3. MATERIALS AND METHOD

The insulin-insensitive form of diabetes, type 2 diabetes mellitus (T2DM), characterized by hyperglycemia (elevated blood glucose concentrations), most frequently arises as a consequence of obesity, represents approximately 95% of the overall incidence of diabetes. Additionally, diabetes-related complications exert a heavy toll on patients with poor metabolic control.

Diabetes mellitus is one of the most common endocrine disorders affecting almost 6% of the world's population. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030. More than 97% of these patients will have type II diabetes. India has the dubious distinction of being the ‘diabetes capital’ of the world. The International Diabetes Federation estimates that the number of diabetic patients in India has more than doubled from 1995 till now. Upto 11 per cent of India’s urban population above the age of 15 has diabetes.

Anti-diabetic agents seek to reduce hyperglycemia and, thus, diminish the elevated risk of micro- and macro-vascular disease in T2D patients. Glycogen synthase kinase-3β (GSK-3β) has recently emerged, in the field of medicinal chemistry, as one of the most attractive therapeutic targets for Type II diabetes. The full potential of GSK-3β inhibitors is yet to be realized and the number of drug candidates being developed by both academic centers and pharmaceutical companies has increased exponentially in the last few years.

Glycogen synthase kinase-3β (gsk-3β) is a unique multifunctional serine/threonine kinase that is inactivated by phosphorylation in response to insulin binding; PKB/AKT phosphorylates GSK-3β on serine9, which prevents the enzyme from phosphorylating glycogen synthase. Unphosphorylated glycogen synthase is active & able to synthesize glycogen.

**Regulation of GSK-3 by Insulin and Growth Factors**

The binding of insulin to its receptor in liver, adipose tissue and muscle, triggers the phosphorylation of IRS proteins and their recruitment to the plasma membrane. The IRS proteins in turn become tyrosine-phosphorylated, recruiting PI 3-kinase (PI3K) to the membrane, where it produces the second messenger PtdIns (3, 4,5) P3 (PIP3).

This molecule binds to PDK1 and PKB co-localizing them at the plasma membrane and allowing the former to activate the latter. Active PKB then inhibits GSK3 by
phosphorylating Ser 21(GSK3α) and Ser9 (GSK3β). As a result, the residues on glycogen synthase and the e-subunit of eukaryotic initiation factor 2B (eIF2B, lilac) that are targeted by GSK3 undergo a partial dephosphorylation, thereby increasing their activity and hence stimulating glycogen and protein synthesis.27 Normally insulin induced inactivation of GSK-3 contributes to glucose uptake and glycogen synthesis. Molecular mechanism of insulin signaling via insulin receptor substrate (IRS). IRS-1 is the immediate substrate of insulin receptor tyrosine kinase, which phosphorylates the protein on multiple tyrosine residues in response to insulin. In addition, IRS-1 is predominantly phosphorylated on serine/threonine residues in the absence of stimuli. It appears that this type of phosphorylation of IRS-1 converts the protein from a positive to a negative regulator of insulin receptor signaling. Yet its serine/threonine phosphorylation (pS) results in the opposite effect, presumably by direct interaction of IRS-1 with the insulin receptor. These studies implicated serine/threonine protein kinases as important regulators in insulin resistance. Phosphorylation of IRS-1 on multiple serine residues by GSK-3 impaired insulin receptor tyrosine kinase activity and insulin action in intact cells. This notion fits well with the fact that GSK-3 is constitutively active and phosphorylates IRS-1 in the absence of stimulus. Thus, GSK-3 serves as a ‘gatekeeper’ to limit activation of insulin receptor signaling. In the absence of insulin, GSK-3 maintains the phosphorylation state of the multiple serine residues on IRS-1, thereby limiting insulin receptor signaling. In the presence of insulin, GSK-3 is inhibited, and tyrosine phosphorylation of IRS-1 mediates the downstream insulin signaling pathway. Thus it is clear that GSK-3 inhibits insulin receptor coupled protein IRS-1, which in turn inhibits glycogen synthesis and glucose uptake.

Most kinase inhibitors act by competition with either ATP or metal-binding sites that are involved directly in the catalytic process. However, small-molecular-weight compounds might regulate GSK-3 activity by inhibiting the protein–protein interactions that are necessary for binding of substrate by modulating the Tyr216 (GSK-3β) and Tyr279 (GSK-3α) activation sites and the Ser9 (GSK-3 β) and Ser21 (GSK-3 α) inhibition sites, and by interfering with the intracellular targeting domain of GSK-3. Inhibition of the interaction between the docking protein and the priming kinase might change the substrate specificity of GSK-3.
**Diabetes facts**

WHO predicts that by 21st century most of developing countries will bear the brunt of this epidemic? Currently, more than 71% of people with diabetes live in low and middle income countries round the world.

