RESEARCH PAPER

Targeting the Hexosamine Biosynthesis Pathway: In-Silico and In-Vitro Evaluation of Biochanin A as a GFAT Inhibitor

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ABSTRACT

Glutamine: D-fructose-6-phosphate amidotransferase (GFAT), an enzyme involved in catalyzing important step in hexosamine biosynthesis pathway, results production of metabolites causing insulin resistance. Investigating Glucosamine-6-phosphate synthase inhibitors may lead to the development of compounds reducing insulin resistance with improved safety profiles. In this study, around four lakh compounds were taken from the coconut database and virtual screening was done by using Schrodinger Suite 2024 – 2. Based on the docking scores and MD Simulations, 15 compounds were selected. Among them, Compound 2542 was selected as GFAT inhibitor for in-vitro evaluation after a series of binding energy calculations. GFAT inhibitory assay was performed on L6 and 3T3-L1 cell lines using Elson-Morgan colorimetric method. Biochanin A was tested at concentrations ranging from 0.5 to 2.5µM, with Azaserine used as a positive control standard. Cytotoxicity assays were done and viability of cells was determined using the Sulforhodamine B assay. Additionally, glucose uptake assay was performed. In L6 and 3T3-L1 fibroblast cell lines, Biochanin A exhibited dose-dependent GFAT inhibition achieving 71% and 75% respectively at 2.5µM concentration. IC50 values were determined as 50.58 and 35.20 in the cytotoxicity assays confirming the safety profile of the test compound at therapeutic concentrations. Glucose uptake assay was also performed where a significant increase in glucose uptake was observed with Biochanin A treated L6 and 3T3-L1 cell lines. These findings suggest that Biochanin A can inhibit Glucosamine-6-phosphate synthase enzyme and serve as potential lead for the development of novel antidiabetic agents.

Keywords: Glutamine: D-fructose-6-phosphate amidotransferase (GFAT), hexosamine biosynthesis pathway, virtual screening, glucose uptake assay, Cytotoxicity assay, Sulforhodamine B (SRB) assay, GFAT enzyme inhibition assay.

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GRAPHICAL ABSTRACT

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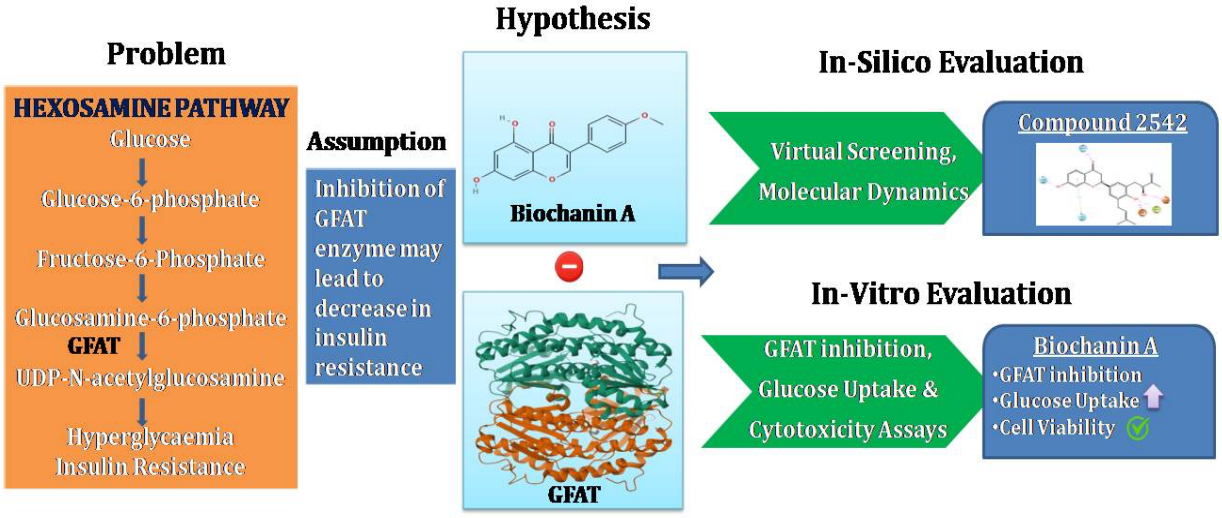


