REGULATION OF LIPID, GLUCOSE METABOLISM AND INSULIN RESISTANCE THROUGH PPAR USING BAICALIN

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IN

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Submitted By

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This is to certify that the dissertation synopsis work entitled **"REGULATION OF LIPID, GLUCOSE METABOLISM AND INSULIN RESISTANCE THROUGH PPAR USING BAICALIN"** submitted by University **A.JEEVITHA (Reg no. 261925906)** is a bonafide work carried out by the candidate under the guidance of **Dr. S Muthukrishnan, M. Pharm., Ph.D.,** Associate professor ,Department of Pharmacology, PSG College of Pharmacy and submitted to The Tamil Nadu Dr. M. G. R. Medical University, Chennai, in partial fulfilment for the Degree of Master of Pharmacy in Pharmacology at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2020-2021.

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DECLARATION

We hereby declare that the dissertation work entitled **"REGULATION OF LIPID, GLUCOSE METABOLISM AND INSULIN RESISTANCE THROUGH PPAR USING BAICALIN"** submitted by University **Reg no. 261925906** to The Tamil Nadu Dr. M. G. R. Medical University, Chennai, in partial fulfillment for the Degree of Master of Pharmacy in Pharmacology at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, was done by self-candidates under the guidance of **Dr. S Muthukrishnan, M. Pharm., Ph.D..,** during the academic year 2020-2019.

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EVALUATION CERTIFICATE

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Examination centre: PSG college of pharmacy, Coimbatore

Date:

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Our dissertation entitled **"REGULATION OF LIPID, GLUCOSE METABOLISM AND INSULIN RESISTANCE THROUGH PPAR USING BAICALIN"** had become successful one with grace of almighty who was there with us throughout our work to lead us in right path and to complete the work on time.

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1.INTRODUCTION

1.INTRODUCTION

1.1 DIABETES MELLITUS:

According to the World Health Organization (WHO) diabetes mellitus is a chronic, metabolic disease characterized by elevated levels of blood glucose due to decreased insulin secretion or inability of tissue to use the insulin, which over time leads to damage of the heart, vasculature, eyes, kidneys and nerves.

Diabetes mellitus is split up into four categories – **Type-1 diabetes** Type –1diabetes is additionally called insulin dependent diabetes mellitus because this disease is characterised by an absolute deficiency of insulin. Beta cells are destructed because of invasion by virus, action of chemical toxins or due to action of autoimmune antibodies. This beta cell apoptosis or necrosis is cause of insulin deficiency and caused Type-1 diabetes. diabetes [1] **Type-2 diabetes** Non- insulin dependent diabetes mellitus or Type-2 diabetes is usually amid organ insulin resistance that limits responsiveness to both endogenous and exogenous insulin [2]**. Type- 3 diabetes**-This type of diabetes is caused by chronic pancreatitis or chronic drug therapy with glucocorticoids, thiazides diuretics, diazoxide, growth hormone and with some protease inhibitors (e.g., saquinavir). **Type- 4 diabetes**- This type of diabetes is observed in approximately 4-5% of all pregnancies, due to placental hormones that promotes insulin resistance [3]**.**

Over 90% of world diabetes mellitus cases are T2DM, a condition portrayed by tissue insulin resistance (IR) and an inadequate compensatory insulin secretory response by beta cells of islet of Langerhans in pancreas [4]**.**

1.2 EPIDEMELOGY:

It is a major Global threat with high prevalence. International Diabetes federation (IDF) estimated that 1 in 11 adults are having diabetes aged between 20-79 years. IDF found that 463.0(9.3%) million people are affected by diabetes mellitus in 2019 globally and estimated to rise to 578.4 million (10.2%) in 2030, 700.2 million (10.9%) in 2045.

Prevalence is lowest among adults aged 20–24 years (1.4% in 2019). Among adults aged 75–79 years diabetes prevalence is estimated to be 19.9% in 2019 and predicted to rise to 20.4% and 20.5% in 2030, and 2045, respectively. The estimated prevalence of diabetes in women aged 20–79 years is slightly less than in men (9.0% vs 9.6%) [5]**.**

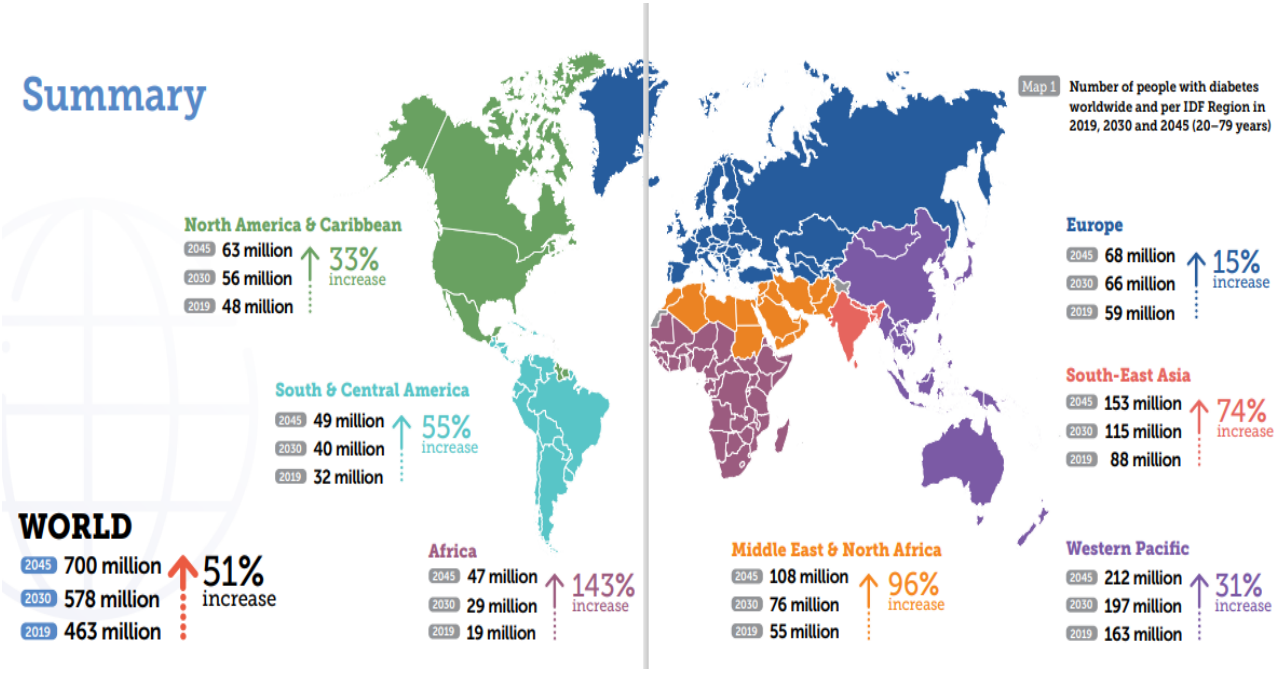

Figure 1. Number of people with diabetes worldwide and per IDF region in 2019, 2030 & 2045

1.3 PANCREAS:

Pancreas is an elongated gland that consist of head, body, tail portion. The head portion is held loop of duodenum and the tail lies in the hilum of spleen. It is double gland, both exocrine and endocrine gland. The exocrine gland secret digestive enzymes and the endocrine gland, islet of Langerhans, a cluster of cells which constitute 1% of pancreas mass secret insulin, glucagon, somatostatin and polypeptides from beta, alpha, gamma and F cells respectively. In humans, alpha and beta cells are found dispersed throughout the islet along blood vessels in association with microcirculation. The beta cells comprise approximately 60% of the endocrine mass of the pancreas and produce both insulin and amylin, the former of which is released in response to elevation in plasma glucose levels. The alpha cells, which comprise about 30% of the endocrine mass of the pancreas, secrete glucagon in response to decreases in plasma glucose levels [6].

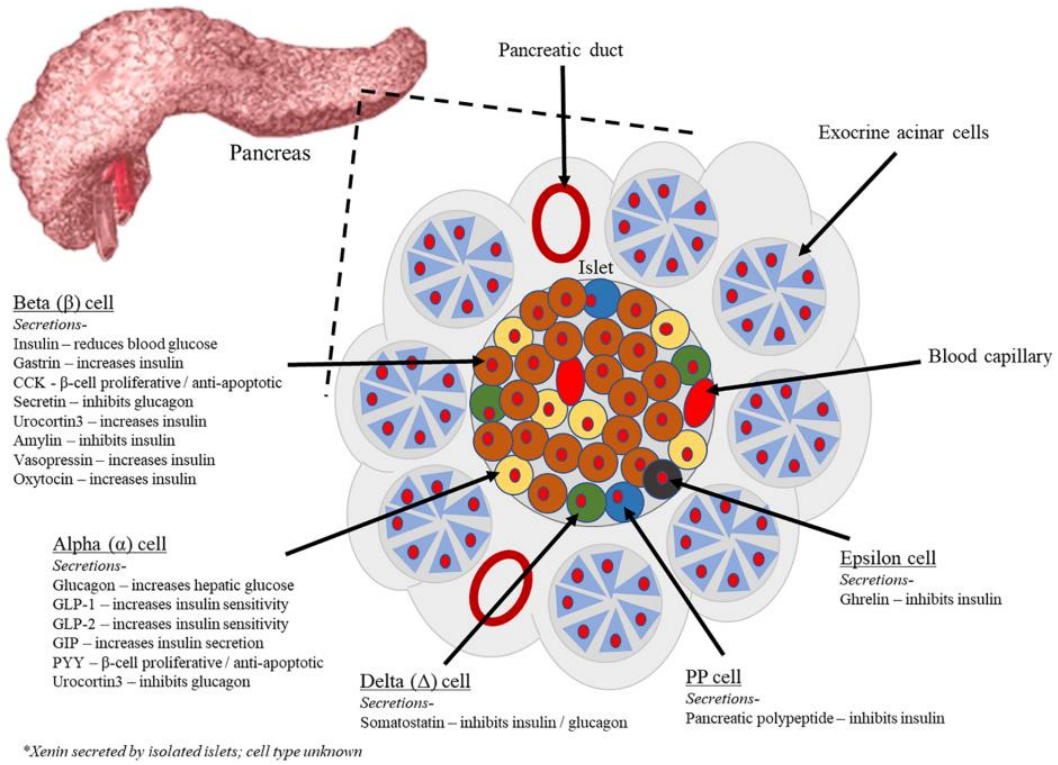

Figure 2. Endocrine pancreatic cell types and their peptide secretions [7]

1.4 INSULIN SYNTHESIS, SECRETION AND REGULATION:

Insulin is a two-chain polypeptide having 51 amino acids and 6000 Dalton of molecular weight. The A chain has 21 while B chain has 30 amino acids connected by disulphide bond.

Insulin is coded on the short arm of chromosome 11 [8] and synthesised within the β cells of the pancreatic islets of Langerhans as its precursor, proinsulin. Proinsulin is synthesised within the ribosomes of the rough endoplasmic reticulum (RER) from mRNA as pre-proinsulin, its precursor. Pre-proinsulin is formed by sequential synthesis of a signal peptide, the B chain, the connecting (C) peptide and then the A chain comprising a single chain of 100 amino acids. Removal of the signal peptide forms proinsulin within the endoplasmic reticulum. Secretory vesicles translocate proinsulin from the RER to the Golgi body, where the proinsulin is converted into insulin by removal of c peptide and forms hexamer with zinc which is enriched in the Golgi body [9]. Insulin forms zinc-containing hexamers which are insoluble, precipitating as chemically stable crystals at pH 5.5. Equimolar amount of insulin and c-peptide are released when mature granules are secreted into the circulation by exocytosis due to stimulus. Proinsulin and zinc typically comprise no more than 6% of the islet cell secretion.

In response to a stimulus such as glucose, insulin secretion is characteristically biphasic, with an initial rapid phase of insulin secretion, followed by a less intense but more sustained release of the hormone [10].

Synthesis and secretion of insulin is controlled by both nutrient and non-nutrient secretagogues, in the context with the environmental stimuli and also the interplay of other hormones. Nutrient secretagogues such as glucose, mannose, galactose appear to trigger insulin secretion from the β cell by increasing intracellular ATP and closing of K+-ATP channels. Generation of cyclic AMP and Ca2+ level in intracellular space also augmented, further enhancing insulin release. Glucose enters into the β cell independent of insulin action (nor do fructose, mannose or galactose). Non-nutrient secretagogues produce its action by neural stimuli such as cholinergic and adrenergic pathways, or through peptide hormones and cationic amino acids [11].

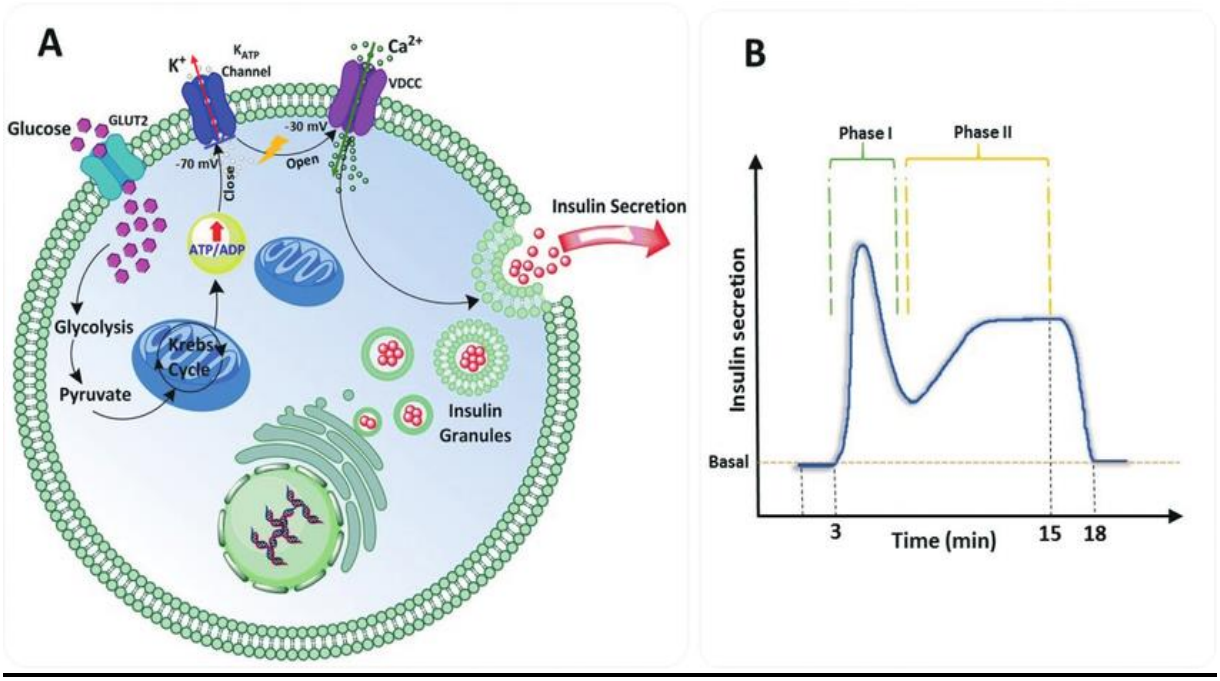

Figure 3. A) Glucose stimulated insulin secretion pathway in pancreatic β-cells and B) biphasic insulin release profile. [12]

1.5 INSULIN RECEPTOR AND CELLULAR SIGNALLING:

Insulin mediates its actions through binding to insulin receptors (transmembrane receptor). The insulin receptor was first characterised in 1971. It is made up of a heterotetramer consisting of 2 α and 2 β glycoprotein subunits linked by disulphide bonds and the α subunit is present extracellular while the β subunit is transmembrane located [13]. The chromosome 19 in

the short arm comprises the gene coding for insulin receptor [14]. Insulin binds to the extracellular α subunit, producing conformational change enabling ATP to bind to the intracellular component of the β subunit [15] triggers Autophosphorylation of the β subunit conferring tyrosine kinase activity. This causes recruitment of intracellular substrate proteins known as insulin responsive substrates (IRS), and tyrosine phosphorylation. Phosphorylated IRS proteins bind specific src-homology-2 domain proteins (SH2), phosphatidylinositol 3-kinase (PI 3-kinase). PI 3-kinase promotes the translocation of glucose transporter proteins, glycogen, lipid and protein synthesis, anti-lipolysis [16] and the control of hepatic gluconeogenesis via serine and threonine kinases such as Akt/protein kinase B (PKB), protein kinase C (PKC) and PI dependent protein kinases1& 2 (PIPD 1&2). The RAS-Akt pathway activates transcription factors and stimulates the growth promoting actions of insulin. Thus broadly, PI 3-kinase mediates insulin's metabolic effects, e.g., cellular glucose uptake, while RAS significantly mediates insulin's mitogenic effects [17].

