Ind. J. Pharm. Edu. Res., 2024; 58(2s):s372-s381.
https://www.ijper.org

Original Article

Chlorogenic Acid, a Potential Glucose-6-Phosphatase Inhibitor: An Approach to Develop a Pre-Clinical Glycogen Storage Disease Type I Model

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ABSTRACT

Introduction: Gluconeogenesis and glycogenolysis are highly regulated metabolic processes that are critical in maintaining blood glucose levels within the physiological range. Any aberration in the regulation of these processes can lead to an inadequate repository or accumulation of excess glycogen in hepatocytes. Glycogen Storage Disease type I (GSD type I), also known as Von Gierke's disease, is categorized under inborn errors of metabolism caused due to either inactivity or complete absence of the Glucose-6-phosphatase enzyme (G6Pase). **Objectives:** This study's focus is to suppress G6Pase, hepatic glucose synthesis, and induce the symptoms of GSD type I in experimental rats. **Materials and Methods:** The *in silico* approach using Chlorogenic Acid (CGA) and G6Pase exhibited a good docking score (-8.9), and promising binding patterns, molecular dynamic simulation studies (at 100 nanoseconds) also confirmed the stability of the docked complex. Based on this *in silico* speculation, the *in vivo* study was designed, where, in the pilot study varying doses (50, 100, 200, 400 mg/kg) and CGA-liposome formulation were scrutinized, **Results:** It was corroborated that the CGA can cause hypoglycemia, hence CGA (200 mg/kg) was chosen for the study, to further augment the other major GSD type I manifestations, metformin (500 mg/kg) was included in the study for 49 days, manifestations like hypoglycemia, suppressed G6Pase activity, elevated hepatic glycogen and lactate dehydrogenase were evident. **Conclusion:** The observations suggest that chlorogenic acid has the potential to induce GSD type I manifestations along with metformin, which can be an alternative animal model to match the genetically modified disease models.

Keywords: Chlorogenic acid, Metformin, Fasting blood glucose, Glucose-6-phosphatase, Glycogen storage disease type I, Glycogenolysis.

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Received: 12-03-2023;
Revised: 28-09-2023;
Accepted: 15-02-2024.

INTRODUCTION

Glycogen Storage Diseases (GSD's) or glycogenoses are a category of inborn errors of metabolism that result due to a deficiency of certain enzymes required to regulate glycogenolysis or gluconeogenesis.[1] The predominant storage sites for glycogen are the liver and muscles. Hence, glycogen breakdown and/or utilization can affect the liver, muscles, or both. Hepatic GSD is characterized by hypoglycaemia, whereas GSDs with muscular involvement are marked by muscle weakness and spasms. These disorders affect about 1 in every 20,000 to 43,000 live births approximately in the United States every year. Moreover, GSD (types I, III, and IX) account for 80% of hepatic GSD's, with type I being the most predominant.[2,3]

DOI: 10.5530/ijper.58.2s.40

GSD type I is caused due to diminished activity or complete absence of glucose-6-phosphatase (G6Pase) and has an incidence of 1 in 1,00,000. It is further classified into subgroups based on the anomaly in the G6Pase system. Around 80% of the clinical GSD type I cases are categorized as GSD type Ia (Von Gierke's disease) which is caused due to diminished activity or absence of glucose-6-phosphatase-α enzyme (G6Pase-α) and 20 % are GSD type Ib caused due to the deficient activity of Glucose-6-Phosphate Translocase/transporter (G6PT).[4] Patients with GSD Type-I encounter life-threatening symptoms if they are left untreated. Despite having intensive dietary therapeutic strategies, they suffer serious untoward effects, like renal failure and liver tumours, as age progresses. Both GSD type Ia and Ib share same manifestations like hypoglycaemia, hyperlipidaemia, hyperuricemia, lactic acidosis, and extensive glycogen accumulation primarily in the liver followed by kidneys, leading to hepatomegaly and nephromegaly (Figure 1).[2,5] Due to hindered glucose mobilization across the cell membrane and within the cell of the polymorphonuclear leukocytes, GSD type Ib patients are

also presented with symptoms like frequent infections, decrease in neutrophil count, and neutrophil dysfunction in addition to the other clinical features of impaired glycogen metabolism.[6]

The clinical presentation of GSD type I hints at the initial diagnosis, a series of biochemical abnormalities in liver biopsy tissue, as well as molecular genetic tests and/or enzymology, which can confirm the diagnosis.[7] Until today, there is no cure for this disorder, and the management of its manifestation is a daunting task. The most common management strategy for this multisystem disorder is dietary therapy. Despite adherence to dietary therapy, GSD type Ia patients suffer from acute and chronic complications.[1] The complex nature of the disorder warrants the need for animal models to provide mechanistic insights that undoubtedly will direct eventual therapies. However, designing and establishing a disease model for GSD-type I is an uphill task. The gene knockout animals and genetically mutated cells serve as perfect disease models, however, the issues of stability, survivability, maintenance, and the cost of these models are a big challenge.[8] Therefore, new animal models which are easier to induce, manage and maintain are required to be explored and contribute to the improvement of the treatment strategies from both pharmacological and nutritional perspectives. Developing a chemically induced animal model could help in overcoming some of the hurdles faced while designing a transgenic model like avoiding the developmental abnormalities caused by the genetic defect (in turn affecting survival). As per the literature review, certain synthetic and phytochemical compounds such as Mumbaistatin,[9] Kodaistatin,[10] for this study, Chlorogenic Acid (CGA) was considered, CGA belongs to a class of hydroxycinnamic acid derivative, which is present in a variety of fruits and abundantly present in green coffee beans[11,12] and its derivatives[13] known to efficiently inhibit the particular enzyme glucose-6-phosphatase complex in the liver and can lead to decrease the fasting blood glucose levels, which can simulate the major manifestation similar of GSD type I, on the other hand, metformin is known to induce lactic acidosis[14] which is also one of the dominant manifestations of GSD type I. The present work is an attempt to determine how best these chemicals mimic the characteristic manifestations of GSD type I.

