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Repurposing an Ancient Drug in New Application

Author:

Abeer Mansour Abdel Al Rasool Hasoon
Prof. Dr. Isam Hamo Mahmood

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By

Abeer Mansour Abdel Al Rasool Hasoon

Prof. Dr. Isam Hamo Mahmood

Personal Information about authors:

Name: Abeer Mansour Abedel Rasool
Date & place of Birth: 1983, Iraq
Gender: Female
Marital status: Married
Nationality: Iraqi
Address: Iraq – mosul
Email: : abeer.mansour@uoninevah.edu.iq.

Education:

1. B. ph. M.S., College of pharmacy Mosul University (2007(
2. M.Sc, Pharmacology / Dental Pharmacology. College Of Dentistry, Mosul
3. PhD, pharmacology /medicine College, Mosul

Experience:

1. worked and have pharmacy in Mosul
2. Boss of patent Iraqi forum of inventors for Mosul and Kirkuk and Salah alden.
3. working in Khansa work in the hospital and after she served as responsible emergency pharmacy sheikhs in the sector responsible sector in Tal Afar after the responsible for pharmacy emergency in al Batool Hospital after finish study for master responsible of the research and knowledge unit in the hamdania sector Then Senior Pharmacist in Pharmacology Science Nineveh Health Directorate, Iraq

Academic Thesis

1. Study of Master in Density Collage, Working in Animals Surgery, In the HPLC and Work in Elisa in the medicine collage, Radiological process working in DEXA, CT scan, Frist student that used image j in histopatholological study of bone in Mosul University

2. The first student on the stage in the master's patented in my field I studied physiology and pharmacology in the College of Dentistry and Pharmaceutical Chemists and toxicology in Mosul University

3. M.Sc. thesis entitled: The Effect of Bisoprolol fumarate gel on the Rate of Bone healing in Rabbits. College of Density Mosul University 2015

4. A book published in German publishing house (LAP LAMBERT Academic Publishing is a trademark of:
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5. Teaching methods course in LFU in Erbil

6. Giving lectures and teaching at Mosul University Collage of Dentistry and medicine collage.

7. Teach in dentistry collage and teaching in medicine collage 2020 study PhD in university of Mosul, pharmacology, collage of medicine2022

8. Now Teach in pharmacy collage Nineveh University

Acknowledgments

I dedicate this humble work…. To

My dear Father….

My beloved Mother….

My dear and beloved husband……

My dear son and daughters…

My dear brothers and sisters….

My dear teachers…

To my lovely friends…

Abeer Mansour

List of Content

List of Tables

List of Figures

List of Abbreviations

A172	glioblastoma cancer cell
AGEs	advanced glycation end products
AKI	acute kidney injury
AKT1	alpha serine/threonine-protein kinase
AMJ13	new breast cancer cell line
AMPK	Activated Protein Kinase
DM	Diabetes mellitus (DM).
DMSO	Dimethyl Sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ELISA	Enzyme-linked immunosorbent assay
GI	Gastrointestinal
GLP-1	glucagon-like peptide-1
H. Pylori	Helicobacter pylori
HCAM	liver cancer cell lines
HeLa	cervical cancer cell lines
HRP	Horseradish peroxidase
IC50	half maximal inhibitory concentration
IL-1 β	Interleukin-1 beta
MALA	metformin-associated lactic acidosis
MATEs	Multidrug and Toxin Extruders (MATEs
MDA	Malondialdehyde

mTOR1	mammalian target of rapamycin complex1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
OCTs	Organic Cation Transporters,
PCOS	poly cystic ovary
PCR	polymerase chain reaction
PFS	'Personal Finance Society'
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PMAT	Plasma Membrane Monoamine Transporter (PMAT).
PRL	prolactin
RAGE	Receptor for advanced glycation end-products
RNA	Ribonucleic acid
ROS	reactive oxygen species
RPMI-1640	Rosswell Park Memorial Institute-1640
T1D	Type 1 diabetes
T2D	Type 2 diabetes
T2M	Type 2 diabetes
TAOS	Total antioxidant status
TNF-α	Tumor necrosis factor-alpha

Summary

Metformin is one of the most widely prescribed oral hypoglycemic agents from the biguanides group used in diabetes mellitus. Metformin pleiotropic effects have recently received increased attention because of its potential cytotoxic effects on a variety of cancers. Cancer is the uncontrolled growth and spread of abnormal cells. There is a growing body of evidence about a link between D.M and the risk of some types of cancer, mainly because diabetes is a state with increased oxidative stress and inflammation, all of which can, in turn, promote carcinogenesis

Aim of study to evaluate the effect of metformin (different doses and times) on inflammatory markers (IL-1β and TNF-α), and oxidative stress on diabetes models, with the major aim is to study the cytotoxic effect of metformin on the growth of cancer cell lines and possible mechanisms of cytotoxic impact. Material and Method designed in two parts, part one: an experimental/intervention study design related to the induction of diabetes models of albino male rats by alloxan 200 mg/kg/dose, to do so, total number 75 rats(15 rat /each) was divided into five groups: control, diabetes, and metformin taking 100, 200, and 300 mg/kg/day after a period of 21, 35 days in order to evaluate the pro-inflammatory and antioxidant effects of different doses of metformin. After the current study's major aim, part two is the laboratory study designs related to the cancer study, the IRAQ Biotech Cell Bank provided cancer cell lines (HeLa, HCA, HBL100, A172, AMJ13) by the maintenance of cell cultures. All cells were kept in RPMI-1640 supplemented with 10% fetal bovine, 100 units/mL penicillin, and 100 g/mL streptomycin. Trypsin-EDTA was used to passage the cells. Reseeded at 50% confluence twice a week and incubated at 37°C with 5% CO2, then MTT assay was used to determine cytotoxicity of

metformin IC_{50} for all cancer lines with study cytomorphological death apoptosis assay, Reactive oxygen species and gene expression cellular study.

Result From part one, the findings metformin-treated diabetic rat showed significant changes observed in terms of body weight and food intake behavior. Metformin treatment dropped serum blood glucose levels in diabetic rats. The metformin groups showed a reduction of blood glucose level at day 35 as compared with day 7 of the three groups P=0.000, Serum TNF- α level significant difference between of three groups at day 7, 21and 35 day with a difference in dose 300,200 mg/kg /day than 100 mg /kg /day. TNF-α tissue liver showed significant difference related to dose and time. Comparison of IL-1β related to serum and liver value of five groups showed significantly difference of five groups support by histological study.

Regarding oxidative stress the present study showed a significant elevation of TAOS in metformin-treated groups at 21 and 35 days compared to a diabetes group that matched the result histological pathological pancreas with reduction of MDA significantly after treatment metformin, DPPH increased in a concentration- time dependent manner reflect antioxidant activity, IC 50 of metformin (498.0 µg/ml) while ascorbic acid IC50 (µg/ml 29.62) respectively at 60 min in different concentration reflected weal antioxidant in vitro.

About part two the results revealed significant reduce viability of cancer cell line had significant cytotoxic effects at levels (P< 0. 01) with in HeLa significantly than, AMJ3, HCAM, A172 when compare with normal cell HBL00 in dose 130 µM. The potential cytotoxic effect of metformin in Hela than other cancer cell line and normal cell 15.081 ± 0.167 and IC 50 µ M for metformin on Hela (7.492 µM). a highly significant selective cytotoxic effect on HeLa, and lesser effect on AMJ3, HCAM, and A172 cells than on normal cells support by assessment of apoptosis immunohistochemistry with morphological changes after that showed significant decreased in ROS production after adding metformin at

dose 130 μ M on Hela cancers cells line. Result of gene the expression of the PIK3CA gene was slightly increased, but this was not significant. Unlike the PIK3CA gene, the expression of the mTOR gene was significantly down-regulated whereas the expression of AKT1 was significantly up-regulated. This indicates that treatment of metformin had effects on both mTOR and AKT1 genes but not PIK3CA.

The current study concludes that metformin has beneficial effects on diabetes mellitus by lowering blood glucose, oxidative stress, and inflammation. Additionally, it has a protective effect on tissue against diabetes-induced cancer. In other words, the beneficial adding of metformin to regmain of anticancer combination lower deleterious effect of chemotherapy that most side effect diabetes after chemotherapy with modify resistance of chemotherapy by impact on mTOR this point summarized the novelty of current study.

1 Introduction & Review of Literature

1.1 Introduction

Biguanides are a group of oral hypoglycemic agents that are utilized for diabetes mellitus treatment, a serious side effect of lactic acidosis by using phenformin treatment led to discontinuity in the United States in 1976, while metformin is currently in common use in many parts of the world, biguanides such as phenformin and metformin are powerful inhibitors of the mitochondrial respiratory complex-1 [1].

Several preclinical investigations have been done to assess the use of biguanides as anticancer therapies that take advantage of neoplastic cells metabolic (2;3).Furthermore, clinical researches have demonstrated that metformin has a considerable clinical response in a variety of cancers [4].

Metformin

Metformin is a biguanides derived from the herb *Galega officinalis French lilac*, also known as goat's rue or Italian Fitch, it is utilized to treatment type 2 diabetic patients [5].

Because of its specific effects in treating type 2 diabetes T2D, metformin is a frequently utilized medicine in today's society, metformin reduces fasting and post-fasting sugar levels. Despite the popularity of metformin in diabetes treatment, the exact mechanism underlying the glucose level-lowering effects of this medication still remains poorly understood [6].

Metformin mechanisms are still debated. In fact; in the past decade demonstrated a simple picture of how metformin works by activating activated protein kinase (AMPK) more complex picture reflecting its different mechanisms in different cells and tissues and metformin works by inhibiting complex1

mitochondrial electron transport chain subsequently activation of AMPK [7]. The activation of AMPK leads to suppression of the mammalian target of rapamycin complex1 (mTORC1) inhibits cancer cell development [8;9]. The indirect mechanism reported for its ability to inhibit the gluconeogenesis genes transcription of liver with stimulate glucose uptake in muscle thus increasing insulin sensitivity and reducing blood glucose and lowering insulin levels [8].

Metformin differs from other anti-diabetic medicines in that it is inexpensive, safe and has few adverse effects additionally there is evidence of increased survival among people taking this prescription [10].

Another advantage is that it may be used along with other anti-diabetic drugs [11]. Metformin's anti-diabetic effects have been studied extensively, and this includes interactions with various pathways in tissues that reflex metformin's pleiotropic effects [12].Metformin impacts decrease the growth, survival and metastasis of a range of tumor cells including breast, liver, bone, pancreas, endometrial gut, kidney and lung cancer [13].

Diabetes mellitus

Metabolic disorder characterized by a high blood sugar accompanied by alteration in the metabolism of carbohydrates, fats and proteins origin due to defects in either insulin secretion, response or both at some point during disease [10]. There is a link between high blood glucose levels with increased reactive oxygen species (ROS) which is associated with increased oxidative stress and pro-inflammatory leading to damage to pancreatic cells and endothelial dysfunction, there is relationship between high blood glucose levels with increased ROS in diabetes and inflammation [12 ;14].

Cancer

Cancer is the second most common cause of death in the world, cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells [15]. The accumulation of genetic and epigenetic alterations in tumor suppressor genes causes cancer [16]. Cancer therapy aims to kill cancer cells without harming the normal cells [17].

Cancer and Oxidative Stress.

The pathogenesis of cancer is a complex process relating to cellular and molecular disputes caused with both endogenous and external stresses, DNA mutation is one of those triggers for free radical that responsible for a serious mechanism in the initial development of cancer [18].

Relationship between Diabetes and Cancer

The complexity of the link between diabetes and cancer studies has shown mitochondrial dysfunction accelerates hyperglycemia generation of free radicals and other reactive molecules such as ROS which stimulate the formation of advanced glycation end products (AGEs) [19]. The AGEs receptor is present in various types of cancer cells, such as neurons, immune cells, activated endothelial cells, osteoblasts and vascular smooth muscle cells, furthermore, AGEs cause persistent inflammation which is linked to many cancer-related signaling pathways [20].

Although many inflammatory cytokines are associated with cancer development, interleukin and tumor necrosis factor α are the major inflammatory cytokines related to diabetes and cancer at the same time [21].

TNF-blockade suppresses the expression of programmed cell death ligand 1 in cancer cells inhibiting tumor development according to an animal experiment, the underlying cause of the diabetes-cancer link, however is still being investigated and thus not fully understood, there have been numerous possibilities, molecular mechanisms are proposed to explain the causal relationship, excessive ROS generated accumulation of DNA lesions affects the

interpretation and transmission of genetic information which leads to changes in genetic material and is steps involved in carcinogenic mutagenesis and tumor transformation [22].

Metformin inhibits ROS production by acting on complex 1 of the respiratory chain which reduces electron entry into the chain [23].

Metformin inhibits the inflammatory response's initial activation which is linked to cellular transformation and cancer stem cell development [24].

Combining metformin with anticancer medications may optimize their effectiveness and reduce adverse side effects, however, few studies revealed the exact cytotoxic effect of metformin on tumor cells and their morphological changes after exposure for different concentration with different periods, a large number of papers have reported the anti-cancer properties of metformin but the mechanisms have not been fully discovered [25].

1.2 Review of literature

1.2.1 Biguanides Overview.

Biguanides such as phenformin and metformin are drugs that are often used to treat T2D as mitochondrial respiratory complex-I inhibitors [1].

"Drugs in this class"

Some forms of biguanides may still be available in some countries, buformin was developed in Germany in 1957 but was never sold in the U.S [1]. It was also found to cause an increased risk of lactic acidosis and was withdrawn in the 1970s., other types called proguanil and chlorproguanil, are used as antimalarial drugs [26;27]. Metformin was helpful in treating a local influenza outbreak [28;29].

"Benefits"

Numerous preclinical studies have assessed the use of biguanides as anticancer therapies in addition to anti-diabetes, however due to its toxicity serious lactic acidosis has been withdrawn from approval in many countries, biguanides have anticancer properties according to findings from retrospective epidemiological research in diabetic communities and preclinical laboratory models suggesting that they could be reused for cancer prevention and therapy, however, a better understanding of how these biguanides act as antitumor agents is required to guide their improved applications in cancer therapy, which has generated a growing interest in their pharmacology [29;30].

New research that helps us to understand the molecular pathways that connect diabetes treatment and cancer is essential for developing primary preventive efforts and potentially successful antineoplastic and their possible applicability to oncology [31].

1.2.2 Biguanides Mechanisms of Action and Their Possible Applicability to Oncology.

Biguanides are a class of drugs that modify metabolism to prevent tumor growth and modulating immune-activity, biguanide is thought to cause energy stress by inhibiting oxidative phosphorylation, energy metabolism is a topic for researchers [32].

Biguanides: (From Diabetes to Cancer)

According to evidence from retrospective population-based studies. We summarized related diabetes to cancer as the following:

- Metformin use was linked to a lower incidence of cancer and cancer the relation death in diabetes patients [33].
- Both metformin and phenformin have antitumor activity in cancer cells lines and animal tumor models (solid tumor) for trying to repurpose them for cancer avoidance and treatment [34].

The anticancer activity of biguanides was elucidated by the mechanism of action [35;36]as figure showed (1.1)

- Biguanides inhibition of mitochondrial complex I these mechanisms highlight a major target for biguanides as a main target.
- Biguanides can activate AMPK and inhibit mTOR signaling.
- Biguanides modulate antitumor immunity by targeting immune cells in the tumor microenvironment.

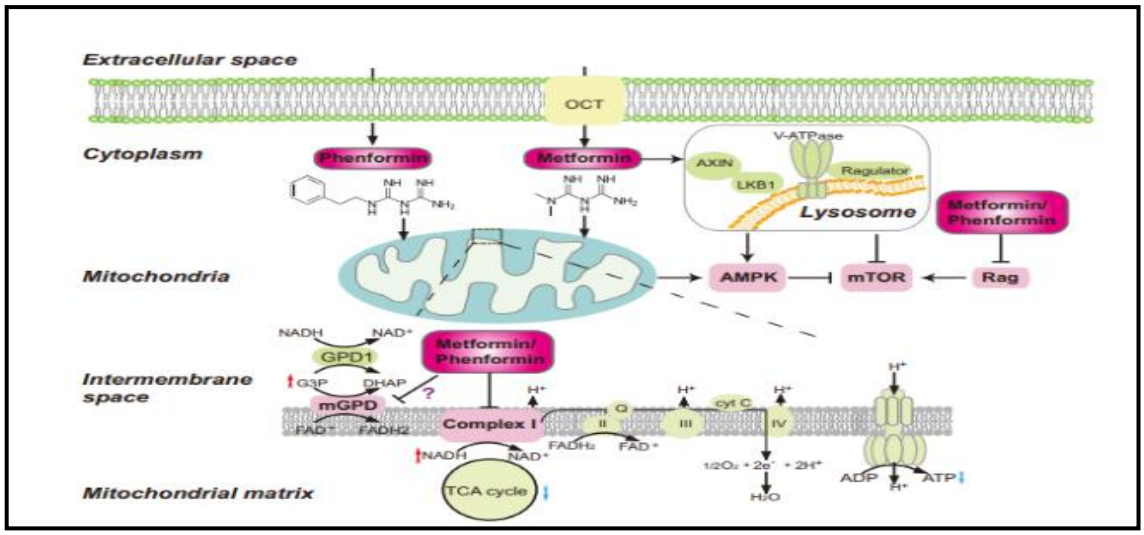


Figure (1.1): Molecular Targets of Biguanides describe the Antitumor Activities. Biguanides enter cells directly (phenformin) or that via organic cation transporters as(metformin) and focusing on target mitochondrial complex I, **AMPK**=activated protein kinase, **mTOR**= mammalian target of rapamycin. Inhibition of mitochondrial complex I by biguanides triggers an increase in the AMP/ADP: ATP ratio, leading to activation of AMPK and subsequent effect of mTOR signaling. Finally, class biguanides may activate AMPK and inhibit mTOR independent of mitochondrial complex I status [36].

Because of their broad range of biological actions biguanides are currently of great interest to drug discovery scientists [23]. As shown in figure (1.2).

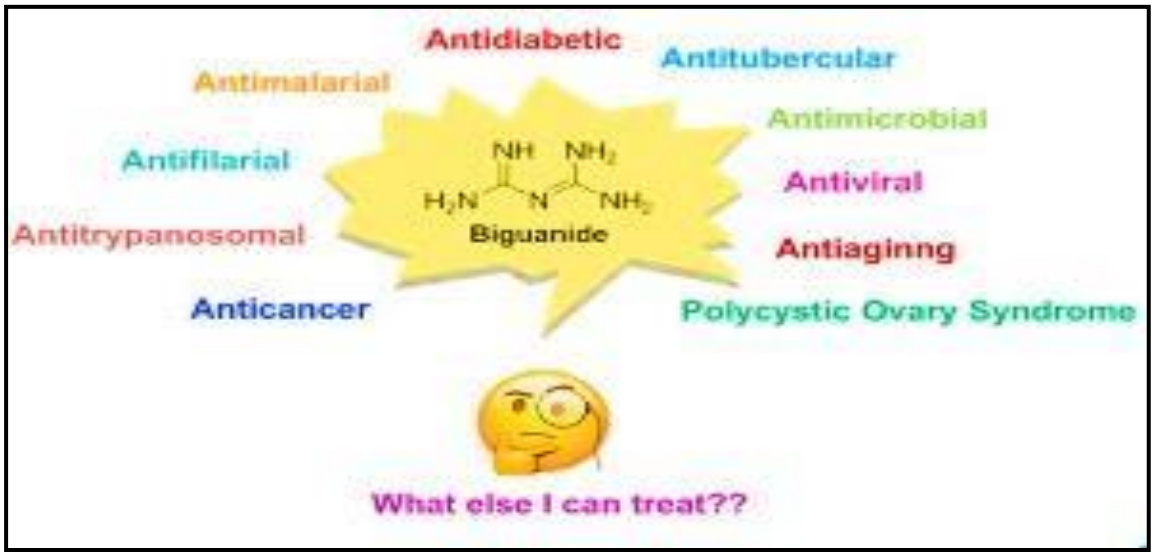


Figure (1.2): Biological Effects of Wide Range of Biguanides [23]

1.2.3 Metformin

Metformin (dimethylbiguanide) has become the preferred first line of an oral blood glucose lowering agent to manage diabetic mellitus, its history is linked to *Galega officinalis* (also known as goat's rue), a traditional herbal medicine in Europe as figure (1.3A) [5;29]. The chemical structure of metformin hydrochloride as shown in figure (1.3 B) [37].

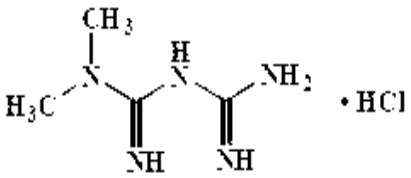


Figure (1.3): (A) *Galega officinal* [11], **(B)** Chemical Structure of Metformin HCl [37].

1.2.3.1 Pharmacokinetics

Metformin is not metabolized [38;39]. It is excreted uncharged in urine with a half-life of five hours [40]. Organic cation transporters (OCTs) transport the drug through the body including the intestine, liver and kidney [41]. Metformin bioavailability is about 50-60% and it has a large volume of distribution in the small intestine [42].

1.2.3.2 Mechanism of action of metformin

The exact mechanism of metformin is still not fully understood because it was discovered from a plant source and was not originally known synthesized to bind to a specific target [11]. The current study summarized the mechanism of action as the following:

- Metformin is the first-choice drug for the treatment of T2D particularly associated with obesity by inhibiting mitochondrial complex I function, followed by inhibiting ATP and activating AMPK [43]. As outlined in Figure (1.4), AMPK is a fuel level enzyme that becomes activated in conditions of high energy consumption inhibiting gluconeogenesis and increasing fatty acid oxidation [44;45].
- Metformin's glucose-lowering effect according to the study, occurs in the gastrointestinal tract rather than in the circulation metformin affects the gut microbiome enhancing glucagon-like peptide-1(GLP-1) release and improving glucose homeostasis according to study [46].

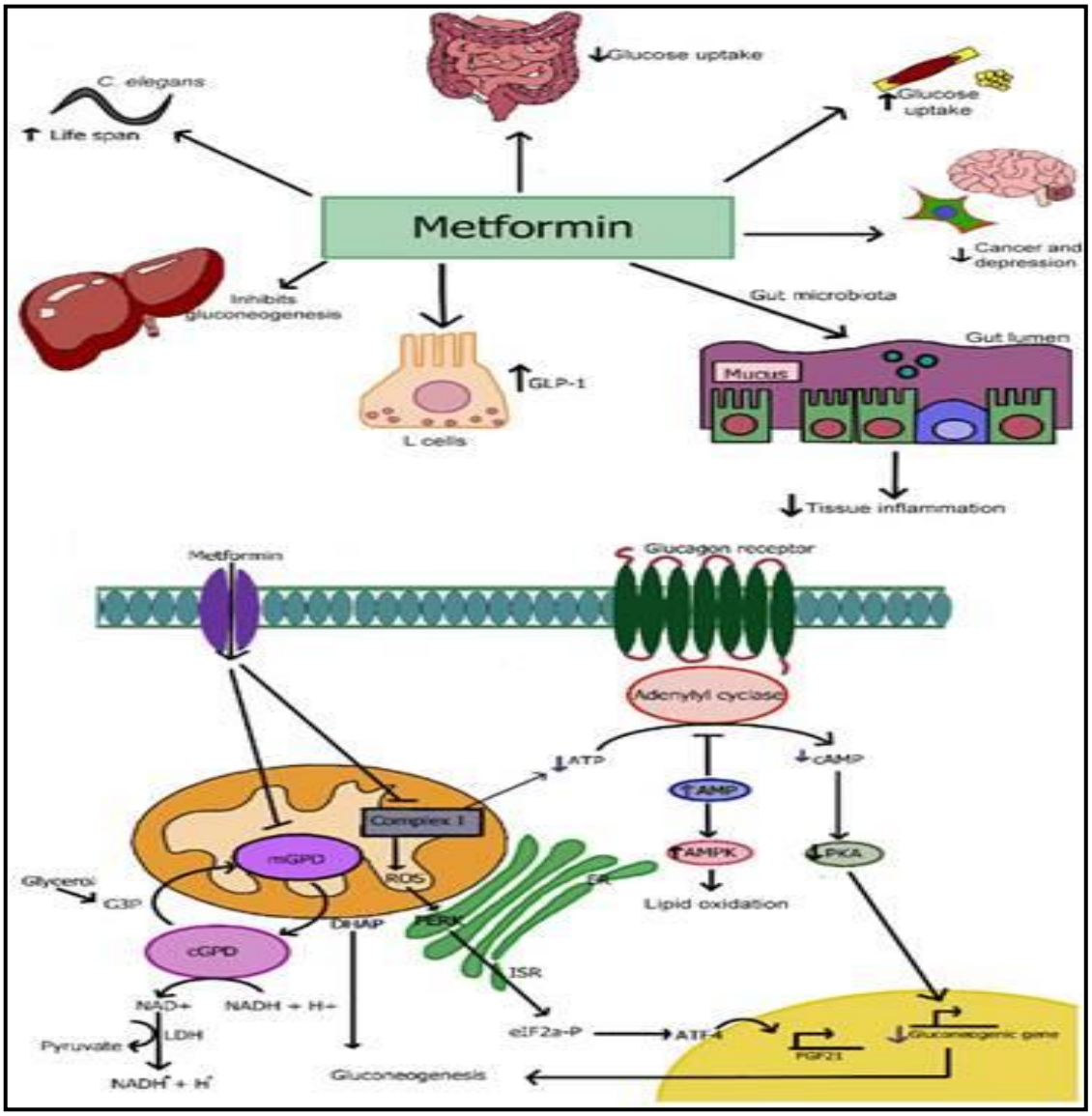


Figure (1.4): Demonstrated Mechanism of Action of Metformin [46].