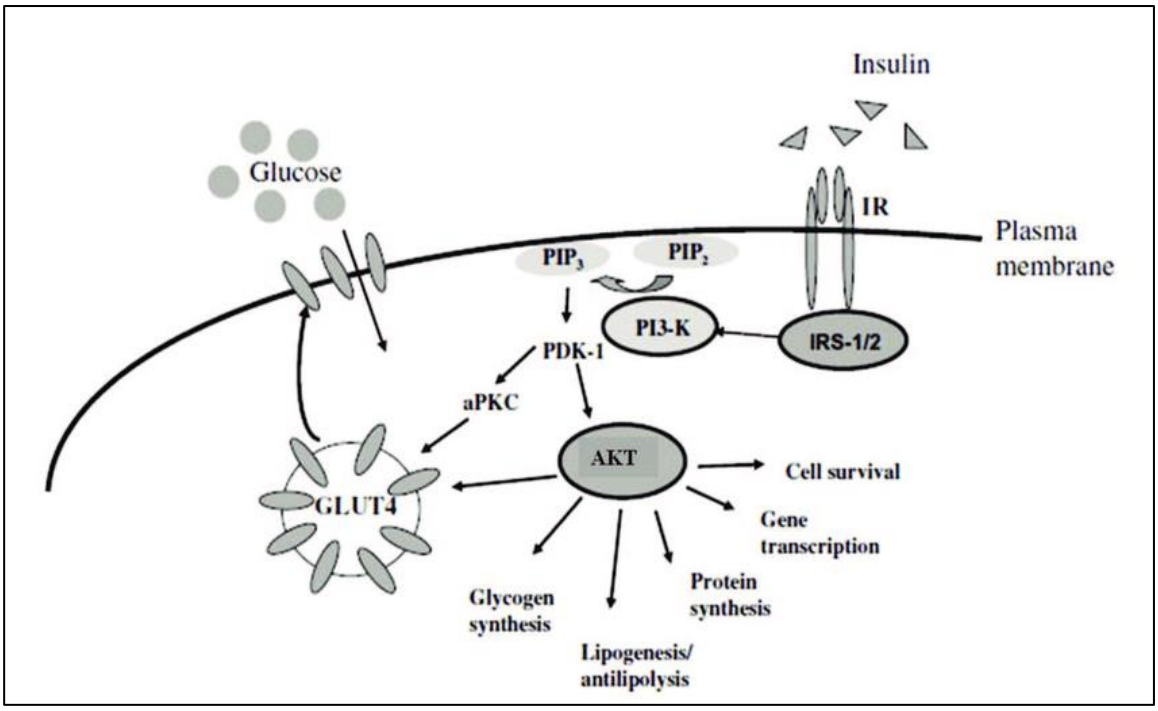

Figure 4. Insulin signalling pathway [18]

1.6 PATHOPHYSIOLOGY OF T2DM:

Type 2 diabetes mellitus (T2DM) has long been categorized as a complex and multifactorial metabolic syndrome that is characterized by the abnormal metabolism of carbohydrates, fats and proteins which leads to the augmented levels of glucose and lipids within the blood [19]. It is very difficult to describe the pathophysiology of T2DM for 2 reasons.

First, our understanding of this important disease is changing almost daily as research progresses. Second, T2DM is not a single disease but is a group of diseases, i.e., a syndrome with different genetics and pathophysiology but similar symptoms and outcome [20].

In simplified terms, T2DM is a combination of insulin resistance and insulin deficiency. Several mechanisms have been put forward, comprising increased non-esterified fatty acids, inflammatory cytokines, adipokines, and mitochondrial dysfunction for insulin resistance, and glucotoxicity, lipotoxicity, and amyloid formation for β-cell dysfunction [21].

Insulin resistance is probably the first defect in T2DM and begins many years before the onset of symptoms or developing a blood glucose level high enough to make the diagnosis. Insulin resistance occurs in the peripheral cells (primarily muscle and fat cells) of the body and the liver. It is caused by genetic factors in the peripheral cells and liver cells as well as environmental factors. The environmental factors include aging, sedentary lifestyle, and obesity. Progressive insulin deficiency is the other defect of T2DM. It is different than the insulin deficiency of T1DM in that it is not mediated by the immune system. The insulin deficiency may be due to β cell exhaustion from the hypersecretion of insulin, glucose and lipid toxicity to the β cells, or genetic factor. It has been estimated that 50% of β cell insulin production is lost by the time the diagnosis of T2DM is made. The loss of β cell function continues.

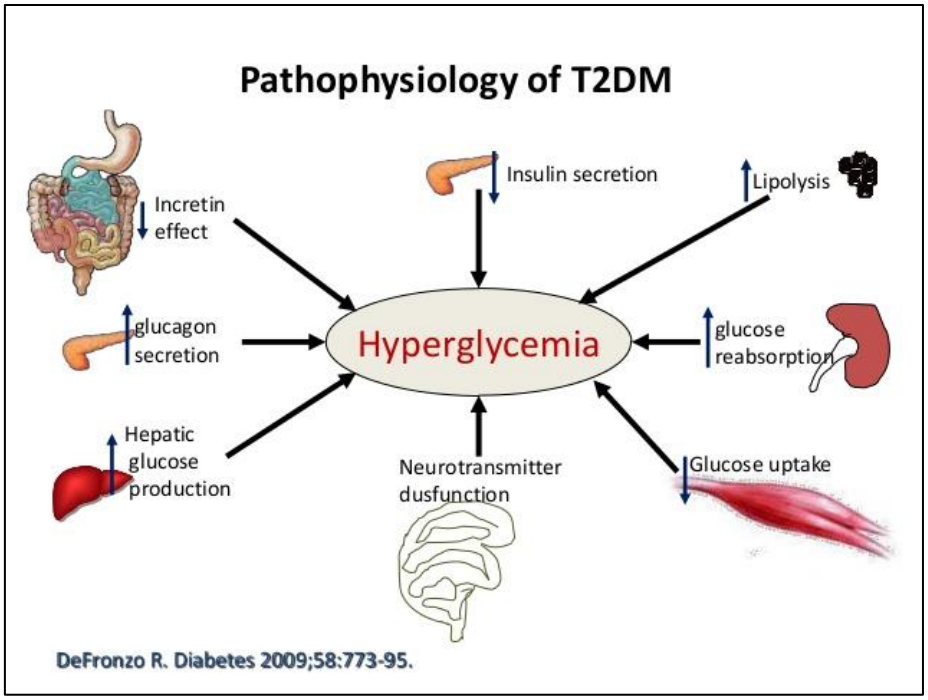

Figure 5. Pathophysiology of T2DM [22]

1.7 PEROXISOME PROLIFERATOR ACTIVATOR RECEPTOR:

The peroxisome proliferator-activated receptors (PPARs) are a subfamily of ligand initiated nuclear receptors/transcription factors that fit into the superfamily of nuclear receptors [23]. The PPAR subfamily comprises of three isotypes: PPAR-α (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3), which are encoded in the separate genes.

PPARs balance the genes that regulate energy balance, glucose homeostasis, TG and lipoprotein metabolism, fatty acid synthesis, oxidation, storage and export, cell proliferation, inflammation and vascular tissue function dysregulation of these metabolic processes contributes to the pathogenesis of metabolic diseases such as obesity, Metabolic Syndrome, diabetes, NAFLD and atherosclerosis.

Similar to other members of the nuclear receptor family, PPAR contains five main domains [24].

The five domains are:

- ✓ **NH2 -terminal end**,
- ✓ **A/B domain**: ligand independent transactivation domain
- ✓ **C domain**: 70-amino-acid long PPAR DNA binding domain (DBD) which has two highly conserved zinc finger motifs and promotes the binding of the receptor to a DNA sequence in the promoter region of target genes known as the peroxisome proliferator response element (PPRE);
- ✓ **D domain**: The hinge region acts as a docking site for cofactors and also connects the C-terminal and E/F domain,
- ✓ **E/F domain**: ligand-binding domain (LBD), which is responsible for ligand specificity, Activation of PPAR binding to the PPRE that leads to increased expression of target genes, assist to the dimerization of the receptor with retinoid X receptors (RXRs) and is a site for binding of co-activators and co-repressors;

There are two activation domains: one in the amino terminus (**AF-1**) and one in the carboxyl terminus (**AF-2**). AF-1 activation alone is in general weak, but synergizes with AF-2 upon ligand binding, leading to an increased gene transcription and expression.

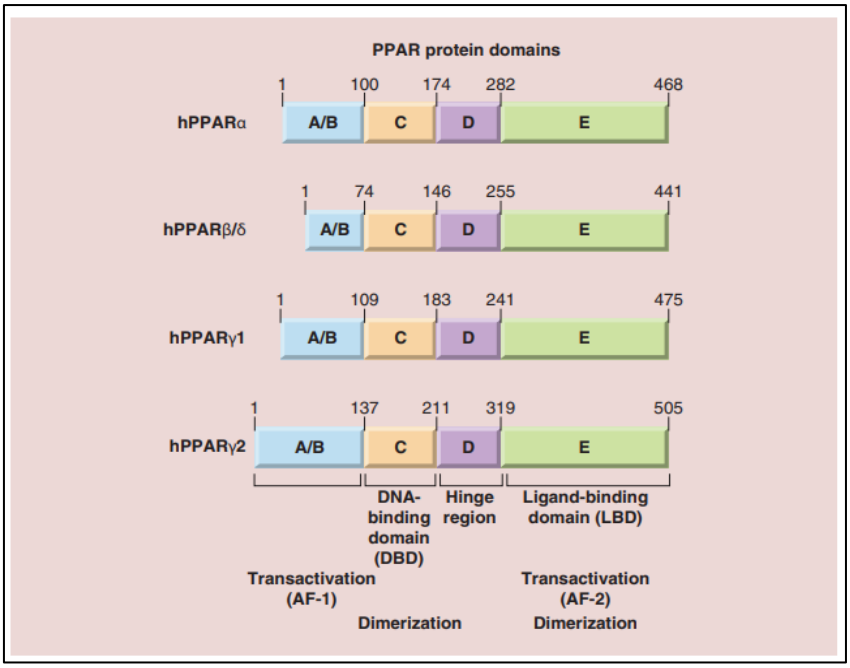

Figure 6. Structure of PPAR [25]

PPARs forms heterodimers with RXRs (α, β, γ) and binds to a consensus cis-element, PPRE, in target DNAs, which consists of a two-hexanucleotide, AGGTCA (or a related sequence), separated by one base pair (AGGTCANAGGTCA), called direct repeat 1**.**

In the absence of ligand state, PPAR/RXR heterodimers are bound to multicomponent repressors containing histone deacetylase activity, which are nuclear receptor corepressor and the silencing mediator for retinoid and thyroid hormone receptor, causing inhibition of gene transcription.

Following PPAR activation, PPAR/RXR heterodimers dissociate from co-repressors, and recruit co-activators such as steroid receptor co-activator-1 and the PPAR-binding protein with histone acetylase activity, and subsequently bind to PPRE target genes to modulate gene transcription. [26]

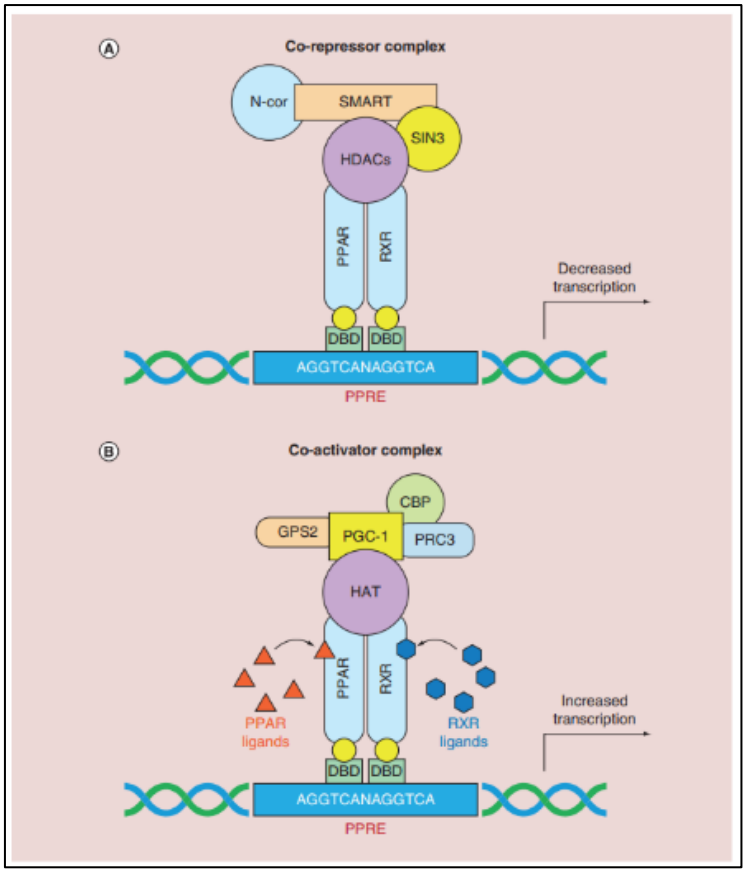

Figure 7. Corepressor and Coactivator [25]

The PPAR-α is transcribed from the human PPARA gene located at chromosomal region 22q12-q13, [27]. consisting of eight exons. Human PPARD is confined at chromosomal region 6p21.2-21.1 [28] and comprised with nine exons. Human PPARG has nine exons and is confined at chromosome 3p25 [29].

PPAR-α is expressed most prominently in high-energy requiring tissues for instance skeletal muscle, heart, liver and brown adipose tissue [30]. Remarkable expression of PPAR-α also occurs in the proximal tubules of the kidney, intestinal mucosa, the adrenal gland and brown adipose tissue and most cell types present in the vasculature including endothelial cells (ECs), smooth muscle cells and monocytes/macrophage.

PPAR-β/δ is ubiquitously expressed in mouse, rat and human tissues.

PPAR-γ is highly expressed in adipose tissue, where it plays an essential role in the regulation of adipocyte differentiation, survival and function, insulin sensitivity, lipid metabolism, lipid storage, lipid transport glucose metabolism and regulate transcription of a number of genes involved in these metabolic processes [31]. The two major isoforms of PPAR-

γ rare **PPAR-γ1** which is expressed at highest level in brown and white adipose tissues, but low-to-moderate levels in other tissues and **PPAR-γ2**.