MATERIALS AND METHODS

In silico study

Molecular Docking

In order to obtain a clean protein structure, water and heteroatoms were removed from the Glucose-6-phosphatase with

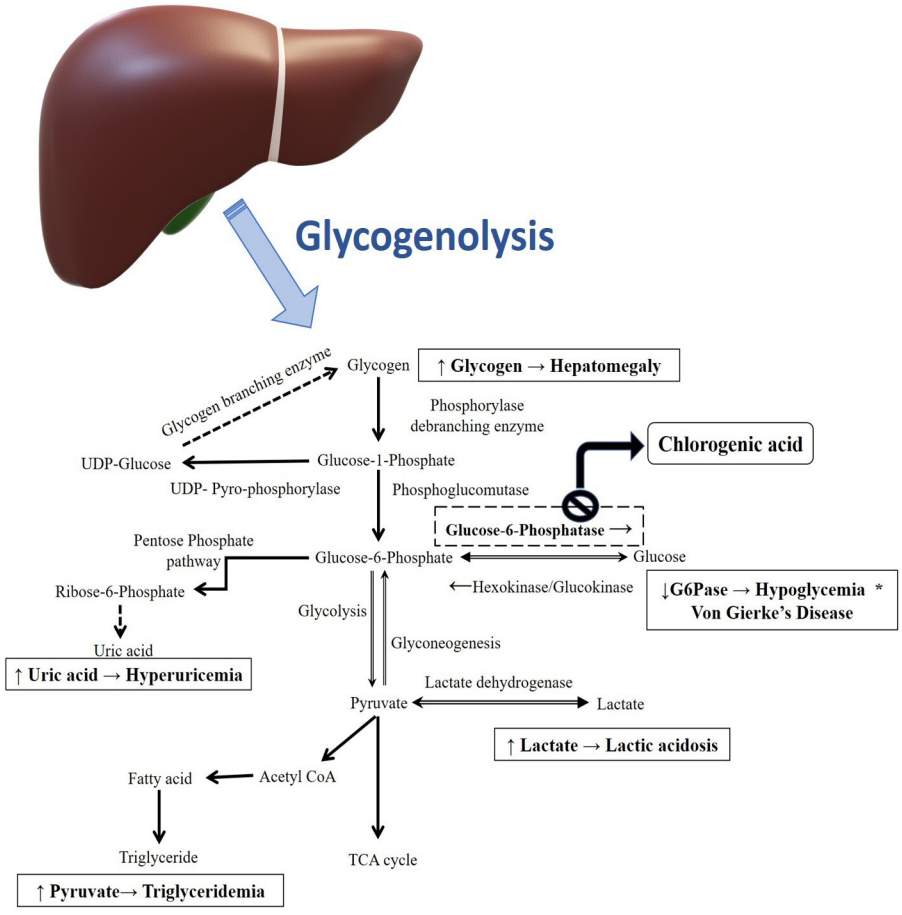

Figure 1: Graphical abstract emphasising the pathway explaining the manifestations of GSD type I upon Glucose-6-Phosphatase inhibition.

the modelled structure. Molecular binding sites are considered in receptor cavity method docking calculations for ligand molecules bound in interactions. The study of receptor-ligand docking was conducted with AutoDock Vina with a box size of 40 x 40 x 40.

Molecular Dynamics-Simulation

The chosen protein-ligand complex was run through a molecular dynamic's simulation in Gromacs-2019. 4. To obtain the force field coordinates, the specified ligand topology was obtained from the PRODRG server (PMID: 15272157). The vacuum minimization technique was used to prepare the system with 1500 steps with the steepest descent algorithm. Subsequently, in a cubic periodic box of 0.5 nm, the complex structures were solvated using a Simple Point Charge (SPC) water model. Subsequently the complex systems were maintained with the optimum concentration of salt at 0.15 M by adding suitable numbers of Na^+ and Cl^- counter ions. Based on a previously published paper, the system preparation was postulated.[15] Each resultant structure from the Number of particles, Pressure, and Temperature (NPT) equilibration phase was subjected to a final production run in the NPT ensemble for 100 ns simulation time (PMID: 31514687). The GROMACS simulation suite of Protein RMSD, RMSF, RG, SASA, and H-Bond was used to perform the trajectory analysis (PMID: 32567989).

Chemicals and Drugs

The chlorogenic acid (Sigma Aldrich, USA) was procured and maintained at 8 to 25°C as per the manufacturer's instructions and metformin tablets (USV India) were maintained at room temperature. All other common laboratory chemicals were of analytical grade and procured from various manufacturers.

Experimental animals and ethical consideration

The investigation was carried out on male albino Wistar rats (n=24), weighing between 150-200 g. Animals were equally distributed into four different groups, housed in polypropylene cages, and the temperature of 27°C±2°C was maintained, with 12 hr, light and dark cycle for acclimatization for about a week. Standard/ routine rat feed (Gold Mohur Lipton India Pvt. Ltd.), and potable water (*ad libitum)* was fed to the animals throughout the study. The proposed animal study was reviewed by the Committee for Control and Supervision of Experiments on Animals (CCSEA) in India and was approved (Approval No. 0/KLECOPH/19) by the Institutional Animal Ethics Committee (IAEC) and ARRIVE guidelines were followed for all the experimental methods.

Induction of GSD type I manifestations

Chlorogenic acid was chosen for this study, considering its safety, availability, and effectiveness. A pilot study was carried out for 14 days to establish the efficient dose of CGA required decreasing the fasting blood glucose level in the test animals. For this experimental setup, the rats were housed into five different groups, each group consisting of six rats. All animals were allowed standard rat feed and water *ad libitum*, except those in the control group. The animals were subjected to daily doses of CGA at varying doses (50, 100, 200, and 400 mg/kg) for two weeks in adjuvant to their daily feed schedule.

