A DISSERTATION ON

Insulin resistance reversal Potential of selected medicinal Plants derivedleads against TNF-α induced insulin resistance in Skeletal muscle Cells.

SUBMITTED TO THE

DEPARTMENT OF BIOENGINEERING

FACULTY OF ENGINEERING

INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULLFILLMENT

FOR THE

DEGREE OF B.TECH.- M.TECH.

IN BIOTECHNOLOGY

By

MOHD RASHID KHAN

DEPARTMENT OF BIOENGINEERING

INTEGRAL UNIVERSITY

UNDER THE SUPERVISION OF

Dr. D.U. Bawankule

Senior Principal Scientist, Division of Pharmacology

CSIR-CIMAP, LUCKNOW- 226021 (UP)

DECLARATION FORM

I, **MOHD RASHID KHAN**, a student of **B.Tech-M.Tech** (V year/X semester) Integral University have completed my six months dissertation work entitled **"*Insulin resistance reversal Potential of Skeletal medicinal Plants derived leads against TNF-α induced insulin resistance in Skeletal muscle cells*"** successfully from Dr.D.U Bawankule, Senior Principal Scientist Bioprospection and product development, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow .I, hereby affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

Name and Signature of Student with Date

Name and Signature of Course Coordinator with Date

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Human Resource Development Programme

CERTIFICATE

This certificate is issued to the candidate by ***CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow.***On successful completion of his/her M.Tech/M.Pharma dissertation work-

Name of the Candidate : MOHD RASHID KHAN

Institution : Integral University Lucknow

Category of Training : Graduate Training

Training Department : Bioprospection & Product Development division

Title of Training:**"*Insulin resistance reversal Potential of Skeletal medicinal Plants derived leads against TNF-α induced insulin resistance in Skeletal muscle cells*".**

Duration of Training : From 15/02/2022 – 15/07/2022

The institute wishes the candidate success in his/her future endeavors.

Head of Department Supervisor

(Dr. D Saikia) (Dr.DU Bawankule)

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This is to certify that **MOHD RASHID KHAN**, a student of **Dual Degree Biotechnology** (5th Year/ 10th Semester), Integral University has completed his six months dissertation work entitled "**Insulin reversal Potential of Skeletal medicinal Plants derived leads against TNF-α induced insulin resistance in Skeletal muscle cells"** successfully. He has completed this work from Department of Botany under the guidance of **Dr. DU Bawankule**. The dissertation was a compulsory part of his Dual Degree Biotechnology Program.

I wish him good luck and bright future.

Dr. Alvina Farooqui
Head
Department of Bioengineering
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TO WHOM IT MAY CONCERN

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Dr. Alvina Farooqui
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Acknowledgement

After an intense period of six months, today is the day: writing this note of thanks is the finishing touch on my dissertation. It has been a period of intense learning for me, not only in scientific area but also on a personal level. Writing this dissertation had a big impact on me. I would like to reflect on the people who have supported and helped me so much throughout this period.

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At last I would like to thank all the people who directly and indirectly were involved in my dissertation work.

Finally, I am thankful to God the Almighty for successful completion of my dissertation.

MOHD RASHID KHAN

Abbreviations

%	:	Percent
@	:	at the rate of
b.wt	:	Body weight
DMSO	:	Dimethyl Sulphoxide
ELISA	:	Enzyme linked immunosorbant assay
g	:	grams
HA	:	Heamagglutination
HRPO	:	Horse Radish Peroxidase
Hb	:	Hemoglobin
IFN – γ	:	Interferon Gamma
IL- 1β	:	Interleukin 1 – Beta
IL – 6	:	Interleukin 6
IPR	:	Intellectual property rights
i.p	:	intra peritoneal
i.v	:	intra venous
i.d	:	intra dermal
IVT	:	In-Vitro or In-Vivo
mg	:	Milligrams
ml	:	Millilitre
NC-Paper	:	Nitrocellulose Paper
mm	:	Millimeter

mM : Milli moles

MTT : (3-4, 5-dimethyl thiazole -2yl)-2-5-

diphenyl tetrazoliumbromide)

N : Number of Animals

Nm : Nanometer

O.D : Optical Density

26: ˚C : Degree Celsius

27: PBS : Phosphate Buffer Saline

RBC : Red Blood Corpuscles

RPM : Revolutions per minute

RPMI : Rose Well Park Memorial Institute1640

RBC's : Red blood corpuscles

RRBC : Rabbit Red Blood Corpuscles

SOD : Superoxide Dismutase

SDS : Sodium Dodecyl Sulphate

TBST : Tris Buffer Saline Tween-20

TNF : Tumour Necrosis factor

WBC's : White blood corpuscles

WE : Water extract

µg : Microgram(s)

U/ l : Units per liter

C O N T E N T S

Introduction

Insulin resistance (IR) defined as a diminished ability of cells or tissues to respond to physiological levels of insulin. It is crucial and characteristic of pre-diabetes and is the detectable abnormality in non-insulin-dependent diabetes mellitus i.e. **type 2diabetes mellitus (T2DM)** (Khodabandehloo et al., 2016). The incidence of T2DM is rapidly increasing. In 2019, nearly half a billion people (9.3% of adults 20–79 years) are living with diabetes worldwide. Prominent features of T2DM include low-grade chronic inflammation, hyperglycemia, hyperlipidemia and pancreatic β-cell dysfunction. Chronic inflammation results in insulin resistance in the primary insulin target organs, such as muscle, adipose tissue, and liver (Soomro et al, 2019). Activated macrophages are important mediators of inflammation primarily in the skeletal muscle, adipose tissue and β-cell islets (Olefsky and Glass, 2010). Inflammatory cytokines secreted by macrophages disrupt insulin signaling in skeletal muscle cells, adiposities and hepatocytes by inducing inflammatory pathways. Over the past decade extensive research has focused on the role of macrophages as key mediators of inflammation in T2DM. It is also known as "non-insulin dependent diabetes" or "adult diabetes", is a chronic-degenerative disease characterized by the presence of **insulin resistance**, a condition where cells that usually respond to insulin stop doing it, and due to relative deficiency of this hormone in the body. Elevated levels of pro-inflammatory cytokines, such as tumor necrosis factor- (TNF-α), interleukin-6 (IL-6), interleukin-1 β (IL-1β) have been reported in various diabetic and IR states.Diabetes mellitus (DM) is the commonest endocrine disorder that affects more than 100 million people worldwide (6% of the population) and in the next 10 years it may affect about five times more people than it does now (WHO/Acadia, 1992, ADA, 1997). In India, the prevalence rate of diabetes is estimated to be 1–5% (Patel et al., 1986, Verma et al., 1986, Rao et al., 1989). Complications are the major cause of morbidity and mortality in DM.

Historical accounts reveal that as early as 700–200 BC, DM was a well-recognized disease in India and was even distinguished as two types; a genetically based disorder and other one resulting from dietary indiscretion (Oubre et al., 1997). In India, indigenous remedies have been used in the treatment of DM since the time of Charaka and Sushruta (6th century BC) (Grover and Vats, 2001).Plants have always been an exemplary source of drugs and many of the currently available drugs have been derived directly or indirectly from them. The ethno-botanical

information reports about 800 plants that may possess anti-diabetic potential (Alarcon-Aguilara et al., 1998). Several such herbs have shown anti-diabetic activity when assessed using presently available experimental techniques (Saifi et al., 1971, Mukherjee et al., 1972, Coimbra et al., 1992, Ajitkar et al., 1999, Jafri et al., 2000). A wide array of plant derived active principles representing numerous chemical compounds has demonstrated activity consistent with their possible use in the treatment of non-insulin-dependent diabetes (**NIDDM**) (Bailey and Day, 1989, Ivorra et al., 1988, Marles and Farnsworth, 1995). Among these are alkaloids, glycosides, galactomannan gun, polysaccharides, peptidoglycans, hypoglycans, guanidine, steroids, carbohydrates, glycopeptides, terpenoids, amino acids and inorganic ions. Even the discovery of widely used hypoglycemic drug, metformin came from the traditional approach of using *Galega officinalis*. Thus, plants are a potential source of anti-diabetic drugs (and others too) but this fact has not gained enough momentum in the scientific community. The reasons may be many including lack of belief among the practitioners of conventional medicine over alternative medicine, alternative forms of medicine are not very well-defined, possibility of quacks practising such medicine providing alluring and magical cures and natural drugs may vary tremendously in content, quality and safety.

Although, oral hypoglycemic agents/insulin is the mainstay of treatment of diabetes and is effective in controlling hyperglycemia, they have prominent side effects and fail to significantly alter the course of diabetic complications (Rang and Dale, 1991). As the knowledge of heterogeneity of this disorder increases, there is needed to look for more efficacious agents with lesser side effects. Though development of modern medicine resulted in the advent of modern pharmacotherapeutics including insulin, biguanides, sulfonylureas and thiazolidinediones, there is still a need to look for new drugs as no drug (except strict glycemic control with insulin) has been shown to modify the course of diabetic complications. In relation to plants also, barring a few studies (Grover et al., 2000, Rathi et al., in press a, Srivastava et al., 1988, Karunanayake et al., 1990, etc.), most of the studies have not assessed the impact of these plants on the course of diabetic complications. In current times, a significant rise in obesity, sedentary lifestyle, and consumption ofcalorie-dense foods has almost led to the development of insulin resistance. There areseveral reports demonstrated that the plant-derived bioactive are capable to improvethe glucose uptake in inflammation-induced insulin resistance in skeletal muscle cells.Recent studies suggest that herbal products are harmless to human being and areuseful to treat insulin

resistance. In the present study, we have standardized TNF-α induced insulin resistance in skeletal muscle cell line using Glucose uptake assay andthe protective effect of selected bioactive were coded as**2271, IVT 2272, and IVT 2273.**

Toperform the blind pharmacological screening to avoid the biasness during experimentalresults-based conclusion. The in-vitro cytotoxicity of all the bioactive **2271, IVT 2272, and IVT 2273**was also performed using (**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide**) MTT assay to see number of viable cells.

Sample received from the chemistry group of institutes CSIR-CIMAP was in coded form as **IVT 2271, IVT 2272, and IVT 2273** for further pharmacology department.

OBJECTIVES

The objectives of this research work are as follow:

1. Standardisation of glucose uptake assay in insulin resistance associated with tumour necrosis factor- α (TNF-α) in skeletal muscle cell line.

2. Glucose uptake profile of **IVT 2271, IVT 2272, and IVT 2273** against insulin resistance associated with TNF-α in skeletal muscle cell line.

3.*In-vitro* toxicity profile of **IVT 2271, IVT 2272, and IVT 2273** by MTT assay.

4. To learn the techniques used in drug discovery and development.

a. To learn the techniques related to preclinical research is in medicinal and aromatic plants.

b. To learn the techniques of acute toxicity.

c. To learn the techniques of sub- acute toxicity.

d. To learn the RNA extraction and Real time PCR.

2.Review of Literature

2.1. Diabetes Mellitus (DM)

Diabetes mellitus (DM) has been defined as a chronic metabolic disease, which is characterized by abnormally high level of glucose in the blood (hyperglycemia). Diabetes can cause long term complications by damaging vital organ, blood vessel and nerves. Diabetes is now a pandemic disease. Moreover, a large number of people with pre-diabetes are at risk for developing frank diabetes worldwide (Chait et al, 2020).

Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the pancreatic β-cells with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues (Alberti et al, 1998).

In the United States, the estimated number of people over 18 years of age with diagnosed and undiagnosed diabetes is **30.2 million.** The figure represents between 27.9 and 32.7 percent of the population.

There are two main types of diabetes, type I **(T1DM**) and type II (**T2DM**). T1DM refers to insulin-dependent diabetes and is caused by insufficient insulin secretion. About 10% of people have this type. T2DM refers to insulin release disorders, or is caused by insulin utilization disorder. Also known as non- insulin dependent or adult onset diabetes. About 90% of people with diabetes have type2.Thus far, type-I and type-II diabetes are the two distinctive clinical forms of diabetes identified.

2.1.1 TYPE- I Diabetes-

T1DM is typically diagnosed in children and young adults. It is an autoimmune disease that arises when the immune system attacks the insulin producing beta cells, the Islets of Langerhans, in the pancreas. Subsequently, there is a lack of the peptide hormone, insulin, produced in the body, which prevents the shuttling of blood glucose into muscle, liver and fat tissues leading to hyperglycemia (Kerru et al, 2018).

Autoimmune destruction of β-cells has multiple genetic predispositions and is also related to environmental factors that are still poorly defined. Although patients are rarely obese when they present with this type of diabetes, the presence of obesity is not incompatible with the diagnosis. These patients are also prone to other autoimmune disorders such as Graves' disease, Hashimoto's thyroiditis, Addison's disease, vitiligo, celiac sprue, autoimmune hepatitis, myasthenia gravis, and pernicious anemia.

2.1.2<u>TYPE-II Diabetes</u>-T2DM is the more common form of diabetes that is predominantly an effect of lifestyle based on diet. It is described by reduced insulin sensitivity or insulin resistance, whereby the signal impulse of insulin to a cell is weakened thus the cell has a reduced uptake of glucose from the blood. This factor combined with reduced insulin secretion and hyperglycemia is the cause of T2DM. Prolonged exposure to high glucose levels in the blood can lead to an irreversible damage to the eyes, nerves, kidneys and heart. The severity of the side effects of T2DM in patients could result in neurological and cardiovascular complications. The major objective of DM management is to maintain favorable glycemic control, by acquiring blood glucose levels as close to normal as possible.

T2DM is a known non-communicable disease. This metabolic disorder causes major health issues, together with social and economic impacts (Nordin et al,2020). It is a complicated disease with βcell dysfunction and insulin resistance that lead to a relative deficiency of insulin. One of the typical characteristics of T2DM is glucose metabolism, resulting in hyperglycemia (Wang, N et al,2018). Recently, many studies have demonstrated that T2DM is accompanied by systemic chronic inflammation, which plays an important role in the process of this disease.

T2DM is known as one of the most prevalent endocrine disorders, and it is estimated by WHO (World Health Organization) that by the year 2030, at least 333 million people or 6.3% of the global population will be affected by T2DM (Asbaghi et al,2020).

Around 30 million people were characterized as diabetic in 1985 worldwide compared to around 171 million cases in 2010 and almost 377 million estimated in 2030. The prevalence of diabetes is the highest in the Middle East, where the number of diabetic subjects reached 15.2 million in 2000 and estimated to be triple by 2030.

According to the World Health Organization (WHO), by 2030, diabetes will be seventh leading cause of death globally. In 2015, 415 million people were estimated to have diabetes, more than 90% of whom had type 2 diabetes, with a projected increase to 642 million by 2040.

Symptoms

The symptoms of high blood sugar in type 2 diabetes tend to appear gradually. Not everyone with type 2 diabetes will notice symptoms in the early stages. If a person does experience symptoms, they may notice the following:

- **Frequent urination and increased thirst**: When excess glucose builds up in the bloodstream, the body will extract fluid from tissues. This can lead to excessive thirst and the need to drink and urinate more.
- **Increased hunger**: In type 2 diabetes, the cells are not able to access glucose for energy. The muscles and organs will be low on energy, and the person may feel more hungry than usual.
- **Weight loss**: When there is too little insulin, the body may start burning fat and muscle for energy. This causes weight loss.
- **Fatigue**: When cells lack glucose, the body becomes tired. Fatigue can interfere with daily life when a person has type 2 diabetes.
- **Blurred vision**: High blood glucose can cause fluid to be pulled from the lenses of the eyes, resulting in swelling, leading to temporarily blurred vision.
- **Infections and sores**: It take longer to recover from infections and sores because blood circulation is poor and there may be other nutritional deficits.

If people notice these symptoms, they should see a doctor. Diabetes can lead to a number of serious complications. The sooner a person starts to manage their glucose levels, the better chance they have of preventing complication.Most people do not experience symptoms in the early stages, and they may not have symptoms for many years. A possible early sign of type 2 diabetes is darkened skin on certain areas of the body, including:

- the neck
- the elbows
- the knees
- the knuckles

This is known as **acanthosis nigricans.** Among 5% to 8% of adults have type 2 diabetes, a disease that is usually asymptomatic at first. The goals of management are timely diagnosis and the prevention of complications**.**

Complications -Diabetes may cause a number of health complications if people do not manage it properly. Many of these are chronic, or long-term, but they can become life-threatening. Others need immediate medical attention as soon as they appear.

Pre-diabetes

A person with blood sugar levels of 100–125 mg/dl will receive a diagnosis of pre-diabetes. This means that their blood sugar levels are high, but they do not have diabetes. Taking action at this stage can prevent diabetes from developing. The range of fasting plasma glucose test from 100-125 is pre-diabetes, 126 & above diabetes and that of oral glucose tolerance test from 140-160 is Pre-diabetes and 200 and above diabetes.

According to a 2016 report published in *The Journal of the American Board of Family Medicine***, 33.6 percent** of people aged 45 years and older had Pre-diabetes in 2012. The CDC estimates that around **84 million** American adults have Pre-diabetes, but most do not know they have it.

