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Original Article

Herb-Drug Interaction Evaluation on Concomitant Administration of IME-9 and Gliclazide in Preclinical Diabetic Rats

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ABSTRACT

Background: Diabetes mellitus is the third most prevalent cause of mortality and morbidity globally. Several complementary or alternative therapies currently used to treat diabetes mellitus. IME-9 is a polyherbal preparation used to treat diabetes and contains numerous phytoconstituents that can alter the cytochrome enzymes, mainly CYP2C8, 2B6, 2D6, 1A2, 2C9, 2C19, and 3A4. Gliclazide is a commonly prescribed antidiabetic drug metabolized primarily by the CYP2C9 and CYP2C19 enzymes. Thereby, it is postulated that the concomitant administration of IME-9 with Gliclazide therapy in diabetic patients may lead to HDI (Herb-Drug Interaction). **Materials and Methods:** In the Pharmacokinetic (PK) and Pharmacodynamic (PD) Herb-Drug Interaction (HDI) studies, GL was given to rats orally at 8.22 mg/kg, followed by administration of IME-9 at a dose of 132.24 mg/kg for 21 days. On the 1st day and after 21 days of the study period, *in vivo* PK/PD parameters were evaluated. *In silico* molecular docking studies were carried out (Autodock Vina1.2.3) to explore the binding affinity of the 46 major phytoconstituents present in IME 9 with the CYP2C9 and CYP2C19 enzymes. The toxicity was studied by MTT assay. **Results:** The PK analysis showed that co-administration of (IME-9+GL) for 21 days altered the PK parameters, increasing the Gliclazide (GL) bioavailability. This study throws light on the occurrence of hypoglycemia following the concomitant administration of (IME-9+GL) post 21 days in rats, leading to significant HDI, which may further lead to deleterious effects such as hypoglycemic coma. Furthermore, it was observed that the co-administration did not restore the body weight but restored the plasma TC and TG levels to normal. Histopathology studies revealed pathological alterations in the pancreas of diabetic control rats, while the treated rats (GL, IME-9) showed moderate degeneration with focal necrosis and atrophy in the islet of Langerhans. The *in silico* study confirms the critical involvement of the cytochrome enzymes in the observed HDI. **Conclusion:** This preclinical study can help to identify potential HDI and take the appropriate measures to improve clinical outcomes and reduce the associated adverse effects.

Keywords: Diabetes Mellitus, Gliclazide, HDI, IME-9, Pharmacodynamic, Pharmacokinetic.

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INTRODUCTION

Diabetes mellitus is the third most prevalent cause of mortality and morbidity globally, next to cancer and cardiovascular disorders.[1] As stated by the International Diabetes Federation's Diabetes Atlas report for 2021, 1 in 10 adults worldwide suffer from diabetes, which has affected 537 million adults of the age 20-79 years (10.5% of all adults) across the globe. According to IDF (2021), half of the world's diabetes population is located in Asian countries (India, China and Pakistan) and the United States.[2] On the global scale, the epidemiology of diabetic patients is projected to increase phenomenoly by 2030 (643 million i.e. 11.3%) and 2045 (783 million i.e. 12.2%). Therefore, while the population of the world is anticipated to increase by 20% during the same timeframe, diabetic patients are predicted to increase by 46%. As per demographic assessments, by 2030, compared to developed countries (48 million), developing countries will have 82 million more adults over the age of 64 who have diabetes. According to forecasts, sub–Saharan Africa, India, and the Middle East crescent will have the most significant relative rise.

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By 2030 worldwide, WHO predicts that diabetes will be the 7th major cause of mortality.[3]

Many complementary or alternative therapies are currently being used to treat diabetes mellitus in addition to the clinically used oral hypoglycemic agents.[4] When traditional medicinal herbs and prescription drugs are consumed at the same time, it may lead to Herb-Drug Interactions (HDIs).[5] The mode of action for such HDI may increase the induction/inhibition of the cytochrome 450 enzymes, P-gp transport proteins, alteration of the plasma protein binding and changes in renal functions. HDI's may affect the pharmacokinetic behaviour or pharmacodynamics of the drug consumed. Pharmacokinetic HDI occurs when the herbs modulate another drug's systemic concentration or bioavailability, while pharmacodynamic HDI results in synergistic, agonistic, or antagonistic effects.[6] Drug-Drug Interaction and Drug-Food Interactions studies are conducted during drug development, however HDI studies are neglected. It is vital to note that trustworthy data are required to accurately evaluate the HDI potential of herbal treatments.

IME-9 tablet (approved by the AYUSH Ministry, India) is a herbal preparation that the Government of India is currently promoting for in the treatment of diabetes. However, most such herbal preparations are consumed by patients without the prescription of doctors, raising the probability of the occurrence of HDI. IME-9, a polyherbal preparation, contains *Mangifera indica* Linn., *Momordica charantia* Linn., *Gymnema sylvestre* (Retz.) *R.Br. ex Sm., Syzygium cumini* (L.) Skeels, and Asphaltum as the major herbs. *Mangifera indica* is reported to inhibit the CYP2C9 (IC_{50}-107.48 µg/mL), 2B6 (IC_{50}-57.83 µg/mL), 2D6 (IC_{50}-67.39 µg/mL) and 2C8 enzymes (IC_{50}-37.93 µg/mL). Human CYP2C9, 2C19, 2A6, 1A2 and 3A4 activities were inhibited by *Momordica charantia* leaf and stem extracts at low IC_{50} value (3.3 to 34.1 µg/mL). Bera *et al*., studied *Gymnema sylvestre* extracts for the inhibitory effects on human: CYP2C9, 2C8, 2D6,1A2 and 3A4 with the IC_{50} value's 0.56, 0.16, 0.19, 0.89 and less than 0.1 µg/mL, respectively, using LC-MS/MS. The fruit of *Syzygium cumini* inhibits human cytochrome P450 enzymes, with IC_{50} values of 76.69 µg/mL for 2C9, 359.02 µg/mL for 3A4, and 493.05 µg/mL for 2D6. These phytomolecules can alter the cytochrome enzymes, mainly CYP2C9, 2C19, 2C8, 2B6, 2D6, 1A2, and 3A4.[7]

Gliclazide is a commonly prescribed antidiabetic drug metabolized mainly by the CYP2C9 and CYP2C19 enzymes.[8] In the literature review it was found that the individual herbs were studied for the HDIs, but formulation is not investigated. Thereby, it is postulated that the concomitant administration of IME-9 with Gliclazide therapy in diabetic patients may lead to development of HDI. The current research aims to assess the possibility of any significant HDI on concomitant administration of IME-9 and Gliclazide through a preclinical Nicotinamide-STZ induced diabetic model in Wistar rats.

MATERIALS AND METHODS

Drugs and Chemicals

IME-9 tablets were acquired from a local Ayurvedic store. GL (purity ≥ 99.5%) and Glimepiride (IS) (purity ≥ 99.5%) were provided as gift samples from the IPCA Laboratory Limited, Kandiwali West, Mumbai, India. HPLC grade water was obtained using the Millipore water purification system (Milipore, Sigma). Streptozotocin and Nicotinamide were purchased from Sigma Aldrich, Mumbai, India. Umbilical cord mesenchymal cells were sourced from the Regenerative Medicine Laboratory, Dr. D. Y. Patil Vidyapeeth, Pune, India. HPLC grade solvents and other analytical reagent grade solvents were obtained from Merck Chemicals, Mumbai, India.

HPLC Analysis

The concentration of GL in rat plasma was measured using a Kromasil 100 C_{18} (250x4.6x5.0) column, mobile phase [acetonitrile:0.03M potassium dihydrogen phosphate (pH 4 adjusted with 1% orthophosphoric acid)] in a 55:45 ratio (v/v)], flow rate-1 mL/min with UV detection at 228 nm. The developed method was validated in accordance with the USFDA bioanalytical method validation guidelines for Industry, Center for Drug Evaluation and Research, 2018.

Study Animals

Crystal Biological Solutions, Pune, India, provided male Wistar rats used in this study (200±250 g, 6-7 weeks old). Prior to experimentation, the protocol was approved (DYPIPSR/ IAEC/ May 17-18/P-24), by the Institutional Animal Ethics Committee (CPCSEA Reg. No.: 198/PO/Re/S/2000/CPCSEA) Pune, India. One-week prior, acclimatized animals to the laboratory conditions were fasted overnight to induce diabetes. The environment in the animal house was maintained at 24±3°C, relative humidity of 50±5%, 12-hrs, light and dark cycle, and animals were fed with a standard diet. According to the guidelines of the "Committee for the Purpose of Control and Supervision of Experiments on Animals," Ministry of Animal Welfare Division, Government of India, New Delhi, all animals were handled, and procedures were followed.

Induction of Diabetes

Diabetes was induced in male Wistar rats as per the procedure mentioned in our previous HDI research studies.[9] Accordingly, animal with fasting blood glucose levels>250 mg/dL (measured using an Accu-Chek glucometer) were classified as diabetic and selected for Group II, III, IV and V in this study.[10]

Experimental Design for the PK/PD Study

Group I (*n*=6)-Normal (N-0.5% CMC in distilled water (1 mL/kg, p. o.).