An oral dose of (CGA 200 mg/kg) bodyweight was observed to be effective and showed the promising capability to induce fasting hypoglycaemia/decreased fasting blood glucose level in albino Wistar rats. However, it was observed that CGA had a shorter biological half-life, due to which its ability to maintain hypoglycaemia was limited to 1.5 to 2.5 hr. To overcome this situation the Chlorogenic Acid Loaded liposomes (CAL) were formulated based on the literature review for this study.[14] However, the formulation did not achieve the desired result. Subsequently, for the main study, based on the literature, the green coffee extract[11,12] consisting of 36% (CGA 200 mg/kg) was admixed in the drinking water and was fed routinely to the animals in the treatment groups, to sustain the CGA effect. In order to induce further manifestations of GSD, like lactic acidosis, metformin[16] was administered along with CGA in a test group of animals.

The animals were divided into three different testing groups for the main study, each with six animals, Group I, or the control group was provided with normal feed and drinking water. In Group II the animals were fed with normal feed and drinking water in addition to 95% CGA which was administered daily by oral gavage (200 mg/kg body weight for 49 days/7 weeks). Group III animals received green coffee bean extract (36% CGA) admixed in drinking water (200 mg/kg body weight orally daily) in addition to metformin 500 mg/kg and 95% CGA (200 mg/kg body weight for 49 days) along with normal feed, administered daily by oral gavage. During the measurement of blood glucose, the maintenance dose of 100 mg CGA (95%) at 45 min interval was administered to sustain hypoglycaemia (Figure 2C).

Collection of blood and tissue samples

Blood collection was performed by tail prick method with the help of a sterile lancet and the blood glucose was measured by glucometer (Arkray Glucocard). Glucometer and test strips were stored and used at an appropriately controlled temperature and humidity throughout the study duration. Subsequently, for the estimation of other blood parameters the animals were anaesthetised with ketamine and xylazine before retro-orbital blood withdrawal, and for euthanasia high dose of ketamine and xylazine was subjected as per the guidelines, post which the liver tissues were isolated. Blood was blotted from the liver tissues and rinsed using a cold physiological saline solution (consisting of 0.9%, w/v NaCl). For staining processes, the tissues were weighed and preserved in 10% v/v neutral buffered formalin.

Biochemical assessments

Assessment of hypoglycemia

The fasting blood glucose level for the experimental rats throughout the study period was estimated with the help of a glucometer.

Glucose-6-phosphatase estimation

The glucose-6-phosphatase enzyme was estimated by the method described by[17,18] with the following experimental conditions: Temperature of 37°C, pH=6.5, Absorbance at 660nm, with Lightpath=1 cm, Method adopted: Spectrophotometric Stop Rate Determination.

Liver glycogen

The liver glycogen was estimated as described by Montgomery, (1957) immediately after the autopsy.[19]

Serum Lactate dehydrogenase

Lactate dehydrogenase levels were estimated using commercial kits procured from Erba Transasia BioMedicals Ltd., India following the instructions provided along with the kit. A semi-automated clinical chemistry auto analyser (Erba Transasia BioMedicals Ltd., India, Model Chem7) was used to record the measurements.