- An estimated of around 285 million people, corresponding to world's 6.4% of the adult population, will be identified with diabetes in 2010. The number is expected to grow to 438 million by 2030, corresponding to 7.8% of the world adult population.

- The global prevalence of diabetes is 6.4%; this prevalence usually varies from 10.2% in the Western Pacific to 3.8% in the African region. However, the African subcontinent is expected to experience the highest increase.

- 70% of the current cases of diabetes generally occur in low- and middle income countries. With an estimate of around 50.8 million people living with diabetes, India would be having the world's largest diabetes population, followed by China with 43.2 million.

- The biggest age group currently affected by diabetes is between 40-59 years. By 2030 this “record” is expected to move to the 60-79 age groups with some 196 million cases.

**Causes for Diabetes Mellitus**

Genetic Defects of β-Cell Function

Insulin Gene: Mutations in the insulin gene have been described, but are extremely rare causes of diabetes. In the few families where such mutations have been found, the result is very mildly abnormal glucose control, inherited in an autosomal-dominant manner. Maturity-Onset Diabetes of the Young: Maturity-onset diabetes of the young is a group of diabetes types caused by mutations in single genes. Mutations in six genes have been identified, each of which results in deficient insulin secretion. These mutations result in autosomal dominantly inherited forms of diabetes that have an early onset, usually before the age of 25. Because these patients retain some insulin secretion, albeit insufficient to prevent hyperglycemia, these patients are, in general, not ketosis prone, and so are not dependent on insulin treatment for survival.
Mitochondrial DNA Mutations: Mutations in the mitochondrial genome can cause diabetes mellitus, the most common involving the mitochondrial tRNA. Due to the maternal origin of mitochondria, this type of diabetes is maternally inherited. The pancreatic b-cell is rich in mitochondria and has a high rate of oxidative metabolism; the mitochondrial mutations result in a b-cell defect that causes diabetes through deficient insulin secretion. This form of diabetes is frequently associated with neurosensory deafness.

b) Genetic Defects in Insulin Action

Insulin Receptor Mutations: Insulin resistance can be caused by mutations of the insulin receptor, although these are rare as a cause of diabetes. Most of the subjects identified with insulin resistance and insulin receptor mutations have mutations of both alleles. Some cases of mild insulin resistance have been described in which only one allele carries a mutation, acting as a dominant negative inhibitor of the wild-type allele, due to their combination within the hetero tetrameric structure of the receptor.

c) Secondary Diabetes Mellitus

A number of systemic disorders can cause marked insulin resistance, which can lead to secondary diabetes mellitus. Excess growth hormone, as in acromegaly, excess glucocorticoid, as in Cushing’s disease or exogenous glucocorticoid treatment, or excess catecholamines, as in pheochromacytoma, as well as other diseases such as uremia and

Diabetes is one of the major reasons of premature illness and death worldwide. Non-communicable diseases including diabetes account for 60% of all deaths worldwide.10,11

3.1 Rational designing of new chemical entities as Glycogen synthase kinase-3β inhibitors:

On the basis of results obtain from exhaustive literature survey; it was found that following properties are the prerequisite for a compound to be designed as Glycogen synthase kinase-3β inhibitors:
1. **Glycogen synthase kinase-3β**

Presently, three distinct regions on the GSK-3 molecule are being targeted to suppress enzyme activity\(^\text{22}\):

- metal ion (Mg\(^{2+}\)) binding site;
- substrate interaction domain, and;
- ATP-binding pocket

![Fig 3.1: Potential sites for inhibition of glycogen synthase kinase 3 (GSK-3)](image)

Most kinase inhibitors act by competition with either ATP or metal-binding sites that are involved directly in the catalytic process. However, small-molecular-weight compounds might regulate GSK-3 activity by inhibiting the protein–protein interactions that are necessary for binding of substrate by modulating the Tyr216 (GSK-3β) and Tyr279 (GSK-3α) activation sites and the Ser9 (GSK-3 β) and Ser21 (GSK-3 α) inhibition sites, and by interfering with the intracellular targeting domain of GSK-3. Inhibition of the interaction between the docking protein and the priming kinase might change the substrate specificity of GSK-3.\(^1\),\(^2\)

3.2 **Promising selectivity and potency of Phenyl methylene hydantoin & Rhodanine as effective pharmacophore**

