Comparative analysis of dietary supplementation of extra-virgin olive oil and canola oil available in commercial market of Pakistan against streptozotocin induced diabetes in wistar rats

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DECLARATION

I, Fareeha Iqbal, declare that all of the work presented in this thesis is my own. I confirm that any information obtained from other sources has been mentioned in the thesis. The work presented here was completed while I was a postgraduate student at NUST Atta-ur-Rahman School of Applied Biosciences, working under Dr. Tahir Ahmad's supervision.

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May Allah bless all of them with long, happy and peaceful lives, **Ameen.**

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DEDICATION

This thesis is dedicated to

My beloved Parents

(Muhammad Iqbal and Razia Parveen)

And

Respected Supervisor

(Dr. Tahir Ahmad)

They always supported me from the dawn of my personal stories to the inevitable dusk, it taught me a better way to acquire knowledge and live wisely.

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T2DM	Type 2 diabetes mellitus
CVD	Cardiovascular disease
GLP-1	Glucagon-like peptide 1
DPP4	Dipeptidyl peptidase inhibitor
HbA1c	Glycated hemoglobin
FIS	Fasting insulin secretion
TI	Total insulin
IRSs	Insulin receptor substrate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
ALDH	Aldehyde dehydrogenase
ARK	Aldo keto reductase
MDA	Malondialdehyde
CAT	Catalase
SOD	Superoxide dismutase
ALT	Alanine transaminase
ALP	Alkaline phosphatase

Abstract

The antidiabetic potential of extra-virgin olive oil (EVOO) and canola oil (CaO) consumption have been shown in recent studies. The goal of this study was to evaluate how effectively dietary supplementation of commercially available vegetable cooking oils (EVOO and CaO) performed against Streptozotocin (STZ) induced diabetes in wistar rats. A number of 30 rats (males and females aged 8-12 weeks) were used in this study and divided into five groups including controls (C), diabetic control (DC), diabetic rats treated with metformin (DM), diabetic rats given EVOO (DO) and diabetic rats given Cao (DCa). For biochemical analysis (lipid profile, liver functions, and kidney functions), oxidative stress markers of the liver (CAT, SOD, and MDA), and histopathological analysis of vital organs (liver, spleen and kidney) were used to assess the protective effect of these supplemental oils. The dietary supplementation of EVOO significantly reduced the serum ALP (P=<0.0001), ALT (P=0.001 in male wistar rats), bilirubin (P=<0.0001), cholesterol (P=<0.0002 in male wistar rats), TG (P=<0.0001) and uric acid (P=0.0058) than CaO. In DC, antioxidant enzymes CAT (P=0.0497, P=0.0462) and SOD (P=0.0017, P=0.0269) are reduced considerably in male and female wistar rats respectively, while decreased MDA level (P=<0.0001, P=0.0002) in EVOO treated group of male and female wistar rats respectively. The diabetic hepatic, renal, and splenic sections from the diabetic wistar rats revealed a number of histological abnormalities. EVOO and CaO supplementation improved the reported physiological, molecular, and histopathological changes. The result of the current study shows that consumption of EVOO is more effective against diabetes and utilized to minimise the risk of diabetes as a prophylactic measure.

Key words: Diabetes Mellitus, Extra-virgin olive oil, Canola oil, Catalase, Superoxide dismutase

Chapter 1

Introduction

1.1 Introduction

Diabetes mellitus (DM) is a metabolic condition characterised by elevated blood glucose levels due to a decrease in insulin production or sensitivity. Diabetes mellitus is becoming more common, causing a severe health threat. According to the International Diabetes Federation, about 345 million people worldwide have diabetes (Cho *et al*. 2018). Secondary effects such as diabetic retinopathy and neuropathy, cardiovascular disease, ulcers, and amputations in hyperglycemic individuals are caused by a variety of factors such as insulin failure, hyperlipidemia, and oxidative stress. Reactive oxygen species compromise the structural integrity of macromolecules such as proteins, polysaccharides, lipids, and DNA. Free radicals are highly unstable substances having an unpaired electron that can be transformed into free radicals through a series of chain reactions. Superoxide anions are harmless to species with widespread antioxidant systems for eliminating them under normal conditions (Punthakee, Goldenberg, and Katz 2018). Unfortunately, various factors like as smoking, air contamination, water contamination, radiation (X-beams, UV beams), pesticides, life stress, exorbitant exercise, and others significantly increment the convergence of free radicals in our environmental elements. Excessive levels of free radicals can lead to chronic diseases like diabetes, CVD, and cancer. High blood glucose level causes protein glycosylation and glucose oxidation, resulting in the production of free radicals. The presence of manifestations like polyuria, polydipsia, and unexplained weight reduction for the most part demonstrate the presence of diabetes, which is affirmed by estimation of strange hyperglycemia. With a growing number of pharmacological medicines on the market, rising concerns about their possible side effects, and increasing doubts regarding the benefits of strict glycemic control on macrovascular problems, glycemic

management is becoming more complicated. In type2 DM has become progressively intricate and, to comparatively, provocative. As a result, many professionals are bewildered about the best treatment options for their patients (Alkhatib, Tsang, and Tuomilehto 2018).

The prevalence of type 2 diabetes increasing rapidly and is a major concerning heath problem causing a socioeconomic health challenge in the world. According to IFD (International diabetes federation) estimated diabetic population in 2013 is 382 million young grown-ups aged 20-70 years have type 2 diabetes in which eighty percent are those individuals who spend their lives with low or middle income. Expectations are to increase the number of patients is 592 million in 2035 (DeFronzo *et al*. 2015). According to the report of IFD, occurrence of type 2 diabetes in provinces of Pakistan, higher rate is observed in Sindh, Punjab, Baluchistan and then KPK. In Sindh, Punjab and Baluchistan, disease is more dominant in male population than female. The rising disease incidence is a concerning trend that necessitates and ensures early detection, prevention efforts, and that every patient has access to care therapy (Zheng, Ley, and Hu 2018).

The main target of different therapies is lower the blood glucose level in body; play a vital role in treating and delaying the progression of the disease but the response of these therapies different in patients according to their complexities. For treatment different factors to be considered like age, weight, cost, efficacy, CVD, hypoglycemia, side effects and other life threating events. Life styles changes can help in reduce the progression of disease. There are various exogenous anti-oxidant compounds which are used to reduce the beta cell damage and improve the insulin secretion in body such as Nicotinamide, desferrioxamine, metformin, troglitazone, sulfonylureas, meglitinides, thiazolidinediones, dipeptidyl peptidase-4 (DPP-4) inhibitors, biguanides, glucagon-like peptide-1 (GLP1) receptor agonists, α-glucosidase inhibitors, amylin analogues and sodium glucose transporter 2 inhibitors. Metformin is used to decrease the insulin resistance,

inflammatory response, fatty acid oxidation and gluconeogenesis in liver. It is involved in improve the functioning of beta cells, increase the insulin sensitivity and translocation of GLUT4. There are other various medications such as cycloset, welchol and afrezza (powder form of insulin used for inhalation) (Rehman and Akash 2017)(Chaudhury *et al*. 2017). For T2D metformin is used as primary agent but it includes different side effects including gastrointestinal infections, such as anorexia, nausea, abdominal discomfort and diarrhea. Other side effects include reduced intestinal absorption of vitamin B12. There are different side effects that have been observed by using sulfonylureas and meglitinide such as ineffectiveness of drug, hypoglycemia and the sign of increased weight indicate the major side effects. By using the insulin, patients feel dizziness, anxiety, hunger and loss of consciousness. As all of the currently used first or second line therapies having certain side effects, it's require to identify the novel therapeutic approach with less side effects (Marín-Peñalver *et al*. 2016).

The quantity of clinical trials completed with therapeutic plants as antidiabetic specialists are very restricted and few of them are created with natural detailing. Different investigations incorporate explicit therapeutic plants or extract, and measure some trademark boundary, for example, glycated hemoglobin in patients with type 2 diabetes. Nonetheless, these examinations were of low quality with indistinct techniques for randomization, dangers to blinding, and absence of standard socioeconomics. As of late, some fascinating articles on regular anti-hyperglycemic activities, counting herbal plants utilized in society medication and phytotherapy, have been accounted for. These plants are, walnut tree (*J. regia*), bitter melon (*M. charantia*), nattle (*U. dioica*), sage (*S. officinalis*), and oil extract from seeds like olive, canola, soya oil are broadly utilized in people medication and some clinical contemplates were additionally evolved (Xu *et al*. 2018). There are various plants having medicinal properties containing

phytochemicals, anti-oxidant, flavonoids and phenolic compounds help in reducing the oxidative stress like nigella sativa, coffee, ginger, olive leaves and onion. Flavonoids and phenolic compounds are having more powerful anti-oxidant activity than others (Rehman and Akash 2017)(Chaudhury *et al*. 2017).

Olive oil and canola oil is fundamentally made by glycerides, tocopherols, phenols, and carotenes having cancer and diabetes preventing agent properties helpful to protect it. They also have positive consequences on the human wellbeing. Canola oil contains high amount of monounsaturated fatty acids (MUFA) and having less concentration of saturated fatty acids. Note that the compounds and nature of virgin olive oil (VOO) are affected by numerous components, as geological creation region, climatic conditions pervasive in the time of creation, the kind of development, what's more, the extraction interaction (Atefi, Pishdad, and Faghih 2018)(Antunes *et al*. 2020).

Higher concentrations of MUFA, particularly oleic acid in EVOO, have a fundamental function in modulating the immune reaction. Phenolic compounds in olive oil have anti-inflammatory, anti-tumor and immune modulating activities. Canola oil is most widely used cooking oil all over the world. It contains significant amount of polyunsaturated and monounsaturated fatty acids showed good effect on lipid profiles as well as anti-inflammatory activity. There is a minor difference in the composition of oils due to their production method. Olive oil contain high amount of phenolic compounds, phytosterols and hydrocarbons. The nature of the oils is reliant upon their substance organizations, similar to the level of unsaturation. Rancidity of oils can deliver possibly harmful mixtures related with long haul wellbeing impacts like neurological problems, heart and malignancy. Oils with a serious level of unsaturation are profoundly helpless to oxidation when contrasted with immersed oils (Ditano-Vázquez *et al*. 2019).

According to the report of IFD -2019, Pakistan have increasing rate of diabetes and more dominantly appear in male than females. The major factors involve in growing increasing rate is age, hypertension, family history and obesity which causes the major economic burden on country. To overcome the economic burden and reduce the increasing rate of disease are more focused on natural products that are easily accessed to everyone. In consent of above mentioned problems caused by diabetes and large socioeconomic burden the present experiment was designed to investigate the **Comparative analysis of dietary supplementation of extra-virgin olive oil and canola oil available in commercial market of Pakistan against streptozotocin induced diabetes in wistar rats.**

1.2 Objectives

- Designing of a diabetic model to access the effect of extra-virgin olive oil (EVOO) and canola oil (CaO) use as dietary supplements.
- Comparative analysis of collected information that was applied to determine the effect.
- Access the diabetic complications at a cellular level by histopathological examination.

Chapter 2

Literature Review

2.1 Diabetes

Diabetes mellitus (DM) is heterogeneous metabolic disorder caused by high blood glucose level (HBG) called hyperglycemia. Blood glucose level is the main source of energy in human body. Insulin is the pancreatic peptidyl hormone produced by beta-cells which regulate the carbohydrate, fats and protein metabolism by stimulating the absorption of glucose from blood into the liver. When body do not respond to secreted insulin, defective insulin action or any defect in insulin secretion leads to high blood glucose. Chronic diabetic condition acutely effect on other body parts like eyes, kidney and heart leading to CVD (Canivell and Gomis 2014)(Punthakee *et al*. 2018). Diabetes is differentiate in to different classes but major are two;

- **Type1 diabetes:** Because of the annihilation of pancreatic beta islets cells body can't deliver sufficient insulin prompts ketoacidosis.
- **Type2 diabetes:** It refers as insulin resistance due to defective signaling pathways of insulin receptors (Tangvarasittichai 2015).
- **Gestational diabetes:** It alludes to high blood glucose level during pregnancy at any stage yet generally happens in second or third trimester and vanishes in the wake of conceiving an offspring (Baynest 2015).
- **Other specific types:** It refers the genetic based diabetes or due to the association of any disease or drug (Baynest 2015).

2.2 Type2 Diabetes

T2DM is complicated and intensive metabolic disorder due to dysfunction of pancreatic beta-cells causes the insulin resistance. In various cases, diabetic population has high blood glucose in

which environmental and genetic factors are involved. The reason behind insulin resistance is defective signaling pathways at the level of insulin receptors and downstream. The major risk factors are overweight and obesity. Fundamental biochemical features of T2D is hyperglycemia causes the activation of inflammation pathways and oxidative stress in human body which leads to death. To regularize the blood glucose level in body, insulin is required in bulk however in obese people liver produced HBG which leads to prediabetes. Due to physical inactivity accumulation of fats in liver, pancreas leads to dysfunction of beta-cells. Early detection, changes in daily routine and medication (glucose lowering) contribute to reduce the progression of diabetes (Zaccardi *et al*. n.d.) (Dendup et al. 2018).

2.3 Epidemiology

In 2015 the prevalence of the disease recognized in 415 million people, estimation of increasing the disease level is round 642 million people in 2040. In 2017 approximately 387 million people diagnosed globally (Zheng *et al.* 2018). Mortality rate and global burden increased due to the complications of T2DM. In 2013 it is the 9^{th} major health risk as in 2010, 3.96 million deaths recorded and in 2015 it increased 5.0 million which is equalent to one death every six second. Undetected population are at high risk is 174.8 million (Zheng *et al*. 2018)(DeFronzo *et al*. 2015). The prevalence of T2D is on top in china and India than other countries due to low BMI in younger age than western population (DeFronzo *et al*. 2015). In 2019 38 million more adults spend their lives with T2D worldwide. The disease level upgraded from 17.1% to 148% and 19 million adults in which 8.5 million are undiagnosed. Asian population has high body fat, abdominal obesity, and poor nutrition in childhood, over-nutrition later contribute more towards disease; diabetes is dominant in males than females. The increasing disease rate is an alarming

condition, it must require and assure the early diagnosis, preventing strategies and every patient have access to the care therapy (Cho *et al*. 2018) (Zheng et al. 2018).

2.4 Pathophysiology of type2 diabetes

Micro and macrovascular complications in body caused due to dysfunctioning of beta cells, insulin resistance and chronic inflammation which increased the BG level as well as environmental and genetic factors also involved (Zheng *et al*. 2018).

2.4.1 Genetic factors

T2DM is a heritable condition, and the risk of having it is higher if the mother has it than if the father does. The risk of the disease also increased by increasing the BMI greater than 30 or NFG concentration is greater than 5.5mmol-1. Various genes are involved in type 2 diabetic complications; in 2007 only SNPs are reported in TCF7L2 but later other SNPs were discovered which have link with the prevalence of disease like *SLC30A8*, *FTO*, *CDKAL1*, *CDKN2A*, *CDKN2B*, *HHEX*, *IGF2BP2*, *GCKR* and others. Rather mostly variants are introns with it few have exons which interfere in gene function; cultured human cells reveal that these SNPs destroy the beta cell function (DeFronzo *et al*. 2015). There are few genes functions;

- ***SLC30A8:*** Encodes a zinc carrier that is fundamental for amassing/store insulin
- ***KCNJ11:*** Encodes an ATP-subordinate potassium channel
- ***GCKR:*** Encodes a glucokinase administrative protein

Intronic part of genes may interfere in the expression of nearby genes having less number than exonic part. For example;

- ***MTNR1B* gene:** A melatonin receptor is encoded by this gene.

Unhealthy insulin production linked with epigenetic modifications and microRNA patterns which increase the inheritance related to pathogenic factors. There are two types of groups with high risk and second is with low risk.

High risk: High-risk people could not increase their insulin secretion to fulfill the needs forced by insulin resstance and thusly created T2DM and risk alleles are greater than 12.

Low risk: All people turned out to be more obese and subsequently insulin resistant regardless of high or low genetic risk and risk alleles are less than 8 (Liang *et al*. 2020).

The greater part of heritability (85%) can't be explained by the at this point perceived SNPs. Substitute choices to explain the heritability are: ailment heterogeneity (T2DM may not be an innately uniform disease), quality environment affiliations (ecological elements) and epigenetic frameworks (DNA methylation and chromatin changes). A couple of varieties, similar to those for KCNQ1 (which encodes a voltage-gated potassium channel), show solid parent-of-origin impacts; KCNQ1 is methylated besides, imprinted in fetal yet not in adult life when procured from the mother (Dirice *et al*. n.d.) (Kohata *et al*. 2020).

2.4.2 Beta cell function

T2DM doesn't happen, aside from those beta cells that can't discharge a sufficient measure of insulin. Different variables are tangled in beta cell brokenness like maturing, hereditary anomaly, incretion chemical GLP-1 and GIP, lipotoxicity, glucotoxicity, insulin opposition prompting beta cell stress, hypersecretion of IAPP (islet amyloid polypeptide), responsive oxygen stress and setting off of provocative pathways (Gerber and Rutter 2017)(Marroqui *et al*. 2015).

2.4.3 Beta-cell physiology

Beta cells comprise 60% of cells and fallen with 30% of glucagon-delivering α cells, 10% of somatostatin-creating δ-cells and 1% of pancreatic polypeptide-delivering cells. Cells express their functional activity through gap intersections. Collectively 1 million of beta islets cells communicate with each other via connexin protein and adhesion complex for releasing the hormone into the blood. Each cell having 100-500 μU of insulin so whole pancreatic cells having weight 0.9gram contain supply for 10 days for healthy adults (Do and Doctor 2016).

2.4.4 Role of β cells in T2DM

The beta cell mass in type 2 diabetic patients is decreased by 30-40% than healthy person caused by apoptosis and dysregulated autophagy. In diabetic or non-diabetic patients, the proliferation of beta cells is similar in nature regardless the regeneration of tissues is reduced in T2DM. Beta cell structure is changed by vascular disarray and accumulation of amyloid protein. As indicated by criticism system insulin opposition in β-cell by expanding its ideal level; more insulin is released at any serum level of glucose. This continuous change is apparently intervened by little augmentations in flowing glucose levels, (for instance, those that occur in large typical glucose-lenient people) similarly as by different elements, as raised levels of free unsaturated fats (FFAs) (Marroqui *et al.* 2015)(Oh *et al*. 2018) (Galicia-Garcia *et al*. 2020).

2.4.5 Insulin secretion

For insulin regulation the beta cells interact with different substrates like glucose, free fatty acids, fructose, arginine, amino acids and hormones. Different concentration of insulin is required to sustain the blood glucose level; a normal healthy person require 0.5U insulin for 75g of glucose over two hours where as in obese body 45U insulin to accomplish the similar task.

The level of incretion hormones increased which activate GSI (glucose Stimulated insulin) in healthy individual but in diabetic patients insulin level increased which cannot be changed by lowering the plasma glucose. As a result of meditation PGC (plasma glucose concentration), FIS (fasting insulin secretion) and TI (total insulin) is decreased in response to glucose as compared to untreated conditions reveal that several beta cells are active but 'shocked' or 'masked', and in this way agreeable to being renewed by intercession (Oh *et al*. 2018).

2.4.6 Insulin resistance

In type 2 diabetes mellitus body cannot respond to insulin at certain concentrations. Insulin sensitivity is decreased due to defective signaling pathways, gene mutation, mitochondrial dysfunction, physical inactivity and obesity etc. Insulin resistance activates the alpha cells over beta cells due to progressive loss of beta cells via apoptosis because genetic irregularities put stress on beta cells which leads to decrease the insulin secretion. In cell culture experiment observed that infected cells needs high threshold of glucose for the secretion of insulin (Nolan and Prentki 2019)(Hameed *et al*. 2015).

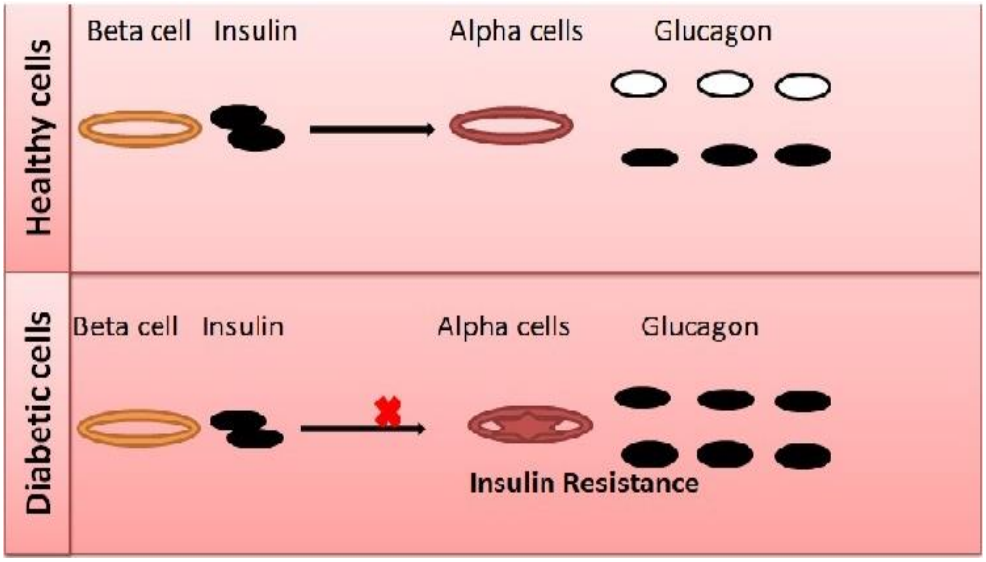


Figure 2.1: Insulin resistance: Insulin resistance is remembered to control glucagon yield in pancreatic cells. Insulin emission from pancreatic cells directs glucagon discharge from pancreatic cells in sound individuals. Insulin opposition in diabetic pancreatic cells, then again, keeps insulin from smothering glucagon discharge, bringing about glucagon hypersecretion. In diabetes, theoretical confusing glucagon hypersecretion has been seen.

2.4.7 Mechanism of insulin resistance

Beta cells produce a peptide like hormone called insulin in pancreatic tissues which regulates the carbohydrate and fat metabolism. In cell insulin binds to its receptor which activates the tyrosine kinase which leads to the process of phosphorylation of IRSs (Insulin receptor substrate) mainly IRSS1 and IRS2. These proteins involve in the activation of P13K signaling pathway which promote translocation of GLUT4. GLUT4 absorb or metabolize the glucose from skeletal muscles furthermore phosphorylates and inactivates the transcription of FOXO1 as well as modifying the downstream pathways. Insulin similarly invigorates the RAS-mitogen-activated protein kinase (MAPK) pathway. IR in T2DM has generally been associated with the PI3K pathway. Insulin resistance is regularly associated with drawn out serine phosphorylation of IRS proteins, which obstructs tyrosine phosphorylation, advance the insulin opposition. Some of the time, serine phosphorylation expands the corruption of IRS further adding to the insulin obstruction. The purposes behind expands the serine phosphorylation is multifactorial, including lipid aggregation, mitochondrial disorder and endoplasmic reticulum (ER) stress (Zatterale *et al.* 2020)(Demir *et al*. 2021).