Diagnosis and treatment - A doctor can diagnose type 2 diabetes with blood tests that measure blood glucose levels. Many people discover they have high blood sugar during a routine screening test, but anyone who experiences symptoms should see a doctor.

Treatment aims to keep blood glucose levels stable at a healthy level and prevent complications. The main ways to do this are through lifestyle measures. These include:

- following a healthful diet
- reaching and maintaining a healthy weight and body mass index (BMI)
- doing physical activity
- getting enough sleep
- avoiding or quitting smoking
- Taking medications or insulin as the doctor recommends.

Diabetes Management- There is currently no cure for diabetes, but most people with the condition can lead a healthful life by managing their condition properly. People who maintain a healthy weight, follow a healthful diet, and do regular exercise may not need medication. Taking these steps can help manage blood sugar levels.Routine screening can alert a person to high blood sugar levels in the early stages, when there is still time to slow, stop, or reverse the progress of diabetes.

Current guidelines recommend regular screening from the age of 45 years, or younger if an individual has other risk factors, such as obesity. A doctor can advise on individual needs. (https://www.medicalnewstoday.com/articles/317462).

Insulin Resistance-

Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the pancreatic β-cells with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from in adequate insulin secretion and/ or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action.

Insulin resistance is defined as the reduced ability of cells or tissues to respond to physiological levels of insulin and is a characteristic condition of most patients with type 2 diabetes mellitus (T2DM). Studies in human insulin resistance have revealed a clear association between the chronic activation of pro-inflammatory signaling pathways and decreased insulin sensitivity. Expression of tumor necrosis factor a (TNF-a) was markedly increased in adipose tissue and muscle of insulin-resistant human and animals

During a meal, glucose is disposed in skeletal muscle, and to a lesser extent in fat tissues and liver. Insulin stimulates glucose uptake in muscle and adipocytes by signaling the translocation of GLUT4 glucose transporters from intracellular membranes to the cell surface. GLUT4 is largely sequestered inside the cell away from the plasma membrane (PM). Insulin, released to the circulation during a meal, binds to the muscle surface, sending signals that ultimately increase GLUT4 abundance at the membrane.

In the early stages of type 2 diabetes, impaired glucose uptake in muscles is a major contributor to insulin resistance. Because skeletal muscle is responsible for approximately 85% of postprandial glucose disposal, it is not surprising that a change in insulin sensitivity within muscle cells has a significant impact on whole-body glucose–insulin metabolism. Insulin resistance is also a major cause of diabetes.Insulin resistance includes insulin production by beta cells and impairment of insulin sensitivity in peripheral tissues (Kwon et al,2020). Therefore, increasing glucose uptake in insulin target tissues can be a therapeutic strategy to treat diabetes.

Mechanisms Linking Inflammation to Insulin Resistance

Insulin resistance (IR) is a complicated condition in which three primary metabolic issues that are sensitive to insulin, skeletal muscle, liver, and white adipose tissue (WAT) become less sensitive to insulin and its downstream metabolic actions under normal serum glucose concentrations (Chawla A et al 2011). IR is closely associated with obesity, hypertension, hyperglycemia, polycystic ovary syndrome, and metabolic syndrome. As the key component of metabolic syndrome, IR is also closely associated with nonalcoholic fatty liver disease (NAFLD). The antilipolytic effect of insulin is decreased in insulin-resistant conditions, which may promote hepatic triglyceride synthesis. Another feature of insulin resistance is an increasing release of free fatty acid. As we know, FFA could be taken up by organs and accumulated as ectopic fat, such as hepatic and cardiac lipids (Gaggini M., et al 2013). And hepatic lipids including triglyceride deposition are involved in the pathogenesis and development of NAFLD. Several factors are implicated in the pathology of obesity-related NAFLD, including complex interactions between glucose and lipid metabolism, genetic predisposition, environmental conditions, and modulation of the intestinal microbiota. IR encompasses a wide spectrum of disorders, such as defective insulin receptor signal transduction and mitochondrial function microvascular dysfunction and inflammation. Obesity, characterized as a state of chronic low-grade inflammation caused by over nutrition, is a major cause of decreased insulin sensitivity, which makes obesity a major risk factor for IR. Obesity, also manifested as excess adiposity, is a main cause of NAFLD. NAFLD is recognized as a typical feature of metabolic syndrome and manifested as a series of hepatic injuries including steatosis, nonalcoholic steatohepatitis (NASH), and even hepatocellular carcinoma. Obesity causes lipid accumulation in adipocytes, which activates c-Jun N-terminal kinase (JNK) and nuclear factor-kappa B (NF-κB) signaling pathways and might subsequently increase the production of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). In most cases, adipose tissue (AT) is an important site of obesity-induced IR, and it can also affect the liver and muscle by releasing cytokines, including adipokines such as TNF-α (Shoelson S. E., Lee J., Goldfine A. B. 2006). AT consists of several cell types. Among these, adipocytes and immune cells, such as macrophages and dendritic cells (DCs), have attracted significant attention as contributors that link inflammation to IR.

Cytokines That Link Inflammation to Insulin Resistance

TNF-α

Studies of TNF-*α* in the 1990s first analyzed the relationship between inflammation and IR (. Lee B.-C., Lee J. 2014). TNF-*α* is an adipose tissue-derived proinflammatory cytokine that causes insulin resistance by enhancing adipocyte lipolysis and increasing the serine/threonine phosphorylation of IRS-1 (insulin receptor substrate-1). Several signaling pathways, including the IKK*β*/NF-*κ*B pathway, are involved in the pathogenesis of IR. It was reported that TNF-*α* can increase glucose uptake in both visceral and subcutaneous adipocytes by activating the adenosine monophosphate activated protein kinase (AMPK) pathway, whereas it triggers insulin resistance in visceral adipocytes by activating JNK1/2. Because of the depot-specific effects of TNF-*α* on glucose uptake, approaches to treat IR by modulating TNF-*α* signaling are ongoing (. Fernández-Veledo S., et al 2009). However, studies of therapies such as the TNF-*α* superfamily member sTWEAK (soluble tumour necrosis factor-like weak inducer of apoptosis), which aims to block TNF signaling to treat IR, have demonstrated that TNF-*α* plays a role in IR (Vázquez-Carballo A et al 2013). Interestingly, the plasma levels of TNF-*α* are higher in males than in females, as well as in obese individuals compared with lean ones. This suggests that obese males are more likely to suffer from IR and related diseases such as cardiovasculardisease.

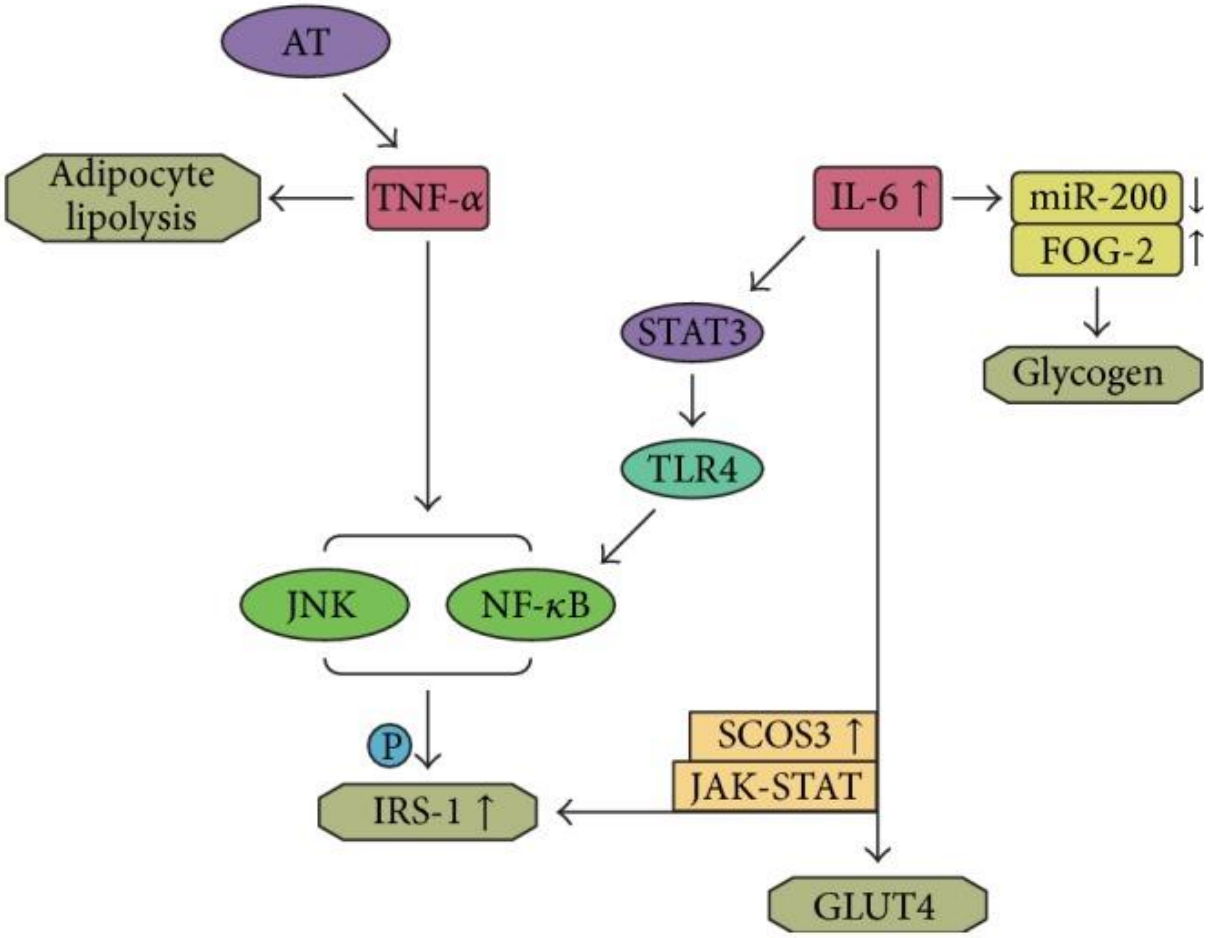


Fig1. Influence of the inflammatory cytokines on the status of insulin resistance. TNF-α causes insulin resistance by enhancing adipocyte lipolysis stimulating JNK and IKKβ/NF-κB pathway which may increase serine/threonine phosphorylation of IRS1. IL-6 induces IR by reducing the expression of GLUT4 and IRS-1 by activating the JAK-STAT signaling pathway and increasing SOCS3 expression, and IL-6 can also lead to IR in skeletal muscle by inducing TLR-4 gene expression through activation of STAT3; besides, TLR4 is suggested to be major upstream molecules in the activation of NF-κB. Besides, IL-6 is also found to induce IR by impairing the synthesis of glycogen through downregulating the expression of miR-200s and up regulating that of FOG-2.

IL-1*β*

Interleukin-1*β* (IL-1*β*) is a proinflammatory cytokine whose secretion is regulated by inflammasome activity. IL-1*β* contributes to IR by impairing insulin signaling in peripheral tissues and macrophages, which leads to the reduced insulin sensitivity of *β*-cells and possible impaired insulin secretion (Su D., Coudriet G. M., et al 2009). The levels of IL-1*β* in various cells such as endothelial cells and monocytes are increased during hyperglycemia. IL-1*β* also plays a vital role in initiating and maintaining inflammation-induced organ dysfunction in type 2 diabetes mellitus (T2DM). IL-1*β* might increase systemic inflammation and inhibit insulin action in the major insulin-target cells, such as macrophages (Hardaway A. L. et al 2013).

IL-6

IL-6 is secreted by multiple tissues, particularly adipose tissue, and is recognized as an inflammatory mediator that causes IR by reducing the expression of glucose transporter-4 (GLUT-4) and insulin receptor substrate-1 (IRS-1). These effects are exerted by the activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway (see Box 1) and increased the expression of suppressor of cytokine signaling 3 (SOCS3). Therefore, hybrid training can ameliorate insulin resistance by suppressing serum IL-6 in skeletal muscle (Kawaguchi T. et al 2011). IL-6 also induces IR by blocking the phosphoinositide 3-kinase (PI3K) pathway and impairing glycogen synthesis by downregulating the expression of microRNA-200s (miR-200s) and upregulating that of friend of GATA 2 (FOG-2). It was suggested that IR in human skeletal muscle is related to IL-6 stimulation, which induces toll-like receptor-4 (TLR-4) gene expression by activating STAT3.

Leptin

Leptin is a protein that is derived primarily from white adipose tissue (WAT) (Pedroso J. A., et al 2014). It suppresses appetite and increases energy expenditure by repressing anabolic neuronal circuits and activating catabolic neuronal circuits. In addition, leptin levels are affected by nutriture . Leptin-mediated appetite and energy homeostasis are associated with the progression

of IR .Furthermore, a state called leptin resistance, which was disputed lately by the concept of hypothalamic leptin insufficiency, is often observed in the obese individuals, and weight loss simultaneously reduces serum leptin levels. This suggests that leptin might have a role in regulating IR. Consistent with this, the stimulation of PI3K signaling by leptin is essential for modulating glucose metabolism and the function of pancreatic β-cells. It is likely that an increased concentration of leptin, an anti-inflammatory cytokine, during inflammation in AT is associated with leptin resistance in obese individuals. Interestingly, leptin was recommended as a biomarker for *in utero* insulin resistance based on the link between maternal and fetal leptin and IR. Leptin is a potential treatment for IR because it improves glycometabolism, insulin sensitivity, and lipometabolis.

Adiponectin

Adiponectin is produced mainly by WAT. Its levels reduce in obesity, IR, or T2DM, where it acts as an anti-inflammatory cytokine, but increase in osteoarthritis (OA) and type 1 diabetes mellitus (T1DM), where it acts as a proinflammatory cytokine (Stofkova A. Leptin and adiponectin 2009). Two receptors are involved in the glucose metabolism that links adiponectin to the amelioration of IR. Adiponectin receptor 1 (AdipoR1) is likely to reduce the expression of the genes that encode hepatic gluconeogenic enzymes and molecules involved in lipogenesis by activating AMPK. In contrast, adiponectin receptor 2 (AdipoR2) increases the expression of the genes that contribute to glucose consumption by activating peroxisome proliferator activated receptor-alpha (PPAR-α) signaling. AdipoR1 and AdipoR2 are expressed at high levels in skeletal muscle and the liver, respectively. In brief, adiponectin ameliorates hepatic insulin resistance by reducing glycogenesis and lipogenesis, as well as increasing glucose consumption.

Resistin

The production of resistin is complex. In rodents, it is generated from adipocytes, whereas it is produced mostly by macrophages in humans. Its concentrations increase concurrently with the levels of inflammatory mediators (Reilly M. P., et al 2005). It was suggested that resistin participates in the pathogenesis of IR and that its levels might be elevated due to obesity and IR.

Resistin promotes IR by regulating the expression of proinflammatory cytokines, including TNF-α and IL-6, in macrophages via an NF-κB-dependent pathway. It also plays roles in inflammation and IR by binding directly to TLR4 receptors in the hypothalamus to activate JNK and mitogen-activated protein kinase (MAPK) signaling pathways.