- ✓ **Pre-diabetes:** The prevalence of pre-diabetes is rapidly rising worldwide as "International Diabetes Federation", these individuals are often overweight or obese high risk to T2DM and are prone to develop cardiovascular disease including coronary heart disease cardiovascular death and stroke [47].
- ✓ **Diabetes mellitus:** Metformin is a valuable therapy for reducing weight and preventing diabetes, metformin therapy is a good option for

overweight individuals with mild hyperglycemia, particularly those who concentrate on style modification [48].

- ✓ **Gestational diabetes:** Another use of metformin was to treat conditions regarding glucose metabolism as a pregnancy category B medicine, metformin is safe to use if there is a therapeutic need [49].
- ✓ **Poly cystic ovary syndrome**: Using Metformin with patient's poly cystic ovary (PCOS) for improving metabolic reproductive abnormalities of PCOS patient raise the chance of conception and preventing abortion[50].
- ✓ **Cancer:** Using metformin suggested reduce cancer risk in patients with type two diabetic [51]. Mechanism of metformin antineoplastic remained partially understood [52]
- ✓ **Antipsychotic-induced weight gain**: Metformin recommended as second line option after non-pharmacologic strategies for managing weight gain in patients with mood disorders and is recognized as often being used as a secondary prevention strategy for antipsychotic-related weight gain [53].

1.2.3.4 Adverse effects

There are few adverse influences to the usage of metformin and these include:

- ✓ **Gastrointestinal disturbance:**

Gastrointestinal (GI) symptoms such as abdominal discomfort, nausea and diarrhea [54]. A majority of patients treated with metformin are gastrointestinal symptom-free but upwards of 25% of patients experience dose-limiting side effects though discontinuation can be within the order of 5%, GI disturbances can be of a brief common but in a few cases they are determined [55]. Conservative beginning dosing and gradual dosage titration may attenuate metformin's affinity to cause GI disturbances [56].

✓ **Vitamin B12 deficiency:**

Vitamin B12 deficiency up to 30% of patients with T2DM receiving metformin treatment [57]. Metformin reported to be associated with decreased folate concentration, although the mechanism of this effect has not been elucidated, finally decreases in both folate and vitamin B12 concentrations might increase in homocysteine concentrations an independent risk factor for cardiovascular disease especially among individuals with T2DM [58]. Combining Proton pump inhibitors or H2 receptor blockers with metformin has been demonstrated to increase the risk of vitamin B12 insufficiency leading to peripheral neuropathy and megaloblastic anemia, should be monitored when taking drug together [59].

✓ **Lactic acidosis (Serious Side Effects):**

Conservative beginning dosing and gradual dosage titration may attenuate metformin's affinity to cause GI disturbances

✓ **Altered taste (frequent)**

Accumulation and secretion of metformin in saliva causing taste disturbance [61].

✓ **Allergic reactions (infrequent)**

Metformin rarely causes systemic allergic reactions, it can be used in asthmatics who are hypersensitive without worsening the risk of associated outcomes, such as hospitalizations, exacerbations or trips to the emergency department due to asthma [62]. Cutaneous allergic reactions have been described rarely but clinicians should be aware of their existence [63].

✓ **Hypoglycemia**

In a meta-analysis metformin was found to be safe and effective in monotherapy as a first-line agent, the risk of hypoglycemia was lower than with sulfonylurea monotherapy [11].

1.2.3.5 Current contraindications to metformin use

Metformin therapy may not be suitable for elderly, anorexic, or underweight older patients and also those with congestive heart failure, renal or hepatic insufficiency, high alcohol intake, dehydration, sepsis or severe infection, these conditions diminish the ability to metabolize lactate as in the case of hepatic failure or alcohol abuse or increase lactate production in the case of heart failure, respiratory failure, peripheral muscle ischemia or severe infection [64]. Metformin is also contraindicated in patients with a creatinine clearance of less than 60 mL/min [65;66].

Precautions with iodinated X-ray contrast media are not an independent risk factor for patients taking metformin , but are a concern only if post-contrast acute kidney injury (AKI) should develop [67].

The Food and Drug Administration documented that prescription information for metformin should be temporarily withdrawn from patients undergoing studies using IV. iodinated contrast media that cause acute renal failure and promote the accumulation of metformin and lactic acidosis [68].

1.2.3.6 Dose

Metformin could be taken in doses ranging from 500 mg to 2.55 g a day with the lowest effective dose being prescribed, since ingestion of more than 1000 mg at one time normally results in severe GI side effects, dosage should always be divided [37].

1.2.3.7 Drug interaction

At physiological pH, metformin is a cation, a strong base, therefore, the absorption, distribution and excretion of metformin depend on transporters such as OCTs, Multidrug And Toxin Extruders (MATEs) and Plasma Membrane Monoamine Transporter (PMAT), OCTs possibly mediate metformin oral absorption and hepatic uptake [69]. As Metformin is not metabolized, it is not expected to be involved in many drug–drug interactions, however, inhibitors of metformin transporters MATEs and OCTs may diminish metformin elimination

and elevate its plasma concentrations, increasing the risk of metformin-associated lactic acidosis [70].

- Metformin interactions with acid suppressing agents such as: H2 receptor blockers, cimetidine is a broad-spectrum inhibitor of transporter including OCT2and a powerful inhibitor of MATE1 of proximal tubular epithelial cell OCT2, metformin excretion is reduced resulting increased risk of metformin associated lactic acidosis [69]. Metformin renal clearance was decreased as a result of ranitidine a potent inhibitor of MATE1 [71].
- Interactions with Antimicrobials: Trimethoprim slows metformin clearance, inhibiting OCTs and MATEs although co-administration of both medicines should be done with caution in individuals with renal failure or those taking larger metformin doses [59]. Cephalexin inhibit the elimination of metformin [72].
- Interaction Ranolazine: Chronic angina is treated with ranolazine, ranolazine decreases glucagon release by blocking sodium channels in pancreatic cells and decreasing electrical activity [73].Metformin plasma concentrations may be increased by co-administration of ranolazine to reduce metformin elimination by inhibiting the OCT2 transporter [74].
- Interaction with Anticancer Drugs: Tyrosine kinase inhibitors such as imatinib, nilotinib, gefitinib and erlotinib inhibiting OCTs and MATE transporters may reduce the elimination of metformin at clinically relevant concentrations [75]. Vandetanib is a drug that is used to treat medullary thyroid carcinoma. It is a potent MATE1 and MATE2K transporter inhibitor, vandetanib with metformin is monitored for metformin toxicity [76].
- Interaction β-adrenergic blockers: Metformin plasma concentration may be elevated as a result of impaired elimination caused by atenolol which lowers renal blood flow and competitively inhibits OCT2 [77].

1.2.4 Diabetes Mellitus (DM).

A metabolic disorder characterized by the chronic presence of high blood sugar accompanied by varying degrees of impairment the metabolism of carbohydrates, fats, and proteins, diabetes has many different causes, but they all involve defects in either insulin secretion, response, or both at some point during the disease [14].

Classification of diabetes the American Diabetes Association Standards of Medical Care for Diabetes 2021recommends the following classification [78]:

- ✓ **Type 1 diabetes (T1D):** Linked to autoimmune-cell destruction which results in absolute insulin deficiency, including latent autoimmune diabetes in adulthood.
- ✓ **Type 2 diabetes (T2D):** Due to a progressive loss of adequate β-cell insulin secretion frequently on the background of insulin resistance.
- ✓ **Diabetes caused by a variety of additional factors:** Monogenic diabetes syndromes such as neonatal diabetes and maturity-onset diabetes of the young, diseases of the exocrine pancreas such as cystic fibrosis, pancreatitis drug or chemical-induced diabetes such as glucocorticoid use in the treatment of HIV/AIDS.
- ✓ **Gestational diabetes mellitus:** Diabetes that diagnosis during the second or third trimester of pregnancy which was not readily noticeable pre pregnancy.

1.2.5 Relationship Between Diabetes Mellitus, Oxidative Stress and Inflammation

Based on the analysis of clinical and experimental studies, the role of oxidative stress in the pathogenesis of T1D and T2D diabetes mellitus and its complications, oxidative damage to pancreatic and endothelial dysfunction are all identified as a source of increased ROS formation in diabetes, there is a connection between oxidative stress and inflammation [12].

In recent decades pro-inflammatory indicators have appeared as a potently interfering factor in the pathophysiology of age-related diseases and major chronic diseases in developed countries, such as cardiovascular disease, diabetes, arthritis and a variety of cancers[13]. The contribution of ROS is a major source of concern, free radical highly reactive molecules is produced by normal cellular processes, environmental stresses and UV irradiation, in the other words, the reaction of ROS, at high concentration with biological components lead to damage in DNA, carbohydrates, proteins, and lipids causing injury on the cellular and tissue level by which can lead to inflammation and several disease states, including cancer, diabetes and atherosclerosis [79]. As figure (1.5).

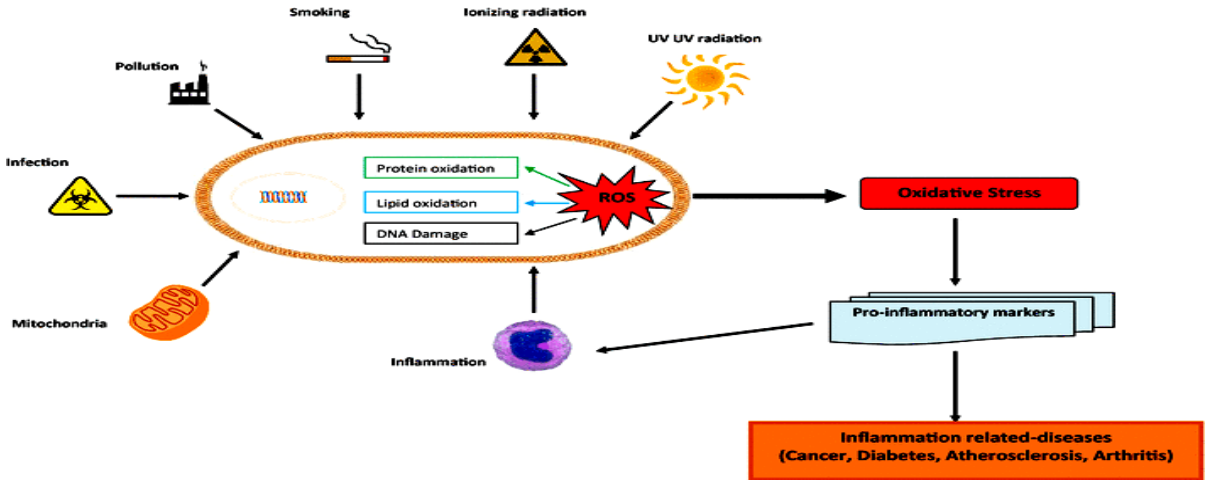


Figure (1.5): Relationship Reactive Oxygen Species (ROS) , Pro- Inflammatory Indicators And Diabetic[79].

1.2.6 Cancer

1.2.6.1 Cancer definition

Defined as a group of diseases characterized by the uncontrolled growth and spread of abnormal cells which normal cells start multiplying uncontrollably ignoring signals to stop and accumulating to form amass that is generally termed a tumor [80].

Cancer is now recognized in both humans and animals as arising from several different causes[24] including:

- Specialized viruses.
- Radiation.
- Chemicals.
- Certain highly irritative parasites (inflammation) [81].
- Infection may contribute to cancer indirectly, for example when inflammatory responses increase mutations or proliferative signals [82].
- Number of other factors, such as specific genetic defects present in individual humans and possibly in every member of a colony of specially bred animal models [81].

1.2.6.2 Classification of Tumors

Tumors are divided into two classes: -

- **Benign Tumors**: These are not cancerous, they either cannot spread or grow, or they do so very slowly, most benign tumors are not harmful, and they are unlikely to affect other parts of the body however, they can cause pain or other problems if they press against nerves or blood vessels or if they trigger the overproduction of hormones, as in the endocrine system, the most common cases of benign tumors are fibroids in the uterus and lipomas in the skin. require surgical removal. For example, colon polyps (another name for an abnormal mass of cells) can become malignant and are therefore usually surgically removed [83].

- **Malignant Tumors:** Malignant tumors are cancerous, these types of tumors can develop rapidly and spread to other parts of the body in a process called metastasis to become life- threatening [84].

1.2.6.3 Pathogenesis of cancer

The complex pathophysiology of cancer, pathologists are principally concerned with the study of disease in all its aspects, this includes the trigger of the disease, diagnosis and how the disease progresses (pathogenesis) in addition to the mechanism of the disease, there is a common pathophysiological process underlying cancer development in the organism, regardless of the histological and physiological differences across cancer types [85]. As figure (1.6).

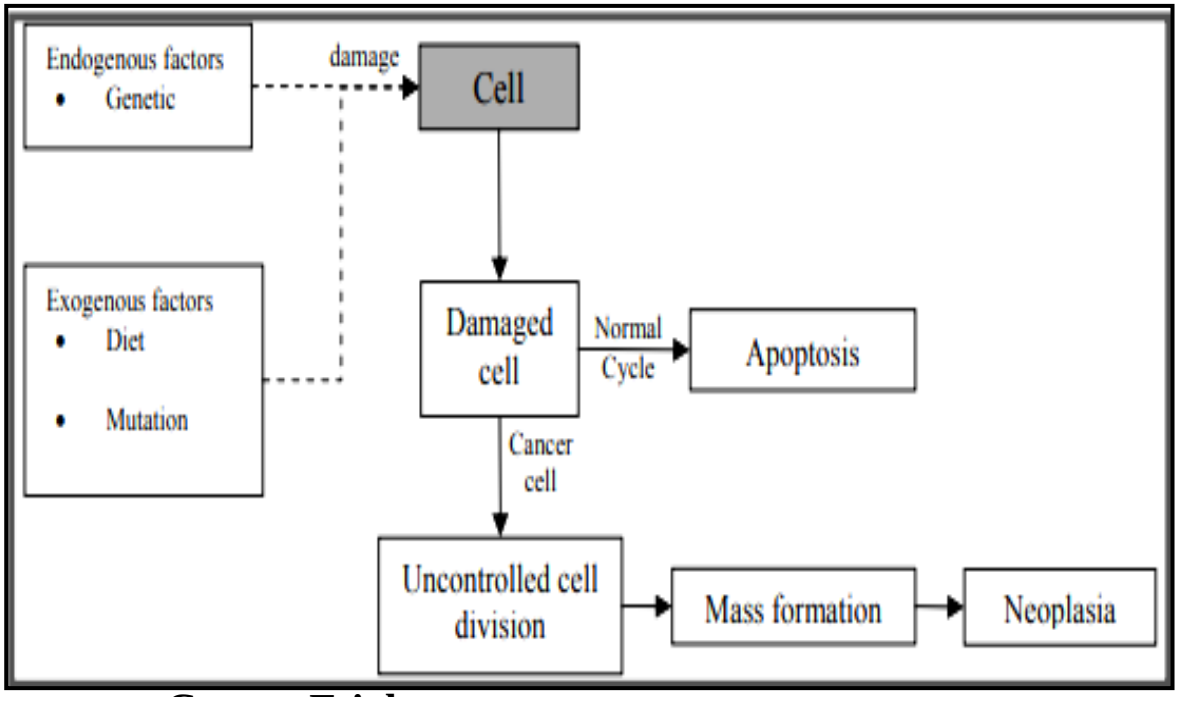


Figure (1.6): Pathophysiology of Cancer in a Schematic [85]

logy, the most widely recognized cancer is the mutation in the genetic material of cancer cells [86]. The etiology of cancer is summarized by the following**:**

- ✓ **Chemical carcinogens**: Carcinogens are mutagens that cause cancer [87]. Particular substances have been linked to specific types of cancer, tobacco

smoking is associated with lung cancer and the bladder, alcohol is an example of a chemical carcinogen [88].

- ✓ **Physical factors**: Specific physical factors have been associated with carcinogenesis such as radon gas, which can cause cancer [89]. Prolonged exposure to ultraviolet radiation from the sun can lead to melanoma and other skin malignancies [90].
- ✓ **Immune system dysfunction**: Human immunodeficiency virus (HIV) is linked to a variety of malignancies including Kaposi's sarcoma, non-Hodgkin's lymphoma and AIDS-defining illnesses have long included these diagnoses, the increased incidence of malignancies in human immunodeficiency virus patients points to the breakdown of immune surveillance as a possible etiology of cancer [91].
- ✓ **Biological factors**: In particular, retroviruses are the most important biological agents in the spread and development of cancer, there are retroviruses that can cause a shift in the start of tumors when they enter the body [92]. As the virus rodent's murine leukemia virus, this causes leukemia and Burkitts Lymphoma [93]. Repeated injury Bilharziasis parasite Schistosoma haematobium lead to injury of epithelial cells squamous cancer Squamous cell carcinoma and the secretion of parasite material nitrosomine carcinogens[94].
- ✓ Helicobacter pylori (H. Pylori) are responsible for the majority of stomach malignancies, H. pylori does not mean you will get stomach cancer, but it does increase your risk. [95]. Chlamydia trachomatis is an intracellular obligate gram-negative bacterium which has been related to cervical cancer etiology [96].
- ✓ **Hormonal imbalances**: A well-established example is the role of hyperactive estrogenic states in promoting endometrial cancer [97]. Studies indicate a role for prolactin (PRL) in breast epithelial proliferation,

differentiation and tumor genesis, women with higher PRL circulating levels have an increased risk of breast cancer [98].

1.2.7 Cell Lines

In vitro models cancer cell lines play a significant role both in cancer research and medication discovery, called" cell lines" are widely used in a variety of medical fields because they provide a supply of biological material for research and they are incredibly useful in developing an understanding of cancer cell biology [99].

1.2.7.2 **Advantages of cell lines:**

The principal advantages of the cell culture method are [100].:

1. Supplies a good tool to study cell metabolism and examine the physiology and biochemistry of cells.
2. Investigate the cytotoxic effect of many compounds or drugs on specific types of cells such as liver cells, through the study of biology and origin of the cells obtained from homogenous cell culture. specific proteins can be synthesized in large quantities from genetically modified cells in large-scale cultures.
3. Reproducibility of the outcomes.

1.2.7.3 **Disadvantage of cell lines [100;101].**

The main disadvantages of using cell culture in basic and advanced research are**:**

1. Expenditure and understanding that cell culture is a specialized technique that requires aseptic conditions, costly equipment, and trained personnel.
2. Differentiation: cell characteristics are changed after a period of continuous growth of cells in cultures, leading to differentiated properties compared to the original cell strain.

3. A disadvantage is the lack of standard protocols and the difficulty in comparing results in addition to the increasing complexity and variety of models accessible to researchers will exacerbate this challenge.

1.2.8 Cancer and Oxidative Stress

Cancer manifestation in humans is a complex process that involves cellular and molecular disputes brought on by both endogenous moreover external stimuli, oxidative DNA mutations are one of those triggers responsible for a serious mechanism in the initial development of cancer [102].

1.2.9 Relationship Between Diabetes and Cancer

Diabetes elevated the risk of developing a variety of severe life-threatening health complications as a result of dysfunction of several organs kidneys, hearts, skin, blood vessels or nerves resulting in both micro and macro vascular complications such as nephropathy, diabetic retinopathy and neuropathy as well as atherosclerosis, hypertension and atherosclerosis [103].Additionally, raised blood glucose levels have been established to accelerate cancer cell proliferation and development, in addition to these consequences, studies have found a strong link between diabetes mellitus and cancer [104;105].

Hyperglycemia produces epigenetic changes in the form of DNA methylation and chromatin remodeling, which leads to abnormal gene expression, furthermore, aberrant gene expression promotes tumor growth by increasing cancer cell metastasis, proliferation and chemo resistance [106].

The proliferation of cancer cells generated by hyperglycemia, related to diabetes is mediated in part by the following, inflammatory responses, production of ROS in addition to hyperglycemia [107].

Hence, the complexity of the link between diabetes and cancer, as well as the interaction these two diseases have become unclear although the increase in cancer risk in diabetic patients may be minor to moderate the global epidemic of

diabetes, the socioeconomic implications of this positive connection may be difficult [108].

Studies have also reported hyperglycemia accelerates mitochondrial dysfunction and the production of free radicals and other reactive molecules, such as ROS stimulation the formation of AGEs and activating protein kinase C isomers [19]. AGEs receptor is present in various types of cancer cells, such as neurons, immune cells, activated endothelial cells, osteoblasts, and vascular smooth muscle cells, AGEs are responsible persistent inflammation, which is linked to many cancer-related signaling pathway [109].

Although many inflammatory cytokines is associated with cancer development, interleukin and tumor necrosis factor α as the major inflammatory cytokines related to diabetes and cancer at the same time [110]. TNF-blockade inhibiting tumor development [22].

Furthermore, despite increased baseline of inflammatory cytokines levels in diabetics the generation of cytokines during immunological defense is impaired according to expert complement-dependent phagocytic activities and chemotactic phagocytosis of macrophages are also suppressed resulting in immunological failure, making infection easier and providing a better environment for the tumor to survive [111].

Diabetes-cancer link is still being investigated and thus not fully understood molecular mechanisms proposed to explain the causal relationship [112].

Additionally, food, exercise, aging, and obesity are all risk factors for both of these diseases because of lifestyle changes and higher life expectancy, the incidence of diabetes and cancer is expanding quickly over the world, diabetes and cancer are global issues, therefore worldwide health professionals or organizations should produce standards for diabetes and cancer prevention, diagnosis, and treatment to alleviate the social cost many problems remain

unresolved because of the inherent heterogeneity of both diabetes and cancer, making research difficult to conduct [113].

1.2.10 Oxidative Stress

Oxidative stress is revealed to as excess production and/ or insufficient elimination of highly reactive molecules such as ROS oxygen species and reactive nitrogen species, the production and accumulation of ROS in cells and tissues as well as the biological system's ability to detoxify these reactive products are out of balance. synonyms, it is responsible for a variety of diseases [114].

Protecting against the effects of ROS / Reactive Nitrogen Species (RNS) has attracted scientists' attention in recent years to the mechanism of action of various antioxidants, the importance of oxygen-derived pro-oxidants and antioxidants which are essential in both normal metabolism and a variety of clinical disease states is becoming increasingly recognized damage to cells of the body can result in cellular mutation tissue and compromised immunity that due to many diseases such as cancer, diabetic, cardiovascular disease, aging, degenerative disease [115]. As shown as in figure (1.7).ROS is generating by free radicals of oxygen such as superoxide anion ($\bullet O_2^-$), hydroxyl radical (•OH) reactive non radical oxygen species such as hydrogen peroxidase(H_2O_2) and singlet oxygen ($1O_2$) [116].

