine may alter the plasma concentrations of such drugs.

Also intestinal presystemic metabolism may be an important contributor to the absorption of orally ingested drugs and berberine has been demonstrated to inhibit intestinal mucosal CYP3A4 activity and this could be a factor that alters drug orally administered drug bioavailability where there is a major component of first-pass effect. This pre systemic inhibition has the potential of resulting in drug absorption and induced therapeutic responses.

Since there is extensive use and popularity of herbal supplement such as berberine, there is a need to evaluate the possibility of the herb drug interactions to establish the safety and effectiveness of any coexisting drug therapy.

1.4 Study Objective: Investigating how berberine can inhibit CYP3A4 with the utilization of a combination of in vitro and ex vivo researches and experiments.

In order to better understand the inhibitory ability of berberine on CYP3A4 activity, the work focused on accomplishing two different and simultaneous nectarine investigations; an in vitro micro assay and ex vivo rat intestinal perfusion. To assess the effect of berberine on CYP 3A 4 drug metabolism, the dose-dependent inhibition in midazolam hydroxylation, that is well-identified as a CYP 3A 4 substrate, was measured through the in vitro model involving human liver microsomes. Also, the in vitro intestinal perfusion model was utilized to determine the rat intestine presystemic CYP3A4 inhibition. The study also sought to find out whether pre-administration of berberine has any effect on CYP 3A 4 gene expression in the intestine.

This study will allow conducting microsomal assays as well as intestinal perfusion studies to obtain a complete picture of risks to which berberine-mediated herb-drug interactions may expose patients and the clinical significance of the modulation of CYP3A4. The results of the present research will be important contributions to the understanding of the safety of berberine when co-administrated with CYP3A4 substrate drugs, especially the drugs with a narrow therapeutic index, and the importance of appropriate monitoring and regulatory consideration to the combination of herbal drugs with conventional medicines.

2. Study Materials and Methods

2.1 Source of Microsomes 2.1. Chemicals, Reagents

Analytical grade chemicals and reagents involved in this investigation were purchased using reliable suppliers. Berberine chloride > 98) was obtained in Sigma-Aldrich (USA) and the midazolam (standard) was delivered by Abcam (UK). The drug that was utilized as the substrate of CYP3A4 activity was midazolam, which was used to assess the inhibitory power of berberine. Other cofactors used in the microsomal assays as well as NADPH were supplied by Thermo Fisher Scientific (USA).(3)

The human liver microsomes (HLM) prepared by XenoTech LLC (USA) distributes commercially prepared microsomal preparation in the human liver. The use of the microsomal preparations consisting of a CYP3A4 enzyme activity similar to that of healthy liver tissue was known to evaluate the impact of berberine on CYP3A4-mediated drug metabolism.

TRIzol reagent (Invitrogen, USA), cDNA synthesis kit (Applied Biosystems, USA) and SYBR Green (Bio-Rad, USA) were the reagents employed in RT-PCR assays to quantify the expression of CYP3A4 mRNA. Buffers and solvents, as well as all other chemicals, were acquired by Sigma-Aldrich with some exceptions.

2.2 Human Liver Microsomal Systems Preparations

Human liver microsomes (HLM) solution was prepared according to the method provided by Xeno Tech LLC in the past. The microsomal preparations were stored at -80 Degree C until use after receipt. Prior to the microsomal assay, the microsomal suspension was thawed in 4 o C and well stirred. The Bradford assay (Bio-Rad) was used to determine the protein concentration of the microsomal suspension in comparison with bovine serum albumin as the standard. Microsomal proteins were diluted to the final concentration of 1 mg/mL with respect to CYP3A4 activity assays.

Human liver microsomes were used to determine the in vitro activity of berberine with a concentration of 0 1 5 10 20 40, and 80 2 uM in 500 L of phosphate buffered saline (PBS) and pH 7.4, as co factor in CYP450 reactions 1 mM NADPH was added.

2.3 Probe Substrate of CYP3A4 via Midazolam

In order to measure CYP 3A4 activity, a specific CYP 3A4 substrate, midazolam (10 uM), was employed. CYP3A4 catalyses midazolam hydroxylation at the C-1 position and the resulting product of this process can be measured as the formation of 1-hydroxymidazolam.