MCP-1

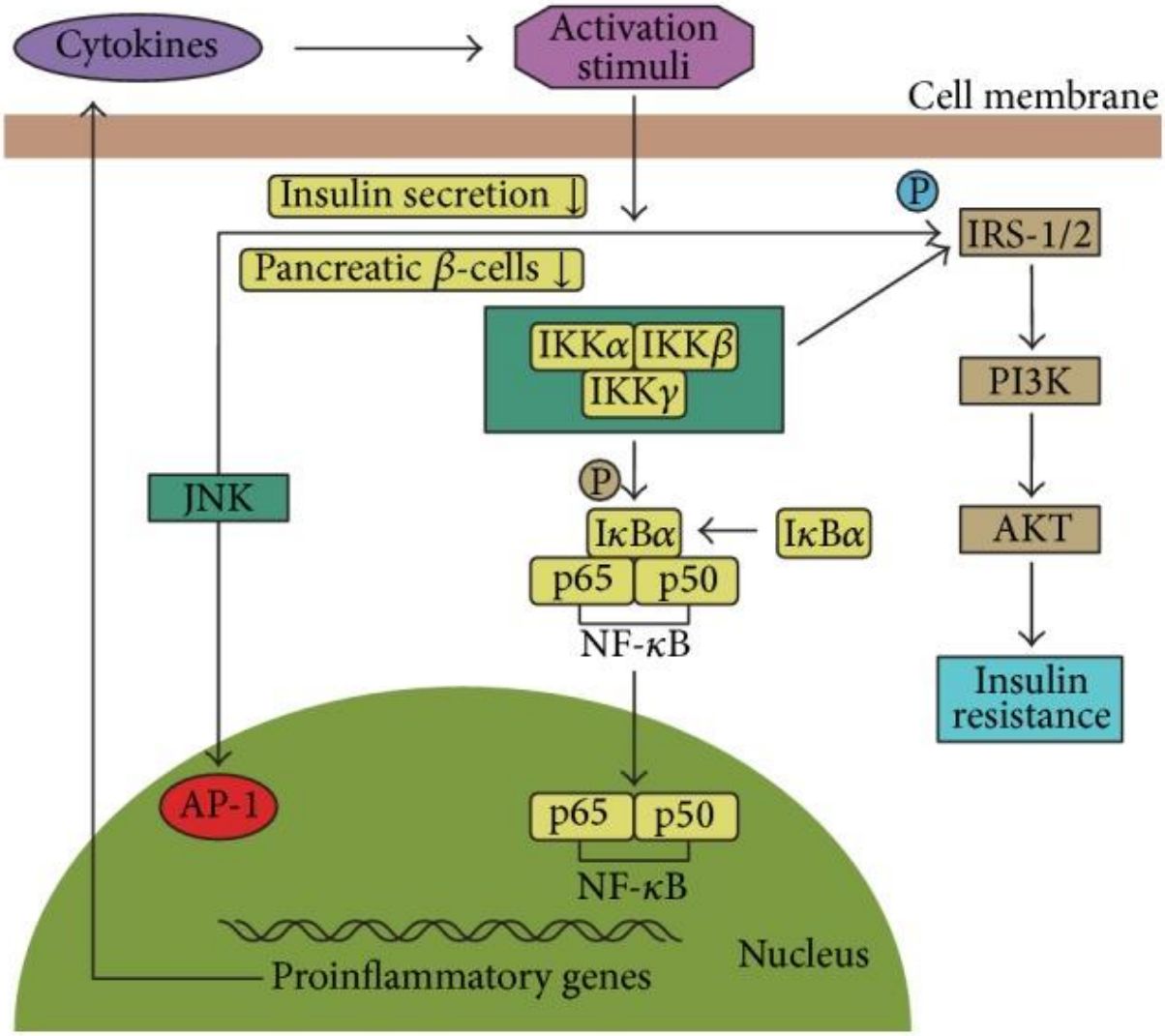
Monocyte chemoattractant protein-1 (MCP-1) is a proinflammatory chemokine produced by adipocytes, macrophages, and endothelial cells, which might lead to the recruitment of macrophages, DCs, and memory T cells (Kanda H., et al 2006). Adipocytes and macrophages are the main source of proinflammatory cytokines. However, the expression of MCP-1 increases during adiposity, which might stimulate the recruitment of macrophages and DCs, which further increases the expression of cytokines to exacerbate inflammation-induced IR. The expression of MCP-1 increases during obesity, particularly in visceral fat areas, which might contribute to the pathogenesis of IR, particularly in the liver. It plays a role in IR by regulating the inflammatory response, insulin sensitivity, lipid metabolism, macrophage polarization and infiltration, and the phosphorylation of extracellular signal-regulated kinase-1/2 (ERK-1/2) and p38 MAPK. C-C motif chemokine receptor 2 (CCR2) is a vital MCP-1 receptor. In adipose tissue of CCR2 knockout mice, macrophage content and inflammatory profile were reduced. CCR2 deficiency also ameliorated hepatic steatosis and improved insulin sensitivity. This suggests that MCP-1 plays a crucial role in the development of both inflammation and IR.

Signaling Pathways Linking Inflammation to Insulin Resistance

IKKβ/NF KB Pathway

NF-κB is a transcription factor comprised of Rel family proteins such as p65/RelA, RelB, c-Rel, p50/p105, and p52/p100. It is involved in a series of pathological processes such as inflammation and innate and adaptive immune responses (Rahman M. M et al 2011). NF-κB is sequestered in the cytoplasm bound to IκB proteins in normal circumstances, which prevents the nuclear localization of NF-κB. After stimulation with various pathogenic stimuli, such as those in obese

individuals, the IKK complex that contains two subunits (IKKα and IKKβ) is activated, which triggers the phosphorylation of IκBα on Ser32 and 36. This leads to the degradation of IκBα, exposes the nuclear localization sequence of NF-κB, and triggers its translocation to the nucleus and the upregulation of target genes that encode inflammatory mediators such as TNF-α, IL-1β, and IL-6. IKKβ deficiency in adipocytes completely prevented the free fatty acid- (FFA-) induced expression of TNF-α and IL-6, whereas the activation of IKKβ inhibited the expression of anti-inflammatory cytokines such as leptin and adiponectin (Jiao P., Ma J., Feng B., et al 2011). According to this, the deletion of IKKβ improved glucose tolerance and insulin sensitivity. In addition, treatments that inhibit NF-κB always improve IR, which suggests that the NF-κB pathway plays an important role in inflammation-associated IR. NF-κB is also a vital intermediary that couples IR to the proinflammatory cytokine IL-1β in IR-related diseases such as obesity and T2DM.



Inflammatory pathways linking inflammation to insulin resistance. Activation of JNK and NF-κB pathways causes serine kinase phosphorylation of IRS-1 or IRS-2, which may block insulin signaling and finally lead to the occurrence of IR. In addition, JNK and NF-κB pathways are involved in the production of proinflammatory cytokines which may in turn become activation stimuli of the pathways.

JNK Pathway

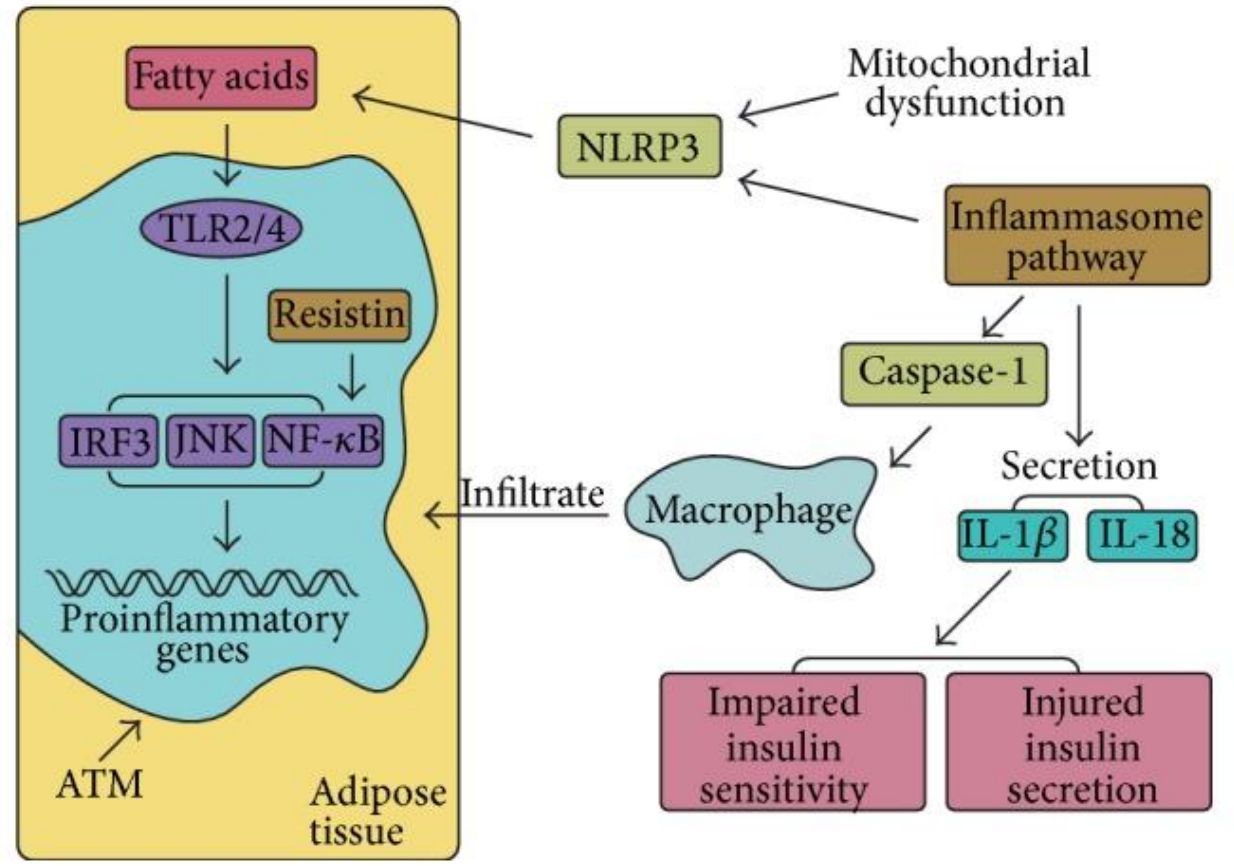
There are three different JNK isoforms (JNK-1, -2, and -3), which belong to MAPK family. JNK contributes to inflammation and metabolic syndrome (MS), obesity, and IR by regulating the

production of proinflammatory cytokines, karyomitosis, and cellular apoptosis (. Pal M et al 2014). JNK can also be stimulated by endoplasmic reticulum (ER) stress, which leads to the serine phosphorylation of IRS-1. JNK plays a role in the phosphorylation of the c-Jun component of activator protein (AP-1) transcription factor, but there is no evidence of a direct relationship between this transcriptional pathway and JNK-reduced IR. The JNK pathway can be activated under diabetic conditions, which might increase IR. Conversely, suppressing the JNK pathway improves IR and glucose tolerance (. Nakatani Y., et al 2004). JNK plays an important role in IR by inhibiting insulin secretion from pancreatic β-cells via proinflammatory stimuli including IL-1. Moreover, the excessive activation of JNK in peripheral insulin-sensitive tissues promotes IR. It was demonstrated that inhibiting JNK reduced the release of IR-related proinflammatory cytokines such as TNF-α and MCP-1. Interestingly, JNK-1 deficiency in adipose tissue protects against hepatic steatosis and promotes glucose intolerance, insulin clearance, IR, and hepatic steatosis. In skeletal muscle, JNK-1 does not affect the development of obesity and IR. However, JNK in isolated rat skeletal muscle plays a vital role during oxidant-induced IR because insulin-stimulated glucose transport activity was improved by the selective inhibition of JNK (Santos F. R., et al 2012). Taken together, these studies suggest that further studies are needed to analyze the effects of JNK in IR.

Inflammasome Pathway

The inflammasome consists of a large group of cytosolic protein complexes and plays roles in inflammation by regulating the secretion of IL-1β and IL-18. Therefore, it is important in innate immunity and metabolic syndromes such as obesity and IR. NOD-like receptor proteins (NLRPs), neutrophilic alkaline phosphatases (NALPs), apoptosis associated speck-like protein (ASC), and caspase-1 are the essential components of inflammasome complexes (Lee B.-C., Lee J. 2014). Inflammasome NLRP3 (nucleotide-binding domain, leucine-rich-containing family, and pyrin domain-containing-3), which links saturated FFAs to chronic inflammation, is being studied extensively because it is highly sensitive to nonmicrobial stress. It can be activated by mitochondrial dysfunction. In addition, the reduced expression of NLRP3 in obesity results in enhanced insulin signaling, decreased inflammation, and improved insulin sensitivity. Caspase-1 is a cysteine protease that contributes to IR by counteracting the metabolic function of adipose

tissue to impair insulin sensitivity and also mediates the infiltration of macrophages into adipose tissues. It was reported that the elimination of ASC and caspase-1 lowers the plasma levels of insulin, leptin, and resistin. Moreover, ASC deficiency might protect individuals against HFD-induced IR, hepatic steatosis, and adipocyte hypertrophy. In addition, caspase-1-deficient mice have high energy expenditure. Taken together, these studies suggest that the inflammasome plays a vital role in obesity-induced IR and that it is an important therapeutic target for the treatment of IR (Stienstra R., et al 2011).



Inflammasome pathway and macrophages are involved in development of insulin resistance. The secretion of IL-β and IL-18 can be regulated by inflammasome pathway. Inflammasome consists of a large group of cytosolic protein complexes including NLRP3 and caspase-1. NLRP3 can be

activated by mitochondrial dysfunction through causing ROS accumulation, and NLRP3 is also a novel molecular link between saturated FFA and chronic inflammation. Caspase-1 mediates macrophages that infiltrate into adipose tissues. Dietary saturated fatty acids lead to activation of TLR2 and TLR4 in ATMs, giving rise to the activation of IRF3, JNK, and NF-κB.

Other Factor Linking Inflammation to IR

Macrophages

Macrophages infiltrate and reside in adipose tissue, named ATMs, and usually play an important role in obesity-induced IR. There are two types of ATM: classically activated (M1) in obese animals and alternatively activated (M2) in lean species (Fujisaka S., Usui I., Bukhari A., et al.). ATMs have an important role in the development of chronic inflammation, including obesity-induced inflammation, because they are the primary source of cytokine production. In addition, obesity might change the number of ATMs by increasing the triple positive CD11b + F4/80 + CD11 + ATM subpopulation. As well as using CD11c as an M1 marker, Fujisaka et al. used CD206 rather than CD209 and CD301 as M2 markers by flow cytometry to demonstrate that IR might be regulated by the number of M1 ATMs and the M1: M2 ratio. In addition, intervention with pioglitazone could reduce inflammation and ameliorate IR by upregulating the expression of IL-10, which might contribute to the reduction of M2 quantity. In another study, it was suggested that the MCP-1/CCR2 axis might contribute to a shift from M2 to M1 polarization, which is an important cause of IR as it leads to the production of inflammatory factors such as TNF-α and IL-6 (Lumeng C. N., et al 2007). During obesity, dietary saturated fatty acids lead to the activation of TLR2 and TLR4 in ATMs, which is followed by the activation of interferon regulatory factor-3 (IRF3), JNK, and NF-κB and subsequent inflammatory signaling.

hs-CRP

C-reactive protein (CRP) is an acute-phase protein synthesized by the liver. It is an inflammatory marker whose expression is increased significantly during inflammation, mainly due to its regulation by proinflammatory cytokines such as IL-6 and TNF-α. In most clinical and scientific

studies, CRP is measured using high-sensitivity assays and is known as high-sensitivity CRP (hs-CRP) (. Hanyu O., Yoshida J., Abe E., et al. 2009). It was suggested that increased hs-CRP levels might be caused by an insufficient insulin-induced suppression of CRP synthesis. Moreover, CRP might contribute to vascular inflammation by activating complement proteins and increasing the production of thrombogenic components bound to the membranes of injured vascular cells, which contributes to the development of IR. In addition, elevated CRP expression is a potential risk factor and indicator for T2DM. However, there is no apparent causality between serum CRP, IR, and diabetes, which suggests that CRP is more likely to be a downstream marker rather than an upstream effector that links inflammation to IR (Brunner E. J et al 2008). Nevertheless, hs-CRP is closely associated with IR, and thus its expression should be assessed during investigations of IR.

The JAK-STAT Signaling Pathway

The Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling pathway are a cytokines-activated cascade involved in many important biological processes including the proliferation, differentiation, and apoptosis of the cells (Xu D., Yin C., Wang S., et al 2014). This signaling pathway contains three components: tyrosine kinase associated receptor, Janus kinase and signal transducer, and activator of transcription. To date, four members of JAK kinase family have been consists of seven proteins (STATs 1, 2, 3, 4, 5A, 5B, and 6). The signaling pathway is initiated through binding of ligands to membrane-bound receptors which may lead to receptor dimerization and then activate the JAK kinases; in turn, the activation of JAK kinases phosphorylates the tyrosine residues with the receptors (Croker B. A., et al 2008). As a result, STAT proteins are phosphorylated by JAK, then dimerize via their src-homology 2 (SH2) domains, and translocate to the nucleus where they regulate transcription of specific target genes involved in multiple diseases including leukemia, rheumatoid arthritis, cancer, and diabetic nephropathy.

L6 cells

Skeletal muscle is recognized as the major tissue of insulin stimulated glucose disposal.Because isolation of skeletal muscle requires intact excision to preserve membrane integrity and the tissue preparation has relatively short survival in vitro, appropriate cell lines are vital to research.

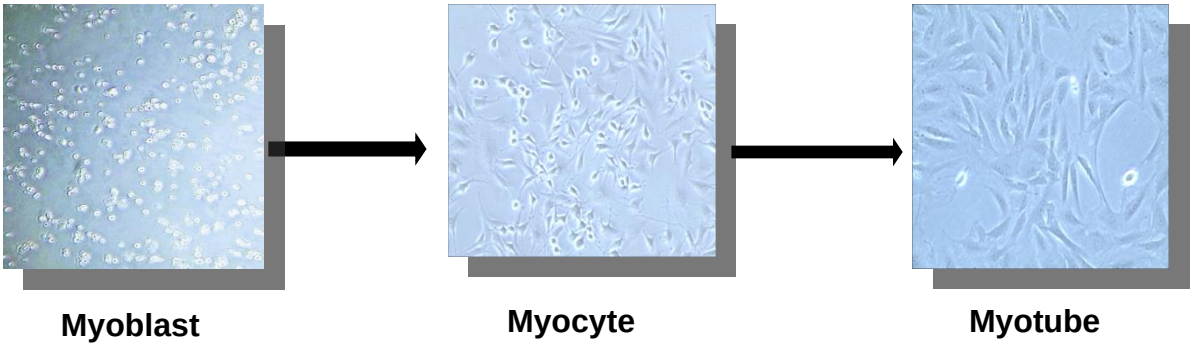


Fig Differentiation of Myoblast to Myotube

Rat L6 myoblast cells, derived from the rat skeletal muscle cell lines, express the morphological, bio-chemical and metabolic properties of isolated skeletal muscle including insulin responsiveness. Rat L6 myoblast cells, derived from the rat skeletal muscle propagate as mononucleated myoblast but can differentiated into multinucleated primary myotubes. These features of L6 myotubes are important since GLUT4 is responsible for insulin dependent glucose uptake in mature skeletal cell.