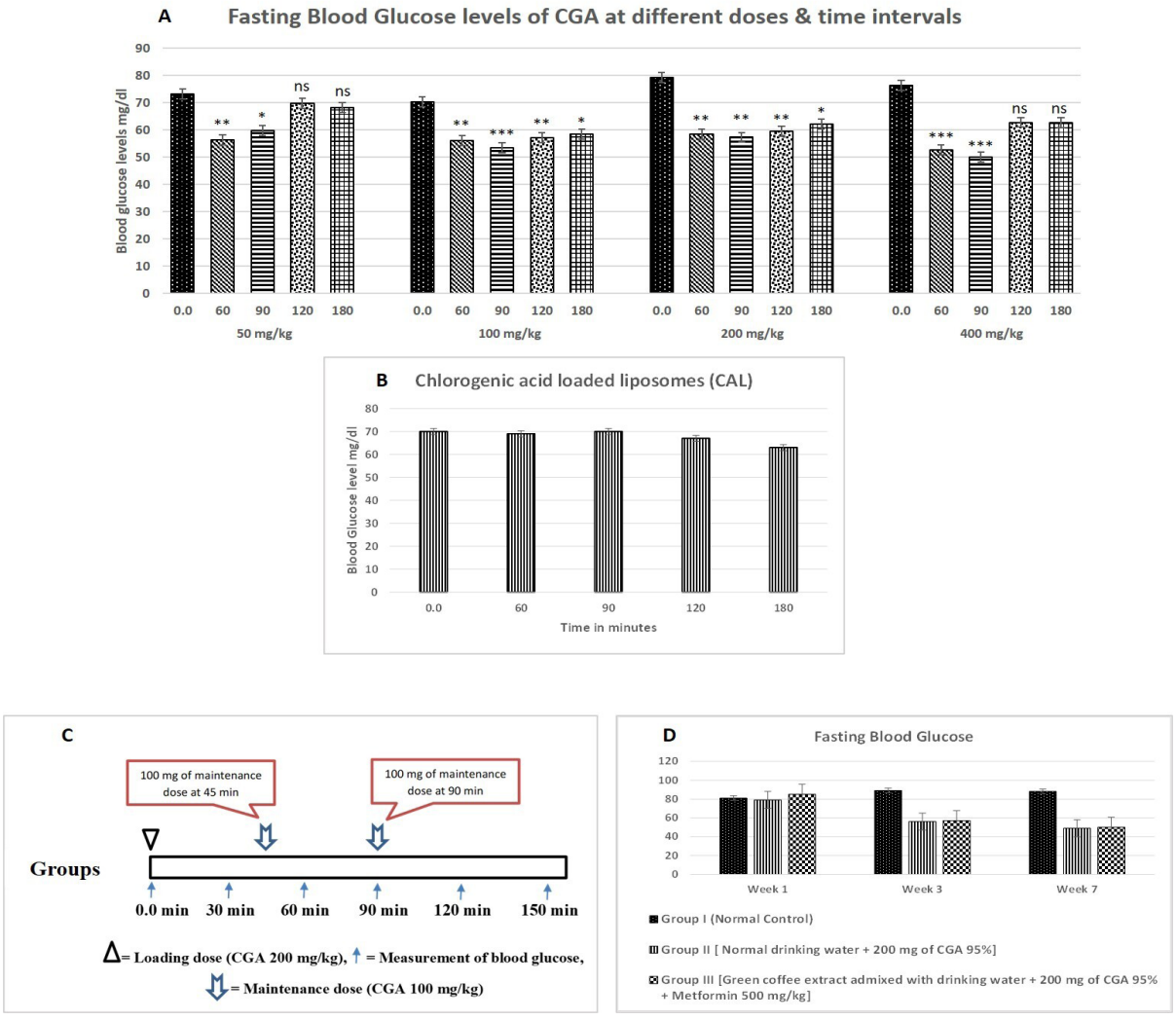

Figure 2: Fasting blood glucose levels, (A) Pilot study to assess the fasting blood glucose levels at different time intervals, ranging from (0.0 min to 180 min), (B) Effect of Chlorogenic acid loaded liposomes on blood glucose levels. The values obtained are expressed as mean±standard error of mean (n=6) in each group, there was no significant decrease in the blood glucose levels (p>0.05), (C) Representative depiction of the experimental set-up, where maintenance dose of CGA 100 mg was administered at every 45 min interval to sustain the enzyme inhibition, during the biochemical estimations, (D) The fasting blood glucose was measured at fixed intervals. The values obtained are expressed as mean±standard error of mean (n=6) in each group, the statistical significance was analysed by one-way ANOVA followed by Bonferroni's post-hoc test during the 1st week there was no significant decrease in the blood glucose, whereas in 3rd week, Group II and III showed significant decrease in the blood glucose levels (**p<0.01) and in 7th week (***p<0.001) when compared to Group I.

Serum Uric acid

Serum Uric acid levels were measured as per Trivedi and Kabasakalian method.[20,21]

Biometric assessment

Liver weight

The liver of the individual animals was surgically isolated, and its weight was measured on the last day of the study.

Statistical analysis

All the data were analysed using the statistical program GraphPad Prism version 6.0 and expressed as mean SEM. Where, Analysis of Variance (ANOVA) was used to examine the data, followed by Bonferroni's multiple comparison *post hoc* test, where p values of <0.05) were set to be considered as statistically significant.

RESULTS

In silico study

Molecular Docking

The binding patterns of Glucose-6-phosphatase were evaluated using AutoDock vina by triplicated docking approach. The intermolecular interactions between G6Pase protein (PRO) and CGA ligand (LIG) is shown in Tables 1 and 2 respectively. The docking images of PRO with the LIG are represented in Figure