Group II (*n*=8)-Diabetic Control (DC).

Group III (*n*=12)-(GL treated group- 8.2 mg/kg, p.o.

Group IV(*n*=12)-(IME-9 treated group- 132.24 mg/kg, p.o.

Group V (*n*=12)-The combination treated group (GL+IME-9), IME-9 132.24 mg/kg orally) followed by GL 8.2 mg/kg orally).

This study was carried out for 21 days. The doses for GL and IME-9 were calculated based on the body surface area of the rats.[9]

The Sample Extraction Method

In polypropylene centrifuge tubes, 100 µL separated plasma and 100 µL aliquot of GL working standard solution (25 ng/mL-1000 ng/mL) were added. It was then vortexed for 7 min with 100 µL of IS and 500 µL of methanol, further centrifuged (3500 rpm, 20 min). The clear supernatant was then collected and dried in a vacuum oven. The resulting residue was reconstituted with the mobile phase to a final volume of 1 mL. Suitable aliquots (20 µL) were injected into the Shimadzu-HPLC system.

In vivo Pharmacokinetic (PK) Investigations

On the 1st day and after 21 days of the study period, blood (250 -300 µL) was withdrawn from each rat (retro-orbital plexus) fasted for 14-16 hrs, into Eppendorf with 50 µL anticoagulant (0.5M EDTA) from animals of groups III, IV and V at preset intervening time of 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 24, and 48 hrs. Plasma was separated and kept at -20°C until further pharmacokinetic analysis. During blood withdrawal, as per the reported study, the animals were sedated with ketamine (90 mg/kg) and xylazine (10 mg/kg), with doses calculated according to the animals' body weight. Ketamine solution 0.9 mL (10%) and Xylazine solution 0.5 mL (2%) in mixture with 0.6 mL of sterile saline was used. The combination of Ketamine and Xylazine (KX) gives a generally safe anesthetic that can be delivered without the need for specific equipment.[11]

In vivo Pharmacodynamic (PD) Investigations

Fasting Blood Glucose Levels (FBGL) and rat body weight were measured on the first, seventh, fourteenth and twenty-first day of the study. After 21 days of treatment period, the lipid levels (TC and TG), histopathological examination were conducted (Crystal Biologicals pathology facility in Pune, India).

Statistical Analysis

WinNonlin software was used to calculate individual animals' plasma concentrations versus time profiles (Pharsight Corporation, Mountain View, CA, USA). The C_{max} and T_{max} of GL were calculated from the PK curve. The other PK parameters studied were AUC_{0-48}hr, Vd, Ke, $t_{1/2}$, CL (in mean±SD). GraphPad Prism software version 8, CA, USA was used for statistical analysis, which included 1/2-way ANOVA, Tukey's multiple comparison tests, and t-tests. The data was considered significant at $* p<0.05$, $** p<0.01$, and $*** p<0.001$.

In silico Molecular Docking Studies

The literature suggests that the primary herbs found in IME-9 act as CYP inhibitors, affecting enzymes such as CYP2C8, 2B6, 2D6, 1A2, 2C9, 2C19, and 3A4. However, evidence on the cytochrome inhibition by individual phytoconstituents is not available. Molecular docking analysis was conducted using AutoDock Vina 1.2.3 to investigate the binding affinity of phytoconstituents/ligands present in IME-9 with CYP2C9 and CYP2C19 enzymes. The analysis computed the ligand poses, binding affinity, interacting amino acid residues, and interactions with the target's active site. Ligands were ranked based on their binding energy (kcal/mol), serving as a measure of binding affinity for the targets.[12]

Molecular Docking

Target Protein Retrieval and Preparation

The target proteins CYP2C9 and CYP2C19 containing OXV and Flurbiprofen as co-crystalized ligand, (PDB ID: 1R9O and 4GQS) were selected, respectively, from the RSCB protein data repository for molecular docking investigations. (https://www.rcsb.org/). The 2D structures for Flurbiprofen(2-(2-fluorobiphenyl-4-yl) pro-panoic acid) and OXV(4-hydroxy-3,5-dimethylphenyl) (2-methyl-1-benzofuran-3-yl)methanone) were prepared using ChemDraw Ultra 12.0 software.

The CYP2C9 and CYP2C19 enzymes (Homo sapiens taxon) were obtained from RCSB database. Employing the Basic Local Alignment Search Tool (BLAST) tool, the PDB was screened, and the top most 10 similar biological sequences were selected based on criteria including percentage identity, E value and better query coverage. Any molecule, maybe a micromolecule or macromolecule, must be optimized and minimized before docking study to understand the active sites on amino acid residue in the binding pockets. Residues in the binding pocket were verified using the PDB sum server, a pictorial presentation of the 3D structure of the native ligand in the active pocket of a target in the Protein Data Bank. This helps us to understand the interactions of the native ligand (standards) with the residues in the act pocket. Prior to the docking study, the absent residues in the protein structures were added, side chains were generated (CHIMERA v1.16) before undergoing optimization and minimization. Further, protein optimization was performed using Steepest Descent steps (1000) with Steepest Descent size (0.1 Å). This was then followed using conjugate gradient steps (100 with conjugate gradient size of 0.1 Å). Hydrogens, including slower ones, were added, and charges were assigned for both standard and nonstandard residues (force field: AMBER ff14SB and AM1-BCC respectively). For all nonstandard residues, net charges were stabilized, allowing their partial atomic charges to

be computed (Antechamber charges). Following optimization and energy minimization, nonstandard residues (cocrystal ligands, water molecules, and unnecessary chains) were removed from the protein. The selected proteins were evaluated based on the Ramachandran plot. It displayed that no. of residues in the favored and allowed region is about 99.9%, which confirms that the target selected is of good quality.

Grid Generation

In silico tools such as Chimera, AutoDock and Maestro were employed for identifying receptor grids to define the binding pockets. The prepared enzymes were visualized in the workspace and using the CASTp server (Computed Atlas of Surface Topography of Proteins), the grid dimensions were determined. An enclosing box was chosen to match the shape and characteristics of the protein's active site, along with the standard ligands expected to dock within the binding pockets. The XYZ coordinates were set at 41.81 X 23.95 X 240.11, and the size of the grid box was established at 20 X 20 X 20 Å. The amino acids present in active pocket of 1R9O and 4QYS were VAL113A, PHE114A, ILE205A, VAL208A, ASP293A, GLY296A, ALA297A, GLU300A, THR301A, ILE362A, LEU366A, PHE476A having area 1786 Å2 (angstrong square) and volume: 1144 Å3 (angstrong cube)and ARG108A, ASN204A, VAL113A, PHE114A, ILE205A, LEU208A, VAL237A, MET240A, VAL292A, ASP293A, GLY296A, ALA297A, THR301A having area 9328 Å2 (angstrong square) and volume: 21016 Å3 (angstrong cube) respectively.

Preparation of Ligands

The major phytoconstituents found in the herbs (46) present in IME-9 formulation were chosen as the ligands for the investigation. The 2D structures of these ligands were created using ChemDraw Ultra 12.0 and Marvin Sketch v21.13 and utilized in the study (3D MOL2 format). All ligands underwent processing and optimization using default parameters.

Molecular docking of ligands with target proteins

Using an in-house bash script created with AutoDock tools 1.5.6 for ligands and ADFR suit for proteins, their structures were converted to the pdbqt format. All the rotatable bonds of the ligands were free to rotate while the receptors were maintained rigid. Employing AutoDock Vina 1.2.3, the high-resolution grid box (spacing of 0.375 Å between grid points) was centered on the active site of selected targets to understand the binding interactions of the ligands with identified receptors. The docking operations were performed at default configurations (CPU: 23, number of modes: 9, exhaustiveness:32, energy range:3). The docking setup utilized in earlier dockings was repeated multiple times to validate the docking pose, using Root Mean Square Deviation (RMSD) between subsequent docking iterations.[13,14]

Visualization

Following the Autodock Vina docking processing, the outcomes were visualized using Biovia Discovery Studio visualizer. Using Maestro 12.3 (academic edition) and LigPlus 1.22D, the 2D/3D images of the complexes were created. The interactions and binding energies of the test ligands were then compared with those of standard inhibitors chosen for the investigation.