Phenyl methylene hydantoin (PMHs) & Phenyl methylene Rhodanine forms strong interactions with the hinge region of GSK-3β; carbonyl oxygen at position 2 form a H-bonding with backbone nitrogen of Val135 and the NH at position 3 to the carbonyl oxygen of Asp133. The hydantoin ring was sandwiched between Ala83, on top, and Leu188, on the bottom. The aromatic ring is rotated out of plane from the hydantoin plane, allowing extensive interactions with the nucleotide-binding loop. Furthermore, the substituted benzylidene ring system builds an H-bonding interaction with the guanidine moiety of Arg141. Targeting Arg141 is important to improve the activity in
the process of designing new derivatives because it is considered the selectivity residue for GSK-3β.3

**Fig 3.2: Phenyl methylene hydantoin**

Design of potent and selective GSK-3β inhibitors should consider the following important hot spots²²:

- H-bonding interaction with the hinge region of Asp133 and Val135.
- Targeting Arg141 and Gln185 amino acids.
- Filling the Val70, Lys85 and Cys99 hydrophobic pocket. For example, keeping the hydantoin ring and chemical moiety at benzylidene ring system can afford potent and selective GSK-3β inhibitor.
Table No. 3.1: - Library of compounds proposed for docking studies

<table>
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<tr>
<th>S.NO</th>
<th>Substitution</th>
<th>Docking score</th>
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Table 3.2: - Library of compounds proposed for docking studies

![Chemical structure](image)

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### Table 3.3: - Library of compounds proposed for docking studies

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### Table 3.4: - Library of compounds proposed for docking studies

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3.3 Docking Study

The interaction studies were carried out between a) synthesized analogs- glycogen synthase kinase -3β; the molecular docking was performed using Molegro Virtual Docker (MVD) 2006.1.5 and CS Chem Office version 11.0. The docking scoring function of MolDock that we use is based on a piecewise linear potential (PLP) including new hydrogen bonding and electrostatic terms. This docking method is fast and accurate and consists of mainly two steps

✔ Adding a Molecular Surface
✔ Predicting the Binding Site
✔ Defining the Region of Interest

A) Ligand preparation

Structures of all the compounds were sketched using builder module of the program. The sketched structures were subjected to energy minimization using molecular mechanics (MM2) until the RMS gradient value became smaller than 0.1 kcal/molÅ°. The energy minimized molecules were subjected to re-optimization via Austin model-1 (AM1) method until the RMS gradient attained a value smaller than 0.01 kcal/mol Å°A using MOPAC The descriptor values for all the molecules were calculated using “compute properties” module of program. The minimized molecule was saved as MOL file format 4.

B) Protein preparation

The X-ray crystal structure of all the proteins Glycogen synthase kinase -3β (PDB-IQ4L) was retrieved from protein data bank. All the proteins were prepared by removing water molecules and metal ions. Hydrogen atoms were added to the proteins so as to satisfy the valences and bond order assigning during optimized steps.

Following Steps were used for docking through MVD:

C) Docking

Phenyl methylene Hydantoin derivatives forms strong interactions with the hinge region of GSK-3β; carbonyl oxygen at position 2 form a H-bonding with backbone nitrogen of Val135 and the NH at position 3 to the carbonyl oxygen of Asp133. The hydantoin ring was sandwiched between Ala83, on top, and Leu188, on the bottom. The aromatic ring is rotated out of plane from the hydantoin plane, allowing extensive interactions with the nucleotide-binding loop. Furthermore, the substituted benzylidene ring system builds an H-bonding interaction with the guanidine moiety of Arg141.
Targeting Arg141 is important to improve the activity in the process of designing new derivatives because it is considered the selectivity residue for GSK-3β.\(^4\)

**Fig 3.3** The prototype poses of docked structure of a Hydantoin analog

Interaction of 4-((4-oxo-2-thioxothiazolidin-5-ylidene) methyl) phenyl 2-chlorobenzoate with GSK-3β.

**Fig 3.4** Interaction of 4-((4-oxo-2-thioxothiazolidin-5-ylidene) methyl) phenyl 2-Chlorobenzoate with GSK-3β
Interaction of \((4-((2,5\text{-dioxoimidazolidin-4-ylidene})\text{ methyl})\text{ phenyl }3\text{-methylbenzoate.}\)

**Fig 3.5 Interaction of \(-((2,5\text{-dioxoimidazolidin-4-ylidene})\text{ methyl})\text{ phenyl }3\text{-methylbenzoate}**

Interaction of 3-methoxy,4-hydroxy) benzylidene) hydantoin With GSK-3β

**Fig 3.6 Interaction of 3-methoxy,4-hydroxy)benzylidene)hydantoin**
3.4 Synthesis

**Instruments and Chemicals**

All chemicals used in the synthesis are of synthetic grade and they were procured from Loba Chemie, HiMedia and Merck. Thin layer chromatographic method was used for monitoring of the reaction progress and product formation. Thin layer chromatography for compounds was performed using silica gel-G on glass plate in different solvent systems. Iodine vapor and UV light (long wavelength) were used as detecting agents. The purification of intermediates and final compounds was carried out through recrystallization. The melting points of synthesized compounds and intermediates were determined by open capillary method on melting point apparatus and Thieles apparatus, which were uncorrected.