PPAR-α regulate the expression of a broad range of hepatic genes encoding enzymes/proteins involved in fatty acid uptake and intracellular transport, fatty acid activation (acyl-CoA formation), fatty acid oxidation, lipogenesis, ketogenesis and lipoprotein/cholesterol metabolism. mRNA levels of PPAR-α strongly connect with mRNA levels of several genes involved in lipid metabolism in skeletal muscle such **as CD36, UCP-2, UCP-3, LPL and CPT-1.**[32] In ECs, PPAR-α agonists intrude with the metabolic processes involved in engage and adherence of inflammatory cells, and protect against vascular inflammation and injury. Moreover, PPAR-α agonists induce NOS expression and increase NO production in vascular ECs, suggesting a Vaso protective effect.

From various studies it is now that PPAR-β/δ regulates the transcription of genes involved in brown or white adipose tissue fatty acid transport, oxidation and thermogenesis**.**[33] GW501516 a potent PPAR-β/δ agonist, treatment caused a dramatic dose-dependent increase in plasma HDL-C and decreased plasma triglyceride, LDL-C and insulin levels.[34] PPAR -β/δ activation induced genes that participate in fatty acid oxidation in brown adipose tissue such as **CPT1, ACOX and LCAD** and thermogenesis/energy expenditure such as **UCP1** and **UCP3**. As a whole, these data suggest that PPAR-β/δ regulates adiposity by promoting fat combustion. Gene array analyses proposed that increased glucose metabolism via pentose phosphate pathway, which is to enhance de novo fatty acid synthesis (lipogenesis), may be one potential mechanism by which PPAR-β/δ ameliorates hyperglycaemia.[35]

PPAR-γ agonists have been shown to effectively lower elevated plasma free-fatty acid levels, ameliorate excessive lipid accumulation in peripheral tissues such as liver, skeletal muscle and heart and hyperinsulinemia/insulin resistance. It also modulates the expression of adipokines and inflammatory cytokines that impact hepatic and muscle metabolism and whole-body insulin sensitivity.[36]

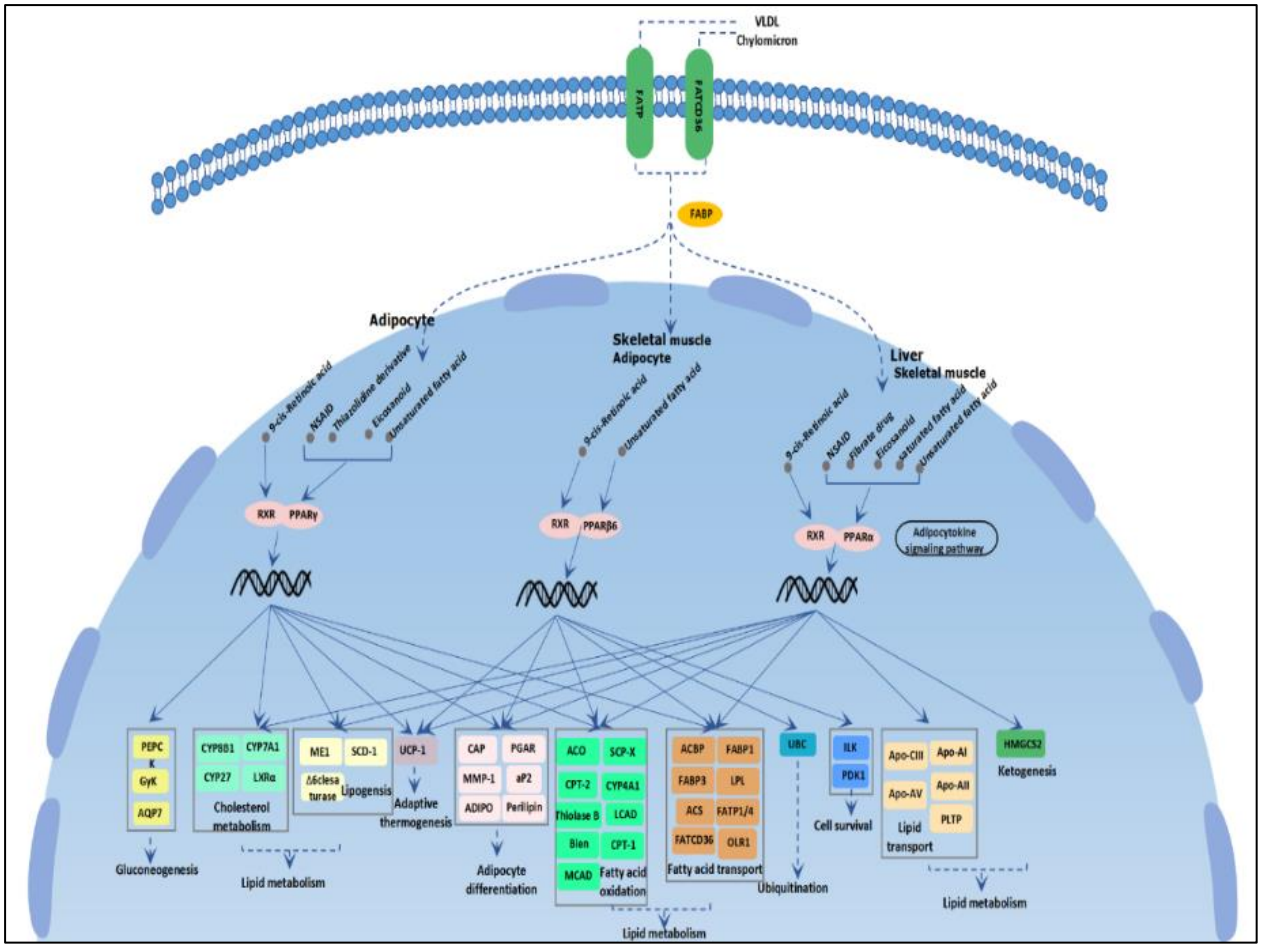

Figure 8. PPAR Action [37]

1.8 BAICALIN:

Baicalin (5,6-dihydroxy-2-phenyl-4H-1-benzopyran-4-one-7-O-D-β-glucuronic acid) is a flavonoid compound refined from the medicinal plant *Scutellaria baicalensis* and other plants of genus *Scutellaria*. The crops of Scutellaria plant species are mostly located in China, Korea, Japan, eastern Russia, Mongolia and Siberia. Baicalin and its aglycone, baicalein are the major flavonoid constituents in the plants of genus *Scutellaria* (Lamiaceae) namely *Scutellaria baicalensis Georgi* (SBG), *S. lateflora L., S. galericulata, and S. rivularia Wall* as well as in *Oroxylum indicum* (L.) *Kurz* (OI, Bignoniaceae). The roots of *Scutellaria baicalensis* are mostly used in Chinese medicines for prevention and treatment of type 2 diabetes, hyperlipidemia, atherosclerosis and hypertension.[38]

The antioxidant and anti-inflammatory effects of this flavonoid have been demonstrated in various disease models, including diabetes, cardiovascular diseases, inflammatory bowel diseases, gout and rheumatoid arthritis, asthma, neurodegenerative-, liver- and kidney diseases, encephalomyelitis, and carcinogenesis. Baicalin possesses antithrombotic activities.[39]

Baicalin produce direct anti-tumour effects on human prostate cancer cells.[40] Baicalin perform as a prooxidant and induces caspase-3 activation and apoptosis via mitochondrial pathway.[41]

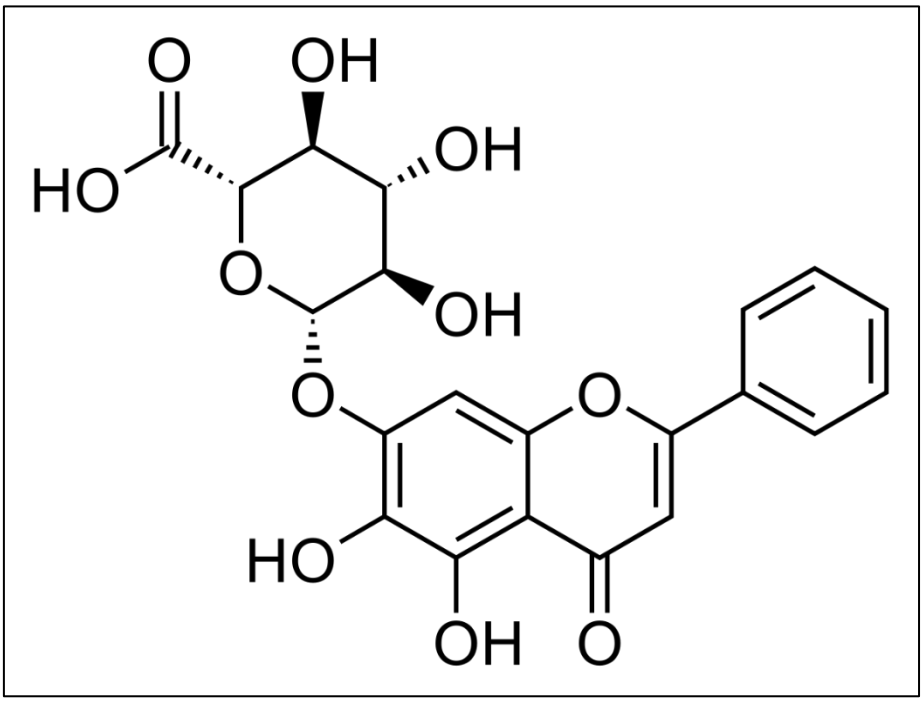

Figure 9. Structure of Baicalin

2.REVIEW OF LITERATURE

Review of literature

2.1 **Waisundara VY et al., (2008)** studied the combined effect of metformin and ethanolic extract of *Scutellaria Baicalensis* in STZ induced diabetic rats. Wistar rats weighing 200-250g are treated with STZ and the diabetic rats are grouped into 4 groups each containing 6 animals namely metformin 500 mg/kg, S. baicalensis 400 mg/kg, metformin 500 mg/kg + S. baicalensis extract 400 mg/kg and diabetic control. The rats treated with S. baicalensis and metformin + S. baicalensis had elevated hepatic activities of the antioxidant enzymes — superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) compared to the vehicle- and metformin treated diabetic groups. Plasma and hepatic lipid peroxide concentrations in the herb-treated and herb + metformin-treated groups were also significantly reduced. In addition, the combined treatment caused significant elevations of plasma and pancreatic insulin levels and reductions of plasma and hepatic triglycerides (TG) and cholesterol levels. The study thus showed that *S. baicalensis* enhanced the antidiabetic effect of metformin in STZ-induced diabetic rats by improving the antioxidant status. It also increased pancreatic insulin content as well as improved the lipid profile in these rats.[42]

2.2 **Zhang X et al., (2009)** investigated the effect of Baicalin on inflammatory mediator levels and in microcirculation disturbance in severe acute pancreatitis (SAP) rats and explore its therapeutic mechanism. Male Sprague Dawley rats weighing 200-250 g were randomised into 3 groups as diseases control, sham control operated with laparotomy and baicalin treatment group, each group consist of 45 rats which are subgroup as 3h, 6h, 12h each containing 15 rats. SAP is induced with 3.5% of sodium taurocholate. At 3, 6 and 12 hours after operation, rats were killed to examine the mortality rates of rats, the ascites volume and pancreatic pathological changes in each group, to determine the contents of amylase, PLA2, TXB2, PGE2, PAF and IL-1β in blood as well as the changes in blood viscosity. Baicalin exerts therapeutic action against SAP by inhibiting inflammatory mediator, lowering blood viscosity, and reducing pancreatic damage.[43]

2.3 **Vázquez-Carrera M et al., (2009)** The nuclear receptors Peroxisome Proliferator-Activated Receptors (PPAR) and PPAR are therapeutic targets for hypertriglyceridemia and insulin resistance, respectively. Evidence is now emerging

that the PPARβ isotype is a potential pharmacological target for the treatment of disorders associated with metabolic syndrome. PPARβ activation increases lipid catabolism in skeletal muscle, heart and adipose tissue and improves the serum lipid profile and insulin sensitivity in several animal models. In addition, PPARβ ligands prevent weight gain and suppress macrophage-derived inflammation. The data indicate that PPARβ ligands may become a therapeutic option for the treatment of metabolic syndrome.[44]

2.4 **Serrano-Marco L et al., (2011)** examined whether the peroxisome proliferator activated receptor (PPAR)β/δ agonist GW501516 prevented activation of the IL-6–STAT3–suppressor of cytokine signalling 3 (SOCS3) pathway and insulin resistance in human hepatic HepG2 cells. Studies were conducted with human HepG2 cells and livers from mice null for PPPAR β and wild-type mice. GW501516 prevented IL-6-dependent reduction in insulin-stimulated v-akt murine thymoma viral oncogene homologue 1 (AKT) phosphorylation and in IRS-1 and IRS-2 protein levels. In addition, treatment with this drug abolished IL-6-induced STAT3 phosphorylation of Tyr705 and Ser727 and prevented the increase in SOCS3 caused by this cytokine. Moreover, GW501516 prevented IL-6-dependent induction of extracellular-related kinase 1/2 (ERK1/2), involved in serine STAT3 phosphorylation; the livers of PPARβ/δ-null mice showed increased Tyr705- and Ser727-STAT3 as well as phospho-ERK1/2 levels. Furthermore, drug treatment prevented the IL-6-dependent reduction in phosphorylated AMP-activated protein kinase (AMPK), a kinase reported to inhibit STAT3 phosphorylation on Tyr705. The PPARβ/δ activator GW501516 prevents IL-6-induced STAT3 activation by inhibiting ERK1/2 phosphorylation and preventing the reduction in phospho-AMPK levels. These effects of GW501516 may contribute to the prevention of cytokine-induced insulin resistance in hepatic cells.[45]

2.5 **Wilson RD & Islam MS (2012)** Developed an alternative non-genetic rat model for type 2 diabetes (T2D). Six-week-old male Sprague-Dawley rats (190.56 ± 23.60 g) were randomised into six groups, namely: Normal Control (NC), Diabetic Control (DBC), Fructose-10 (FR10), Fructose-20 (FR20), Fructose-30 (FR30) and Fructose-40 (FR40). The NC and DBC control groups receive normal drinking water while the fructose groups receive 10, 20 30, and 40 % fructose respectively, all groups were fed with normal diet ad libitum. After two weeks, all groups except the NC group received a single injection (i.p.) of streptozotocin (STZ) (40 mg/kg b.w.) dissolved in

citrate buffer (pH 4.4). The NC group received only a vehicle buffer injection (i.p). One week after the STZ injection, animals with non-fasting blood glucose levels > 300 mg/dl were considered as diabetic. This animal model shows Insulin resistance and partial pancreatic beta cell damage similar to that of the T2DM patients which is the characteristic of the disease. Thus, Fructose-10 and STZ (40mg/kg) model can be used as a new experimental model for T2DM. [46]

2.6 **Lim HA et al., (2012)** investigated the anti-inflammatory action of baicalin, which depends on its ability to activate PPAR gamma, and subsequently to suppress NF-κB. They examined in baicalin-treated kidney tissue from 24-month-old Fischer 344 aged rats (10 or 20 mg/kg/day for 10 days) and baicalin-fed mice (10 mg/kg/day for 3 days) for in vivo investigations, and YPEN-1 cells for in vitro studies. In the baicalin- fed aged rats, enhancement of both nuclear protein levels and DNA binding activity of PPAR gamma, and a decreased expression of NF-κ B target genes (VCAM-1, IL-1b, and IL-6) compared with non-baicalin-fed aged rats were observed. Furthermore, to confirm the anti-inflammatory action of PPAR γ activated by baicalin, we used lipopolysaccharide (LPS)-treated cells and mice. The results showed that baicalin induced PPAR γ-selective activation in YPEN-1 cells, and that the effects of baicalin were blocked by the PPAR γ receptor antagonist, GW9662. In addition, baicalin treatment prevented RS generation, NF-κ B activation and the expression of proinflammatory genes, whereas it increased PPAR γ expression in LPS-treated cells and mouse kidney. Our data suggest that baicalin-induced PPAR γ expression reduced age-related inflammation through blocking pro-inflammatory NF-κ B activation. These results indicate that baicalin is a novel PPAR γ activator and that this agent may have the potential to minimize inflammation. [47]