Cell cytotoxicity assay

The *in vitro* cytotoxicity tests were carried out on human mesenchymal stem cells derived from umbilical cord (UCMSCs), cultured in α-MEM medium with Fetal Bovine Serum (FBS) and 1% anti solution (Gibco). At a density of $0.5x10^5$ cells/well, UCMSCs were seeded into 96-well plates. After 24 hrs, fresh medium and various doses of GL, IME-9, and (GL+IME-9) were added at 10, 100, and 500 µg/mL, respectively. Culture mediums without drugs were kept as control. Post 48 hrs, MTT assay (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was employed for the cytotoxicity assessments. Each well received 50 µL of MTT reagent (5 mg/mL MTT in phosphate buffered saline) and was then incubated for 4 hrs. Further DMSO (200 µL) was added to each well to dissolve the formazan crystals and absorbance measurements were performed at 560 nm (microplate reader).[15]

RESULTS

RP-HPLC Bioanalytical Method Validation for the HDI Investigation

The chromatograms (Figure 1) representing blank rat plasma, blank rat plasma spiked with GL (25 ng/mL), IME-9 formulation, and plasma sample collected after co-administration of GL+IME-9 demonstrated the selectivity of the developed analytical method. No interference was detected in the blank plasma (Figure 1 B) chromatograms using the optimal chromatographic conditions, where the Retention Times (RTs) were 9.038±0.5 min for GL and 13.955±0.4 min for IS (Figure 1 A). The selectivity of the analytical method is demonstrated by these chromatograms. They show that the method can distinguish between the blank plasma, plasma with spiked GL, plasma with IME-9, and plasma with both GL and IME-9 without interference from other components, confirming the ability of the method to accurately analyze the substances of interest. The calibration curve for GL exhibited linearity in 25-1000 ng/mL, showing a correlation coefficient (r^2) of 0.9935 and a LLOQ of 25 ng/mL. The method demonstrated acceptable precision and accuracy across all QC levels. For intra-day precision, %CV ranged from 1.65 to 9.81%, while inter-day %CV ranged from 1.03 to 6.09%. Accuracy values (expressed as %RE) remained within ±20%.GL was stable under all test conditions. Short-term, freeze-thaw, and long-term stability data demonstrated %CVs below 20%. This method

enabled the determination of GL concentration in rat plasma samples, in the pharmacokinetic HDI studies.

Pharmacokinetic HDI study following the treatment with GL, IME-9, and (GL+IME-9)

In diabetic Wistar rats, the PK parameters were measured on day 1 and day 21 in group III, the combination treated group V [GL at a dose (8.20 mg/kg p.o.) and the IME-9 formulation at a dose (132.24 mg/kg p.o.]. The concentration of GL in plasma versus time profiles. According to the PK data, the co-administration of GL and IME-9 (Group V) on day 1 showed a slight increase in the C_{max} of GL. However, the other PK parameters were not significantly altered. On day 21 of the study, combination therapy resulted in a C_{max} of 6240.39±353.01 ng/mL for GL at the T_{max} 2 hrs. The Vd value was 34.34±0.79 L/kg with CL of 2.455±0.200 L/hr and a t1/2 of 9.722±0.59 hr. Combination therapy group V (GL+IME-9) increased the C_{max} of GL from 1461.58±88.93 ng/mL to 6240.40±353.011 ng/mL. However, no shift in T_{max} (2 hrs) was revealed. Consequently, a slight reduction in V_d (138.37±7.87 to 34.344±0.79 L/kg), CL (7.74±0.32 to 2.45±0.200 L), and $t_{1/2}$ (12.407±1.14 to 9.722 hr) was observed. The PK profile also revealed a slight rise in $AUC_{0\text{-}48hr}$ (28568.33±347.19 h*ng/mL to 66337.66 ±4719.9 h*ng/mL). Table 1 summarizes the PK parameters.

Pharmacodynamic HDI study following the treatment with GL, IME-9 and (GL+IME-9)

Effect of GL, IME-9 and GL+IME-9 Treatment on FBGL

In the PD studies, in the DC group (412±3.11 mg/dL), a significant increase (p<0.001) in the FBGL in comparison to the NC group (106±3.94 mg/dL) was obtained. Both in the GL treated group and IME-9 treated group, a significant reduction in FBGL (p<0.001) 160.66±12.65, 158.5±13.6 mg/dL, respectively was observed on the day 21 of the study (Figure 2). While in the (GL+IME-9) treatment group, a significant (p<0.001) reduction of FBGL (75.83±16.87 mg/dL) was observed when compared to the DC group. Also, the reduction in FBGL was highly significant (p<0.001) in group V (GL+IME-9) when compared to the monotherapy with GL and IME-9 treated groups (p<0.001).

Effect of GL, IME-9 and GL+IME-9 treatment on the rat body weight

In this study, Wistar male rats weighing between 225±250 g was used. Throughout the study, it was observed that rats in the NC group gained weight (270.33±2 g) steadily over the 21st day treatment period. However, the body weight of the DC group showed a significant decrease (109.83±13.8 g) (p<0.001) in comparision to the NC group. From the 7th day till the end of the treatment period in GL and IME-9 treated groups, a significant gain (p<0.001) in body weight (200.66±1.5 g and 214.33±2.60 g respectively) was observed, compared to the DC group. By the 21st day, the body weight of rats in the GL and IME-9 treated groups had approached that of the NC group (Figure 2). However, in the combination (GL+IME-9) treated group, body weight consistently decreased throughout the 21-day study period.

Effect of GL, IME-9, and GL+IME-9 treatment on TG and TC levels

On comparison of the plasma TC and TG levels of the NC group (63.16±4.88 mg/dL and 64.64±5.48 mg/dL respectively), in the DC group, a significant elevation (p<0.001), both TC and TG levels were observed (170.66±6.25 mg/dL and 177.12±6.88 mg/dL respectively). After 21 days, Group IV showed reduced plasma total cholesterol and triglyceride levels (57.31±6.89 mg/dL and 61.92±5.35 mg/dL, respectively) than DC group rats (Figure 3). Further, the GL treatment also reduced plasma TC and TG levels to 122.83±13.76 mg/dL and 100.94±3.99 mg/dL, respectively. In the combination (GL+IME-9) treated group, a significant decrease in (p<0.001) plasma TC and TG levels (44.67±3.07 mg/dL and 47.81±2.18 mg/dL, respectively) were observed when compared to both the monotherapy groups (GL/IME-9 alone).

Table 1: Pharmacokinetic HDI parameters of GL in diabetic rats treated with GL (Group III) and GL+IME-9 (Group V).

Pharmacokinetic parameters	Day 1		Day 21	
	GL treated group	GL+IME-9 treated group	GL treated group	GL+IME-9 treated group
C_{max} (ng/mL)	447.88±8.51	507.05±39.49**	1461.58±88.93	6240.40±353.01***
T_{max} (h)	2hrs	2 hrs	2hrs	2 hrs
$AUC_{(0\text{-}48\ hr)}$ ng*hr/mL	11247.09±399.85	9843.58±1792.70**	28568.33±347.19	66337.66±4719.9***
Elimination rate constant (k_e) hr-1	0.043±0.0067	0.045±0.0045*	0.05618±.005	0.0714±.0044**
Elimination half-life ($t_{1/2}$) h	17.21±1.73	15.40±1.46*	12.40±1.15	9.72±0.59**
Volume of distribution (V_d) L	379.77±8.50	396.68±23.65*	138.38±7.88	34.34±0.79***
Clearance of drug (CL)L/hr	15.39±1.50	17.99±2.41*	7.75±0.32	2.46±0.200***
MRT	24.83±2.49	22.23±2.11*	17.90±1.66	14.03±0.856**

*Data expressed as mean±SD. p-value<0.05 is considered as significant; *p<0.05, **p<0.01, ***p<0.001 compared to the GL alone treated group.

Effect of GL, IME-9, and GL+IME-9 treatment on histopathological alterations of pancreas

Microscopic examination of the pancreas from the normal group did not reveal any lesions of pathological significance, and both the exocrine and endocrine pancreas (Islets of Langerhans) displayed normal histological architecture.

However, the microscopic examination of the pancreas from disease control showed pyknotic nuclei, degeneration of exocrine pancreatic acini, and atrophy of the islet of Langerhans. The