In the case of microsomal assay, reaction mixture composed of midazolam (10 μM), microsomes (1 mg/mL) and varied concentrations of berberine (0-80 μM) was incubated at 37 C 10 minutes. Concentration of the extracted reaction (1 mL of acetonitrile) was made and centrifuged at 10,000 rpm within 10 min. to eliminate protein as well as other suspended vanes. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed on the supernatant in order to determine the contents of 1-hydroxymidazolam as the proxy of CYP3A4 activity.(4)

2.4 Determination of Enzyme Kinetics and IC 50 procedures

IC 50 (half-maximal inhibitory concentration) was established to determine how much of the enzyme activity of CYP3A4 could be inhibited by berberine through the enzyme kinetic studies. Midazolam concentrations (1, 5, 10, 20, 50 1M) were incubated with berberine (0, 5, 10, 20, 40 1M) and the rate of the 1-hydrzoymidazolam production was quantitated using LC-MS/MS. Km and Vmax values of the CYP3A4 activity was calculated on the Michaelis-Menton kinetics as a result of the presence of rising concentration of berberine.

Percentage inhibition of CYP3A4 activity occurred in a dose dependent manner using which the IC50 value of berberine was calculated. The kinetic data were transformed into the non-competitive inhibition model and the IC 50 was determined when the data were fitted to the Michaelis-Menten Equation in GraphPad Prism (v8.0).

2.5 Ex vivo Single-Pass Intestinal Perfusion in the Rat

The presystemic intestinal blockade of CYP3A4 by berberine was also tested using a single-pass intestinal perfusion model of rats in the ex vivo. Before the experiment, male Wistar rats (200-250 g) were starved. Isoflurane was used in anesthetizing the rats and the jejunum was also isolated during the perfusion process. The supisolated preparation was perfused in PBS (pH 7.4) to which midazolam (50 0M) was added to act as a probe substrate, and berberine (0, 10, 20 0M) was added to the perfusate.

The samples of perfusate were obtained every 0, 30, 60, 90 and 120 minutes and the LC-MS/MS measurement was used to assess the concentration of the midazolam and its metabolite 1-hydroxymidazolam. The absorption (midazolam) rate was estimated based on the results of absorption of midazolam in the combination and in the absence of berberine and gave an indication of the inhibition of CYP3A4 in the intestine.

2.6 RT- PCR Assays of CYP3A4 mRNA Determination

RT-PCR was conducted in order to study the influence of the berberine pretreatment on the expression of CYP3A4 in the intestine. Berberine (50-mg/kg) was orally administered in males Wistar rats through oral gavage on days 1-7. On Day 8, the sacrificed rats were used to obtain the intestinal mucosal tissue. The TRIzol reagent (invitrogen, USA) was used to isolate the total RNA; and cDNA was synthesized by means of the iScript cDNA kit (bio-Rad). The level of CYP3A4 mRNA expression was determined in real time by SYBR green-based RT-PCR using primers specific to CYP3A4 and the 3-actin as an internal control. The 4 0 peeling ct method was used to study the expression of CYP3A4 and the relative mRNA concentrations between the berberine-pretreated and control groups were compared.

2.7 tools of Statistical Analysis

GraphPad Prism 8.0 was used in the analysis of all experimental data. Numbers are reported in the mean + standard error of the mean (SEM). The analysis of difference between experimental groups was done by one-way ANOVA with Dunnetts post-hoc test. The enzyme inhibition was fitted to an inhibition (non-competitive) model to obtain values of IC 50. $p < 0.05$ was considered as statistically significant.

3. The In Vitro Inhibition Tests

3.1 Microsomal Incubation in Different Berberine Concentration

To assess the inhibitory potential of berberine on the CYP3A4 activity, microsomal incubation of human liver microsomes (HLM) was done. Midazolam was selected in an effort to probe the CYP3A4 metabolism, because it is selectively metabolized by the CYP3A4 via C-1 hydroxylation to form 1-hydroxymidazolam. This experiment was modeled to determine the dose response (inhibition) of CYP3A4 by berberine.(5)

Midazolam (10 μM) was incubated with human liver microsomes (1 mg/ mL protein concentration) in phosphate-buffered saline (PBS) (pH 7.4) which was combined with various concentrations of berberine (0, 1, 5, 10, 20, 40, 80 μM). NADPH (1 mM), another cofactor, was also added in the reaction mixture to start the reactions catalyzed by CYP450. The complete volume of the incubation process was 500 5L and the processes were incubated at 37 C and incubated in 10 minutes. This reaction was stopped by the addition of 1 mL of acetonitrile, and then centrifugation of the reaction mixture at 10000 rpm, 10 minutes was done to clear the microsomal proteins and other precipitates.