**Synthetic Scheme 1**

**General Synthesis of Derivatives (Step I):**

Benzoic acid derivatives (0.01 moles) were refluxed with thionyl chloride (10 ml.) for 3- 4 hrs and progress of the reaction was monitored through TLC. On completion of the reaction, excess of thionyl chloride was removed under vacuum. The residue was used as such in next step.

**Step I – Synthesis of acid chloride**

\[
\begin{align*}
\text{R} & \quad \text{SOCl}_2 \\
\text{C} & \quad \text{Reflux 3 hr} \\
\text{C} & \quad \text{Cl}
\end{align*}
\]

Substituted benzoic Acid Derivatives \hspace{2cm} Acid Chloride
Mechanism

Fig 3.7: Mechanism of chlorination

Step-II: Synthesis of aldehyde ester:

**General Synthesis of derivatives (Step II):**
Benzoyl chloride analogs were taken into 20 mL of dichloromethane in RBF and cooled to 0°C. To this reaction mixture triethylamine (0.03 mole) was added slowly with constant stirring. Followed by p-hydroxy benzaldehyde (0.01 mole), was added with stirring. The reaction mixture was stirred at 0°C for another 2 hrs. and stirring continued at RT for overnight. Progress of the reaction mixture was checked through TLC. Then reaction mixture washed with saturated solution of sodium bicarbonate, brine solution and water. Organic phase was separated and pass through anhydrous Na₂SO₄. Solvent was removed under vacuum and recrystallized by ethanol.
Mechanism: - The Steglich Esterification

DCC (dicyclohexylcarbodiimide) and the carboxylic acid are able to form an O-acylisourea intermediate, which offers reactivity similar to the corresponding carboxylic acid anhydride:

The alcohol may now add to the activated carboxylic acid to form the stable dicyclohexyl urea (DHU) and the ester:

Step III: Synthesis of Phenyl Benzoate Derivatives:

General Synthesis of derivatives (Step III):

Rhodanine (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the 4-chlorobenzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5).
Mechanism of Reaction

The mechanism follows the principle of Knoevenal condensation reaction.

**Step-I** - Ethanolamine abstracts a proton from the heterocyclic ring & a carbanion ion is generated (enol).

**Step-II** - Product formed in step-I react with the aldehyde in the second step to form an aldol intermediate.

**Step-III & IV** - Product formed in step-II abstracts a proton and with the removal of water molecule converted into final product.
1. Step-I(Z)-4-((4-oxo-2-thioxothiazolidin-5-ylidene)methyl) phenyl 2-chlorobenzoate

Rhodanine (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5)

2. 4-(4-oxo-2-thioxothiazolidin-5-ylidene)methyl) phenyl 3-nitrobenzoate

Rhodanine (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5)
3. **4-((4-oxo-2-thioxothiazolidin-5-ylidene)methyl) phenyl 4-fluorobenzoate**

![Chemical Structure](image)

Rhodanine (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5).

4. **(Z)-4-((4-oxo-2-thioxothiazolidin-5-ylidene)methyl) phenyl 4-nitrobenzoate**

![Chemical Structure](image)

Rhodanine (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5).
5. **Z)-4-((4-oxo-2-thioxothiazolidin-5-ylidene)methyl) phenyl 4 (trifluoromethyl)benzoate**

![Chemical Structure]

Rhodanine (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5)

6. **(Z)-4-((4-oxo-2-thioxothiazolidin-5-ylidene)methyl) phenyl benzoate**

![Chemical Structure]

Rhodanine (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5)
7. \( \text{Z)-4-((4-oxo-2-thioxothiazolidin-5-ylidene)methyl) phenyl 3-fluorobenzoate} \)

\[
\begin{array}{c}
\text{O} \\
\text{C} \\
\text{O} \\
\text{S} \\
\text{N} \\
\text{H} \\
\text{O} \\
\end{array}
\]

Rhodanine (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5)