2.7 **Chung HY et al., (2012)** attempted to elucidate the molecular modulation of antioxidant baicalin on the insulin induced FoxO1 inactivation. They used HEK293T cultured cells and kidney tissue isolated from 24-month-old Fischer rats treated with baicalin at a dose of 10 or 20 mg/kg/day for 10 days. Baicalin enhanced catalase and suppressed RS production in cell system and in isolated kidney tissue in contrast to the nontreated aged rats. Results also showed activation of insulin signaling (PI3K/Akt), FoxO1 phosphorylation/acetylation and the downregulation of catalase and manganese superoxide dismutase, both of which are FoxO1-targeting genes. Furthermore, baicalin-treated rats showed a decreased FoxO1 phosphorylation via

PI3K/Akt cascade and FoxO1 acetylation by the cAMP-response element-binding protein binding protein (CBP). These results strongly suggest that treatment with baicalin influenced phosphorylation/acetylation of FoxO1 by up-regulating PI3K/Akt signalling through insulin in aged rats. Our results further reveal that baicalin regulated FoxO1 phosphorylation via PI3K/Akt by insulin and FoxO1 acetylation by the interaction of CBP and SIRT1, leading to changes in catalase gene expression during aging.[48]

2.8 **Luo Z et al., (2013)** investigated the effects of telmisartan on insulin signaling and glucose uptake in cultured myotubes and skeletal muscle from wild-type and muscle-specific peroxisome proliferator–activated receptor (PPAR) delta knockout (MCKPPARd2/2) mice. Telmisartan increased PPARβ expression and activated PPAR β transcriptional activity in cultured C2C12 myotubes. In palmitate-induced insulin-resistant C2C12 myotubes, telmisartan enhanced insulin-stimulated Akt and Akt substrate of 160 kDa (AS160) phosphorylation as well as Glut4 translocation to the plasma membrane. These effects were inhibited by antagonizing PPARβ or phosphatidylinositol-3 kinase, but not by PPAR γ and PPARα inhibition. Palmitate reducing the insulin stimulated glucose uptake in C2C12 myotubes could be restored by telmisartan. In vivo experiments showed that telmisartan treatment reversed high-fat diet–induced insulin resistance and glucose intolerance in wild-type mice but not in MCK-PPARd2/2 mice. The protein levels of PPARβ, phospho-Akt, phospho-AS160, and Glut4 translocation to the plasma membrane in the skeletal muscle on insulin stimulation were reduced by high-fat diet and were restored by telmisartan administration in wild-type mice. These effects were absent in MCK-PPARd2/2 mice. These findings implicate PPARβ as a potential therapeutic target in the treatment of hypertensive subjects with insulin resistance.[49]

2.9 **Fang P et al., (2017)** studied whether baicalin ameliorate obesity-induced insulin resistance and to explore its signal mechanisms in skeletal muscles of mice. Diet-induced obese (DIO) mice were given 50mg/kg baicalin intraperitoneally (i.p.) once a day for 21 days, and C2C12 myotubes were treated with 100, 200, 400 μM baicalin for 12 h in this study. They discovered that administration of baicalin decreased food intake, body weight, HOMA-IR and NT-PGC-1α levels, but enhanced GLUT4, PGC-1α, pP38MAPK, pAKT and pAS160 contents, as well as GLUT4 mRNA, PGC-1α mRNA, PPAR γ mRNA, GLUT1 mRNA expression in skeletal muscles of obese

mice and myotubes of C2C12 cells, and reversed high fat diet-induced glucose and insulin intolerance, hyperglycemia and insulin resistance in the mice. These results suggest that baicalin is a powerful and promising agent for treatment of obesity and insulin resistance via Akt/AS160/GLUT4and P38MAPK/ PGC1α/ GLUT4 pathway.[50]

2.10 **Kumar DP et al., (2020)** tested the hypothesis that saroglitazar, a PPAR α/γ agonist would improve NASH in the diet-induced animal model of NAFLD. Mice received chow diet and normal water (CDNW) or high fat western diet and ad lib sugar water (WDSW). After 12 weeks, WDSW fed mice were randomized to receive (1) WDSW alone, (2) WDSW+vehicle, (3) WDSW+pioglitazone or (4) WDSW+saroglitazar for an additional 12 weeks. Compared to mice on WDSW and vehicle controls, mice receiving WDSW + saroglitazar had lower weight, lower HOMA-IR, triglycerides, total cholesterol, and ALT. Saroglitazar improved steatosis, lobular inflammation, hepatocellular ballooning and fibrosis stage. Transcriptomic analysis confirmed increased PPAR target expression and an anti-inflammatory effect with saroglitazar. Lipidomic analyses demonstrated that saroglitazar also reduced triglycerides, diglycerides, sphingomyelins and ceramides. These preclinical data provide a strong rationale for developing saroglitazar for the treatment of NASH in humans.[51]

3. AIM AND OBJECTIVE

AIM AND OBJECTIVE:

Aim:

- Assessing Baicalin for amelioration of insulin resistance via activation of PPAR.

Objective:

- To find PPAR agonist through in-silico studies.
- To induce T2DM by high fructose STZ model.
- To assess change in the body weight, Food & Water intake, blood glucose level during the experimental period.
- To evaluate changes in the lipoprotein level in serum.
- Histopathological studies in liver, pancreas and adipose tissues.
- To elucidate the mechanism of Baicalin for its anti-diabetic activity.

4. PLAN OF THE STUDY

PLAN OF THE STUDY

Phase I

- Phytoconstituents structures are selected for *in silico* docking studies
- Phytoconstituents are screened through molecular docking to find a potential PPAR agonist.
- Phytoconstituents are selected based on docking score and MMGBSA was done.
- One or two phytoconstituent were selected and most stable compound is found by MD.

Phase II

- Optimization of Fructose- STZ diabetic model.
- Diabetic animals are grouped as standard group, low dose baicalin and high dose baicalin treatment and treated for 2-4 weeks.
- Assess body weight, food and water intake, and blood glucose level weekly.
- Euthanasia by high dose Ketamine and Xylazine.
- Blood was collected by cardiac puncture.
- Liver, pancreas and adipose tissue are preserved.

Phase III

- Biochemical analysis of samples for serum lipid level.
- H&E staining of liver, pancreas and adipose tissue.

Phase IV

- Collection of data
- Statistical analysis

5. MATERIALS AND METHODS

5.MATERIAL AND METHODS

5.1. Reagents and consumables

Table 1: Chemicals used and its details

Item Description	Vendor
Fructose	Gem health care
Streptozotocin	Hi-media
Pioglar	Sun pharmaceuticals
Triglyceradies (S.L)	Agappe
Cholesterol (S.L)	Agappe
HDL	Agappe

5.2 Equipment and Software:

Table 2: Equipment used and its details

Equipment and Software	Manufacturer & Details
Schrodinger	Schrodinger, LLC, Newyork, NY,2019-1
Gluco One	Morepen laboratories
Centrifuge	Remi R-8C

5.3 METHODOLOGY

5.3.1 *IN SILICO* STUDIES

a) Protein preparation and structure validation

The X-ray crystal structure of PPARα (PDB code: 2ZNN), PPARβ (PDB code:2ZNQ), PPARγ (PDB code:5U5L) complexed with agonist ligand were extracted from protein data base, RCSB, with resolution of 2.01Å, 2.65Å, 2.55Å Respectively. Proteins were prepared by protein preparation wizard of Schrodinger suite (Schrodinger, LLC, New York, NY, 2019-1). [52] It involves three processes, import and process, review and modify, and refine. In first step, the protein was pre-processed, in which the bond orders were aligned, hydrogen atoms were added which it lacks. Followed by proper coordinates for missing side chains and missing loops proteins were generated. All water molecules beyond 5° A from co-crystallized ligand were removed and below 5° A from co-crystallized ligand were kept to ensure the presence of water for ligand interactions. As, with the LigPrep, Epik v4.0 was used to generate possible ionization and tautomeric states for all het groups at pH 7.0 ± 2.0. In the second step, the unwanted structures in the PPARα, PPARβ, PPARγ proteins were removed. In the last step, optimization of hydrogen bonding network was carried out by reorienting hydroxyl and thiol groups, water molecules, amide groups of asparagines (Asn) and glutamine (Gln), and the imidazole ring in histidine (His). Protonation states of histidine, asparagines, and glutamine were also predicted in order to optimize the hydrogen bonding network. Finally, the energy minimization process was carried to restrain the heavy atoms. A RMSD of 0.30Aº was selected for the atom displacement in order to terminate the minimization step. OPLS3e was used for energy minimization for the refinement of PPARα, PPARβ, PPARγ receptors.

b) Receptor grid generation

Once the protein preparation was completed, receptor grid was generated by specifying active binding sites using OPLS3e force field by by using Receptor Grid Generation Panel in GLIDE (Schrodinger LLC, New York, NY, 2019-1). It allows defining the receptor structure and determining the position, size of the active site. Ligand docking was carried out after grid generation. Options in receptor grid generation panel gives idea about receptor structure by excluding co-crystallized ligand. Panel includes five tabs: receptor tab which gives information about which receptor grid to be taken, Van der Waals radii scaling, per atom Van der Waal radius and charge scaling. For non-polar atoms a Van der Waal radius of 1.0Aº and the default is 0.25 Aº were used. The site tab determines where the scoring grids are arranged, GLIDE constraint tab consists of six types of constraints, positional, NOE (nuclear over Hauser effect),

hydrogen bond, metal, meta co-ordination and hydrophobic. A maximum of ten constraints can be used for a given grid. A grid of 20 Aº was created inside the selected co-crystallized co-ordinates of PPARα, PPARβ, PPARγ. In this both internal and external receptor grid boxes were generated using co-crystal ligands bound to proteins. With this Receptor grid generation, co-crystal ligand was excluded from P-ligand interactions and favours the further molecular docking of ligand library.

c) Ligand preparation

During ligand preparation, the structure of phytoconstituents that are proved to have anti diabetic activity and their similar structures were retrieved as 2D Format and incorporated into ligand preparation using lig prep software pack of glide v7.7 (Schrodinger LLC, New York, NY,2019-1). The refinement process was performed as converting 2D into minimum 3D molecular structure using OPLS3e force field. Structures with tacit hydrogen atom were rectified before the 3D structure minimization step. By default, counter ions in salts and water molecules were removed, charged groups were also neutralized by adding or removing hydrogen ions. Ionization states were generated at pH 7.0 ± 2.0. As with ionization, tautomeric states were generated using Epik. Input structures given in 2D often associated with indicated chiralities. This chirality information was discarded from chirality properties in the input file and from the 3D geometry, and internally generates all possible configurations that results from the combination of chiralities on each chiral centre. Maximum of 50 stereoisomers were generated for each ligand. A relative energy window of 10KJ/mol and minimum 1 Aº of atomic deviation were kept to filter every minimized conformer.

d) Molecular Docking

Once the above computer simulation was completed, a powerful prediction environment was created for ligand-receptor docking. GLIDE v6.2 was used for ligand-receptor docking study.[53] All the ligands were docked within the grid generated area of protein structure of PPARα, PPARβ, PPARγ. The SP, XP mode of docking was done to scrutinize the hits and core setting constraints, torsional constraints were kept default. After docking, top compounds are selected based on G- score, docking score and nature of binding interactions were subjected using GLIDE v7.7 of Schrodinger suite (Schrodinger LLC, New York, NY,2019-1). Glide uses the formula below to calculate the docking score.

Gscore= 0.05 vdW + 0.15 Coul + lipo + Hbond + Metal + Reward + RotB + Site

Where vdW, coul, Lipo, H bond, Metal, Reward, RotB and the site denote van der Waals energy, Coulomb energy, lipophilic contacts, hydrogen bonding, metal- binding, penalty for buried polar groups, penalty for freezing the rotatable bonds, and polar interactions with the residues in the active site, respectively. The docking score consists of a sum of the Glide Score, measured from the SP or XP scoring functions and used for comparing different ligands.

e) Molecular mechanics (MMGBSA)

The relative binding energy of selected agonists was calculated from structural information in bimolecular complexes using Prime module version 4.2 of Schrödinger software.[54] Prime MM-GBSA (Molecular Mechanics, the Generalized Born model and Solvent Accessibility) is a physics-based method that computes the force field energies in implicit solvent of the bound and unbound molecules involved in the binding process. The MM-GBSA binding energy includes terms such as protein-ligand van der waals contacts, electrostatic interactions, ligand desolvation, and internal strain (ligand and protein) energies. Prime calculates binding energies using VSGB2.0 implicit solvent model with the OPLS3 force field. The collective hits obtained from docking on all three targets were subjected to the MM-GBSA analysis individually. During these calculations, the active site of both receptors was set to adjust itself up to a distance of 5 Å for ligand accordingly. These operation imports XP docking "out. maegz" file of protein and ligand complex, which results in ranking of the ligands based on calculated binding energies. The binding energy will be calculated based on the following equations.

$$\Delta G = E_{\text{Complex (Minimized)}} - [\, E_{\text{Ligand (Minimized)}}]$$

For MM-GBSA calculations, all the protein atoms were kept rigid while relaxing the atoms of the compounds

f) **Molecular dynamics**

The molecular dynamic simulations are carried out using **DESMOND v5.2**, a module of Maestro v11.4 (Schrödinger, LLC, New York, NY, 2019-1). Molecular dynamics simulations are fundamental computational tool that analyse complex stability ligand induced changes of protein active site, RMSD and interaction of selected ligands. During simulation, the atoms of Protein-ligand complex were incorporated in an orthorhombic stimulation box solvate with TIP3P (Transferable inter molecules potential with 3pts) explicit water model. The overall change was neutralized by adding counter ions and also salt concentrated of 0.15m NaCl of 20°A away from ligand to mimic the iso-osmotic environment. The system was

assisted using OPLS3e force field, subjecting to molecular dynamics simulation for 100ns using NPT ensemble at 300k temperature and 1.01325 atmospheric pressure for small molecule interactions. During simulation time, Protein-ligand complex was recorded as trajectory frames at 100ps and total of 1000 frames were saved. Finally molecular dynamics were analysed using simulation interaction diagram tool panel.[55]

5.3.2 *IN VIVO* STUDIES

a) Animal Procurement:

The Sprague Dawley rats were supplied by PSG Institutional Animal house facility after obtaining ethical approval (Approval number: 500/IAEC/2021). 7 days before the experimentation procedures animals were acclimatized. Animals were allowed free access to food and water ad libitum. After Acclimatization, the animals were subjected to the induction of Diabetes.

b) Induction of Diabetes:

Six-week-old Sprague Dawley rats of body weight between 200-250g of both sexes are used in this study. A total number of 30 rats are used. The animals are grouped into five groups each containing 6 rats. Animals were housed in polycarbonate cages as four rats per cage with wood chip bedding, and maintained in an air-conditioned animal room (temperature: 24°C, relative humidity: 55% ± 5%) with a 12 h light/dark cycle. The rats were fed a commercially available rat pellet diet ad libitum throughout the experimental period. Control groups were supplied with normal drinking water ad libitum while other groups were supplied with 10% fructose solution ad libitum for the initial 2 weeks only, then supplied with normal drinking water during the remaining period of the experiment.