RNS is producing by free radicals such as nitric oxide (•NO) and nitrogen dioxide (•NO2) as well as no radicals like peroxynitrite (ONOO-) nitrous oxide (HNO2) and alkyl peroxynitrates (RONOO) [117].

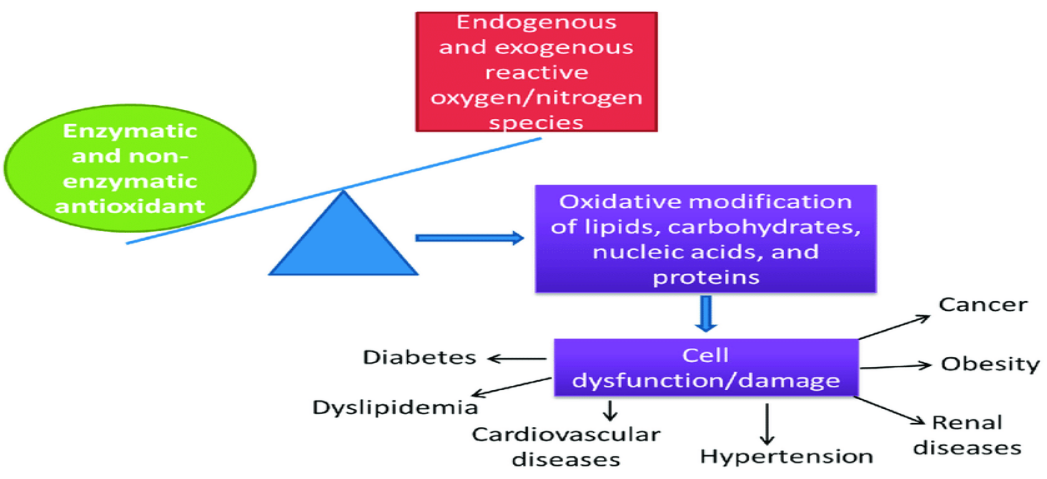


Figure (1.7): Cell injury is caused by an imbalance between free radicals and antioxidant mechanisms, which leads to organ/system pathogenesis [114].

1.2.10.1 Reactive Oxygen Species.

Radical of oxygen superoxide anion ($\bullet O_2$-), hydroxyl radical (•OH) reactive non radical oxygen species such as hydrogen peroxidase (H_2O_2) and singlet oxygen ($1O_2$), ROS is part of a controlled inflammatory reaction and by being exposed to environmental factors and generated in vivo as an integral part of metabolism [116]. Any species able of independent existence with one or more unpaired electrons is referred to as a free radical [119]. Non-radical oxygen derivatives are able to formation of radical in present intra and extracellular environments [120].

Crucial role of oxidative stress in the etiology of many clinical diseases antioxidant therapy could positively impact on improving the history of diseases, but more investigation is required to determine the efficacy of therapeutic intervention, excess production of ROS in oxidative stress has harmful effects including proteins, lipids and DNA [121].

1.2.10.2 Reactive Nitrogen Species Nitric oxide

Nitric oxide (NO°) is a common reactive radical that plays a significant role as an oxidative biological signal in various physiological processes, including smooth muscle relaxation, neurotransmission and Immune regulation [122]. Vascular endothelium, neutrophils and macrophages by using enzyme nitric oxide synthase to produce nitric oxide from arginine ,"L-arginine" is an amino acid involved in a number of biological processes [123].

- ✓ **Effect of Oxidants on Macromolecule:** Unstable molecule that contain oxygen and that reacts easily with other molecules in a cell, a buildup of ROS in cells causing damage to DNA, RNA and proteins [124]. ROS are

free radicals, free radicals are well reported for playing a dual part in our body as both deleterious and useful species, in low/moderate concentrations free radicals are involved in normal physiological functions but excess production of free radicals or decrease in antioxidant level leads to oxidative stress [125].

- ✓ **Oxidative Damage to DNA**: Free oxidizing radicals readily attack DNA causing damage and mutations in the genetic materials or even actual breakage of DNA strands, the body can repair some of these damaged strands, but imperfect repair leaves altered DNA and can cause cancer [126]. The study by [127], analyzed the role of DNA damage and DNA repair mechanisms in colorectal carcinogenesis and ROS overproduction can induce oxidative stress and oxidative DNA damage can cause mutations ultimately to cancer.
- ✓ **Oxidative Damage to Lipids:** The cellular membranes and LDL contain polyunsaturated fatty acids [128]. Lipid peroxidation is initiated when free radicals steal electrons from the lipid materials of the cell membrane, free radical will steal a single electron from the hydrogen-related double bond with carbon, this leaves the carbon with an unpaired electron and hence becomes a free radical, in order to stabilize the carbon-centered free radical, molecular rearrangement occurs, the newly arranged molecule is called a conjugated diene, conjugated diene then very easily reacts with oxygen to form a peroxy radical and steals an electron from another lipid molecule in a process called propagation, this process is then repeated in a chain reaction [129]. The products of lipid peroxidation are easily detected in blood plasma and have been used as a measure of oxidative stress, the most commonly measured is MDA, the unsaturated aldehydes produced by these reactions have been linked to the modification of cellular proteins. and other molecules [130].

- ✓ **Oxidative Damage to Proteins:** Oxidative damage can have a harmful effect on the structure and activity of proteins, and may even lead to cell death, the sulfur-containing amino acids cysteine and methionine are particularly susceptible to ROS and reactive chlorine species RCS, which can damage proteins [131].

1.2.11 Inflammation

Inflammation is typically the reaction of the body to a tissue injury. edema, redness, heat, pain and loss of function are all signs of inflammation, the inflammatory process results in a sequence of well-regulated humoral and cellular events that contribute to the localization of harm in the case of tissue injury caused by minor trauma or surgical treatment noxious agents are removed physical damage is repaired and the wounded tissue's function is restored [112]. On the other hand, uncontrolled acute inflammation can become chronic leading to a variety of chronic inflammatory diseases [132].

1.2.12 Cytokines

Cytokines are small released proteins (40 kDa) that every cell generates to regulate and influence immune response [133]. The release of pro-inflammatory cytokines induces immune cell activation and production as well as the release of more cytokines [134]. However, several studies show that in any immune response the simultaneous release of pro- inflammatory cytokines is required [135].

Cytokines are pleiotropic polypeptides that operate on cells to regulate inflammatory and immunological responses, they have a crucial role in the pathophysiology of a variety of diseases, including diabetes [134].

Moreover, clinical and experimental research have revealed that hyperglycemia causes the accumulation of AGE in diabetes patients' tissues which

bind to the cellular receptor, receptor for advanced glycation end products RAGE, the interaction between AGE and RAGE triggers a signaling cascade that results in an increase in nuclear transcription factor NF-κB. As a result, oxidative stress and the production of pro-inflammatory cytokines increase much more [136].

Pro-inflammatory cytokines such as Interleukin and TNF- α play a key role in the adjustment of β-cell function and the aggravation of diabetes symptoms [137;138].

When pancreatic beta cells are exposed to IL-1 or IL-6 plus TNF-α, apoptotic cell death is induced by downstream activation of the NF-κB signaling pathways [136]. NF-κB is a pleiotropic oxidant and a sensitive transcription factor that has been linked to oxidative stress caused by hyperglycemia. Previous research has shown that NF-κB plays an important role in the etiology of diabetes [139].

Tumor necrosis factor-alpha: Proliferation, differentiation, apoptosis, immunity regulation and inflammation induction stimulated by tumor necrosis factor-alpha [140]. TNF-α is a homotrimer protein consisting of 157 amino available in two forms soluble and transmembrane, this cytokine is a cell signaling protein that controls a variety of cell functions consequential in necrosis or apoptosis [141]. TNF-α binds to two different receptors, which initiate signal transduction pathways ,these pathways lead to various cellular responses including cell survival, differentiation and proliferation [142].

Interleukin-1 beta (IL-1β): is a cytokine protein that is encoded by the IL-1β gene in humans, the interleukin 1 cytokine family includes IL-β, this cytokine plays a key role in the inflammatory responses are generated in stimulate to inflammatory agents, infection and microbial endotoxins by a wide range of cells[143]. Furthermore, in case of chronic inflammation, persistent IL-1β may

increase tumor induction and later tumor propagation through many mechanisms [144].

Variety of anti-tumor therapies induce IL-1β production, highlighting the multifaceted biological activities of this key cytokine under pathologic conditions with a focus on tumor development [145].

1.2.13 A Review of the Different Mechanisms of Metformin's Action in Cancer.

Metformin's anti-diabetic effects have been studied extensively and this includes interactions with various pathways in tissues that reflex the metformin's pleiotropic effects. [48]. As shown in the figure (1.8).

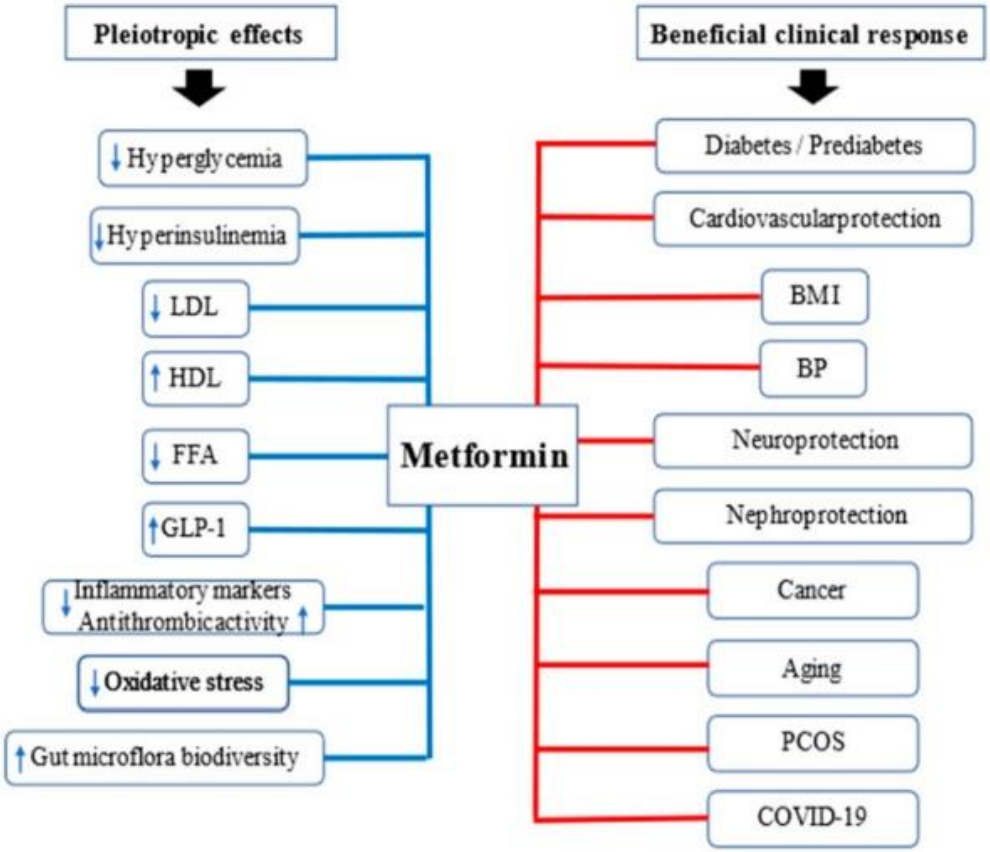


Figure (1.8): Metformin's Pleiotropic Effects have Beneficial Clinical Effect [48].

The mechanisms underlying the actions of metformin appear to be complex and responsible for the multidirectional effects of metformin, these mechanisms remain the subject of much debate, indeed, in the past decade we have gone from a simple picture, that metformin acts via the liver's 5′ AMP-activated protein kinase to more complex picture reflecting its different mechanisms in different cells and tissues early studies demonstrated metformin acts by inhibition of complex1 in mitochondrial electron transport chain subsequently activation of AMPK [107].Metformin inhibits the mTORC1, mTORC1 is a multiprotein complex made up primarily of the mTOR protein kinase and the raptor scaffolding protein [146]. Increased activation of mTOR leads to an elevation in inflammatory cytokines levels through differentiating

macrophages [147]. Inflammatory pathways that is triggered by mTOR activation [8;148].

Metformin also decreases both pro-inflammatory cytokines and NF-κB and improves the immune response to cancer cells The activation of the AMPK pathway called AMPK- dependent mechanisms of metformin "direct acting" [149].

In other hand metformin inhibited Ras-induced ROS production and DNA damage provide a novel mechanism to explain the reduced cancer incidence associated with metformin therapy and raise the possibility of novel applications [33].

AMPK-independent mechanisms of metformin, the indirect effects of metformin are decreased in glucose level, hyperinsulinemia, and Insulin-like Growth Factor1level [9].As showed in figure (1.9).

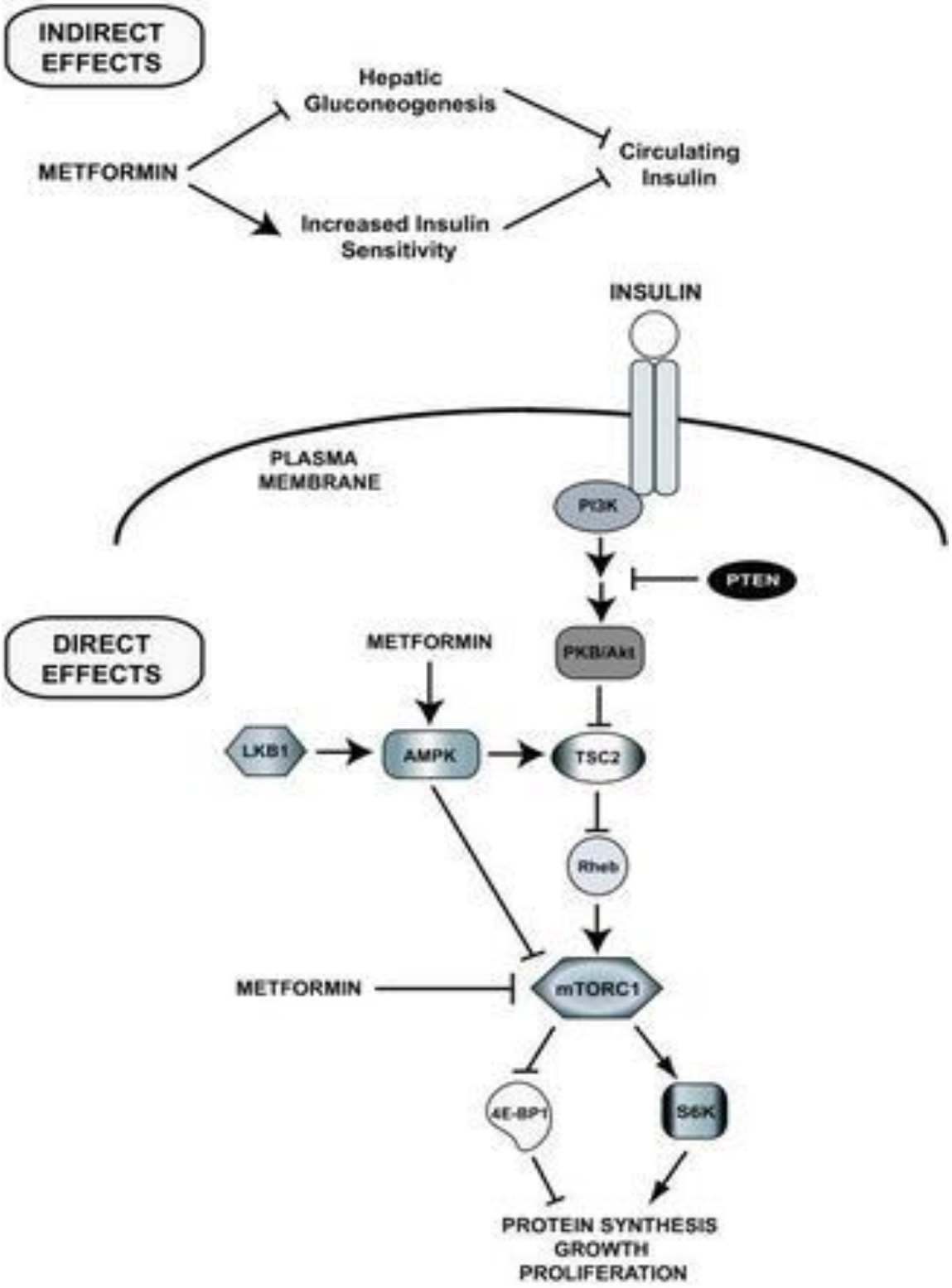


Figure (1.9): Mechanism of Action of Metformin Direct and Indirect Acting [9]

1.2.14 Metformin and Inhibition of Generation of Reactive Oxygen Species

In several forms of cancers ROS signaling pathways are significantly increased resulting in abnormal proliferation and differentiation peroxides, superoxides, hydroxyl radicals, singlet oxygen and alpha oxygen are examples of ROS [150].

The role of hydrogen peroxide, a typical example of ROS is implicated in reversible oxidation of tyrosine phosphatases, tyrosine kinases and transcription factors [151]. Excessive ROS-generated effect on DNA causing interpretation and transmission of genetic information which leads to changes in genetic material and is a step involved in carcinogenic mutagenesis and tumor transformation [23].

Metformin inhibits ROS production by acting on complex 1 of the respiratory chain which reduces electron entry into the chain [152].

Oxidative stress plays an important role in the pathogenesis of T2DM. ROS also plays important role in cancers, metformin has been shown to decrease oxidative stress and thus can prevent cancers [149].

1.2.15 Metformin and Inhibition of Inflammation.

Metformin also reduces inflammation, which is a crucial factor in carcinogenesis' initiation and development [153].

There is an association between diabetic mellitus and cancer development related to a pathogenic state of chronic inflammation, the state of poor metabolic control causes a permanent pro-inflammatory condition that promotes inflammatory cytokines with other proteins involved in cancer development and progression [24]. Metformin inhibits the inflammatory response's initial activation, which is linked to cellular transformation and cancer stem cell development [154].

1.2.16 Review of Clinical Trials for Metformin on Cancer.

The suppression of tumor proliferation and growth is one of the benefits of metformin which has been shown in many animal models to have a significant potential for advancing treatment response in cancer cells, metformin for example that shown to inhibit the growth of cancer cells in the lungs, prostate and colon [154]. Metformin is a low-cost diabetes medication that does not cause hypoglycemia or weight gain [155]. Metformin treatment decreases the mortality rate in people with T2D congestive heart failure and moderate to severe chronic renal disease [156]. Metformin can be safely used with other diabetes medications [157].

Researchers investigated the possibility of metformin having a cytotoxic effect in non-diabetic patients with cancers such as lung cancer, breast cancer and prostate cancer however, the results are controversial [158]. Clinical trials testing metformin as an adjuvant treatment are still being conducted [159].

Metformin has been shown to activate the AMPK pathway, enhance cellular apoptosis, and inhibit the mTOR/AKT pathway [160].

Metformin's potential benefits for cancer prevention and treatment have been thoroughly researched [4]. Diabetes may be linked with an increased risk of cancer in specific sites [161].

Metformin use was linked to improved clinical outcomes in cervical cancer patients with T2D according to Hanprasertpong [162].

However, not all of the investigations into the prognostic importance of metformin in cervical cancer patients currently underway observed no connection between metformin use and the survival of cervical cancer survivors [163].

Metformin use has been linked to a better prognosis or lower death in cancer patients in several studies. metformin may be related with a considerable reduction in mortality in older women with diabetes and cervical cancer [164].

Another study of Metformin in combination with radiation has been demonstrated to boost intrinsic sensitivity of cells in several investigations [165].

Metformin that used with radiotherapy or chemotherapy enhanced the sensitivity of cancer cells to that treatment demonstrate the growth of tumor with volume and weight decrease [4]. The combination of radiotherapy and metformin lowered the risk of death by 48 % [6].

Metformin has been suggested as a preventive drug against chemo brain in several studies, metformin appears to prevent chemo brain in patients receiving cyclophosphamide or cisplatin, but not in those receiving doxorubicin, the survival rate of animal models undergoing acute doxorubicin treatment can be increased with metformin; however, the survival rate is reduced with chronic doxorubicin, cyclophosphamide [166].

AMPK inhibits mTOR which have important role in cell survival, it also inhibits NF-κB which plays a major role in elevating the TNF-α by its ability to activate the promoter region for the of TNF-α gene that lead to inflammation, metformin inhibits the NF-κB that decrease inflammation [167].

Metformin has a number of benefits, including the ability to lower the risk of cancer, decrease cancer-related mortality, when combining radiotherapy and chemotherapy to increase the response to treatment in cancer cells, optimize tumor mobility and malignancy decrease and reduce the relapse [4]. Dose-time dependent relationship of metformin use in patients with DM was associated with a decreased risk of colorectal cancer (CRC) in a dose-dependent manner in this nationwide cohort study [168]. Duration of metformin treatment has a profound impact on antitumor effects [169].

1.2.17 According to an Overview of Clinical Trials Studied of Metformin's Impact on Cancers

Numerous clinical studies have been accompanied to improve metformin's therapeutic benefit , the objective of all of these studies was to see if metformin

could be used to treat cancer. National Clinical Trial number (NCT) is defined " Clinical Trials.gov assigns"[170].

The NCT number is in the format "NCTXXXX" ,the study is not registered until an NCT number is assigned according to a web site (https://clinicaltrials.gov/ct2/manage-recs/faq)

A current study reported certain trail related to the possible effect of metformin on certain type of cancer as the following:

- This trial was number NCT01101438, “Metformin Versus Placebo on Recurrence and Survival in Early Stage Breast Cancer” is completed the study about this research in early 2022 that research investigates (3;582) people and examined perhaps metformin in patients with a primary stage, non-metastatic breast cancer is superior to placebo, patients received metformin twice a day for 5 years after diagnosis, patients with prior use of metformin, insulin, or other oral hypoglycemia were excluded, results of these trail reported that metformin improved patients related to ‘Personal Finance Society’ PFS, these trials showed whether metformin in patients with a primary stage, non-metastatic breast cancer is superior to placebo, patients received metformin twice a day for 5 years after diagnosis, patients with prior use of metformin, insulin, or other oral hypoglycemia were excluded, the results of the study showed that metformin improved patients PFS [170].
- Another trial was number NCT01210911 that demonstrated metformin combined with chemotherapy for pancreatic cancer showed that metformin enhanced patients with combined chemotherapy compared to combined chemotherapy alone [171].
- Metformin in Castration-Resistant Prostate Cancer number of trail NCT01215032 (https://clinicaltrials.gov/ct2/show/NCT01215032), the result under processing.

- The other number of trials NCT02028221, planned to determine whether metformin reduces obesity-related breast cancer risk and is due to be completed in mid-2021, a phase III trial two publication for this study the last by [172]. We demonstrate that in women with metabolic imbalance, metformin administration led to beneficial alterations in anthropometric measures of obesity and a borderline decrease in non-dense breast volume, to understand how metformin affects reducing breast cancer risk, additional study is required, the study should assistance determine the potential breast cancer preventive activity of metformin in a growing population at risk for multiple diseases [173].
- The indication of clinical trials related effect of metformin on cancers.