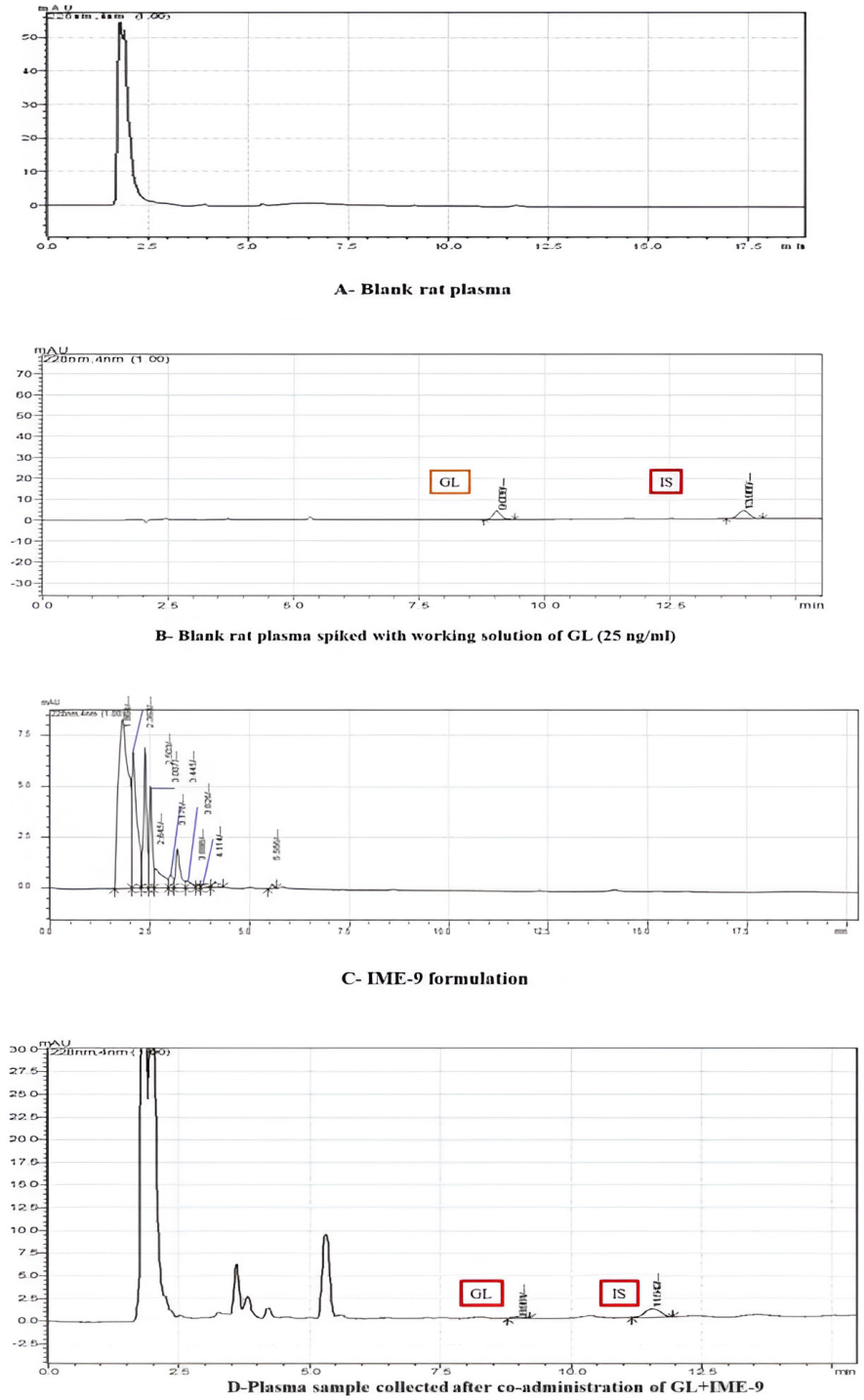


Figure 1: Chromatograms A-Blank rat plasma, B-Blank rat plasma spiked with working solution of Gliclazide at the concentration of 25 ng/mL, C-IME-9 formulation, D-Plasma sample collected after co-administration of GL+IME-9.

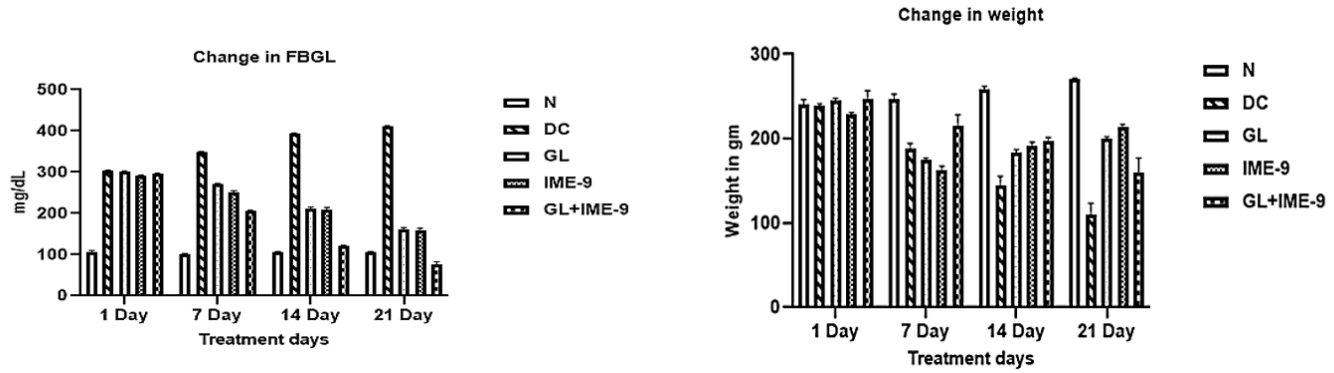


Figure 2: Effect of GL, IME-9 and (GL+IME-9) treatment on FBGL and change in body weight of diabetic rats. Values are expressed as Mean±SEM.

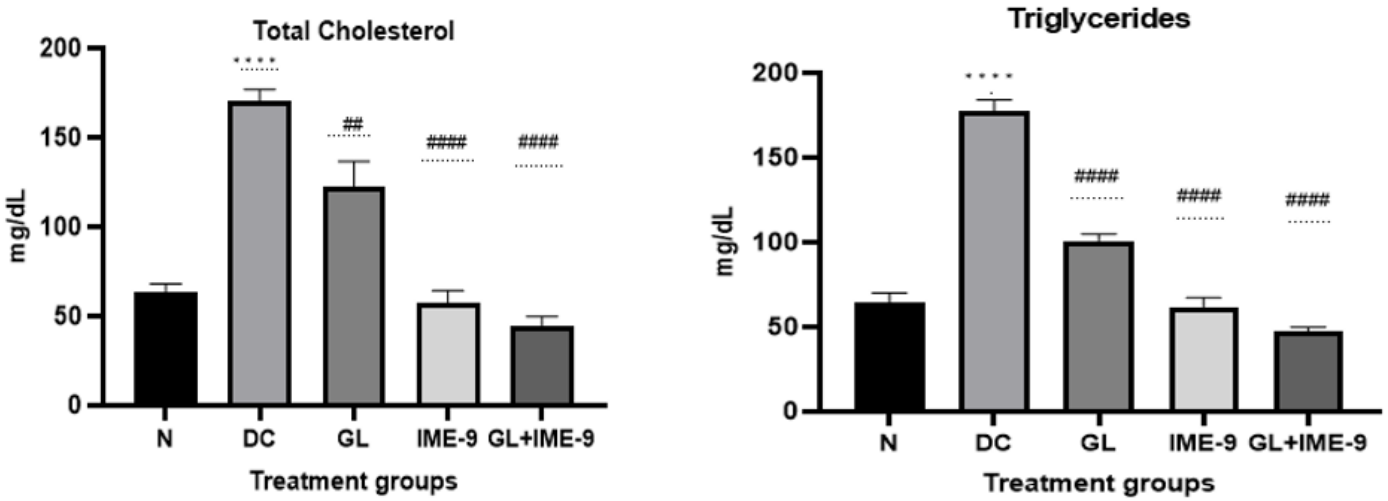


Figure 3: Effect of GL, IME-9, and (GL+IME-9) administration on TC and TG levels. Values are expressed as Mean±SEM (n=6); p-value <0.05 is considered as significant; *p<0.05, **p<0.01, ***p<0.001 compared to the control group; #p<0.05, ##p<0.01 and ### p<0.001, compared to the diabetic control group.

severity and incidence rate of these lesions were reduced in the rats treated with the GL, IME-9, and combination treated groups (GL+IME-9) compared with the disease control group after the 21 days of treatment, as shown in the microphotographs. Pancreatic histopathological changes are shown in (Figure 4).

In silico Molecular Docking Studies

Interaction with the CYP2C19 Enzyme

Based on the binding energy, the affinity of the phytoconstituents present in IME-9 for the CYP2C19 enzyme was computed and presented in (Table 2). Also (Figure 5 A) depicts the binding interaction of the best 03 ligands (P1, P2, and P3) with the selected target.

Binding interaction of OXV (4-hydroxy-3,5-dimethylphenyl) (2-methyl-1-benzofuran-3-yl)methanone (A), Beta-Amyrin (B), Gymnema Saponin IV (C) and Gymnema Saponin V (D)with CYP2C19 enzyme.

The standard ligand OXV (4-hydroxy-3,5-dimethylphenyl) (2-methyl-1-benzofuran-3-yl) methanone) forms the multiple hydrophobic interactions with the several active amino acid residues within the binding pockets with bond distances of 3.32 to 4 Å, as shown in (Figure 5 (a)). These multiple hydrophobic interactions resulted in the highest affinity (binding energy of -9.923 Kcal/mol) of ligand OXV compared to the test ligands (P1-P46). Among the phytoconstituents studied, beta-amyrin (P1) shows the highest affinity (binding energy of -9.695 Kcal/mol) with the hydrogen bonding interaction with the amino acid ALA103A with the bond distance of 3.99Å. Additionally, it forms fewer hydrophobic interactions with the active amino acid residues within the binding pocket, as shown in (Figure 5 (b)). Also, Gymnema Saponin IV (P2) displayed strong affinity (binding energy of -9.495 Kcal/mol) with multiple hydrogen bonding interactions with the active amino acid residues having bond distances in the range of 2.7-3.97Å with additional hydrophobic interactions as shown in (Figure 5(c)).

The phytomolecule Gymnema saponin V(P3) also showed strong affinity (binding energy of -9.137 Kcal/mol) with the multiple hydrogen bonding interaction with the active amino acid residues

(having bond distances of 2.37-4.07 Å) along with hydrophobic interactions as shown in (Figure 5 (d)).