INTRODUCTION

Diabetes mellitus is a long term disorder of metabolism where the patient suffers from increased glucose in blood due to insulin deficiency[1] with a prevalence history of 400 million people worldwide over the past 30 years[2] and according to the prediction of International Diabetes Federation, the number of people suffering from diabetes might reach up to 552 million by 2030[3]. The onset age of the diabetes on an average is determined as 42 years and it is recorded as 8% of children are suffering from diabetes[4]. Diabetes is mentioned as an apocalypse, a threat to human health[5]. WHO has released its first global report in 2016 on diabetes[6]. Diabetes mellitus leads to several microvascular complications like nephropathy, neuropathy etc and macrovascular complications like stroke, ischemic heart disease etc[7] Although many drugs are useful in the treatment of diabetes, the benefits of those drugs are always accompanied with the side effects like hypoglycemia, liver injuries, gastrointestinal disturbances and weight gain etc [8]

It is evident that hexosamine pathway leads to generation of uridine 5'-diphospho-N-acetyl-D-glucosamine increasing the blood glucose levels in mammals[9,10] GFAT is known to be involved in hexosamine pathway[11] showing that increased enzyme activity leads to development of hyperglycemia and insulin resistance[12] Hence, focusing on the natural compounds inhibiting GFAT enzyme may serve as an alternative therapeutic agent to inhibit insulin resistance[13] In addition to this, GFAT enzyme is known to be involved in the synthesis of chitin[14] which is an important material in the microbial cell wall synthesis, hence the inhibitors of GFAT enzyme can also serves as antimicrobial agents. It is also known that the end products formed from the hexosamine pathway facilitates the cancer cells proliferation[15] and their growth[16] So inhibiting the GFAT enzyme, there by inhibiting the hexosamine pathway could provide anti-cancer effect[17] On the other hand, Biochanin A which is abundantly found in many natural products including red clover[18] cabbage, chick pea[19] etc known to modulate several enzymes and can be tested as potent drug candidate for various diseases[20] Biochanin A is tested for its anti-inflammatory, antioxidant, antimicrobial properties and it is proven to be effective [21] It has been popular for ages among menopausal women in controlling symptoms [22]

Here, in this study, we have performed In-Silico screening of more than 4 lakh compounds to find out the most suitable GFAT inhibitor, further in-vitro assays like GFAT inhibitor assay was performed in both L6 and 3T3-L1 cell lines to check whether Biochanin A can inhibit GFAT enzyme or not. Cytotoxicity assay was also performed to evaluate the safety profile and toxic effect of the compound on the living cells. In addition to these, Glucose uptake assay was also performed to evaluate to measure the glucose uptake level by cells.

2. Methodology

2.1 In-Silico Evaluation

2.1.1 Virtual Screening[23]

3D structure of human GFAT enzyme and Glucose-6-phosphate was taken using Scrodinger 2024-3. Water molecules were removed and hydrogen atoms were added. Virtual screening of almost 4 lakh compounds was done which were downloaded from the COCONUT database. Duplicate structures were removed and

hydrogen atoms were added to prepare the ligand. After the preparation, Ligand was subjected to optimization at pH 7 approximately. By following the docking protocol, low energy conformers were docked. A Glide HTVS, Standard and Extra Precision docking were performed having top 10% compounds after each docking step with default parameters

2.1.2 Post docking minimization (MMGB-SA)[24]

Prime MMGB-SA was used to analyze the enthalpy and entropy of components towards ligand – protein complex binding including the estimation of OPLS4 force field. Calculation of relative binding affinities was done for final XP docked poses keeping VSGB 2.0 Solvation model.

2.1.3 Molecular Dynamics[25]

Extra precision docked structure was taken for the compound 2542/6R4F complex to perform MD simulation using Desmond software. The system was neutralized with sodium and chloride ions and further minimized to 25 kcal/mol/Å gradient threshold with OPLS3e force-field. Particle-mesh-Ewald method was used for the electrostatic interactions of long range. Van der Waals and Coulomb interactions were measured with 9 Å cutoff radius. The entire system is placed in 100 ns simulation at 300 K temperature and 1 bar pressure. With RESPA integration algorithm the bonded, near nonbonded, and far nonbonded interactions were calculated with time steps of 2, 2, and 6 fs, respectively. The data was collected for every 100ps. 3D structures and Molecular dynamics trajectory were analyzed using Maestro graphical interface.