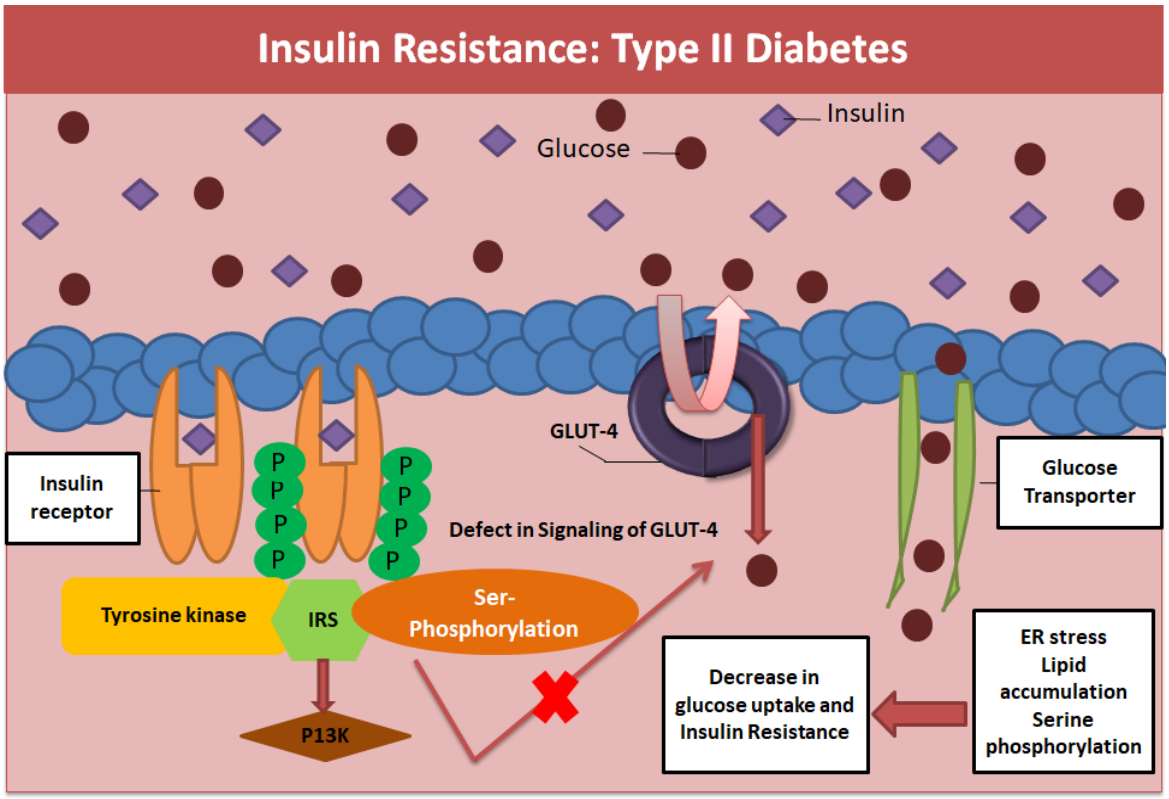


Figure 2.2: Mechanism of insulin resistance: Insulin in the bloodstream binds to its receptors, activating the phosphorylation of tyrosine kinase implicated in IRS activation; the P13K pathway is activated as a result of these processes, and is involved in the translocation of GLUT4. Instead of tyrosine, aberrant cells promote serine phosphorylation, which suppresses GLUT4 signalling pathways and leads to insulin resistance.

2.5 Diabetic complications

Diabetes is a complicated disorder due to high blood glucose level in body and effect other major organs like kidney, liver, cardiovascular and nervous system. By using different medications and interventions reduced the risk of complications such as the kidney disease, microvascular complications and cardiovascular mortality rate reduced by 0.04%, 0.28% and 1.5% respectively. Now the question raised either diabetogenic complications involved in complications or not. The

late diabetic complications caused due to ROS produced as a result of dysregulation of cellular metabolic activities as well as hyperglycemia (Demir *et al.* 2021)(Amutha and Mohan 2016).

Diabetic complications explained by Brownlee hypothesis that high blood glucose level increases the production of reactive oxygen species (ROS) which interfere in the function of glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH); inhibition of GAPDH divert the upstream glycolysis metabolites into other different pathways which involved in mitochondrial dysfunction.

- Polyol pathway
- Protein kinase C (PKC) pathway
- Advanced glycation end product (AGE) formation pathway
- Hexosamine pathway

The main driver of diabetic entanglements is mitochondrial brokenness which expands the ROS creation prompts movement of infection. A significant disadvantage of this theory is the truth that ROS have an especially short half-life and spatially amazingly limited exercises. Notwithstanding the way that there are a couple of examinations that show patients with diabetes have raised ROS, ROS levels don't actually change between patients with and without diabetic intricacies. Despite the fact that mitochondrial impedance is huge to the extent pathogenesis of diabetes and diabetic intricacies, the evidence for ROS-provoked mitochondrial brokenness that prompts diabetic intricacies similarly remains interesting (Amutha and Mohan 2016).

As both test and clinical philosophies disregard to give solid and unsurprising evidence to help Brownlee hypothesis, experts are investigating various pathways or metabolites that may accept part in diabetic confusions. Methylglyoxal (MG) is one of these responsive metabolites; the levels of which increase upon hyperglycemic change and incapacitated detoxification. One of the

mixtures that accept part in MG detoxification is Glyoxalase 1 (Glo1). Glo1 knockout flies have raised levels of MG, which animates type 2 diabetes. Also, Glo1 knockout alongside diet-incited chubbiness raises MG levels and induces type 2 diabetes like articulation in zebrafish. In help of these discoveries, MG is in like manner satisfactory to invigorate retinopathy like wounds in rat models without inciting hyperglycemia, recommending that total of MG is making a simple course to make diabetes-like total without hyperglycemia. Aside from Glo1, MG can likewise be handled either by aldo-keto reductases (AKR) to hydroxyacetone or by aldehyde dehydrogenase (ALDH) to pyruvate. Compensatory MG detoxification by extended AKR and ALDH practices is more critical in vertebrates, as not in any way like in drosophila and zebrafish, lack of Glo1 don't lift MG levels in mice (Kim et al. 2016).

2.5.1 Diabetic kidney disease

Diabetic kidney disease is a microvascular complication with dominance rate 30-40%; the major cause of death is end stage renal disease (ESRD) with prevalence rate of 30-47%. Glucose maintenance in blood decrease the disease progression rate but somehow patients with controlled blood glucose having DKD due to lipotoxicity and oxidative stress. Ion homeostasis, filtration and blood pressure rely on kidney cells which contain epithelial cells, mesangial cells and podocytes. In DKD alter the function of cells as well as the morphology like ways the extracellular matrix accumulate in glomerular basement membrane causes thickness which leads to glomerular sclerosis and permanent damaging of kidney function (Mitrofanova *et al*. 2019)(Ms, Pongchaidecha, and Lungkaphin 2019)(Hojs *et al*. 2020).

2.5.2 Cardiovascular complication

A big part of the deaths of type 2 diabetes caused because of the cardiovascular infection with commonness rate are 30%. The explanation of endothelial cell work is hyperglycemia which eventually prompts atherosclerosis. Hyperglycemia animate the initiation of AGEs (Advanced glycation finished results) adjusts the capacity of proteins and lipids by creating bond with them. These adjusted proteins and lipids initiate the AGEs receptors at last increment the statement of vascular cell attachment atom 1 (VCAM-1) and expands restricting of monocytes which penetrate into the extracellular metrix in the middle of the EC (Endothelial Cells) and SMC (Smooth muscle cells). Calm vascular smooth muscles cells (VSMC) present under the endothelial layer impel on account of high blood glucose even out and disregard to contract, move and duplicate. Monocytes changed into froth cells due to started VCMCs which related with the take-up of LDL causes plaque development loaded with fats at the conduit dividers (De Rosa *et al*. 2018).

2.5.3 Retinopathy complications

Type 2 diabetic patients have common complication called retinopathy which mainly involved in the dysfunction of two types of cells. Endothelial cells of the retinal microvasculature and pericytes that lie underneath the endothelial cells to help and manage endothelial cell work. The induction of apoptosis in pericytes and detachment caused due to high blood glucose level and oxidative stress leads to increase the permeability of blood to the retina barrier. High level of oxidized lipoprotein, AGEs and free radicals are main cause of chronic inflammation as well as diabetic retinopathy (Schlotterer *et al*. 2019)(Halim and Halim 2019)(Fahmy *et al*. 2021).

2.5.4 Diabetic neuropathy

Practically half of diabetes patients experience multifaceted nature in their autonomic and fringe tactile framework, known as diabetic neuropathy. A significant part of the time, diabetic neuropathy impacts the end points of fringe tangible nerves in hands and lower limbs causing torture, consuming, shuddering tendency similarly as deadness. As the contamination spreads, engine touchy spots at lower uttermost focuses get hurt, causing loss of offset and numb foot with loss of sensation. Because of harmed fringe nerves, there are also circumstances where diabetic neuropathy makes at the proximal areas like the thigh or pelvic and presents a proximal-to-distal angle. The fringe sensory system primarily contains schwann cells; high blood glucose level adjusts their capacity, for example, myelin issue, debilitated axon conduction, and compromised recovery in diabetic neuropathy (Kim *et al.* 2016).

2.6 Metabolic biomarkers involved in T2D

In type 2 diabetes different types of biomarkers and metabolites are involved based on their preventing strategies by integrating in metabolic pathways. Insulin regulate in body by two main factors one is nutrients like carbohydrates, proteins and lipids and second is hormones and neurotransmitters. In type 2 diabetes metabolic abnormalities are found in metabolic pathways of carbohydrates, lipids and proteins via metabolomics techniques at the onset of disease for proper treatment (Ma *et al*. 2018)(B *et al*. 2018).

2.6.1 Biomarkers related to protein metabolism

High level of amino acids in blood plasma specifically branched chain amino acids (Val, Leu and Ile) and aromatic amino acids (Phe, Trp and Try) are involved in obesity and insulin resistance.

Few amino acids have negative correlation with the onset of diabetes like glutamine and glycine. These amino acids increased the risk of diabetes from 5-7 folds high as well as the reason of hyperglycemia is linked with alanine. During breakdown of amino acids in metabolic pathways occurred in body for energy maintenance various metabolites have high concentration indicating the impaired fasting glucose like ketoacid 3-methyl-2-oxovalerate. The metabolic products produced during the down streaming process of branched chain amino acids were also increasing the risk of type 2 diabetes. Increased concentration of α-hydroxybutyrate which is produced during amino acid catabolism involved in insulin resistance via oxidative stress. Reduced expression of few amino acids linked with insulin resistance like glycine and glutamine. Patients with insulin resistance have reduced level of betaine (homocysteine methylation and detoxification). These amino acids are targeted biomarkers treated by sulfonylurea therapy. Cellular mechanism of insulin can activated by branched chain amino acids which further trigger the tor-dependent pathways as well as impede the insulin signaling leads to insulin resistance (B *et al.* 2018)(Ejaz *et al.* 2016)(Gonzalez-Franquesa *et al.* 2016).

2.6.2 Biomarkers related to lipid metabolism

The process of lipolysis in adipose tissues released free acids which is the cause of obesity. Metabolites derived from adipose tissues involve in insulin resistance. Type 2 diabetic patients with obesity have increased level of heptadecane, oleate, palmitoleic acid, palmitate, stearate, acetoacetate and 3-hydroxybutyrate. Beta oxidation of fatty acids is the main source of energy due to the lack of insulin leads to increase the ketone bodies in patients. The lipid biomarkers in type 2 diabetic patients are palmitic acid, stearic acid, oleic acid, linolenic acid, and linolenic acid. Defective oxidation of long chain fatty acids stimulate the NF-κB which leads to insulin resistance, inflammation and oxidative stress. The risk of type2 diabetes can be predicted by the

presence of sphingomyelin, 9 phosphatidyl cholines, lysophosphatidylcholine, decanoyl carnitine and lysolecithin. Biomarkers to predict the onset of disease are hemolytic phosphatidylcholine and acetylcarnitine as well as the increased concentration of diacylphosphatidylcholine and decreased concentration of alkylphosphatidylcholine and sphingomyelin. The patients with T2D have high cholesterol and phospholipids are also involved in pathogenesis but mechanism is not clear yet (Swisa, Glaser, and Dor 2017)(Erion, Park, and Lee 2016).

2.6.3 Biomarkers related to carbohydrate metabolism

The critical wellspring of energy in human body is glucose; changed over into pyruvate to acetyl co-A then, at that point, go into TCA cycle to meet energy necessity for human body. In type 2 diabetic patients, the centralization of pyruvate, lactate, citrus extract and citrate is higher in blood serum. A compound have comparable design of glucose atom called 1, 5-anhydroglucitol (1, 5-AG) is a non-physiologically powerful polyol in the body. It is reabsorbed in the renal tubule, which is truly limited by glucose. It keeps a steady state level through renal filtration and reabsorption at normal blood glucose levels. In any case, with the extension of blood glucose obsession (>180 mol/L), glucose can't be completely reabsorbed by the kidney and it truly limits the reabsorption of 1, 5-AG by the renal tubules, which achieves a decreasing in serum 1,5-AG found that 1,5-AG is a sensitive marker of postprandial hyperglycemia and various patients who are especially compelled by the glycemic control record HBc similarly have colossal postprandial hyperglycemia (Giesbertz *et al.* 2015)(Ohara *et al.* 2016).

2.7 Metabolic pathways

There are different metabolic pathways that play important role in diabetes, these are the following;

2.7.1 Biosynthetic pathway of different amino acids

There are metabolic pathways of different amino acids like serine, aromatic amino acids, glutamic, aspartate and alanine. The concentration of 3-phosphoglycerate is increases when the glucose level is higher in blood which changed into serine after a sequence of reaction. Serine converted into amino acid in the presence of glutamic acid. In diabetic patients, synthesis of serine in smaller amount is due to decreased concentration of glutamate which leads the reduction in glycine production. Decreased production of glycine leads to ROS (reactive oxygen species) production because it's having antioxidant activity in body as well as the expression of phospho-serine aminotransferase 1 regulatory homolog 3 is decreased which induce the insulin resistance (Wang *et al*. 2018).

During pentose phosphate pathway, phosphate ribose is produced which changed into shikimic acid via shikimic acid pathway. Shikimic acid converted into phenylalanine derivatives which impede the dipeptide kinase action and protect the incretion molecule from degradation. Incretin is a compound which actively involved in excess release of insulin which ultimately the cause of insulin resistance. Phosphate ribose is also produced histidine in the presence of glutamine. The amount of glutamine is less in diabetic patients which means the less concentration of histidine causing the inflammation and oxidative stress (Rehman and Akash 2017).

In human body, pyruvate content is increased by the increased concentration of glucose in blood. By following a series of biochemical reactions pyruvate is converted into leucine and valine. In white adipose tissues, the fats movement is reduced due to high level of leucine as well as in brown adipose tissues the expression of uncoupling protein is decreased. The decreased expression associated with less energy production and increased fat accumulation. The increased concentration of leucine and valine involved in decreased the insulin sensitivity and fat

movement as well as inhibit the mTOR or AMPK signaling pathway. In type 2 diabetic patients, glutamine produced in less amount which leads to inadequate secretion of insulin in body. The main function of glutamine in body is increase the expression of glucagon-like peptide-1 (GLP-1) which is responsible for increase the secretion of insulin (Wang *et al*. 2018).

In aspartate biosynthetic pathway, during transaminase reaction oxaloacetic induce the production of aspartic acid which further converted into methionine and isoleucine. Higher production of isoleucine and methionine leads to insulin resistance by inhibiting the mTOR and AMPK signaling pathway and by increasing food intake reduced the energy use in body respectively (Rehman and Akash 2017).

2.7.2 Biosynthetic pathway of fatty acids

Higher concentration of glucose in blood increases the glycolysis which produced pyruvate up to the maintained level which further converted into acetyl co-A via oxidative decarboxylation. Acetyl co-A produced fatty acid by following different biochemical reactions. Higher concentration of free fatty acids leads to diabetes by causing defects in beta cells and minimize the glucose uptake from muscles (Rehman and Akash 2017).

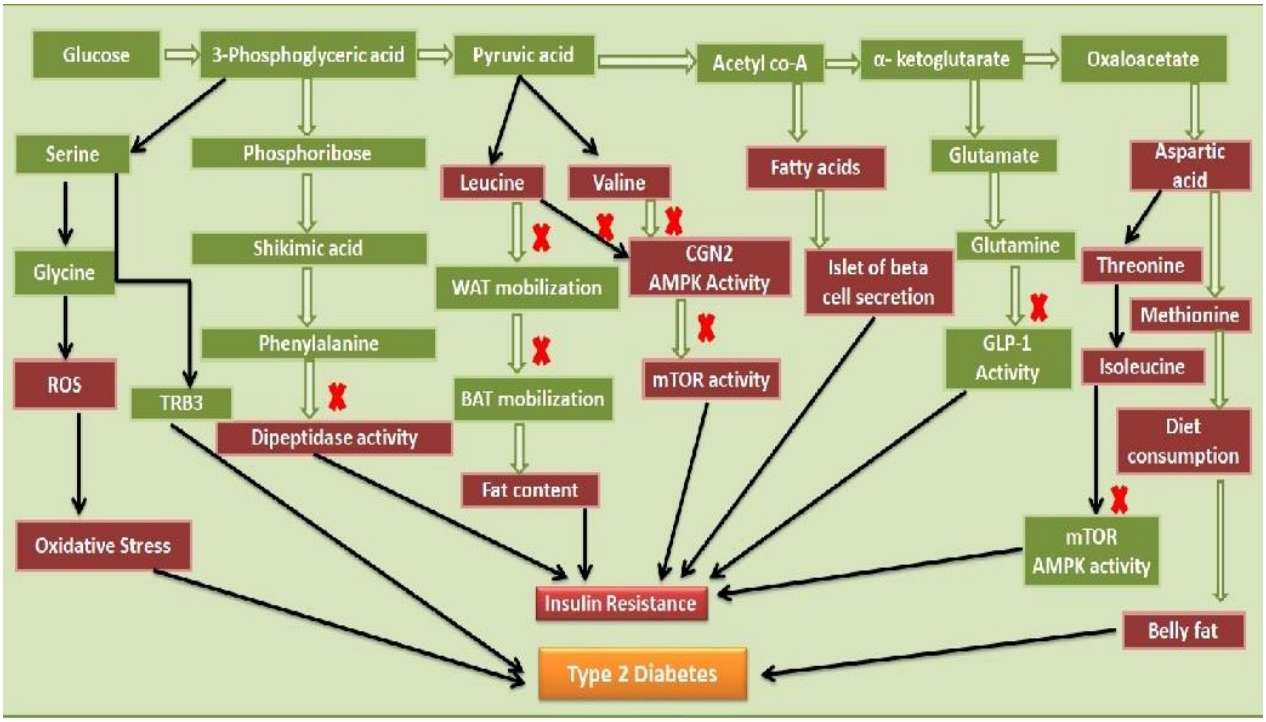


Figure 2.3: Metabolic pathway of carbohydrate, fatty acids and amino acids: There are six metabolic pathway associated with insulin resistance leading to type 2 diabetes. Metabolites in green are up-regulated and remaining are down-regulated, considered as active biomarkers.

2.8 Oxidative stress and T2D

Oxidative stress is intensely associated with T2D; total antioxidant enzyme activity is decreased with the severity of disease while concentration of peroxide enzymes has been increased. Oxidative stress is considered as one of the major factor that is responsible for insulin resistance, inflammation, hyperglycemia, endothelial dysfunction and defect in insulin secretion (Rehman and Akash 2017).

2.8.1 Mechanism of oxidative stress

The fundamental cause of oxidative stress is the production of reactive oxygen species (ROS) which is produced due to the generation of electron molecules in the membrane of mitochondria

further it form the superoxide anion (O2-). The enzyme called NADPH oxidase which is responsible for the production of superoxide anion also called reactive oxygen species. There are some other factors which are responsible for the production of superoxide anion like nitric oxide (NO) generation, endothelial disorder that rapidly involved in the oxidation of carbohydrates, lipids and proteins. The molecules of lipid peroxides are produced due to the oxidation of polyunsaturated fatty acids (Rehman and Akash 2017) (Dhounchak *et al*. 2021) (Asmat, Abad, and Ismail 2016)(Wang *et al*. 2020). The generation of reactive oxygen species is involved in stimulating the various biochemical pathways like production of advanced glycation end-product, Hexosamine pathways and PKCβ1/2. Higher generation of reactive oxygen species disrupt the stability of oxidant and anti-oxidants enzymes. There are different pathways which contribute in the progression of disease such as NH2-terminal Jun kinases, p53 MAPK and nuclear factor kappa B (NF-κB) (Yaribeygi *et al*. 2020)(Trpkovic *et al*. 2015)(Burgos-Morón *et al*. 2019)(Liang *et al.* 2021).

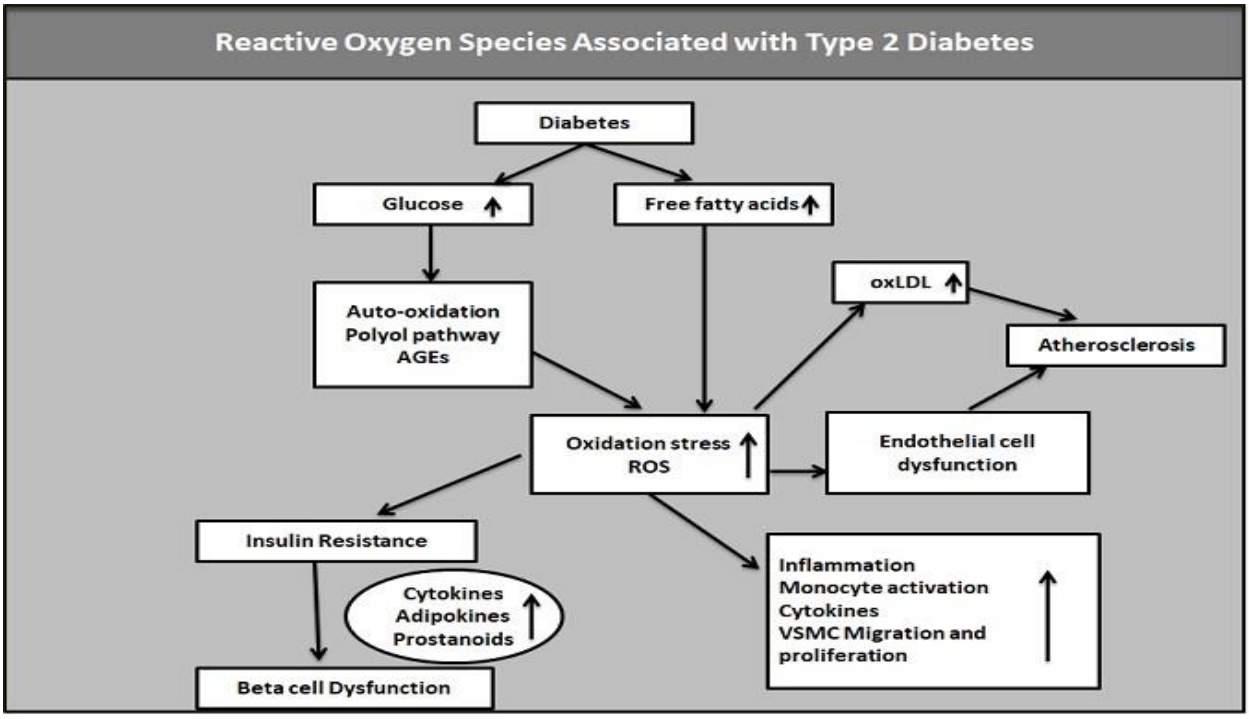


Figure 2.4: ROS associated with T2DM: Hyperglycemic and high concentration of free fatty acids is the cause of oxidative stress which leads to insulin resistance. Reactive oxygen species are also activating the production of oxidized low density lipoprotein that leads to atherosclerosis (Tangvarasittichai 2015).