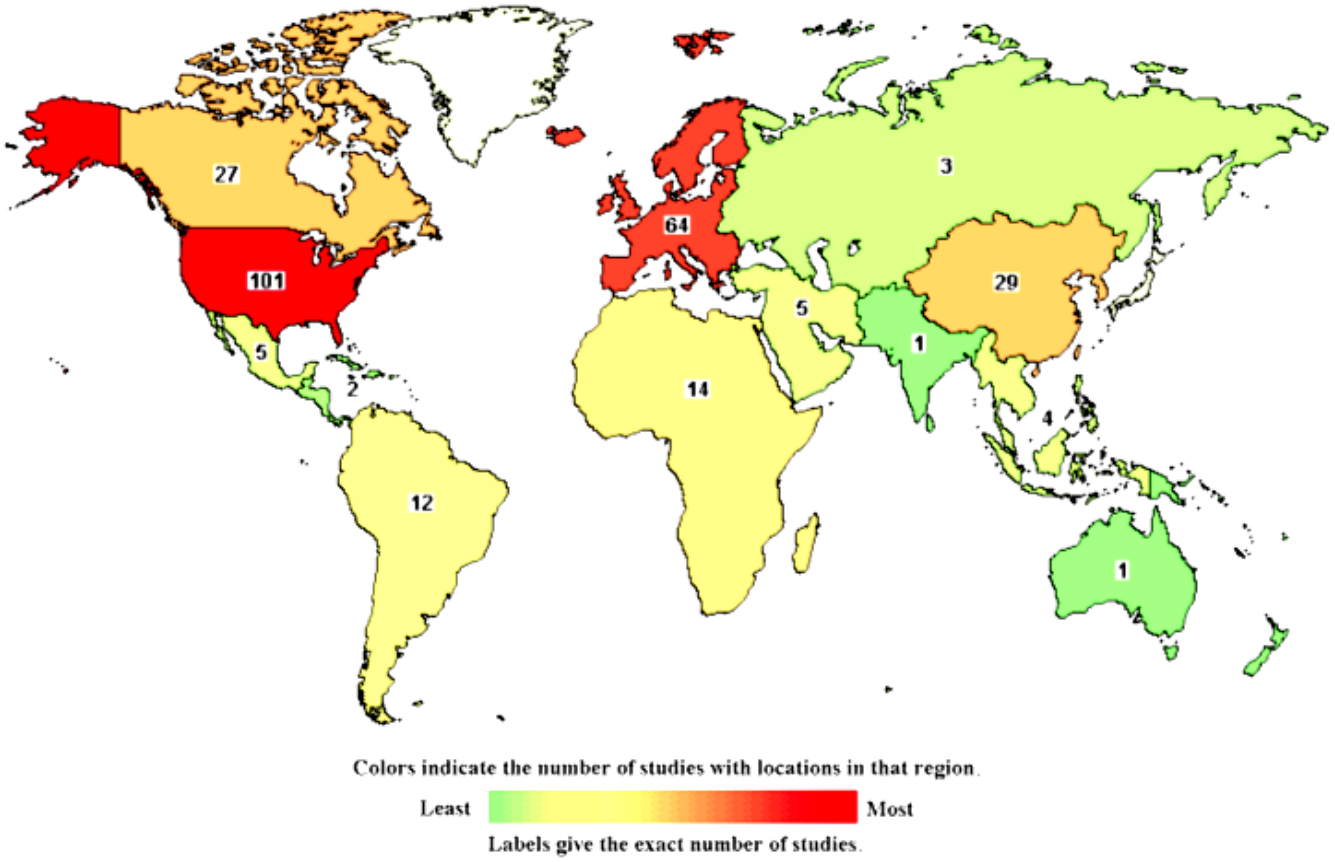
As summarized in table (1.1) on other hand summarized the updated clinical trial of metformin in ovarian cancer is in table (1.2).

Table (1.1): Overview of Clinical Trials Studies of Metformin's Impact on Cancers

Study	Period	Cancer Type	Intervention	Outcome
Phase III In early stage breast cancer ,a randomized trial of metformin vs. placebo	2010 until 2022to	Breast cancer	Drug: metformin Two time aday for 5 years after diagnosis orally Other: placebo orally	Metformin improved patients' PFS
Metformin Combined with Chemotherapy for Pancreatic Cancer (GEM)	2010 until2021	Locally Advanced Pancreatic Cancer Metastatic Pancreatic Cancer	Drug: gemcitabine Drug: erlotinib Drug: metformin Drug: placebo	Metformin improved patients with combined of chemotherapy compared to combined chemotherapy alone
Metformin's Effect in Patients With Metastatic Castration-Resistant Prostate Cancer (Phase II)	**Date start**: June 23,2021 **Completion Date**: April 2024 Completion Date August 31, 2024	Metastatic Prostate Cancer	Experimental Metformin+ADT+abiraterone Patients in will be treated with metformin plus ADT and abiraterone	After extensive research, there is no published results from prospective randomized trials evaluating the effect of metformin in combination with ADT and abiraterone among patients with mCRPC
Metformin vs. Placebo in Early Stage Breast Cancer: A Phase III Randomized Trial.	First, 2016 to 2021	Prostate Cancer	Drug: Metformin Radiation: Salvage Radiotherapy SRT	The main objective of the trial is to explore the efficacy of salvage radiotherapy (SRT) plus metformin compared to SRT in the endpoint of time to progression after prostatectomy failure. Under processing
Phase II Study of Metformin for Reduction of Obesity-Associated Breast Cancer Risk	2014to2021`	Breast Cancer Prevention	Drug: Metformin Drug: Placebo	The study will evaluate whether metformin can result in favorable changes in breast density, select proteins and hormones, products of body metabolism, and body weight and composition. The study should help determine the potential breast cancer preventive activity of metformin in a growing population at risk for multiple disease

Table (1.2): Updates on the Metformin Clinical Trials and Ovarian Cancer.

Disease Condition	Treatment	Summary	Trial Phase Status	Clinical Trial Number
Advanced stage OvCa	Metformin with carboplatin/paclitaxel	Metformin with carboplatin/ paclitaxel mTOR pathway inhibition ,p53-induced apoptosis	Phase1	NCT02312661.
Advanced epithelial OvCa in Stages IIIa–-IV	Metformin, acetylsalicylic acid, olaparib, and letrozole	Women with advanced (stage IIIa-IV) OvCa of the histologic subtype high-grade serous carcinoma are going through diagnostic laparoscopy. They will receive treatment with a study agent for 10–14 days before surgery. The study is randomized	Early Phase 1	NCT03378297
Complex endometrial hyperplasiawith atypi agrade1 endometrial endometrioid adenocarcinom	Levonorgestrel and metformin	Metformin is effective treatment for early-stage endometrial cancer and endometrial hyperplasia with atypia.	Phase 2 up to 2025	NCT01686126
Cancer	Metformin, atorvastatin, doxycycline, and mebendazole	To evaluate a regimen of selected c therapies for people with cancer in a realistic setting and to perform exploratory analysis on the association between the level of response and alterations in biochemical markers (such as glucose and lipid levels).	Phase 3	NCT02201381



Region Name Number of Studies
World 266
Africa 14
Central America 2
East Asia 29
Europe 64
Middle East 5
North America 125
Canada 27
Mexico 5
United States 101
North Asia 3
Pacifica 1
South America 12
South Asia 1
Southeast Asia 4

Aim of the study

The present work was designed to find the following:

1. Study the effect of metformin at different doses on different periods on an albino diabetic rats model and estimation of biochemical markers in serum blood and tissue as following:

- ✓ Rats pro- inflammatory cytokine as Tumor necrosis factor (TNF-α) and interleukin-1β(IL-1β) for rats ELISA kit on serum blood and liver tissue.
- ✓ Total anti-oxidant status(TASO) for rat ELISA kit on serum blood and pancreatic tissue and Malondialdehyde (MDA).
- ✓ Histological Examination for pancreas and liver that involved descriptive in addition to a pathological score of the pancreas and inflammatory score for the liver. Detecting the antioxidant activities of metformin through the DPPH-scavenging assay (In Vitro)

2. The more specific objectives of the present study were to detect the possible cytotoxic effect of metformin at different doses on different periods on a variety of cancer offered human cervical cancer cell lines (HeLa), liver cancer cell lines (HCAM), glioblastoma cancer cell (A172) and a new breast cancer cell line (AMJ13) then compare with normal human cell lines (HBL100) to determine the half maximal inhibitory concentration (IC_{50}) values for each cell line then cytotoxic effect of metformin compare with drug paclitaxel injection on the same cancer cells lines and normal cells.

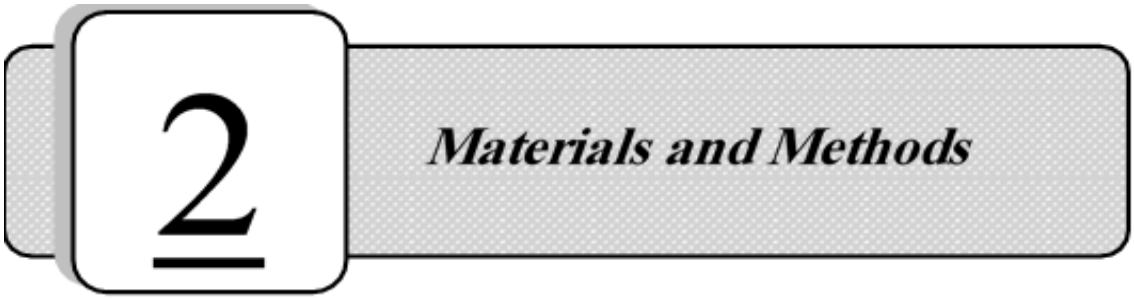
3. Study the morphological changes in cancer cell lines and normal cell.

4. Investigate the apoptosis induction on cancer cell lines and normal cells.

5. Detect the cytotoxicity pathway of metformin by:

- ✓ Evaluate the effect of metformin on the production of reactive oxidative damage by using a ELISA kit designed to measure ROS on cancer cells lines.

- ✓ Assess the effect of metformin on expression of genes by quantitative polymerase chain reaction qPCR on human cervical cancer cell lines (HeLa).



2 Animals, Materials and Methods.

The Council of the College of Medicine at Mosul university research project approval by medical research ethics and the scientific committee of the department of pharmacology at the college of medicine both provided their approval to the study Ref.no: UOM/ COM /MREC/ 20-21.

The current study designed to demonstrate effects of metformin as antioxidant, ant -inflammatory and time-dose dependent in diabetes then major study its possible cytotoxic effect on cancer. According to this purpose, the current study was divided into two parts.

Part one study design experimental/intervention study of the present research was carried out at the animals' house in the College of Veterinary Medicine / University of Mosul with the support and assistance of the Department of pharmacology / College of Medicine / University of Mosul (study related to diabetes measuring pro-inflammatory and oxidative stress on the liver and pancreas).

In part Two laboratory design related to the cancer study the IRAQ Biotech Cell Bank provided cancer cells lines.

"The Iraqi Biotechnology Ltd Co." Founded officially in (2006) study effect by measuring cytotoxicity ratio by MTT assay, IC_{50} for all cancer lines and then study impact of metformin as a cytotoxic and cytomorphological death apoptosis assay, ROS and gene expression.

2.1 Part One Related to Effects of Metformin on Diabetes

2.1.1 Material Used in The First Part.

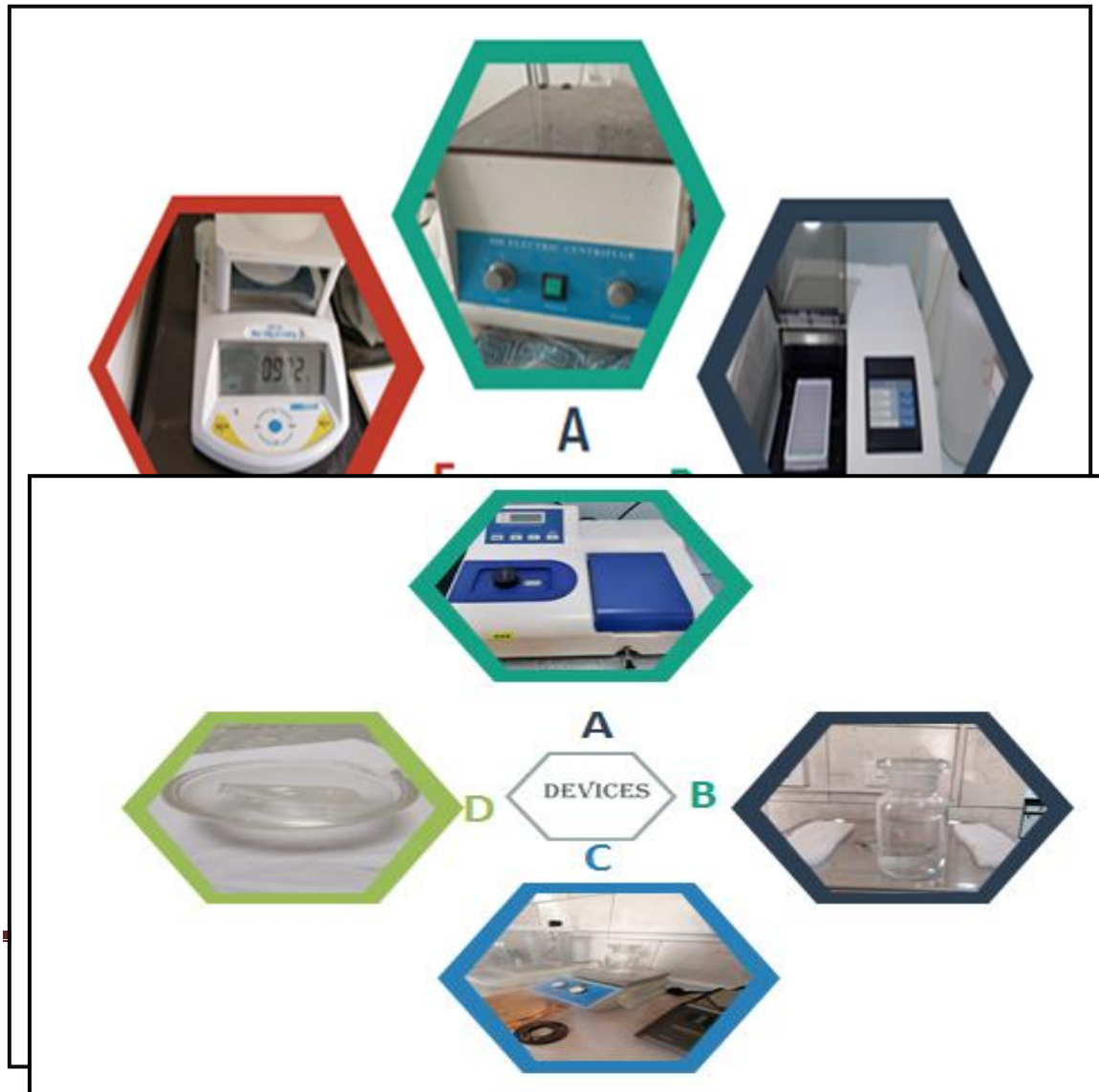
2.1.1.1 Devices and Instruments Used in First Part of Study.

The devices and instruments used in this study to induce diabetes in male albino rats appear in figures (2.1) and figure (2.2). In the current study the general laboratory is used in standard grades. Standardized kits were used for some of the biochemical parameters proposed in this study. Tests were performed by following the instructions for each kit.

Table (2.1): Devices& Instrument Used in First Part of Study.

NO	Devices& instruments	Manufacture
1	Centrifuge	Hermel Labortechnik company, Germany.
2	ELISA Plate Reader	BioTek50TS/USA
3	Blood Glucose Monitoring	toy coo, Hamburg, Germany
4	Micro plate Washer	Biotech/USA
5	pH Meter	knick766 Climatic with Schott N61 pH electrode/ UK
6	Sensitive Balance	ADAM, UK
7	Spectrophotometer Device	Apel Company, Japan
8	Thermostatic Water bath .	Taiwan
9	Incubator	JRAD/Syria
10	Micro plate Washer	USA
11	Vortex Thremo	USA
12	Mantic stirrer with hot plate Cole-Parmer	Germany
13	Mortar and Pestle	China
14	Cylinder	Germany
15	Kidney Dish Stainless Steel.	England.

16	Reusable Oral Gavage Needle	Handmade.
17	Plain Tube	China
18	Gel tube	China.
19	Sterile Surgical Blades No. 15	England
20	Scissors	Germany
21	Artery Forceps	Germany
22	Plain Tube	China
23	Disposal syringe (1cc,3cc,5cc),	China
24	Eppendorff 1.5 ml,	China
25	Howarth Periosteal Elevator	Japan
26	Multichannel pipette 0-250 µl,	Japan.
27	Micro Pipette 10-100 µl,	Japan



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Monitoring. **D.** Micro plate Washer. **E.** pH Meter. **F.** Sensitive Balance

4. Dimethyl Sulfoxide (DMSO Chemcruz , Germany)
5. Ascorbic Acid Ampole. (Spain).
6. Deionized water (Ibn -Sina Teaching Hospital).
7. Normal Saline 0.9% (Pioneer, Iraq).
8. Glucose water 5% (Pioneer, Iraq).

Figure (2.3): **A.** Alloxan, **B.** Metformin**, C.** DPPH**, D.** Dimethyl Sulfoxide, **E.** Deionized water**, F.** Ascorbic Acid Ampule.

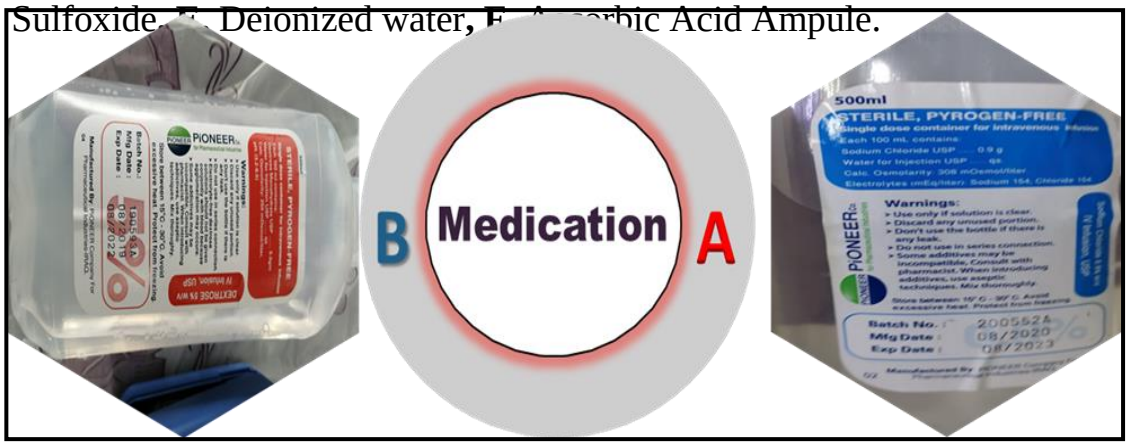


Figure (2.4) A. Normal Saline 0.9%, **B.** Glucose water 5%.

2.1.1.3 Diagnostic Kits Utilized in The First Part of The Current Study

1. Tumor necrosis factor -α TNF-α ELISA kits (Cat No. 0722Ra, assay range 15 mg/L - 300 mg/L, sensitivity 2.8 mg/L, Sun Long Biotech Co., LTD, China) Number two for serum and tissue**.**

2. Interleukin 1beta IL-1*β* ELISA kits (Cat NoSL0402Ra, assay range 1 pg./L to 80 pg. /L, sensitivity 0.1 pg., Sun Long Biotech Co., LTD, China) Number two for serum and tissue**.**

3. Total antioxidant status TAOS ELISA kits (Cat No. SL1402Ra, assay range 0.1U/ml-7U/ml, Sensitivity 0.01U/ml, Sun Long Biotech Co., LTD, China) Number two for serum and tissue.

4. Malondialdehyde MDA ELISA Kit (Cat No E-EL-0060., lot No U35MM81GXX, Sensitivity 18.75 ng/mL, Detection Range 31.25-2000 ng/ml, Elabscience Biotechnology Inc, USA) One kit for serum.

2.1.1.4 Histological Appraisal:

- **Materials for Histological Preparation:**

Buffered Formalin, 40% Scharlau, Germany, prepared the buffered 40% formalin by adding 100 ml of formaldehyde to distilled water 900 ml to sodium phosphate monobasic 4 gm. With sodium phosphate dibasic anhydrous.

1. Sodium Citrate(China).
2. Formic Acid (China).
3. Canada balsam (China).
4. Hematoxilin and Eosin (China).
5. Microscopic Glass Slide (China).
6. Microscopic Glass Cover Slips (China).
7. Xylene.

- **Auxiliary equipment is shown as a figure (2.5)**

1. Autoclave(Germany)
2. Water Bath (Germany).
3. Oven (Memmert, Germany).
4. Microtome (Electron Corporation, Thermo, Germany).
5. Light Microscope (Krüss, Germany).

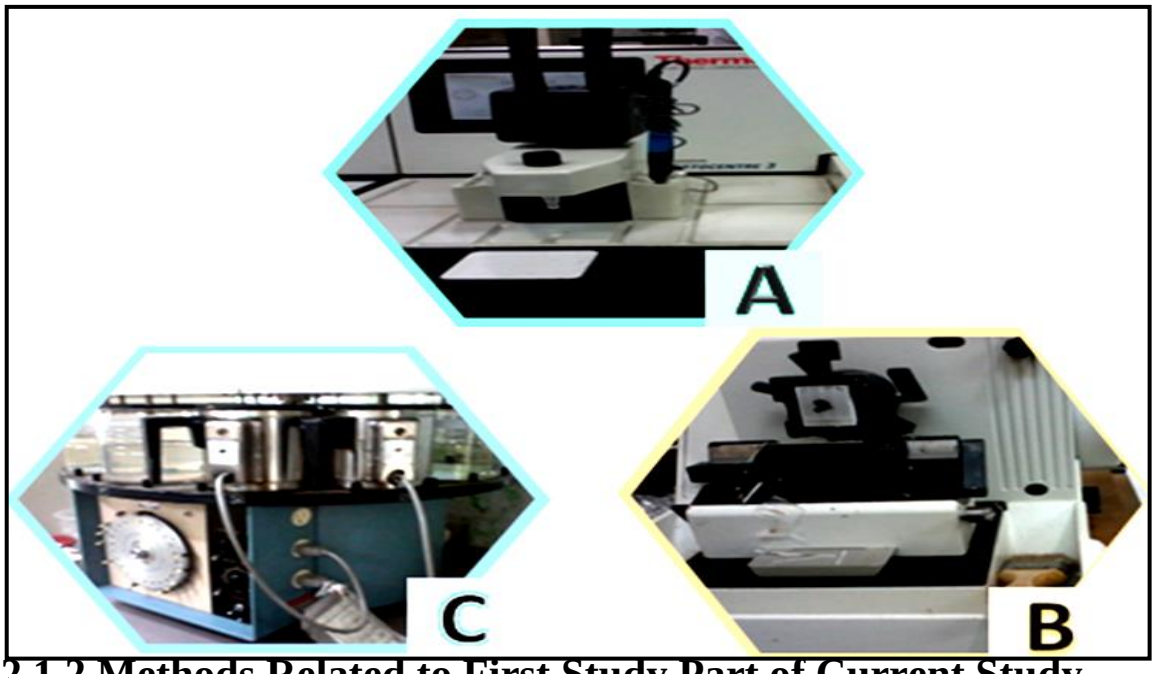


Figure (2.5) (A) Device of Paraffin Embedded **(B)** Microtome **(C)** Device that Cleans Alcohol Solution

The current study was conducted at the University of Mosul College of Veterinary Medicine animal house. Male albino rats were housed in plastic cages with dimensions (30×20×17) cm. The rats were weighing (175-250gm) at (23 ± 2°C) and relative humidity (55%) with a 12 h light/dark cycle was used in the current study as shown as figure (2.6). The number of animals used in study seventy-five each group 15 rats with any lost animals from the trial being replaced selection of sample size supported by [174].

Water and food were freely available with the exception of the fasting period prior to induction, all animal was examined by a veterinarian to assure its

safety, health and absence of disease during a one-week incubation time. Each of these occurrences performed in accordance to medical research ethics rules.

Figure (2.6): Demonstrates the species of albino rats used in the current study.