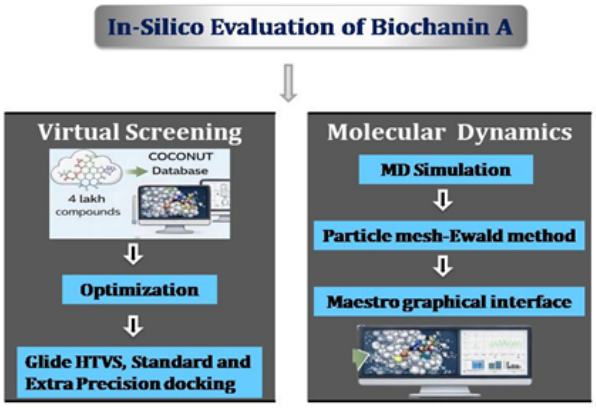


Figure 1: Schematic representation of In-Silico methodology

2.2 In-Vitro Evaluation

2.2.1 GFAT Inhibitory Activity of Biochanin A in L6 and 3T3-L1 Cell Lines [26]

Materials required: L6 and 3T3-L1cell lines**,** Biochanin A, Azaserine, Fetal Bovine Serum (10%), DMEM (high glucose), Penicillin–Streptomycin, L-glutamine, fructose-6-phosphate, Elson–Morgan reagent, RIPA buffer, CO_2 incubator and UV-Visible spectrophotometer.

Method: GFAT Inhibitory activity of Biochanin A was measured in two cell lines namely L6, 3T3-L1. These cells were prepared in DMEM having f bovine serum of foetus and pencillin – streptomycin. Azaserine was taken as a standard, the standard and the test drugs were taken in the concentrations of 0.5, 1, 1.5, 2, 2.5 µM. The cells were treated for 24hrs. After the treatment, cells were lysed by using RIPA buffer and the GFAT activity was analyzed using Elson – Morgan calorimetric method.

2.2.2 Cytotoxicity Assay [27,28]

Materials required: L6 and 3T3-L1 cell lines**,** Biochanin A, Fetal Bovine Serum (10%), DMEM (high glucose), Penicillin–Streptomycin, Amphoterecin-B etc.

Method: Cell lines supplemented with DMEM mixed with fetal bovine serum, 100 IU/ml of Penicillin, 100mg/ml of Streptomycin, 5mg/ml of Amphoterecin-B maintained at 37 °C. The cell lines were differentiated by using trypsin and EDTA. Cells were subjected to 24h incubation until the formation of monolayer. Then these cells were treated with test drug of varied concentrations whereas the control wells receives only normal medium and again subjected to incubation for 72hrs. An inverted microscope was used to find out the changes in cells structure and compared with control cells. Cell viability was examined through SRB assay [29]

2.2.3 Glucose uptake assay [30, 31, 32]

Materials required: L6 and 3T3-L1cell lines**,** Biochanin A, Fetal Bovine Serum (10%), DMEM (high glucose).

Method: Cell lines were taken and incubated for a period of 48hrs until the formation of monolayer. Again the cell lines were subjected to incubation for 18hrs. Later on, the cells were treated with Biochanin A and insulin was used as a standard. The cells were treated with Insulin as standard drug. Both test and standard treated cells were incubated for 30min. The glucose uptake by the cells estimated by measuring the variation between initial and end glucose level in cell lysate by GOD-POD method [33, 34]

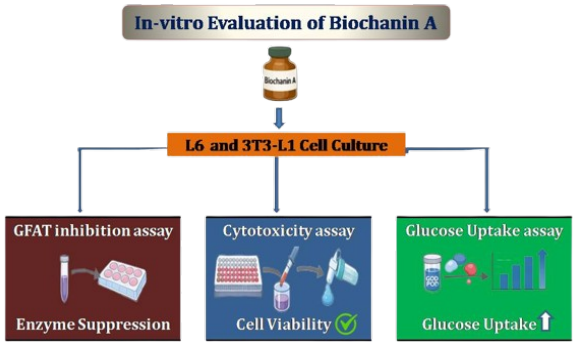


Figure 2: Schematic representation of In-vitro methodology

3. RESULTS AND DISCUSSION

3.1 In-silico virtual screening, molecular docking and MMGB-SA studies

The compounds those showed to be having hydrogen bonding and salt bridges with the 6R4F.pdb were given in Table 1. Compound 2542 showed the glide score of -10.1kcal/mol. It has 1 pi-pi contact and 7 hydrogen bonds with the linker site of 6R4F.pdb. The carbonyl group and hydroxyl group of compound 2542 at 3rd and 7th position of chromen-4-one ring formed three hydrogen bonds with Gln310 and Asn465, Gln315 respectively. When it comes to the second hit compound 4636, it shows the glide score of -10kcal/mol. the 4-OH group on the 4-hydroxyphenyl ring at the 2nd position of 2, 3-dihydrochromen-4-one ring formed hydrogen bonding and salt bridge interactions with Asn465, Asp262 and Gln422 with one hydrogen bond. For both the compounds 2542 and 4636, one pi-pi contact was observed between His463 and aromatic ring of 2, 3-dihydrochromen-4-one (Figure 3).