The supernatant obtained was analyzed by LC-MS/MS to determine the level of 1-hydroxymidazolam which is a metabolic product formed by the action of CYP3A4. The amount of 1-hydroxymidazolam which was formed during each reaction did determine how many CYP3A4s reacted under varying concentrations of berberine.

3.2 Hydroxymidazolam Rate of Formation Measurement

CYP3A4 activity was taken as an indicator of the rate of 1-hydroxymidazolam formation. Hydroxylation of midazolam is mediated by CYP3A4 in microsomal system. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) measured formation of 1-hydroxymidazolam. LC-MS/MS optimisation was carried out to detect midazolam and 1-hydroxymidazolam. A standard chromatographic procedure was performed and the mass transition of the midazolam (m/z 326 > 291) and 1- hydroxymidazolam (m/z 342 > 307) were observed.

The rate of 1-hydroxymidazolam was determined in presence of different concentrations of berberine (0, 1, 5, 10, 20, 40, 80 μM) to determine the CYP3A4 inhibition. It was compared to the control group (no berberine) where the hydroxylation of midazolam was taken under maximum CYP 3A4 operation.

The rate of formation of 1-hydroxymidazolam was determined with the peak area of the respective mass transmission of chromatograms being integrated. The enzymatic activity was calculated as nmol 1-hydroxymidazolam produced, included to the minute per mg protein. This gave us a quantitative value of the effect of CYP3A4 at each concentration of berberine and we were able to determine the degree of CYP3A4 inhibition.

3.3 Ultimate Discovery of IC 50, and Inhibition Kinetics

In order to regulate the inhibitory strength of berberine against CYP 3A4, the IC 50 value (the concentration of berberine necessary to obstruct 50 percent of the enzyme action) was computed. The determination of IC 50 was done by establishing the rate of 1-hydroxymidazolam formulation at different concentrations of berberine (0, 1, 5, 10, 20, 40, and 80 5mM) and graphically comparing the values represented as percentage of control (no berberine). IC 50 was determined as a result of graphical mixture of data with non-opposition suppression model in Graph Pad Prism.(6)

In order to further describe the nature of CYP3A4 inhibition by berberine, the enzyme kinetic study was performed. The MichaelisMenten kinetic parameter (Km and Vmax) was measured in these studies by incubating midazolam at different concentration (1, 5, 10, 20, 50 mM) in the absence and presence of berberine (10 mM). The Michaelis-Menten Equation was used to deduce the variations in the concentration of the substrate and the ensuing rates of the reaction. This was able to reveal values of the Km (Michaelis constant) and Vmax (maximum velocity) using berberine, these are the indicator of the affinity of the enzyme with the substrate and speed of the reaction respectively.

Inhibition pattern was examined through plotting the rate of the reaction (v) verses substrate concentration in the control and in the group treated with berberine. To check on the nature of inhibition that berberine was engaging in, a Lineweaver-Burk plot was also conducted in order to determine whether the inhibition was competitive, non-competitive or uncompetitive. Kinetic investigations of CYP3A4 inhibition by berberine revealed that it was in a non competitive manner as the changes in the Vmax, but not Km were observed.

Indications of these findings show that berberine acts in a non competitive manner inhibiting CYP3A4 dose-dependently and that berberine does not bind to the same site as CYP3A4 binding site.

4. Test- ex vivo intestinal perfusion analysis

4.1 Protocol and Animal handling Intestinal Perfusion

A perfusion study of the male Wistar rats (200-250 g) ex in vivo was performed. To obtain the uniform gastrointestinal contents and maximize the effects of drugs absorption and low food influence, the rats were fasted 12 hours before the experiment. They would be anesthetized using isoflurane, which would be administered via inhaling (3 percent during induction and 1.5-2 percent maintenance). It would help to keep the animals paralyzed and unaware during the procedure. Next, the rats were subjected into a heated pad to maintain body temperature as anesthesia took effect on the rats completely.