**Synthetic Scheme –II**

General synthetic scheme for the synthesis of Phenyl methylene hydantoin derivatives: Phenyl methylene hydantoin derivatives were prepared by reaction of hydantoin with benzaldehyde derivatives in the presence of ethanolamine\(^6,8,10\)

\[
\begin{array}{c}
\text{O} \\
\text{C} \\
\text{O} \\
\text{N} \\
\text{H} \\
\text{N} \\
\text{H} \\
\text{O} \\
\end{array}
\]

**Fig no 3.11: Scheme no II**
8. 5-(3,4,5-(trimethoxy)benzylidene)hydantoin

Hydantoin (9.9 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 7h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5). Compound was purified through column chromatography.

9. 5-(3-methoxy,4-hydroxy)benzylidene)hydantoin

Hydantoin (9.9 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 7h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5). Compound was purified through column chromatography.
10. 5-(2-hydroxy)benzylidene)hydantoin

Hydantoin (9.9 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 7h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5). Compound was purified through column chromatography.

11. 5-(2-nitro)benzylidene)hydantoin

Hydantoin (9.9 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 7h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5). Compound was purified through column chromatography.
12. **5-(4-chlorobenzylidene)hydantoin**

Hydantoin (9.9 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonates solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 7h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5). Compound was purified through column chromatography

13. **5-(4-nitrobenzylidene)Hydantoin**

Hydantoin (9.9 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 7h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5). Compound was purified through column chromatography

14. **5-(4-hydroxy)benzylidene)Hydantoin**
Hydantoin (9.9 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 7h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5). Compound was purified through column chromatography.

15. 5-(3-Chloro)benzylidene)Hydantoin

Hydantoin (9.9 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 7h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5). Compound was purified through column chromatography.

16. 5-(3,4,5-trimethyl)benzylidene)Hydantoin

Hydantoin (9.9 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 7h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5). Compound was purified through column chromatography.
continuous stirring. The reaction was kept under reflux for approximately 7h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5). Compound was purified through column chromatography.

**Synthetic scheme – III**

General synthetic scheme for the synthesis of Phenyl methylene Rhodanine derivatives: Phenyl methylene Rhodanine derivatives were prepared by reaction of Rhodanine with benzaldehyde derivatives in the presence of ethanolamine$^6,8,10$

![Chemical Reaction Diagram](attachment:reaction_diagram.png)

**Fig no 3.12: Scheme no III**

17. 5-(4-(Dimethylamino)benzylidene)rhodadine

Rhodanine (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5). Compound was purified through column chromatography.
18. 5-(4-(Nitro)benzylidene)rhodadine

Rhodanine (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5). Compound was purified through column chromatography.

19. 5-(4-Hydroxy)benzylidene)rhodadine

Rhodanine (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5). Compound was purified through column chromatography.
20. 5-((4-Chloro)benzylidene)rhodadine

Rhodanine (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5). Compound was purified through column chromatography.

Synthetic scheme – IV

**General Synthesis of derivatives. (Step I):**

Benzoic acid derivatives (0.01 mole) were refluxed with thionyl chloride (10 ml.) for 3-4 hrs and progress of the reaction was monitored through TLC. On completion of the reaction, excess of thionyl chloride was removed under vacuum. The residue was used as such in next step.

**Step I – Synthesis of acid chloride**

Substituted benzoic Acid Derivatives

Acid Chloride

**Step-II: Synthesis of aldehyde ester:**

General Synthesis of Compound (Step II):

Benzoyl chloride analogs were taken into 20 mL of dichloromethane in RBF and cooled to 0°C. To this reaction mixture triethylamine (0.03 mole) was added slowly with constant stirring. Followed by p-hydroxy benzaldehyde (0.01 mole), was added with stirring. The reaction mixture was stirred at 0°C for another 2 hrs. and stirring
continued at RT for overnight. Progress of the reaction mixture was checked through TLC. Then reaction mixture washed with saturated solution of sodium bicarbonate, brine solution and water. Organic phase was separated and pass through anhydrous Na$_2$SO$_4$. Solvent was removed under vacuum and recrystallized by ethanol.