Interaction with the CYP2C9 enzyme

Based on the binding energy, the affinity of the phytoconstituents in IME-9 for the CYP2C9 enzyme was computed and shown in Table 3. Additionally, (Figure 5 b) shows the binding interactions of the top three ligands (S1, S2, and S3) and the chosen target.

The standard ligand Flurbiprofen ((2S)-2-(3-fluoro-4-phenyl-phenyl) propanoic acid) forms hydrogen bonds interaction with amino acid ASN204A with the bond distance 3.07 Å and hydrophobic interactions with the active amino acid residues within the binding pockets with bond distances in the range of 3.44 to 3.98 Å as shown in (Figure 5 (e)). Out of 46 compounds studied, beta amyrin (S1) (binding energy of -10.141 kcal/mol) displayed binding energy more than that of the standard ligand Flurbiprofen as shown in (Figure 5 (f)) through the formation of hydrophobic interaction with many active amino acid residues within the binding pocket (bond distances in the range of 2.56-3.68 Å). Gymnema Saponin IV and Corilagin showed binding energies of -9.701 and -9.401 kcal/mol, respectively. Gymnema Saponin IV (S2) forms multiple hydrogen bonds with the active amino acid residues (2.57-4.05 Å) with hydrophobic interactions, as shown in (Figure 5 (g)). While Corilagin (S3) also forms many hydrogen bonding interactions with the active amino acid residues (3.11-4.08 Å) with additional hydrophobic interactions, as shown in (Figure 5 (h)).

Cell Cytotoxicity Study

The cell viability assay was performed to check the concentrations of the GL, IME-9, and (GL+IME-9) toxic to the cells. The cytotoxic activity of GL, IME-9, and GL+IME-9 was evaluated using MTT assay, indicated that GL (10,100 and 500 µg/mL), IME-9 (10,100 and 500 µg/mL) and GL+IME-9 (10,100 and 500

Table 2: Binding affinities of the phytoconstituents in IME-9 with CYP2C19 enzyme.

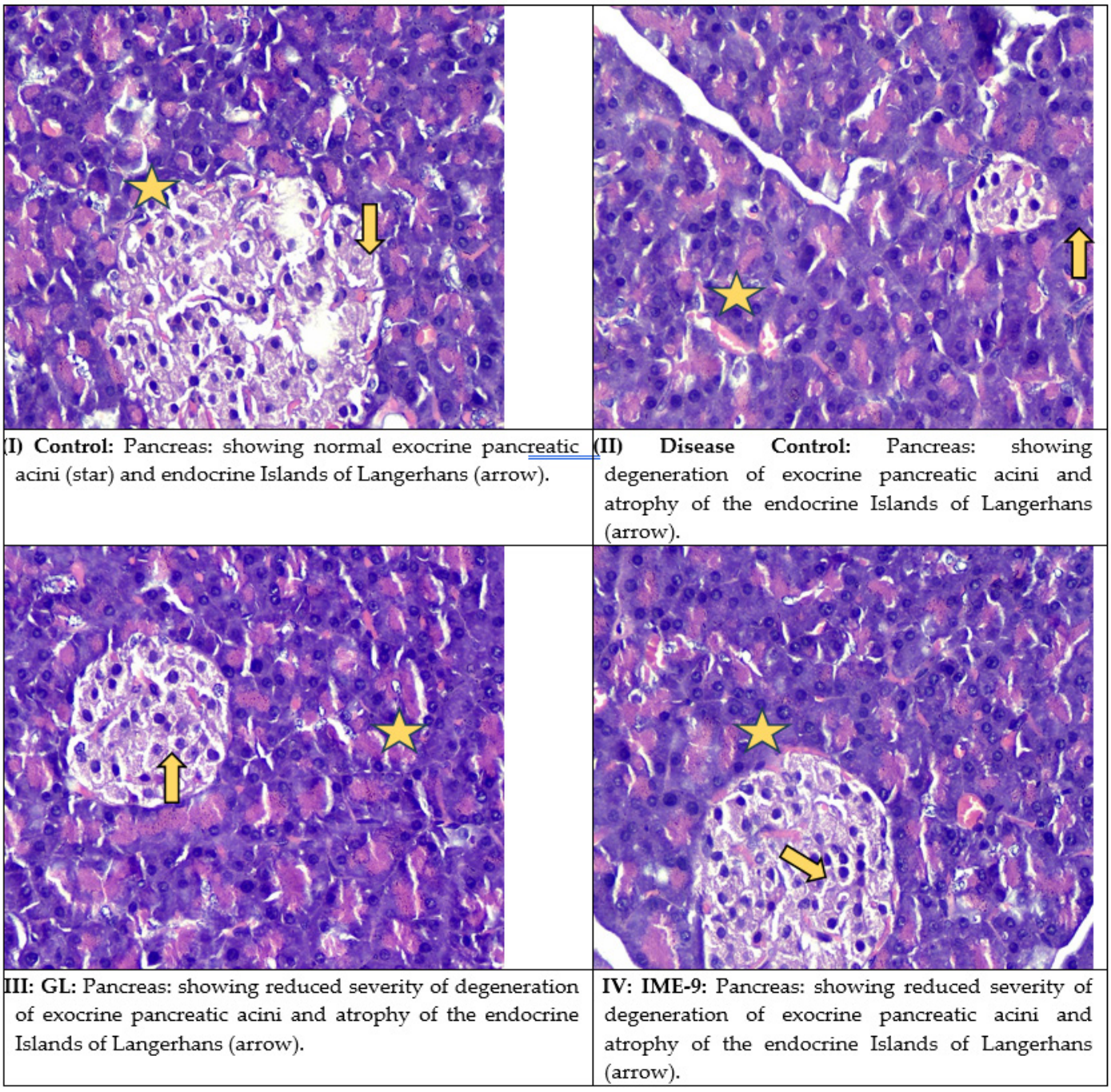
Molecule No.	Molecule	Binding Energy (kcal/mol)	Molecule No.	Molecule	Binding Energy(kcal/mol)
Standard	OXV (4-hydroxy-3,5-dimethylphenyl) (2-methyl-1-benzofuran-3-yl) methanone).	-9.923	P24	Gymnemic-acid-V	-7.789
P1	Beta-Amyrin	-9.695	P25	Homomangiferin-	-7.769
P2	Gymnemasaponin-IV	-9.495	P26	Gymnemic-acid-IV	-7.739
P3	Gymnemasaponin-V	-9.137	P27	Fisetin	-7.728
P4	Campesterol	-9.059	P28	Gymnemic-acid-III	-7.718
P5	Gymnestrogenin	-8.913	P29	Kaempferol	-7.691
P6	Gypenoside-XXXVII	-8.885	P30	Gypenoside-XXVIII	-7.673
P7	Gymnemagenin	-8.824	P31	Humulene	-7.615
P8	momordicoside-F2	-8.727	P32	Cedrol	-7.535
P9	Gymnemasaponin-II	-8.711	P33	Gymnemic-acid-II	-7.503
P10	Gymnemic -acid -VIII	-8.605	P34	Indicoside-A	-7.497
P11	Corilagin	-8.604	P35	Momordicoside-K	-7.465
P12	Gynosaponin	-8.535	P36	Manglupenone	-7.451
P13	Momordicine-II	-8.494	P37	Mangiferin	-7.448
P14	Momordicoside_-I	-8.462	P38	Momordicoside	-7.276
P15	Friedelin	-8.309	P39	Momordicine I	-6.908
P16	Momordicoside -C	-8.278	P40	(-)-Germacrene -A	-6.838
P17	Gymnemic-acid-X	-8.199	P41	Charantin	-6.65
P18	Mangiferonic-acid	-8.138	P42	Gallic -acid	-5.988
P19	Momordicoside-A	-8.07	P43	Myrtenol	-5.915
P20	Momordicoside-F1	-8.052	P44	Myristic -acid	-5.284
P21	Myricetin	-8.047	P45	Guaiacol	-5.146
P22	Narcissin	-7.966	P46	Dotriacontane	-3.645
P23	Gymnemic-acid-I	-7.926			

μg/mL) were able to maintain the viability of UCMSCs in the range of 76.48-97.57%. [GL (90.54%, 82.04%, 76.487%), IME-9 (97.57%, 95.27%,92.76%,) and GL+IME-9 (94.77%, 92.83%, 87.73%)]. This study helped to establish the treatments' safety in the tested concentration range.

DISCUSSION

Herb-drug interactions continue to pose significant challenges and may alter the therapeutic efficacy of any drug treatment. Today, with a rise in the occurrence of diabetes mellitus, many patients resort to traditional medicinal therapies in addition to the prescribed modern medicine. However, many patients fail to inform their medical practitioner, posing concerns about HDI occurrence. Many HDIs, as reported in the literature, may not be serious. However, cases of significant HDI on the concomitant administration of Trazodone, an antidepressant drug with herbs like *Ginkgo biloba* are reported, leading to fatal effects like coma. In this purview, it was thought appropriate to study the possibility of any HDI on the concomitant administration of GL and IME-9.

GL is one of the most commonly prescribed drugs for treating diabetes mellitus. It is primarily metabolized by the CYP2C9 and CYP2C19 enzymes in the liver, resulting in the formation of methyl hydroxyl gliclazide and 6 β-hydroxy gliclazide metabolites.