2.8.2: Oxidative stress related to hyperglycemia and hyperlipidemia

Hyperglycemia showed their detrimental influence on beta cells ultimately promote the oxidative stress in cells. Beta cells are more susceptible to produce oxidative stress against hyperglycemia in type 2 diabetes mellitus. There are few modified proteins having higher concentration are to be observed during animal trials like high level of HNE and 8-hydroxy-deoxyguanosine indicate that the major factor of oxidative stress and dysfunction of beta cells are due to hyperglycemia. When body has severe hyperglycemic condition, the superoxide anion produced as a deficiency of superoxide dismutase is changed into H2O2 which further produce toxic radicals after interacting with heavy metals called hydroxyl radicals (Jain and Sark 2016). In type 2 diabetes, excessive generation of FADH2 and NADH are related to high blood glucose level which further leads the higher production of protons in mitochondria. There are two main compartments which involve in the generation of superoxide from released electrons such as cytochrome c reductase and NADH dehydrogenase (Besseling *et al*. 2015)(Kahal *et al*. 2020).

In normal human body, almost 30-35% of glucose metabolize during polyol pathway, defect in polyol biochemical pathway increased the production of superoxide. There are two enzyme related abnormalities such as glucose converted into sorbitol in the presence of aldose reductase by using NADPH. Excessive generation of NADPH decreased the accessibility of NADPH which promotes the insignificant production of reduced glutathione and NOS synthase. Sorbitol

converted into fructose in the presence of sorbitol dehydrogenase with production of NADH. When NADH is produced in excess; it is used by NADH oxidase to generate oxidative stress. Diacylglycerol involved in the activation of PKCβ ½ which further lead the production of VEGF, matrix protein and variate the permeability of retinal blood. During the process of glycation the reducing sugars binds with free amino acids produced amadori product which further rearrange and formed AGEs. Increased level of AGEs can activate the redox sensitive transcription and intracellular redox signaling which are the main factors of inflammation (Daya, Bayat, and Raal 2017)(DiNicolantonio, Bhutani, and O'Keefe 2015).

The prolonged exposure of free fatty acids to beta cells is the major cause for the generation of ROS and oxidative stress. Hyperlipidemia is involved in the reduced function of insulin gene expression leads to decreased glucose related insulin secretion; damaged the beta cell structure due to accumulation of long chain fatty acyl co-A activate the β-cell K+-sensitive ATP channels. Insulin resistance and superoxide generation is majorly associated with increased the lipid synthesis in liver as well as the presence of VLDL, TG in higher concentration with decreased HDL concentration. In hyperlipidemic condition, higher concentration of significant oxidative stress biomarkers 8-oxo-dG and oxidized GSH are to be observed. Oxidative stress activates the production of various chemokines and cytokines such as IL-β, IL-6, TNF-α, and IL-1β *(Nowotny et al*. 2015)(Aouacheri *et al*. 2015)(Tangvarasittichai 2015).

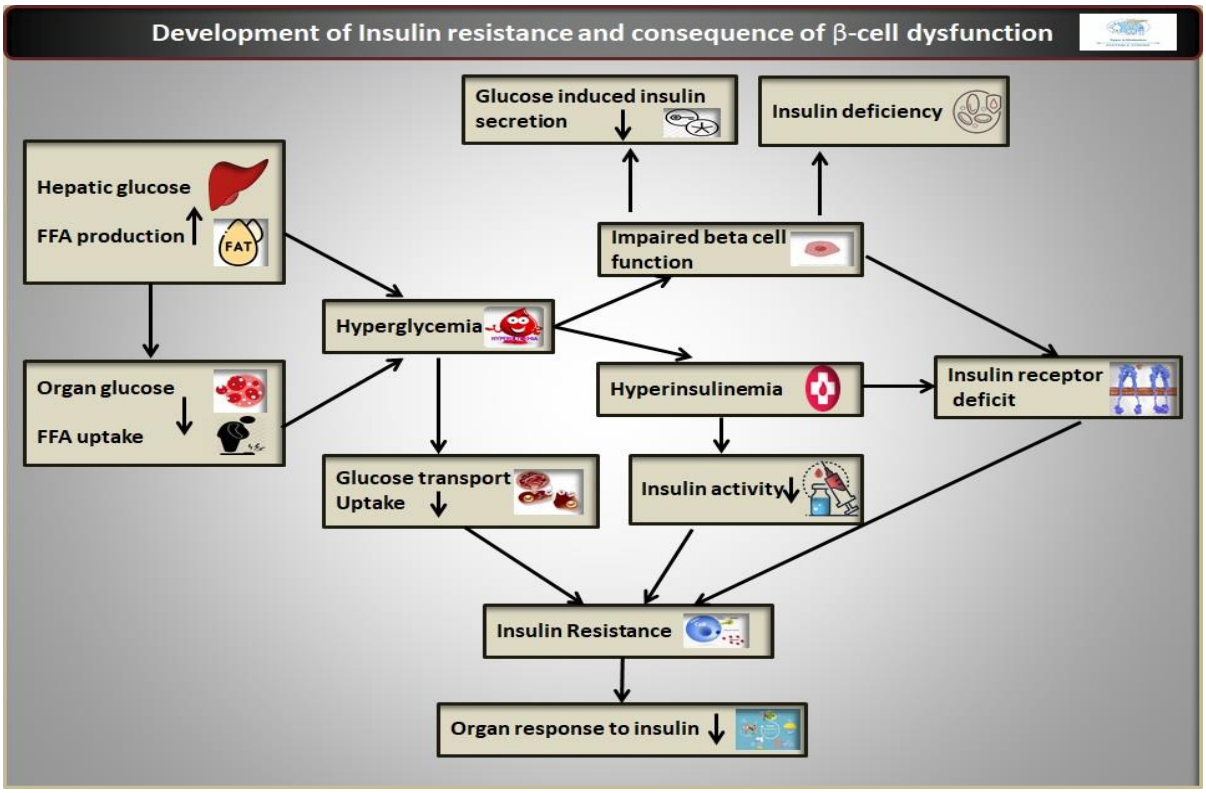


Figure 2.5: Insulin resistance and consequences of beta cell dysfunction: Glucose stimulates the insulin production, in hyperglycemic conditions beta cells do not functioned properly, defect in insulin receptors causes the insulin resistance, means that body organs did not respond to insulin for glucose metabolism (Tangvarasittichai 2015).

2.9 Oxidative stress biomarkers

There are different oxidative stress biomarkers which studied to understand the pathogenesis mechanism, insulin resistance and for diagnostic purpose.

2.9.1 Glycated proteins and Lipid peroxidation

In diabetic condition, high blood glucose is responsible for the oxidative stress which activates the protein glycation. Glycated hemoglobin and fructosamine level in blood or serum is used for

the approximation of protein glycation. The oxidative stress in type 2 diabetes is increased due to the abnormal function and change in structure of anti-oxidant enzyme which leads to the production of free radicals. A compound called 3, 3-dityrosin act as a bridge between same or different protein molecules considered as oxidative stress biomarker for protein oxidation (Asbaghi *et al*. 2019)(Jamuna Rani and Mythili 2014).

During the pathogenesis of type 2 diabetes, the disorganized level of lipid concentration in body is the main reason of lipid peroxidation. Poly unsaturated fatty acids are a part of cell membrane having several bonds more susceptible for the production of lipid free radicals like lipid hyper peroxides is noxious radical for the induction of oxidative stress. Lipid peroxidation is oxidative stress biomarker; malondialdehyde (MDA) is produce as a result of lipid oxidation is used to measure the value of lipid oxidative stress by reacting with thiobarbituric acid (Yeda *et al*. 2017)(Zhang *et al.* 2020).

2.9.2 Glutathione, catalase (CAT) and superoxide dismutase (SOD)

There are two classes of glutathione one is glutathione reductase and other is glutathione peroxidase both considered as major oxidative stress biomarkers which process the peroxides into water molecule in T2DM pathogenesis. Hydrogen peroxide (H2O2) is a lethal compound functioned as to damage the DNA, RNA and lipid molecules causing severe modification in body. Catalase is an enzyme that's help in neutralizing the hydrogen peroxide molecule into water and oxygen. Catalase insufficiency in body is more prone to oxidative stress and beta cell show damage is impropriate for the production of insulin. The main reason for downregulation of catalase enzyme gene is hyperglycemia (Bigagli and Lodovici 2019).

Superoxide dismutase (SOD) is an antioxidant enzyme functioned as detoxify the damaging oxygen species into less toxic compound. The enzyme provides major protection mechanism in body for breaking the superoxide molecule in to oxygen and peroxide which are very harmful and toxic compound activate the production of reactive oxygen species in type 2 diabetes mellitus pathogenesis (Malik *et al*. 2020).

2.9.3 Vitamins, oxidized low-density lipoprotein and protein thiols

Vitamins play a crucial role as antioxidants in body to detoxify the lethal oxygen species. The decreased level of vitamins likes A, C and E activates the production of pro-oxidants that are toxic for body. The changed level of these vitamins is act as biomarkers for oxidative stress (Balducci, Stefano, Sacchetti, Massimo, Haxhi, Jonida, Orlando, Giorgio, D'Errico, Valeria, Fallucca, Sara, Menini, Stefano, Pugliese 2014)(Xu *et al*. 2018). The level of oxidized low density lipoproteins and glutathionylated proteins (glutathionylated hemoglobin) are increased in diseased condition specially related to oxidative stress like diabetes and obesity and act as special biomarker T2DM (Cojic *et al.* 2021)(Ragheb *et al.* 2020).

2.9.4 4-Hydroxynonenal (HNE), malondialdehyde (MDA) and 3-nitrotyrosine

MDA concentration in blood serum observed has to be increased with age factor. The oxidative stress produced due to MDA and HNE has been decreased by using oil of *Eruca sativa* seeds in alloxan associated diabetic rat model. Oxidative stress is associated with tyrosine molecule when nitro group is replaced the C3 hydrogen atom and itself make bond with tyrosine formed 3-nitrotyrosine called tyrosine nitration. In diabetic patients it is observed as an important biomarker (Schaft n.d.) (Chaudhury *et al*. 2017).

2.10 Phenolic compounds in olive oil

Olive oil is perceived similar to an intense pharmacological specialist that contributes essentially to work on the wellbeing. Extra-Virgin Olive oil is fundamentally used to lessen the death rate in patients with CVD and incendiary illnesses like disease, hypertension and endothelial capacity. It is the new olive juice comprises of 98% mono and poly unsaturated fats and staying 2% is containing minor mixtures which is up to 230 in number like (aliphatic and triterpene alcohols, sterols, hydrocarbons, unpredictable mixtures and cancer prevention agents). In extra-virgin olive oil, monounsaturated unsaturated fats are available in high substance just about 56-84% like oleic acid and remaining portion is 3-21% having polyunsaturated unsaturated fats like linoleic corrosive which contribute in decreasing the LDL-cholesterol action (Morvaridi et al. 2020). Ongoing examinations exhibit those phenolic compounds in additional virgin olive oil playing a critical part to fix the cardiovascular illnesses. A few investigations showed that 5mg/day utilization of olive phenolic compounds for medical advantages in light of the fact that the phenolic compounds diminish the peroxidation of blood lipids (Valls et al. 2015). The mixtures like lipophilic and hydrophilic phenols (optional metabolites) with modest quantity of carotenoids demonstrating more noteworthy cell reinforcement movement in additional virgin olive oil. The main gatherings of phenols found in EVOO were phenolic acids, including caffeic, vanillic, syringic, p-coumaric, o-coumaric, protocatechuic, sinapic, p-hydroxybenzoic and gallic corrosive. Before, phenolic alcohols, mainly addressed by (3, 4-dihydroxyphenyl) ethanol (3, 4-DHPEA) and (p-hydroxyphenyl) ethanol (p-HPEA), were found in EVOO. As of late, flavonoids, for example, luteolin and apigenin have been found (Morvaridi *et al.* 2020)(Paulo and Santos 2021)(Umeno *et al*. 2016).

Table 2.1: Phenolic compounds in olive oil and their derivatives: Phenolic compounds and their derivatives have antioxidative property as well as flavonoids and seciriodiods (Loizzo et al. 2011).

Phenolic Compounds and Derivatives	**Phenolic Alcohols**	**Flavonoids**	**Secoiridoids**
Vanillic acid	(3,4-Dihdroxyphenyl) ethanol (3,4 DHPEA)	Apigenin	Dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA (3,4 DHPEA-EDA)
Syringic acid	p-Hydroxyphenil) ethanol (p-HPEA)	Luteolin	Dialdehydic form of decarboxymethyl etenolic acid linked to p-HPEA (p-HPEA-EDA)

p-Coumaric acid/ o-Coumaric acid	(3,4-Diidrossifenil)etanolo-glucoside		Oleuropein aglycon (3,4 DHPEA-EA)
Gallic acid			Ligstroside aglycon
Caffeic acid			Oleuropein
Protocatechuic acid			Dialdehydic form of Oleuropein aglycon
p-Hydroxybenzoic acid			Dialdehydic form of Ligstroside aglycon
Ferulic acid			
Cinnamic acid			

4-(acetoxyethil)-1,2-Dihydroxybenzene			
Benzoic acid			

2.10.1 Antioxidant activity of hydrophilic phenols in extra-virgin olive oil

Antioxidants play an important part in the shelf life of extra virgin olive oil by delaying their oxidation process. Phenolic compounds in olive oil donating their radical hydrogen to alkylperoxyl. It is produced as a reaction of lipid peroxidation and phenolic compounds act as chain breaker because they have biological properties. Oil rich in phenolic compounds reduces the NF-κB activation by increasing the activation of IκB-alpha in cells. These phenolic compounds decrease the lipopolysaccharide plasma concentration and act as active inflammatory biomarker (Tome-Carneiro *et al*. 2020)

The effects of phenolic compounds on Caco-2 cells demonstrate that phenolic compound decreases the IL-8 expression in cells as well as change the inflammatory response in intestinal epithelial tissues. Phenolic compound also inhibit the tumor necrosis factor alpha that induced matrix metalloproteinase 9 in monocyte cell line. The valuable impacts of phenolic compounds on wellbeing have been explored broadly; furthermore, late exploration upholds prior proof that these parts apply helpful impacts on physiological cycles identified with wellbeing and illness. Various examinations both in vivo and in vitro exhibit that phenolic compounds successfully change inflammatory response and have valuable impacts on markers of malignant growth,

atherosclerosis and furthermore qualities identified with weight and metabolic disorder (Ganesan, Sukalingam, and Xu 2018) (Vlavcheski, Young, and Tsiani 2019).

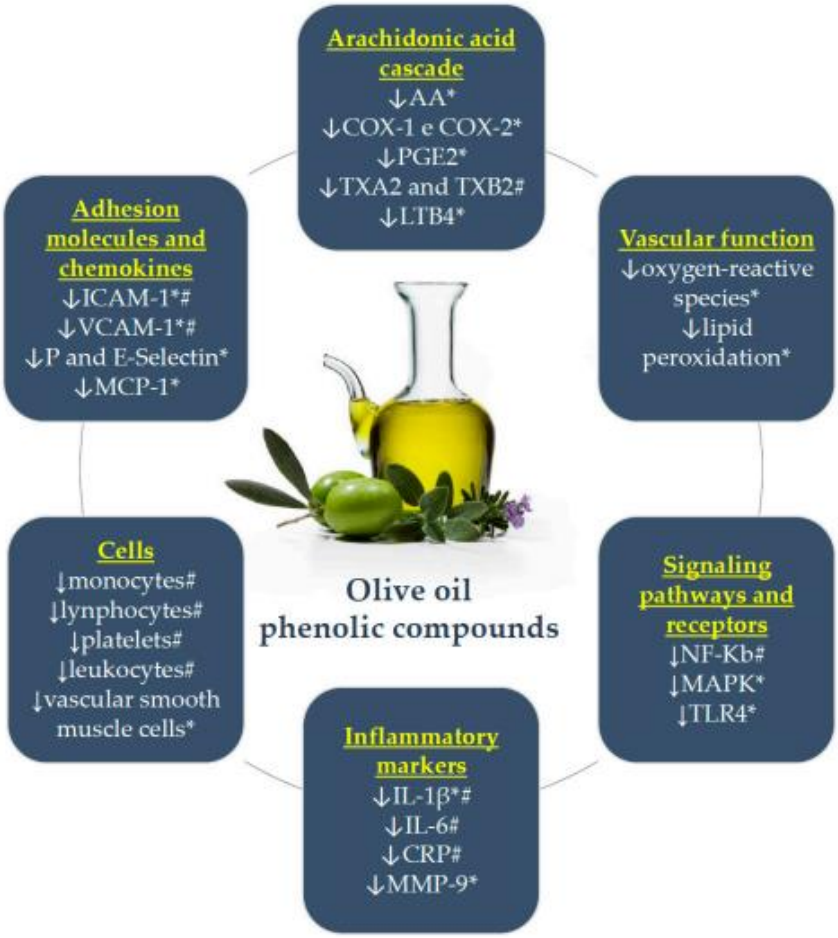


Figure 2.6: Effect of phenolic compounds in human health: Phenolic compounds improve the cardiac health by activating different metabolic pathways like aracdonic acid cascade and other immune related cells (Tome-Carneiro *et al*. 2020).

2.10.2 Phenolic compounds in canola oil

Due to its superior nutritional qualities and significantly higher content, canola (*Brassica napus*) is in high demand for processing into animal feed and vegetable oils as compared to other

oilseeds in terms of phenolic compounds. Sinapic acid, protocatechuic acid, vanillic acid, syringic acid, and -hydroxybenzoic acid are phenolic acids, ferulic acid, -coumaric acid, caffeic acid, and -courmaric acid has been discovered in canola seed. Phenolic acids of varied kind, the hydroxycinnamic acids (sinapic acid, sinapic acid, sinapic acid, sinapic acid, sinapic acid, sinapic acid, sinapic acid, sinapic acid, coumaric acid, caffeic acid, cinnamic acid, chlorogenic acid, and ferulic acid, among others) have drawn a lot of attention (Amiri *et al.* 2019). They've gotten a lot of importance because of their diverse biological functions, which include action of antioxidants. Sinapic acid, in particular, and its derivatives in canola, derivatives are the most abundant phenolic antioxidants. Seed and they're well-known for being just as effective as synthetics. Antioxidants are beneficial to the body. As a result, canola seed may play a significant role in the food business as well as a natural antioxidant source as in the case of livestock and medicines (Cano-Europa *et al*. 2016)(Li *et al*. 2019).

2.10.3 Anti-diabetic property of olive and canola oil

Oxidative stress in the body increases during pre-symptomatic stages, borderline diabetes, or the beginning of type 2 diabetes. Lipid oxidation levels in the blood are high in the early stages of type 2 diabetes. HODE, the product, has been boosted. A rise in HODE levels implies oxidative stress in general and, in particular, oxidative stress in the liver radicals and singlet oxygen are involved. As a result, oxidative stress inhibition could be beneficial. In the early phases of type 2 diabetes, including pre-symptomatic states, it is beneficial in preventing the disease. When type 2 diabetes first appears, it causes chronic blood sugar increase and insulin resistance observed. As a result, regular consumption of polyphenols may help to reduce oxidative stress and, as a result, improve health. Reduce your chances of getting type2 diabetes. In vitro, polyphenols have a high

antioxidant activity. Polyphenols have anti-oxidant properties, As a result of the elimination of reactive oxygen, oxidative stress is reduced (Zhuang *et al.* 2020)(Santangelo *et al.* 2016).

This is the principal study to check out the neuroprotective and against diabetic properties of phenol-rich concentrates from EVOOs. Different proteins engaged with Alzheimer's, Parkinson's, significant burdensome issue, and diabetes mellitus could be repressed by phenolic bioactive substances that work as multi-target ligands. Both phenol-rich concentrates from EVOOs had the option to hinder BuChE, LOX, hMAO-A, and hMAO-B in a portion subordinate way, and could be considered as promising normal items for treating CNS diseases like AD, MDD, and PD. Throb chemical was likewise repressed by oil extricate. This double ChE restraint can possibly work on the adequacy of treatment for an assortment of CNS illnesses. Since they showed more prominent restraints, phenol-rich concentrates from EVOOs could be considered as an effective extra treatment for postprandial hyperglycemia (Samarji and Balbaa 2014). Analysts recommend that both Nigella sativa (NS) and olive oils can assist with lessening diabetic entanglements how much enzymatic movement. It was believed that their instrument of activity was because of cell reinforcement movement. Canola oil, then again, adversely affected diabetic confusions and enzymatic changes are normal (Samarji and Balbaa 2014).

According to research, hydroxytyrosol (HT) exerts insulin-like effects on insulin target cells such as adipocytes, hepatocytes, and muscle cells, and has anti-diabetic benefits in animals. T2DM models are a type of diabetes mellitus. Furthermore, HT has been shown to protect against oxidative damage, inflammation, and cancer. Chemically, genetically, and dietary-induced hyperglycemia and hyperlipidemia in animal models T2DM is a kind of diabetes mellitus. Despite the paucity of current research on HT toxicity, evidence suggests that HT is harmful is well-accepted oral administration of pure HT at doses of 5, 50, and 500 mg/kg/day was found to

be effective in a study. There were no negative consequences in wistar rats after 13 weeks; including no changes in micro or macro organs also morbidity or mortality more notably; the European food safety authority was established in 2011 (EFSA). The usage of HT (5 mg/day) or its derivatives to protect against oxidative damage has been approved and inflammation, as well as lowering the risk of cardiovascular disease and diabetes. More systematic long-term human investigations, however, are required (Ditano-Vázquez *et al.* 2019) (Jun, Wiesenborn, and Kim 2014).

Chapter 3

Materials and Methods

Materials and Methods

3.1 Materials collection

3.1.1 Collection of cooking oils/ drugs and chemicals

Extra-virgin olive oil (EVOO) and canola oil (CO) was purchased from CSD (Canteen Stores Department) NUST, Islamabad. Streptozotocin (STZ) was bought from Sigma Aldrich. Metformin as Glucophage (250mg tablet) was purchased from local pharmacy. Biochemical test are done by using diagnostic kits purchased from Bio Research and CHEMELEX S.A diagnostic reagents. All other chemicals were of analytical grade used in this research purchased from Sigma Aldrich, USA.

3.1.2 Experimental model

The Experiment was done by using male and female wistar strain of albino rats, 8-12 weeks old, weighing female rats 195-249g and male rats 164-347g. The rats were housed in plastic cages at a controlled temperature of 25°C with a 12-hour light/dark cycle. Animals were fed with standard chow and supplied with drinking water as per desired condition. Rats were habituate to the laboratory conditions for one week prior to the initiation of experiment. The study lasted for a total of 5 weeks. Wistar rats were taken from the animal house facility of Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad, Pakistan. All of the animal procedures and experiments were conducted after taking the approval of institutional review board (Ref. IRB No. 11-2020-01/01) and all of the experimental procedures were according to the guidelines described by the laboratory animal house (LAH), ASAB, NUST.

3.1.3 Animal diet

Animal diet consists of total 23% of proteins, 4-5% of fats and 4% fibers.