L6 cells and rat skeletal muscle cells have been shown to have similar sensitivities to the harmful effects of inflammatory cytokines including TNF-a. Hence, L6 cells are used as a tool in exploring the mechanisms involved in the pathogenesis of diabetes.

Skeletal muscle is crucial for whole-body glucose homeostasis, as it is responsible for approximately 80-90% of the insulin-stimulated glucose uptake. When circulating insulin binds to its cell surface receptor, a cascade of intracellular signaling events ultimately leads to the exocytosis of glucose transporter type 4 (GLUT4) to the Plasma-membrane, thus increasing glucose uptake in adipose and muscle tissue (Fujimoto et al,2019).

While the molecular mechanism of targeted intracellular GLUT4 trafficking has been extensively studied in cultured adiposities, less is known about the factors regulating GLUT4 trafficking in skeletal muscle cells. This is especially relevant for a better understanding of the pathophysiology of diabetes and insulin resistance where insulin- induced GLUT4 trafficking and consequently, glucose transporters areimpaired. Deciphering the molecular mechanisms of insulin-dependent GLUT4 delivery and glucose uptake will further our knowledge of how skeletal muscle contributes to glucose homeostasis.

Different remedies and drugs have been used to treat T2DM, including insulin before knowledge of its mechanism of action. Some of them have been included in the therapeutic arsenal of medicine, and others are used as complementary therapy in patients with hyperglycemia. Several of these compounds have been obtained from plants or from microbes. Classic examples are galegine, phenolics compounds and medicinal plant described with a clear antidiabetic effect was *Galega officinalis* L. (Fabaceae), which has been prescribed since the Middle Age to treat diabetes mellitus. From this plant, also called goat's rue, French lilac, or Italian fitch, a guanidine derivative, galegine, was isolated. This compound, whose chemical structure is quite similar to the antidiabetic drug metformin, is responsible for the lowering of blood glucose pycnogenol derived from plants, and acarbose, miglitol, and voglibose from microbes.(Bedekar A, Shah K,2010)

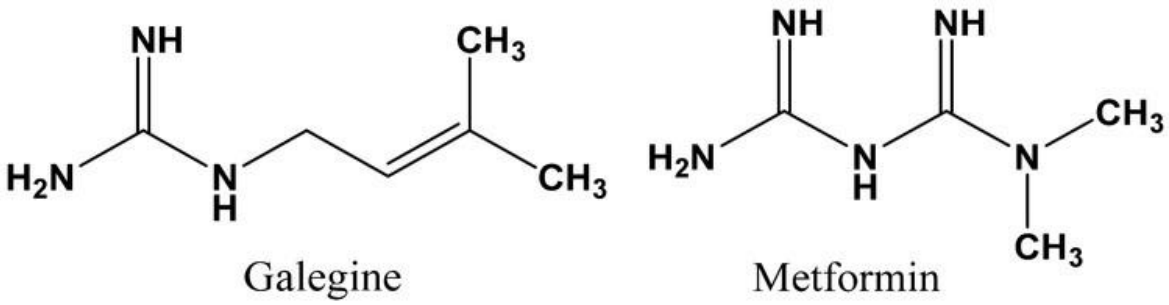


Fig Chemical structures of galegine and metformin.

Different 0henolic compounds such as flavonoids and antocyanins have positive effects on diabetes (Nelson-Dooley C,et al 2005). For example, different anthocyanins from *Ipomoea batatas* (L.) Poir. (Convolvulaceae) and *Pharbitis nil* (L.) Choisy (Convolvulaceae) are effective inhibitors of intestinal α-glucosidase/maltase activity and can reduce glycemia after starch-rich meals. Inhibitory effects of anthocyanins depend on their structure, as their potency as α-glucosidase inhibitors is higher in acylated anthocyanins than in deacylated derivatives (Bedekar A, Shah K, 2010).

A third example of a natural product with antidiabetic properties is pycnogenol, a patented water extract obtained from French maritime pine bark (*Pinus pinaster* Aiton, Pinaceae) rich in polyphenols. Antidiabetic properties of pycnogenol are due to its digestive enzyme inhibitory activity, especially against α-glucosidase (Schafer A, Hogger P.2007)of the compounds present in the mixture, only (+)-catechin and procyanidins are able to inhibit α-glucosidase. Pycnogenol also competitively inhibited α-amylases from the human salivary glands and the porcine pancreas.Pycnogenol (100 mg/day for 3 months) in addition to conventional treatment with oral antidiabetic drugs reduced blood glucose levels and improved endothelial function in patients with T2DM. Moreover, this reduction in glycemia was dose dependent without increasing insulin secretion.

Materials

Table No.2: List of chemicals used

Chemicals	Catalog No.	Provider Company
Cyanmethemoglobin	25ST201-31	Cogent
Dimethylesulphoxide	D8418	Sigma- Aldrich
Drabkins	D5941	Sigma-Aldrich
Ethanol	K40839983	Merck millipore
Fetal Bovine Serum	10082-147	Life technologies (Gibco)
HEPES	AC74570146	Merck millipore
Giema stain powder	GRM128	Himedia
Glacial acetic acid	100056	Merck millipore
D- Glucose	108342	Merck millipore
Minimum essential medium eagle	M0643	Sigma-Aldrich
Peptone	136136	Thomas Baker
TMB Substrate Reagent	555214	BD OptEIA
Potassium chloride	13305	Qualigens
Potassium dihydrogen phosphate	PCT0009	Himedia
Sodium bicarbonate	RM 467	Himedia
Sodium chloride	GRM853	Himedia
Sodium hydroxide	RM467	Himedia
TMB stop solution	1857852	Thermo scientific
Streptopenicillin antibiotic	RM3063	Himedia
Tris buffer	GRM262	Himedia
TMB stop solution	1857852	Thermo scientific
Trypsin	T2600000	Sigma-Aldrich

Table No.3: Kits

Kits	**Catalogue No.**	**Provider Company**
Stop solution	51-2608KZ	BD OptEIA
Substrate reagent A	51-2606KZ	BD OptEIA
Substrate reagent B	51-2607KZ	BD OptEIA
Mouse TNF-α ELISA kit	558534	BD Pharmingen

Table No.4: Instruments

Instruments	Provider Company
Autoclave	Lab guard
p H meter	Labman
Carbon Dioxide Incubator	Esco
Centrifuge	Eppendorf, Sigma
Cryofreezing container	Indian oil
Cryo box	Tarson
Vortex	TarsonSpinix
Weighing Balance	Sartorius
Spectramax plate reader	Molecular Devices
Freezer (-80°C, -20°C, 4°C)	New Brunswick scientific, Blue star Celfrost
Ice Machine	AngelantoniIndustrie
Inverted Microscope	Nikon
Laminar air flow chamber	Haier
Light microscope	Leica
Magnetic stirrer	Spinot

Table No.5: Plastic wares and glass wares

Materials	Provider Company
Beakers	Borosil
75 mm^2 ,25 mm^2 Culture flasks	Corning
Falcon tubes	Axygen,Tarson
Filter unit (0.22µm)	Millipore
Hemocytometers	Rohem
Cryopreservation vials	Corning
Measuring cylinders	Borosil
Microscopic glass slides	Blue star
Pipettes	Eppendorf, Thermoscientific
Schott Bottles	Duran
Single use syringes	Dispovan
Syringe filters	Nalgene
Tissue culture 96,48,24,6 well plates	Axygen,Greiner bio-one

Table No.6: List ofMedia and buffers

Dulbecco's modified eagle's medium for in-vitro cell culture of primary peritoneal macrophages and cell lines

Components	Quantity
Minimum essential medium Eagle	9.6 gm
HEPES	2.25 gm
$NaHCO_3$	2.2 gm
FBS (10%)	100 ml
Streptopencillin (1%)	10 ml

All the components were dissolved in 800 ml of autoclaved DW, volume was made upto 1 L and filter sterilized by 0.22 µ filter in laminar flow.

- **For glucose media add 4.5 g D-glucose**

Table No.7: Cryopreservation Buffer

Components	Quantity
FBS	90 ml
DMSO (10%)	10 ml

Table No.8: Phosphate Buffer saline solution (PBS)

Reagents	Quantity
NaCl	160 gm
Na_2HPO_4	23.6gm
KH_2PO_4	4gm
KCl	4gm

All the reagents were mixed in 800ml DW and final volume was made upto 1000ml.The p H was adjusted to 7.4.The prepared solutions were autoclaved and stored at 4°C.

Table No.10: RBC diluting fluid

Reagents	Quantity
Formalin	1 %
Tri sodium citrate	31.3gm

For preparing 500ml RBC diluting fluid, 15.65 gm of tri sodium citrate was dissolved in 500ml of distilled H2O +10ml of 1% formaldehyde solution.

Table No.11: WBC diluting fluid

Reagents	Quantity
Glacial acetic acid	2ml
Gentian violet solution	2ml

For preparing gentian violet solution, 0.1gm of gentian violet was dissolved in 100ml distilled water.

Table No.12: Trypsin-EDTA

Reagents	Quantity
EDTA 0.5M	1%
Trypsin	0.1gm

→ First of all 1% EDTA solution was prepared. For this 0.2gm of EDTA was dissolved in 20ml PBS and p H was maintained 7.2-7.4

→ 0.1gm trypsin was added to 20ml EDTA solution and finally volume was made up to volume 100ml with PBS

→ The solution was filtered with syringe filter 0.22µm and stored at -20°C by making aliquots.

Table No.13: Drug solutions

Prepared fresh or taken from the stock solutions stored at 0^{o}C.

Drug Solution	Stock	Solvent
IVT 2271, IVT 2272, and IVT 2273	10 mg/ml	DMSO

Methods

Preparation of stock solutions

The pure molecule was dissolved in DMSO to produce 10mg/ml stock solution. The suspensions were sterilized using 0.22 µM Millipore nylon syringe filters before using for in-vitro experiments. Hence the different concentrations of pure molecule were prepared in laminar hood and tested.

Cell Culture and differentiation

Rat L6 myoblast cells, derived from the rat skeletal muscle propagate as mononucleated myoblast but can differentiated into multinucleated primary myotubes. These cells are adherent in nature and fuse in culture to form multinucleated myotubes. Myotubes represent mature muscle fibers and express GLUT4. These can therefore be used as a tool to measure the kinetics of glucose transport. The degree of fusion of cells decreases with passaging. Hence, the freezing of cells was performed at low passage number.

Rat-derived L6 myoblasts were obtained from National Centre for Cell Science (NCCS), Pune, India for screening in glucose uptake assay. Rat L6 cells were cultured in 96 well culturing plates & were grown in DMEM 25 cm^2 culturing flasks in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS and 1% v/v antibiotic–antimycotic solution (10,000 units/ml penicillin G, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B) at 5% CO_2 and 37°C until the confluence reached by 80%. For myotubes formation, the cells were cultured in DMEM containing 2% FBS for 5 days. The media were changed every 48 h and cells were used at the stage of myotubes (60–70%) when expression of GLUT4 level is highest.

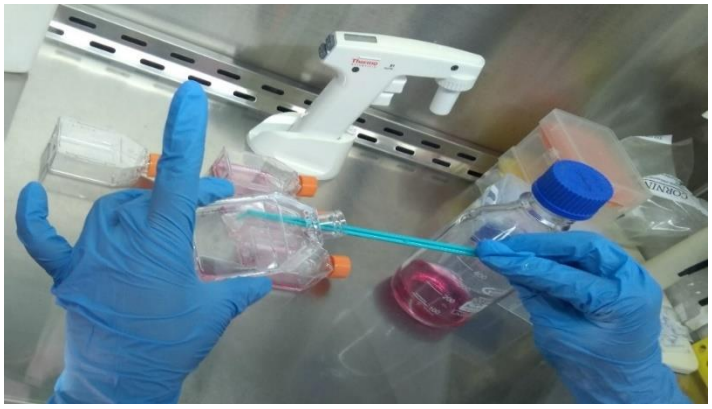


Figure 3.2.1. Cell culture

3.2.2. Sub-culturing of cells

Before sub-culturing of cells, the degree of confluency (80-90%) was access using an inverted microscope and it is necessary to confirm that there are no bacterial and fungal contaminations in the cultured flask. The media was removed after the cells were found attached in the flask and the cell monolayer was washed with PBS. The cells were detached from the flasks by addition of trypsin-EDTA solution. The flask was kept at 37ºC for 10-15 minutes. Cells were observed under the microscope to ensure that all cells were detached and floating. The cells were resuspended in a small volume of fresh medium with the help of pipette and the required number of cells was transferred to new flask containing fresh media. The culture flask was kept in incubator at 37 ºC with 5% CO_2.The cells are treated and further the supernatant is collected for standardization of glucose uptake assay.

3.2.3. Cell Counting

The cell counting was done with the help of hemocytometer. To prepare the hemocytometer the mirror like polished surface is carefully cleaned with ethanol and is placed under the microscope. The coverslips should also be cleaned and placed over the mirror surface prior to addition of cell suspension. The cell suspension was introduced into the grid with the help of pipette. The counting chamber was placed on the microscope stage and the counting area was focused at 40X objective. The sample was allowed to settle for about 30-50 seconds without disturbing the coverslip. The counting was performed by counting the cells inside the four corner squares and the middle grid. The main divisions separate the grid into 9 large squares. Each square has a surface area of $1mm^2$; the depth of the chamber is 0.1mm. Each square of hemocytometer (with cover slip in place) represents a total volume of $0.1mm^3$or 10^{-4} $cm^{3.}$ Since 1 cm^3=1ml, the subsequent cell population per ml is calculated using the following formula.

$$\text{Total Cells/ml} = \frac{\text{Total cell counted} \times \text{dilution factor} \times 10000}{\text{No of Squares}} \quad \text{cells/ml}$$

3.2.4. Cell seeding and differentiation

Seeding density of the cells was decided based on literature and initial optimisation studies. This was also dependent on the assay format. For glucose uptake assay performed in 96 well plates, the seeding density used was $2.5x10^5$ cells per ml.

2×10^5 cells/ml were seeded in T-75 flasks. Myotube differentiation was initiated 48 hours after seeding by reducing serum percentage in the complete medium from 10 percent to 2 percent. The medium was replenished every 48 hours till the seventh day by when myoblasts were completely differentiated into myotubes. All studies were performed at this stage i.e. on the myotubes Differentiated L6 myotubes in 96-well plates were studied.Count the cells. Dilute the volume by serial dilution to get 100 cells in 1ml.For ex if you get1 million count resuspend in 10 ml. Take 100 µl from 1st dilution and make 1ml. Take 10 µl from dilution 2 and make them to 1 ml. you get 100 cells in 1ml. Distribute100 µl per well. Distribute in 96 well plate. 80% well can have single cells. Some wells have no cells. Some wells may have 2 cells. omit the wells with no cells and more than 1 cell. In serial dilution, mix well otherwise cells get settle in bottom.

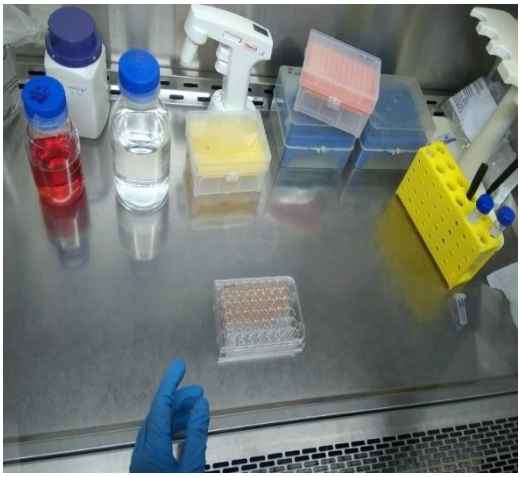


Figure 3.2.4. Cell seeding

Cryopreservation of cells

Before cryopreservation of cells, the cell viability (>90%) was assessed and the presence of contamination was examined. The media was completely removed and the cell monolayer was washed with PBS. The trypsinization was done as mentioned in section. The cells were counted by Hemocytometers .100µL of cryopreservation buffer and 900µl of cells ($1x10^6$ cells/ml) was added to cryovial .The cryovials were kept in 4°C for 1 hour, then shifted to 0°C for 2 hours and left overnight in -20°C.Finally the next day the cells were transferred to the liquid nitrogen container -196°C.