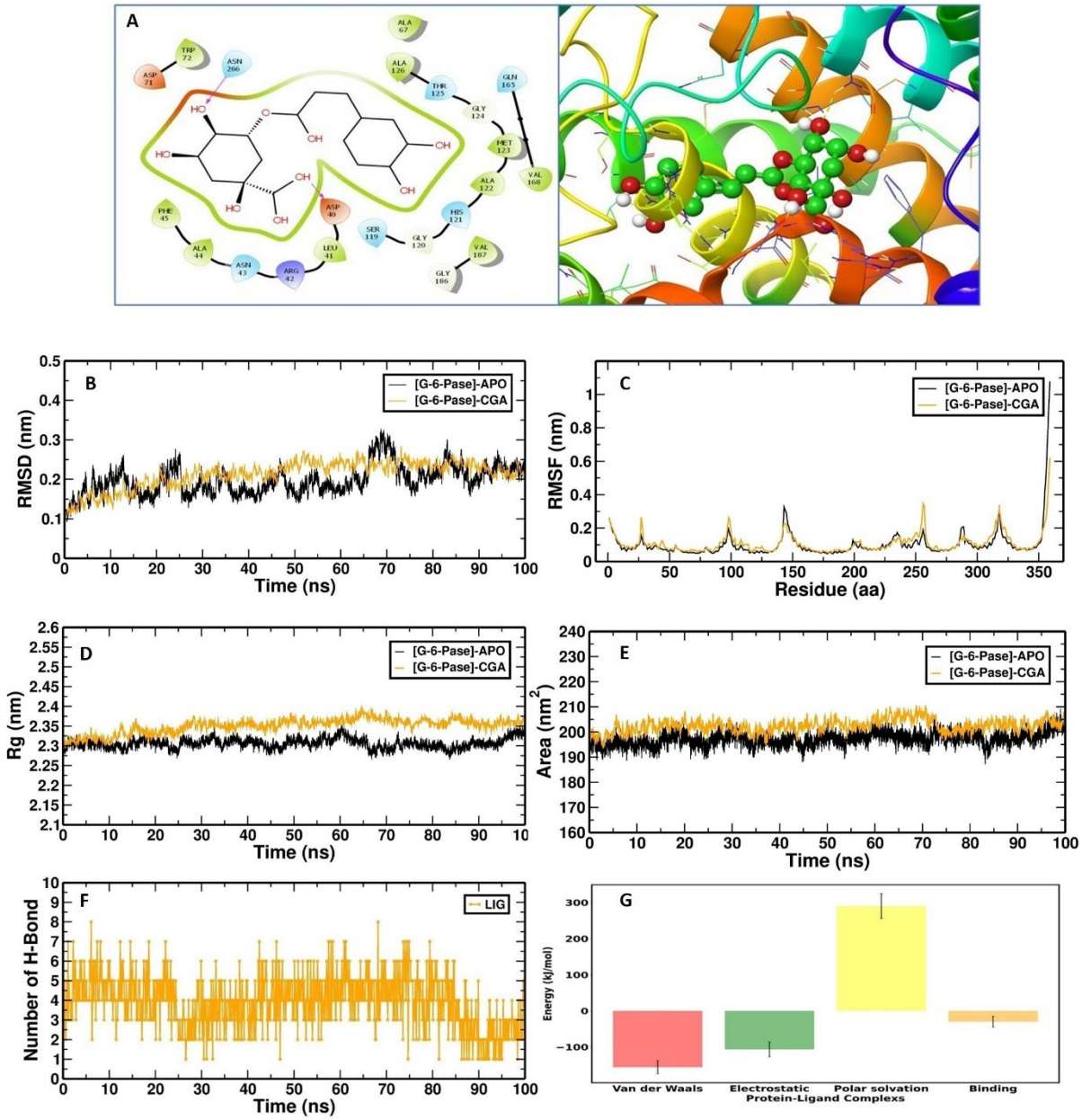

Figure 3: Molecular docking and Molecular dynamics simulation, (A) 2D and 3D interaction of Protein and ligand, (B) Root means square deviation of backbone atoms, (C) Root means square fluctuation of c-alpha atoms, (D) RG of backbone atoms, (E) SASA of backbone atoms of with Protein-Substrate and Protein-Ligand, (F) H-Bond, and (G) Determination of relative binding affinity of Protein-Ligand by MM-PBSA method.

3A. The stability of the complex is greatly influenced by the carbon-hydrogen interaction, Vander Waal's force, and typical hydrogen bonds.

Molecular Dynamics

To comprehend the stability of the protein-ligand complexes, an all atom MD simulation lasting 100 nanoseconds (ns) was performed. (i) Protein alone [PRO-APO], and (ii) Protein with ligand [PRO-LIG]. The average value of RMSD, RMSF, Rg and SASA has been tabulated in Table 3, this information describes the structural properties of Protein-Ligand interaction. The "Average RMSD" (root mean square deviation) and "Average RMSF" (root mean square fluctuation) values are measures of the overall conformational stability and flexibility of the protein. A lower RMSD value indicates that the protein has a more stable structure, while a higher RMSF value indicates that the protein is more flexible. The "Radius of gyration" value is a measure of the size of the protein, with a larger radius indicating a larger protein. The "Average SASA" (Solvent Accessible Surface Area) value is a measure of the amount of surface area of the protein that is exposed to the solvent (such as water). A larger SASA value indicates that more of the protein is exposed to the solvent.

Root mean square deviation

It is a crucial factor in figuring out how the two confirmations differ from one another. The greater the RMSD value, the more will be the deviation. The RMSD values are calculated against the simulation timescale of 100 ns (Figure 3B). The average RMSDs from 0 to 100 ns for PRO-APO, PRO-LIG protein complex proteins were 0.22+/-0.03 nm, 0.21+/-0.04 nm respectively. The relative stability of chemical complexes during the simulation is represented by these RMSD values. The complexes were also stable throughout the simulation.

Root mean square fluctuation

The amino acids in the protein that produce the most vibrations are identified by RMSF analysis, causing the protein to become unstable in both the presence and absence of the ligands. The RMSF values are calculated against the simulation timescale of 0 to 100 ns. The average RMSFs from 0 to 100 ns for PRO-APO, PRO-LIG, protein complex proteins were 0.4+/-0.07 nm and 0.3+/-0.06 nm respectively. The results for PRO-APO, PRO-LIG complexes as depicted in Figure 3C. The result suggested that there are no significant structural changes during the 100 ns simulation.

Radius of Gyration (RG)

The radius of gyration can be well described as the mass-weighted root mean square distance of atoms from their centre of mass. The Rg plot shows the competency, shape folding, and overall structure at varying times during the trajectory, illustrated in Figure 3D. Throughout the simulation, PRO-APO, PRO-LIG complexes exhibited a similar pattern of Rg value. The average RG value from 0 to 100 ns for PRO-APO, PRO-LIG protein complex proteins were 2.36+/-0.03 nm and 2.35+/-0.01 nm respectively.

Solvent Accessible Surface Area (SASA)

The hydrophobic core's compactness was evaluated by SASA. The change in SASA of the PRO-APO, PRO-LIG protein with time is shown in Figure 3E. The average SASA value from 0 to

Table 1: The docking score of CGA with G6Pase.

Compound Name	Affinity (T1)	Affinity (T2)	Affinity (T3)	Average Docking Score
LIG	-8.9	-9.0	-8.9	-8.9

Table 2: Hydrogen Bonding information.

Compound	H-Donor	H-Acceptor	Distance	Type
LIG	A: ASN43:HN	B: UNK0:O	3.0318	Conventional Hydrogen Bond
	A: ASN266:HD22	B: UNK0:O	2.38869	Conventional Hydrogen Bond
	B: UNK0:H	A: ASP40:OD1	1.31199	Conventional Hydrogen Bond
	B: UNK0	A: MET123	5.24331	Pi-Alkyl

Table 3: Molecular Dynamics report at 100 ns.

Sl. No.	Protein	Average RMSD (nm)	RMSF (nm)	Radius of gyration (nm)	Average SASA (nm2)
1	PRO-APO	0.22+/-0.03	0.4+/-0.07	2.36+/-0.03	205.07+/-5.20
2	PRO-LIG	0.21+/-0.04	0.3+/-0.06	2.35+/-0.01	203.07+/-4.30

Table 4: The relative binding strength of PRO-LIG computed via the MM-PBSA method.