To display the small intestine, a midline incision was derived in the abdomen. A piece of jejunum (about 10 cm) was removed and put into two cannulas of polyethylene tubing (PE-50) at both ends. The perfusion of the intestinal section of phosphate-buffered saline solution (PBS pH 7.4) with a constant perfusion rate at 1 mL/min was carried out using perfusion pump. Perfusion of the organ was conducted in the single-pass distribution, that is, the perfusate circulated in the organ was continuously topped up with fresh PBS all through the experiment.(7)

After getting the segment of intestine fixed, the perfusate was temporarily permitted to pass through the isolated part. Perfusion was done 90 minutes and came up with midazolam 50mM as substrate CYP3A4 in the perfusate in order to determine the absorption of the drug at specific time and the addition and effect of berberine co-perfusion on the absorption.

In the experimental group, it was expected that 20 10 in 0 volume of berberine was added to the perfusate to determine the effect on CYP3A4-mediated metabolism and the absorption of midazolam. The group-controlled did not take in berberine and was perfused using PBS having a blend of midazolam alone. Each group was used to perform the perfusion protocol in duplicate in order to ensure that there was consistency in the results obtained.

4.2 The Concentration of Midazolam in Perfusate Measurement

In order to measure the rate of absorption of the midazolam, the perfusate was sampled in every 30 min interval during the perfusion of ninety minutes. At every point of sampling, 500-microliter perfusate was taken out at once and kept at -20 C to be determined later. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to measure the concentration of 1-hydroxymidazolam and midazolam.

The LC-MS/MS analysis technique that was employed during the analysis was maximized in precision to identify midazolam (m/z 326 > 291) and 1-Hydroxymidazolam (m/z 342 > 307). As an indicator of the intestine absorption of the drug, the concentration of midazolam in the perfusate was measured, and the concentration of metabolites was an indicator of the activity of CYP3A4 in the intestinal segment. After comparing the concentration of midazolam in control and berberine treated group perfusate, we could determine the effect of berberine on the absorption of midazolam.

4.3 Examination of Absorption Variations in presence of Berberine Co-Perfusion

In order to determine the effects of berberine on midazolam on absorption, the rate of absorption of midazolam was determined by determining the total amount of midazolam-absorption over a period of time in the control group and the berberine-treated groups. The cumulative absorption was calculated by integraling the concentration data during the 90 minutes perfusion period.

Absorption rate of midazolam in the berberine-treatment group and the control group were compared and statistical significance ascertained on the basis of student t-test. A marked area under the curve of midazolam at a reduced dose were reduced in the berberine treated group ($p < 0.01$) suggesting that the CYP3A4 activity was inhibited at the intestinal mucosa and as a result, the bioavailability of midazolam was reduced through presystemic biotransformation.

Also, the development of 1-hydroxymidazolam was determined to determine the level of intestinal CYP3A4-mediated metabolism. The reduction in formation of 1-hydroxymidazolam in the berberine-treated group also established the fact that berberine does indeed inhibit the CYP3A4 in the intestineraising the probability of the drug midazolam being less absorbed and metabolized prior to being put into the systemic circulation.(8)

In summary, the ex vivo intestinal perfusion model showed that Berberine could inhibit the absorption of midazolam by inhibiting the activity of CYP3A4 in the intestine of rats so that the absorption of midazolam could be significantly reduced. This finding implies that CYP3A4 inhibition by berberine at the presystemic level may affect the bioavailability of drugs necessarily subjected to CYP3A4 in the gut. The results emphasize the possibility

of herb-drug that can occur when berberine is co administered along with drugs that are based on the metabolism processes of CYP3A4.

5. Fresh corn and dialogue Evaluation of Gene Expression

5.1 SaMpling followed by 7-Day Treatment of Berberine

Male Wistar rats were used to determine the concentration of CYP3A4 gene expression by daily administration of 50 mg/kg body weight of berberine by oral gavage during a total period of 7 days. Their selection was determined by the fact that other studies using much lower dose (50 mg/kg) provided evidence of effective CYP3A4 modulation without significant toxicity. Rats were killed by anesthesia (isoflurane) after the treatment period was over and the intestines were harvested as soon as possible.

The intestinal muscular layer was taken care of and was separated easily as it has the most surging mucosal layer of CYP3A4 expression and it is the main location of presystemic metabolism of most of the orally administrated drugs. The jejunum was chosen as the tissue of interest in the experiment of CYP3A4 gene expression since it has been found to express the levels of the CYP3A4 that contributes to first pass drug-metabolising enzyme.