Diabetes induced in the morning after fasting .The rats were injected with a single dose to induce diabetes. Alloxan intra-peritoneal injection (AVONCHEM.CAS NO.50-71-5, made in UK) at the dose of (200 mg/kg/day) of the body weight overnight fasted rats. Before fixed to this dose there are many trials pilot study that used to reach appropriate dose to Alloxan was dissolved in 1ml of normal saline animal species, route of administration and nutritional status have been considered to play a role in determining the dose of allowance appropriate for induction of diabetes before experiments draws the blood day 0 [175]. as showed as figure (2.7). Rats were allowed to drink 5% glucose solution overnight to prevent drug-induced hypoglycemia that blood glucose multiphasic response to Alloxan injection begins in the first few minutes with a transient hypoglycemic phase that lasts maximally for 30 min [176].The animals blood were kept until diabetes for a week and during this time, fluctuations in blood glucose levels were noticed until blood glucose levels were stable Animals with random blood glucose values ≥200 mg/dl were defined as diabetic rats [177]. The use of Alloxan is best to induce diabetes in these models due to the therapeutic intervention can be determined that causes diabetes via a process that involves

partial degradation of pancreatic islet beta cells, resulting in selectively a reduction in the quality and quantity of insulin through its ability to generate ROS resulting in the selective necrosis of beta cells [175].

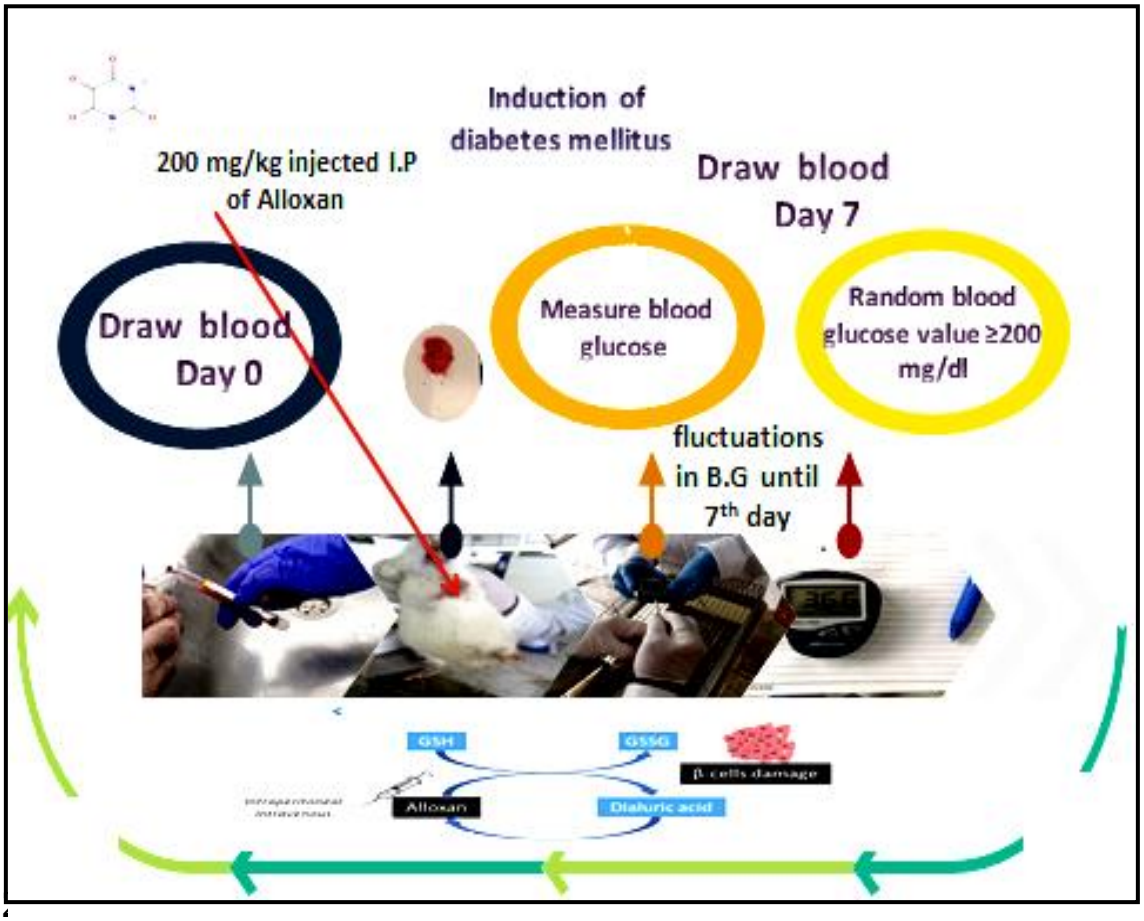


Figure (2.7): Explain process Induction of Diabetes Rats of Current Study.

od

all

groups, current study rats divided into five group:

- Control Group(C): It is the healthy control group that was injected with normal saline which is composed of fifteen healthy rats who were routinely fed were left all alone in cages until the end of the experiment and clear water was provided to them.
- Diabetic Group(D): It's the diabetic group which consists of fifteen healthy rats who were given alloxan injections and kept in cages for 30 days.

- Metformin Group (M 100): A group of diabetic rats was given oral Metformin at a dose of (100 mg/kg/day)
- Metformin Group (M 200): Diabetic animals were administered 200 mg/kg/day using oral Metformin.
- Metformin Group (M 300): A group of diabetic rats who were given oral Metformin at a dose of (300 mg/kg/day).

The dose of metformin administered to rats in this study was calculated based on body surface area. Doses used in the current study 100, 200, and 300 mg/kg/day [178]. Metformin (Samara company) dissolved in deionized water was given by oral gavage at (8:30~ 9:00 a.m. every day of the study) as shown as figure (2.8).

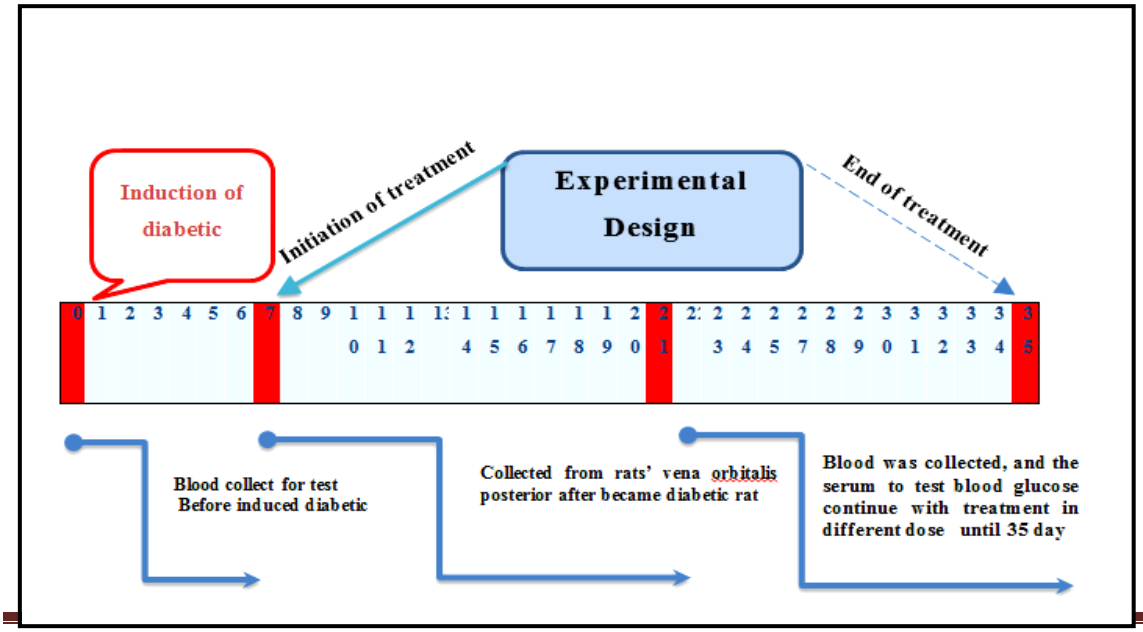


Figure (2.8): Illustrates scheme of work for experimental study

2.1.2.3 Animal Surveillance.

The animals living conditions were constantly monitored in order to offer a comfortable living environment for them on a regular basis. The rats had feed and water sufficiently regular with cleaning and removal of feces together along with feed in their cages in order to provide good hygiene [179].

2.1.2.4 Random Blood glucose determinations

Blood samples were collected from the rat tail vein blood sample was obtained from the tip of the rat tail after injection of Alloxan for the determination of blood glucose according to using a blood glucose monitoring Toycoo, Hamburg, Germany [175] as shown as figure (2.9).

2.1.2.5 Body Weight Determinations:

Changes in body weight for each group were determined in a period and the difference between the initial weight and the final weight was taken. Signs of abnormalities were observed in the experimental animals throughout the study period [180].

Figure (2.9): Measure Blood Glucose by Glucometer Strip.

2.1.2.6 Food and Water Intake Measured:

Rats were housed in each cage, along with the 200 grams of feed that had been weighed and placed in each cage. On the second day, the remaining feed was withdrawn from the tray at the same time, and the quantity was measured. The overall food intake of the rat is equal to the amount of feed added minus the remaining amount. The total food Intake of the rats was divided by the number of rats in each cage then the mean "calculate the average food intake per rat in the cage"

Drinking Bottles were placed in each cage containing 150 ml of tap water. On the next day, the water was collected into a graduated cylinder and the remaining water was read. The amount of water added minus the remaining amount is the total amount of water in the caged rats. The total. The total water in the cage was divided by the number of rats, which represents the average consumption of water per rat in the cage [181].

2.1.2.7 Samples Collection

Blood and tissue collected liver tissue and pancreas tissue were analyzed for the biochemical parameters collected at different period 0, 7, 21and 35 day.

(A) Blood samples: They were collected in clean dry centrifuge tubes from the orbital plexus of vein by serum was separated after centrifugation at 1500 rpm for 15 minutes.

(B)Tissue collection: Rats were euthanized by cervical dislocation at each period. The livers and pancreas were then removed, isolated from the fat immediately and carefully washed by the procedure of rinsing tissues in Phosphate-buffered saline PBS (pH 7.4) is commonly used in biological research. The tissue samples were homogenized with a glass homogenizer then collected the supernatant carefully after centrifuging for 20 min at 2,000-3,000

rpm. The supernatant for the ELISA assay and future use is frozen at -8°C. For about 20 minutes, centrifuge at 2000-3000 RPM [182].

The tissues and serum samples were maintained at -80°C. Another part of tissue preserved in a 10% formalin solution for histological analyses should be kept for a day according to the procedure[183], then washing the tissue for half hour to removal the excess of tissue, then step dehydration. At this point the samples were passed a series of alcohol as the following (50%, 70%, 90%, 100) for two hours for each concentration in order to withdraw water from the samples then receive xylene that was used at this stage for the purpose of clearance in order to withdraw the alcohol and make it more transparent then infiltration. The samples were placed in a mixture of xylene and paraffin wax in a ratio (1: 1) at 60 C0 then placed the samples in two stages of molten wax After impregnating in paraffin wax. Paraffin Embedded Section (PES)of 4-5 microns by Electron Corporation, Thermo, Germany. Then cut with a rotary microtome and mounted on a glass slides containing 5-6 section, each sections were prepared, then stained with standard Haematoxylin and Eosin for blinded histological assessment then final step mounting put drop of canda balam to each slide and cover by slide cover Each slide was examined under light microscope by pathologist [184]**.**

2.1.3 Biochemical Assessment

2.1.3.1 Determination of Rats Pro-Inflammatory Cytokine (TNF-α) and (IL-1β)

2.1.3.1. A Determination Rats Tumor necrosis factor- alpha ELISA kit

Blood and tissue were analyzed as mentioned in sample collection and use plate layout to record standards and sample assayed then analyzed for pro-inflammatory cytokines using ELISA kits TNF-α (Cat No. 0722 Ra, Assay Range 15 mg/L –300 mg/L, sensitivity 2.8 mg/L, Sun Long Biotech Co., LTD, China) used for Research use only as shown as figure (2.11).

- **Purpose of kit:**

Tumor necrosis factorα ELISA kit is to assay TNF-α levels in rat serum, tissue, plasma, culture media or any biological fluid. The current study used serum and liver tissue for this kit.

- **Principle of kit:**

The Sandwich-ELISA method is applied by this ELISA kit. The Micro Elisa Strip plate provided in this kit has been pre-coated with an antibody specific to TNF-α. standards or samples are added to the appropriate wells and combined to the specific antibody The optical density is measured spectrophotometric ally at a wavelength of 450nm [185].

- **Procedure according to kit [186].**

Before starting the used kit, we take all the regents at room temperature and read the procedure carefully. After that, dilution of the standard into small tubes is the first step, then pipette 50 µl from each tube to a microplate well two wells per tube for a total of ten wells. In the Micro Elisa strip plate, leave a well Empty as blank control. In sample wells 40µl buffers and 10µl samples are added (dilution factor is 5) the samples should be Loaded onto the bottom without touching the well then we mix well with gentle shaking after that we incubate at 37℃ 30 min sealed with a closure plate then dilution with distilled water for 30 times. Then washing for 30 sec then repeat the wash for five times after washing should add 50 µl Horseradish peroxidase HRP- Conjugate reagent to each well, except the blank control well then incubated and washed after all adding (50 µl Chromogen Solution A) and (50 µl Chromogen Solution B) to each well shacked gently and incubated at 37℃ for 15 min. In this step of coloring avoid light during the coloring as figure (2.10), then automatically plotting standard curve the average OD for each standard on the vertical Y axis against the concentration on the horizontal X axis. as in figure (2.12).

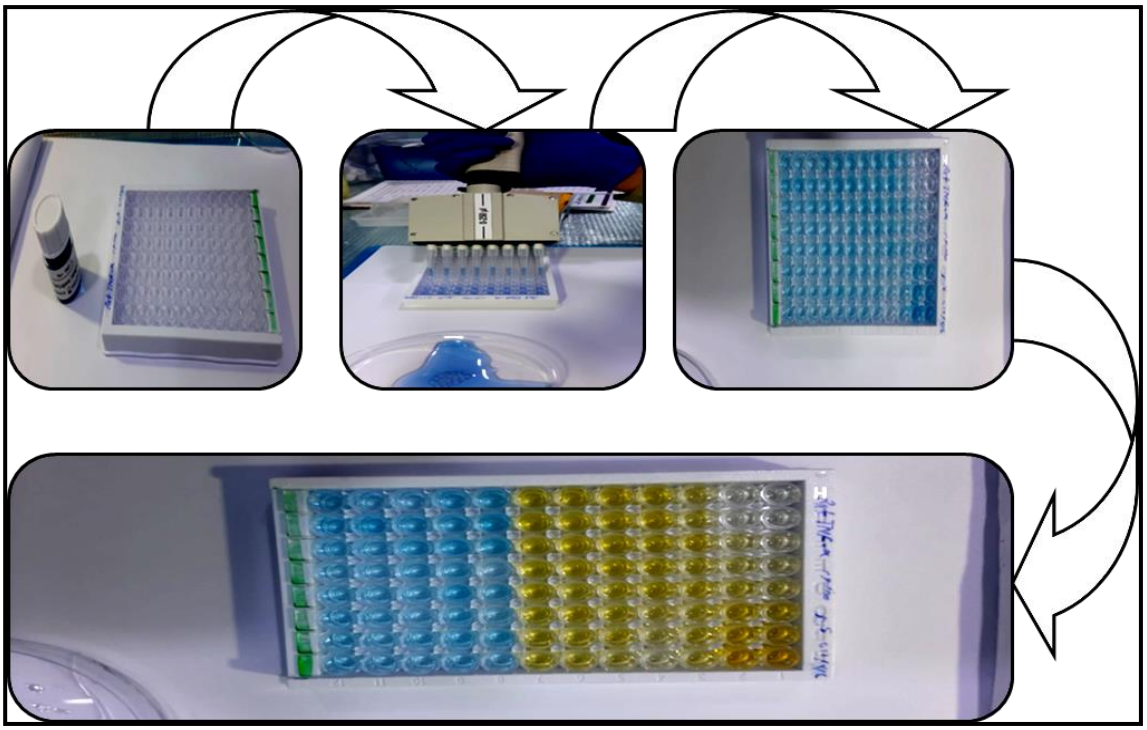


Figure (2.10): Change Color of TNF- α Kit

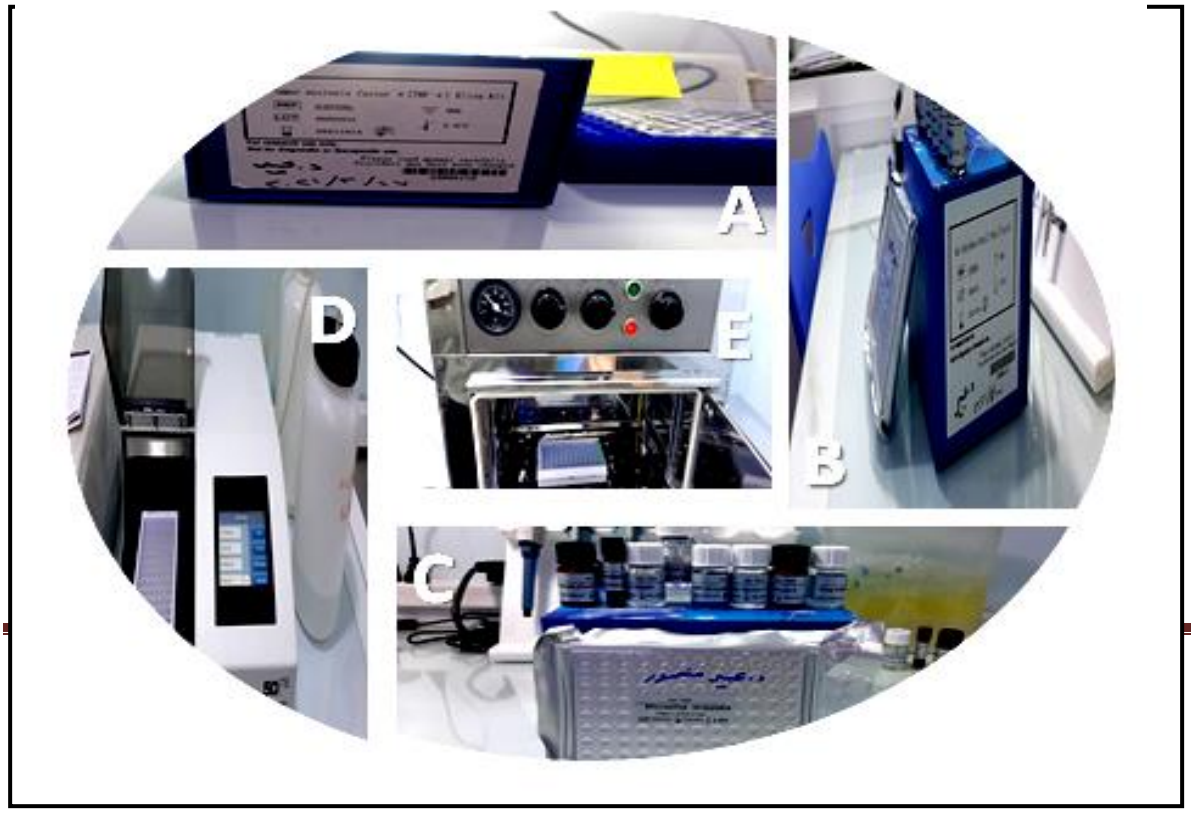


Figure (2.11): Explain the Procedure of TNF- α **A & B**: Rat TNF- α

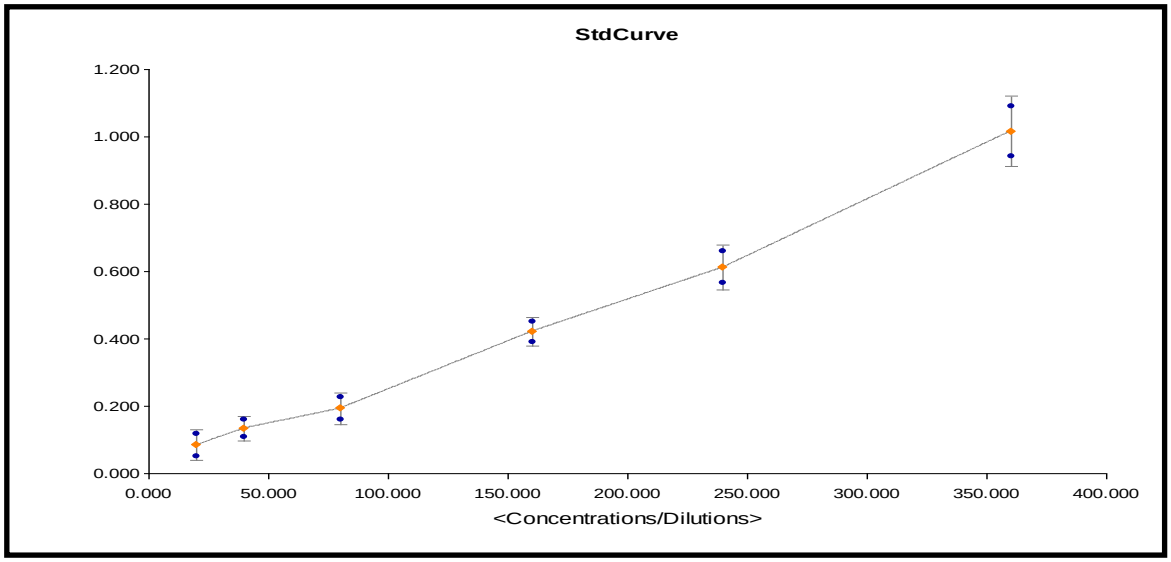


Figure (2.12): Standard Curve of TNF- α Kit by ELISA Device.

2.1.3.1. B. Determination Rats Interleukin- 1beta IL-1*β* ELISA Kit:

The Interleukin- 1beta ELISA kits that were used in the current study (Cat NoSL0402Ra, assay range 1 pg./L-80 pg./L, sensitivity 0.1 pg. Sun Long Biotech Co., LTD, China) as shown in figure (2.13).

- **Purpose:**

Rat Interleukin -1beta Kit is used to determine the levels of IL-1*β* in rat serum, tissue, culture media, or any other biological fluid sample. The same preparation as before was used in this investigation for serum and liver tissue.IL-1*β* are expressed in tissue macrophages in the lung, digestive tract, and liver [145].

- **Principle:**

This ELISA kit uses Sandwich-ELISA as the method. The Microelisa strip plate pre-coated with an antibody specific to IL-1β Standards or samples are added that contain antigen .Then HRP-conjugated antibody specific for IL-1beta is incubated. Free components are washed away after that coloring step .Only those wells that contain IL-1beta and HRP conjugated IL-1beta antibody will appear blue in color and then turn yellow after the addition of the stop solution. [187].

- **Procedure:**

First, we used the manufacturer's procedures. The first step was to dilute the standard in small tubes first, then pipette the volume of 50 from each tube to the microplate well. After that, we added 50µl HRP-Conjugate reagent to each well except the blank control well. and incubated at 37°C for 15 minutes then added 50 µl Chromogen solution A and 50 µl Chromogen solution coloring agent to each well. Result showed the change in color then we add 50 µl stop solution to each well to terminate the reaction. The color in the well should change from blue to yellows in figure (2.14) and read absorbance at 450 nm automatically with the curve shown in figure (2.15).