STZ was dissolved in a citrate buffer (pH 4.4). All animals were fasted overnight and each of the fructose-fed animal groups were injected (i.p) with a low dose STZ (40 mg/kg b.w) whilst the animals in NC group were injected with vehicle buffer only.[46]

c) Drug Treatment:

The animal in the Group III receives the standard drug pioglitazone (20mg/kg/day) orally [56] Group IV and V receive treatment of baicalin of dose 80mg/kg/day [57] and 120 mg/ kg/day [58] for the treatment period of 28 days through intraperitoneal route. Fasting blood glucose test was done on 1, 7, 14, 21 days for treatment period and OGTT was performed at 28 days. At the end of 28 days treatment the animal was sacrificed by high dose of Ketamine

and Xylazine. Blood was collected from cardiac puncture. The serum was stored at -20°C for further biochemical analysis. The liver, pancreas and adipose tissue were isolated for further studies.

Table 3: Animal Grouping and Treatment

GROUPS	TREATMENT	NO. OF ANIMALS
I	Control(saline)	6
II	Negative control (10% fructose + STZ 40mg/kg, i.p)	6
III	Standard group (pioglitazone20mg/kg, Orally)	6
IV	Low dose baicalin (80mg/kg, i.p)	6
V	High dose baicalin (120mg/kg, i.p)	6

d) Random blood glucose level:

The Random Blood Glucose level of rats in each group were measured on 1, 7, 14, 21 day of the treatment period using glucometer.[46]

e) **Oral glucose tolerance test (OGTT)**

OGTT was performed in the week 6 of the experimental period. After an overnight fast (12 h), rats were orally dosed with a D-glucose solution (2.0 g/kg b.w.) and blood glucose concentrations were subsequently measured at 0 (just prior to oral glucose dosing), 30, 60, 90, and 120 min after the oral dosing of glucose. [46]

f) Food and fluid intake, Body weight changes:

Daily food and fluid intake and weekly body weight changes were measured during the entire experimental period. [46]

Biochemical parameters analysis:

5.4.1 Determination of serum Cholesterol Level:

The cholesterol level of samples received from each group is analysed by Semi Auto analyser using Agappe kit.

Table 4: Reagents used for Cholesterol analysis

	Blank	Standard	Sample
Working reagent	1000µL	1000µL	1000µL
Standard	-	10 µL	-
Sample	-	-	10 µL

Mix and incubate for 5mins at 37°C. Measure the absorbance of sample and standard against reagent blank.

Calculation:

Cholesterol Conc(mg/dL) = Absorbance of sample/Absorbance of standard × 200

5.4.2 Determination on serum Triglyceride level:

The Triglyceride level of samples received from each group is analysed by Semi Auto analyser using Agappe kit.

Table 5: Reagents used for TAG analysis

	Blank	Standard	Sample
Working reagent	1000µL	1000µL	1000µL
Standard	-	10 µL	-
Sample	-	-	10 µL

Mix and incubate for 5mins at 37°C. Measure the absorbance of sample and standard against reagent blank.

Calculation:

Triglycerides Conc (mg/dL) = Absorbance of sample/Absorbance of standard × 200

5.4.3 Determination on serum HDL level:

The Triglyceride level of samples received from each group is analysed by Semi Auto analyser using Agappe kit.

Table 6: Reagents used for HDL analysis

	Blank	Standard	Sample
Working reagent	1000µL	1000µL	1000µL
Standard	-	10 µL	-
Sample	-	-	10 µL

Mix and incubate for 5mins at 37°C. Measure the absorbance of sample and standard against reagent blank.

Calculation:

HDL Conc (mg/dL) = Absorbance of sample/Absorbance of standard × 200

5.4.4 Calculation for VLDL and LDL Level:

Serum very low-density lipoprotein cholesterol (VLDL-C) and low-density lipoprotein cholesterol (LDL-C) concentration were calculated according to **Friedewald et al. 1972.**[59]

LDL−C conc(mg/dl) = Total cholesterol – (HDL +TAG/5)

VLDL−C conc(mg/dl) = TAG/5

5.5 Histopathological studies:

The formalin preserved pancreatic tissue, adipose tissues and liver were treated according to a standard laboratory protocol for paraffin embedding. Sections were cut at a size of 3 μm. Then, slides were deparaffinized in p-xylene and rehydrated in changes of ethanol (100%, 90%, 80%, 70%, 50%) and rinsed in water. Slides were stained in hematoxylin for 5 min and rinsed with water. Slides were counterstained in eosin, mounted in DPX, cover-slipped and viewed under a light microscope connected to a computer.[46]

6.RESULT

6. RESULTS

6.1 *IN SILICO* STUDIES

6.1.1 Protein identification:

Protein structures of PPAR and its various isoforms PPARα, PPARβ, PPARγ were downloaded from protein data bank. The three dimensional X-ray crystal structure of PPARα (PDB ID:2ZNN), PPARβ (PDB ID:2ZNQ), PPARγ(PDB ID: 5U5L) with co-crystallised native agonist ligand and having resolution of 2.01Å, 2.65Å, 2.55Å respectively were identified and used for docking studies. The ligand binding site identified were selected as a flexible residue for molecular docking studies.

6.1.2 Molecular docking studies:

For screening through molecular docking studies 417 Ligand structures were obtained from PubChem which are proven to be anti-diabetic and their similar structures. Ligprep was done with GLIDE v7.7 and 4568 ligands conformers were created. The ligand structures were docked with PPARα in the SP(standard precision)mode and 719 were screened for PPARα agonist activity. These 719 compounds were screened for PPARβ agonist activity from which 578 were docked with better docking scores. Then these 578 compounds were docked with PPARγ and 257 compounds were screened.

Top 20 compounds were selected for each protein based on their docking score and interactions. Then the compounds which were common for all three proteins were selected.

From the selected compounds Baicalin is the only compound that shows better docking score and interaction with all three isoforms of PPAR (PPARα, PPARβ, PPARγ).

Table No: 7 Docking score and G Score of Baicalin with PPAR Isoforms

Protein	**PPARα**		**PPARβ**		**PPARγ**	
Ligand	**D Score**	**G Score**	**D Score**	**G Score**	**D Score**	**G Score**
64982(Baicalin)	-8.238	-8.245	-9.072	-9.079	-12.435	-12.442
Pioglitazone	-7.534	-7.944	-10.513	-10.953	-11.570	-12.053

6.1.3 Molecular dynamics simulation:

The preferrable hit that have been finalised from selectivity evaluation was assigned for MD to obtain stability facts over the given particular period. The Root Mean Square Deviation (RMSD) is used to measure the average change in displacement of a selection of atoms for a particular frame with respect to a reference frame. It is calculated for all frames in the trajectory. The RMSD for frame *x* is:

$$RMSD_x = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(r_i'(t_x)) - r_i(t_{ref}))^2}$$

where *N* is the number of atoms in the atom selection; *tref* is the reference time, (typically the first frame is used as the reference and it is regarded as time *t*=0); and *r'* is the position of the selected atoms in frame *x* after superimposing on the reference frame, where frame *x* is recorded at time *tx*. The procedure is repeated for every frame in the simulation trajectory.

Protein RMSD: The RMSD plot shows the RMSD evolution of a protein in Y axis(left *Y*-axis). All protein frames are first aligned on the reference frame backbone, and then the RMSD is calculated based on the atom selection. Monitoring the RMSD of the protein can give insights into its structural conformation throughout the simulation. RMSD analysis can indicate if the simulation has equilibrated, its fluctuations towards the end of the simulation are around some thermal average structure. Changes of the order of 1-3 Å are perfectly acceptable for small, globular proteins. Changes much larger than that, however, indicate that the protein is undergoing a large conformational change during the simulation. It is also important that your simulation converges the RMSD values stabilize around a fixed value. If the RMSD of the protein is still increasing or decreasing on average at the end of the simulation, then your system has not equilibrated, and your simulation may not be long enough for rigorous analysis.

Ligand RMSD: Ligand RMSD (right *Y*-axis) indicates how stable the ligand is with respect to the protein and its binding pocket. In the above plot, 'Lig fit Prot' shows the RMSD of a ligand when the protein-ligand complex is first aligned on the protein backbone of the reference and then the RMSD of the ligand heavy atoms is measured. If the values observed are significantly larger than the RMSD of the protein, then it is likely that the ligand has diffused away from its initial binding site.

LP Contacts: Protein interactions with the ligand can be monitored throughout the simulation. These interactions can be categorized. Protein-ligand interactions (or 'contacts') are categorized into four types: Hydrogen Bonds, Hydrophobic, Ionic and Water Bridges.

The PL-RMSD value of 64982 (Baicalin) and 2ZNN(PPARα) ranges between 2.1-4.2 Å throughout the simulation as well as careers essential interaction in which O-LYS358, OH-LYS358 accounts for 96% and 55% of hydrogen bonds respectively and H2O-GLN has 34% water bridge interaction

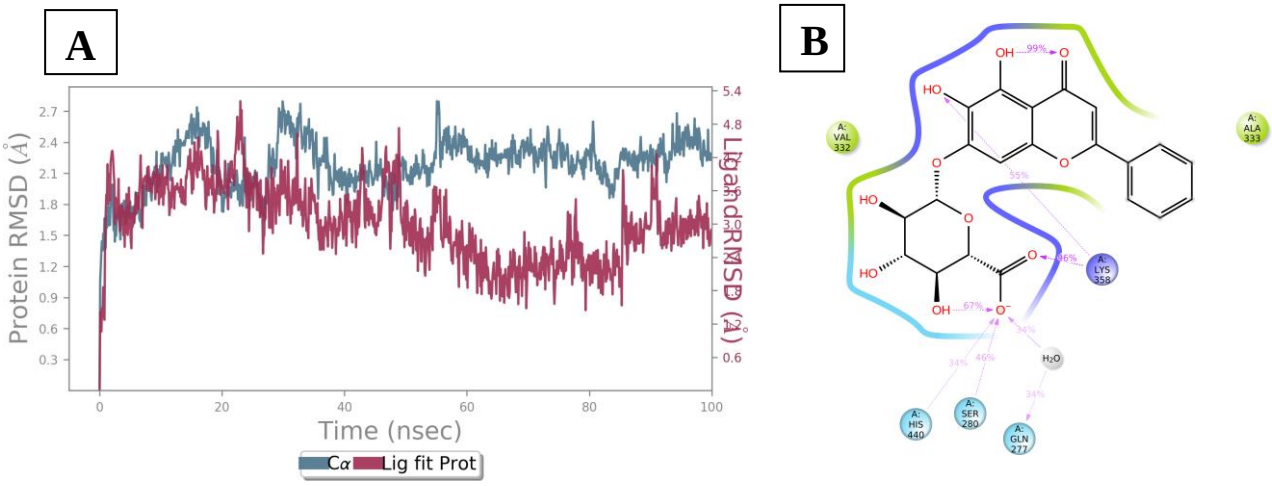

Figure 10: A. Protein- Ligand RMSD graph of baicalin with 2ZNN(PPARα) B. Ligand ineraction diagram of Baicalin and 2ZNN(PPARα)

The PL-RMSD value of 64982(Baicalin) and 2ZNQ(PPARβ) ranges 3-5 Å in 0-50ns of the simulation period and ASN227-OH, MET228-O, THR288-O contributes 93%, 99% and 75% of hydrogen bond interaction. ASN343-H2O, ARG284, TRP264, GLU291-O accounts 59%, 53%, 34%, 35% of water bridge interaction.

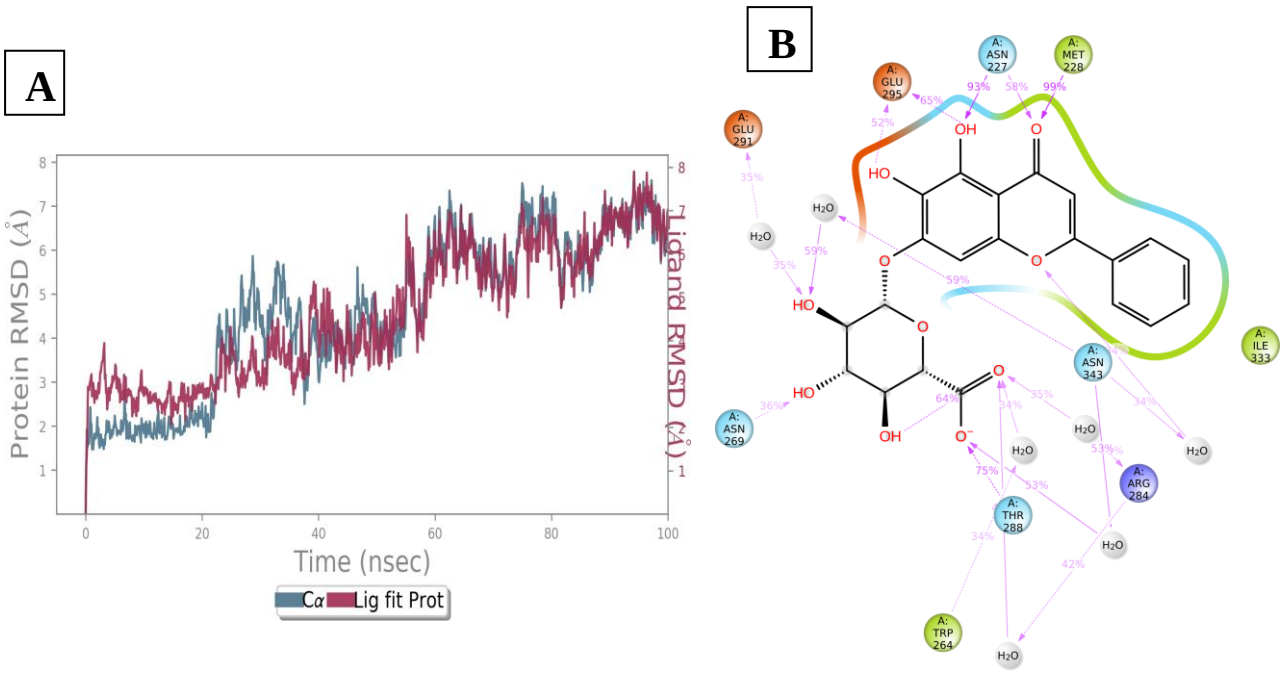

Figure 11: A. Protein- Ligand RMSD graph of baicalin with 2ZNQ(PPAR*β*)
B. Ligand ineraction diagram of Baicalin and 2ZNQ(PPAR*β*)

The PL-RMSD value of 64982(Baicalin) and 5U5L(PPARγ) ranges between 2-2.4 Å during simulation. SER289-O, TYR473-O accounts 98%, 76% of hydrogen bond interaction. ARG288-O, TYR327-OH, LYS367-OH Contributes for 86%, 60% and 56% of water bridge interaction.