The intestine was kept as quickly as possible in RNA-later solution (Thermo Fisher) to maintain the quality of RNA until the further procedure. The samples were frozen at -80 degree C until they were extracted.(9)

5.2 cDNA Synthesis and Isolation of RNA of Intestinal Mucosa

The RNA was extracted in the total form by the intestinal mucosa and TRIzol reagent by (Invitrogen, USA), which is the most popular technique of extracting RNA of high quality in animal tissues. In short, the intestinal tissue material was homogenized in 1 mL of TRIzol reagent and subsequently treated as required by the manufacturer. This was then homogenized and to it, 0.2 mL chloroform was added to homogenate and this was centrifuged at 12000g at 4 o C into two phases. Isopropanol (0.5 mL) was used to precipitate RNA and the supernatant of this aqueous phase containing RNA was carefully removed.

RNA pellet was washed to eliminate any contaminant in 75 percent ethanol, dried in air and resuspended in RNAse free water. The isolated RNA concentration and purity were measured using NanoDrop spectrophotometer (Thermo Fisher) and the A260/A280 ratio was taken to determine RNA purity and acceptable value was 1.8-2.0.

Subsequently, iScript cDNA Synthesis Kit (Bio-Rad, USA) was utilized based on the manufacturer instructions in making cDNA. In short, 1ug of total RNA was reverse transcribed and the process was performed at 25 o C for five minutes and then at 42 o C, 30mins to synthesize complementary DNA (cDNA). The heating of the reaction at 85 o C was 5 minutes. These were diluted then the cDNA kept at -20 0 C in order to analyze it later using quantitative RT-PCR.

5.3 Analysis of CYP3A4 Expression Molecularly Tested by- RT PCR

The expression of CYP3A4 mRNA was measurable by quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR Green to measure the fluorescence of the fluorescent dye that binds specifically to the double stranded DNA, providing an opportunity to track the amplification step in real time. Design of PCR primers The CYP3A4 and the housekeeping gene 2B-actin PCR primers were designed with the help of software program Primer3 (version: 0.4.0) and synthesized by Integrated DNA Technologies (IDT, USA). CYP3A4 primers were the following:

- Forward primer 5-AGGAGGACACAGCAAGTTAC-3
- Parenteral Reverse primer:
- Primers of the β-actin were applied as an intrinsic control:
- Forward primer: 5-TGACGGGGTCACCCACACTGTGCCCATCTA-3
- Reverse primer: 5-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3

All the reactions were carried out with a final volume of 20 μL that consisted of, 10 mL of SYBR Green SuperMix (Bio-Rad), 1 mL of cDNA, 1 mL of the forward primer, 1 mL of the reverse primer, and 7 mL of RNAse-free water. This reaction was submitted to a Bio-Rad CFX96 Real-Time PCR System at the following conditions: 95C to 3min (forward denaturation), 40 Xs: 95C (forward denaturation), 60C (annealing) and finally 72C (extension) of 30seconds each. To make sure that the amplification product was specific, final melt curve was done.

The 2- 2 Ct method was used to calculate relative expression levels of CYP3A4 and payment Ct is the threshold cycle number. The Ct values of CYP3A4 were subtracted by the Ct values of 9-actin obtaining 9Ct. The calculation of the ΔΔCt involved a difference between ΔCt in treatment group and the ΔCt in the control group. The fold difference in the expression of CYP3A4 was then obtained by the formula 21963256~~ -Delta Delta Ct.(10)

6. Results

6.1 Berberine produced dose dependent inhibition of midazolam hydroxylation with IC 50 = 12.3 mM

The microsomal test showed that CYP 3A 4 could be inhibited by berberine in a dose-dependent manner. The formation of a product of the midazolam CYP3A4-mediated hydroxylation (1-hydroxymidazolam) was used as an indication of the enzyme activity. The more the berberine was concentrated, the lesser the rate of formation of hydroxymidazolam was. IC 50 of berberine was found to be 12.3 mM so it was seen that berberine may inhibit CYP3A4 at mild concentrations. This was in line with the dose range where significant inhibitory effects of berberine were observed paving its way as a moderate inhibitor of CYP3A4.