System	van der Waal energy	Electrostatic energy	Polar solvation energy	Binding energy
PRO-LIG	-155.826+/-18.129 kJ/mol	-106.616+/- 20.776 kJ/mol	290.413+/- 34.240 kJ/mol	-30.141+/- 14.943 kJ/mol

100 ns for PRO-APO and PRO-LIG protein complex proteins were 205.07+/-5.20 nm and 203.07+/-4.30 nm respectively. This indicates that there is no change in structural level protein throughout the simulation.

Hydrogen Bond (H Bond)

Hydrogen bonds are formed to stabilise protein-ligand complexes. In our study, the simulation analysis supports the hydrogen bonds established in the molecular docking analysis. The complex's H-Bond outcome with PRO-LIG. The complex's H-Bond outcome with PRO-LIG is depicted in Figure 3F.

Molecular Mechanics Poisson–Boltzmann Surface Area (MM - PBSA)

In order to determine the binding affinity of PRO-LIG, we examined the relative binding strength within the protein of summery energy. Table 4 and Figure 3G compares the binding strength of PRO-LIG concerning inhibitors computed via the MM-PBSA method. Across a stable simulation trajectory, we calculated residue level contributions to the interaction energy.

Assessment of the effect of chlorogenic acid on fasting blood glucose level

Assessment of the fasting blood glucose level on day 14 (Pilot study)

The blood glucose levels were measured at different time intervals (i.e., at 0.0, 60, 90, 120, and 180 min) on day 14, to fix the dose of CGA. The fasting blood glucose levels consistently decreased at the dose of 200 mg/kg throughout the time interval (**p<0.01, up to 120 mins and **p<0.5 at 180 min) and even 400 mg/kg dose showed good results (***p<0.001, up to 90 min), however, the hypoglycaemia was not persistent post 90 min interval (Figure 3A).

Assessment of the role of Chlorogenic Acid loaded Liposome (CAL) on fasting blood glucose level to sustain hypoglycaemia

The administration of CAL formulation did not show any success in sustaining the hypoglycaemic property of chlorogenic acid (Figure 2B).

Assessment of the fasting blood glucose levels at different stages of the study

The blood glucose levels were measured on the 1st, 3rd and 7th week of the study. There was a considerable drop in blood glucose levels in Group II and III (**p<0.01) at 3 weeks interval, whereas at the 7th week interval, significant decrease in the blood glucose levels was evident (***p<0.001) when compared with control group animals (Figure 2D).

Glucose-6-phosphatase estimation

The glucose-6-phosphatase activity was measured as the amount of inorganic phosphate liberated. by using the standard curve approach. Group II showed no significant inhibition (p<ns), whereas, Group III showed a significant (***p<0.001) drop in glucose-6-phosphatase activity when compared to the normal control group (Figure 4A).

Estimation of hepatic glycogen

The estimated glycogen level was analysed by one-way ANOVA followed by Bonferoni's *post hoc* test. Group II showed no significant elevation (p<*ns*), whereas, in comparison with the normal control, Group III showed a significant elevation in liver glycogen levels when compared to the normal control group (**p<0.01) as well as when compared with Group II ($^{\Psi\Psi}p$<0.01) (Figure 4B).

Measurement of serum lactate dehydrogenase

The Lactate dehydrogenase levels increased considerably in Group II (*p<0.05), whereas in Group III there was significant increase when compared to Group I (***p<0.001) and Group II ($^{\Psi\Psi\Psi}p$<0.001) (Figure 4C).

Measurement of Serum Uric acid

Serum Uric acid levels did not implicate any substantial difference between the control and experimental groups (^{ns}p>0.05) (Figure 4D).

Liver weight

The liver weight of the rats was measured after 7 weeks/49 days and it was observed that the liver weight of Group II did not increase significantly (^{ns}p>0.05), whereas Group III showed considerable increase in liver weight when compared to Group I (*p<0.05) and group II ($^{\Psi}p$<0.05) (Figure 4E).

Histological study

The liver tissues showed clear signs of abnormalities associated with GSD type I, in Group II, swollen hepatocytes and ballooning degeneration were observed and in Group III, significant mosaic pattern, glycogen nuclei, glycogen, swollen hepatocytes, ballooning degeneration and sinusoidal congestion were evident (Figure 5, A, B, and C).