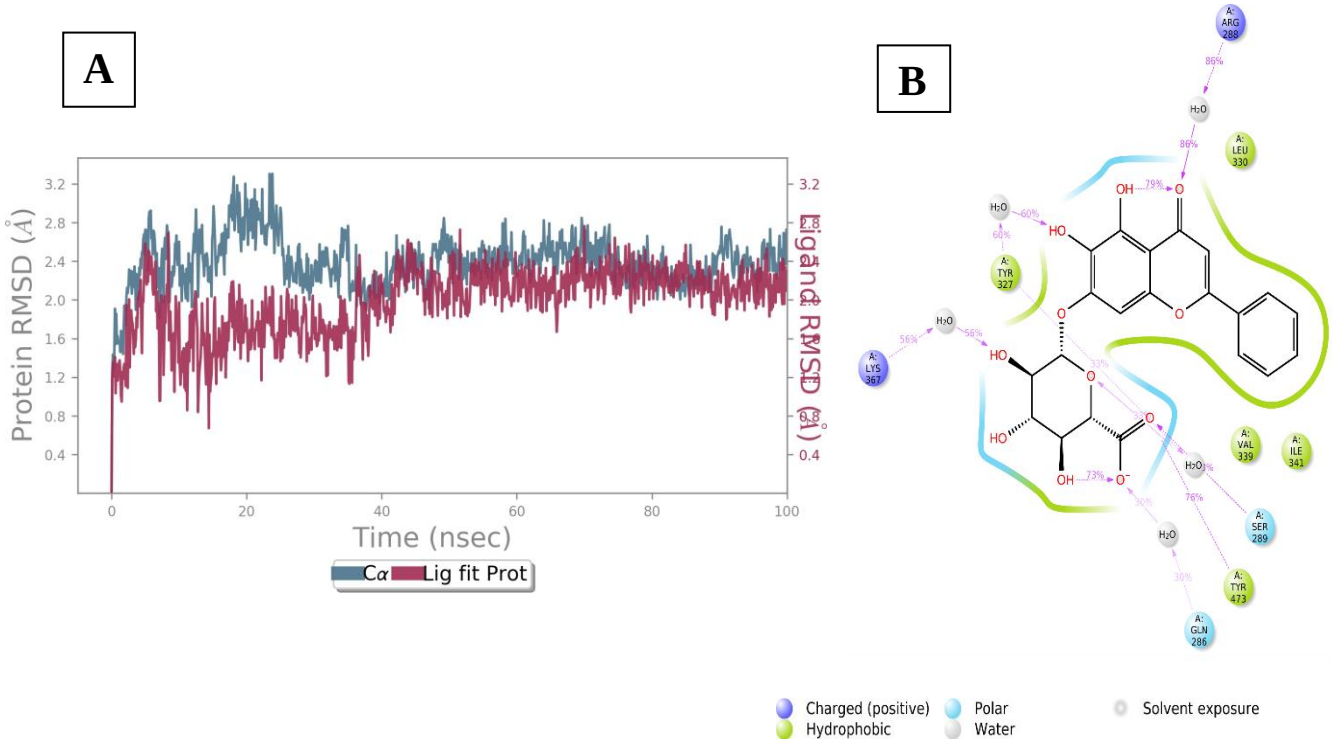

Figure 12 A. Protein- Ligand RMSD graph of baicalin and 5U5L(PPARγ) B. Ligand ineraction diagram of Baicalin with 5U5L(PPARγ)

6.1.4 MMGBSA

Further validation of the selected hit molecule(64982) was done by carrying out the Molecular mechanics study using Prime v5. Here, numerous energy properties were generated including binding free energy (MM-GBSA Dg bind). These properties were in accordance with a ligand, receptor and complex structures as well as energy differences relating to strain and binding, and are broken down into contributions from various terms in the energy expression. The ligand and the receptor must be properly prepared beforehand, for example by using Ligprep and the protein preparation Wizard. Water and ions must be removed from the protein, as an implicit solvent model is used. The ligands must be pre-positioned to the receptor, and the receptor must be prepared as for a Prime refinement calculation.

The Hit molecule(64982) has the MMGBSA dG bind value of -20.79 with 2ZNN(PPAR α), -33.86 with 2ZNQ(PPARβ) and -40.77(PPARγ).

TABLE NO: 8 MMGBSA dG Bind of BAICALIN and PPAR isoforms

Protein-ligand	MMGBSA dG Bind
2ZNN (64982)	-20.79
2ZNQ (64982)	-33.86
5U5L (64982)	-40.77

6.2.1 Change in weekly Food and Water intake:

The daily Food and Water intake was measured during the drug treatment period. The **Figure 13 and 14** shows the change in weekly Food and water intake over a period of 4 weeks. There is no significant difference between the groups for Food and Water intake level.

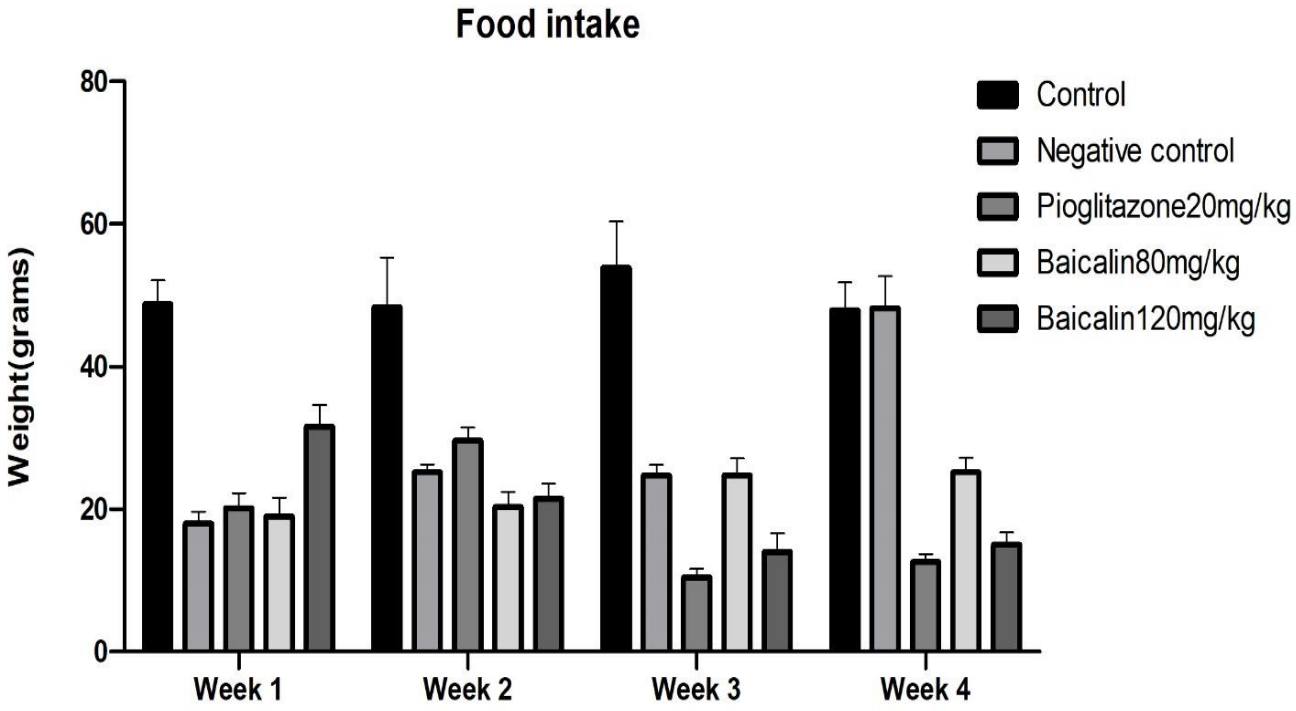

Figure 13: Changes in daily food intake of Control, Negative control and treatment groups. Data's are expressed as mean± SD. Statistics were done by Two-way ANOVA and post hoc analysis were done by Tukey's method. a,b,c show significance of p<0.05,p<0.01,p<0.001 with control group and x,y,z shows significant of p<0.05, p<0.01,p<0.001 with Negative control group

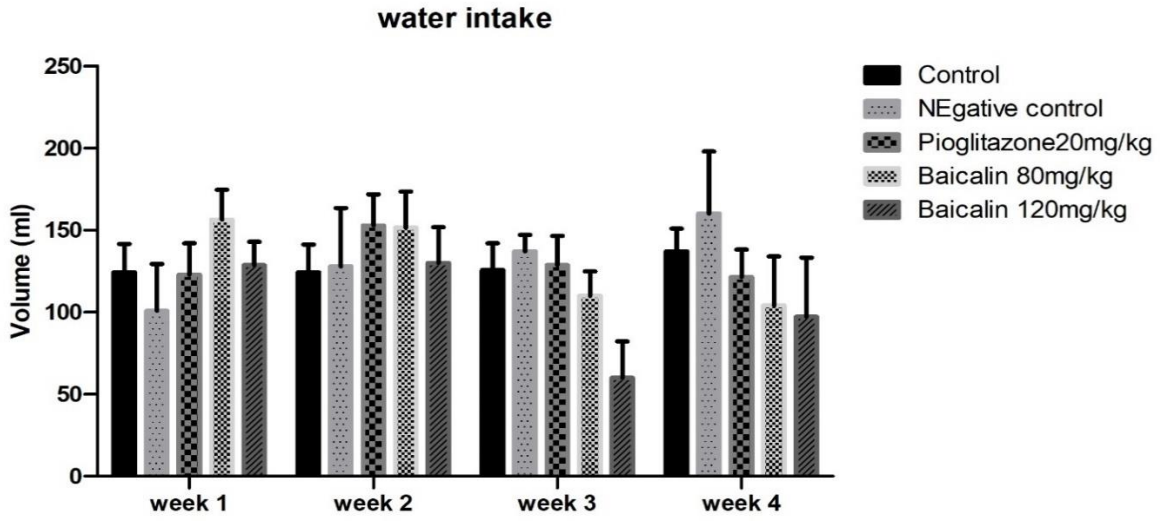

Figure 14: Changes in daily water intake of Control, Negative control and treatment groups. Datas are expressed as mean± SD. Statistics were done by Two-way ANOVA and post hoc analysis were done by Tukey's method. a,b,c show significance of p<0.05,p<0.01,p<0.001 with control group and x,y,z shows significant of p<0.05, p<0.01,p<0.001 with Negative control group

6.2.2 Change in Body weight:

Weekly body weight change was measured during the entire experimental period. Th**e** **Figure 15** show the weekly change in body weight. When control group compared to negative control group, Pioglitazone(mg/kg), low dose Baicalin (80mg/kg), High dose baicalin(120mg/kg) shows significant difference p(<0.001) throughout the study period. When Negative control group compared to Pioglitazone(20mg/kg), low dose Baicalin (80mg/kg), High dose baicalin(120mg/kg) does not shows significant difference.

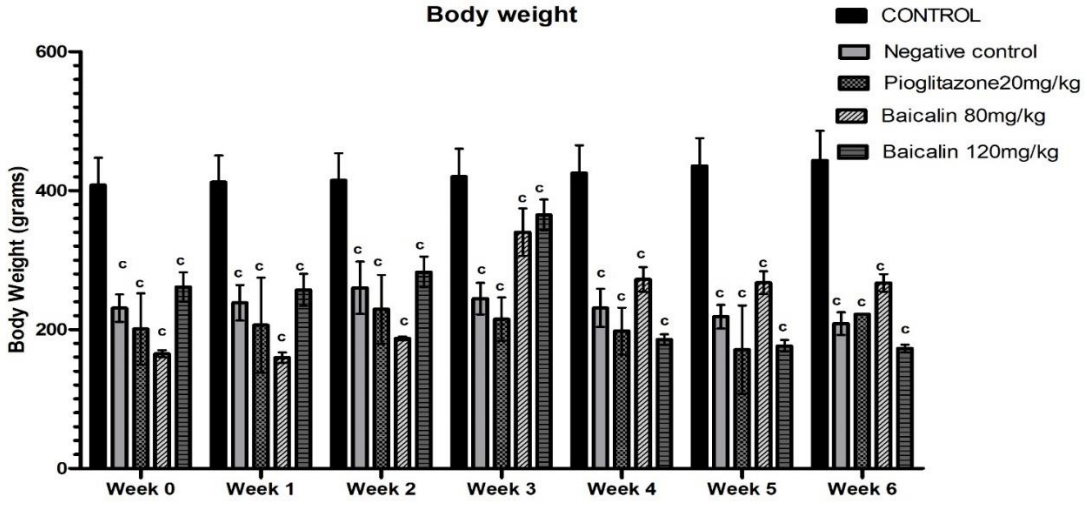

Figure 15:Changes in Body weight of Control, Negative control and treatment groups. Data's are expressed as mean± SD. Statistics were done by Two-way ANOVA and post hoc analysis were

done by Tukey's method. a,b,c show significance of p<0.05,p<0.01,p<0.001 with control group and x,y,z shows significant of p<0.05, p<0.01,p<0.001 with Negative control group

6.2.3 Weekly blood glucose level:

Figure 16 shows the weekly blood glucose level of animals in different groups. When control group compared to negative control group, show significant difference p(<0.001) in week 0, week 1, week 2 and week 3. High dose baicalin(120mg/kg) and pioglitazone(20mg/kg) does not show significant change when compared to control at week2 and week 3 but shows significant difference p(<0.001)when compared to negative control group.

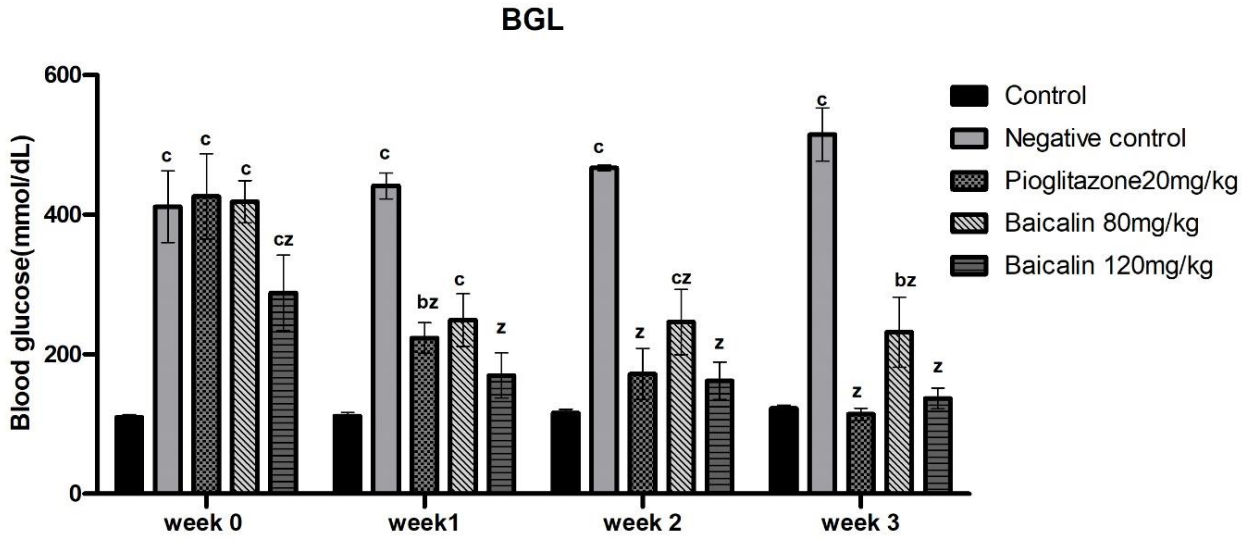

Figure16: Changes in weekly blood glucose level of Control, Negative control and treatment groups. Datas are expressed as mean± SD. Statistics were done by Two-way ANOVA and post hoc analysis were done by Tukey's method a,b,c show significance of p<0.05,p<0.01,p<0.001 with control group and x,y,z shows significant of p<0.05, p<0.01,p<0.001 with Negative control group.

6.2.4 Oral Glucose Tolerance Test:

Figure 17 shows the change in Blood glucose at a time of 0, 30, 60, 90, 120 minutes in different groups. The blood glucose level of control group is compared with all other groups shows a significant difference at time 0, 30, 60, 90,120 minutes p(<0.001). When negative control group is compared to Pioglitazone(20mg/kg) treated group, High dose Baicalin(120mg/kg) is shows significant at time 0, 30, 60, 90,120 minutes p(<0.001). When Low dose baicalin is compared to negative control groups is shows significance at 90, 120 minutes p(<0.001).