3.2 Methods

3.2.1 Induction of diabetes

In 0.05M citrate buffer, streptozotocin was dissolved (pH 4.5). A single intraperitoneal dose of streptozotocin (40mg/kg) by body weight was used to cause diabetes. Non-diabetic control group is injected with citrate buffer instead of streptozotocin. After streptozotocin injections, rats were given 10% (weight/volume) fructose solution ad libitum for consecutive 3 days and free access to normal feed. Diabetes mellitus was permitted to create and settle in these STZ-treated rats over a time of seven days. Diabetes mellitus was defined by determination of rat tail blood glucose level by using glucometer (On call Glucometer EZ II). Blood glucose level 250-300mg/dl considered as diabetic.

3.2.2 Experimental group design

The duration of these experiments was 5 weeks. A total of 30 rats including normal (n=6) and diseased rats (n=24) were divided into 5 groups. Each group consist of 6 rats (3 males and 3 females) were housed in separate cages. Nature of each group taken in this study is group 1 is healthy control animals (C) chow with normal feed and water. Group 2 contains diabetic control rats (DC) chow with normal feed and water. Group 3 (DM) contains diabetic rats treated with metformin 300mg/kg body weight/day orally. Group 4 (DO) consist of diabetic rats treated with extra-virgin olive oil 2.5ml/100g /day orally. Group 5 (DCa) contains diabetic rats treated with canola oil 2.5ml/100g /day orally.

3.2.3 Measurement of body weight

Rat's body weights were evaluated before induction of diabetes then 1st, 15th and 35th day of treatment by using a digital balance. The weights of the experimental rats were taken at the same time in the morning. Throughout the trial, signs of abnormalities in body weight were observed.

3.2.4 Blood sampling

The experimental animals were starved for 12 hours at the end of the 5-week period, but water was not restricted. Direct cardiac piercing was used to collect blood samples, which were then placed in clotting blood tubes. Blood samples were centrifuge at 4000rpm for 5 minutes and serum were separated and stored at -20°C for biochemical analysis and organs are stored in 10% formalin and stored at -80°C for further analysis.

3.3 Biochemical analysis

3.3.1 Glucose test

Glucose is the key source of energy in human body. Body converts carbohydrates into glucose (simplest sugar), if the blood glucose level in body is high it considered as diabetic conditions. Glucose test is done to optimize the blood glucose level; sometimes it is done to check the hypoglycemic conditions known as when blood glucose level is too low. Blood glucose level is also measure in other abnormal conditions like pancreatic cancer, hyperthyroidism, and acromegaly and Cushing syndrome. Here it is done for diabetic conditions (Walvekar, Ambekar, and Devaranavadagi 2015). Glucose was measured in serum samples using Glucose (SL), GOD-PAP CS008 (Bioresearch diagnostic kit). Measurement was taken according to manufacturer's instructions by using Chemistry Analyzer (CHEMREADER Smart-N SE250).

3.3.2 Lipid profile

Diabetic dyslipidemia mostly occur in diabetic patients as well as in cardiovascular diseases. One of the most prevalent secondary causes of hyperlipidemia is type 2 diabetes. Because both tend to occur with increased frequency in type 2 DM, the link between hyperlipidemia and vascular complications of diabetes has long been of interest. Insulin resistance and obesity promote dyslipidemia and hyperglycemia, which increase the risk of cardiovascular disease. Patients with diabetes mellitus should be treated as if they already have coronary artery disease. Complications in diabetes patients are associated with increased lipid fractions (TGs, cholesterol, and LDL-C) and decreased HDL-C. This implies that there may be a link between the onset of various vascular issues (both micro and macro vascular) and the presence of lipid abnormalities. It's difficult to pinpoint a single cause because the presence or progression of these issues is determined by a complex web of interconnected elements. Because good diabetes control has been found to keep lipid levels in the normal range, it looks crucial to aim for critical DM control to prevent or at least delay the onset of different problems (Prajapati *et al*. 2019).

Cholesterol, High density lipoprotein, Low density lipoprotein and Triglycerides were measured in serum using Cholesterol (SL), CHOD-PAP CS005, LDL Cholesterol CS011 and Triglyceride (SL), GPO-PAP CS016 (Bioresearch Diagnostic kits) respectively. Measurements were taken according to manufacturer’s instructions by using Chemistry Analyzer (CHEMREADER Smart-N SE250).

3.3.3 Determination of liver function test

Liver enzymes are proteins that help your body's chemical reactions move faster. Producing bile and compounds that help your blood coagulate, breaking down food and pollutants, and combating illness are all examples of chemical reactions. The following are some of the most

common liver enzymes: Alkaline phosphatase (ALP), Alanine transaminase (ALT), Aspartate transaminase (AST), Gamma-glutamyl transferase (GGT). When your liver is harmed, it produces enzymes that are released into your bloodstream (most commonly ALT or AST) (Mathur *et al*. 2016).

Alkaline phosphatase (ALP), Alanine amino-transferase (ALT), Albumin (ALB) and Bilirubin (BIL) were measured in serum using Alkaline Phosphatase (SL), DGKC, (CZ001), ALT/GPT (SL), UV IFCC, (CZ003), Albumin, BCG, (CS001)(Bioresearch diagnostic kits) and BILIRUBIN T&D-DMSO DMSO, Colorimetric (CHEMELEX S.A Diagnostic reagents, ref # 30157). Measurements were taken according to manufacturer's instructions by using Chemistry Analyzer (CHEMREADER Smart-N SE250).

3.3.4 Determination of kidney function test

Diabetes is the most well-known reason for ongoing kidney infection (CKD) around the world. Arising information proposes that serum uric acid (SUA) is an indicator of kidney harm in both non-diabetic and diabetic patients lately. A high SUA level is connected to an expanded danger of creating CKD and end-stage renal sickness (ESRD). SUA is connected to a lower glomerular filtration rate (GFR) and an expanded danger of early moderate renal capacity misfortune in individuals with type1 diabetes. In type 2 diabetic patients, a high SUA was likewise connected to diminished renal capacity. In patients with type 2 diabetes and unblemished renal capacity, both hyperuricemia and a high-ordinary SUA show the turn of events and movement of CKD. Creatinine is a side-effect of the energy-producing exercises in your muscles. Creatinine is taken out from the blood by sound kidneys. Creatinine is discharged in the pee as a byproduct. At the point when the glomerular filtration rate is essentially decreased or pee end is impeded, serum creatinine levels rise. Before a spike in serum creatinine can be seen, around half of renal

capacity should be lost. Thus, serum creatinine fills in as a late sign of intense renal injury (Moinuddin and Awanti 2016).

Creatinine and uric acid were measured in serum using Creatinine (SL), KINTEIC, (CS006) and Uric Acid (SL), URICASE-PAP (CS018) (Bioresearch diagnostic kits), respectively. Measurements were taken according to manufacturer's instructions by using Chemistry Analyzer (CHEMREADER Smart-N SE250).

3.4 Antioxidant enzyme activities from liver tissue

There are various enzyme systems that catalyze free radical and reactive oxygen species neutralization processes. Among these enzymes are:

- Superoxide dismutase
- Catalases
- Lipid peroxidation MDA

These are the body's natural defensive systems against free radical-induced cell damage. Antioxidant enzymes such as catalase and superoxide dismutase (SOD) metabolise oxidatively damaging intermediates. These enzymes also require co-factors like selenium, iron, copper, zinc, and manganese for maximum catalytic activity. It's been suggested that if you don't get enough of these trace minerals in your diet, your antioxidant defence mechanisms will be less effective. Consumption and absorption of these critical trace elements may decrease as people age (Noeman, Hamooda, and Baalash 2011).

3.4.1 Tissue lysate

Tissue homogenate was made by dissolving 500 mg of liver tissue in 1ml of 0.1M cold phosphate buffer (PBS) pH 7.4. In a chilly jacket, all tissues were homogenised with a pestle and mortar. The homogenates were transferred to tubes without increasing the heat, and then centrifuged for 20 minutes at 4 °C at 4,000 rpm. Following this technique, the generated supernatants were kept at -80°C and employed in lipid peroxidation and antioxidant enzyme assays such as SOD and CAT.

3.4.2 Lipid peroxidation (MDA)

Lipid peroxidation assay (also named as TBARS) basically tells about the oxidative degradation of lipids. Oxidative stress results in generation of free radicles that takes electrons from lipids (especially from lipids of membrane), this ultimately damages the cell. In order to measure the oxidative stress, quantification of lipid peroxidation is an important assay which is performed by Satoh method:

- 0.5ml of tissue homogenate with 1.5ml of 10% TCA solution.
- Incubate the mixture solution at room temperature for 10 minutes.
- Add 1.5ml of supernatant of above solution into 2ml of 0.67% TBA solution.
- Boiling in water bath for 30 minutes
- Cooling it for 20 minutes on ice.
- Add 1.2 ml of N-butanol and centrifuge for 5 minutes at 2000rpm at 4°C.
- Record absorbance at 532nm.

Calculation

Concentration in milimole= Change in Abs/155 *sample volume

3.4.3 Catalase (CAT)

Catalase is an anti-oxidant enzyme that protects the cell against reactive oxygen species (ROS) that are produced as a result of oxidative stress. This enzyme is primarily responsible for catalyzing the synthesis of water and oxygen from hydrogen peroxide H_2O_2. Catalase is present in the tissue sample is degraded, resulting in the formation of water and oxygen molecules. Catalase assay was carried out by Aebi method;

- Reaction mixture containing 0.1ml tissue homogenate with 0.85ml of potassium phosphate buffer (0.05M, pH 7.0); incubate at room temperature for 10 minutes.
- Add 0.05ml of H_2O_2 (30mM, pH 7.0).
- Record the decrease in absorbance at 240nm for 3 minutes.

Calculations

Catalase concentration (M): Change in absorbance/36mM-1cm-1

3.4.4 Superoxide dismutase (SOD)

Superoxide dismutase (SODs) assumes a basic part in the body's cell reinforcement safeguard against oxidative pressure. The compound is a viable treatment for sicknesses brought about by responsive oxygen species. The capacity of O2• to communicate with NBT and diminish the yellow tetrazolium inside the gel to a blue hasten is the premise of this measure. Grass makes a reasonable region (colorless groups) in regions where it is dynamic, rivaling NBT for O2•. SOD was done by following procedure;

- Reaction mixture containing 0.5ml of potassium phosphate buffer (0.1M, pH. 7.8), 0.2ml of 0.5M EDTA (pH. 7.8 adjust with NaoH), 0.1ml of 13mM methionine, 0.1ml of 20uM of riboflavin, 0.1ml of 750uM of NBT with 50ul of enzyme extract (Tissue lysate).
- Measure absorbance at 560nm.

Calculation

Control OD-Treated OD/Control OD*100= X% of inhibition

X% of inhibition is equal to 1/50*X =Y unit

Y unit in 50ul of enzyme extract

1000ul of enzyme extract contain SOD units= n value

So 1ml of enzyme extract from 100mg tissue = n value/ 100mg= SOD units/mg

3.5 Histopathological examination

1. Fresh specimen stored in 10% formalin on ice.
2. Heat paraffin wax and store it at 65 to 70° C.
3. Cut the tissue to a thickness of 3 - 4 mm.
4. Put it in cassette and label it with pencil.
5. Dehydrate it in ethanol soln. of 50, 70, 90 and 100% for 20 minutes in each solution.
6. Clear it with two changes of xylene for 30 minutes each.
7. Infiltrate in paraffin wax for 2 hours in paraffin 1 and 15 min in paraffin 2.
8. Pour the wax in mold and place tissue in proper orientation
9. Allow it to solidify and cool it at room temperature

10. Trim the tissue to remove excess paraffin
11. Place in Freezer for further processing
12. Apply mordant on slide and incubate at 45°C for 10 min.
13. Set the water bath at 55° C.
14. Switch on microtome and adjust the position of cassette.
15. Cut few sections to make tissue appear from the wax, and place in ice
16. After 10 min, adjust it again and take ribbons.
17. Place in water bath and quickly take the tissue over the slide. Remove excess with a tissue paper
18. Place it to dry in incubator at 45°C for 20 minutes.
19. Deparaffinize in 3 changes of xylene for 2min each.
20. Rehydrate in 100% of ethanol in 3 changes for 2min each then in 95% and 70% for 2 min each.
21. Wash with distilled water for 2 min.
22. Dip in Hematoxylin Solution for 2-3 min.
23. Wash with water for 5 minutes at room temperature.
24. Counter stain with eosin for 3-5 minutes.
25. Dehydrate with 95% ethanol (dip 20 times in it) then place in 95% for 2 min.
26. Place slides in 2 changes of absolute alcohol for 2 min.
27. Place in 3 changes of xylene for 2 minutes in each.
28. Dry the slides and mount the cover slips after applying dppx.
29. Observe under microscope.

3.6 Statistical analysis

All the data were analyzed using GraphPad Prism version 8.0.1 software. The significant values among different groups were determined by using TWO WAY ANOVA Test and significant value considered as when p value is less than 0.05.

Chapter 4

Results

4.1 Effect of EVOO and CaO on body weight in STZ-induced type 2 diabetic rats

The body weight in all diabetic, non-diabetic and treated groups are represented in figure 4.1.The fluctuation in body weight has been observed separately in male and female wistar rats with increasing and decreasing trend respectively. The significant difference has been observed in male wistar rats than female. When diabetic group (DC) group compared with (C) shows significant decrease (P=0.0489, P=0.0345) in male and female rats respectively. In male wistar rats when diabetic control (DC) compared with treated groups more significant results were observed DO than DCa (P=0.0066, P=0.0335). Moreover, in female wistar rats, there is non-significant results were observed in treated rats DM, DO and DCa (P=0.5718, P=0.3547, P=4302) respectively.

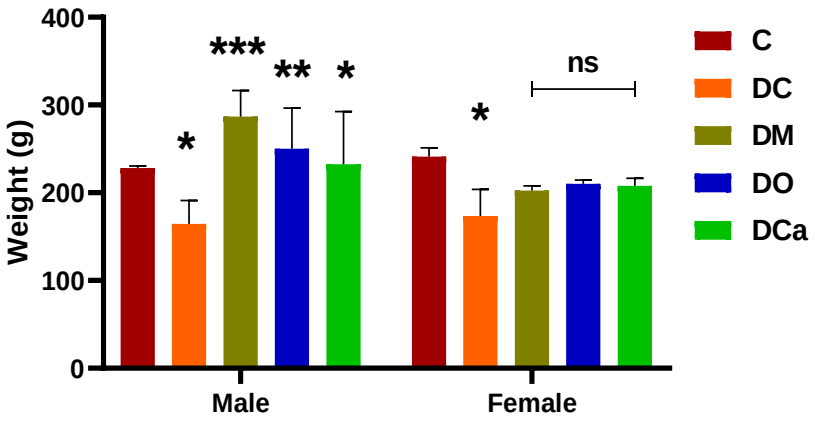


Figure 4.1: Changes in body weight gain: Variations in body weight gain during the period of study. Decrease in body weight gain has been observed in DC group (P=0.0489, P=0.0345) in male and female wistar rats.

4.2 Effect of EVOO and CaO on blood glucose level in STZ-induced type 2 diabetic rats

The blood glucose level in all diabetic, non-diabetic and treated groups is represented in figure 4.2. Moreover, Treated groups compared to their respective control (DC), both in males and females wistar rats observed a significant increase in blood glucose levels as a result of STZ treatment (P=<0.0001). A significant difference in blood glucose level has been observed in both male and female treated groups. By applying the TWO-WAY ANOVA when diabetic control group is compared with metformin its showed non-significant results in both male and female wistar rats. Significant results has been observed in those groups that treated with EVOO and CaO but CaO show more significant results in male wistar rats (P=<0.0001) than EVOO (P=0.0011). In female wistar rats EVOO exhibited more significant values (P=0.0050) than CaO (P=0.0229).

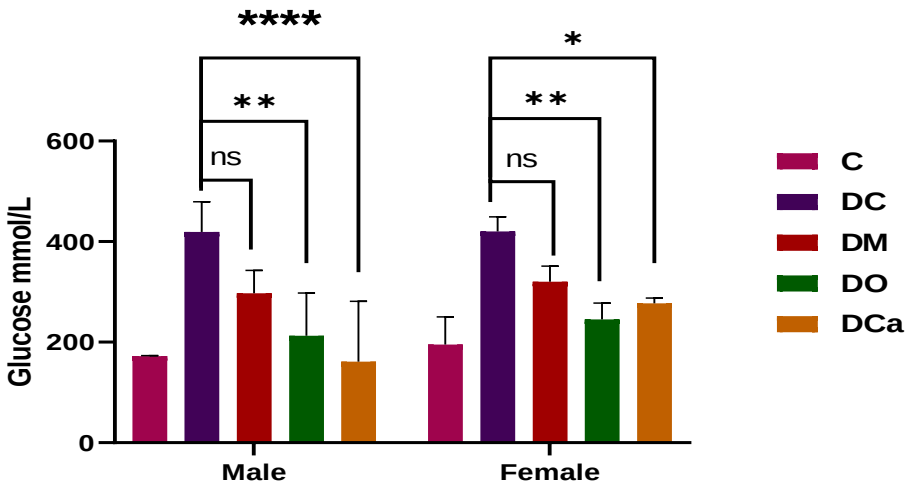


Figure 4.2: Blood Glucose level: Changes in the Blood glucose level in diabetic rats or treated rats after 35th day of treatment. Significant values were observed with P=<0.0001 in both male and female wistar rats.

4.3 Effect of EVOO and CaO on the biochemical parameters of STZ-induced type 2 diabetic rats

4.3.1 Effect on liver functions

4.3.1.1 Alkaline phosphatase (ALP)

The ALP level in all diabetic, non-diabetic and treated groups is represented in figure 4.3. Diabetic control group showed elevated levels of ALP as compared to control group (P=<0.0001) due to insulin resistance, kidney and liver damage. More Significant results have been observed in male wistar rats when DC group compared with treated groups DM, DO and DCa (p=<0.0001, P=<0.0001 and P=<0.0001) correspondingly than female because diabetic group treated with metformin DM showed less significant results than EVOO and CaO (P=0.0023, P=<0.0001, P=<0.0001) respectively.

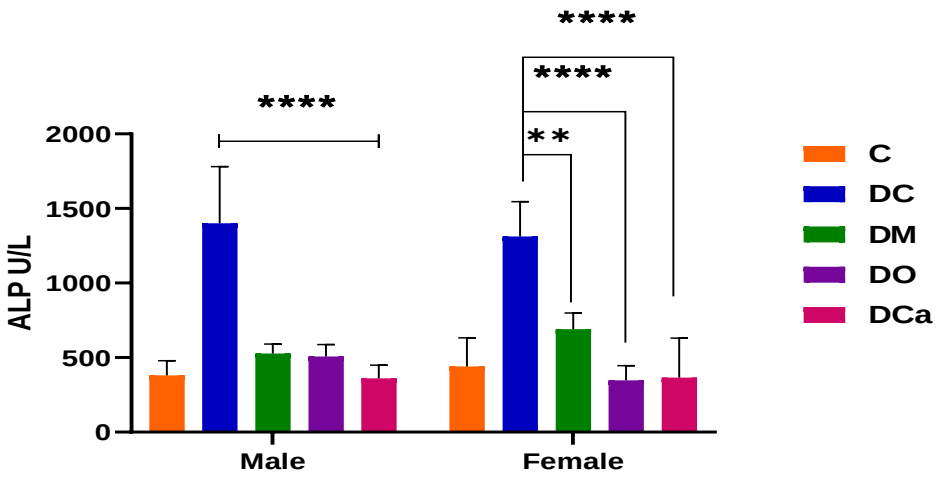


Figure 4.3: ALP level: Changes in the ALP level in diabetic rats or treated rats after 35th day of treatment. Significant values were observed with P=<0.0001 in both male and female wistar rats.

4.3.1.2 Alanine transaminase (ALT)

The ALT level in all diabetic, non-diabetic and treated groups is represented in figure 4.4. Diabetic control group (DC) showed elevated levels of ALT as compared to control group (C) (P=<0.0001). The elevated level of ALT is a risk factor for type 2 diabetes and that the liver may play a role in the disease etiology. Aminotransferases are considered as hepatic health markers. More significant results have been observed in male wistar rats when DC group compared with treated groups DM, DO and DCa (p=<0.0001, P=<0.0001 and P=<0.0001) correspondingly than female because diabetic group treated with CaO DCa showed non-significant results than EVOO and metformin (P=0.0966, P=0.0054, P=0.0194), respectively.

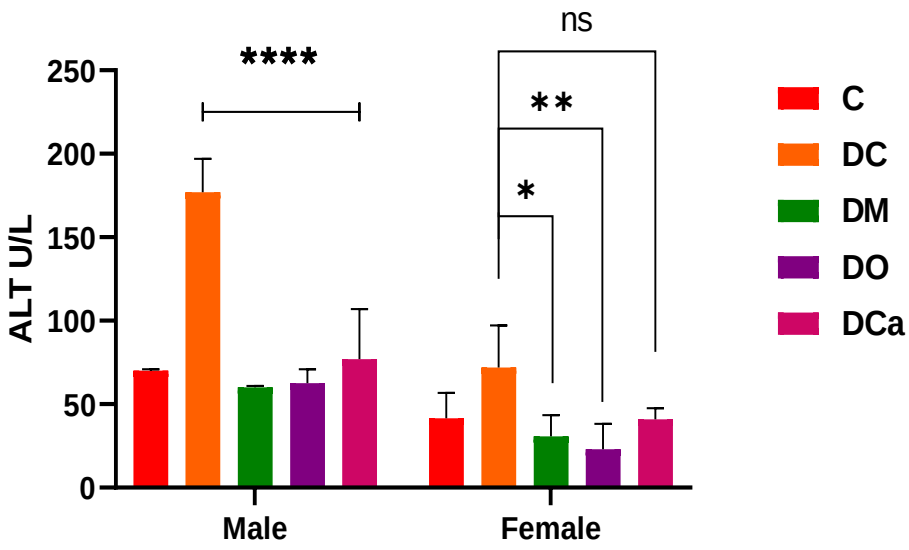


Figure 4.4: ALT Level: Changes in the ALT level in diabetic rats or treated rats after 35th day of treatment. More Significant values were observed with P=<0.0001 in male wistar rats than female wistar rats.

4.3.1.3 Bilirubin

The Bilirubin level in all diabetic, non-diabetic and treated groups is represented in figure 4.5. A natural antioxidant called bilirubin is linked to a lower incidence of type 2 diabetes (T2D). Higher-than-normal bilirubin levels can indicate a variety of liver or bile duct problems. A faster rate of red blood cell disintegration might sometimes cause higher bilirubin levels (hemolysis). Diabetic control group showed raised values of Bilirubin as compared to control group (P=<0.0001). More significant results have been observed in male wistar rats when DC group compared with treated groups DO and DCa (p=<0.0001, P=<0.0001) and non-significant results was observed in diabetic group treated with metformin (P=0.5315) correspondingly than female because diabetic group treated with CaO (DCa) and metformin (DM) showed non-significant results and EVOO have significant results (P=0.0619, P=0.3433, P=<0.0001) respectively.