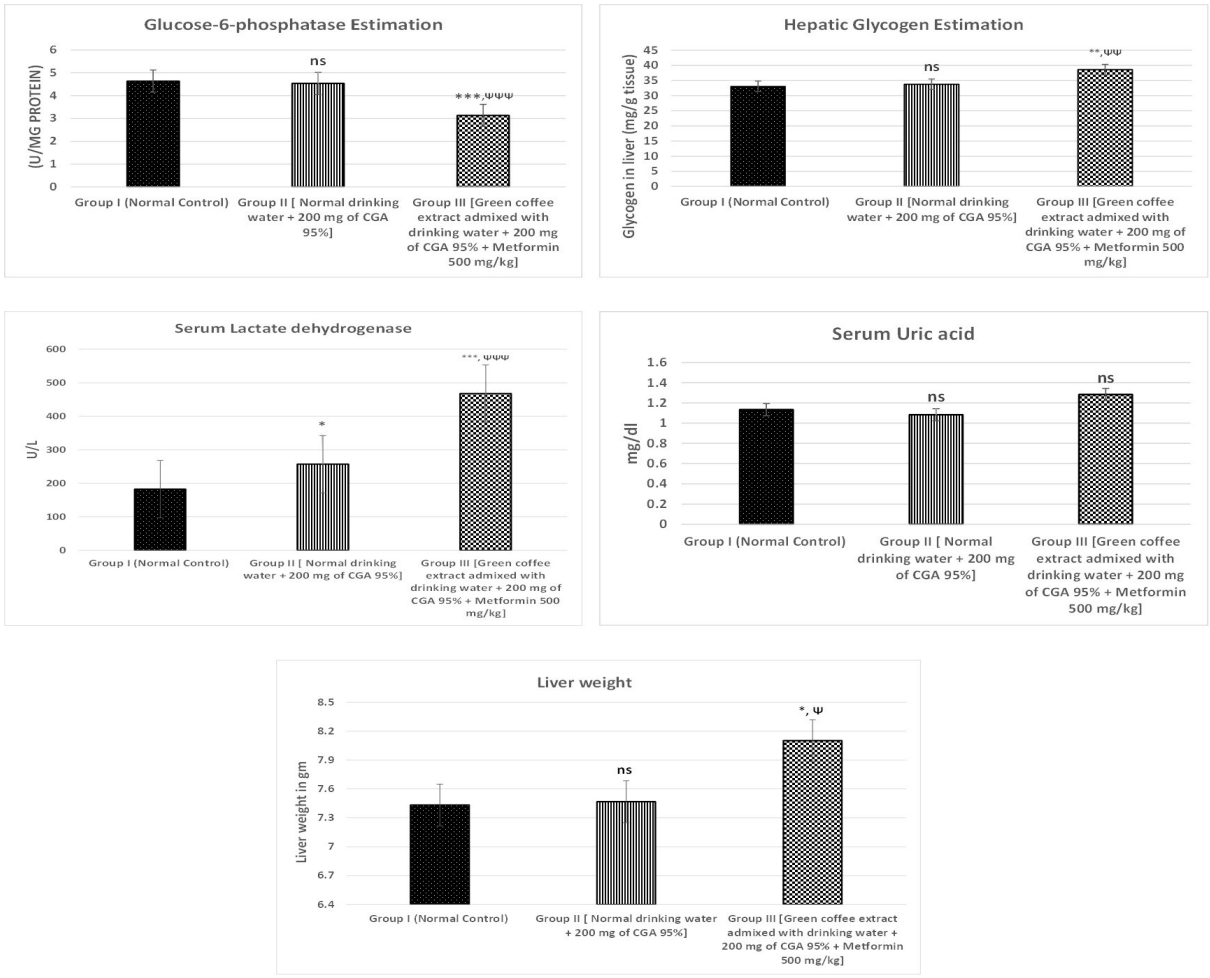

Figure 4: GSD Manifestations (A) The glucose-6-phosphatase estimation, where Group III presented significant decrease in the blood glucose levels (***p<0.001) when compared with Control group and ($^{\Psi\Psi}p$<0.001) when compared to Group II. (B) Hepatic glycogen content estimation, where all the values obtained are expressed as mean±standard error of mean (n=6), Group III showed a significant elevation in the hepatic glycogen (**p<0.01) when compared to Group I and ($^{\Psi\Psi}p$<0.01) when compared to Group II. where all the values obtained are expressed as mean±standard error of mean (n=6). (C) The lactate dehydrogenase levels significantly increased in Group II (*p<0.05), and in Group III (***p <0.001) high significance was observed when compared to the Group I respectively and ($^{\Psi\Psi\Psi}p$<0.001), when compared to Group II respectively. (D) The serum uric acid was non-significant (ns, p>0.05), when compared to the both, Group I and Group II. (E) The liver weight of the Group III is increased (*p< 0.05) when compared Group I and Group II ($^{\Psi}p$<0.05), whereas Group II did not present significant changes when compared to Group I.

All the results obtained are expressed as mean±standard error of mean (n=6). The statistical significance was analysed by one-way ANOVA and Bonferroni's post-hoc test where *p<0.5, **<0.05 and ***p<0.01.

DISCUSSION

The absence of endogenous glucose synthesis resulting in acute hypoglycaemia after a short duration of fasting is a characteristic feature of GSD type I. This pathophysiology is induced due to the deficiency of enzyme complex glucose-6-phosphatase (G6Pase), involving the G6Pase catalytic subunit which is encoded by the (G6PC1) gene and the glucose-6 phosphate transporter or translocase subunit (G6PT), which is encoded by (*SLC37A4*) gene. The complex macromolecules like glycogen are fundamental in the maintenance of glucose levels within the physiologic range. The regulation of synthesis or degradation of glycogen is highly systematized and complex. Anomaly in the degradation of glycogen is referred to as Glycogen Storage Disorders (GSDs) are rare diseases yet they make up a major category of inborn errors of metabolism or autosomal recessive illnesses and there is no specific treatment available for the disorders. GSD type I renders the patients vulnerable to other long-term complications, such as hyperuricemia, lactic acidemia and hepatomegaly and glycogen build up in liver, along with other chronic and serious complications like hepatocellular adenomas and renal impairment. Animal models which can be easily manipulated and maintained are essential in providing mechanistic insights that can direct eventual therapeutic strategies. To elucidate the biochemical basis of GSD type Ia and to assess the advent of gene therapy approach to rectify or improve *G6pc* deficiency have been studied on two animal models of GSD type Ia. By using *G6pc* knockout mouse, created by Dr. Janice Chou presented with low birth weight, but

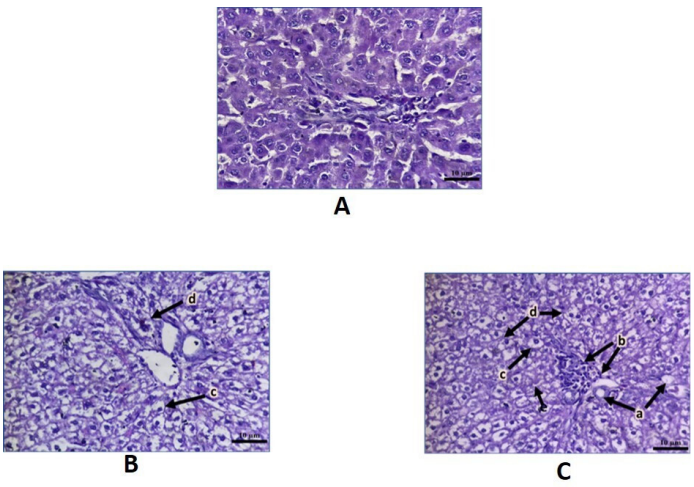

Figure 5: A: Periodic Acid-Schiff stain (PAS) of Group I showed normal architecture with intact hepatocytes and no Glycogen accumulation. B: Group II; CGA (100mg/kg) treated group PAS stained, showed significant swollen hepatocytes[c] and ballooning degeneration [d]. C: Group III; CGA (200mg/kg) treated group PAS stained, showed significant Mosaic pattern, Glycogen nuclei [a], Glycogen [b] swollen hepatocytes[c], ballooning degeneration [d] and sinusoidal congestion [e].