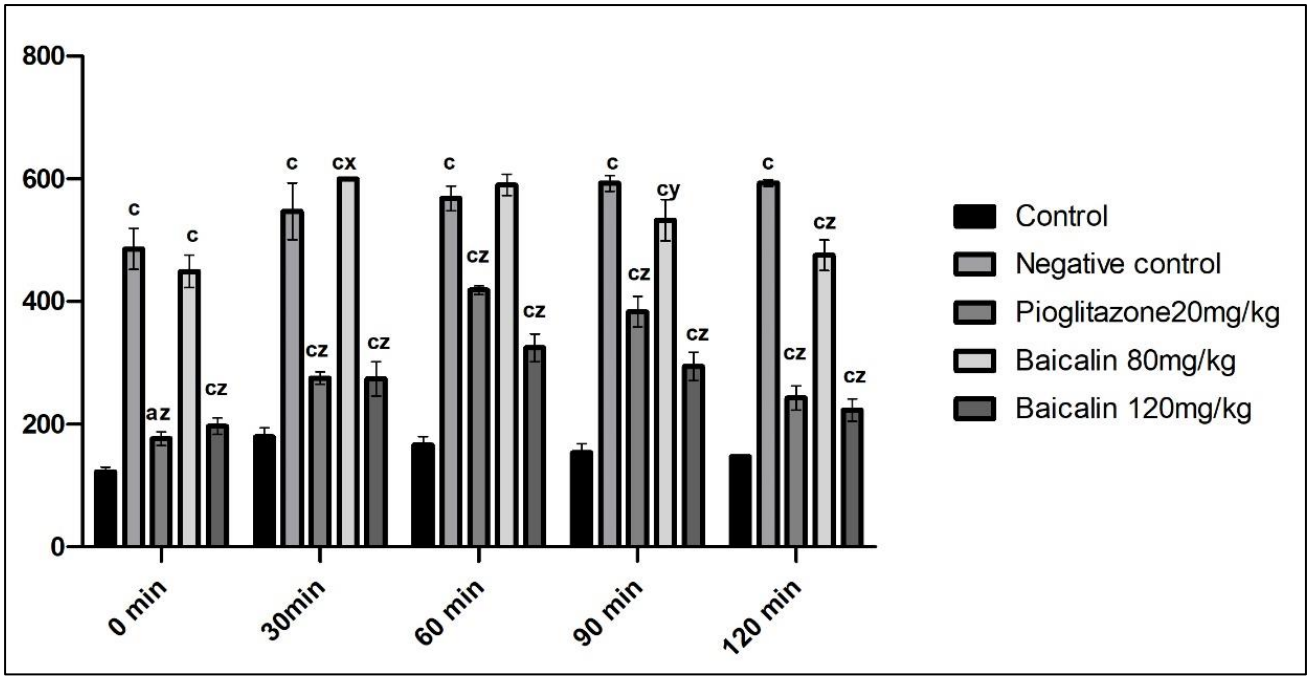

Figure 17: Changes in Blood glucose level of Control, Negative control and treatment groups at 0, 30, 60, 90, 120minutes. Datas are expressed as mean± SD. Statistics were done by Two-way ANOVA and post hoc analysis were done by Tukey's method a,b,c show significance of p<0.05,p<0.01,p<0.001 with control group and x,y,z shows significant of p<0.05, p<0.01,p<0.001 with Negative control group

6.2.5 Biochemical parameter:

After 28 days of treatment, the animals were euthanized and blood samples were collected and used for serum lipids analysis. **Figure 18** shows the difference in the serum lipid profile among the groups. There is significant difference in the total cholesterol level when control group is compared to other groups($p<0.001$) and also between Negative control and treatment groups($p<0.001$).When triglyceride level of control is to negative control and treatment groups it shows significant difference $p<0.001$ and $p<0.01$. The control shows significance with negative control and Baicalin(80mg/kg) treated group ($p<0.001$) for HDL levels and Negative shows significance of $p<0.01$ and $p<0.05$ with Pioglitazone and Baicalin(120mg/kg) treated group respectively. The control group shows significance $p<0.001$, $p<0.05$, $p<0.01$ with negative , Pioglitazone and Baicalin treated groups respectively for LDL, VLDL level and the negative control shows significance of $p<0.001$ with the treatment groups.

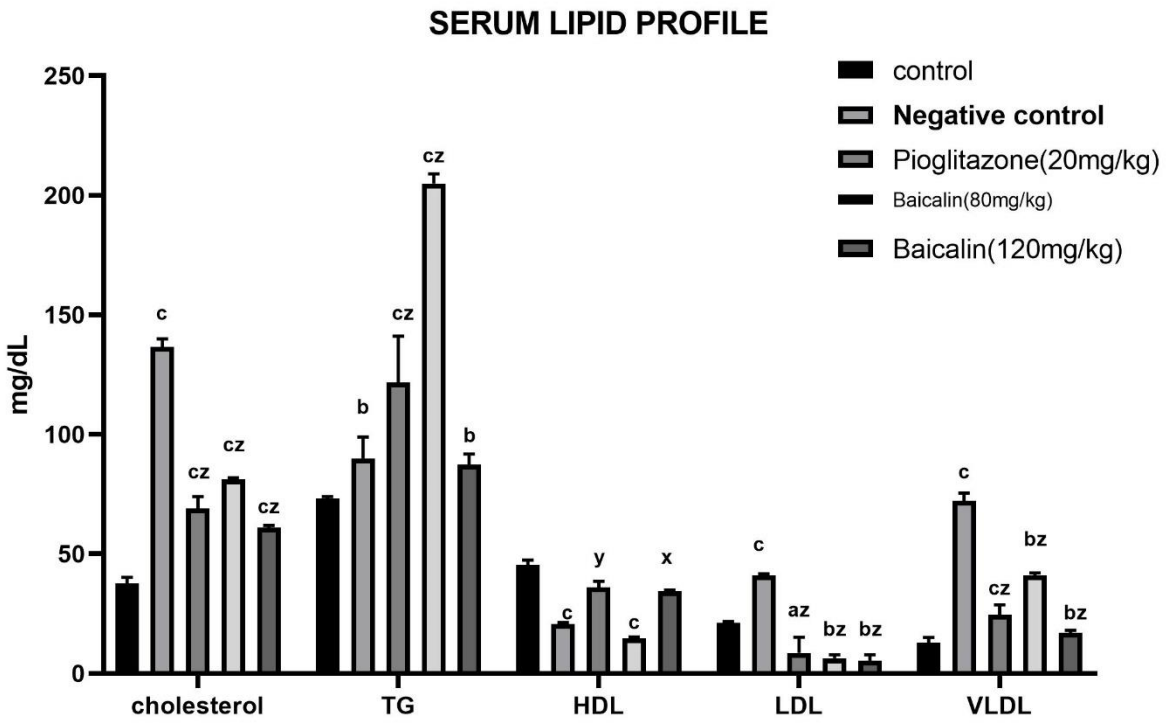

Figure 18: Changes in Total Cholesterol, triglycerides, HDL, LDL, VLDL of Control, Negative control and treatment groups. Datas are expressed as mean± SD. Statistics were done by Two-way ANOVA and post hoc analysis were done by Tukey's method a,b,c show significance of p<0.05,p<0.01,p<0.001 with control group and x,y,z shows significant of p<0.05, p<0.01,p<0.001 with Negative control group

6.2.6 Histopathological results:

a) Histopathology of Pancreas:

The histopathology of Pancreas of experimental animals are shown in **Figure 19**. The H&E staining of the control group shows normal islets number and size. The Negative Control group shows complete destruction of pancreatic islets and acinar cells. The Standard Group treated with Pioglitazone(20mg/kg) shows reduced number of islets compared to normal group. In the Baicalin (80mg/kg) treated group the pancreas shows small islets but in Baicalin (120mg/kg) treated there is normal acini and small islets are seen.

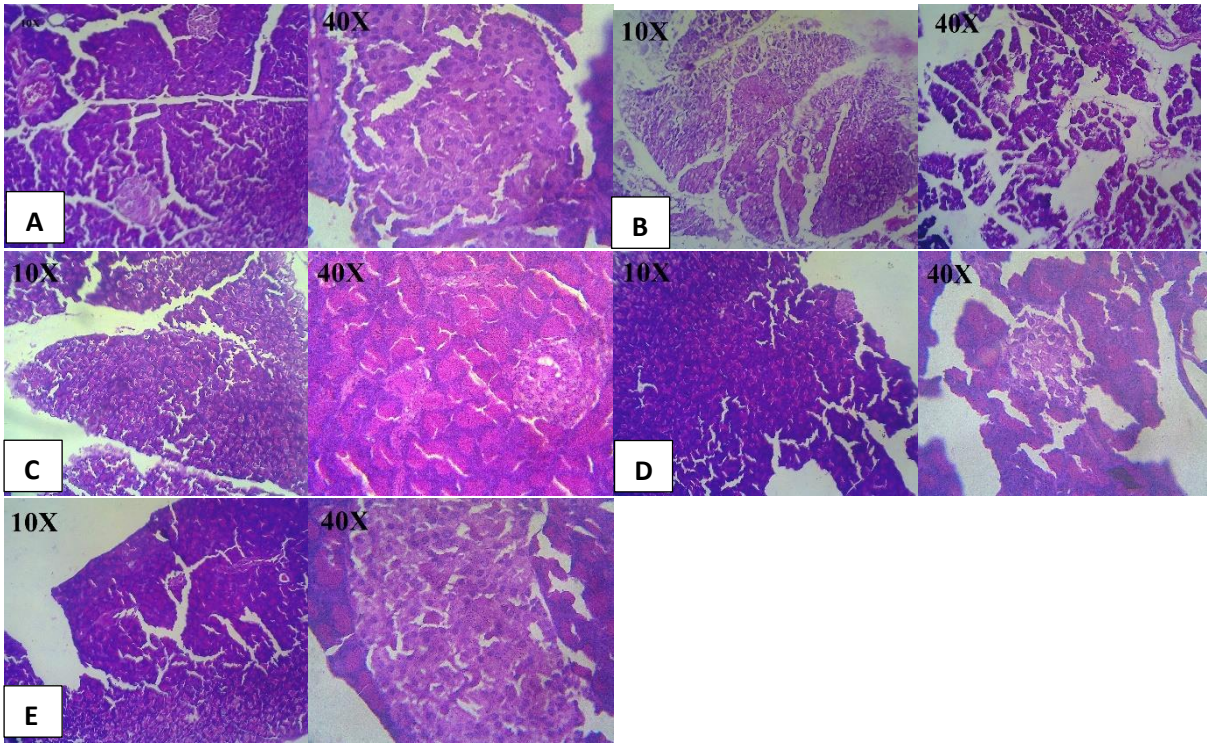

Figure 19: Changes in histopathology of pancreas of experimental animals A) Control shows islets that are normal n number and size, B) Negative Control show completely destructed islets and acinar cells, C) Pioglitazone(20mg/kg) shows reduced islets number, D) Baicalin (80mg/kg)shows small islets, E) Baicalin (120mg/kg)shows normal acini and islets at 10X and 40X

b) Histopathology of Liver:

Figure 20 shows the changes in the histopathology of the liver in the experimental animal groups. Control group shows normal lobular architecture and normal hepatocytes with no significant pathology with central vein congestion, where in the Negative Control, it shows altered lobular architecture and necrosis along with central vein congestion. In the Pioglitazone(20mg/kg) treated group the histology of liver shows interface hepatitis. The Baicalin (80mg/kg) treated groups shows altered lobular architecture and cytoplasmic vacuolation, where Baicalin (120mg/kg) shows normal hepatocytes and no significant pathology

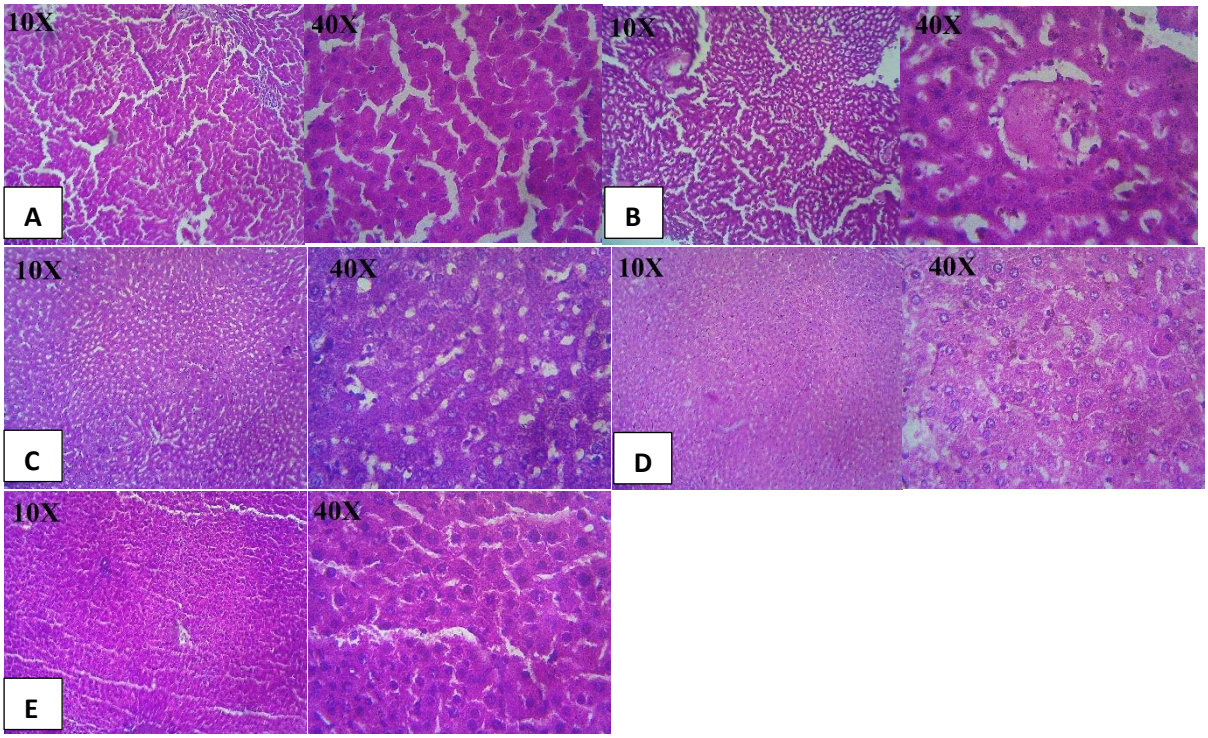

Figure 20: Changes in histopathology of liver of experimental animals A) Control shows lobular architecture and normal hepatocytes, B) Negative Control shows altered lobular architecture and necrosis, C) Pioglitazone(20mg/kg) shows interface hepatitis, D) Baicalin (80mg/kg) shows altered lobular architecture and cytoplasmic vacuolation, E) Baicalin (120mg/kg) shows normal hepatocytes and slight interface hepatitis at 10X and 40X

c) Histopathology of Adipose Tissue:

Figure 21 shows the histopathology of the adipose tissues in experimental animal, the control groups show the sheets oof adipose tissue separated by fibrous stroma with no inflammation seen where as in Negative control adipose tissue show increase in the size of adipose tissue. The Pioglitazone treated groups shows the increase in adipose tissues with dense inflammation seen in the stroma. The baicalin (80mg/kg) treated group shows adipose tissue separated by fibrous stoma with mild inflammation and Baicalin 120mg/kg shows scattered inflammations and adipose tissues are separated by stroma.