**Step III: Synthesis of Phenyl Benzoate Derivatives**:

General Synthesis of Compound (Step III):

Hydantoin (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5)

![Scheme IV](image)

**Fig no 3.13: Scheme no IV**
21. 4-((2,5-dioxoimidazolidin-4-ylidene)methyl)phenyl benzoate

Hydantoin (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5)

22. 4-((2,5-dioxoimidazolidin-4-ylidene)methyl)phenyl 3-methylbenzoate

Hydantoin (7.4 mM) was dissolved in 10 mL water at 70°C on oil bath with continuous stirring. After complete dissolution the pH was adjusted to 7.0 using saturated sodium bicarbonate solution. The temperature was then raised to 90°C after the addition of 0.9 mL ethanolamine. Equimolar quantity of the substituted benzaldehyde solution in 5 mL ethyl alcohol was then added drop wise with continuous stirring. The reaction was kept under reflux for approximately 8h. The reaction was monitored by TLC. After complete depletion of the starting aldehyde, the mixture was cooled and the precipitate was filtered and washed with ethanol/water (1:5)
### 3.5 List of Synthesized Compounds

Table 3.3: List of Synthesized Compounds

<table>
<thead>
<tr>
<th>STRUCTURE</th>
<th>IUPAC NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>(Z)-4-((4-oxo-2-thioxothiazolidin-5-ylidene)methyl) phenyl 2-chlorobenzoate</td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>(Z)-4-((4-oxo-2-thioxothiazolidin-5-ylidene)methyl) phenyl 3-nitrobenzoate</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>(Z)-4-((4-oxo-2-thioxothiazolidin-5-ylidene)methyl) phenyl 4-fluorobenzoate</td>
</tr>
<tr>
<td><img src="image4.png" alt="Structure 4" /></td>
<td>(Z)-4-((4-oxo-2-thioxothiazolidin-5-ylidene)methyl) phenyl 4-nitrobenzoate</td>
</tr>
<tr>
<td><img src="image5.png" alt="Structure 5" /></td>
<td>(Z)-4-((4-oxo-2-thioxothiazolidin-5-ylidene)methyl) phenyl 4-(trifluoromethyl)benzoate</td>
</tr>
<tr>
<td><img src="image6.png" alt="Structure 6" /></td>
<td>(Z)-4-((4-oxo-2-thioxothiazolidin-5-ylidene)methyl) phenyl benzoate</td>
</tr>
<tr>
<td><img src="image7.png" alt="Structure 7" /></td>
<td>(Z)-4-((4-oxo-2-thioxothiazolidin-5-ylidene)methyl) phenyl 3-fluorobenzoate</td>
</tr>
<tr>
<td><img src="image8.png" alt="Structure 8" /></td>
<td>5-(3,4,5-(trimethoxy)benzylidene)hydantoin</td>
</tr>
<tr>
<td>Chemical Structure</td>
<td>Name</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td><img src="https://example.com/structure1.png" alt="Structure 1" /></td>
<td>5-(3-methoxy,4-hydroxy)benzylidenehydantoin</td>
</tr>
<tr>
<td><img src="https://example.com/structure2.png" alt="Structure 2" /></td>
<td>5-(2-hydroxy)benzylidenehydantoin</td>
</tr>
<tr>
<td><img src="https://example.com/structure3.png" alt="Structure 3" /></td>
<td>(E)-5-(2-nitrobenzylidene)Hydantoin</td>
</tr>
<tr>
<td><img src="https://example.com/structure4.png" alt="Structure 4" /></td>
<td>(E)-5-(4-chlorobenzylidene)Hydantoin</td>
</tr>
<tr>
<td><img src="https://example.com/structure5.png" alt="Structure 5" /></td>
<td>(E)-5-(4-nitrobenzylidene)Hydantoin</td>
</tr>
<tr>
<td><img src="https://example.com/structure6.png" alt="Structure 6" /></td>
<td>(E)-5-(4-hydroxybenzylidene)Hydantoin</td>
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<td><img src="https://example.com/structure7.png" alt="Structure 7" /></td>
<td>(E)-5-(3-chlorobenzylidene)Hydantoin</td>
</tr>
<tr>
<td><img src="https://example.com/structure8.png" alt="Structure 8" /></td>
<td>(E)-5-(3,4,5-trimethylbenzylidene)Hydantoin</td>
</tr>
<tr>
<td><img src="https://example.com/structure9.png" alt="Structure 9" /></td>
<td>5-(4-(Dimethylamino)benzylidene)rhodadine</td>
</tr>
</tbody>
</table>
3.6 Biological Evaluation

3.6.1 In-vitro Biological Evaluation

a) Inhibition assay for α-amylase activity:
A concentration of 5 mg/mL of synthesized compounds was prepared by dissolving in double distilled water and further diluted to produce different concentrations. A total of 500 µL of prepared compound solution and 500 µL of 0.02M sodium phosphate buffer (pH 6.9 with 0.006M sodium chloride) containing α-amylase solution (0.5mg/mL) were incubated for 10 minutes at 25°C. After pre-incubation, 500 µL of 1% starch solution in 0.02M sodium phosphate buffer (pH6.9 with 0.006M sodium chloride) was added to each tube at 5s intervals. This reaction mixture was then incubated for 10 minutes at 25°C. DNSA colour reagent 1mL was added to stop the reaction. These test tubes were then incubated in a boiling water bath for 5 minutes and cooled to room temperature. Finally this reaction mixture was again diluted by adding 10 mL distilled water following which absorbance was measured at 540 nm.
The percentage inhibition and 50% inhibitory concentration (IC$_{50}$) value was calculated using formula $^{11-14}$

Inhibition (%) = (control−test/control) 100.