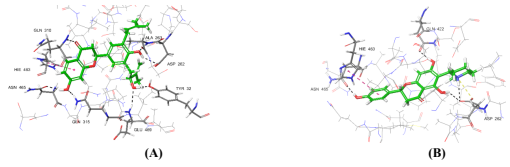


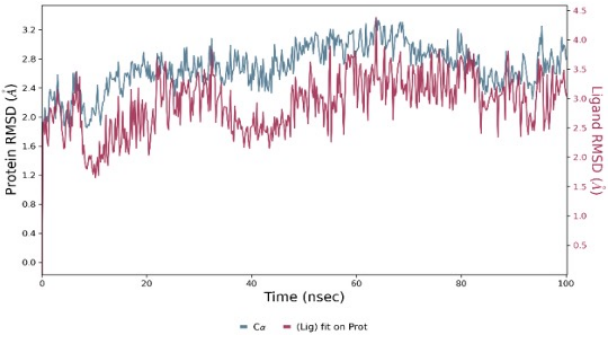
Figure 3 representing 3D interaction of top hits (A) 2542 and (B) 4636

S. No	Compound code	Glide gscore	MMGB-SA ΔG Bind	MMGB-SA ΔG Coul	MMGB-SA ΔG Hbond	MMGB-SA ΔG Lipo	MMGB-SA ΔG$_v$ dW
1	2542	-10.1	-92.02	21.73	-7.97	-24.53	-10.26
2	4636	-10.0	-91.86	-61.93	-6.76	-25.80	-50.88
3	556	-9.84	-88.54	-26.52	-5.86	-25.86	-52.32
4	12698	-9.76	-88.38	-17.26	-7.26	-22.38	-31.12
5	7064	-9.68	-86.57	-25.30	-7.25	-23.42	-44.62
6	11840	-9.62	-86.04	-101.36	-10.02	-21.68	-34.79
7	9286	-9.59	-82.62	-2.87	-4.53	-25.76	-38.63
8	5827	-9.54	-81.09	-96.35	-10.25	-18.03	-22.31
9	11834	-9.52	-81.0	1.21	-4.9	-19.62	-56.21
10	5829	-9.52	-76.52	-58.53	-10.56	-14.02	-21.92

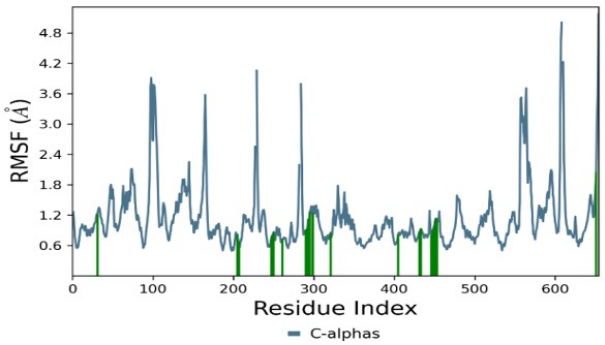
Table 1: Top 10 hit compounds in the catalytic pocket of human GFAT-1 6R4F.pdb

Molecular dynamics studies

100ns molecular dynamics simulation was conducted for the 2542/6R4F.pdb complex. As shown in the figure 4, RMSD of Cα atoms was equilibrated and fluctuated in between 2.55 to 3.08 Å for the first 20 nanoseconds and the RMSD values of ligand were found to be in between 2.45 to 3.8 Å. RMSF values of protein are below 1.2 Å indicating the protein and ligand complex stability. It was also found that the hydrogen bonds were formed with amino acids and more importantly His463 formed pi-pi bonds at half of 100ns simulation time. The timeline representation of Protein-Ligand interaction contacts was illustrated in the Figure 5 representing the stability of compound 2542.