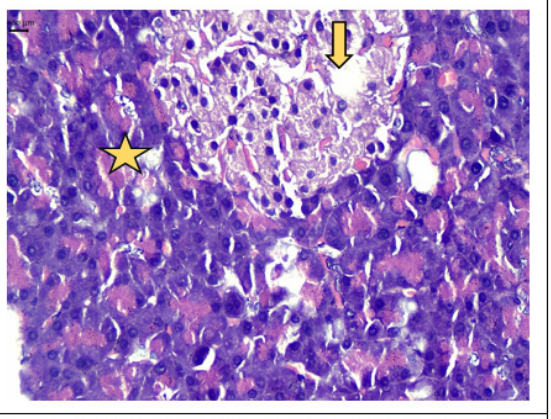


(I) Control: Pancreas: showing normal exocrine pancreatic acini (star) and endocrine Islands of Langerhans (arrow).

(II) Disease Control: Pancreas: showing degeneration of exocrine pancreatic acini and atrophy of the endocrine Islands of Langerhans (arrow).

III: GL: Pancreas: showing reduced severity of degeneration of exocrine pancreatic acini and atrophy of the endocrine Islands of Langerhans (arrow).

IV: IME-9: Pancreas: showing reduced severity of degeneration of exocrine pancreatic acini and atrophy of the endocrine Islands of Langerhans (arrow).



V: GL + IME-9: Pancreas: showing reduced severity of degeneration of exocrine pancreatic acini and atrophy of the endocrine Islands of Langerhans (arrow).

Figure 4: Histopathological changes of rat pancreas after administration of -C: Control group, DC: Diabetic control group, GL: GL treated group, IME-9: IME-9 treated group, GL+IME-9: combination treated group.

Table 3: Binding affinities of the phytoconstituents in IME-9 with CYP2C9 enzyme.

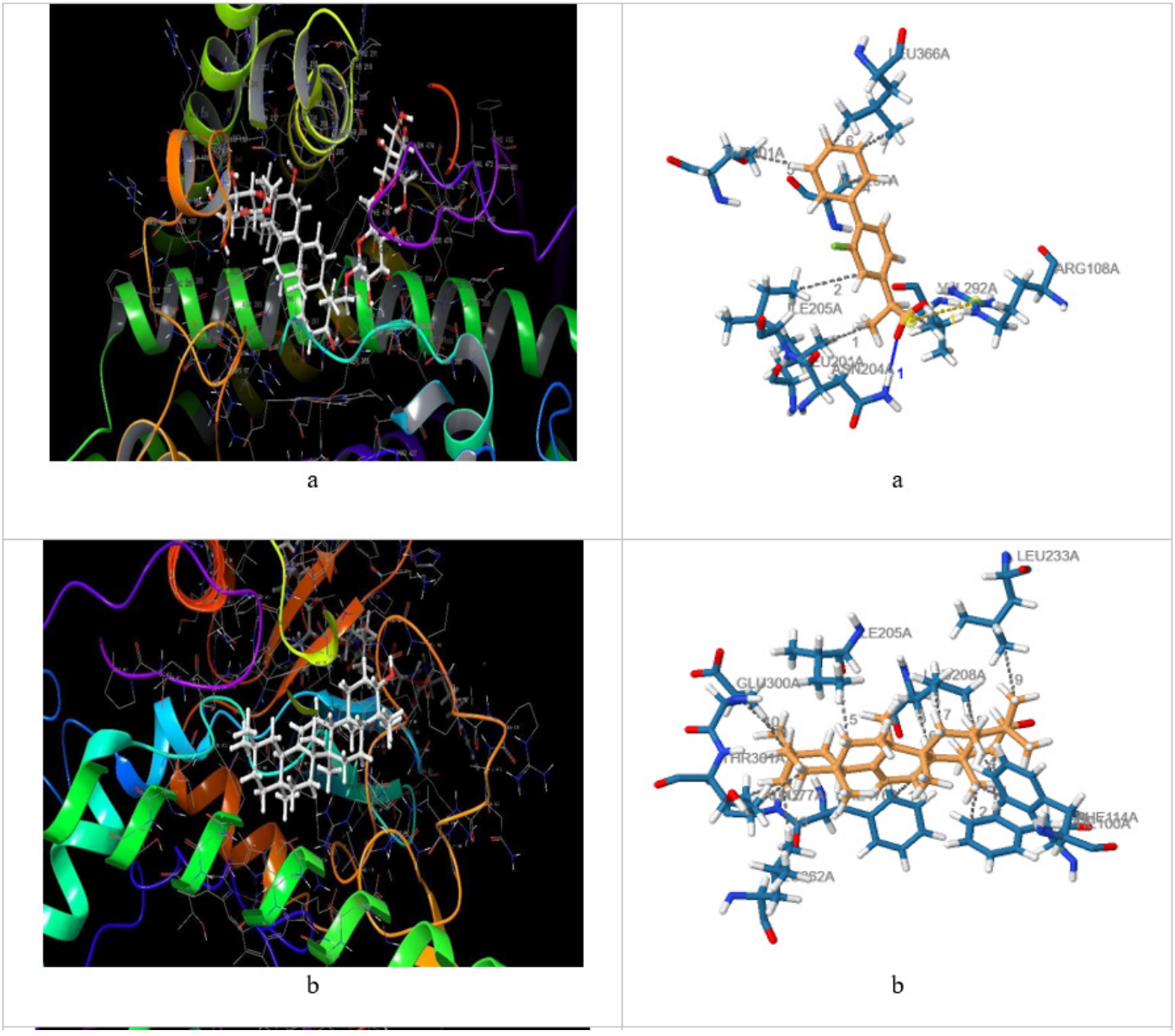
Molecule No.	Molecule	Binding Energy (kcal/mol)	Molecule No.	Molecule	Binding Energy (kcal/mol)
Standard	Flurbiprofen((2S)-2-(3-fluoro-4-phenyl-phenyl) propanoic acid)	-9.737	S24	Momordicoside F2	-8.005
S1	Beta-Amyrin	-10.141	S25	Myricetin	-7.997
S2	Gymnemasaponin IV	-9.701	S26	Gymnestrogenin	-7.989
S3	Corilagin	-9.401	S27	Gymnemic acid -I	-7.961
S4	Momordicine II	-9.098	S28	Gymnemic acid -V	-7.961
S5	Gymnemagenin	-9.086	S29	Gymnemic acid III	-7.958
S6	Gymnemasaponin II	-8.946	S30	Gynosaponin	-7.878
S7	Gypenoside XXXVII	-8.926	S31	Narcissin	-7.73
S8	Gymnemasaponin V	-8.83	S32	Kaempferol	-7.712
S9	Gymnemic acid IV	-8.732	S33	Mangiferin	-7.688
S10	Gymnemic acid VIII	-8.533	S34	Gymnemic acid II	-7.684
S11	Campesterol	-8.414	S35	Homomangiferin	-7.529
S12	Charantin	-8.402	S36	(-)-Germacrene A	-7.477
S13	Gymnemic acid X	-8.227	S37	Indicoside A	-7.467
S14	Momordicoside A	-8.19	S38	Momordicoside	-7.339
S15	Momordicoside C	-8.153	S39	Mangiferonic acid	-7.326
S16	Humulene	-8.124	S40	Gypenoside XXVIII	-7.287
S17	Friedelin	-8.098	S41	Cedrol	-7.251
S18	Momordicoside F1	-8.075	S42	Dotriacontane	-6.696
S19	Fisetin	-8.051	S43	Myrtenol	-5.904
S20	Momordicoside I	-8.038	S44	Gallic acid	-5.812
S21	Momordicine I	-8.033	S45	Myristic acid	-5.402
S22	Manglupenone	-8.032	S46	Guaiacol	-5.251
S23	Momordicoside K	-8.013			