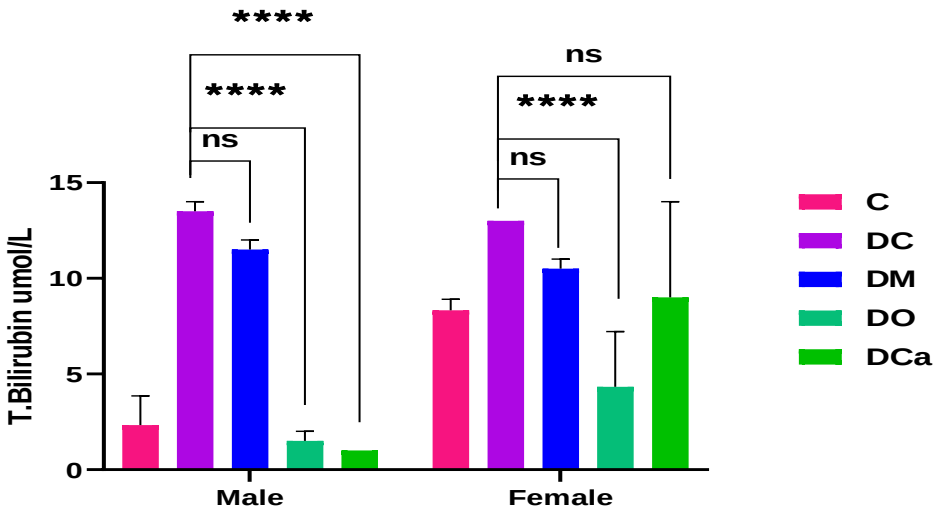


Figure 4.5: Bilirubin level: Changes in the Bilirubin level in diabetic rats or treated rats after 35th day of treatment. Significant values were observed with P=<0.0001 in both male and female wistar rats by comparing DC to control.

4.3.1.4 Albumin

The Albumin level in all diabetic, non-diabetic and treated groups is represented in figure 4.6. High levels of albumin are one of numerous markers of chronic kidney disease (CKD), a common consequence of both type 1 and type2 diabetes. Acute infections and stress can all raise albumin levels. Diabetic control group showed raised values of albumin as compared to control group (P=0.0010). More Significant results have been observed in female wistar rats when DC group compared with treated groups DM (P=0.0003) and non-significant results was observed in diabetic group treated with EVOO and CaO (P=0.7270, P=0.9654) respectively. In male wistar rats non-significant results was observed in diabetic group treated with metformin (P=0.1198) and significant results are reported in diabetic group treated with EVOO (DO) and CaO (DCa) (P=0.0158, P=0.0052). More significant results are observed in male wistar rats than female wistar rats.

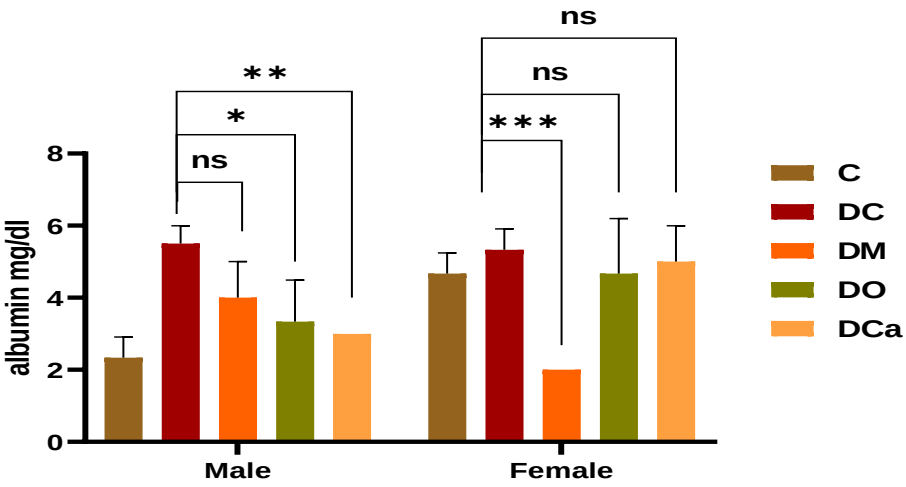


Figure 4.6: Albumin Level: Changes in the Albumin level in serum of diabetic rats or treated rats after 35th day of treatment. Significant values were observed with P=0.0010 by comparing DC with C.

4.3.2 Effect on lipid profile

4.3.2.1 Cholesterol

The Cholesterol level in all diabetic, non-diabetic and treated groups is represented in figure 4.7. Patients have elevated cholesterol levels if they have type2 diabetes. Your body doesn't control or utilize glucose (sugar) properly if you have type2 diabetes. This can result in blood glucose levels that are too high. High blood glucose levels can exacerbate other health problems, such as high cholesterol. Diabetic control group showed raised values of Cholesterol as compared to control group. More significant results have been observed in female wistar rats when DC group compared with treated groups DM, DO and DCa (P=<0.0001, P=0.0002, P=0.0129) and non-significant results was observed in male wistar rats when diabetic group DC compared with DO and DCa (P=0.4398, P=0.3485). Significant results were observed when diabetic group compared with diabetic group treated with metformin DM (P=0.0032).

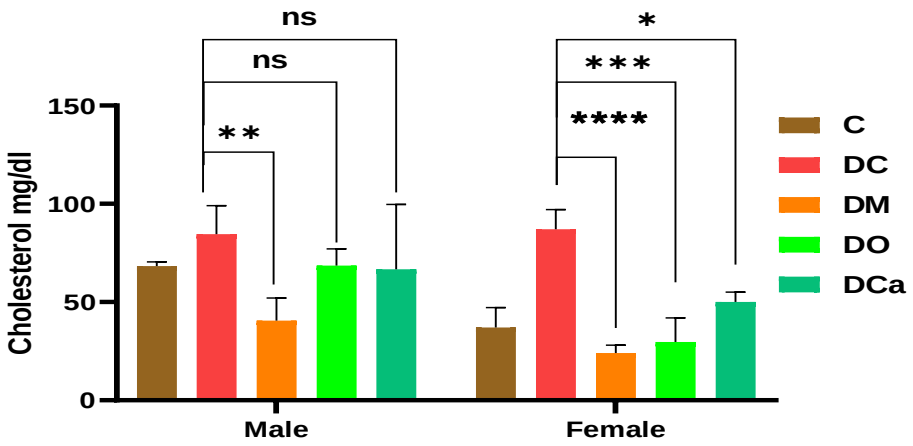


Figure 4.7: Cholesterol level: Changes in the Cholesterol level in serum of diabetic rats or treated rats after 35th day of treatment. More Significant values were observed in female wistar rats (P=<0.0001)

4.3.2.2 High density lipoprotein (HDL)

The HDL level in all diabetic, non-diabetic and treated groups is represented in figure 4.8. Diabetes is linked to changes in the amount of circulating lipids, most notably an increase in triglycerides, an increase in LDL, and a decrease in HDL. HDL, like other lipoproteins, suffers major structural and functional alterations as a result of diabetes. Overall the significant results were observed (P=0.0047). Non-significant results were observed when diabetic control group compared with treated group in both male and female wistar rats.

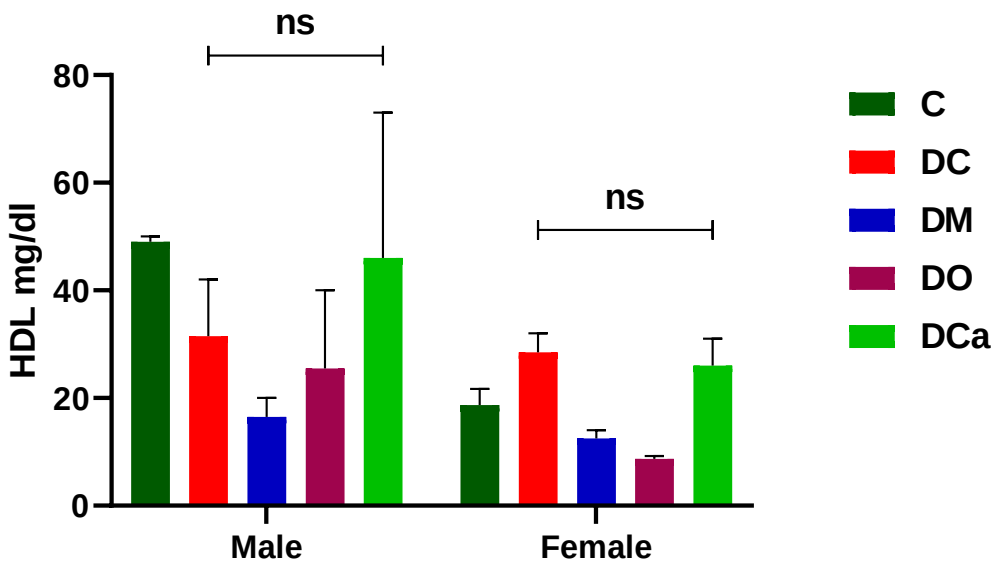


Figure 4.8: HDL level: Changes in the HDL level in serum of diabetic rats or treated rats after 35th day of treatment. Overall non-significant results were observed in both male and female wistar rats by comparing with diabetic control.

4.3.2.3 Triglycerides (TG)

The TG level in all diabetic, non-diabetic and treated groups is represented in figure 4.9. Insulin resistance and type2 diabetes are connected to a large number of interconnected lipid and lipoprotein anomalies in the blood, including low HDL cholesterol, a high commonness of little thick LDL particles, and high fatty substance levels. Significant results were observed when diabetic control group (DC) compared with treated group DM, DO and DCa (P=<0.0001) in both male and female wistar rats.

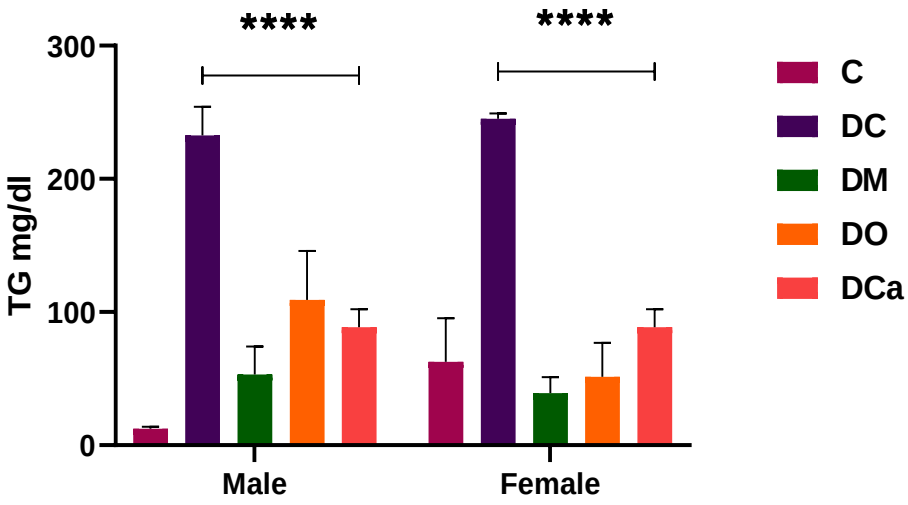


Figure 4.9: Triglyceride level: Changes in the TG level in serum of diabetic rats or treated rats after 35th day of treatment. Significant values were observed with P=<0.0001 by comparing with control in both genders.

4.3.2.4 Low density lipoprotein (LDL)

The Low density lipoprotein level in all diabetic, non-diabetic and treated groups is represented in figure 4.10. Diabetes raises LDL "bad" cholesterol levels while lowers HDL "good" cholesterol levels in the body. Controlling one's cholesterol levels is critical for lowering one's risk of heart disease. Non-significant results were observed when diabetic control group compared with treated group DM, DO and DCa in both male and female wistar rats.

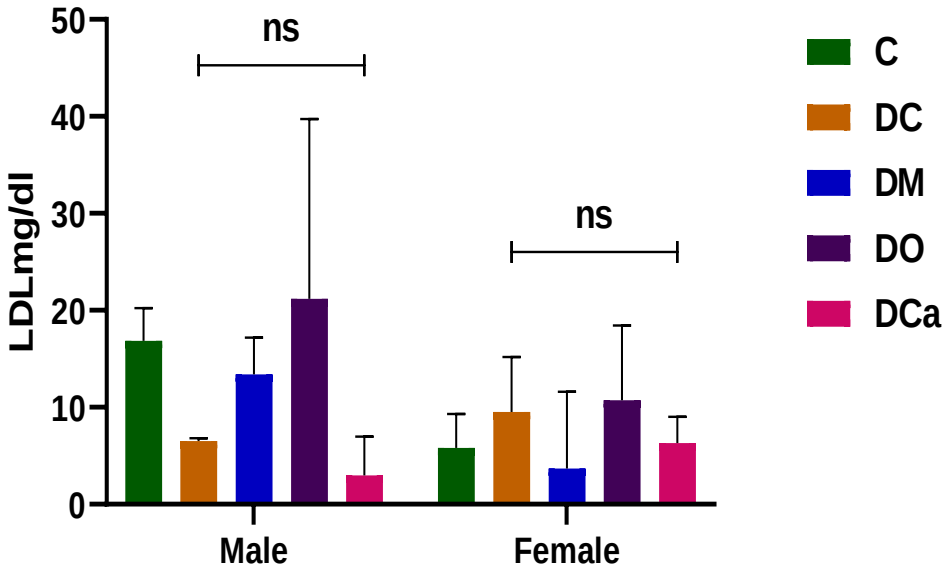


Figure 4.10: LDL level: Changes in the LDL level in serum of diabetic rats or treated rats after 35th day of treatment. Non- Significant values were observed.

4.3.3 Effect on kidney functions

4.3.3.1 Creatinine

The Creatinine level in all diabetic, non-diabetic and treated groups is represented in figure 4.11. A lower serum creatinine level was linked to a higher risk of type 2 diabetes. Participants with diabetes had lower mean creatinine levels than those without diabetes in men and women of all age groups. Non-significant results were observed in male wistar rats when diabetic control group compared with treated groups DM, DO, and DCa (P=0.8249, P=0.3982, P=0.5980) respectively. Significant results were observed in female wistar rats when diabetic control group compared with diabetic group treated with CaO (DCa) (P=0.0054) and non-significant results with other groups.

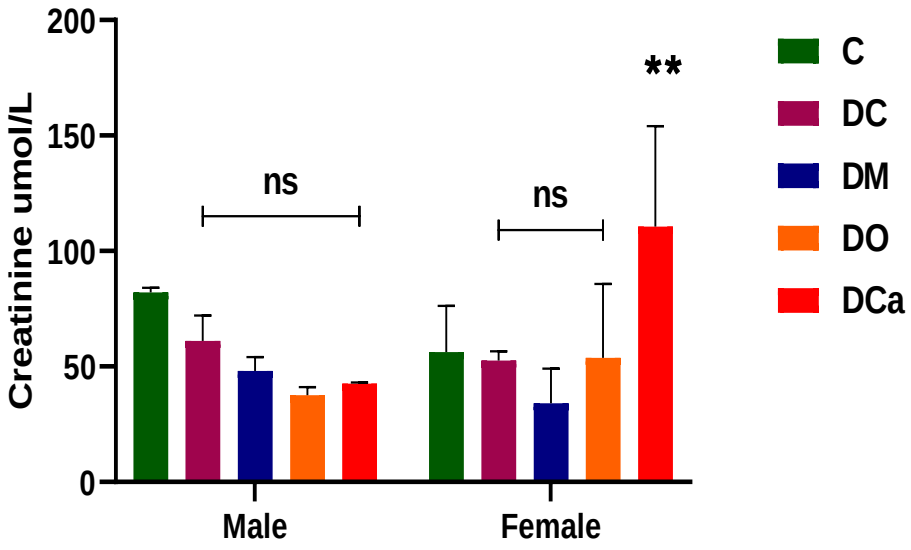


Figure 4.11: Creatinine level: Changes in the creatinine level in serum of diabetic rats or treated rats after 35th day of treatment. Non-Significant values were observed by comparing DC rats with treated rats.

4.3.3.2 Uric acid

The Uric acid level in all diabetic, non-diabetic and treated groups is represented in figure 4.12. High measures of uric acid in the blood are normal in people with type2 diabetes, which could be connected to abundance fat. Your body creates more insulin assuming you are overweight. This makes it harder for your kidneys to wipe out uric acid, maybe prompting gout. In the present study decreased level of uric acid has to be observed in diabetic control (DC) group. More significant results were observed in male wistar rats when DC group compared with DM and DO (P=0.0143, P=0.0058) and non-significant results with CaO. In female wistar rats non-significant results are observed.

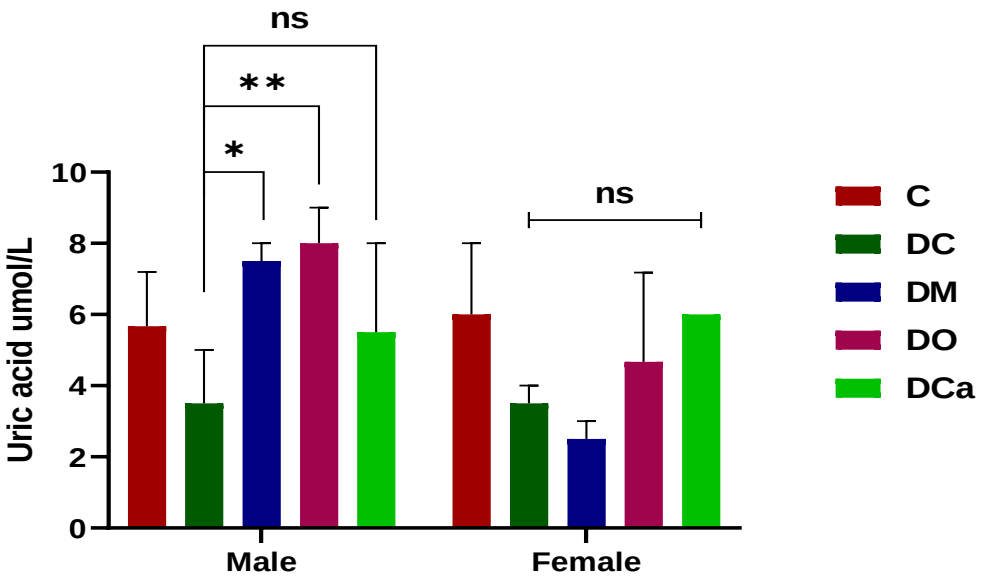


Figure 4.12: Uric acid level: Changes in the uric level in serum of diabetic rats or treated rats after 35th day of treatment. Significant values were observed in female wistar rats.

4.4 Oxidative stress markers and antioxidant system in STZ-induced type 2 diabetic rats

4.4.1 Catalase activities in in liver

The catalase activity in all diabetic, non-diabetic and treated groups is represented in figure 4.13. Catalase is a crucial enzyme that feeds on hydrogen peroxide, a non-radical ROS. This enzyme is in charge of neutralizing hydrogen peroxide by breakdown, ensuring that the molecule remains at an optimal level in the cell, which is also important for cellular signalling activities. Significant results were observed when diabetic control group compare with control group in both male and female wistar rats (P=0.0497, P=0.0462) respectively. Non-significant results were observed when DC compared with DM, DO and DCa in both genders.

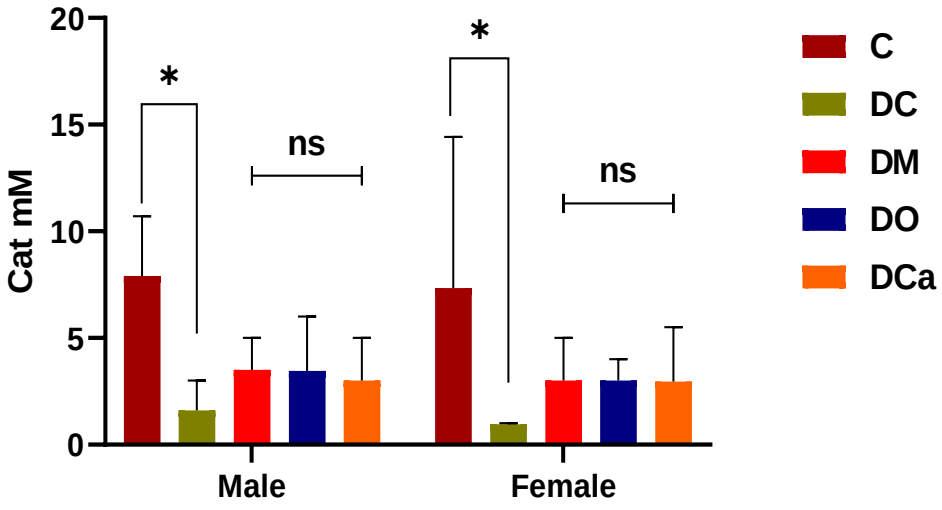


Figure 4.13: Catalase activity: Changes in the catalase level in liver tissue lysate of diabetic rats or treated rats after 35th day of treatment. Significant decrease was observed in DC rats.

4.4.2 Superoxide dismutase activities in liver

The SOD activity in all diabetic, non-diabetic and treated groups is represented in figure 4.14. Superoxide dismutase is a cellular enzyme that aids in the breakdown of potentially damaging oxygen molecules. This may help to prevent tissue injury. It's being studied to determine whether it can help with diseases where damaging oxygen molecules are thought to play a role. Over all significant results has been observed (P=0.0005). Significant results were observed when diabetic control group compare with control group in both male and female wistar rats (P=0.0017, P=0.0269) respectively. Non-significant results were observed when DC compared with DM, DO and DCa in both male and female wistar rats.

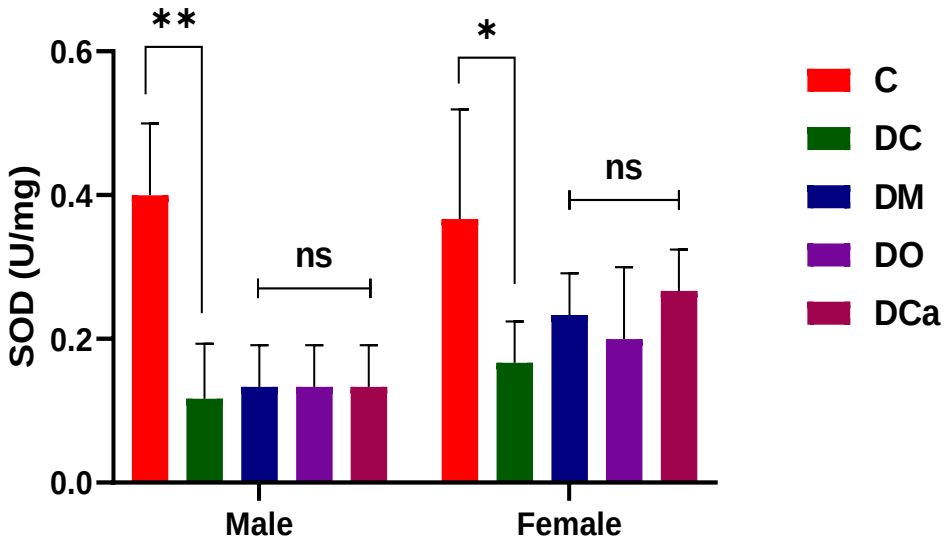


Figure 4.14: SOD activity: Changes in the SOD level in liver tissue of diabetic rats or treated rats after 35th day of treatment. Significant decrease was observed in DC rats than control group.