(A)



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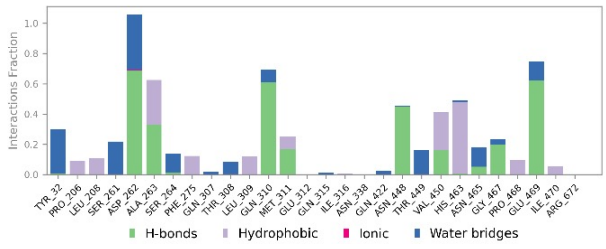
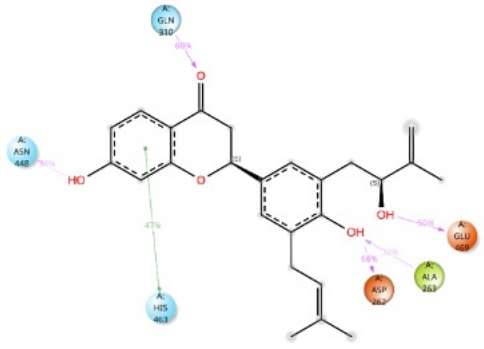


(C)



(D)

Figure 4 (A) RMSD plot representing simulated positions of Cα atoms of 2542/6R4F.pdb complex **(B)** Protein RMSF values of 2542/6R4F.pdb complex **(C)** Bar diagram representing the interactions of inhibitor 2542/6R4F.pdb complex **(D)** 2D trajectory interaction of 2542/6R4F.pdb during 100ns MD simulation

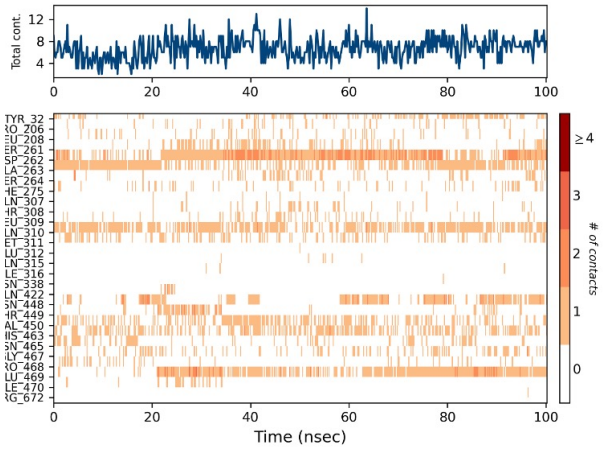


Figure 5 representing Protein-Ligand interaction contacts in 100ns simulation trajectory of 2542/6R4F.pdb.

3.2. GFAT Inhibitory Activity of Biochanin A in L6 and 3T3-L1 Cell Lines

Entering of glucose molecules in to hexosamine pathway leads to the generation of metabolites developing insulin resistance in adipocyte cell culture [35] This hexosamine pathway is catalyzed by the enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT) [36] Hence, inhibition of GFAT provides opportunity for the development of target compound reducing insulin resistance. In this study, we have carried out. Biochanin A produced a dose-dependent inhibition of GFAT activity in L6 fibroblast cells. At higher concentration, Biochanin A was found to inhibit the GFAT enzyme by 71%, where as the inhibitory effect of azaserine at the same concentration was found to be 74% as shown in Figure 6. The inhibitory effect was comparable but slightly lower than that of the standard inhibitor azaserine. The Log IC_{50} value calculated from the regression equation ($y = 27.688x - 4.516$) was 1.97, IC_{50} value of 93.3 (unit).

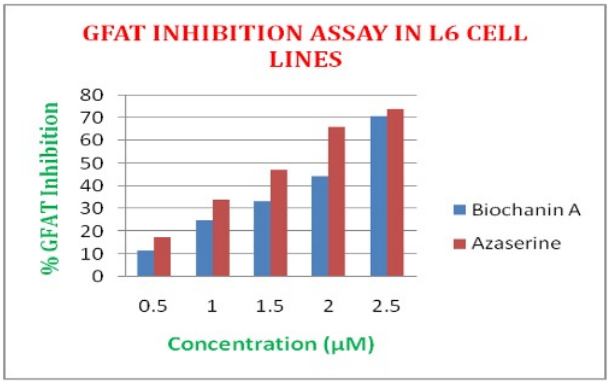


Figure 6: Graph representing GFAT Inhibitory Activity

of Biochanin A in L6 Cell Lines

Biochanin A produced a dose-dependent inhibition of GFAT activity in 3T3-L1 fibroblast cells. At 2.5 µM, Biochanin A inhibited GFAT activity by 75% as shown in Figure 7, demonstrating strong enzyme suppression. The inhibitory effect was comparable but slightly lower than that of the standard inhibitor azaserine. From the regression equation (y = 30.418x − 2.141), the Log IC_{50} value was calculated as 1.71, IC_{50} of 51.3 (unit).