developed severe hypoglycaemia instantaneously and gradually exhibited a pronounced increase in the serum cholesterol and triglyceride levels. Nonetheless, they did not generally manifest lactic acidosis. Similarly, a canine (Maltese breed) model of GSD type Ia having a natural mutation in the *G6pc* gene was discovered and used to delineate the pathology of the disease. The similar canine model was manipulated by crossbreeding a *G6pc* mutation carrier Maltese breed with a beagle breed. These canine models presented with peculiar and specific symptoms similar to that of human disease, including lactic acidemia, however, these models proved ineffective for investigation of the long term or chronic complications observed in GSD type Ia. GSD type Ia mice models that only target deletion of *G6pc* in liver, and/or kidneys, or gut have been recently developed with a purpose of monitor the development of long term diseases or manifestations that emerge in GSD type Ia patients and to assess the potential treatment strategies.

The primary goal of this work was to find out if the chemically induced GSD model can be developed, which could be more reliable and economical. Several *in vitro* studies have claimed that CGA and its derivatives can inhibit or attenuate the activity of glucose-6-phosphate enzyme complex (G6Pase and G6PT) resulting in altered metabolism. which we substantiated with *in silico* studies. Based on this evidence, we carried out a pilot study, where it was evident that a dosage of 200 mg of CGA per kg of body weight was optimum to induce hypoglycaemia in rats. However, since CGA has a shorter biological half-life, its ability to maintain hypoglycaemic was limited to 1.5 to 2.5 hr. Literature suggested the formulation of Chlorogenic Acid loaded Liposomes (CAL) can prolong the effects of CGA. However, there was no significant difference in the outcome using the liposome formulation. Hence, to augment the effect of CGA, green coffee extract consisting of 36% (CGA 200 mg/kg) was admixed with drinking water to be fed continuously to the animals in the treatment groups along with the daily dose of 200 mg/kg of 95% CGA via oral gavage. Glycogen levels were found to be altered in CGA treated rats which is probably due to the suppressed activity of glucose-6-phosphatase, further contributing as a factor for hepatomegaly and significant increase in the liver weight. In addition to CGA, metformin was employed, as an attempt to replicate the lactic acidosis generally observed in GSD type I cases. Thus, it indicates that along with CGA, administering other drugs or chemicals can be used to induce and augment the manifestation of GSD type I like symptoms in the rats.

CONCLUSION

The article discusses a pre-clinical study aimed at inducing the symptoms of Glycogen Storage Disease Type I (GSD type I) in rats through the inhibition of the glucose-6-phosphatase enzyme using Chlorogenic Acid (CGA) and metformin. The *in silico* study, demonstrated that CGA had good docking scores and binding patterns with G6Pase, followed by an *in vivo* pilot study to determine the effective dose. This study concludes that CGA was evidently responsible to induce major manifestations of GSD type I, and when it was combined with metformin, further there was significant augmentation of the manifestations. Thus, this model can serve as an alternative to genetically engineered animal models, that can promote research on GSD like diseases. However, there is future scope to explore other combinations of chemicals/drugs which can contribute to induce other GSD type I manifestations such as, hyperlipidaemia, neutropenia, hepatic fibrosis etc. in experimental animals.

ACKNOWLEDGEMENT

We gratefully acknowledge the Principal, KLE College of Pharmacy, Hubballi, Karnataka for providing research facilities.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Santosh B Patil: Conceptualization, Methodology, Investigation, Visualization and Project administration; Pramod C Gadad: Supervision, Validation and Data curation.

ABBREVIATIONS

GSD: Glycogen Storage Disease; **CGA:** Chlorogenic acid; **CAL:** Chlorogenic acid Loaded Liposomes; **PRO-LIG:** Protein and Ligand; **PRO-APO:** Protein Alone; **RMSD:** Root mean square deviation; **RMSF:** Root mean square fluctuation; **RG:** Radius of gyration; **SASA:** Solvent accessible surface area; **MM-PBSA:**

Molecular mechanics Poisson-Boltzmann surface area; **G6Pase:** Glucose-6-phosphatase; **G6PT:** Glucose-6 phosphate transporter.

SUMMARY

This study explored the possibility of inducing GSD type-I manifestations in rats using chlorogenic acid (CGA) and metformin, where the *in silico* studies revealed promising binding patterns between CGA and Glucose-6-phosphatase on the other hand, the *in vivo* experiments potentiated the role of CGA and metformin in inducing the major manifestations of GSD type-I, like hypoglycemia, suppressed G-6-Pase activity, mimicking GSD type-I symptoms. Addition of metformin further augmented these manifestations. The study offers an alternative chemically induced model to genetically engineered animal models for GSD research. Future studies could explore additional chemical combinations to induce other GSD type-I manifestations like hyperlipidaemia and hepatic fibrosis. This approach demonstrates the potential of simple chemically induced models to comprehend the manifestations of complex metabolic disorders, paving the way for additional research and therapeutic advances.

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Cite this article: Patil SB, Gadad PC. Chlorogenic Acid, a Potential Glucose-6-Phosphatase Inhibitor: An Approach to Develop a Pre-Clinical Glycogen Storage Disease Type I Model. Indian J of Pharmaceutical Education and Research. 2024;58(2s):s372-s381.