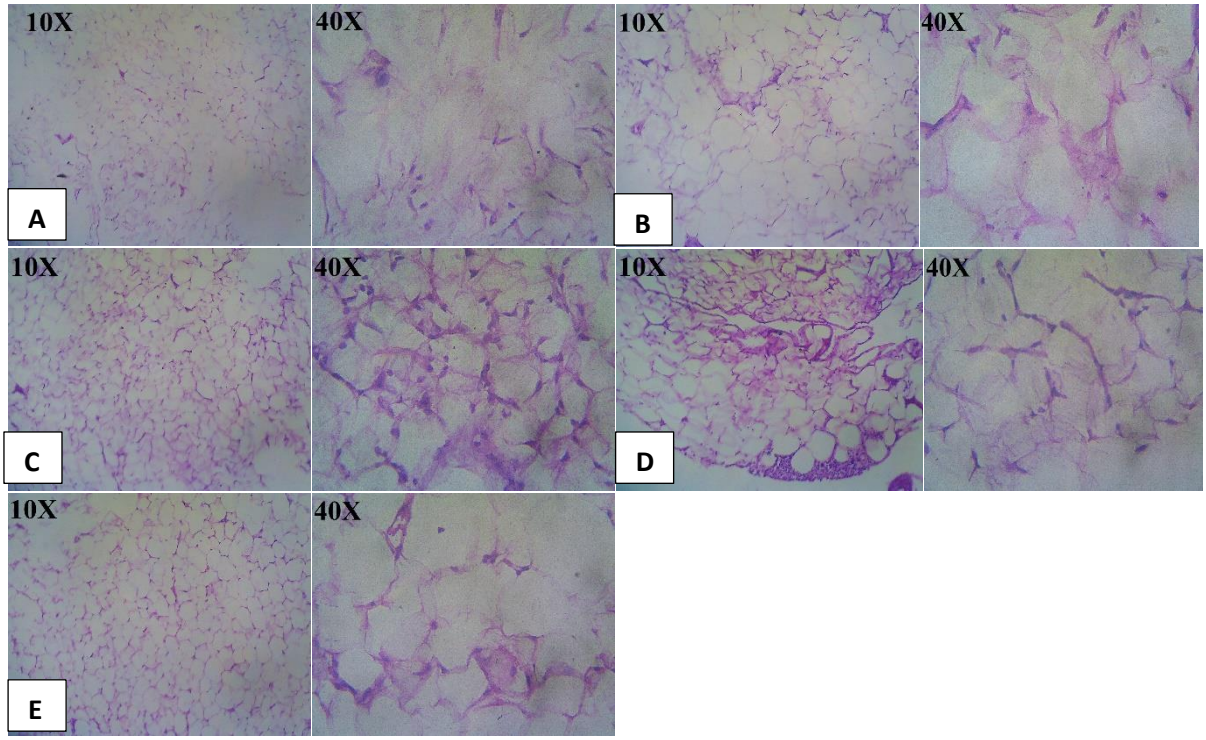

Figure 21: Changes in histopathology of adipose tissues of experimental animals A) Control shows adipocytes, B) Negative Control shows increase in adipose size, C)Pioglitazone(20mg/kg)increase in the size of adipocytes and cytokine infiltration, D) Baicalin (80mg/kg) shows normal adipose tissue, E) Baicalin (120mg/kg) shows the normal adipocytes separated fibrous stroma at 10X and 40X

7. DISCUSSION

DISCUSSION:

Diabetes is a serious, long-term condition that occurs when the body cannot produce any or enough insulin or cannot effectively use the insulin it produces. It is a major Global threat with high prevalence affecting 463.0 million people globally. There are several drugs for the treatment of diabetes but still there is need of better treatments.

PPAR are nuclear receptors that play a vital role to regulate numerous aspects of metabolism through gene transcription. Whereas PPARγ is adipocyte enriched and tends to regulate lipid storage, PPARα and PPARβ are expressed most highly in oxidative tissues and regulate genes encoding enzymes involved in mitochondrial biogenesis and metabolism, fatty acid oxidation, and triglyceride takeover.

Numerous natural and synthetic PPAR agonist have been studied for therapeutic efficacy including agonist that can activate two or three PPAR isoforms. Rosiglitazone a PPARγ agonist has proven its effectiveness in reducing insulin resistance . However, some meta-analyses indicated that among patients with impaired glucose tolerance or type 2 diabetes the use of rosiglitazone for at least 12 months was associated with a significantly increased risk of myocardial infarction and heart failure, as well as with an elevated risk of cardiovascular mortality**[60]**. Pioglitazone (PPARα/γ agonist) is an Insulin sensitizer but it has fewer adverse effects which lesser than the adverse effects of Rosiglitazone **[61]**. Lanifibranor, A PAN PPAR agonist significantly improved glycemic profile, in a randomized controlled trial comprising 42% diabetes patients **[62]**. So, in this study we screened 421 phytoconstituent from Pub chem database for Pan PPAR agonist activity. In this Baicalin shows a promising docking score than Market available PPAR agonist Drug Pioglitazone. It also shows greater stability and lesser energy in the Molecular dynamics and MMGBSA studies

Baicalin is a Phytoconstituent present in *Scutellaria baicalensis*, a Traditional Chinese medicine that is used for diabetes treatment. The extract of *Scutellaria baicalensis* in which baicalin and baicalein are major constituents, *was* proven to have anti-diabetic activity. A study suggests that baicalin is a powerful and promising agent for treatment of obesity and insulin resistance via Akt/AS160/GLUT4 and P38MAPK/PGC1α/GLUT4. I.p. administration of baicalin significantly decreased plasma glucose levels in a dose-dependent manner in streptozotocin-nicotinamide induced diabetic rats **[63, 64]**. In accordance with these, we found that i.p. injection of baicalin reduced circulating glucose levels in DIO mice too, further manifesting that baicalin had the antihyperglycemic effects.A study attempted to elucidate the

molecular modulation of antioxidant baicalin on the insulin-induced FoxO1 inactivation and the results revealed that baicalin regulated FoxO1 phosphorylation via PI3K/Akt by insulin and FoxO1 acetylation by the interaction of CBP and SIRT1, leading to changes in catalase gene expression during aging. These anti-obesity, anti-oxidant and anti-inflammatory activity of baicalin also helps in the better treatment of diabetes.

The primary objective of the study is to evaluate anti diabetic activity of the compound Baicalin screened through *in silico* studies and to elucidate the mechanism of action as PPAR agonist.

In this study diabetes was induced by high fructose-STZ model as done by Wilson D et al., the increase in the Blood glucose level after STZ administration at the end of 2 weeks shows the induction of diabetes in the animal model.

According to American Diabetes Association, Weight loss is a common condition seen in diabetic patients. There was a decrease in body weight in the negative control group than the control group showing the induction of diabetes and in the treatment groups there is slight increase in body wight when compared to Negative control. The characteristic loss of body weight associated with diabetes is due to increased muscle wasting in diabetes **[65]**. The decrease in the Blood glucose level shows increase in insulin sensitivity. There is a decrease in blood glucose level over the period of drug treatment indicating that the drug increased insulin sensitivity.

The fundamental mechanism underlying hyperglycemia involves over-production (excessive hepatic glycogenolysis and gluconeogenesis) and decreased utilization of glucose by the tissue. Persistent hyperglycemia, the common characteristic of diabetes can cause most diabetic complications. In all patients, treatment should aim to lower blood glucose to near-normal levels **[66].** In our investigation, the oral glucose tolerance revealed that the baicalin has the capacity to lower blood glucose levels.

Insulin resistance promotes lipoprotein abnormalities, qualitative and kinetic abnormalities, which results together towards more atherogenic lipid profile **[67]**. Existing evidence demonstrated that the availability of free fatty acids (FFA) for oxidation by muscles and other tissues may lead to impairment of carbohydrate oxidation as well as to glucose intolerance as seen in obesity and obese diabetics **[68, 69].** In addition, FFA can stimulate hepatic gluconeogenesis and alter pancreatic insulin release as well as pathways of glucose metabolism **[70, 71]**. Therefore, the reduction of circulating TG and TC is an important aspect

of the treatment of diabetes. Also, the excess cholesterol from peripheral cells is removed and transported to liver by HDL. This activity is reduced in insulin resistance condition where increase in hepatic lipase activity leads to HDL clearance **[72].** All these effects together lead to increase in serum lipids (TG, LDL and VLDL) and decrease in HDL, the "good cholesterol". Baicalin reduced plasma and hepatic TG and TC contents and increased the HDL level.

Histopathological studies of Pancreas, Liver and Adipose shows that Baicalin containing extract produced considerable changes which may be due to the antidiabetic effect of the compound. The pancreatic tissues of the normal control animals showed high intensity of stained β-cells of the islets and preserved β-cell numbers compared to untreated diabetic animals. This supports the fact that chronic uncontrolled hyperglycaemia in T2D causes impaired insulin secretion **[73, 74]** with subsequent distortion of pancreatic architecture, β-cell integrity and functions **[75].** The Baicalin(120mg/kg) treated animals, depicted moderate β-cells comparable to the pioglitazone treated animals. The improved pancreatic β-cell integrity and functions could be mediated through the antioxidant potentials of Baicalin**,** because oxidative stress is the major causal factor for the pancreatic β-cell damage in T2D**[76].** Liver the largest organ in the body is vulnerable to hyperglycaemic condition due to insulin resistance**[77].** In this state, the liver was affected by Oxidative stress, lipid accumulation, inflammation and necrosis which was seen in negative Group. Interestingly, the Baicalin treated groups shows reduced damage which may be due to their anti-inflammatory, anti-oxidant activity.

Adipose tissue exists in adipocytes and a vascular-stromal fraction in which macrophages, fibroblasts, endothelial cells and pre-adipocytes are present**[78].** The essential roles of adipose tissue are exemplified by the fact that total absence of adipose tissue results in non-viability as occurs in homozygous peroxisome proliferator-activated receptor gamma (PPAR-γ) knock-out mice. During evolution, fat tissue presumably acquired an intermediary role between nutritional status and essential body functions such as feeding behaviour, growth, metabolism, and even fertility**[79, 80]**. There was an increase in the size of adipose tissue in negative control group which is in accordance with the fact that Adipose tissue expandability in response to positive energy balance has been considered traditionally an adaptive passive process. However recent evidences suggest that the expandability of the adipose tissue is not an unlimited process. In fact, adipose tissue expandability may be an important factor determining the appearance of obesity associated co-morbilities **[81].** In this regard hypertrophy could be a marker of failure in the mechanisms of preadipocyte recruitment. Despite normal energy intake, lack of adipose

tissue results in insulin resistance, elevated serum free fatty acids (FFA) and accumulation of FAs in tissues such as the liver, skeletal muscle and the pancreas. Insufficient attempts by oxidative tissues to dispose the excess of FAs leads to chronic FA deposition and lipotoxicity, followed by insulin resistance, fatty liver and beta-cell failure**[82].** Conversely individuals expanding adipose tissue at the expense of increasing the number of adipocytes through a hyperplastic response remain insulin sensitive and healthier. Hyperplastic changes have the advantage of retaining insulin sensitivity and a favourable pattern of signalling molecules secretion**[83]** Which was seen in the Baicalin Treatment groups.

Collectively, these data suggest that the compound Baicalin has anti-diabetic effect by regulating the lipid glucose metabolism and amelioration of insulin resistance in the diabetic induced rat model. Molecular level studies in future such as examination of gene expression of PPARγ, PPARβ, PPARα, and also the gene expression regulate by PPAR isoforms like GLUT4, ACOX, C/EBP α, AP2, UCP1, TNF α, LPL, PGC α,may pave the way for finding the exact mechanism behind the anti-diabetic effect of Baicalin.

8. CONCLUSION

CONCLUSION

The present study proved that high fructose diet-streptozotocin (40mg/kg/i.p) produces diabetes via insulin resistance and it shows changes in animal body weight and random blood glucose level, insulin sensitivity and also causes alterations in serum lipid levels and histology of liver and pancreas. Thereby Baicalin treatment showed favourable effects on diabetic rats. This study proved the regulative effect of Baicalin on lipid glucose metabolism and insulin resistance in diabetes induced animals which may be due to Pan PPAR agonist activity of the compound. This may be a promising source for the discovery of novel antidiabetic agents. Further studies are needed to explore the safety and molecular mechanisms in the treatment of diabetes mellitus.

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ANNEXURE

PSG Institute of Medical Sciences & Research
Institutional Animal Ethics Committee

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This is to certify that the project proposal no. **500/IAEC/2021** entitled **"*Regulation of lipid, glucose metabolism and insulin resistance through PPAR using Baicalin.*"** submitted by **Ms.A.Jeevitha** has been approved/recommended by the IAEC of PSGIMS&R (Organization) in its meeting dated **01.06.2021** and **6 Weeks old, BW 200-250g, 30 Male & Female Sprague Dawley rats** (No. of animals) have been sanctioned under this proposal for a duration of next 12 Months.

Authorized by	Name	Signature	Date
Chairman:	Dr.S.Ramalingam The Chair Person, CPCSEA IAEC of PSGIMS&R Coimbatore-641 004.		01.06.2021
Member Secretary:	Dr.N.Ramanujam Dr. N. RAMANUJAM, MEMBER - SECRETARY, PSG-INSTITUTIONAL ANIMAL ETHICS COMMITTEE (PSG-IAEC)		01.06.2021
Main Nominee of CPCSEA:	Dr.C.Kathirvelan Main Nominee, CPCSEA IAEC of PSGIMS&R Coimbatore-641 004.		01.06.2021

(Kindly make sure that minutes of the meeting duly signed by all the participants are maintained by Office)

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Certificate Of Analysis

Product Name: Skullcap Extract
Batch Number: HN/SE/110205
Quantity: 0.5 Kg
Manufacture Date: July 21
Exp Date: June 24

Analysis	Specification	Results
Appearance	Brown fine Powder	Complied
Odor	Characteristic	Complied
Taste	Characteristic	Complied
Extracts Assay:	20% Baicalin by HPLC	20.05%
Loss on Drying	≤5.0%	2.88%
Sieve Analysis	Pass 60 mesh	Complied
Bulk Density	45-55g/100mL	Complied
Heavy Metal	NMT 20ppm	Complied
As	NMT 2ppm	Complied
Microbiology		
Total Plate Count	NMT 500cfu/g	Complied
Yeast & Mold	NMT 100cfu/g	Complied
E.Coli	Negative	Complied
Salmonella	Negative	Complied

Conclusion Conform with specification
Storage Store in cool & dry place. Keep away from strong light and heat.
Shelf life 3 years when properly stored

The information herein is correct based on our knowledge; please evaluate the raw material prior to use in a finished product

Computer generated certificate, valid without signature.

RAMAIAH Institute of Technology | RAMAIAH UNIVERSITY OF APPLIED SCIENCES | IEEE EMB MSRIT Student Chapter | SRIGEN SOCIETY OF BIOTECHNOLOGISTS

Certificate Of Participation

This is to certify that **Jeevitha A**

has attended 3 days International e-Workshop on ***"Docking, QSAR and Molecular Dynamics"*** jointly organized by Department of Biotechnology, Ramaiah Institute of Technology and Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Ramaiah University of Applied Sciences, Bengaluru, Karnataka, India in association with IEEE-EMB MSRIT student chapter and SRIGEN-Society of Biotechnologists from 29 to 31 July 2020.

Dr. Bindu S
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Dr. V. Madhavan
Dean, FPH, RUAS

BPNMI CONSORTIUM

The Organizing Committee of the

"International Conference on Medicinal and Food Plant Research & 3rd Sino-CPLP Symposium on Natural Products and Biodiversity Resources", 9-10th April 2021, Hanzhong, China

This is to confirm that

Jeevitha Ambigai Kumar

Attended the International Conference on Medicinal and Food Plant Research & 3rd Sino-CPLP Symposium on Natural Products and Biodiversity Resources

Distinguished Professor at Shaanxi University of Technology
Senior Researcher, CBMA, Univ. of Minho, Portugal
Adjunct Professor, Univ. of Guelph, Canada

Professor at Department of Biology, University of Minho, Portugal

Distinguished Professor at State Key Laboratory of Quality Research in Chinese Medicine, University of Macau