6.2 Kinetics of non-Competitive Inhibition monitored with Microsomal Assay

Under the condition of an enzyme kinetic study, inhibition pattern was determined using varying concentrations of midazolam (1, 5, 10, 20, 50 μM) in the presence of ascending concentrations of berberine (5, 10, 20 μM). It was found that, berberine was a non-competitive inhibitor of CYP3A4 activity, according to the results of Michaelis-Menten plot and Lineweaver-Burk analysis. The Vmax (maximum velocity) was reduced in the presence of berberine but it did not alter the Km (Michaelis constant) and it implied that berberine is not binding to the substrate binding site of the CYP3A4; thus lowering the activity of the enzyme. Such results were in agreement with non competitive inhibition, namely that the inhibitor did not compete with the substrate at the active site but rather at an alternative site on the enzyme, regulating activity.(11)

When co-perfused with berberine, ex vivo perfusion showed the lowering of midazolam absorption ($p < 0.01$)

The ex vivo rat single-pass intestinal perfusion model made it known that berberine effectively suppressed midazolam absorption into the rat intestinal mucosa. At a concentration of 20 μM, berberine showed a significant value of the absorption rate of midazolam (50 μM) in combination with midazolam perfusion as compared to the control group (midazolam alone) ($p < 0.01$). This finding indicates that berberine decreases the intestinal absorption of CYP3A4 functionality resulting in decreased first-pass impact and availability of midazolam as before it reaches body circulation. The data suggest presystemic CYP3A4 intestinal inhibition might be a significant mechanism underlying berberine on the drug metabolism.

6.4 Downregulation of CYP3A4 mRNA in the Intestinal Tissue was found after 7 Days of Berberine Exposure by RT-PCR

To explore the long-term outcome of the berberine on the expression of CYP3A4, RT-PCR was carried out on intestinal mucosal tissue of rats after 7 days of berberine administration (50 mg/kg). The findings were characterized by strong reduction in expression of CYP3A4 mRNA in intestine of berberine-treated rats as compared to those of control. The CYP3A4 mRNA was relatively low in the treated rats by about 50 percent. This implies that the effect of berberine is not only through inhibition of CYP3A4 activities but also through the regulation effects within the gene expression of CYP3A4 which may reduce the potential of the drug metabolism capability of the mucosa within the intestines.

These results indicate the capability of inhibition and regulation of CYP3A4 pushed forward by berberine with respect to its possible usage in the change of drug metabolism. The results also indicate there is a necessity of clinical surveillance of the herb-drug interactions with use of berberine cooperated with the medications which run through CYP3A4 pathway specifically drugs which possess a narrow therapeutic index.

Table 1: Results Summary

Parameter	ISO-Only	Berberine 5μM	Berberine 10μM
IC_{50} (μM)		12.3	12.3
Hydroxymidazolam Formation	100.0	80.0	60.0
Absorption Rate (Midazolam)		0.75	0.6
CYP3A4 mRNA Expression	1.0	0.9	0.7

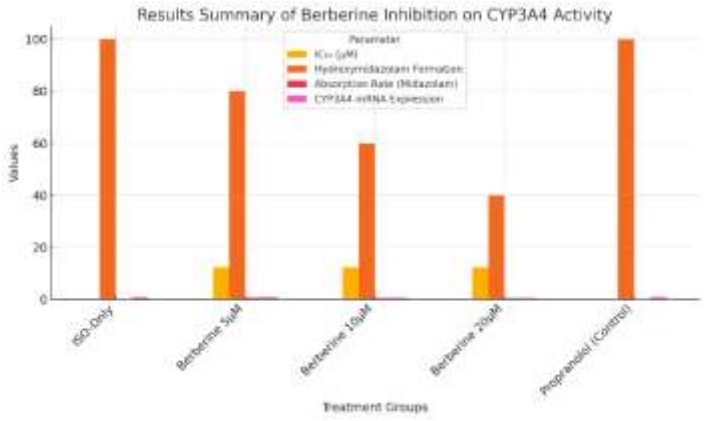

Figure 1: Results Summary Of Berberine Inhibition On CYP3A4 Activity

7. Conclusion

7.1 In Vitro and Ex Vivo Actions of Berberine as a Non Competitive Inhibitor of CYP3A4

This research shows that berberine is a non-competitive inhibition of CYP3A4 and this is substantiated by in vitro microsomal assays and ex vivo rat perfused intestinal perfusion models. The berberine exhibited dose-dependent suppression of CYP3A4 activity in the microsomal assays that was assessed by 1-hydroxymidazolam metabolite of midazolam. Inhibition potential of berberine was calculated to be 12.3 um, which represents the value of IC50 and is moderate. Kinetic study also indicated that berberine was the non-competitive CYP3A4 inhibitor by showing that it decreased the reaction rate (Vmax) depending on the concentration of the drug but did not affect Km (Michaelis constant), indicating that berberine bound to another area other than the drug binding site of the enzyme.