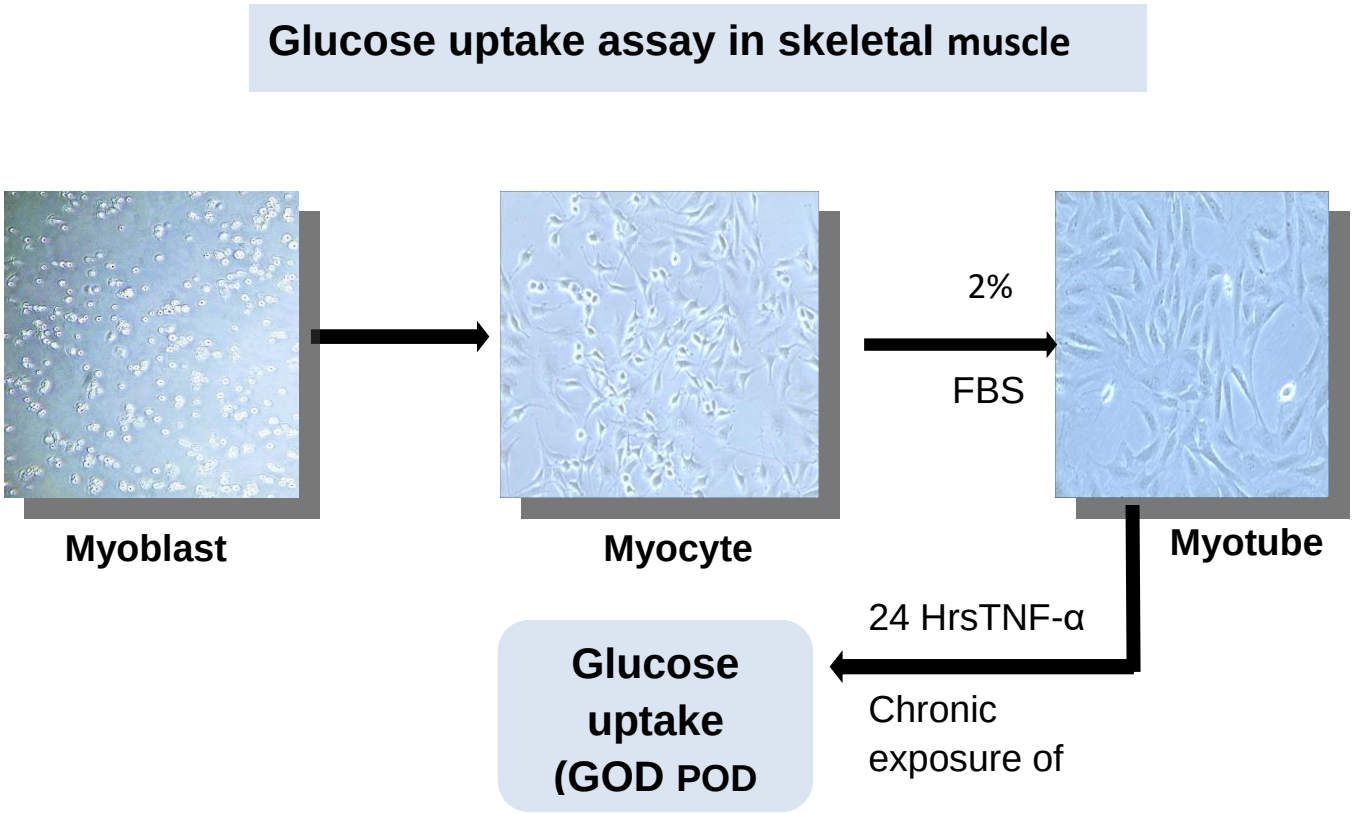


Figure 3.2.5. Glucose uptake assay

3.3. Experimental design

After myotube formation, before initiation of treatment, the cells were starved in serum free medium for 4 hours. The cells were then treated with TNF to evaluate the impact of insulin

resistance; the cells were treated with $Mncl_2$ for 3 days (chronic exposure) at different concentration before compound treatment. After compound treatment for the indicated time period, glucose uptake assay, was performed. DMSO concentration of 0.5% was considered as vehicle control.

3.3.1. Standardization of glucose uptake assay

Glucose consumption was then determined with a glucose assay kit (glucose oxidase-peroxidase method). L6 myoblasts (5×10^5 cells/well) cells will be sub-cultured into 96- well plates and grown for 7 days in 2% FBS until they formed myotubes. Insulin resistance condition will be induced by the cells with chronic exposure of $Mncl_2$ at (50,100 & 500µM)for three days and TNF-α (7 ng/ml) was co-incubated for another 24 h. The myotubes will be then cultured in KHH buffer containing 11 mM glucose in the absence or the presence of 1 µM of insulin for another 4 h. Glucose concentrations in Krebs–Henseleit buffer (KHH) were determined with ERBA Glucose kit (GOD-POD Method, end point) using standard kit and the amounts of glucose consumed were calculated from the differences in glucose concentrations between before and after culture.

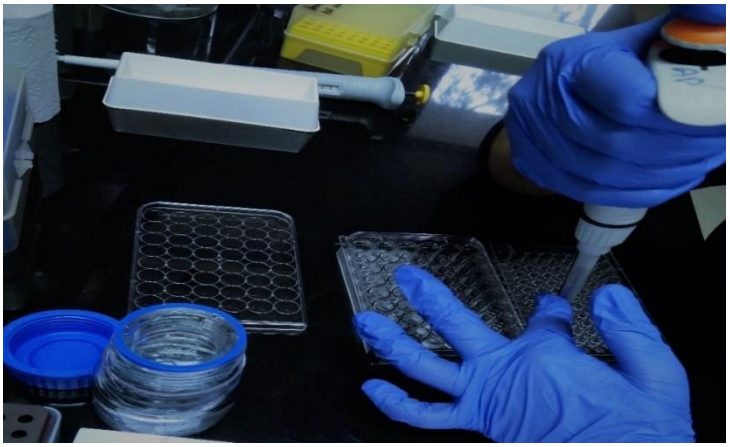


Figure 3.3.1. Standardization of Glucose uptake

3.3.2. Sample preparations

IVT 2271, IVT 2272, and IVT 2273 purchased from Sigma Life-science Pvt Ltd, India. It is found in citrus fruits. It has been found to possess anti-inflammatory, antihyperglycemic,

antihyperlipidemic, anti-oxidant, anti-tumor, antifungal, anti-viral, immune regulation and hepatoprotective effect cardioprotective, antidepressant and neuroprotective effect. The present study was done to examine the effects of sub-chronic, low-dose Mn exposure on indicators of β-cell function and assess the effects of**IVT 2271, IVT 2272, and IVT 2273** in diabetic condition.

Glucose uptake profile of Bioactiveagainst insulin resistance associated with TNF-α in skeletal muscle cell line

Glucose uptake assay was examined by the procedure glucose oxidase-peroxidase method or GOD-POD method. Briefly, L6 myoblasts (5×10^4 cells/well) were sub-cultured into 96-place multi-well plates and grown for 7days in 2% FBS until they formed myotubes. Insulin resistance condition will be induced by the cells with chronic exposure of $Mncl_2$ at (500µM)for three days. Next, TNF-α (7 ng/ml) was co-incubated to induce insulin resistance and IVT-GT1, IVT-GT2 & IVT-GT (1+2)at different concentration of 5 and 10 µg/ml for another 24 h. The myotubes will be then cultured in KHH buffer containing 11 mM glucose in the absence or the presence of 1 µM of insulin for another 4 h. Glucose concentrations in Krebs–Henseleit buffer (KHH) were determined with ERBA Glucose kit [Catalog No-120200, Provider Company- Erba Mannheim] .GOD-POD Method or end point using standard kit and the amounts of glucose consumed were calculated from the differences in glucose concentrations between before and after culture.

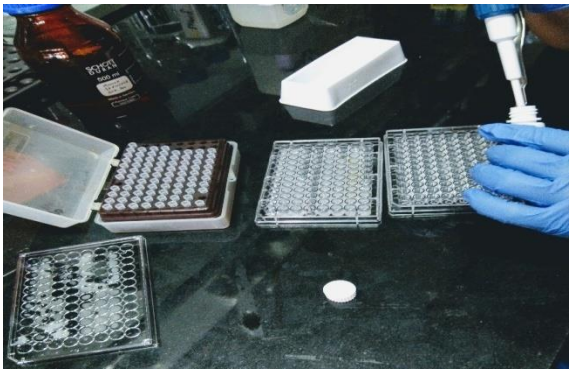


Figure 3.3.3 Glucose ake profile of bioactives

Acute and sub- acute oral toxicity studies in Swiss albino mice-

Acute and oral test toxicity for the potent phytomolecule or potent plant extract was carried out on Swiss albino male/female mice following IAEC approved protocol no and following OECD test guidelines and methodology published from the lab (Chanda et al.,2009) for acute oral toxicity and (Singh et al., 2019) for sub-acute oral toxicity . The experimental mice (20-22g) were obtained from the breeding facility of in-vivo testing lab of the institute. The animals were maintained at 22 ± 5°C with humidity control and were given standard mouse pellet and ad libitum access to drinking water. The animal was acclimatized to the experimental environment for a period 5-7 days before commencement of actual experiment.

Briefly, in acute oral toxicity 15 male and 15 female Swiss albino mice were taken and divided into five groups each group were treated as single acute oral dose after suspending the groundnut oil while controls animals received only matching volume of vehicle. The animal was absorbed for mortality, morbility and any sign of ill health for a period of 7 days. At the end of experimental period , sample were harvested and analyzed for hematology and serum biochemistry using diagnostic kits and following the prescribed methodology in UV-visible micro plate reader 9 spectra max 384 molecular device USA) The cell counts in hematology were carried out manually using light microscope . The gross pathological observation of vital organ like liver, heart, kidney, spleen, lungs, and brain were recorded including their gross and relative organ weight.

In the sub- acute oral toxicity, fractional doses of the maximum tolerated dose from acute oral toxicity was considered and phytomolecules was given at once daily through oral route for 28 days to Swiss albino mice and control animals were treated with vehicle .Like acute oral toxicity each group have 3 male and 3 female mice and animals were observed for 28 days for morbidity, mortality and any sign of ill health as started in acute oral toxicity.

Haematology, serum biochemistry and gross pathology were studied as described in the acute oral toxicity test.

4. Result

In-vitro Insulin study

Objective 1: Standardization of Glucose Uptake Assay in Insulin Resistance associated Tumour Necrosis factor-α (TNF-α) in Skeletal Muscle Cell.

L6 myoblast (2 x 10^5 cells/ml)were sub cultured into 96 well and grow for 7 days in 2% FBS until they form myotube. Insulin resistance condition will be induced by the cells with chronic exposure of and TNF-α (7 ng/ml) was co-incubated for another 24 h.The myotubes will be then cultured in KHH buffer containing 11 mM glucose in the absence or the presence of 1 μM of insulin for another 4 h. Glucose concentrations in Krebs–Henseleit buffer (KHH) were determined with ERBA Glucose kit (GOD-POD Method, end point). TNF- α significantly decreased insulin stimulated glucose uptake by 70% compare to the one with insulin alone.

Sample	TNF-α	Insulin	Glucose Uptake(μg/dl)
Normal	-	-	33.41 ± 9.39
Insulin(1μM)	-	✓	140.36± 6.07#
Insulin+ TNF-α (1μM + 10 ng/ml)	✓	✓	41.38± 13.76*

Table 1 TNF-α significantly decreased insulin stimulated glucose uptake by 50% compare to the one with insulin alone. Each value represents the Mean ± SEM n=3 $p<0.05$ #Normal Vs insulin alone, *Insulin Vs Insulin +TNF-α. ANOVA, Tukey's Multiple Comparison Test

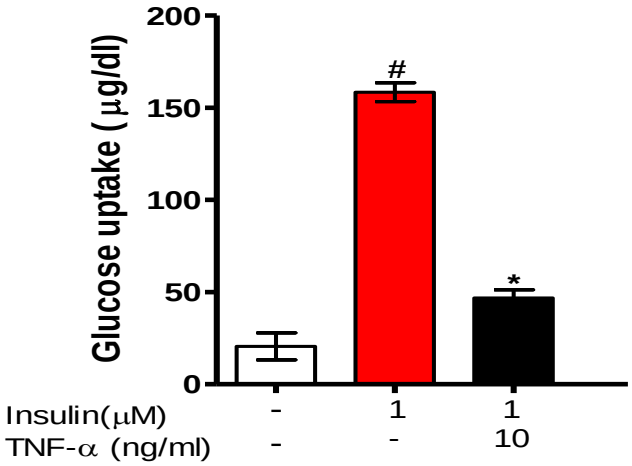


Fig. 1 TNF-α significantly decreased insulin stimulated glucose uptake by 50% compare to the one with insulin alone. Each value represents the Mean ± SEM n=3 $p<0.05$ #Normal Vs insulin alone, *Insulin Vs Insulin +TNF-α. ANOVA, Tukey's Multiple Comparison Test

Objective 2: Glucose uptake profile of Bio-actives against insulin resistance associated with TNF-α in skeletal muscle cell line.

L6 myoblasts (5×10^4 cells/well) were sub-cultured into 96-place multi-well plates and grown for 7days in 2% FBS until they formed myotubes. Insulin resistance condition will be induced by the cells with chronic exposure of TNF-α (7 ng/ml) was co-incubated to induce insulin resistance and **IVT 2271, IVT 2272, and IVT 2273** at different concentration of 1, 3 and 10 µg/ml for another 24 h TNF-α significantly decreased insulin stimulated glucose uptake by 70% compared to the one with insulin alone. However, pre-incubation of**IVT 2271, IVT 2272, and IVT 2273** prior to TNF-α treatment significantly restored the decreased glucose uptake.

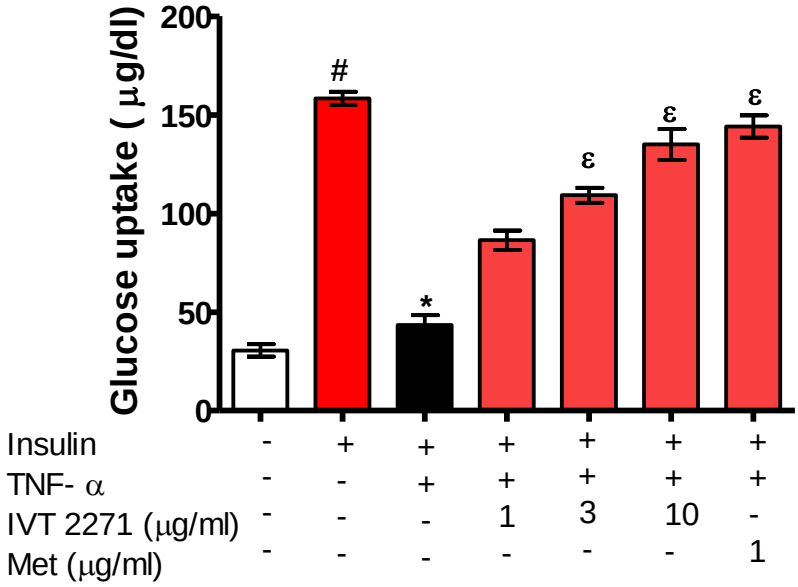


Fig. 2. Glucose uptake assay in differentiated L6 myotubes in response to IVT 2271 treatment. Each value represents the Mean ± SEM n=3 $p<0.05$ [#]Normal Vs insulin alone, *Insulin VsInsulin +TNF-α; [i]nsulin + TNF-α Vs Insulin +TNF +Treatment ANOVA, Tukey's MultipleComparison Test.

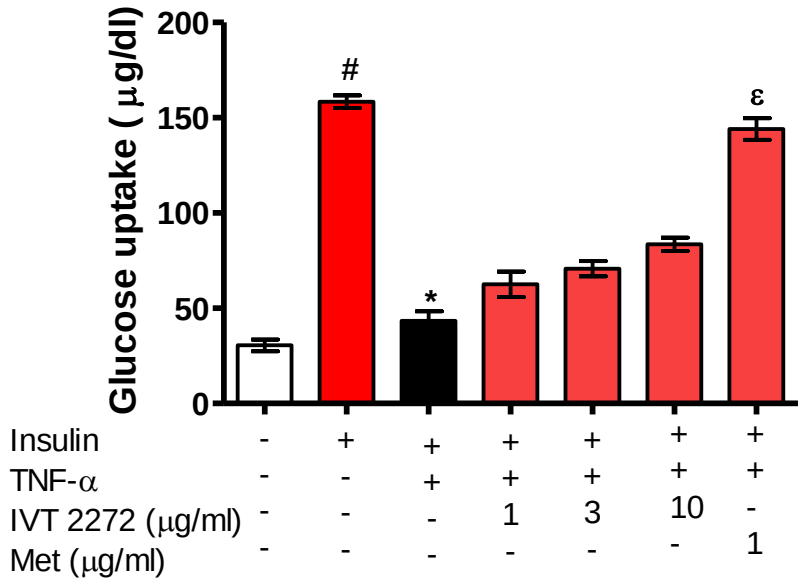


Fig. 3. Glucose uptake assay in differentiated L6 myotubes in response to IVT 2272 treatment. Each value represents the Mean ± SEM n=3 $p<0.05$ [#]Normal Vs insulin alone, *Insulin VsInsulin +TNF-α; [i]nsulin + TNF-α Vs Insulin +TNF +Treatment ANOVA, Tukey's MultipleComparison Test.