4.4.3 Lipid peroxidation activities in liver

The MDA activity in all diabetic, non-diabetic and treated groups is represented in figure 4.15. Malondialdehyde is produced when polyunsaturated fatty acids undergo lipid peroxidation. It is a key component of thromboxane A2 synthesis, in which platelets and a variety of other cell types and tissues convert arachidonic acid to prostaglandin H2 via cyclooxygenase 1 or cyclooxygenase 2. Over all significant results has been observed (P=<0.0001). More Significant results were observed when diabetic control group compare with treated groups DM, DO and DCa in male wistar rats (P=<0.0001, P=<0.0001, P=<0.0001) respectively. Less significant results were observed when DC compared with DM, DO (P=0.0001, P=0.0002) and more significant with DCa (P=<0.0001) female wistar rats.

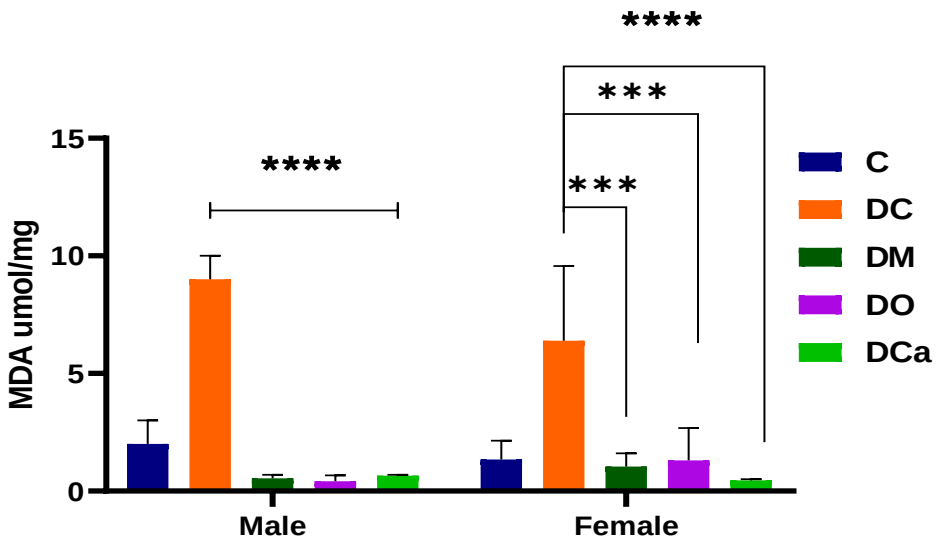
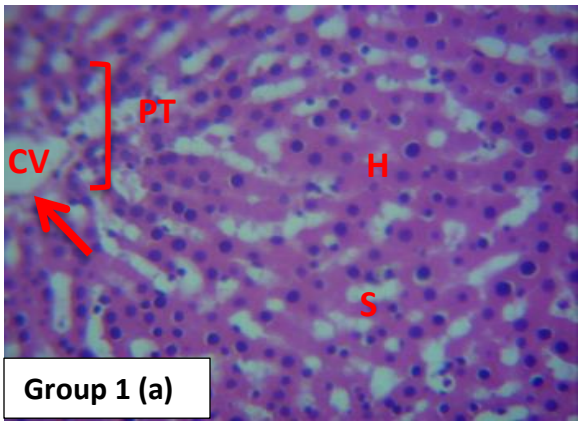


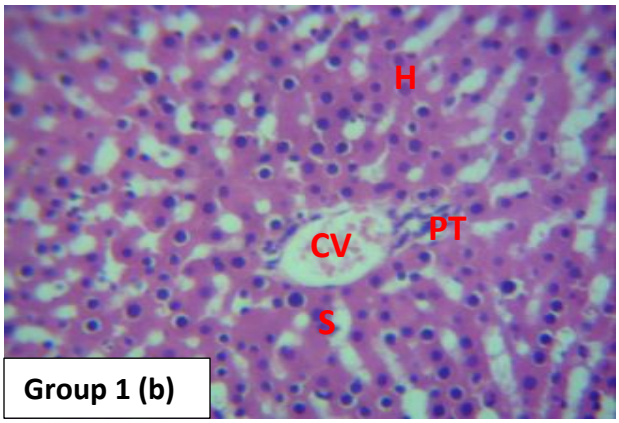
Figure 4.15: MDA activity: Changes in the MDA level in liver tissue of diabetic rats or treated rats after 35th day of treatment. Significant increase was observed in DC rats. Significant results were observed in treated rats (P=<0.0001).

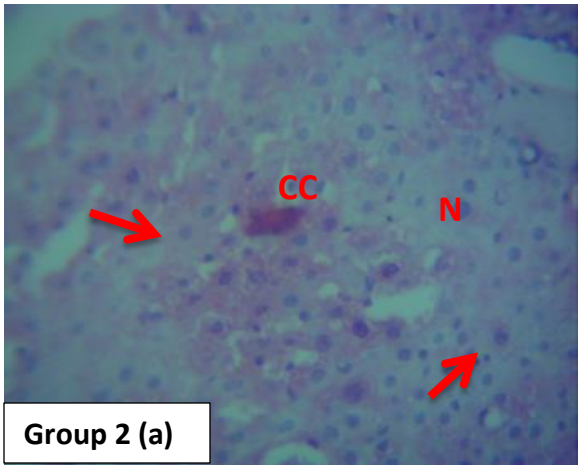
4.5 Effect of EVOO and CaO on the histopathology of liver, kidney and spleen

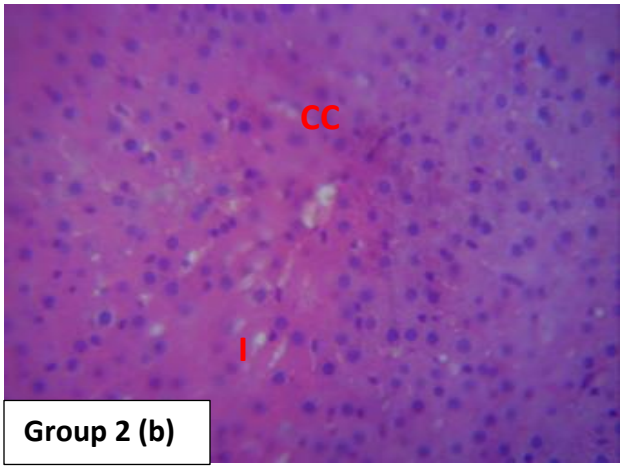
4.5.1 Effect on the histopathology of liver

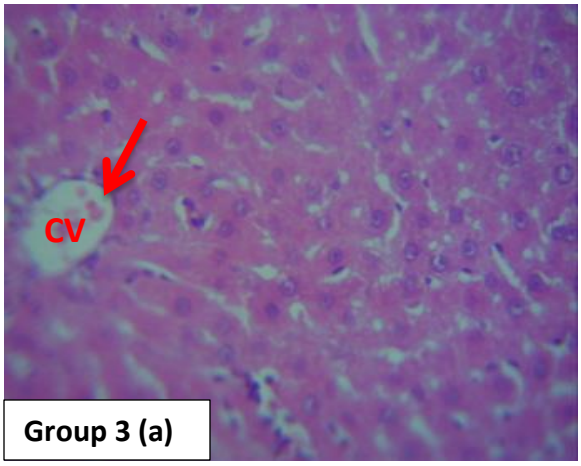
In order to determine the effect of EVOO and CaO on STZ-induced diabetic liver tissue in wistar rats, light microscopy was utilized to conduct a histological study. In group 1, tissue structure and cell physiology is normal. The portal triad, which comprises of the hepatic artery, portal vain, and interlobular duct, runs around the periphery of each lobule, forming a hexagonal configuration plate. Hepatocytes are organized radially around the central vein. The nuclei of macrophages (kupffer cells) were stained darkly. Hexagonal plates were divided by thin blood veins termed sinusoids. In group 2, observed that a diabetic rat's liver with total (severe) hepatocyte death, nuclear condensation, hepatic lobule loss, blocked hepatic inflammation in severe congestion, fibrosis and leukocyte penetration near central vain. Necrosis is clearly observed in diabetic rats with dark stained irregular hepatocytes and damaged nuclei. In treated group 3, hepatocytes are healthy and radially arranged toward central vain. In group 4 treated with EVOO cell structures healthy as it C and group 5 treated with CaO hepatocytes were almost normal and radially arranged toward central vain. Dark stained macrophages were observed in greater number than DC control. A few abnormalities were observed the hepatocytes are showing ballooning the process called degeneration of hepatocytes with remarkable protection of the cells against diabetes are outwardly arranged to central vain.

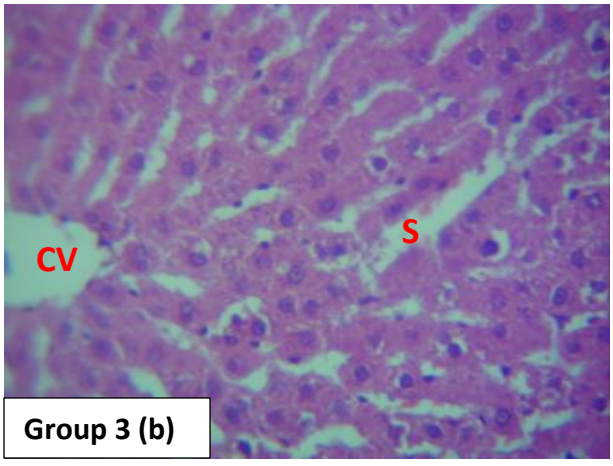

PT
CV
H
S
Group 1 (a)


H
CV
PT
S
Group 1 (b)


CC
N
Group 2 (a)


CC
I
Group 2 (b)


CV
Group 3 (a)


CV
S
Group 3 (b)

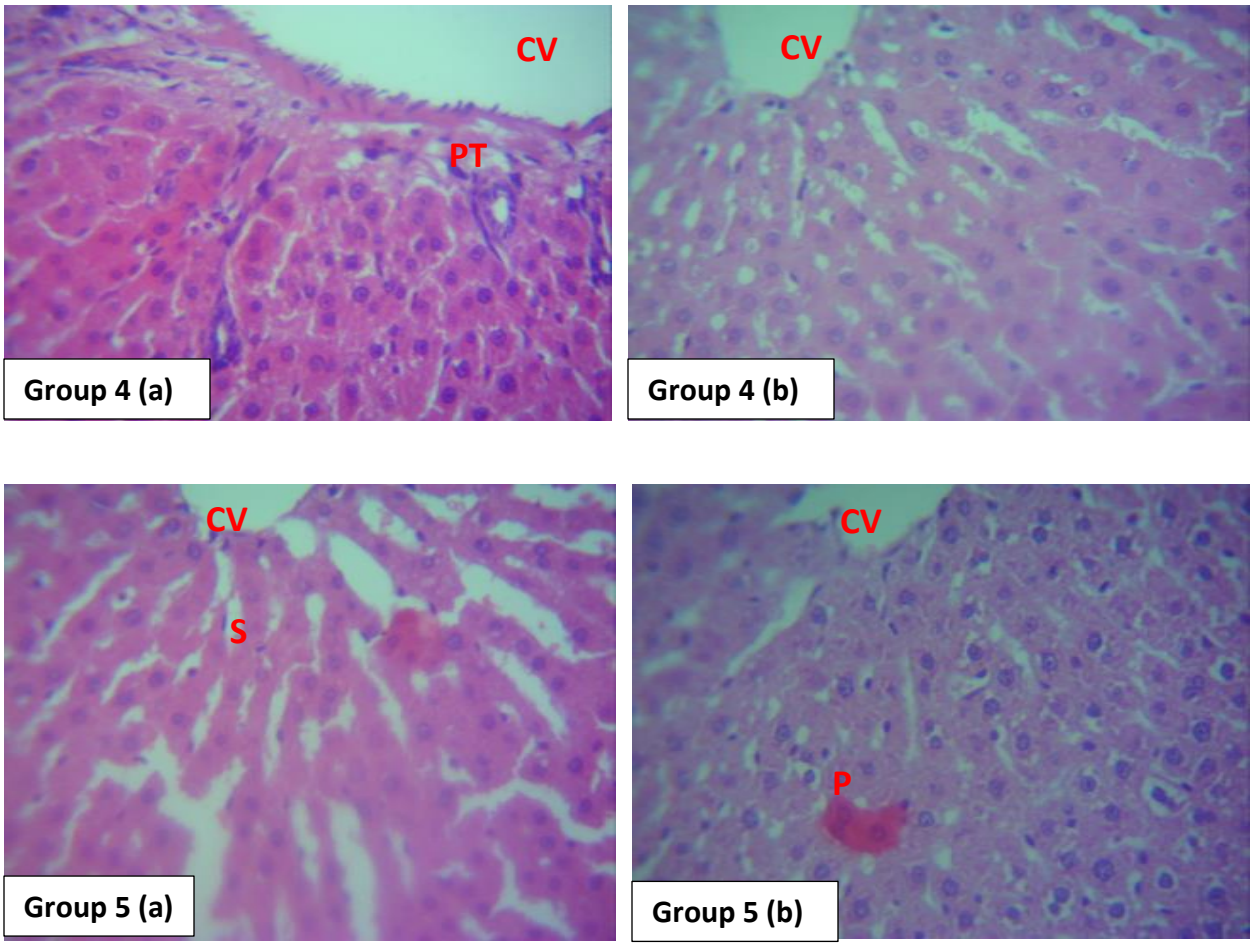
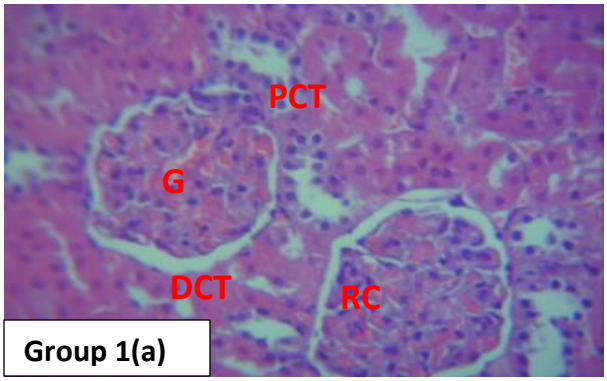
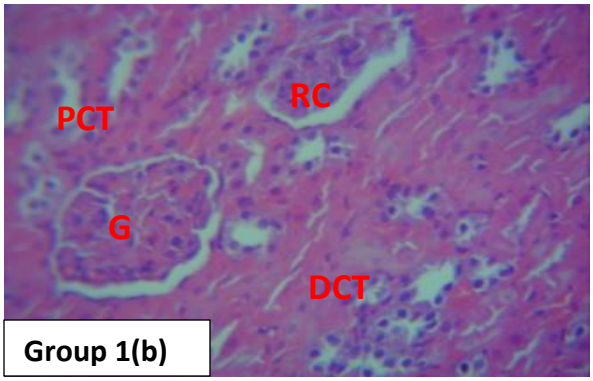


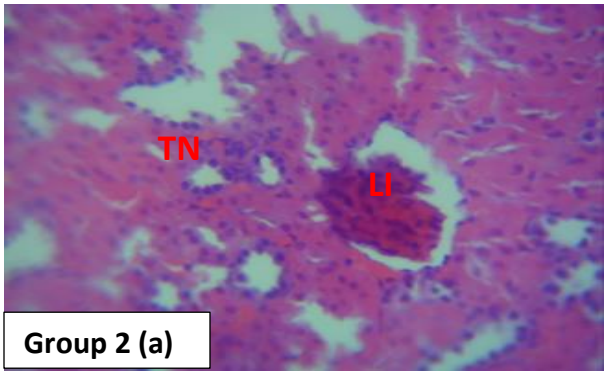
Figure 4.16: Photomicrographs of sections of the hepatic tissue from wistar rats: (a: male wistar rat b: female wistar rats) at 40X resolution. Group 1-C group showed normal hepatic lobules; dark stained kupffer cells and hepatocytes are radially arranged toward central vain. Group 2-DC showed necrosis and sign of inflammation, leukocytes penetration and nuclear condensation. Group 3-DM and 4-DO hepatocytes are radially arranged toward central vain. Group 5-DCa showing few abnormalities like pigmentation and loss of hepatocytes as well as most of the cells are healthy. CV (central vain), PT (portal Triad), S (Sinusoids), H (Hepatocytes), CC (Cells clump), I (Inflammation), N (Necrosis).

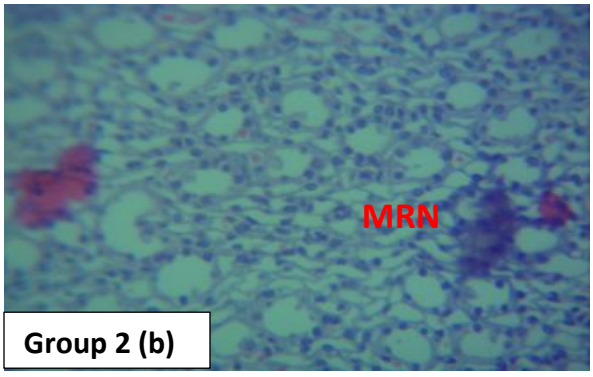
4.5.2 Effect on the histopathology of kidney

Normal control rats in group 1 renal histopathology revealed a well-organized cell structure. Proximal convoluted tubules are systemically placed between two renal corpuscles called Bowman's capsule, as seen in microtomed renal sections of control rats. The glomerulus of Bowman's capsule and distal convoluted tubules are normal in the cortex area, with no signs of nephropathy. In the non-treated diabetic group 2, there was cell condensation and tubule inflammation in the glomerular portion. Tubular necrosis and leukocyte infiltration are seen in the medullary area known as medullary ray tubular necrosis. The cell structure of group 3, 4(a) and 5 is similar to that of the control group. In group 4 (b) cells, a condensation called macula dense near Bowman's capsule is found, but there is no evidence of disease.









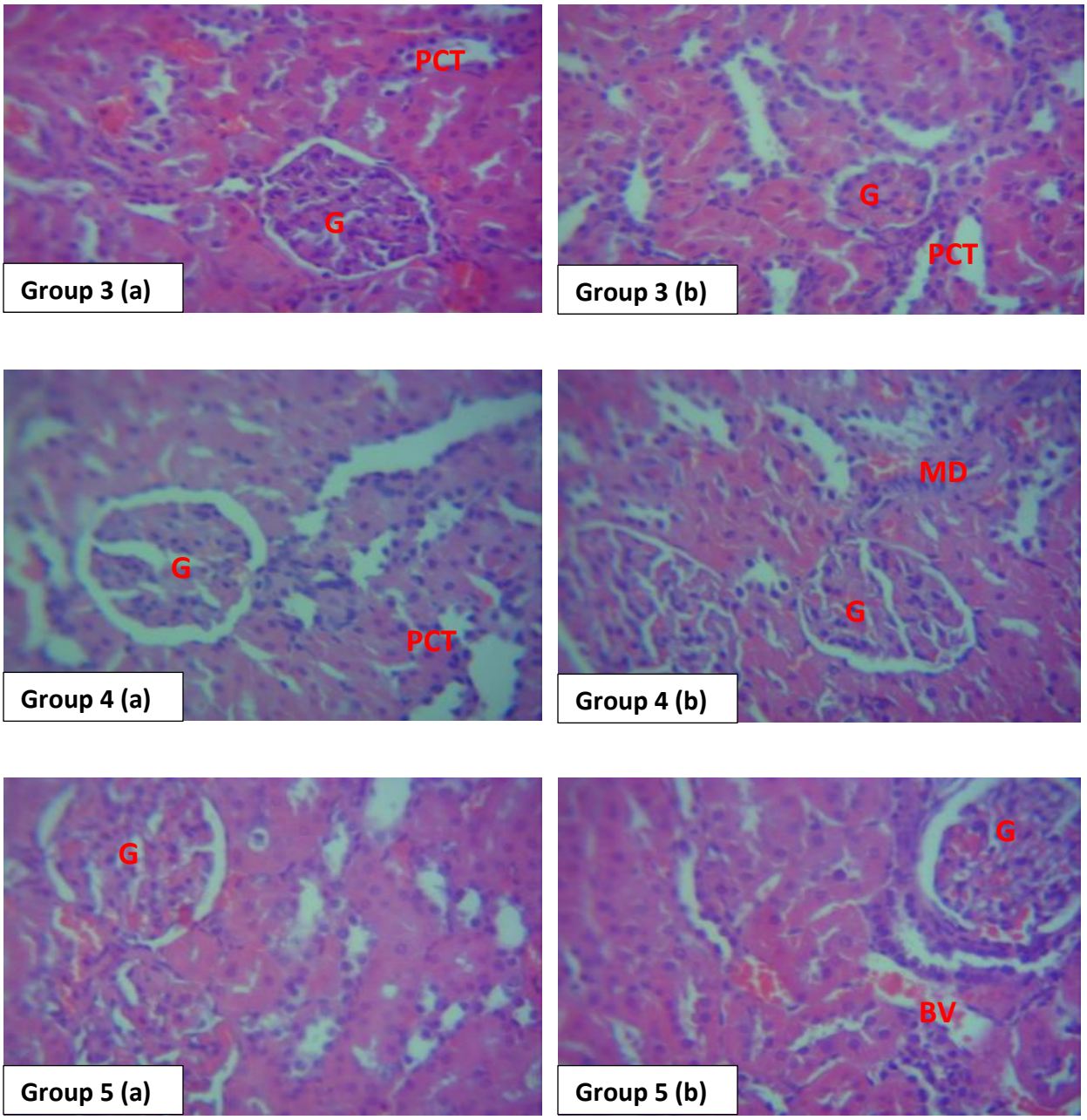
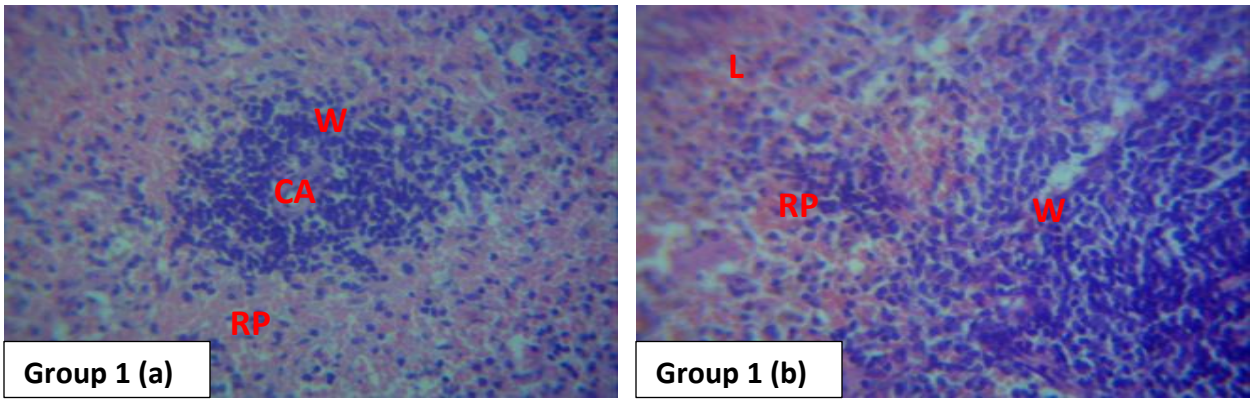


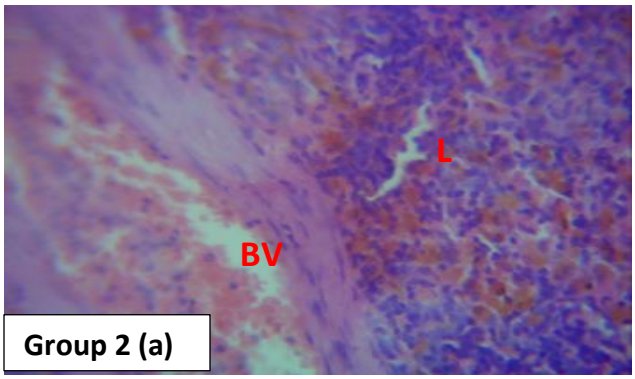
Figure 4.17: Photomicrographs of sections of the renal tissue from wistar rats: (a: male wistar rat b: female wistar rats) at 40X resolution, Group 1-C group showed normal renal capsule with proximal convoluted tubules and distal convoluted tubules. Group 2-DC showed necrosis and sign of inflammation, leukocytes penetration and nuclear condensation in Bowman's capsule and medullary ray. Group 3-DM Renal capsule are well arranged like control group. Group 4-

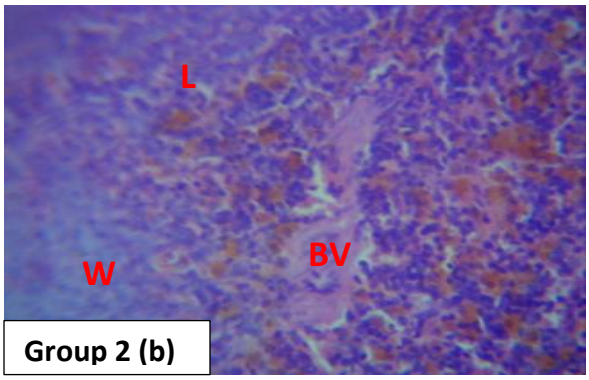
DO cell structure healthy as it C and in group 4(b) cells condense near renal capsule, Group 5-DCa observed most of the cells are healthy. G (Glomerulus), PCT (Proximal Convoluted tubules), DCT (Distal Convoluted Tubules), RC (Renal Capsule), MRN (Medullary ray necrosis), BV (Blood vessels), TN (Tubules necrosis), LI (Leukocytes infiltration).