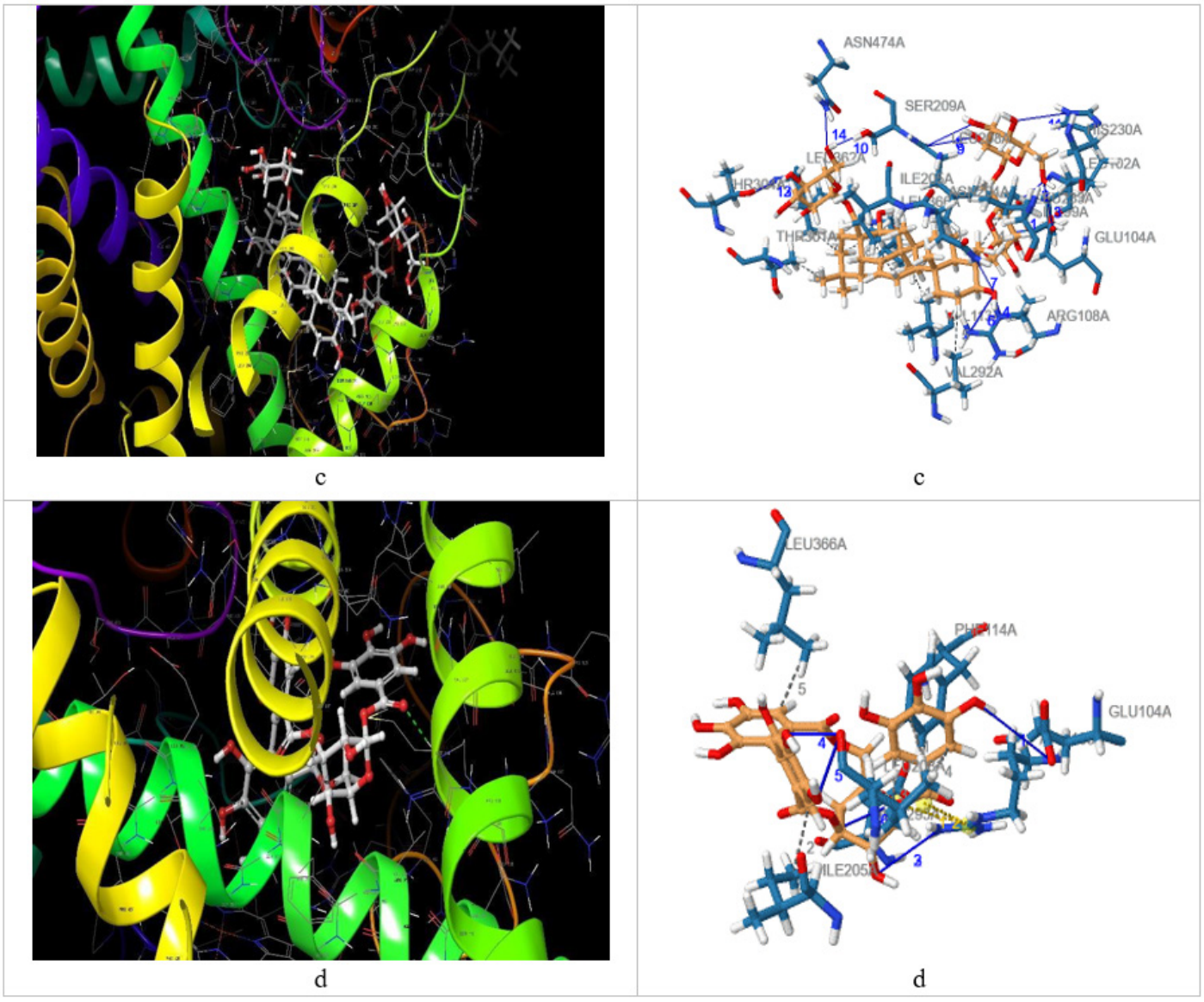
*Nomenclature of the phytomolecules has been changed for understanding the interactions with CYP2C9.

IME-9 contains several herbs, including *Mangifera indica, Momordica charantia, Gymnema sylvestre, Syzygium cumini* many of which are studied and reported to possess inhibitory effects on the CPY2C9/2C19 enzymes.[15] The *in vitro* investigations of aqueous extracts of *Mangifera indica*, as reported in the literature, indicate that it is capable of inhibiting the CYP2C8, 2B6, 2D6, 1A2 and 2C9 enzymes. The *Momordica charantia* leaf and stem extracts exhibit inhibitory effects on the CYP2C9,2C19, 1A2, 2A6 and 3A4 activities.[11] *In vitro* inhibitory assay of the extract of *Gymnema sylvestre* also showed that it is a potent inhibitor of CYP2C9,1A2,2C8, 2D6, and 3A4.[16] Further another study reported that the fruit of *Syzygium cumini* inhibits the CYP2C9 with IC_{50} of 76.69.[17]

The study was undertaken based on the hypothesis that as many of the herbs present in IME-9 are potent inhibitors of the cytochrome enzymes, they may alter GL's PK/PD behaviour, leading to undesired side effects or therapeutic failures.[18] This was confirmed through the *in silico* studies that most of the phytomolecules present in the herbs in IME-9 are potent inhibitors of both the CYP2C9 and CYP2C19 enzymes, raising concerns about HDI.

In the PK-HDI studies, it was observed that co-administration of IME-9 herbal formulation with GL for 21 days altered the PK parameters, resulting in a significant 4-fold increase in C_{max} of GL, increasing the GL bioavailability. This modification in pharmacokinetic parameters may be attributed to the inhibitory potential of the IME-9 formulation on CYP2C9 and CYP2C19 activity. Consequently, a decrease in Ke, CL, MRT, and t1/2 increased GL's bioavailability, due to reduced elimination, increased higher plasma concentration, increased half-life, and increased potential for accumulation. which may be responsible





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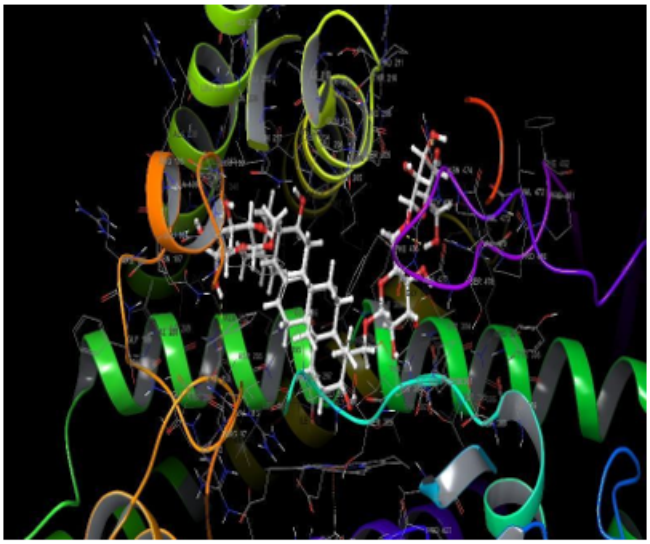

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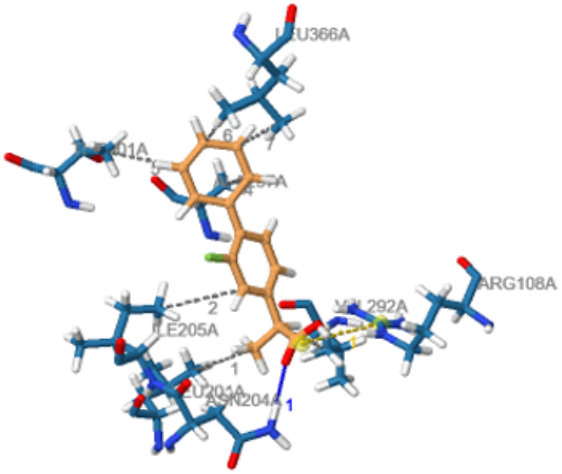
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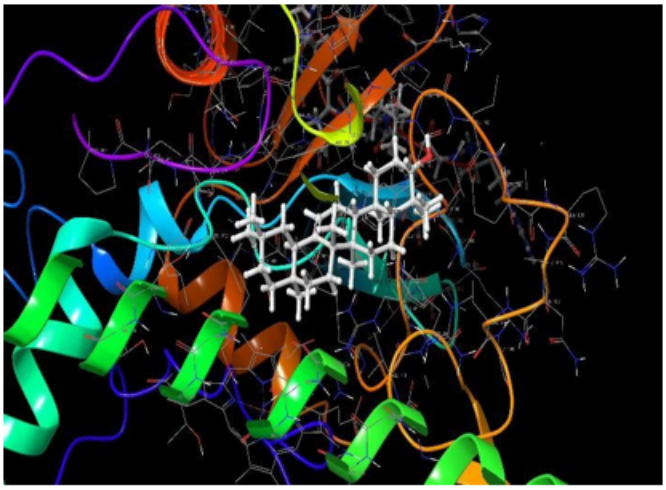