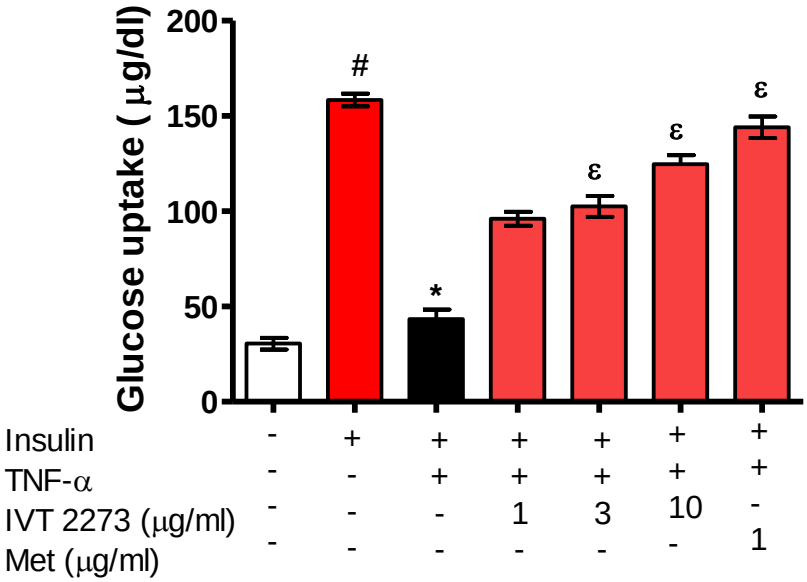


Fig. 4. Glucose uptake assay in differentiated L6 myotubes in response to IVT 2273 treatment. Each value represents the Mean ± SEM n=3 $p<0.05$ [#]Normal Vs insulin alone, *Insulin VsInsulin +TNF-α; [i]nsulin + TNF-α Vs Insulin +TNF +Treatment ANOVA, Tukey's MultipleComparison Test.

Objective 3: In-vitro toxicity profile of IVT 2271, IVT 2272, and IVT 2273 by MTT assay.

To examine the direct effect **IVT 2271, IVT 2272, and IVT 2273** to the cells, we first examined the cell viability using the MTT assay. DAC, GYM and GENIN at the dose of 1,3,10 and 100 did not affect the cell viability suggesting these doses of **IVT 2271, IVT 2272, and IVT 2273** did not of direct toxic effect to the cell.

Treatment	Dose (µg/ml)	% Cell viability
		Skeletal Muscles Cells (L6)
IVT 2271	1	99.16 ± 2.54
	3	98.04 ± 0.61
	10	96.22 ± 0.98
	100	44.12 ± 0.78
IVT 2272	1	99.16 ± 2.54
	3	99.12 ± 0.62
	10	97.28 ± 0.23
	100	42.58 ± 1.11
IVT 2273	1	99.76 ± 1.72
	3	98.01 ± 1 .51
	10	97.42 ± 0.75
	100	91.62 ± 1.73

Table 1: Effect of**IVT 2271, IVT 2272, and IVT 2273**on Percent (%) Cell viability In Skeletal Muscles Cells (L6) cells using MTT assay; n=3.

Objective 4: To learn the techniques used in drug discovery and development.

a. To learn the techniques of acute toxicity.

b. To learn the techniques of sub- acute toxicity.

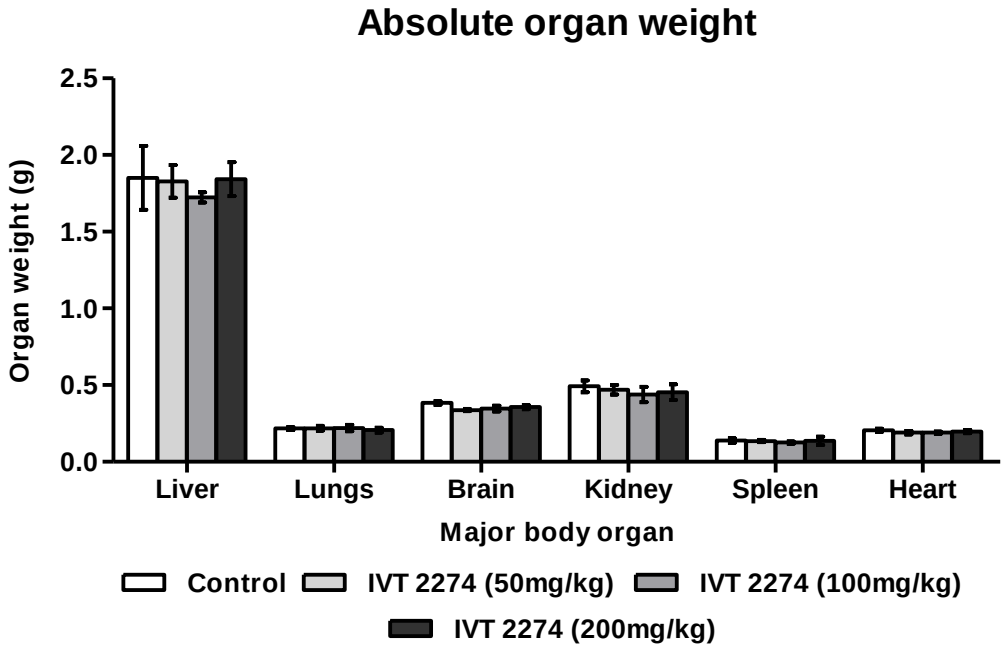


Figure 1. Effect of (IVT 2274) at 50, 100 and 200 mg/kg dose on absolute and relative organ weight in Swiss albino mice (n=6, non-significant changes were found compared to control).

Parameters	Dose of (IVT 2274)at 50, 100 and 200mg/kg body weight.			
	Control	50 mg/kg	100 mg/kg	200 mg/kg
Gain in body weight (gm)	13.63±1.30	12.48±1.61	12.31±1.75	13.56±1.43
Haemoglobin (gm/dL)	7.60±0.48	7.42±0.76	7.27±0.68	8.56±0.31
RBC (million/mm^3)	7.44±0.38	7.27±0.22	7.39±0.32	7.66±0.48

WBC (1000*/mm^3)	10.51±1.64	11.43±1.49	10.54±1.53	10.83±1.19
ALKP (U/L)	254.56±22.26	255.26±37.07	253.20±12.60	253.74±25.08
SGOT(U/L)	24.78±1.93	24.25±2.36	28.52±2.76	26.23±4.40
SGPT(U/L)	15.17±1.45	15.27±1.71	14.82±1.69	15.26±1.48
Creatinine (mg/dL)	0.84±0.01	0.83±0.03	0.82±0.04	0.85±0.03
Triglycerides (mg/dL)	161.45±16.95	161.26±18.08	162.47±20.74	162.98±41.26
Total protein (mg/ml)	7.40±1.35	7.16±0.15	7.56±0.12	7.39±0.23
Cholesterol (mg/dL)	93.26±9.02	93.59±4.34	96.22±0.70	92.36±12.36
Albumin (g/dL)	2.47±0.07	2.75±0.03	2.72±0.05	2.91±0.21
Bilirubin (mg/dl)	0.17±0.05	0.18±0.02	0.20±0.03	0.21±0.02

Table1. Effect of (IVT 2274) as a Sub-acute oral dose at 50mg/kg, 100mg/kg and 200mg/kg on body weight, haematological and serum biochemical parameters in Swiss albino mice (Mean ± SE; n=6; a, $P<0.05$ compared to control).

Discussion

Plant-derived extracts and bioactives are known to have anti-diabetic properties in the traditional systems of medicine as well as peer reviewed publications. Despite considerable progress in the treatment of metabolic disorders includes diabetes and obesity due to Insulin Resistance condition using modern therapeutic agents, search for newer drugs continues because the existing synthetic drugs have several limitations. The aim for this study was to evaluate the Glucose Uptake Potential of Selected Bioactives against Manganese and TNF-α Induced Insulin Resistance in Skeletal Muscle and encoded as IVT-2271, IVT-2272 & IVT-2273 for conducting a blind screening. Control of disease by immunological means has two aspects namely development of preventing agents and development of therapeutic agents to cure the disease. The bioactives selected for this research work are derived from fruit waste. Fruit wastes are one of the main sources of municipal waste and flavonoids are major class of secondary metabolites in fruit waste which have beneficial effects on human health. The fruit residues could be inexpensive and readily available resources of bioactive compounds for use in the pharmaceutical industries.

Insulin carries out vital actions in energy metabolism and Insulin resistance (IR) is defined as a series of clinical manifestations for diminished effectiveness of insulin in lowering blood sugar levels caused by decreased sensitivity to insulin of liver, muscle and adipose tissue. IR is the major contributor to the etiology and pathogenesis of type 2 diabetes mellitus (DM2). (Khodabandehloo et al., 2016). The incidence of DM2 is rapidly increasing and is currently one of the most challenging health problems in the world. According to the International Diabetes Federation (IDF), currently it affects more than 387 million people worldwide, and by the year 2035, potentially it will affect more than 592 million (Gutierrez et al., 2017). DM2, also known as “non-insulin dependent diabetes” or “adult diabetes”, is a chronic-degenerative disease characterized by the presence of insulin resistance, a condition where cells that usually respond to insulin stop doing it, and/or due to relative deficiency of this hormone in the body. Insulin, which carries out vitalfunctions especially in energy metabolism, also participates in the regulation of different processes at the cardiovascular level and in the central nervous system (Shulman, 2000). Inflammation is an immune response of tissues to deal with injuries, which rely upon metabolic support and energy redistribution and the long-term consequences of

prolonged inflammation have detrimental effects (Singh et al., 2014. Intervention studies have demonstrated the occurrence of inflammation in DM2 pathogenesis, especially that with IR (olefsky et L.,2010).Elevated levels of pro-inflammatory cytokines, such as tumor necrosis factor- (TNF-α), interleukin-6 (IL-6)12, interleukin-1β (IL-1β) have been reported in various diabetic and IR states.(Festa et al., 2000).

7. Future Direction

- The further molecular mechanism of IVT-2271, IVT-2272 & IVT-2273 with special emphasis on antidiabetic signalling pathway using In-vitro bioassays.
- It can also be further validated in physiological condition (*in-vivo* system) using standard anti-diabetic linked rat animal models.

4) TECHNIQUES LEARNED

4.1. Techniques involved in in-vitro Experimental Pharmacology

4.1.1. CELL CULTURE

1: **Animal Cell Culture**

Animal cell culture basically involves the in-vitro (in the laboratory) maintenance and propagation of animal cells in a suitable nutrient media. Thus, culturing is a process of growing cells artificially. Cell culture has become an indispensible technology in various branches of life sciences. Animal cells can be cultured either using a completely natural medium or an artificial/synthetic medium along with some natural products.

Equipments for Cell Culture

1. CO_2 Incubator

Water jacketed incubators are required to facilitate optimum cell growth under strictly maintained and regulated conditions, normally requiring a constant temperature of 37°C and an atmosphere of 5-10% CO_2 plus air. The purpose of CO_2 is to ensure that culture medium is maintained at required physiological pH (usually 7.2-7.4).This is achieved by the supply of CO_2 by glass cylinder into the incubator through a wall that is triggered to draw in CO_2 whenever the level falls below the set level. The CO_2 that enters dissolves in the culture medium contain bicarbonate. The latter reacts with H^+ generated from cellular metabolism to form carbonic acid, which is in equilibrium with water and CO_2, thereby maintaining the pH in the medium at approximately 7.2. These incubators are generally humidified by a tray of sterile water at the bottom deck. The evaporation of water creates highly humidified atmosphere which helps to prevent evaporation of medium from culture.

Figure No.14: CO_2 Incubator

2: Microscope:

Inverted phase contrast microscopes are usually employed for visualizing cells in culture medium. They are expensive but easy to operate with light source located above and the objective lenses below the stage on which the cells are placed. Visualization of cells by microscopy provides useful information about morphology and state of the cells. Early signs of stress can easily be identified and thus precautionary measures can be taken.

Figure No.15: Inverted Microscope

3. AUTOCLAVE:

Autoclave is sterilization equipment used to sterilize glassware, culture media, culture waste, etc. It works on the principle of moist heat sterilization in which the article to be sterilized is subjected to the high pressured steam (15psi) at 121^0C, for about 15–20 minutes. Such extreme temperature and pressure eliminates any living organism that can cause contamination. Depending upon its orientation, autoclaves can either be horizontal or vertical.

Figure No.16: Autoclave

4. HORIZONTAL LAMINAR AIR FLOW CABINET:

It is a device which creates a sterile environment within its working area by the virtue of laminar flow of air. Laminar flow is the motion of a medium (fluid or a gas) in parallel layers, with no disruption between the layers. Due to parallel flow, a unidirectional layer of medium is created which takes the contaminants along with itself all the way out. In addition to it, laminar air flow cabinet also deploys HEPA Filters (High Efficiency Particulate Air Filters) to filter out dust particles & contaminants from air.

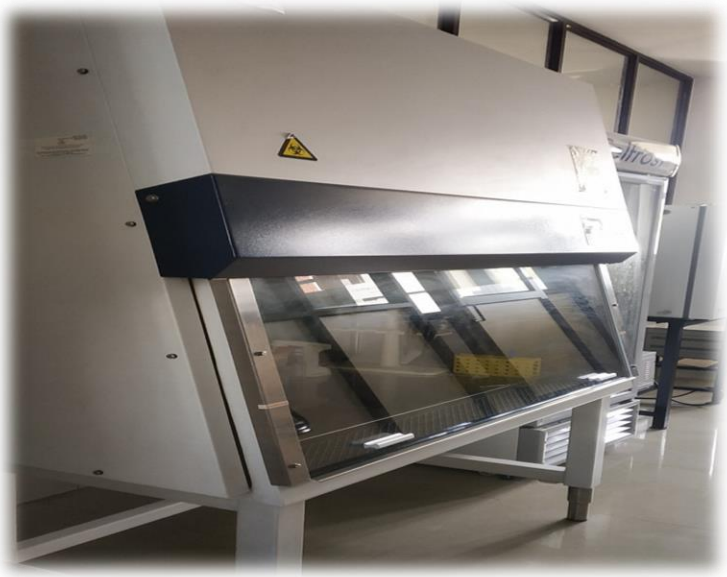


Figure No.17: Biological Safety Cabinet

5. VORTEX MIXER:

A substance in a liquid is mixed by creating a small vortex within the container of the liquid. The device consists of a rubber end which moves in a circular motion at very high speeds. When the container of a liquid comes in contact with it, the motion is transferred to it, creating a vortex within, thus facilitating rapid mixing.

2: Cell Culture Media

Cell culture media are complex mixtures of salts, carbohydrates, vitamins, amino acids, metabolic precursors, growth factors, hormones, and trace elements. The requirements for these components vary among cell lines. Carbohydrates are supplied primarily in the form of glucose. Other carbon sources include amino acids (particularly L-glutamine) and pyruvate. In addition to nutrients, the medium helps maintain the pH and osmolality in a culture system. The pH is

maintained by one or more buffering systems; CO_2/sodium bicarbonate, phosphate, and HEPES are the most common. Sera will also buffer a complete medium. Phenol red, a pH indicator, is added to medium to calorimetrically monitor changes in pH. Commonly used culture media include the following: Eagle's Minimum Essential Medium (EMEM) was among the first widely used media and was formulated by Harry Eagle.

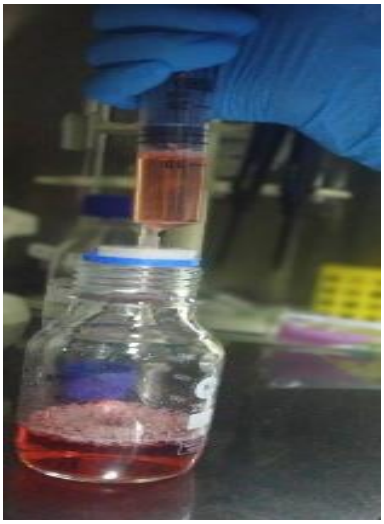


Figure No.18: Cell culture media

COMPONENTS OF CELL CULTURE MEDIA

COMPONENT	FUNCTION
Glucose	Source Of Energy
Amino acids	Building block of proteins
Vitamins	Promote Cell Survival And Growth
Balanced salt solution	Isotonic mixture of ions to maintain optimum osmotic preasure within the cells and provide essential metal ions to act as co-factor for enzymatic reactions, cell adhesion etc
Phenol red dye	Ph indicator the color of phenol red change to orange/red at ph 7-7.4 to yellow at acidic(lower) ph and purple at(higher) basic ph.
Bicarbonate/HEPES buffer	It is used to maintain a balance ph in the media.

Dulbecco's Modified Eagle's Medium (DMEM) has roughly twice the concentration of amino acids and four times the amount of vitamins as EMEM, as well as ferric nitrate, sodium pyruvate, and some supplementary amino.