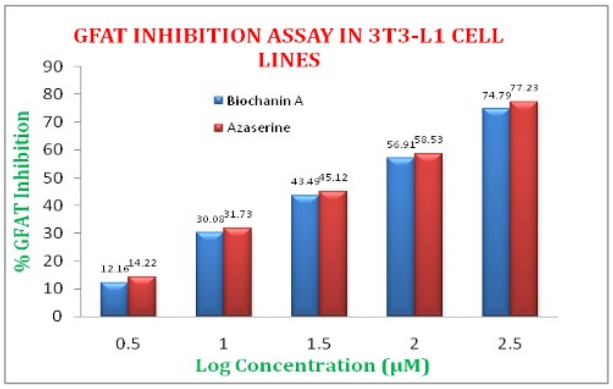


Figure 7: Graph representing GFAT Inhibitory Activity of Biochanin A in 3T3-L1 Cell Lines

3.2 Cytotoxicity Assay

In order to evaluate the safety profile of a compound, it is important to conduct cytotoxicity assays along with their respective test assays[37] and here, we have performed the following assay to rule out cytotoxicity effect of Biochanin A. Through this assay, it was found that the rate of cell proliferation increases with the increase of concentration of Biochanin A in both L6 and 3T3-L1 cell lines as shown in the Figure 8. Biochanin A at 0.01µM concentration, L6 cell lines showed 5.66% proliferation and 3T3-L1 cell line showed 3.66% proliferation. Biochanin A at the concentration of 0.1 µM, the proliferation rate rises up to 11.33% in L6 cell lines and 14.33% in 3T3-L1 cells. At concentration 1µM, rate of proliferation increased to 20.66% in L6 cell lines and 25.66% in 3T3-L1 cells. At the highest test concentration (10 µM), L6 cell lines showed the higher proliferation rate of 32.33% and 3T3-L1 cells showed 36.33%.

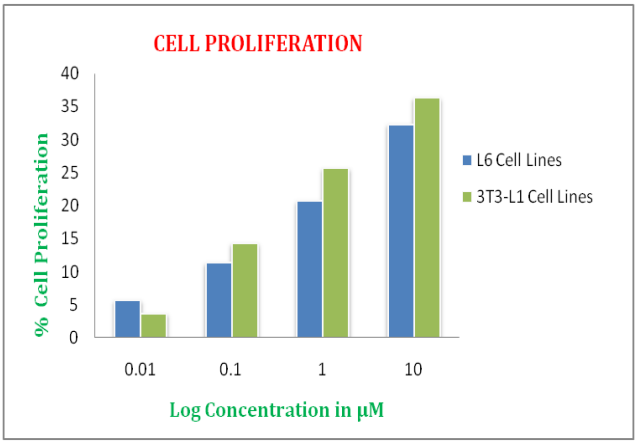


Figure 8: Graph representing % Cell Proliferation of Biochanin A on L6 and 3T3-L1 cell lines

As per the figure 9, Biochanin A showed cytotoxicity in a dose dependant manner in both L6 and 3T3-L1 cell lines. At a minimum concentration of 0.5µM, 11.6% of toxicity was observed in L6 cell lines and 9.44% was observed in 3T3-L1 cells. At 1µM concentration, cytotoxicity raised to 22.7% in L6 cells and to 19.37 in 3T3-L1 cells. As we increase the concentration of Biochanin A up to 1.5 µM, cytotoxicity rose to 38.42% in L6 cells and 39.22% in 3T3-L1 cells. At 2µM concentration, cytotoxicity raised up to 46.12% and 47.35% in L6 and 3T3-L1 cells respectively. At the maximum test concentration (2.5µM), cytotoxicity raised up to 68.15% and 66.3% in L6 and 3T3-L1 cell lines respectively. The IC50 value of Biochanin A on L6 cell lines was found to be 50.58, whereas on 3T3-L1 cell lines were found to be 95.5%.