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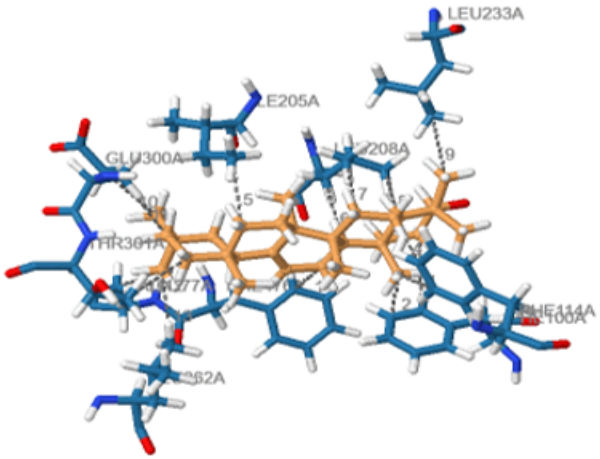
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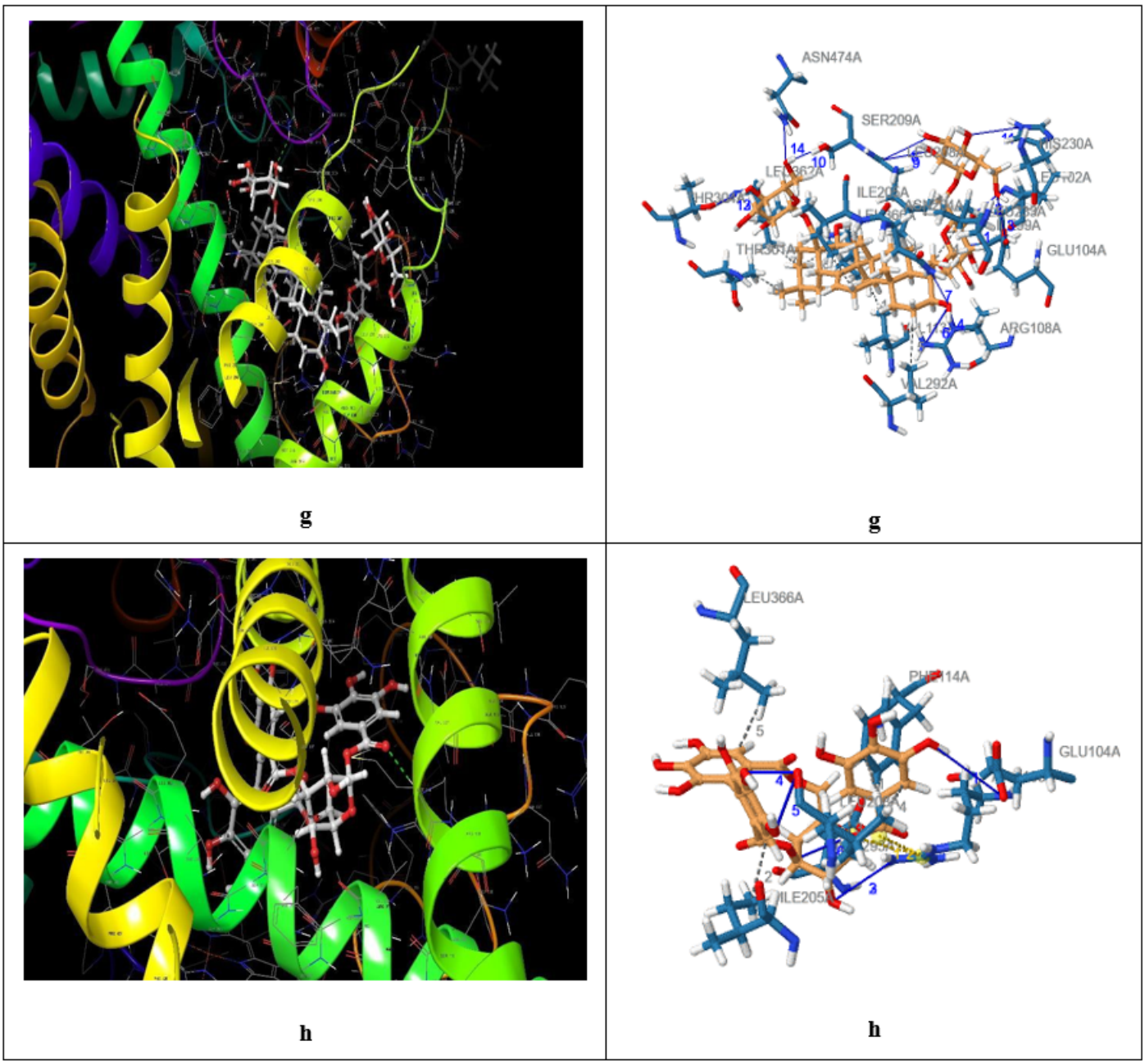

(B)

Figure 5: (A)-Binding interaction of OXV(4-hydroxy-3,5-dimethylphenyl) (2-methyl-1-benzofuran-3-yl)methanone (a), Beta-Amyrin (b), Gymnema Saponin IV (c) and Gymnema Saponin V (d) with CYP2C19 enzyme. (B) Binding interaction of Flurbiprofen ((2S)-2-(3-fluoro-4-phenyl-phenyl)propanoic acid) (e), beta-Amyrin (f), Gymnemasaponin IV (g), Corilagin (h) with CYP2C9 enzyme.

for significantly lowering the FBGL. This may prove to be highly fatal and may lead to hypoglycemic coma if left unaddressed.

Further, the combination therapy (GL+IME-9) was not able to restore the reduced body weight of the rats, which may cause other undesirable effects, as usually observed in diabetic patients. The reduction in Fasting Blood Glucose Levels (FBGL) was highly significant in Group V (GL+IME-9) compared to the groups treated with GL or IME-9 monotherapy. The combination therapy, however, restored the plasma TC and TG levels to normal following the 21 day treatment period and thereby may be of benefit, as many of the diabetic patients may also be hyperlipidemic. As this combination may also provide the added benefit of reducing elevated lipid levels, it may help reduce the burden of taking additional medications to treat hyperlipidemia.

However, the histopathology findings conclude that the rats treated with GL, IME-9 and combination of (GL+IME-9) treatment groups showed ameliorative effects and helped to restore the damaged pancreas's altered architecture.

This study underscores the critical involvement of cytochrome enzymes in the observed HDI.

Among the phytoconstituents analyzed, most compounds found in the herbs of IME-9 demonstrated a strong binding affinity for the CYP2C9 and CYP2C19 enzyme, suggesting a notable potential to inhibit its activity. Nevertheless, there are multiple mechanisms involved in such interactions, such as changes in gastrointestinal function that may affect drug absorption, the induction/inhibition of P-gp transport proteins, alteration in plasma protein binding that may also affect drug distribution, and changes in renal functions that affect the elimination of drugs and their metabolites.[19,20]

CONCLUSION

The PK/PD information obtained throughout the experiment showed pharmacokinetic and pharmacodynamic interactions on the concomitant administration of GL with IME-9. This study throws light on the occurrence of hypoglycemia following the subsequent administration of GL and IME-9 post 21 days in rats, leading to significant HDI, which may further lead to harmful effects such as hypoglycemic coma. However, due to the potential lack of correlation between preclinical animal data and human clinical data, sample case studies involving human volunteers are necessary to validate the outcomes of these preclinical experiments. However, the data obtained from this research is extremely beneficial in understanding the possibility of such complications in diabetic patients, helping to make judicious decisions in dose adjustments, and employing such combinations to treat diabetic subjects.

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ABBREVIATIONS

IDF: International Diabetes Federation; **T2DM:** Type 2 Diabetes Mellitus; **WHO:** World Health Organization; **HDI:** Herb-Drug Interactions; **CYP:** Cytochrome P450; **RP-HPLC:** Reverse Phase High Performance Liquid Chromatography; **STZ:** Streptozotocin; **GL:** Gliclazide; **AYUSH:** The Department of Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homeophaty; **PK:** Pharmacokinetic; **PD:** Pharmacodynamics; **IS:** Internal Standard; **DST-FIST:** Department of Science and Technology Fund for Improvement of Science and Technology; **EDTA:** Ethylenediamine Tetraacetic Acid; **LLE:** Liquid Liquid Extraction; **QC:** Quality Control; **LQC:** Lower Quality Control; **HQC:** Higher Quality Control; **MCQ:** Middle Quality Control; **LLOQ:** Lower Limit of Quantification; **RT:** Retention Time; **FBGL:** Fasting Blood Glucose Level; **DC:** Diabetic Control; **CMC:** Carboxymethyl Cellulose; **AUC:** Area Under Curve; **Vd:** Volume of Distribution; **T_{max}:** Time to Reach Maximum Concentration; **ANOVA:** Analysis of Variance; **CV:** Coefficient of Variation; **C_{max}:** Peak Plasma Concentration; **SEM:** Standard Error Mean; **$AUC_{0-\infty}$:** Area Under Plasma Concentration-Time Curve Extrapolated to Infinity; **AUC_{0-t}:** Area Under Plasma Concentration-Time Curve from Zero to Last Time Point; **TG:** Total Triglyceride; **TC:** Total Cholesterol; **Ke:** Elimination Rate Constant.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

Herb-drug interactions are a vital challenge for the clinical therapeutic efficacy of drug treatment. Herb-drug interactions alter the PK/PD fates of drugs, resulting in therapeutic failure or toxicities. In the present research work the assessment of PK/PD HDI of anti-diabetic drugs GL with commonly used herbal preparation IME-9 in pre-clinical models was established. For the PK study of GL, RP-HPLC methods were developed and validated as per USP guidelines 2018. The pharmacokinetic data attained in the investigation demonstrated that the co-administration of (GL+IME-9) in diabetic rats for 21 days altered the PK parameters resulting in pharmacokinetic interactions. Diabetes is known to be associated with dyslipidemia, the combination group (GL+IME-9) may be beneficial to control elevated TC and TG levels in diabetic patients. It was observed that the

co-administration of IME-9 and GL did not restore the body weight of the rats. In the combination treatment (GL+IME-9) showed highly significant reduction in FBGL as compared to all other groups. This study throws light on the occurrence of hypoglycemia following the concomitant administration of GL and IME-9 post 21 days in rats leading to significant HDI which may further lead to deleterious effects such as hypoglycemic coma. In the histopathological evaluation it was found that the (GL+IME-9) didn't restored normal pancreatic cells.

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