DMEM/F12 Medium is a 1:1 mixture of Dulbecco's modified EMEM and Ham's F-12. It is an extremely rich and complex medium and will support the growth of a broad range of cell types in both serum and serum- free formulations.

3: Culture Vessels and Surfaces Vessels

Culture vessels provide a contamination barrier to protect the cultures from the external environment while maintaining the proper internal environment. For anchorage-dependent cells, the vessels provide a suitable and consistent substrate for cell attachment. Other characteristics of vessels include easy access to the cultures and optically clear viewing surfaces.

Selecting the right vessel

First, match the characteristics of the cells to be grown with the characteristics of the different culturing systems. There are three basic types of cell cultures:

• Anchorage dependent, which must become attached to a surface to grow (for example, human diploid fibroblasts).

• Anchorage independent, which grow in suspension (most blood-derived cell cultures).Cells that can grow either attached or in suspension (many transformed cell lines such as HeLa and BHK-21).

2: CELL PASSAGING

The culture flasks were observed under inverted phase microscope to check for the level of confluency and signs of contamination. The cultures were passaged at the confluency of about 60- 70%. The media in the flask was aseptically aspirated and the cells were rinsed with sterile PBS to remove any traces of media remaining in the flasks. The cells were detached from the flasks by addition of trypsin-EDTA solution. The flasks were swirled well after addition of trypsin and were incubated at 37^0C for 3 minutes. Serum was added to the flask to inactivate the trypsin, followed by washing with complete medium. The cells were then pipetted into sterile 50 ml centrifuge tubes and centrifuged at 1000 rpm for 5 mins. The supernatant was disposed and the pellet obtained was resuspended in appropriate volume of fresh medium. A sample of

resuspended cells was mixed with trypan blue and cell count was performed using Hemocytometer. The cells could be used for seeding or freezing or could be transferred to fresh flask for maintenance.

Figure No.19: Cell scrapping or Trypsinization

3: Examination of Cultures

Observe the morphology and viability of cultures regularly and carefully. Examine the medium in the vessel for macroscopic evidence of microbial contamination. This includes unusual pH shifts (yellow or purple color from the phenol red), turbidity, or particles. Also, look for small fungal colonies that float at the medium-air interface. Specifically check around the edges of the vessel as these may not be readily visible through the microscope. With an inverted microscope at low power (40×), check the medium for evidence of microbial contamination and the morphology of the cells. Bacterial contaminations will appear small, shimmering black dots within the spaces between the cells.

Most some cases, healthy cells will round up and detach somewhat during mitosis and appear very refractile. Following mitosis, they will reattach. Some of these will float free if the culture vessel is physically disturbed. In contrast, dead cells often round up and detach from the

monolayer and appear smaller and darker (not refractile) than healthy cells. Cells in suspension culture grow either as single cells or as clusters of cells. Viable cells appear round and refractile whereas dead cells appear smaller and darker. Occasionally, a portion of the cells will attach and grow on the side of the culture vessel and appear round or flattened. The percentage of attached cells varies with the culture conditions and the cell density. Cellular debris may also be observed in healthy cell populations. Some cell lines grow as mixed adherent and suspension cultures.

4: Cell Counting

Cell counts are necessary in order to establish or monitor growth rates as well as to set up new cultures with known cell numbers. Hemocytometers (also spelled Hemocytometers) are commonly used to estimate cell number and determine cell viability. A Hemocytometers is a fairly thick glass slide with two counting chambers, one on each side. Each counting chamber has a mirrored surface with a 3 × 3 mm grid of 9 counting squares. The chambers have raised sides that will hold a cover slip exactly 0.1 mm above the chamber floor. Each of the 9 counting squares holds a volume of 0.0001 ml.

Count cells as follows:

1. Clean, thoroughly dry, and assemble the Hemocytometers with the cover slip.
2. Transfer a small amount of cell suspension to the edge of each of the two counting chambers. Allow the cell suspension to be drawn into the counting chamber by capillary action.
3. Place the haemocytometer under an inverted microscope and view the cells at 100× magnification.
4. Record the number of cells in each section. Average the number of cells, and multiply by the dilution factor. If the cells have not been diluted, this factor will be 10^4 cells/ml. Any dilution of the sample after it was removed from the cell suspension, such as using vital stain, needs to be included in the calculation.

5: Cryopreservation

Most cell cultures can be stored for many years, if not indefinitely, at temperatures below –130°C (cryopreservation). Advantages of cryopreservation include:

• Generation of safety stocks to ensure against loss of the culture from equipment failures or contamination by microorganisms or other cell lines.

• Elimination of the time, energy, and materials required to maintain cultures not in immediate use.

• Preservation of cells with finite population doublings (that will ultimately senesce).

• Insurance against phenotypic drift in the culture due to genetic instability and/or selective pressure.

• Creating a standard reagent to be used for a series of experiments.

Liquid Nitrogen Freezer Storage

The ultra-low temperatures (below –130°C) required for long-term storage can be maintained by specialized electric freezers or more commonly by liquid nitrogen freezers. There are two basic types of liquid nitrogen storage systems: immersing vials in the liquid and holding vials in the vapor phase above the liquid. The liquid-phase system holds more nitrogen and thus requires less maintenance.

Cryopreservation Procedure

Harvest cells in exponential growth.

1. Check your cell culture for contamination from bacteria, fungi, mycoplasma, and viruses immediately before cryopreservation.

2. Prepare a freeze medium consisting of complete growth medium and 5% DMSO. Do not add undiluted DMSO to a cell suspension as dissolution of DMSO in aqueous solutions gives off heat.

3. Collect cells by gentle centrifugation (10 minutes at 125 × g) and resuspend them in the freeze medium at a concentration of 1 × 106 to 5 × 106 viable cells/mL. Continue to maintain the cells in culture until the viability of the recovered cells is confirmed.

4. Label the appropriate number of vials with the name of the cell line and the date. Allow cells to equilibrate in the freeze medium at room temperature for a minimum of 15 minutes but no longer than 40. This time is usually taken up in dispensing aliquots of the cell suspension into the vials. After 40 minutes, cell viability may decline due to the DMSO.

6. Place the vials into a pre-cooled (4°C), controlled-rate freeze chamber and place the chamber in a mechanical freezer at –70°C (or colder) for at least 24 hours. Alternately, use a pre-cooled (4°C) programmable freezer unit set to cool the vials at –1°C per minute until a temperature below –40°C is achieved and then set to abruptly drop to –130°C.
7. Quickly transfer the vials to a liquid nitrogen or –130°C freezer. Frozen material will warm up at a rate of10°C per minute and cells will deteriorate rapidly if warmed above –50°C.

2: Recovery of Cryopreserved Cells

1. Prepare a culture vessel (T-75 flask) so that it contains at least 10 mL of the appropriate culture medium equilibrated for temperature and pH.
2. Remove the vial from the liquid nitrogen freezer and thaw by gentle agitation in a 37°C water bath (or a bath set at the normal growth temperature for that cell line). Thaw rapidly until ice crystals have melted (approximately 2 minutes).
3. Remove the vial from the water bath and decontaminate it by dipping in or spraying with 70% ethanol. Follow strict aseptic conditions in a laminar flow tissue culture hood for all further manipulations.
4. Unscrew the top of the vial and transfer the contents to a sterile centrifuge tube containing 9 ml complete growth medium. Remove the cryoprotectant agent by gentle centrifugation (10 minutes at 125 × g). Discard the supernatant, taking care not to disturb the soft pellet, and resuspend the cells in 1 ml or 2 ml of complete growth medium. Pipette gently to loosen the pellet and break apart clumps. (If the cells normally grow as clusters, avoid over-pipetting during resuspension.) Transfer the cell suspension into the medium in the culture vessel and mix thoroughly.
5. Examine the cultures after 24 hours and subculture as needed.

Figure No.20: CRYOPRESERVATION

Isolation of Primary Cells

ISOLATION AND CULTURE OF PERITONEAL MACROPHAGES

Macrophages are mononuclear phagocytes that are widely distributed throughout the body. They are a type of White Blood Cells that cleans the body unwanted microscopic particles such as bacteria and dead cells, they are born from White Blood Cells called monocytes which are produced by stem cells in our bone marrow. Monocytes move through the bloodstream and when they leave the blood they mature into macrophages. They are highly phagoctic and in this capacity they are considered to be essential immune effectors cells.

ISOLATION

Peritoneal cavity provides an easily acceptable site for harvesting moderate number of macrophages, macrophages are isolated from mouse peritoneal cavity, and yield of macrophages can be increased by injecting eliciting agents into peritoneum several days prior to cells harvest.

Mice should be breed and housed in a clean pathogen free environment, it is important to use mice that re clean, stress free and uninfected. Mouse should be housed in the facility for at least one week.

MATERIALS

Swiss albino or Balb/c mice, 1ml of 3% peptone, Harvest medium (DMEM),5ml syringe with 27g needle, Styrofoam block and pins for mounting the mouse ,Tray on which the mounting blocked can be placed, Scissors and forceps, Centrifuge, Collection tubes 15 ml to 50 ml, 70 % ethanol, PBS with 3% fetal bovine serum (FBS) (pre chilled and kept on ice).

PROCEDURE

1: Swiss albino mice were sacrificed. Abdomen of the mice was soaked with 70% ethanol. With the help of a sterile scissors small incision along the middle was done.

2: Fill the 10 ml syringe with cold harvest PBS with the leveled end of 20 g needle facing inward, insert needle through peritoneal wall along mouse left side (spleen side) through peritoneal wall and inject 10 ml of cold harvest PBS medium in each mice.

3: Using the same syringe and needle aspirate fluid from peritoneum move needle away from the viscera to cause tenting of the peritoneal cells and withdraw peritoneal fluid.
4: Remove needle from the syringe and dispense peritoneal fluid on a centrifuge tube Centrifuge the peritoneal exudates cells (PEC) in a refrigerated centrifuge 10 min at 400 × g (~1000 rpm in Eppendorf 5810R), 4°C. Discard supernatant and resuspend cell pellet in cold DMEM/F12-10 by gently tapping the bottom of the tube and pipetting up and down.
5: Count cells a Hemocytometers, first diluting 20 µl of cell suspension into 180 µl of DMEM/F12-10 (final dilution of 10×) and then applying 10 µl to a Hemocytometers.
6: Adjust cell concentration in harvest medium to ~1–3 × 106 total peritoneal cells/ml and keep on ice.

A. ELISA (Enzyme Linked Immunosorbent Assay)

ELISA (Enzyme Linked Immunosorbent Assay) is a plate based assay technique designed for detecting and quantifying peptides ,proteins ,antibodies and hormones or other biomolecues it is widely used for detection of Antigen or Antibody. It is extremely sensitive, capable of detecting low antigen concentrations.

PRINCIPE OF ELISA

ELISA is typically performed in 96 well polystyrene plate. The serum is incubated in the well and each well contains different serum. A positive control and negative control serum is included among the 96 samples being tested. Antibodies or antigens present in serum are captured by the corresponding antigen or antibody coated on the solid surface. After sometime the plate is washed to remove serum and unbound antibodies or antigens with a series of wash buffer.

To detect the bound antibodies or an antigens a secondary antibody that are attached to an enzyme such as (HRP) Horseradish Peroxidase or Alkaline Phosphatase are added to each well. After an incubation period the unbound secondary antibodies are washed off. When a suitable substrate is added the enzyme reacts to it to produce a color. The color product is measurable as a function or quantity of antigens or antibodies present in the sample. The intensity of color/optical density is measured at 450 nm. The intensity of color gives an indication of the amount of antigen or antibody.

The procedure of ELISA on cell culture supernatant can be carried out with the help of following steps

1- Dilute the Capture Antibody 1:250 in Coating Buffer and coat micro-wells with 100µlof diluted Capture Antibody per well. Seal plate and incubate over night at 4^0C.
2- Aspirate wells and wash 3 times with ≥300µl/well wash buffer. After last wash, invert plate and blot on absorbent paper to remove any residual buffer.
3- Block plates with ≥200µl/well Assay Diluents. Incubate at room temperature for 1 hour.
4- Aspirate /wash as in step 2.
5- Prepare standard and sample dilutions in Assay Diluents.
6- Pipette 100µl of each standard, sample and control into appropriate well. Seal plate and incubate for 2 hours at RT.
7- Aspirate/wash as in step 2, but with 5 total washes.
8- Dilute the Detection Antibody 1:250 in Assay Diluents and add 100µl of diluted Detection Antibody to each well. Seal plate and incubate for 1 hour at RT.
9- Aspirate/wash as in step 2, but with 5 total washes.
10- Dilute the Enzyme Reagent (SAv-HRP) 1:250 in Assay Diluents and add 100µl of diluted Enzyme Reagent to each well. Seal plate and incubate for 30 minutes at RT. Do not dilute more Enzyme Reagent than is needed for the experiment.
11- Aspirate/wash as in step 2, but with total 7 washes. (Note- In this final wash step soaks wells in wash buffer for 30 seconds to 1 minute for each wash).
12- Add 100µl of TMB Substrate Solution to each well. Incubate plate (without plate sealer) for 30 minutes at room temperature in the dark.
13- Add 50µl of Stop Solution to each well.
14- Read absorbance at 450nm within 30 minutes of stopping reaction.

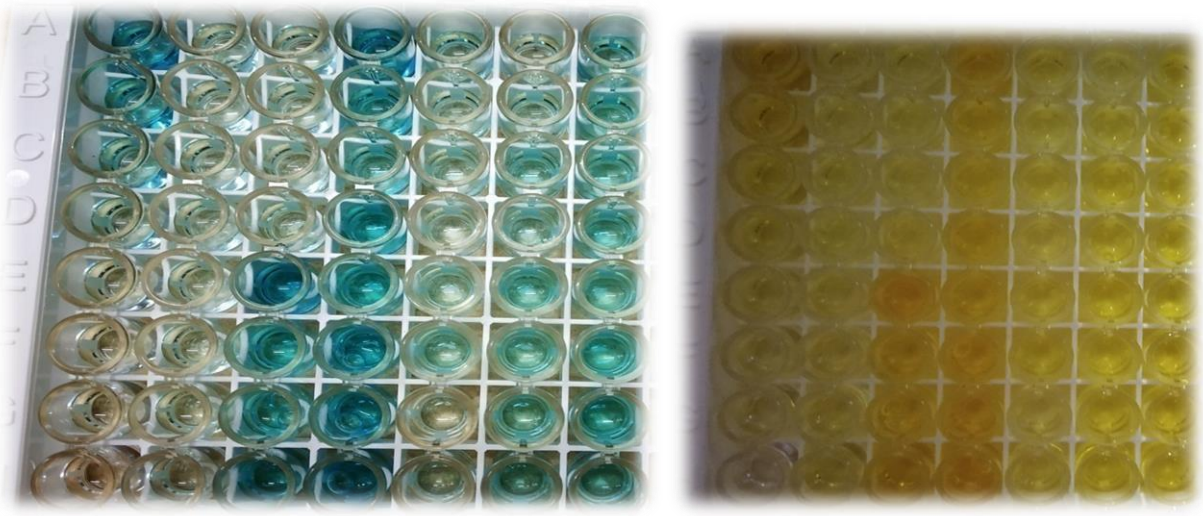


Figure No.21: ELISA (Enzyme Linked ImmunoSorbent Assay)

NON CYTOTOXIC DOSE DETERMINATION VIA MTT ASSAY

The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The number of assay steps has been minimized as much as possible to expedite sample processing. The MTT Reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation.

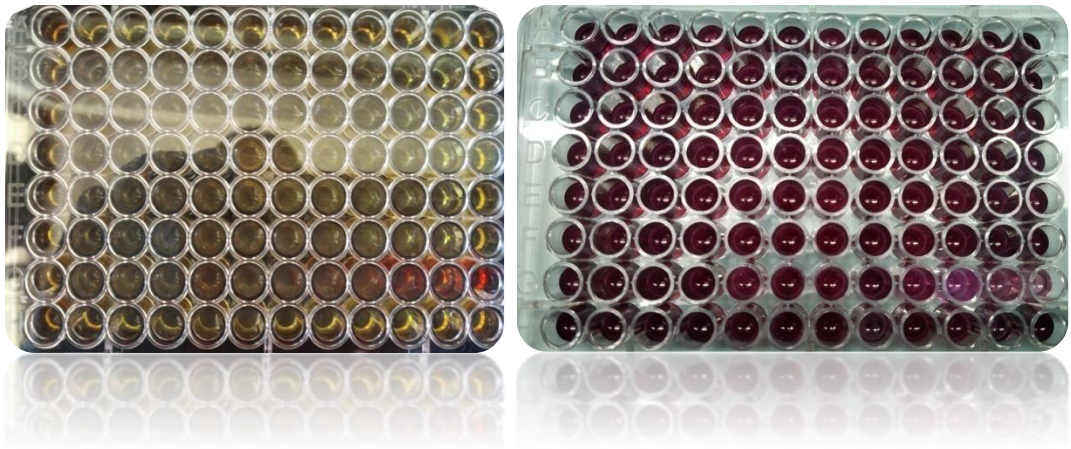


Figure No.22:NON CYTOTOXIC DOSE DETERMINATION VIA MTT ASSAY

Techniques involved in in-vivo Experimental Pharmacology

1) HANDLING OF ANIMALS

Laboratory animals may should be handled and restrained when put into new cages or removed for various experimental purposes. Most domestic and laboratory animals need no restraint for such routine handling but will respond to gentleness; in fact they tend to escape from cages.