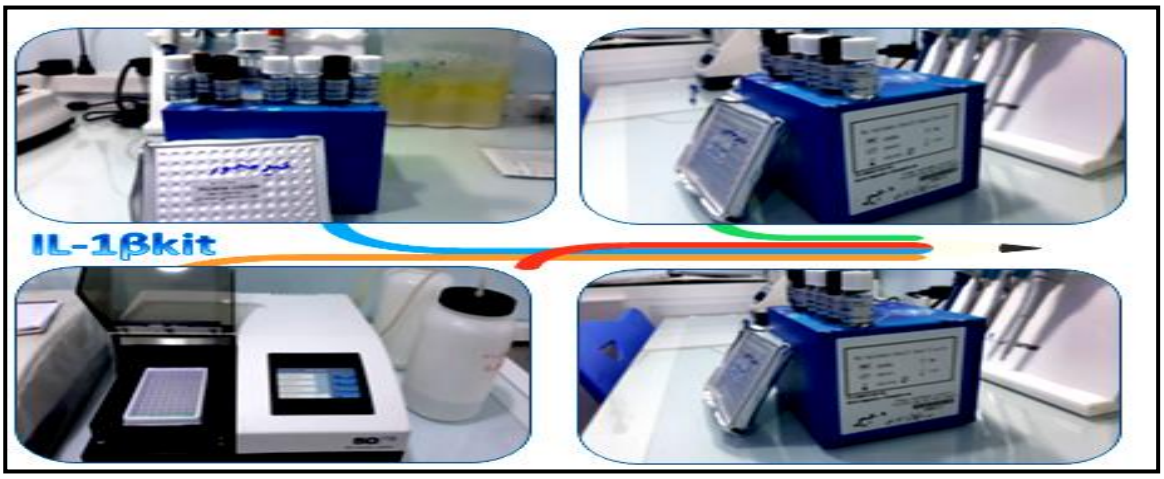
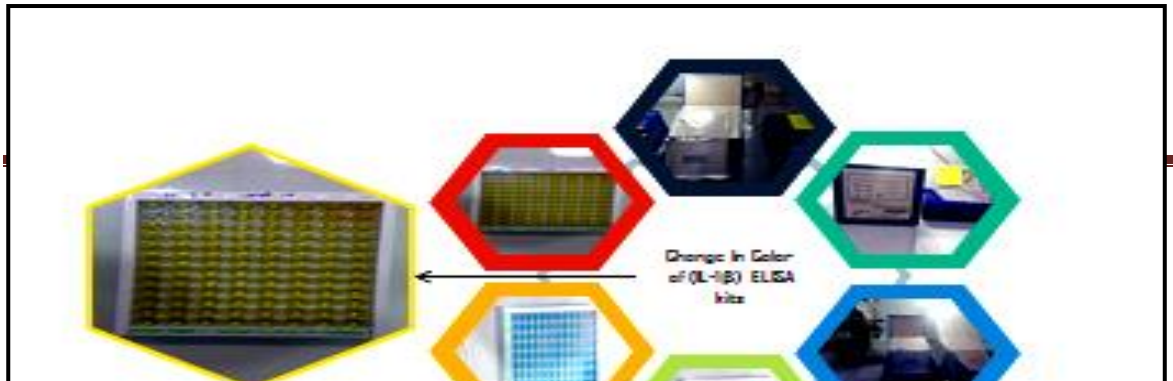


Figure (2.13): Pro-inflammatory cytokines IL-1β ELISA kits



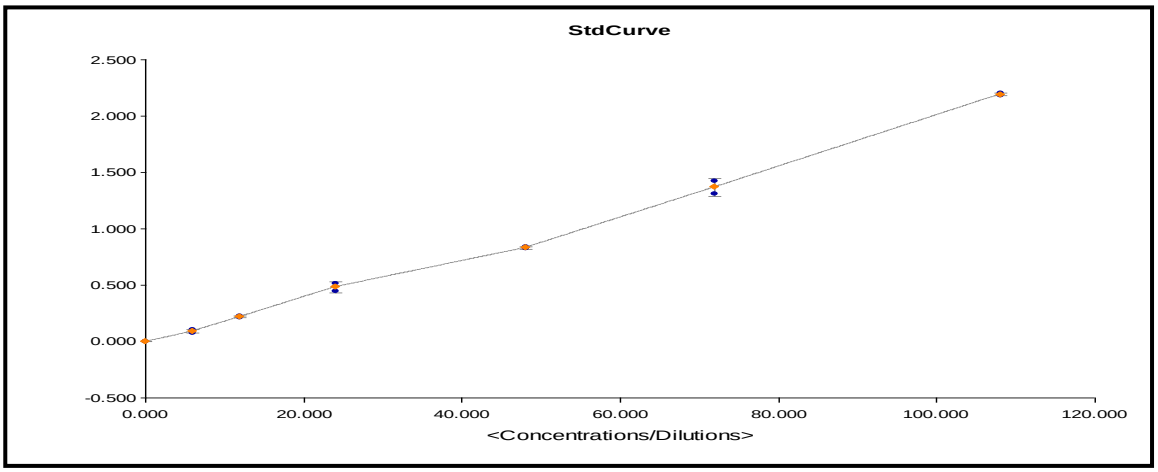


Figure (2.15): Standard Curve of IL-1β Kit by ELISA Device.

2.1.3.2 Determination of Oxidative Stress.

2.1.3.2.A Determination Rats Total Antioxidant Status ELISA Kit.

Analyzed serum and pancreatic tissue TAOS levels by using ELISA kits (Cato.SL1402Ra, assay range 0.1U/ml-7U/ml, sensitivity 0.01U/ml, Sun Long Biotech Co., LTD) according to the manufacturer's protocols. This sandwich kit is for the accurate quantitative detection of rat TAOS in serum, plasma, and tissue homogenates figure (2.16).

- **Purpose**

Our Rat Total antioxidant status ELISA Kit is to assay TAOS levels in Rat serum, plasma, tissue, culture media, or any biological fluid. Current study use serum and pancreas tissue.

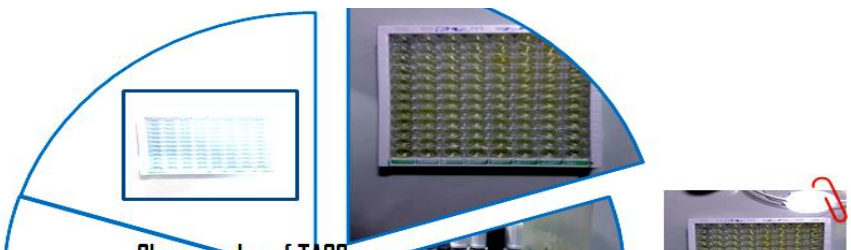
- **Principle**

This ELISA kit uses Sandwich-ELISA as the method. An antibody specific to TAOS standards or samples is added to the appropriate Micro ELISA strip plate wells and combined with the specific antigen specific for TAOS in serum or tissue then added conjugate substance HRP-conjugated antibody specific for TAOS we added chromogen responsible for coloring [188].

➢ **Procedure**

Dilution, incubation and coloring and finally termination by stopping solution are the key steps in the preparation of the TAOS kit by ELISA device automatically. Dilute the standard by small tubes first, then pipette the volume of 50ul from each tube to microplate well, each tube use two wells, total ten wells. . In the Micro Elisa strip plate, leave a well empty as blank control. In sample wells, 40µl sample dilution buffer and 10µl sample are added (dilution factor is 5). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking then incubated, dilution and added HRP-Conjugate reagent to each well except the blank control well, subsequently adding the coloring agent ,the color in the well change from blue to yellow final color at 450 nm as in figure (2.17), then automatically plotting the curve as shown in figure (2.18).

Figure (2.16): Rat Total Antioxidant Status ELISA Kit.



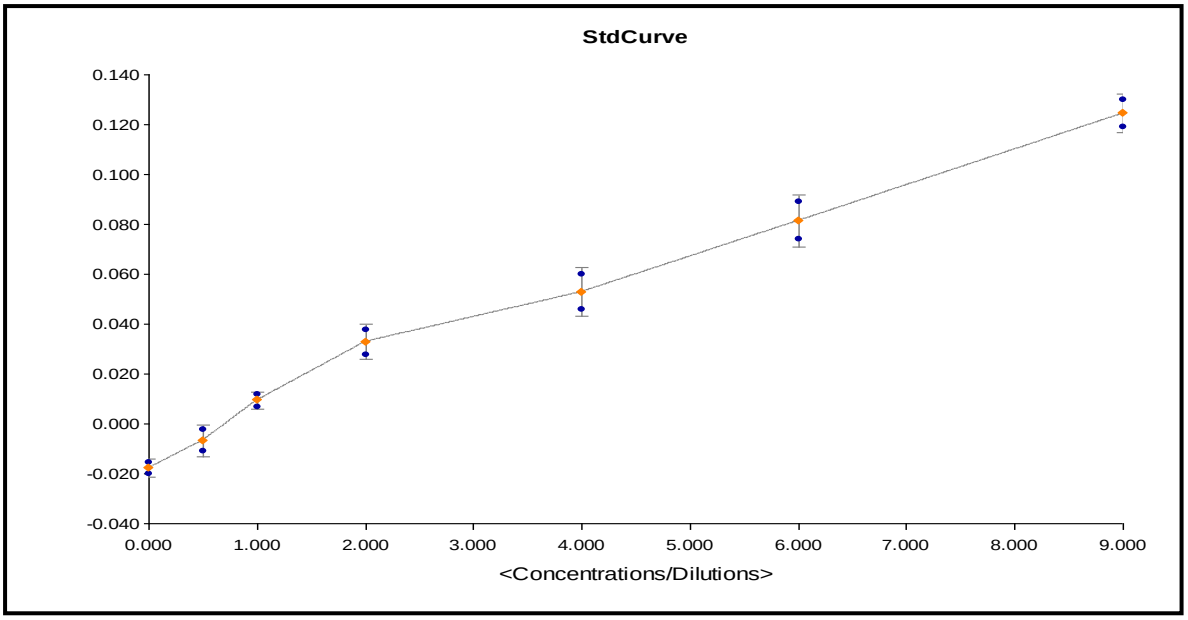


Figure (2.18): Standard Curve of TAO) Kit by ELISA Device.

2.1.3.2.B Determination Malondialdehyde (MDA) ELISA Kit.

This ELISA kit is used to determine the levels of MDA in serum, plasma and other biological fluids in vitro , in current study used serum of rats. Character of MDA ELISA kit such as sensitivity 18.75 ng/ml. Detection Range 31.25-2000 ng/ml, this kit recognizes MDA in samples, Catalog No: (E-EL-0060) Elabscience ,USA.

- **Principle :** The Competitive-ELISA model is utilized by this ELISA kit [189].
- **Assay procedure**: the assay produces as the following [179]:
    1. Immediately we add 50 µL of Biotinylated Detection Ab working solution to each well.
    2. Decant the solution from each well, add 350 µL of wash buffer to each well.
    3. Add 100 µL of HRP Conjugate working solution to each well.
    4. Decant the solution from each well, repeat the wash process for 5 times as conducted in step 2.
    5. Add 90 µL of Substrate Reagent to each well.
    6. Add 50 µL of Stop Solution to each well as shown as (2.19)

Then automatically computer Construct a standard curve by plot-ting the average OD for each standard on the vertical Y axis against the concentration on the horizontal X axis. These calculations best fit line can be determined standerted curve as figure (2.20).

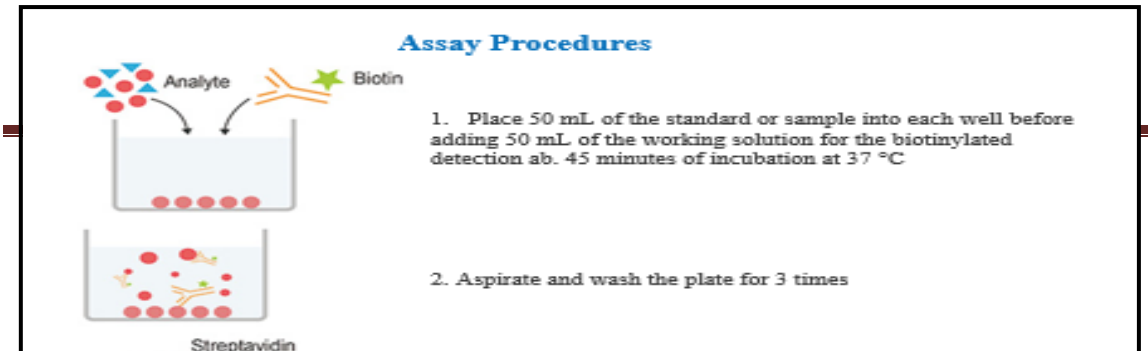


Figure (2.19): Assay procedure for MDA kit by ELISA device

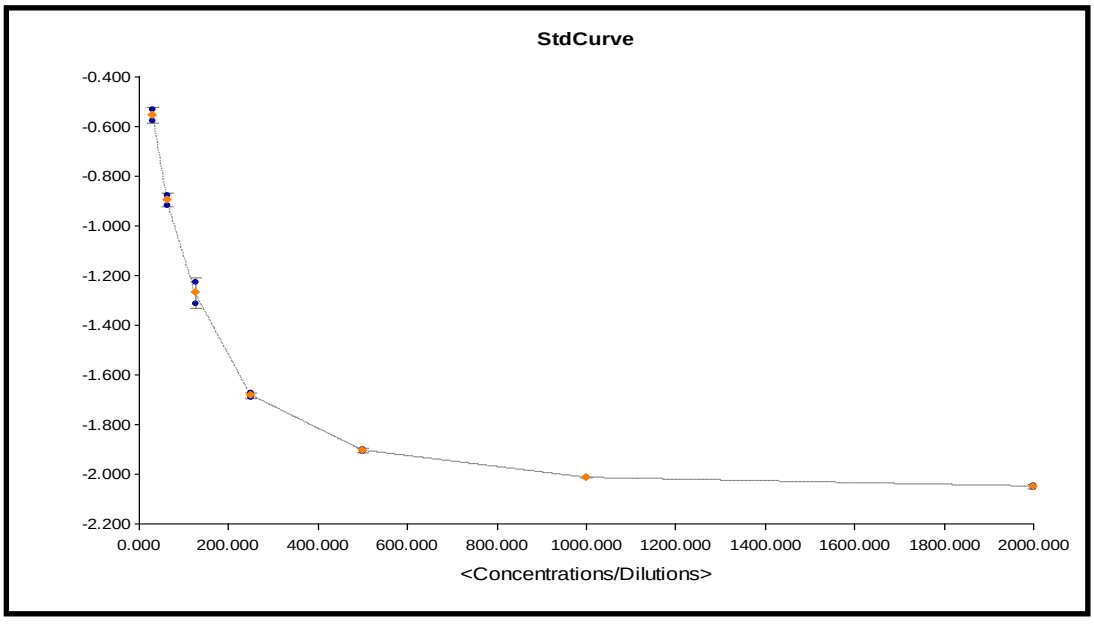


Figure (2.20): Standard Curve of MDA Kit by ELISA Device.

2.1.3.3 Antioxidant Activity Measurement.

Solutions Used in Antioxidant Activity Test:

The following solutions were prepared according to [190].

Methanol-DMSO Mixture 9:1 v/v Solution:

Methanol-DMSO mixture 9:1 v/v solution was prepared by adding 9 volumes of methanol to 1 volume of DMSO**.**

DPPH Radical Solution:

DPPH radical was dissolved in DMSO-Methanol mixture to prepare (0.1mg/ml) DPPH radical stock solution.

Vitamin C Solution

Ascorbic acid powder was dissolved in DMSO-Methanol mixture to prepare a concentration of 0.1mg/ml vitamin C stock solution.

Preparation

DPPH radical scavenging assay according to the procedure described by [190]: as follow:

1. Aliquots (0.5ml) of two-fold serial dilution from metformin and ascorbic acid [250, 500, 1000µg/ml] were added in reaction test tube
2. Simultaneously, 3ml of methanol-DMSO mixture and 0.3ml of DPPH solution were added to each concentration
3. Samples were shaken few seconds and allowed to stand at room temperature for 60 minutes.
4. Radical scavenging activity of samples against the stable DPPH radical was determined spectrophotometrically.
5. The colorimetric changes from deep violet to light yellow when DPPH is reduced were measured at 517 nm.

The % inhibition of radical by the samples was calculated according to the following formula was computed :

% DPPH radical scavenging activity = [(A0-A1/A0)]*100

Where A0 is the absorbance of the negative control consisting of the methanol-DMSO mixture and DPPH solution and A1 is the absorbance of the material examined while ascorbic acid was used as a reference. Then % of

inhibition was plotted against concentration and from the graph, IC_{50} was determined from the graph. The experiment was repeated three times at each concentration.

2.1.4 Histological Examination

Microscopic examination the pathological change score was evaluated by the pathologist who was blinded to the groups evaluation of the animals table (2.2) determined score pathology pancreatic tissue groupings and arranges them in an "ordered" progression of lesion severity [191].

Table (2.2): Demonstrate the pathological score of pancreas tissue according to [192]**.**

Score 0–1	Normal rats' pancreas histology showed the normal manifestation of islets of Langerhans and there were no pathological changes**.**
Scores 2 -3	Normal rats pancreas histology showed the normal manifestation of islets of Langerhans and there were no pathological changes.
Score 4–5	Pathological changes present necrotic changes in slide and small vacuoles and swollen

Inflammation Findings For liver assets the inflammatory score histological slides were prepared from each specimen under the light microscope were 0-1 referred to no sign of pathological (a sign of pathological infiltration changes observed) 2 referred to mild (less than 25% of the sign of pathological infiltration changes), 3 referred to moderate to severe evidence (between 25 and 75% showed sign of pathological infiltration changes) and 4 corresponded to apparent evidence (more than 75% of the showed sign of pathological infiltration changes) [193].

2.2 Part Two Related to The Effect of Metformin on Cancer Cells Lines.

2.2.1 Materials

2.2.1.1 Apparatus and Equipment

List of apparatus, equipment, glasses ware with origin used in second part of current study as shown as table 2.3 & figure (2.21& 2.22).

Table (2.3): Demonstrated list of instruments used in second part of study.

NO	Instruments	Manufacture
1	Autoclave	APln Japan (temp& time system).
2	Beaker 100,50,25 ml	Santa Cruze / USA
3	Biological hood class2	UK
4	Cell Culture plate flat bottom (96) well	Nunc/ Denmark
5	Cell Culture Flask	Denmark.

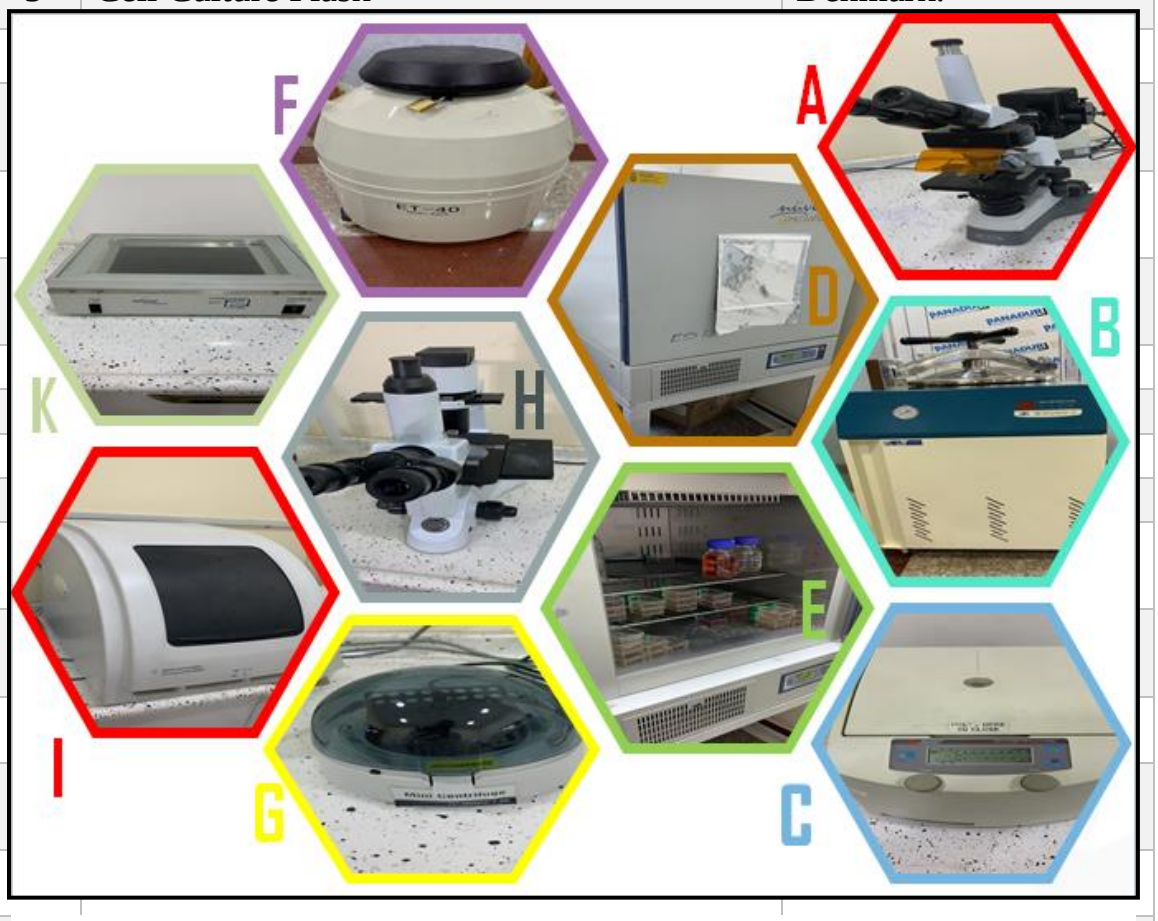


Figure (2.21): Equipment used in current study **A.** Light microscope**, B.** Autoclave **C.** Centrifuge Microfuge, **D.** Incubator**, E.** Incubator with cancer cell line, **F**. liquid nitrogen to preserve cells **H.** Inverted microscope. **G**. Mini centrifuge UV, **I**. qPCR

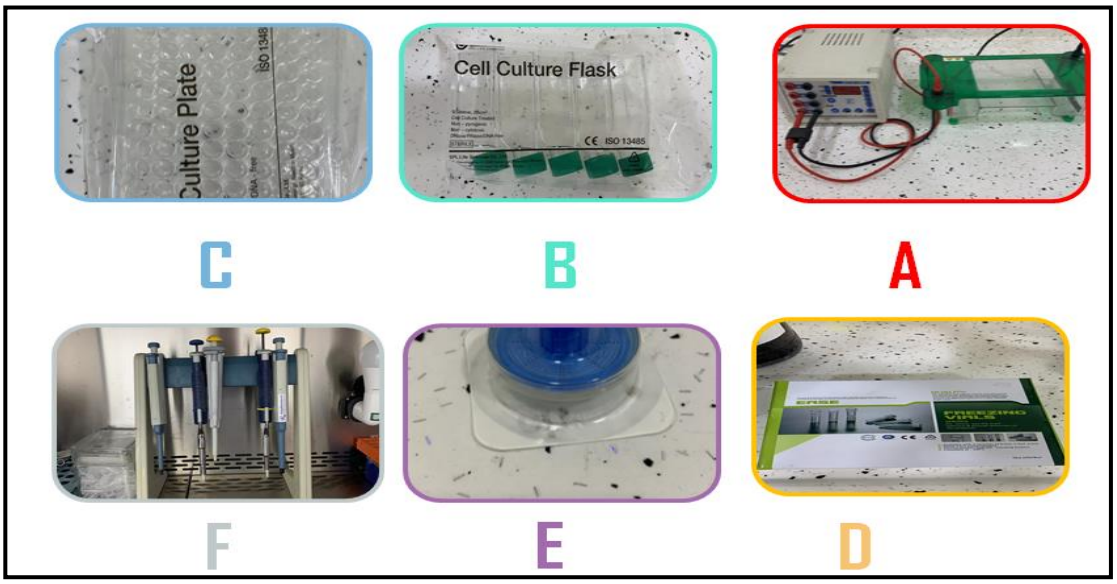


Figure (2.22): A. Gel Electrophoresis **B.** cell culture flask, **C.** cell culture Plate, **D.** Freezing Vail, **E.** Syringe Filters, **F.** Micropipette

2. Trypsin 1:250 / EDTA/ UK Cat.No.27250-018, lot No. A79353.
3. RPMI 1640 Gibco / USA Lot Analysis, L18060701, C18060701.
4. Fetal bovine serum, Cat. No. ECSO0180L, lot No. EUS036147, USA.
5. Trypan blue /Lab use Nanjing duly biotech Co. Ltd.is
6. Thiazolyl Blue/Chem Cruz USA, MTT.
7. Dimethyl Sulfoxide DMSO Chemcruz, Germany.
8. Ethidium Bromide,USA.

2.2.1.3 Medications and diagnostic kits

1. Paclitaxel injection I.P Taxeleon ® 100mg /16.67ml Neon brand. India.
2. Streptomycin, Benzyl Penicillin Ajenta Pharm, India.
3. Human ROMO1, Reactive oxygen species modulator 1. ELISA Kit Catalog No: E-EL-H5430 96T Detection Range: 0.16~10 ng/Ml, Sensitivity: 0.10 ng/mL Elabscience®, USA.
4. GENEzol™ TriRNA Pure Kit Geneaid / Taiwan.
5. AccuPower® RocketScriptTM RT PreMix “Bioneer” USA.