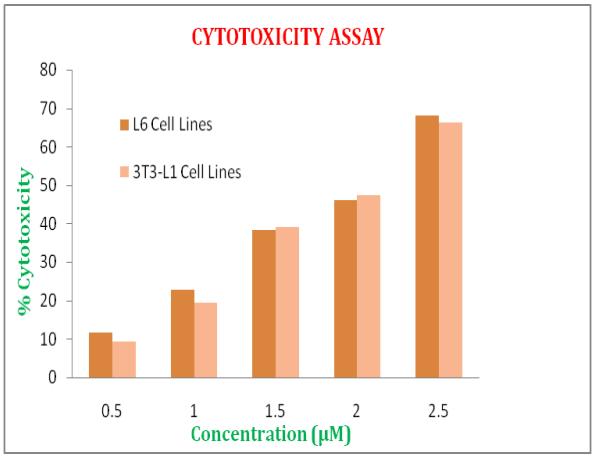


Figure 9: Graph representing % Cytotoxicity of Biochanin A on L6 and 3T3-L1 cell lines

3.3 Glucose uptake assay

To measure the improvement in the insulin sensitivity of test drug treated cells [38] glucose uptake assay was performed. Biochanin A exhibited dose dependant

manner uptake of glucose in L6 and 3T3-L1 cells. Cells have been subjected to Biochanin A varying from 0.5 – 2.5µM concentration. As shown in figure 10, at 0.5µM concentration, 23.36% of glucose uptake was observed in L6 cell lines and 18.28% was observed in 3T3-L1 cell lines. At 1µM concentration, 43.47% and 35.46% glucose uptake was observed on L6 and 3T3-L1 cell lines respectively. As the concentration of test compound increases from 1 to 1.5µM, the glucose uptake also increased to 70.72% and 68.36% in L6 and 3T3-L1 cell lines respectively. At 2µM concentration, 84.84% and 80.6% glucose uptake was observed on L6 and 3T3-L1 cell lines respectively. At the maximum test concentration of 2.5µM, glucose uptake reached to 92.46% in L6 cell lines and 91.24% in 3T3-L1 cell lines.

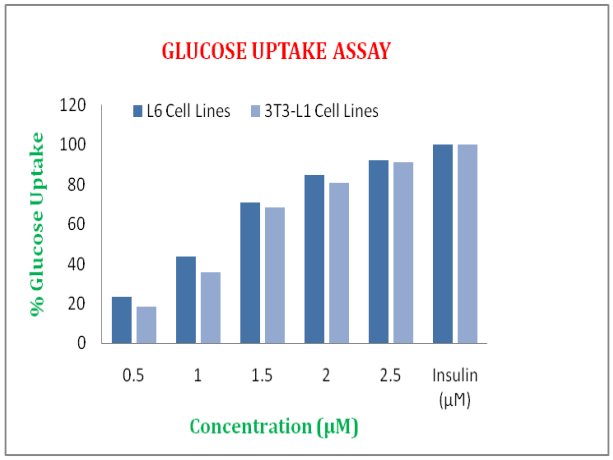


Figure 10: Graph representing percentage of glucose uptake in L6 and 3T3-L1 cell lines

CONCLUSION

Virtual screening was done for more than 4lakh compounds downloaded from the COCONUT database where Compound 2542 has been chosen as the top hit. Compound 2542 showed good glide score representing stronger affinity. Adding to this, the hydrogen bond stability of Compound 2542 is greater than the other hits and it is interacted with many amino acid residues. On the other hand, Biochanin A exhibited GFAT inhibitory effect, acceptable cell viability and maximum glucose uptake in both L6 and 3T3-L1 cell lines. As the Biochanin A was found to be having GFAT inhibitory effect almost comparable to standard GFAT inhibitor Azaserine. Hence, Biochanin A was proved to be having hypoglycemic effect and can be evaluated as potent anti-diabetic agent. Biochanin A also exhibited a clear dose dependant increase of glucose uptake in both the cell lines; the action was same as that of with the insulin at higher concentrations. In addition with the GFAT inhibitory effect, increase in the glucose uptake proves that the Biochanin A plays a potential role in reducing insulin resistance. From the cytotoxicity assays, it was evident that Biochanin A has a good safety profile in its therapeutic concentrations. Overall, the results from my study suggest that Biochanin A can be evolved as a lead anti-diabetic agent inhibiting hexosamine pathway. Anyway, further in-vivo evaluation must be carried out to elucidate its precise therapeutic potential in diabetes treatment.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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