**b) Inhibition assay for α-Glucosidase Activity:**

The α-glucosidase enzyme inhibition activity was determined by incubating 100μL of α-glucosidase enzyme (1U/mL) solution with 100μL of phosphate buffer (pH 7.0) which contains 100μL of enzyme inhibitor such as SP$_1$-SP$_{22}$ and acarbose as reference at concentration 1, 2, 4, 6, 8 and 10μg/mL at 37°C for 60 min in maltose solution. To stop the α-glucosidase action on maltose, the above reaction mixture was kept in boiling water for 2 min and cooled. To this, 2mL of glucose reagent was added and its absorbance was measured at 540nm to estimate the amount of liberated glucose by the action of α-glucosidase enzyme. The activity was performed in triplicate. The percentage inhibition and 50% inhibitory concentration (IC$_{50}$) value was calculated $^{11, 12, 13, 15, 16}$.

**c) Glucose diffusion inhibitory study:**

To test the diffusion of glucose across the bio-membrane, 3 cm strips of the dialysis membrane (12000 MW, HiMedia Laboratories, Mumbai, India) were cut and filled with 1 mL glucose solution (0.22 mM) in 0.15 M sodium chloride) and 1 mL solution of synthesized compound. It was then tied at both ends using nylon thread and it was immersed in a 100 mL beaker containing 40 mL of 0.15 M sodium chloride and 10 mL of distilled water. The control contained 1mL of 0.15 M sodium chloride containing 22 mM glucose and 1 mL of distilled water to equalise the strength of internal and external media. The beakers were then placed on orbital shaker (The I L E Company, Chennai, India) and kept at room temperature. The movement of glucose into the external solution was monitored by taking the sample from each beaker and glucose concentration in them was tested every half hour. Three replications of this were done for 3 hours (Denise and David, 2000); (Pasupathi et al., 2009). The percentage Relative movement value of glucose at time different measured times (minutes) was calculated $^{17, 18}$.

**In-vivo Biological Evaluation**

**a) In-vivo anti-diabetic activity: Evaluation for anti-hyperglycemic activity**

All animals were used from inbred colony which is maintained on standard laboratory and the study protocols were approved by institutional animal ethics committee.
**Induction of non-insulin-dependent diabetes mellitus:** - The non-insulin-dependent diabetes mellitus will be induced in overnight fasted adult Wister Albino rats, weighing 100-200g by single intraperitonal injection of freshly prepared solution of Streptozotocin 60 mg/kg, body weight. After administration of Streptozotocin, blood glucose will be monitored after 72 hours to detect hyperglycemia. Diabetes will be developed and stabilized in the Streptozotocin treated animals over a period of 3-5 days. The rats found with permanent non-insulin-dependent diabetes mellitus (NIDDM) will be used for the antidiabetic study.

**Preparation of Streptozotocin solution:** Streptozotocin solution will be prepared by dissolving Streptozotocin in citrate buffer (pH 4.5) with proper mixing. The preparation will be used immediately within 15-20 minutes.

**Experiment designs for antidiabetic study:** - The rats will be classified into 25 groups, each group consisting of six rats.