Handling of the laboratory animal involves two most important responsibilities on the part of experimenter.

- The animal is handled with utmost care so that it does not suffer any pain.
- A due regard is paid towards the health and well-being of the animal colony. Even when they are killed at the end of experiment, it should be done by a humane method i.e. Euthanasia which means painless killing.

RAT (Rattusnorvegicus) Albino rat (white rat) is one of the commonest laboratory animals suitable for experimental work because of its small size and greater sensitivity to most drugs. It is very sturdy to withstand long periods of experimentation under anesthesia.

Mouse (Mus musculus): The mouse makes an excellent model for human disease because the organization of their DNA and their gene expression is similar to humans, with ninety-eight percent of human genes having a comparable gene in the mouse. They have similar reproductive

and nervous systems to humans, and suffer from many of the same diseases such as cancer; diabetes and even anxiety are used in a vast range of experiments.

2) **Euthanasia:** is resorted to events where an animal is required to be sacrificed on termination of an experiment or otherwise for ethical reasons.

Requirements to be fulfilled:

A: Death, without causing anxiety, pain or distress with minimum time lag phase.

B: Minimum physiological and psychological disturbances.

3) **Procedure for orbital sinus blood**

Requirement includes animal, anesthetic agent, cotton, capillary tube and blood sample collection tissues.

- This technique is used with recovery in experimental circumstances and this method is also called per orbital, posterior-orbital and orbital venous plexus bleeding.
- Blood sample is collected under general anesthesia.
- Topical ophthalmic anesthetic agent is applied to the eye before bleeding.
- The animal is scuffed with thumb and forefinger of the no dominant hand and the skin around the eye is pulled taut.
- A capillary is inserted into the medial can thus of the eye (30 degree angle to the nose).
- Slight thumb pressure is enough to puncture the tissue and enter the plexus/sinus.
- Once the plexus/sinus is punctured, blood will come through the capillary tube.
- Once the required volume of blood is collected from plexus, the capillary tube is gently removed and wiped with sterile cotton. Bleeding can be stopped by applying gentle finger pressure.
- Thirty minutes after blood collection, animal is checked for postoperative and periorbital lesions.

Caution:

- Repeated blood sampling is not recommended.
- Skill is required to collect blood.
- Even a minor mistake will cause damage to the eyes.

4) DOSING OF ANIMALS

Oral administration of substances is a common procedure in scientific experiments using laboratory animals and typically is achieved in conscious animals by using the intragastric gavage technique. Gavage the introduction of a solution into the stomach by means of a tube is and is used clinically and for research. In laboratory animals, dosage by gavage involves removing the animal from its cage, manually restraining it, inserting a small-diameter tube into the esophagus, and delivering the drug directly into the stomach by means of a syringe. Although highly effective, care must be taken to ensure that the tube or needle does not enter the trachea or damage the esophagus or stomach. Oral dosing of mice and rats can be trick.

5: **INJECTION TECHNIQUES**

Routes of administration of injections are subcutaneous, intraperitoneal, intravenous and intra muscular. Each route has a recommended injection site, approximate needle gauge and recommended maximum injected volume at a single time at one site.

Intraperitoneal Injections: intraperitoneal injections are typically made in the lower right or left quadrant of abdomen, not on the midline. A relatively short needle (e.g., 0.5-inch or less) is suggested to help prevent puncture of the intestines or cecum. Once the animal is properly restrained the injection site is visualized, the needle is inserted at approximately a 60° angle to the body wall.

Preparation of Thin-Film Blood Smear with special reference to Malaria parasites

- One of the most classic assays of malaria research is the thin-film blood smear. In this assay, parasitized red blood cells can be visibly identified by the presence of stained nucleic acid, which is present at comparatively low levels in the a nucleate mouse red blood cells. This allows simple assessment of parasitemia – the proportion of red blood cells that are infected – using basic light microscopy.
- Using a pair of sharp surgical scissors, cut off a 0.5 to 1 mm section from the tip of the mouse-tail. The snip should only remove enough of the tail to obtain a drop of blood.

- In one fluid motion towards the distal end of the tail, gently milk the tail to collect a drop of blood at the tip.
- Transfer a 1 to 2 µl drop of blood, directly from the tip of the tail, close to the edge of a microscope slide
- As the blood will begin to coagulate within several seconds, quickly touch the edge of a second slide to the blood sample at a 45° angle, allowing the blood to spread across the edge.
- Apply gentle pressure and evenly spread the blood across the width of the sample slide to obtain a thin-film blood smear. Allow the blood to dry for a few seconds.
- Place the slides into a rack and submerge the slides in methanol for approximately 5 seconds to fix the smears.
- Remove the slides from methanol and allow them to air dry, preferably in front of a fan. While the slides are drying, pipette 1 ml of Giemsa into 250 ml of 1× PBS in a slide staining chamber.
- Incubate the slides in the chamber for 10 min to 1 h, depending on the strength of your staining solution. The slide is sufficiently stained when leukocytes present in the smear have dark blue-purple nuclei.
- Thoroughly rinse the slides with water and place them in front of a fan to dry. If counting will be performed immediately, the rinsed slides can also be gently blotted dry and analyzed using the procedures described below. The stained slides are stable; if counting will not be performed immediately, the dried slides can be stored in a slide box at room temperature indefinitely.

Counting Thin-Film Blood Smears

For routine monitoring, parasite burden is most conveniently calculated as parasitemia. In order to obtain this number, infected erythrocytes must be distinguished from uninfected erythrocytes, leukocytes, and platelets, and care must be taken to differentiate between reticulocytes (immature red blood cells that still harbor some nucleic acid) and parasitized normocytes (fully mature erythrocytes). Different species of malaria parasites have different predilections for different types of red blood cells.

1: Place a small drop of immersion oil directly onto the smear and find the focal plane on a bright field microscope with a 100× objective.

2: Count approximately 500 red blood cells per smear with a tally counter, separately keeping track of infected and uninfected cells. If the parasite life cycle stage is important for your experiment, count the various stages separately.

3: Calculate parasitemia, the percentage of erythrocytes infected with parasites.

5) Hematological parameters

Total Erythrocytes Count (Red Blood Cells)

Principle:

The method involves an accurate dilution of a measured quantity of blood with a fluid which is isotonic with the blood and which will prevent its coagulation. A dilution of 1 to 200 is usually necessary. The diluted blood is placed in accounting chamber and the cells in a circumscribed volume are enumerated under a microscope.

Materials: Red blood pipette: It is a capillary tube, graduated in 10ths, which open into a bulb, with a red glass bead. The bulb when filled to the mark above it (101) will hold hundred times the quantity of fluid contained in 10 divisions of the capillary tube.

Counting chamber: a neubauer counting chamber (hemocytometer) with rating is commonly used.

Dilution fluid:

A solution of 1% formalin, 40 %formaldehyde in 31.3g/l tri sodium citrate.

Procedure:

- The blood should be taken directly from the tail of mice; this procedure should do quickly to avoid coagulation.
- The blood is drown by mouth suction up to the 0.5 mark, the tip of the pipette is wiped clean and the diluents is drawn in until the solution fills the pipette and reaches the '101" mark or 20µl of blood can be diluted in 4.0 ml of the diluting fluid in a test tube.
- The cover slip should be put on the counting chamber and then a small quantity of the diluted blood should be put between the cover slip and ruled the platform of the counting chamber.

- The chamber should not be overflow and there should not be any air bubble in the chamber.
- The solution is allowed to settle for a couple of minutes and then the counting should be done under the high power of a microscope.
- In the Neubauer ruling, the small squares in the central large 1 mm square are used for the enumeration of erythrocytes.
- The number of cells in the 4 corner groups of 16 squares are counted and also one central group including those cells which has lie within the area or on the dividing lines to the left or above the section. If the dilution has been 1 to 200 then the total number of cells in millions per mm^3 of blood.

Calculation:

The smallest square has an area of $0.0025mm^2$ and is 0.1mm deep, being thus $0.00025mm^3$ in volume. Since 80 such squares are counted, a volume of 0.00025 or 0.02 mm^3 has been covered. In order to give the value per mm^3 of blood, the number of cells counted must be multiplied by 50. However, since the dilution is 1 to 200, the multiplication factor is 50 X 200 or 10 000.

Total leucocytes Count (White Blood Cells)

Principle:

The method involves an accurate dilution of a measured quantity of blood with a fluid which is isotonic with the blood and which will prevent its coagulation? A dilution of 1 to 20 is used and the diluents are usually one which destroys the red blood cells. The diluted blood is placed in accounting chamber and the cells in a circumscribed volume are enumerated under a microscope.

Materials:

WBC pipette: Pipette with a white colored glass bead and it's calibrated to give 1 to 20 dilutions.

Counting chamber: a neubauer counting chamber (Hemocytometers) with rating is commonly used.

Diluting fluid: Mix 2.0 glacial acetic acid and 1 drop of gentian violet in 100 ml of water.

Procedure:

- The blood should be taken directly from the tail of mice , this procedure should do quickly to avoid coagulation.
- 20µl of blood can be diluted in 0.4 ml of the diluting fluid in a test tube.
- The cover slip should be put on the counting chamber and then a small quantity of the diluted blood should be put between the cover slip and ruled the platform of the counting chamber.
- The chamber should not be overflow and there should not be any air bubble in the chamber.
- The solution is allowed to settle for a couple of minutes and then the counting should be done under the high power of a microscope.
- The WBC count is made in large (1 mm) corner squares of neubauer chamber. The numbers of cells in the 4 corner groups of 16 squares are counted. If the dilution has been 1 to 20 then the total number of cells in millions per mm^3 of blood.
- Calculation:
- The 1 square has an area of 1 mm^2 and is 0.1mm deep, being thus $0.1mm^3$/0.1µlin volume. Since 4 such squares are counted, a volume of 0.4 mm^3/0.4µl has been covered. In order to give the value per mm^3, the number of cells counted must be multiplied by 2.5. However, since the dilution is 1 to 20, the multiplication factor is 2.5 X 20.
- Cells/mm^3=No. of cell counted in four square X 2.5 X20

6) Hemoglobin Estimation:

Hemoglobin estimation carried out by Randox kits. Hemoglobin in the presence of alkaline potassium ferricyanide undergoes oxidation to form Met hemoglobin, which then reacts with KCN to form cyanmethemoglobin .having characteristic absorbance at 540 nm .intensity of absorbance is directly proportional to hemoglobin concentration.

Reagents:

Drabkin reagent

Potassiumphosphate52mmol/l, Potassium ferricyanide 30.4 mmol/l ,

Pot.cyanide 38.4 mmol/l, Brij-35 solution 25%, Standard methaemoglobin 18 gm/dl.

Procedure

- One ml of the Drabkin reagent provided in the Randox kit was pipetted out into an eppendorf tube.
- 4 µl of blood sample collected from the tail of mice was mixed with the reagent and allowed to stand for 15 min at room temperature.
- Absorbance was measured at 540 nm against reagent blank.
- Concentration of Hemoglobin in the sample was calculated from the standard graph and then the value was multiplied with the dilution factor.

7) Serum Preparation

Collect whole blood in a covered test tube. After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 15–30 minutes. Remove the clot by centrifuging at 1,000–2,000 x g for 10 minutes in a refrigerated centrifuge.

The resulting supernatant is designated serum. Following centrifugation, it is important to immediately transfer the liquid component (serum) into a clean polypropylene tube. The samples should be maintained at 2–8°C while handling. If the serum is not analyzed immediately, the serum should be apportioned into 0.5 ml aliquots, stored, and transported at –20°C or lower.

Pharmacological Studies:-

10: ANALGESIC ACTIVITY (Thermal and chemical induced pain models)

Hot plate analgesic test

Hot plate analgesic test consist of introducing a mouse or a rat into an open ended cylindrical space with a floor consisting of a metallic plate that is heated by a thermode or a boiling liquid.

Plate is heated to a constant temperature that can be measured in terms of their reaction time mainly paw licking and jumping. Animals were placed in a hot plate which consisted of electrically heated surface temperature on a hot plate is measured at 50-55C, responses such as jumping, withdraw of the paws and licking of the paws are seen.

PROCEDURE

1: Animals were divided into two groups they were allowed to acclimatize for a week before starting the experiment.

2: One night before the experiment, animals were fasted over night to avoid any kind of food-drug interaction.

3: Mice were treated with orally with different doses of drugs.

4: The initial latency time of all mice towards thermal heat were noted. The increased latency time of all animals towards thermal heat was recorded. 30, 60, 90 and 120 min

5: The reaction time was taken in seconds for fore paw licking or jumping. A cut off time was 30s to avoid any injury of paws.

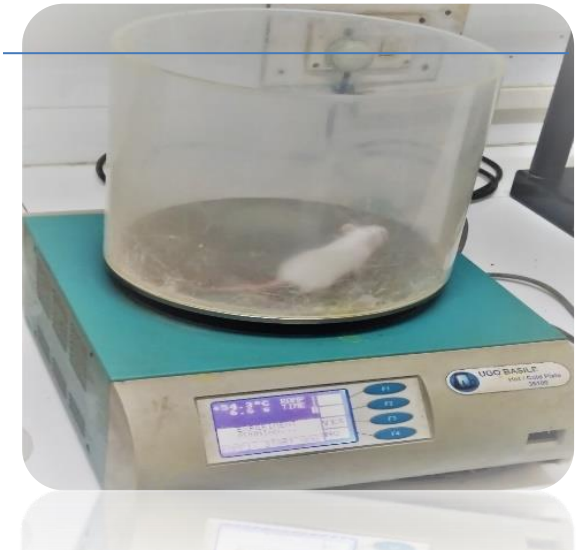


Figure No.23: Hot Plate Technique

%Inhibition = post treatment latency- pre-treatment latency X 100

Cut-off time – pre- treatment latency

Formalin induced paw licking test

The formalin test is a popular chemical assay of injury-produced pain. Thenoxious stimulus is an injection of dilute formalin (1% in saline)under the skin of the dorsal surface of the right hind paw. The response is the amount of time the animals spend

licking the injected paw.

PROCEDURE

1: Animals were divided into two groups they were allowed to acclimatize for a week before starting the experiment.

2: One night before the experiment, animals were fasted over night to avoid any kind of food-drug interaction.

3: Mice were treated with orally with different doses of plant extracts.

4: After 1hour of treatment with plant extract, each mouse was injected into sub plantar region of right hind paw with 20µl of 5% formalin in saline.

5: These mice were individually placed in a transparent observation chamber. The time spent on paw licking by mice was estimated and used as an index to measure the analgesic effect during the 0–5 min period (first phase, neurogenic) and the 5-10 min (second phase, inflammatory) period after formalin injection.

$$\%\text{Inhibition Reaction} = \frac{\text{Reaction time (control)} - \text{Reaction time (treated)}}{\text{Reaction time (control)}} \times 100$$

Tail immersion test

The tail immersion assay is a thermal test for evaluating the analgesic potential of compounds. The procedure is based on the observation that drugs are selectively capable of prolonging the reaction time of the typical tail-withdrawal reflex in mice induced by immersing the end of the tail in warm water of 55 °C.

PROCEDURE

1: Animals were divided into two groups they were allowed to acclimatize for a week before starting the experiment.

2: One night before the experiment, animals were fasted over night to avoid any kind of food-drug interaction.

3: On the day of experiment, mice were placed into individual restraining cages leaving the tail hanging out freely. Mice were allowed to acclimatize to the cages for 5 min before testing.

4: The marked portion of the mice tail was immersed in the water bath having temperature maintained exactly 50°C ± 1°C and filled with fresh water.

5: The preliminary latency time of all animals towards tail immersion test were recorded. The reaction time was determined 30, 60, 90, and 120 min after oral administration of the drug.

6: To avoid tissue injury, the cut off time was 25 second.

$$\%\text{Inhibition} = \frac{\text{post treatment latency - pretreatment latency}}{\text{Cutoff time - pretreatment latency}} \times 100$$

Food and water intake study

24-hour food and water intake study

Food and water intake study was done to determine the food and water intake pattern in mice/rats. Adult mice (22-30mg) were divided into group of four, containing three animals in each cage, providing for average intake/mice. Acclimatization was done before starting the experiment for 24 hours and animals were provided with free access to food and water. The weight of animal was measured and noted down on day of animal issued as well as on the day experiment started followed by taking the weight after every seven days during experiment. The subtraction of weight of feed and water given to mice/rats by weight of feed and water remaining after 24 hours, results in calculation of food and water consumed by the animal.

Food consumed = weight of feed given (gm) - weight of feed remaining after 24 hours (gm)

Water consumed = weight of water given (ml) – weight of water remaining after 24 hours (ml)

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