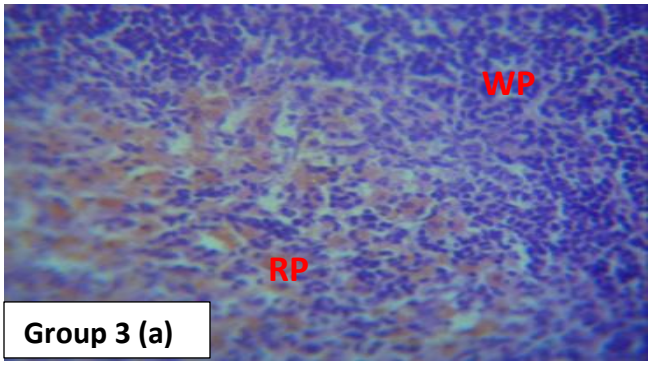
4.5.3 Effect on the histopathology of spleen

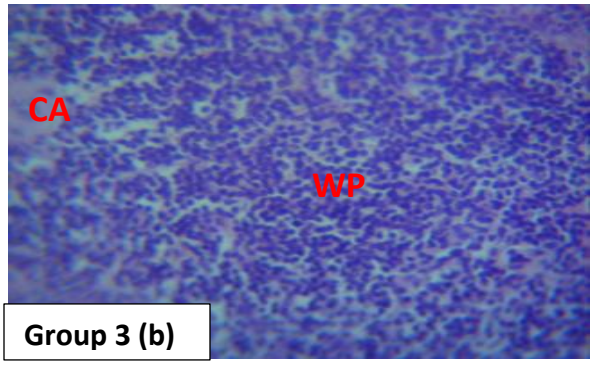
A sample of splenic tissue was histopathological investigated, and it was determined that the pathology of the group 1 control rat was normal, with white and red pulp. Lymphocytes with their central arteriole are found in the white pulp, which is surrounded by numerous arterioles. Lymphocytes, macrophages, and collagen bundles called trabeculae are found in the red pulp. The activation of red pulps is increased in group 2 diabetic rats, as is the accumulation of hemosiderin granules (a hallmark of injured spleen). Splenic tissue blood vessels dilated, while lymphocytes in peripheral sections shrank. It notably protects the tissue from diabetes by maintaining the normal structure in treated groups 3 (metformin), cells are healthy and white pulp arranged central arteriole. In group 4 (EVOO), white pulp is arranged around central arteriole as well as there are some abnormalities related to leukocytes and macrophages. In group 5 (CaO) cells are like healthy cells.

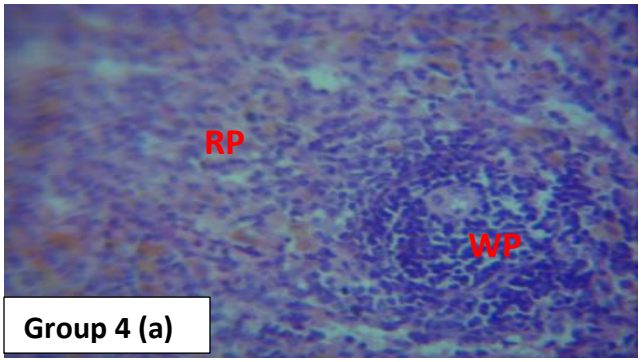


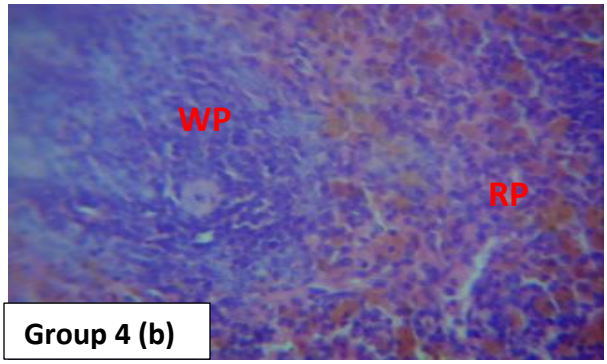

L
BV
Group 2 (a)

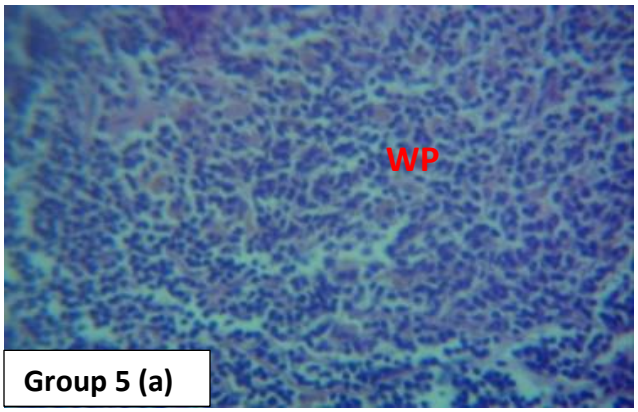

L
W
BV
Group 2 (b)


WP
RP
Group 3 (a)


CA
WP
Group 3 (b)


RP
WP
Group 4 (a)


WP
RP
Group 4 (b)


WP
Group 5 (a)

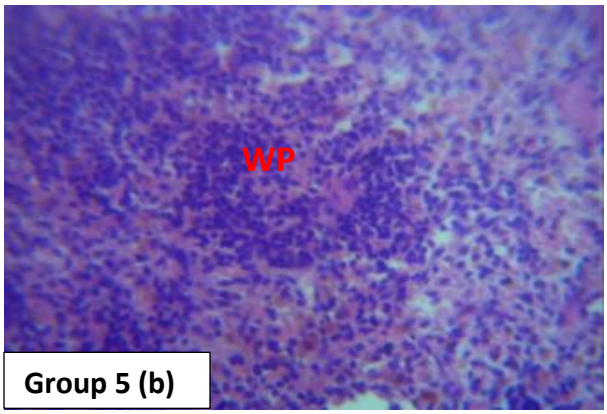

WP
Group 5 (b)

Figure 4.18: Photomicrographs of sections of the splenic tissue from wistar rats: (a: male wistar rat b: female wistar rats) at 40X resolution. Group 1-C group showed normal red pulp and white pulp with macrophages and leukocytes. Group 2-DC showed necrosis and sign of inflammation and dilation in blood vessels. Group 3-DM cells are well arranged like control group. Group 4-DO cell structure healthy as it C and in group 4(b) light stained cells in white pulp, Group 5-DCa observed most of the cells are healthy. WP (White pulp), RP (Red pulp), CA (Central arteriole), L (Leukocytes), BV (Blood vessels).

Chapter 5

Discussion

Discussion

Hyperglycemia, the most common bio clinical manifestation of diabetes, has been connected to the onset of certain difficulties, including the production of oxidative stress in the body and pancreatic injury, and may be to blame for elevated blood glucose in animals. STZ promotes hyperglycemia by killing pancreatic β-cells, which produce insulin from endocrine cells (Rebolledo-Solleiro and Fernández-Guasti 2018)(Laaboudi *et al*. 2016). In previous studies, effect of canola oil, olive oil and sunflower oil were evaluated against lipid profile in randomized controlled study for the prevention of cardiovascular diseases (Khandouzi, Zahedmehr, and Nasrollahzadeh 2020)(Ghobadi *et al*. 2019). In this study, the efficacy of EVOO and CaO available in commercial market of Pakistan against STZ-induced diabetic rats was investigated using histopathological and biochemical markers. The Glucose abnormalities, body weight alterations, biochemical markers including (liver functions, lipid abnormalities and kidney functions) oxidative stress markers (CAT, SOD and MDA) were also investigated in present study.

The loss of beta cells and interruption of insulin secretion in diabetes causes physio-metabolic irregularities such as a decrease in body weight gain and an increase intake of food, as well as an increase in urine volume. Weight loss is a common sign of diabetes caused by STZ. The diabetic rats' weight loss could be attributed to protein waste caused by a lack of glucose as an energy source. Increased food consumption and reduced body weight in diabetic rats imply a polyphagic condition and weight loss due to excessive tissue protein breakdown, as compared to normal rats (Al-Attar and Alsalmi 2019). These changes were also observed in diabetic rats caused by STZ in the current investigation. Our findings are in line with a study that found a reduction in body weight gain in STZ-induced diabetic rats (Rebolledo-Solleiro and Fernández-Guasti 2018).

According to our findings, the treated groups DM, DO, and DCa maintained a considerable stability level of body weight gain. The diabetic group treated with EVOO showed more substantial improvements. Due to hormonal abnormalities, non-significant changes in female wistar rats' body weight have been reported. Other research, however, also found that STZ-induced diabetic rats lose weight significantly (Rebolledo-Solleiro and Fernández-Guasti 2018).

Furthermore, STZ-treated rats (DC) showed a substantial increase in blood glucose levels when compared to controls (C), while treatment groups (DO, DCa) showed a significant drop in blood glucose level by comparing to DC. Male wistar rats responded to CaO more favorably than female wistar rats. EVOO and CaO have anti-diabetic benefits due to their high content of phenolic components. Vegetable oils such as olive oil, canola oil, sunflower oil, sesame and fish oil have also been shown in recent studies to have reduced hyperglycemic activity due to their antioxidant qualities (Parveen *et al.* 2019)(Bouhrim *et al*. 2019). Hyperglycemia, on the other hand, has been associated to a range of abnormalities in the structure of the pancreatic islets in several studies. The amount of circulating insulin, the number of receptors, and the receptor's affinity for insulin all play a role in how well insulin binds to its receptor. In diabetes mellitus and other insulin-resistant diseases like obesity, higher levels of circulating insulin lower insulin receptor concentration in a dose-dependent manner, a process known as down regulation. Insulin receptors tyrosine kinase molecules play a key role in insulin signalling and are required for basic cellular functions such as growth, survival, and metabolism. They serve as connectors between the insulin receptor and a complex intracellular signalling network. At the molecular level, insulin resistance is linked to inadequate insulin signalling. Cell surface components such as insulin receptor, as well as intracellular components such as the IRS-1 docking protein family and other insulin signalling and glucose transport pathway components, can malfunction. Insulin

resistance in the liver is described as a decrease in insulin's ability to activate its receptor kinase and downstream targets, resulting in insufficient hepatic glucose production suppression and, as a result of hyperglycemia. Insulin resistance may be caused by an insulin binding defect caused by a reduction in IR levels, according to the research (Al-Attar and Alsalmi 2019)*(Balamash et al*. 2018)(Bouhrim *et al.* 2019).

The liver function test, When compared to the control group (C), the diabetic control group (DC) have higher levels of ALP (P=0.0001), ALT (P=0.0001), Bilirubin (P=0.0001, P=0.0254), and albumin (P=0.0010). In the treated groups, there was a significant decrease EVOO and CaO both revert the ALP and ALT level to normal except DCa rats showed non-significant value (P=0.0966). For albumin, DO and DCa showed non-significant results in female wistar rats. Furthermore DO showed significant values than DCa. These findings were consistent with a study that found higher ALT and ALP levels in diabetic rats, as well as non-significant results in case of bilirubin and increased albumin levels in olive oil-treated groups. In contrast to the previous findings, the current investigation found a considerable increased level of bilirubin in diabetic rats (Balamash *et al*. 2018)(Bouhrim *et al*. 2019). Abnormal liver function tests in diabetics can be caused by a variety of factors. To begin with, hyperinsulinemia can result in hepatic insulin resistance as well as lipid changes. Hepatocytes are known to be toxic to fat accumulation in the liver, resulting in an increase in transaminases and a reduction in the liver's synthetic capacity (Balamash *et al.* 2018). The second reason, pro-inflammatory cytokines like tumor necrosis factor (TNF) are linked to insulin resistance, which could lead to hepatocellular injury. NAFLD is a hepatic symptom of diabetes mellitus that is linked to metabolic syndrome, and ALT has been used to assess NAFLD. According to new research, hepatitis C virus infection has been associated to aberrant LFTs in diabetes patients. Although rare, statin medication can

result in abnormal liver function tests (Balamash *et al.* 2018)(Singh *et al*. 2019). The upregulation of CYPE1 (cytochrome p450 subfamily 2 peptidase 1) are highly expressed on liver during obesity and insulin resistance that involved in the metabolism of polyunsaturated fatty acids. Excessive metabolism leads to the production of cytotoxic products that further cause mitochondrial ROS which cause the liver injury and inflammation ultimately the reason of abnormal liver functions. A higher value of albumin has been associated to liver and renal failure, as well as a faster rate of water loss in the body. A spike in serum albumin indicates impaired liver function or synthesis, which could be caused by liver cell damage or a decrease in protein intake. The higher concentration of bilirubin was observed in DC wistar rats due to impaired glucose production. In this condition, upregulation of heme-oxxygenase activated the insulin resistance, inflammation in liver as well as increased concentration of bilirubin. (Singh *et al*. 2019)(Sodipo *et al*. 2021).

In terms of lipid profiles, the current study found that the treated groups had significantly lower cholesterol and TG levels than the diabetic control group DC, with non-significant results by comparing with DC for HDL and LDL. Female diabetic rats treated with EVOO exhibited more substantial effects in decreasing cholesterol levels than male wistar rats, and TG levels in treated groups returned to normal (P=<0.0001).These findings are consistent with a prior study that found higher cholesterol, LDL, and TG levels, as well as a decrease in HDL levels in diabetic rats (Al-Attar and Alsalmi 2019)(Balamash *et al*. 2018). Due to the short duration of the trial, the results of HDL and LDL were found to be non-significant either there is an increasing trend was observed in female wistar rats for LDL. The high cholesterol and TG is due to dyslipidemia, major risk factor of CVD. According to recent study, Rapeseed oil is more advantageous than olive oil (Kruse et al. 2015), however in the present study; EVOO exhibited more healthful

positive benefits. Diabetic rat's exhibit altered lipid metabolism as a result of insulin shortage in the body produced by STZ-induced pancreatic cell damage, as seen by these lipid profile abnormalities. Insulin can stimulate lipoprotein lipase, a lipoprotein solver enzyme. In diabetics, the activity of the enzyme lipoprotein lipase is diminished, leading in an increase in lipoprotein levels in the blood (Balamash *et al*. 2018).

Serum creatinine and uric acid levels are nephrotoxicity indicators used in the diagnosis of kidney injury. Except for DCa, there were no significant differences in creatinine levels in the treated groups in this investigation. Creatinine is the waste product depends on muscles mass and excreted out through urination. A low value of creatinine is the indication of type 2 diabetes. Furthermore, uric acid levels were significantly higher in male wistar rats treated with EVOO than in female wistar rats. These results are against with previous reported study where due to short period of the experiment, non-significant findings were seen in the examination of renal functions treated with olive oil (creatinine and uric acid). In diabetic condition, the uric acid level increased that promoted the expressions of interleukins and TNF- α which leads to inflammation. High level of uric acid directly affects the insulin gene expression causing he decrease in insulin secretion (Balamash et al. 2018).

The current study discovered that STZ increased oxidative stress, as demonstrated by reducing the SOD and CAT activity, and increasing the MDA activities in the liver. Furthermore, when DC rats were compared to normal control rats (C), oxidative stress was shown to be significantly higher. The findings of this study were consistent with those of many other studies published in the literature. Glycation of antioxidant enzymes, which happens when blood glucose levels are consistently high, could be linked to a decrease in antioxidant enzyme activity and lipid peroxidation in diabetic individuals. Oxidative stress, on the other hand, is generated by a redox

imbalance between the formation of reactive oxygen species (ROS) and the compensatory reaction of the endogenous antioxidant network. There is no consensus on how antioxidant enzyme activity changes in diabetic rats (Suanarunsawat, Anantasomboon, and Piewbang 2016) (Al-Attar and Alsalmi 2019).

The liver and kidney, in particular, were shown to be target organs for diabetic problems after a review of essential organs. In histopathological analysis, the DC group's liver histology, the presence of numerous dark-stained shrunken hepatocytes and small dark nuclei revealed pathological abnormalities indicating degenerative degeneration (apoptosis). Metformin (DM), EVOO(DO), and CaO (DCa) treatment restored the normal structural organisation of the liver tissue in STZ diabetic rats, demonstrating a protective effect against hepatic damage (Balamash *et al.* 2018) (Anahita, Asmah, and Fauziah 2014) (Gopal *et al*. 2014).

In this study, the kidney tissue of the DC group showed renal corpuscle deformation and glomerular capillary atrophy. While renal tubules showed unstained degraded cytoplasmic regions of the lining epithelium, arterioles showed diffuse muscular media thickening with perivascular edema, fibrosis, and little interstitium lymphocytic infiltration. Rats with STZ-induced diabetes had substantial damage to their kidneys, as well as glomerular sclerosis. The DO and DCa group also showed protection against diabetes-related changes in the current study similar to control group's corpuscles, glomeruli, and tubules appeared to be in regular shape (Anahita *et al*. 2014)(Altinoz *et al.* 2015)(Suanarunsawat *et al*. 2016).

The spleen tissue of the DC group showed dilations in blood vessels, deficient white and red pulp, and leukocyte infiltration. Rats with STZ-induced diabetes had severe damage to their spleens, as well as necrosis of cells. The DO group also showed protection against diabetes-

related changes in the current study. The control group's white pulp, which is clustered around central arterioles, appeared to be healthy. These findings imply that EVOO and CaO can be utilized to minimise the risk of diabetes as a prophylactic measure (Ghosh *et al*. 2018).

Conclusion

In conclusion, each of EVOO and CaO improved the lipid profile, liver and kidney functions as well as show their antioxidant activity against STZ-induced diabetic rats. Comparing these two vegetable oils, EVOO and CaO involve in reducing the ALP, ALT, bilirubin, cholesterol and triglyceride against diabetes but EVOO is more effective than CaO. Results are more significant in male wistar rats than female wistar rats because female's faces certain type of hormonal problem throughout their life. Significant changes were observed during histopathological examination of vital organs in both male and female wistar rats in diseased group or treated groups. These data suggest that EVOO can be used as a preventative approach to cure the risk of diabetes. This study will additionally help in the exploration to reveal the basic area of how to achieve the management of diabetes and counteraction of optional complications emerging out of hyperglycemia.

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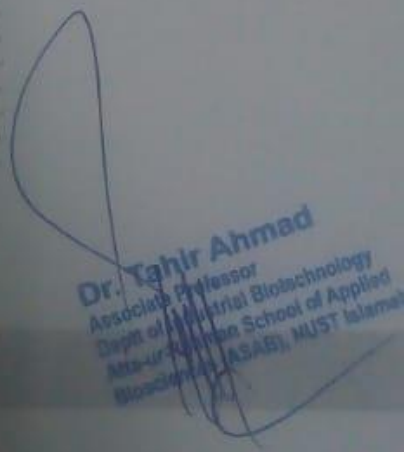