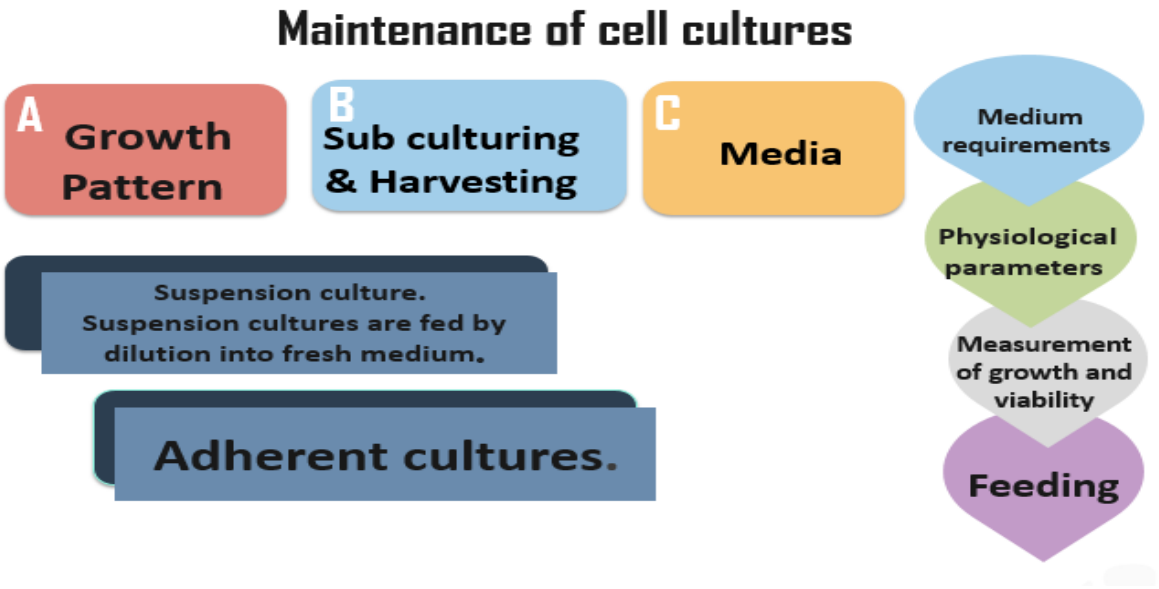
Figure (2.23): A. RPMI, **B.** Trypsin, **C**. Fetal bovine serum, **D & E.** Thiazolyl Blue, **F.** Trypan blue

2.2.3 Method.

2.2.3.1 Maintenance of cell cultures

Maintenance of cell cultures: Lines of normal and cancerous cells the IRAQ Biotech Cell Bank provided human cervical cancer cell lines (HeLa), liver cancer cell lines(HCAM), normal human HBL100 cell lines (HBL100),

glioblastoma cancer cell(A172) malignant brain tumor, and a new breast cancer cell line (AMJ13). All cell kept in RPMI-1640 supplemented with 10% Fetal bovine, 100 unit's/mL penicillin, and 100 g/mL streptomycin. Trypsin-EDTA was used to passage the cells. reseeded at 50% confluence twice a week and incubated at 37°C and 5% Co_2 [194] as shown as figure (2.24)



2.2.3.2 Cytotoxicity Assays

To test the cytotoxic effect, the MTT cell viability assay was performed on 96-well plates. Cell lines were plated at a density of 1* 104 cells per well. After 24 hours and when a confluent monolayer was produced, cells were treated with the tested compound at varying concentrations with Metformin (0,5,10,25,35,45,65,130 μM).

After 48 and 72 hours, cell viability was measure by removing the medium, adding 28 μL of 2 mg/mL solution of MTT and incubating the cells for 2 h at 37 C^0. Following removal of the MTT solution, the crystals in the wells were solubilized and incubating at 37 C0 for 15 minutes with shaking [192]. The absorbency was measured using a micro plate reader at 620 nm test wavelength and the assay was done three times. .

$$\textbf{Cell viability\%} = \textbf{Mean OD/Control OD} * \textbf{100\%}.$$

The half maximal inhibitory concentration (IC_{50}) is a values were calculated from survival-concentration curves using non-linear regression the IC50 was calculated using Graph Pad Prism for metformin after72 hours for all cancer and normal cell line [195].then compare the concentration that more effect and more cytotoxic cancer cells compare with Paclitaxel injection anticancer drug on the same cancer cell line and same concertation & time then focusing about how metformin act to these cell by study other marker such as apoptosis , ROS, Quantitative PCR.as shown as figure (2.25).

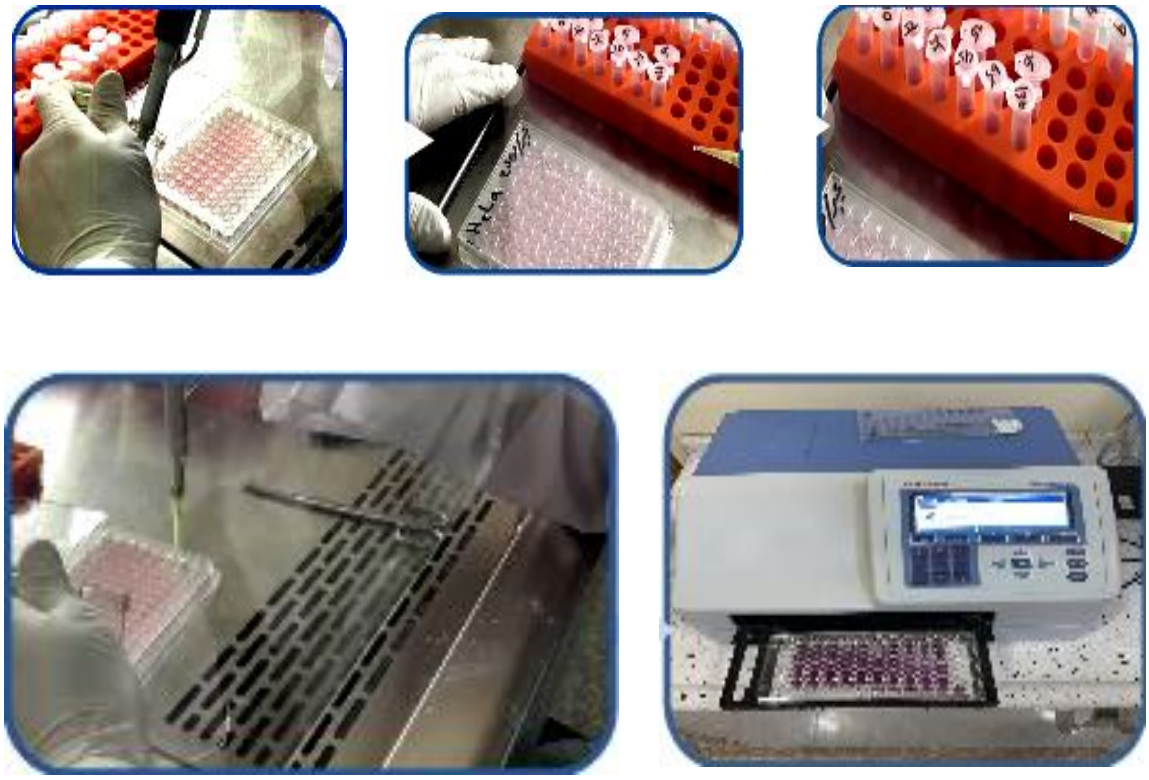


Figure (2.25): Clarify the Procedure of Cytotoxicity Assays

2.2.3.3 Morphological Analysis

To visualize the shape of the cells under an inverted microscope, 100 µl of cell suspension for cell lines were seeded in 96- well microplate at a density of 1x104 cells, and incubated at 37C^0.

After confluence of cells to become monolayer, the media was aspirated and then the cells were exposed to metformin in accordance with separately at 37 C^0 for 72 hrs.

The media was discarded, the plates were stained with 50 µl crystal violet and incubated at 37 C^0 for15 min and the stain was washed off gently with tap water until the dye was removed. The cell was observed under an inverted microscope at 10X, 40X magnification and photographed with a digital camera compare with normal cell [196].

2.3.2.4 Assessment of Apoptosis

In order to prepare the working solution, add Acridine Orange AO staining and Ethidium Bromide and mix well. Store the working solution at room temperature for up to 2 weeks for use after the cells are diluted with an equal volume of AO-EB working solution. Using a fluorescence microscope, you can immediately see cells [147].

 Red Cells = Dead  Green Cells = Live

2.3.2.5 Reactive Oxygen Species Assay

ROS assay was performed to measure the production of oxidative damage using an ELISA kit designed to measure ROS in living cells. The current study was to evaluate the effect of metformin on the production of ROS in the Hela cancer cell line [197].

Intended use for research use only. Not for use in diagnostic procedures. This ELISA kit principle applies to the in vitro quantitative determination of Human ROMO1 concentrations in serum, plasma, and other biological fluids. The character of the kit is :

- ✓ Sensitivity: 0.10 ng/mL
- ✓ Detection Range: 0.16-10 ng/mL
- ✓ Specificity: This kit recognizes Human ROMO1 in samples. No Significant cross-reactivity or interference between Human ROMO1 and analogs was observed.
- ✓ Repeatability: Coefficient of variation is < 10%.

Test principle

Sandwich-ELISA is the principle used in this ELISA kit. The antibody specific to Human ROMO1 has been pre-coated on the micro ELISA plate included in this kit [198].

Assay procedure

1. A standard working solution is added to the first two columns in duplicate, with each concentration of the solution added to one well, side by side. The samples are added to the other wells and then cover on the plate with the cover supplied in the kit. Incubated after that for "90 min at 37 ".
2. Eliminating the liquid out of the well should not wash it. Immediately added 100 µL of Biotinylated Detection Ab working solution to each well. then mixed up gently after that Incubated for 1 hour at 37°C. Aspirate the solution from well, added 350 uL of wash buffer to each well. Then soaked for 1-2 min and aspirated the solution from well. Repeated wash step 3times. Note: a microplate washer can be utilized in step and other wash steps.

- ✓ Added 100 µL of HRP Conjugate working solution to well then incubated for 30 min at37C^0.
- ✓ Decant the solution from well, repeated the washing for 5 times as in step 3.
- ✓ Added 90 µL of Substrate Reagent to well, then incubated for about 15 min at 37C^0
- ✓ Added 50 µL of Stop Solution to well.
- ✓ Estimated the optical density "OD value" of well at 450 nm as shown as figure (2.26)

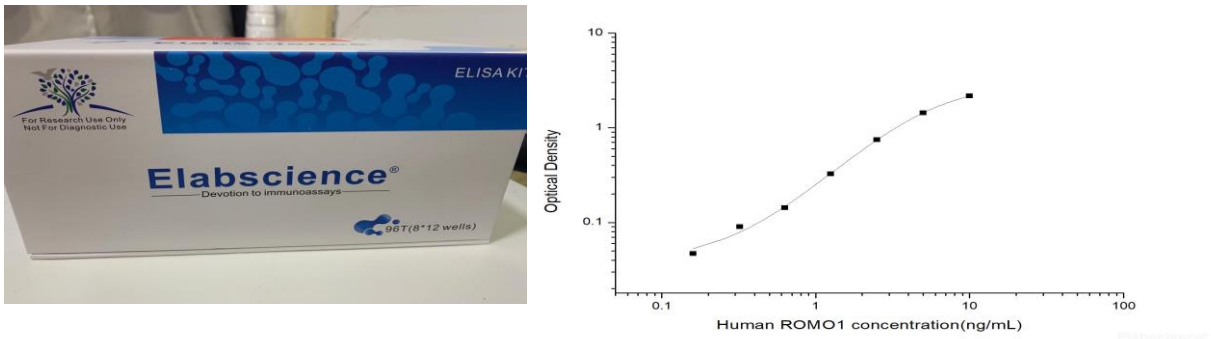


Figure (2.26): Demonstrate Optical Density Value of Well at 450 nm of ELISA kit Human ROMO1

Quantitative PCR (qPCR) was conducted for cell lines Hela cancer cell line that cultured at 100 x 104 cells / well in 12-well plates that exposed to the half-lethal concentrations IC_{50} for metformin RNA was extracted from cell lines according to the GENEzol™ TriRNA Pure Kit (Geneaid) Pure Kit [199].

- **RNA extraction :** according to the manufacturer's protocol. This included on-column digestion with 10 units/column of DNase I (Geneaid) to remove any potentially contaminating DNA from the sample

GENEzol™ TriRNA Pure Kit.

RNA Purification Protocol Procedure

Optional requirements 1 µL of 20 mM EGTA (pH=8.0) for Optional

Step1: Removal of genomic DNA from RNA samples [200].

1. Sample Homogenization and Lysis: -Sample preparation should be evaluated at room temperature.

2. RNA Binding

The sample was centrifuged at (12-16,000 x g for 1 minute) to eliminate cell debris, and the clean supernatant was then transferred to a new 1.5 ml micro centrifuge tube (RNase-free). In GENEzolTM Reagent we add 1 volume of absolute ethanol to 1 volume of sample mixture (1:1) after that vortex the mixture the put 2ml of collection RB Column, then 700 µl of the sample were transferred to the RB Column and centrifuged. Repeated the RNA binding step then transferred the remaining sample mixture to the RB Column Centrifuged for1 minute at 14-16,000 x g for then Placed the RB Column in a new 2 ml collection tube.

3. RNA Wash

Pre-Wash Buffer adding then centrifuged for 30 seconds at 14-16,000 x g. we placed the RB Column 2 ml collection Tube then adding 600 µl of Wash buffer then centrifuged for 30 seconds at 14-16,000 x g then. Centrifuge at 14-16,000 x g for 30 seconds. Replace the RB Column in the 2 ml collection Tube

after discarding the flow-through. Centrifuged at for 3 minutes 14-16,000 x g to dry the column matrix.

4. RNA Elution

Dried RB Column in a clean 1.5 ml microcentrifuge tube (RNase-free) then adding 25-50 µl of RNase-free water into the column matrix. After that we make RNase-free Water is absorbed completely by the matrix to elute the pure RNA use a minute-long centrifugation at 14–16,000 x g.

Optional Step 2: DNA Digestion In Solution DNase I reaction in a 1.5 ml micro centrifuge tube (RNase-free) as follows: Then gently pipette the DNase I reaction solution to mix, then incubate the micro centrifuge tube at 37oC for 15–30 minutes, add stop agent, incubation, and repurify the RNA sample by adding 3 volumes of GENEzolTM. After that, transfer all of the sample mixtures to a new RB Column. As summarized in the figure(2.27)

GENEzol™ TriRNA Pure Kit Functional Test Data (HeLa Cells)

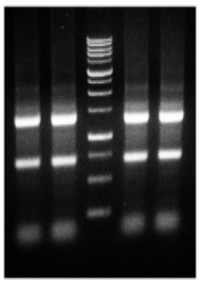


Figure 1. RNA was purified using the GENEzol™ TriRNA Pure Kit in parallel to the similar product from competitor Z. 5 x 10^5 HeLa cells were homogenized using GENEzol™ Reagent and competitor Z tri reagent. RNA was then purified using the corresponding kits spin column procedure. 10 µl from a 50 µl eluate of purified RNA was analyzed by electrophoresis on a 0.8% agarose gel.

Product Test	ng/µl	260/280	260/230	Yield
1. Competitor Z	162.5	2.00	2.07	8.1 µg
2. Competitor Z	160.7	2.03	2.07	8.0 µg
3. Geneaid	164.0	2.00	2.07	8.2 µg
4. Geneaid	161.6	2.03	2.06	8.0 µg

Figure (2.27): GENEzol™ TriRNA Pure Kit Functional Test Data (HeLa Cells) form kit instructions

The concentration of the eluted RNA was determined using an Implennano photometer N60. The RNA was stored at -80 C^0 until it was needed. First strand cDNA synthesis was performed using 1 µg of RNA, extracted as described above together with a AccuPower® Rocket Script TM RT Premix Bioneer according to the manufacturer's instructions. The cDNA synthesis reactions were diluted 5-fold in nuclease free water and then stored at -20 C^0 until we could be analyzed.

- **Quantitative Real-Time Polymerase Chain Reaction**

Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using a GoTaq® qPCR Master Mixkit and an Agilent thermal cycler (USA). Each reaction consisted GoTaq® qPCR. Master Mix and forward and revers primers via to the manufacturer's protocol. (5 µl) of the template cDNA was added to reaction.

The cycling conditions were a pre-denaturation step at 95C^{o} for 2 min followed by either 45cycles of denaturation at 95C^{0} for 15 s annealing at 60 C^{0} for 30s and extension at 72C^{0} for 30s. This was followed by a dissociation curve. Appeared in Figure (2.28)

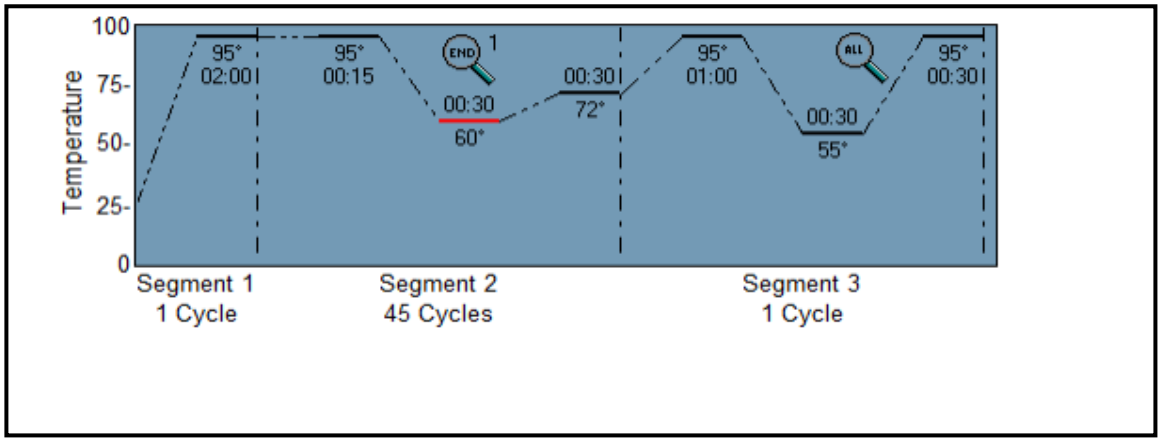


Figure (2.28): Describe the Dissociation curve of Gene Expression

2.3 Statistical Analysis

Data tabulation and coding performed via Microsoft Excel-2010. Descriptive and analytic statistics was performed using SPSS software statistical program. Version 23, prior to statistical analysis, all groups are tested to see if the data is distributed normally in all groups as follow :

1. Normality tests (Kolmogorov-Smirnov, Shapiro-Wilk) if more than 0.05 mean normal distribution parametric test, if not used non- parametric test
2. The descriptive statistics included mean ± Standard error of mean (SEM) for measurable variables, One-way Analysis of Variance test ANOVA-

test with Duncan's multiple comparison test were used to analyze the data *P*-value less than 0.05 was considered to be statistically, less than 0.01 highly significant

3. Unpaired *t*-test was used to compare among two different groups
4. Dependent t-test of two means (paired t test)
5. Kruskal –Wallis test for comparing each group at different time interval with mann whitny test to compare between two groups non- parmartic test
6. The IC_{50} was calculated using Graph Pad Prism version8.

Table (3.1): Monitoring The General Appearance of the Rats.

3 Results

3.1 Part One About Study-Related to Diabetes

3.1.1 : Monitoring of the animals.

Table (3.1) showed the general appearance of five groups of rats during the experiment. Animals in an experiment that die are replaced to complete the

required number of each group during a period that started from day 0, meaning at the start of the experiment before induction disease. After diabetes is confirmed the baseline meaning is day 7. Day 21 for M100, M200, and M300 after two weeks of treatment, day 35 the end of the experiment after four weeks of treatment.

Groups	General Appearance Daily Habit	
Control group (C)	Good response and action. Hair color and luster were fine. No noticeable difference in water and food consumption or urine volume	
Diabetic group (D)	A flabby spirit lags in response and actions. Hair color- disordered and lack luster. Polyphagia, polyuria, loose stools , increase urination	
Treatment with Metformin (M_{100},M_{200},M_{300})	Improvements behavior, food, water intake and response movement.	

3.1.2: Impact of Metform

in on Body Weight

Table (3.2) and Figure (3.1) show the effect of metformin on the weights of the rats. Comparison between the weights of the rats at the start of trial day 0 with those at the end of trial day 35 showed:

1. **Control group (C):** A significant increase in weight (p=0.000).
2. **Diabetic group (D):** A significant reduction in the weight of the rats (p = 0.048)
3. **Metformin group (M 100):** non-significant increase in rat weight (p = 0.133).
4. **Metformin group (M 200):** A significant increase in the weight of rats (p=0.021).
5. **Metformin group (M 300)**: A significant increase in the weight of rats (p=0.003).

Comparisons of weight of rats of five groups at day 0 showed non-significant difference (p=0.293), in contrast when comparisons between the weights of the rats of five groups at day 35, showed a significant difference between groups (p=0.000).

In figure (3.2) shows the difference of rat's weight of each group.

Table (3.2): Effect of Metformin in Body Weight of Rats.

Groups	Weight (gm.) Mean ± SEM			*P*=value
	(Day 0)	(Day 35)	Difference	
Control group (C)	205.40 ± 1.88 (a)	247.10 ± 3.82 (c)	41.70	**0.000****
Diabetic group (D)	204.56 ± 1.71 (a)	184.60 ± 9.47 (a)	-20.20	**0.048***
Metformin group (M 100)	198.75 ± 6.49 (a)	206.25 ± 0.940 (b)	7.50	0.133
Metformin group (M 200)	199.50 ± 1.47 (a)	211.10 ± 4.29 (b)	11.60	**0.021***
Metformin group (M 300)	200.06 ±1.50 (a)	218.50 ± 4.43 (b)	17.90	**0.003****
***P* -value**	0.293	**0.001 ****		

- Prior to statistical analysis, all groups are tested to see if the data is distributed normally. Normality tests (Kolmogorov-Smirnov, Shapiro-Wilk) in all groups
- The values in the vertical column were tested by one- way ANOVA, Post Hoc Duncan test to test the difference between groups. The superscript letters (a, b, and c) the same letter means no difference between groups, while different letters mean a difference between groups.
- Horizontal values were tested using a Paired-sample T test to determine the difference between (day 35-day 0).
- Significant level less than 0.05*, Highly significant level less than0.01**

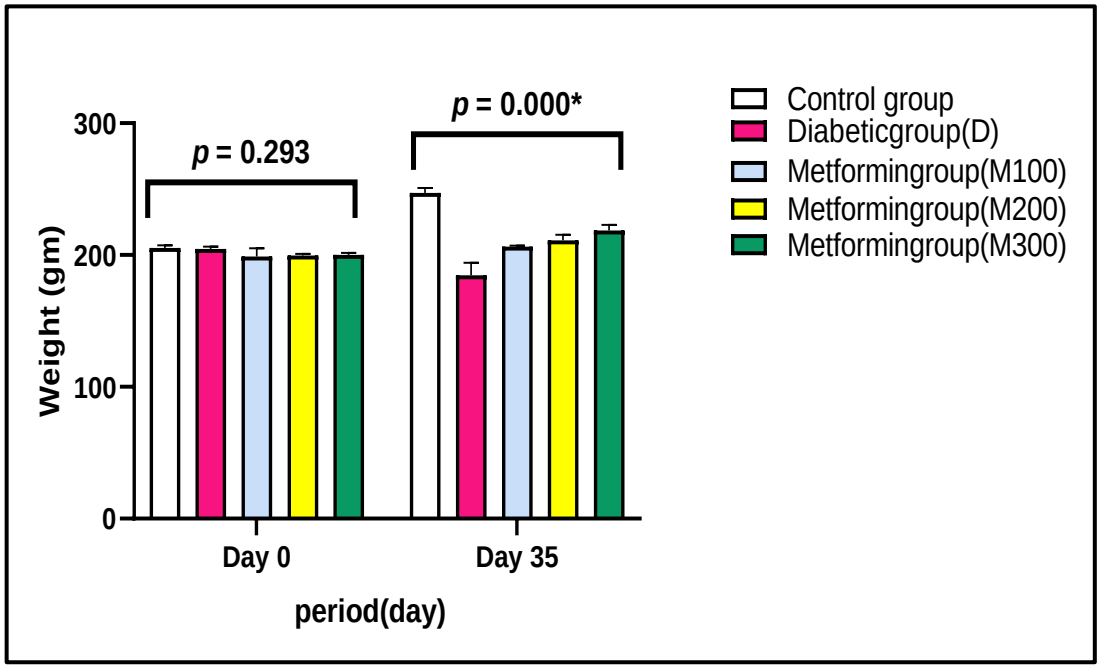


Figure (3.1): Effect of Metformin on Rats Body Weight.