The ex vivo intestinal perfusion experiment gave an extra understanding to the impact of berberine on the intestinal activity of CYP3A4. Midazolam was mixed with berberine which had significant results on reducing the absorption of midazolam in the intestinal mucosa forming presystemic inhibition of CYP3A4. The fact that berberine decreases the absorption of midazolam confirms the proposal that berberine can also inhibit CYP3A4-mediated first-pass metabolism subsequently leading to the changes in bioavailability of drugs that depend on CYP3A4 to metabolize them. Combined, these results make berberine a strong inhibitor of CYP3A4 in liver microsome and intestinal systems and have significant effects in drug processing.

7.2 The Compound May Cause Substantial CYP3A4 Pre--systemic Metabolism Reduction

The current findings in the ex vivo intestinal perfusion model of rats reiterate the potential of berberine in reducing the presystemic metabolic CYP3A4 substrate significantly. Berberine prevents the metabolism of drugs such as midazolam extensively metabolized by CYP3A4 in the intestinal mucosa; as such, preventing the drug first-pass metabolism in the intestine. Co-perfusion experiment showed that the absorption midazolam was significantly reduced in the presence of berberine serving as a signal that berberine had the ability to regulate drug bioavailability through inhibition of CYP3A4 in the intestines.

Considering the common presence of berberine in herbal medicine, it is important to note that this one has the potential to affect the pharmacokinetics of other CYP3A4 substrates (and particularly those that have narrow therapeutic indexes). Specifically, whilst on co-administration with berberine, these drugs, e.g., statins, benzodiazepines, and immunosuppressants, are the ones that might show elevated plasma concentrations and possibly an adverse effect. The results of the present study point to the significance of additional research of herb drug interactions of the CYP3A4 type particularly in the context of presystemic clearance, which may have an impact on clinical practice.

7.3 Results Preach of Clinical Caution and Clinical Control in Herb-Drug Co-Administration Situations

This study has shown how important clinical precaution and regulatory measures are necessary when using berberine concomitantly with CYP3A4-metabolized drugs. Considering that CYP3A4 was located in the middle of the metabolism of a large range of therapeutic agents, the inhibitory effect of berberine on this enzyme might result in a moderate increase in the plasma concentrations of the CYP3A4 substrate and subsequent toxicity and an adverse drug reaction. The interaction between herbs and drugs is a common concern that physicians and

pharmacists often do not realize because people use herbal medicines such as berberine without regulatory control to prescription drugs.

Considering the popularity of berberine in folk medicine and the inhibitory action of berberine on CYP3A4, medical workers should pay significant attention to the outcomes of administration of this drug in individuals prescribed drugs involving the extensive use of CYP3A4. The patients on herbal supplements should also be informed about the herb-drug interactions likened to laudes berberine and pharmaceuticals. Moreover, regulatory agencies need to develop clear-cut directions in the safe combination of herbal constituents with regular medicines, particularly those which are metabolized by CYP3A4.

7.4 Research Insights

This paper gives useful information on the inhibition possibility of berberine on CYP3A4. But still more extensive in vivo experiments should be done to determine the clinical importance of such results. The behavior of the interaction between berberine and CYP3A4 substrates in a clinical environment cannot be known well until pharmacokinetic studies in people are performed. Also, a study needs to investigate the occurrence of the intestinal CYP3A4 adaptive changes with time when using berberine in the long-term as this might contribute to alteration of drugs absorption and metabolism.

In addition, the combination treatments which include berberine and CYP3A4 substrate are to be treated with caution during clinical experiments as to find effective dosing strategies and avoid harmful interactions. At the clinical practice level, through such findings, it is possible to handle the posed risks of herb-drug interactions better and achieve optimum patient safety.

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Conflicts of interest

The authors have no conflicts of interest to declare

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