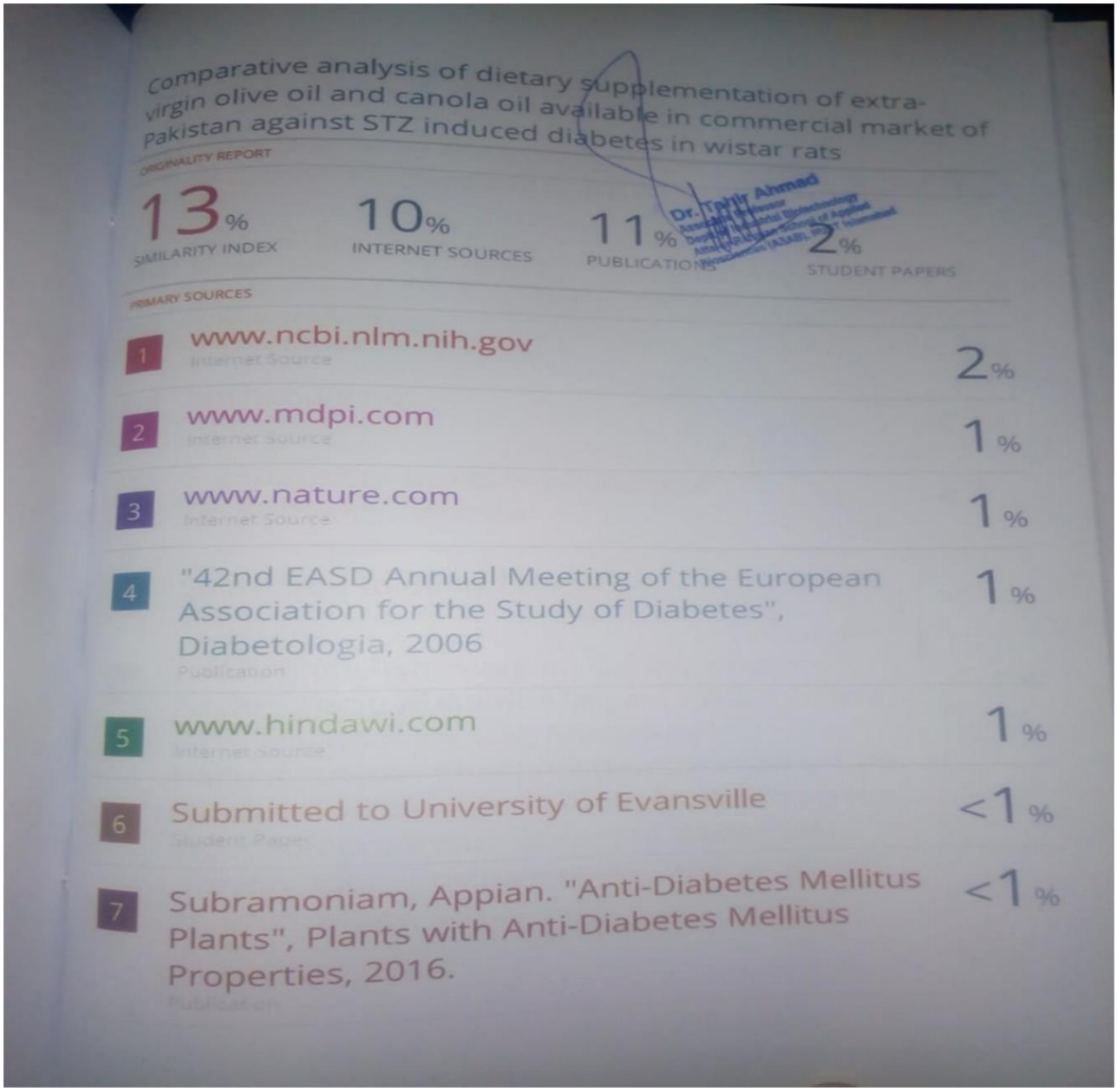

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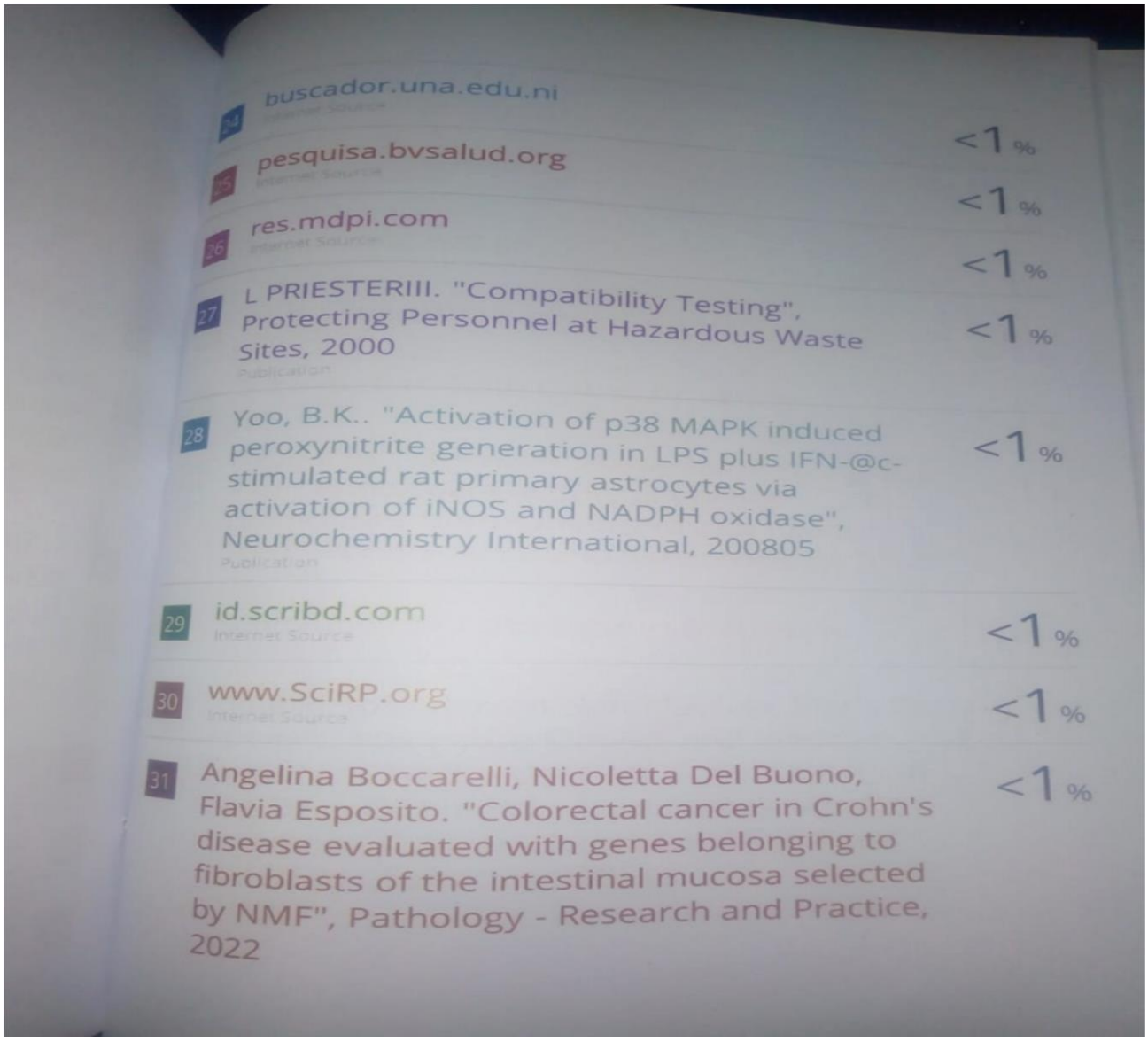

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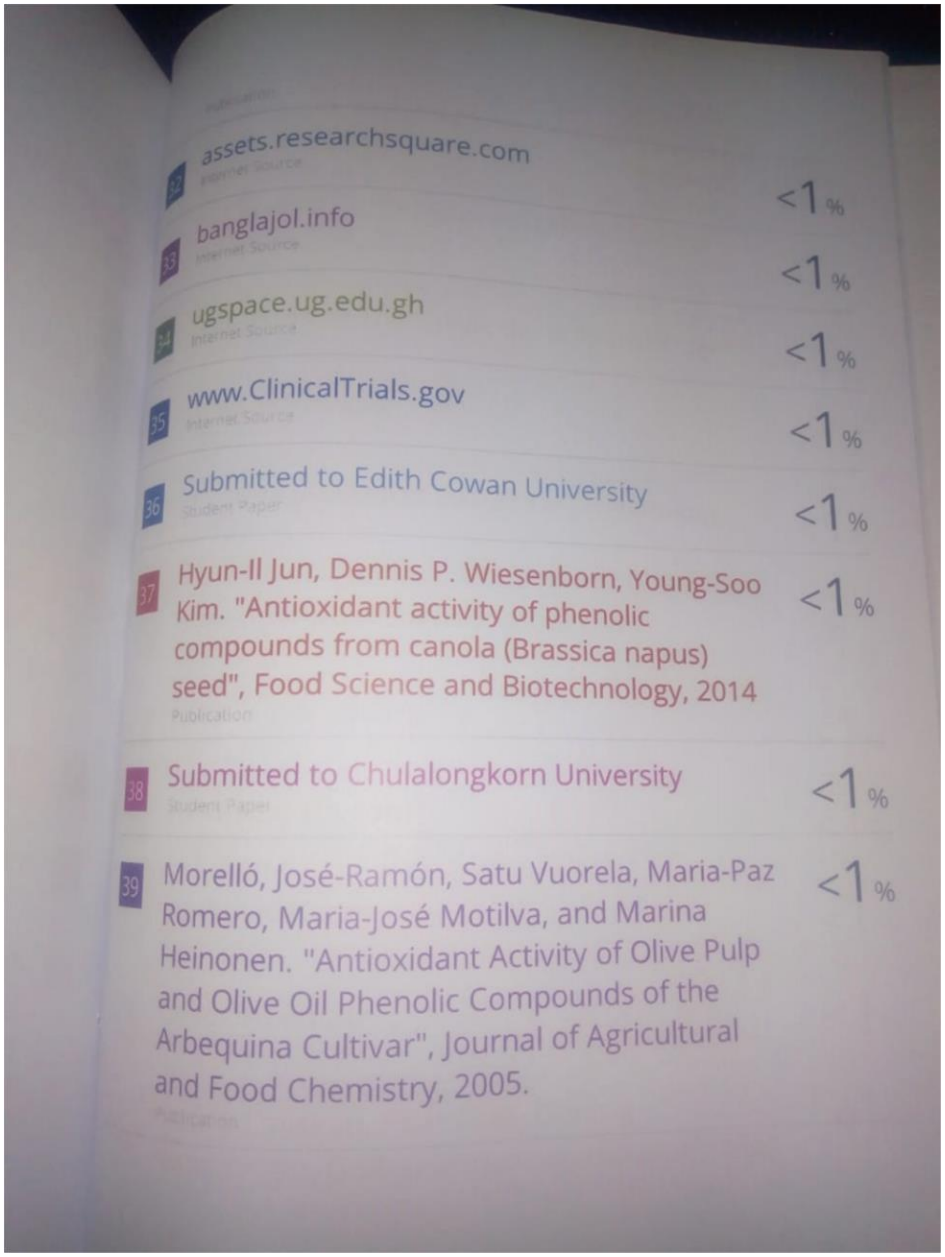

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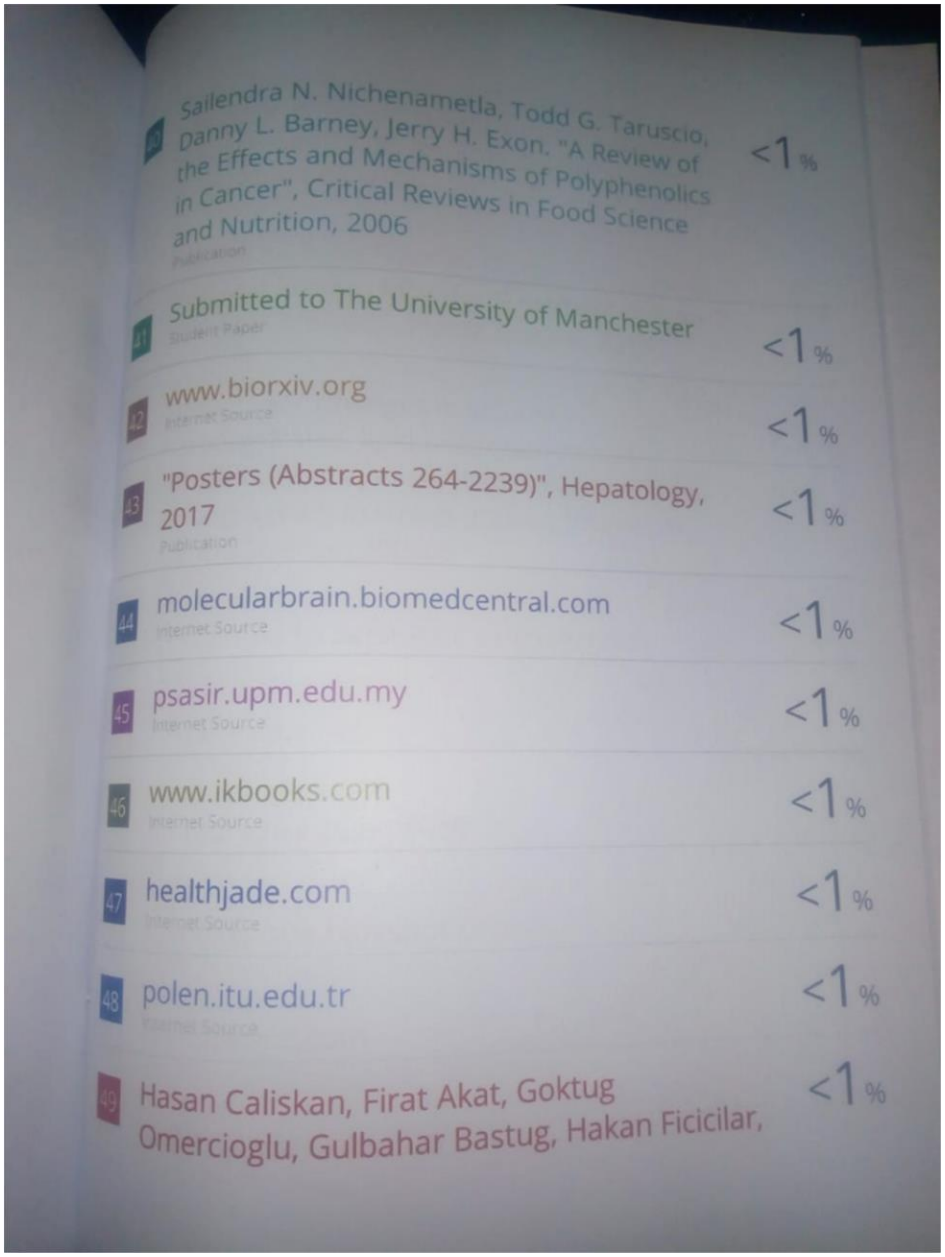

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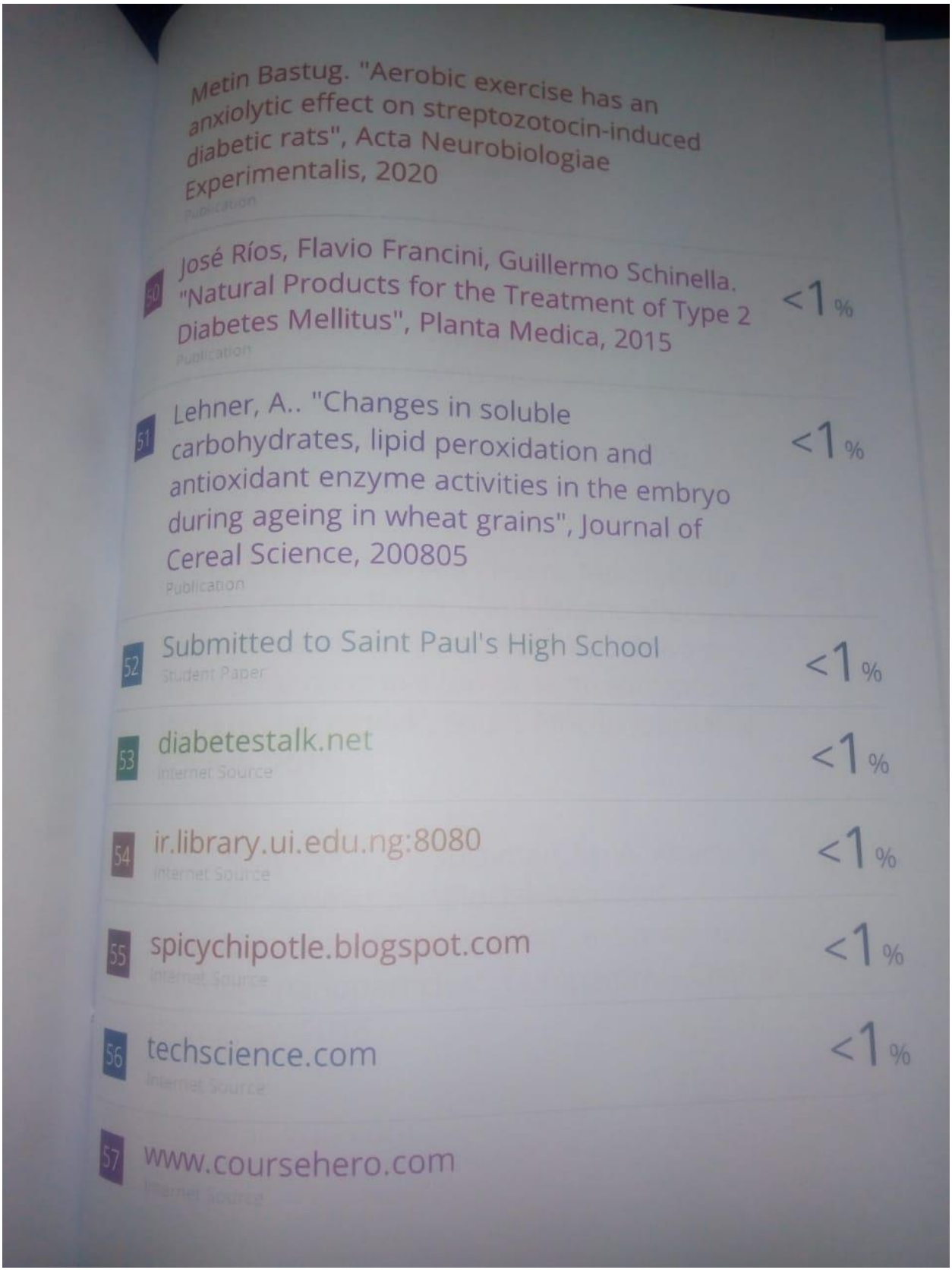

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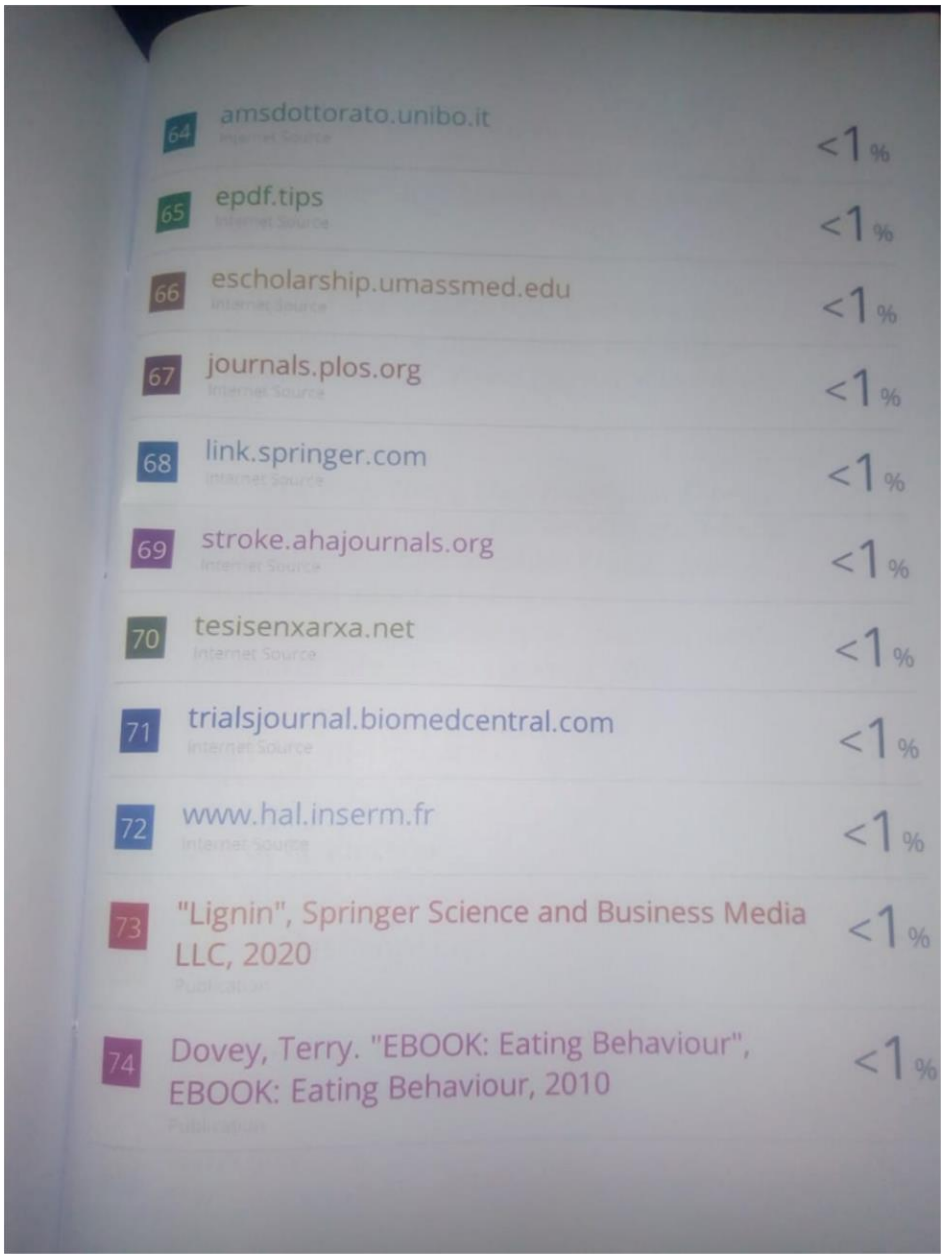

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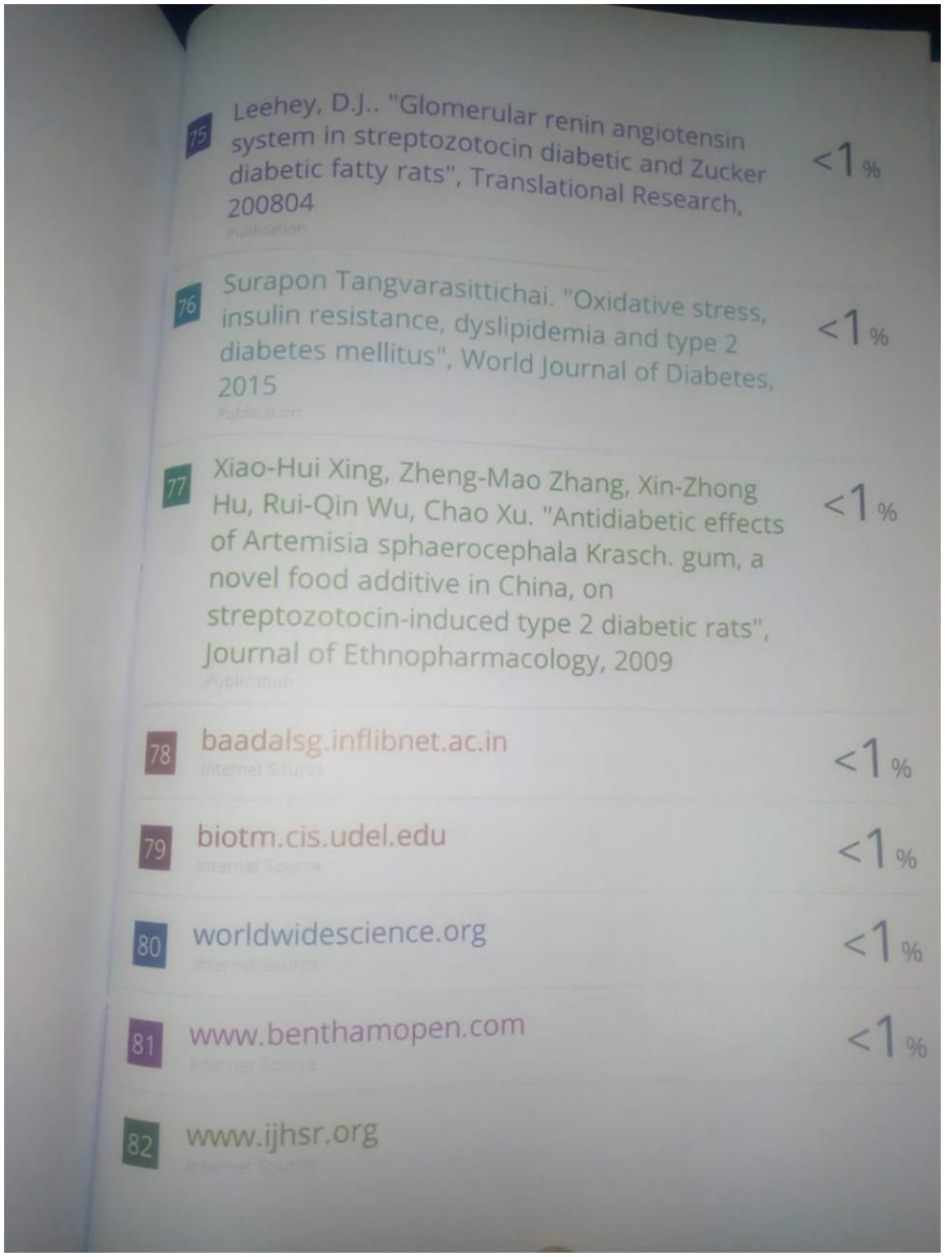

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