- **Group 1:** Serve as normal control (0.9 % w/v Saline 10 ml/kg b.w.)
- **Group 2:** Serve as diabetic control (Streptozotocin 60mg/kg b.w.)
- **Group 3:** Serve as reference standard (Rosiglitazone, 4 mg/kg b.w. by oral route)
- **Group 4:** Serve as test standard (test compound SP-1 15 mg/kg by oral route)
- **Group 5:** Serve as test standard (test compound SP-2 15 mg/kg by oral route)
- **Group 6:** Serve as test standard (test compound SP-3 15 mg/kg by oral route)
- **Group 7:** Serve as test standard (test compound SP-4 15 mg/kg by oral route)
- **Group 8:** Serve as test standard (test compound SP-5 15 mg/kg by oral route)
- **Group 9:** Serve as test standard (test compound SP-6 15 mg/kg by oral route)
- **Group 10:** Serve as test standard (test compound SP-7 15 mg/kg by oral route)
- **Group 11:** Serve as test standard (test compound SP-8 15 mg/kg by oral route)
- **Group 12:** Serve as test standard (test compound SP-9 15 mg/kg by oral route)
- **Group 13:** Serve as test standard (test compound SP-10 15 mg/kg by oral route)
- **Group 14:** Serve as test standard (test compound SP-11 15 mg/kg by oral route)
- **Group 15:** Serve as test standard (test compound SP-12 15 mg/kg b by oral route)
- **Group 16:** Serve as test standard (test compound SP-13 15 mg/kg by oral route)
- **Group 17:** Serve as test standard (test compound SP-14 15 mg/kg by oral route)
- **Group 18:** Serve as test standard (test compound SP-15 15 mg/kg by oral route)
- **Group 19:** Serve as test standard (test compound SP-16 15 mg/kg by oral route)
- **Group 20:** Serve as test standard (test compound SP-17 15 mg/kg by oral route)
| Group 21: | Serve as test standard (test compound SP-18 15 mg/kg by oral route) |
| Group 22: | Serve as test standard (test compound SP-19 15 mg/kg by oral route) |
| Group 23: | Serve as test standard (test compound SP-20 15 mg/kg by oral route) |
| Group 24: | Serve as test standard (test compound SP-21 15 mg/kg by oral route) |
| Group 25: | Serve as test standard (test compound SP-22 15 mg/kg by oral route) |

In antihyperglycemic study treatment will continue with a daily single oral administration of vehicle (saline), standard suspension, Test compounds in morning by oral feeding needle. The blood samples will be collect from rats by retro orbital plexus bleeding method and the fasting blood glucose level was monitored at different times 0 h, 1h, 3h, and 6h by using a glucose oxidase-peroxidase reactive strips and a glucometer (Kannur et al., 2006; Selvan et al., 2008).

3.6.2 In-vivo glycogen content determination test

a) Preparation of test solution:
The effect of test compound on liver glycogen content of Albino rats was investigated. The test compounds were administered orally as acacia suspension (2%). Dose: 15 mg/kg of body weight was used.

b) Determination of liver glycogen:
Six-week old Albino rats with average weight of 200 g were used for this investigation. The animals were randomized and fed ad libitum with standard food and water except when fasting was needed in the course of study. All animals were housed in the same conditions and separated randomly to nine groups. Eight groups (three rats/group) used to investigate test compound were administered orally with the 15 mg/kg dose of test compound and one group as control. On the day of experiment, food and water were removed 6h before the drug administration. Then animal’s livers were immediately removed for glycogen determination.

Liver glycogen content was determined quantitatively following a reported procedure. Briefly, livers were removed immediately after the animals sacrificed and were homogenized using a homogenizer with appropriate volume of 5% trichloroacetic acid over 5 min. The homogenate was centrifuged at 3000 rpm for 5 min. The supernatant fluid was taken and filtered using acid-washed filter paper. The glycogen of 1.0 mL of this filtrate was precipitated using ethanol (95%, 5 mL), incubated in water bath at 37-40°C for 3h, and centrifuged at 3000 rpm for 15 min. The clear liquid is gently decanted from the packed glycogen, and the tubes were allowed to drain in an
inverted position for 10 min. The glycogen was dissolved in distilled water (2 mL) and mixed with 10 mL of the anthrone reagent (0.05% anthrone, 1.0% thiourea in 72% H₂SO₄). The mixture incubated for 30 min, and subsequently, the absorbance was measured at 620 nm by a UV-Vis spectrophotometer. Blank and standard solutions were prepared by adding 10 mL of anthrone reagent to 2 mL of water and to 2 mL of glucose solution containing 0.1 mg of glucose in saturated benzoic acid, respectively.

The liver glycogen content is estimated using the following formula:

Amount (mg) of glycogen liver tissue

= \( \frac{DU}{DS} \times \frac{\text{Volume of Extract (mL)}}{\text{Weight of Liver Tissue (g)}} \times 0.09 \)

Where, DU is the absorbance of the unknown sample and DS is the absorbance of the standard.

Measurement of Biochemical Parameters

The blood glucose, Hb, and HbA1c were estimated using whole blood. The serum total cholesterol (TC), high-density lipoprotein (HDL), triglycerides (TG), and total protein (TP) were estimated using commercially available kits in a semiautoanalyzer (Photometer 5010v5+, Germany). The serum insulin was estimated by radioimmunoassay method.