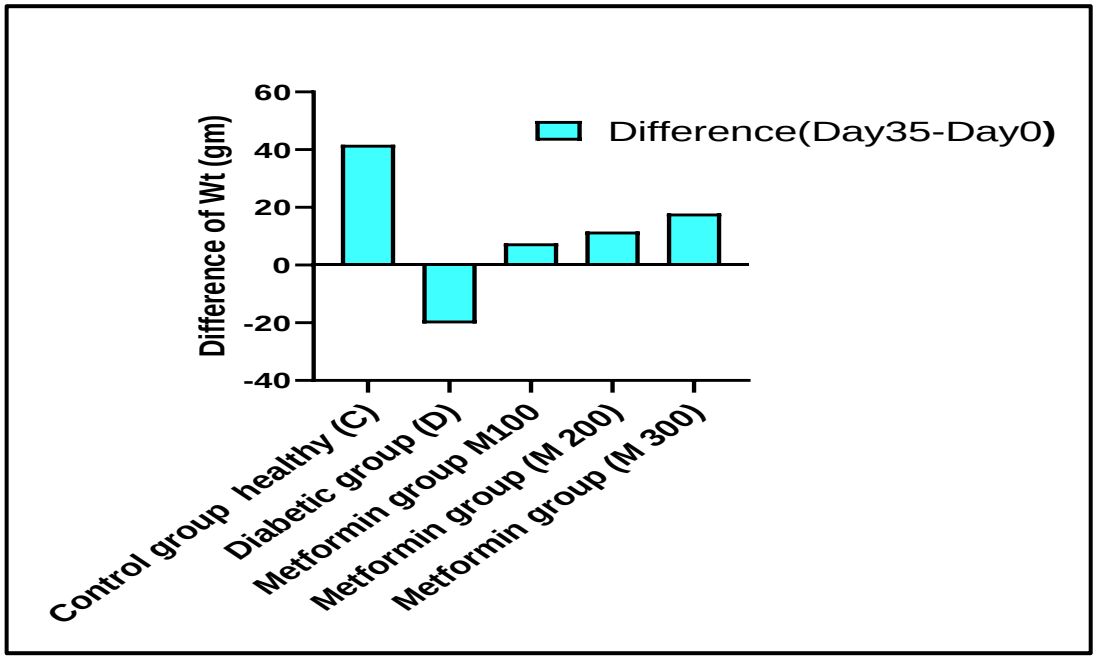


Figure (3.2): Show a Difference in Body Weight Between Rats.

3.1.3: Effect of Metformin on Fluid and Food intake

Table (3.3) and Figure (3.3) show the fluid and food intake of the rats in each of the five groups.

1. **Food intake:** Comparison between the five groups showed a significant difference (p=0.000), the results showed that diabetic group (D) consume more food as compared with other groups. The metformin groups showed higher food intake as compared with the control group but less than the diabetic group.
2. **Fluid intake:** Comparison between the fluid intake of the five groups showed a significant difference (p=0.000). The diabetic group consumed fluid more than the other groups, and the control group is the lowest consumption of fluid.

Table (3.3): Effect of Metformin on Fluid and Food Intake.

Groups	Mean ± SEM	
	Food Intake (gm) /rat	**Fluid Intake(ml)/ rat**
Control group(C)	18.55 ± 0.37 a	21.80 ± 0.41 a
Diabetic group(D)	32.90 ± 0.47 c	115.60 ± 3.13 c
Metformin group (M 100)	22.80 ± 0.44 b	36.10 ± 1.51 b
Metformin group (M 200)	22.40 ± 0.30 b	32.70 ± 0.67 b
Metformin group (M 300)	22.10 ± 0.27 b	32.400 ± 0.16 b
P -value	**0.000****	**0.000****

- ✓ **Prior to statistical analysis, all groups are tested to see if the data is distributed normally.**
- ✓ Values in vertical column tested by one - way ANOVA, Post Hoc Duncan test to test the difference between group the superscript different letter mean difference between groups, similar letter mean non-significant. Significant difference at p <0.05*, Highly significant level less than 0.01**

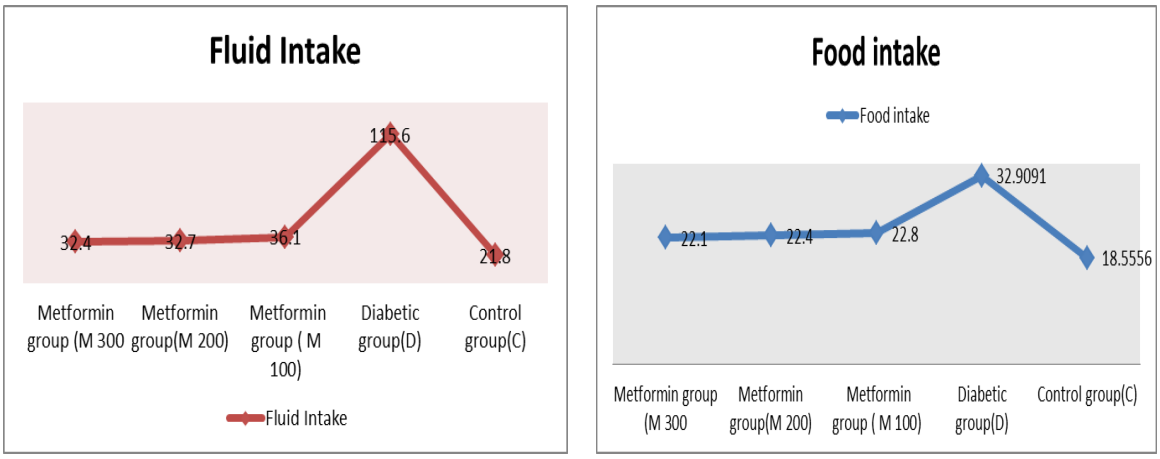


Figure (3.3): Effect of Metformin on Fluid and Food Intake.

3.1.4: Effect of Metformin on Blood Glucose Level.

Table (3.4), figure (3.4) and figure (3.5) show the blood glucose level (mg/dl) of the five groups. Comparison of blood glucose level between day 0, day 7, day 21 and day 35 of five groups, showed non- significant difference for control group (p=0.375) and higher significant for the four groups as compared to day 0 (p=0.000). The metformin groups showed a reduction of blood glucose level at day 35 as compared with day 7 of three groups.

Table (3.4): Effect of Metformin on Blood Glucose Level (mg/dl) at the Different Periods of the Study.

Groups \ Duration	Blood glucose levels (mg/dl) Mean ± SEM				
	(Day 0)	**(Day 7)**	**(Day 21)**	**(Day 35)**	***P*-value**
Control group (C)	98.30 ±1.83 (**A**, a)	102.8 ±2.35 (**A**, a)	100.00 ± 2.49 (**A**, a)	98.00 ±2.48 (**A**, a)	0.375
Diabetic group (D)	97.60 ±2.65 (**A**, a)	344.90 ±24.74 (**B**, b)	346.20 ±30.48 (**B** ,c)	357.10±14.04 (**B**, c)	**0.000****
Metformin group (M 100)	98.90 ±2.33 (**A**, a)	333.75±9..830 (**D**, b)	229.60 ± 8.28 (**C**, b)	179.00 ±16.11 (**B** ,b)	**0.000****
Metformin group (M 200)	99. 30 ±1.31 (**A**, a)	336.10 ± 20.69 (**D**, b)	227.60 ±22.83 (**C**, b)	173.00 ±20.87 (**B**, b)	**0.000****
Metformin group (M 300)	98.50±2.23 (**A**, a)	338.90±26.54 (**D**, b)	223.80 ±26.63 (**C**, b)	157.20 ± 26.82 (**B**, b)	**0.000****
***P* -value**	0.995	**0.000****	**0.000****	**0.000****	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk data in all group ≥ 0.05.
- ✓ Analysis by one way –ANOVA, Post –Hoc Duncan′s test.
- ✓ Vertical Colum different superscript small letter black color significant difference, same letter non-significant.
- ✓ Horizontally mean superscript capital letter blue color same letter significant difference while different letter non - significant
- ✓ * Significant differences at p<0.05, ** Highly significant level <0.01

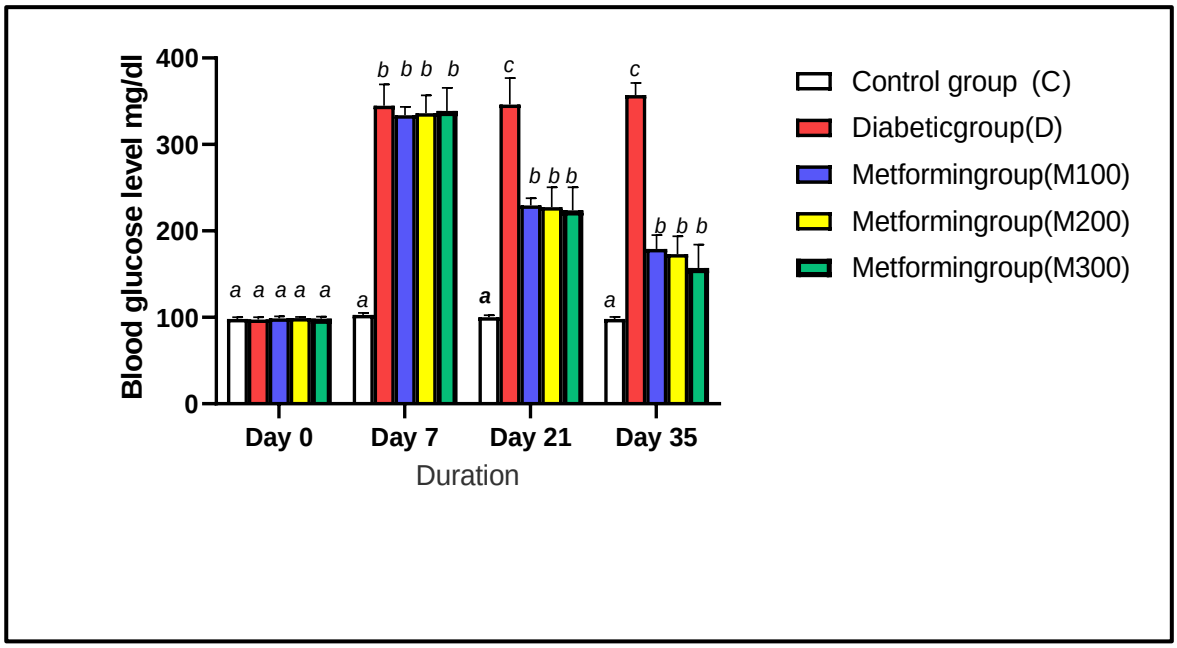


Figure (3.4): Blood Glucose Level (mg/dl) of Different Groups in Same Period (Effect Related to Dose).

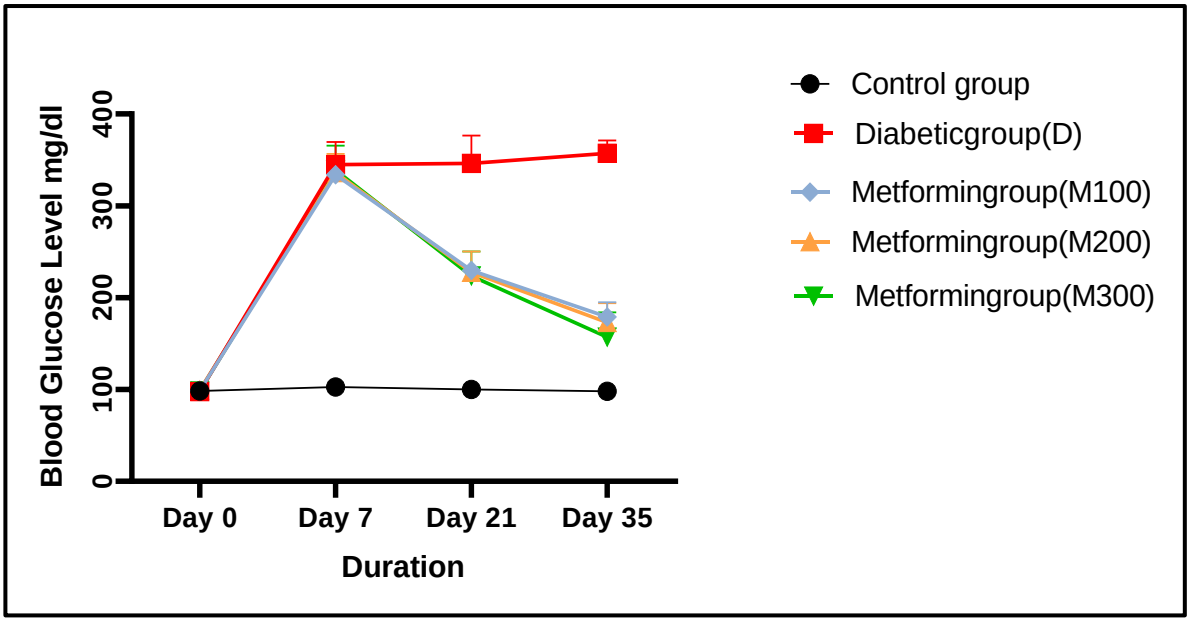


Figure (3.5): Blood Glucose Level (mg/dl) in Different Period at Same Periods of Different Groups (Effect Related to Time).

3.1.5: Biochemical Markers Evaluation:

3.1.5.1: Estimation of Tumor Necrosis Factor-Alpha.

3.1.5.1. A: Results Serum Rats TNF- α.

Table (3.5), figure (3.6) and figure (3.7) show the effect of metformin on serum TNF-α inflammatory marker levels:

1. A comparison of TNF- α values of five groups at day 0 showed non-significant difference (p=0.250).
2. A comparison of between day 0, day 7, day 21 and day 35, showed non-significant difference for control group (p=0.697).
3. A significant increase difference of TNF- α level for the diabetic groups at day 7, day 21, day 35 rather than day 0 but no difference of values at day 7, day 21and day 35.
4. Regarding metformin groups, there is a significant difference between serums TNF- α level of three groups at day 7, day 21and day 35 as compared with day 0, but there is a reduction of values of TNF-α at day 21, day 35 as compared with day 7 indicating a reduction effect of metformin on serum TNF-α Level, when compared between different dose showed significantly decreased M300 rather than M200 and M100 on day 21. Furthermore, M300 significant difference more than M200 and M100.

Table (3.5): Effect of Metformin on Serum TNF-α Level (ng/L) Related to Dose and Time.

Duration / Groups	Serum TNF-α Level (ng/L) Mean ± SEM				
	(Day 0)	**(Day 7)**	**(Day 21)**	**(Day 35)**	***P*- Value**
Control group (C)	82.21 ± 0.27 (A , a)	81.70 ± 0.29 (A , a)	82.11 ± 0.29 (A , a)	82.01 ± 0.39 (A , a)	0.697
Diabetic group (D)	81.81 ± 0.34 (A , a)	195.31±6.53 (B , b)	196.65 ± 3.56 (B, e)	197.75 ± 3.46 (B, d)	**0.000****
Metformin group (M 100)	81.40 ±0.26 (A ,a)	199.15 ± 4.61 (D, b)	142.31 ±3.70 (C, d)	129.50 ±2.19 (B , c)	**0.000****
Metformin group (M 200)	82.01 ±0.33 (A, a)	191.85 ±2.86 (D, b)	129.49 ±3.80 (C, c)	108.63± 3.62 (B , b)	**0.000****
Metformin group (M 300)	82.31 ±0.28 (A, a)	194.55 ±2.601 (D, b)	109.99 ±2.58 (C, b)	101.93 ±1.650 (B, b)	**0.000****
***P* -value**	0 .250	**0.000***	**0.000***	**0.000***	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk data in all group data ≥ 0.05.
- ✓ Analysis by one way –ANOVA, Post –Hoc Duncan′s test.
- ✓ Vertical Colum different superscript small letter black color significant difference, same letter non-significant.
- ✓ Horizontally mean superscript capital letter blue color same letter significant difference while different letter non - significant
- ✓ * Significant differences at p<0.05, ** Highly significant level <0.01.

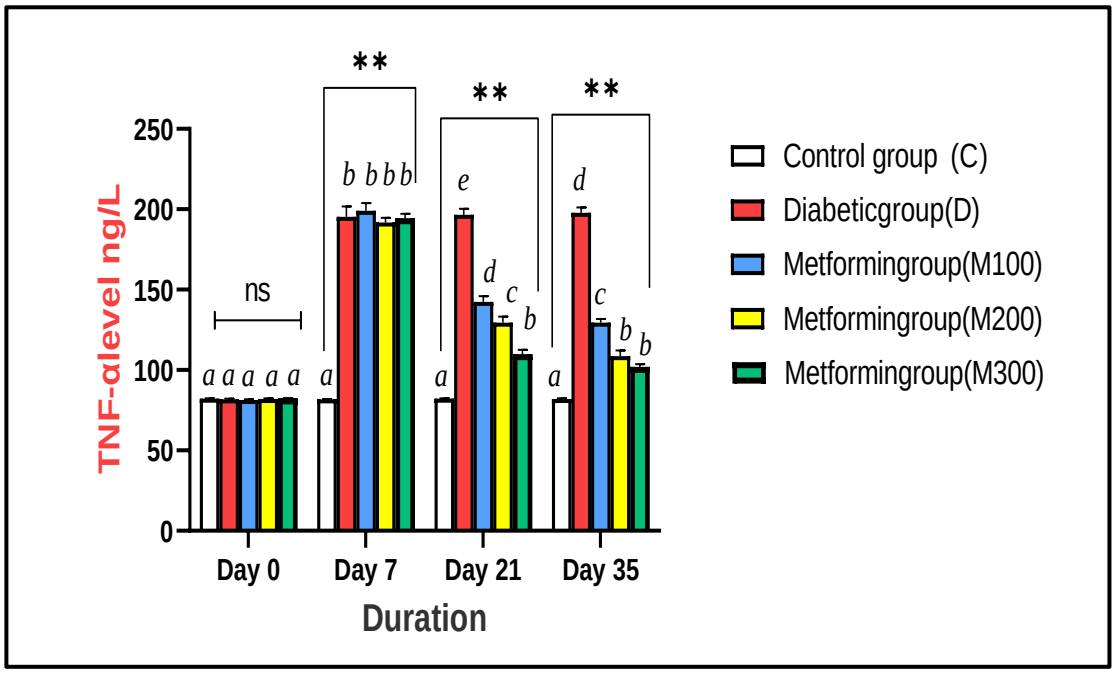


Figure (3.6): Effect of Metformin on serum Level of TNF-α at Same Periods of different Groups (Effect Related to Dose).

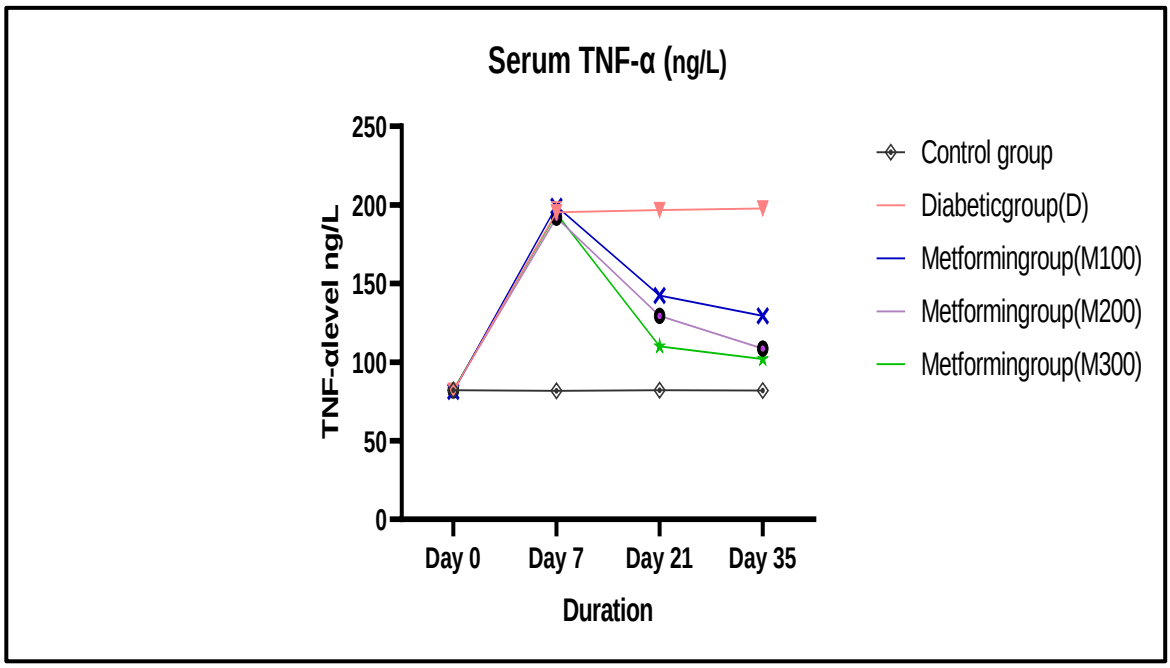


Figure (3.7): Effect of Metformin Drug on Serum Level of TNF-α Different Periods for Each Groups (Effect Related to Time).

3.1.5.1.B Results Rat TNF-α on Liver Tissue.

Table (3.6), Figure (3.8) and (3.9) shows the values of TNF-α tissue liver non-significant difference was obtained at day 0 of five groups (p=0.437).

1. Comparison of TNF – α of control group at day 0, day 7, day 21 and day 35, showed non- significant difference (p=0.697).
2. Comparison of TNF – α level of diabetic groups at day 7, day 21, day 35 with those at day 0 showed a significant difference (p=0.000), but comparison between values of day 7, day 21 and day 35 showed non- significant values.
3. Comparison between TNF- α values of the metformin three groups at day 7, day 21, day 35 with those at day 0 showed significant difference, but comparison of TNF- α values of three groups at day 21, day 35 with those at day 7 showed a significant difference (p=0.000) indicating reduction effect of metformin on the TNF – α values. when compared between different dose showed is significantly decreased M300 M200 than M100 at day 35.

Table (3.6): Effect of Metformin on Liver Tissue TNF- α Level (ng/L) Related to Dose and Time.

Duration / Groups	TNF-α Level in liver tissue (ng/L) Mean ± SEM				
	(Day 0)	(Day 7)	(Day 21)	(Day 35)	*P*- Value
Control group (C)	92.64 ± 0.64 (A , a)	92.91 ± 0.52 (A , a)	93.27 ± 0.83 (A, a)	93.79 ± 0.69 (A, a)	0.657
Diabetic group (D)	93.63 ± 1.10 (A , a)	228.30 ± 14.61 (B, b)	226.55 ±17.11 (B, c)	233.46 ±16.35 (B, d)	**0.000****
Metformin group (M 100)	91.82 ± 0.82 (A, a)	224.19 ± 16.35 (D, b)	155.96 ±1.69 (B, b)	147.22 ± 2.16 (B, c)	**0.000****
Metformin group (M 200)	91.65 ± 1.10 (A, a)	222.42 ± 17.44 (C, b)	152.92 ± 2.84 (B, b)	145.19 ± 1.53 (B, b)	**0.000****
Metformin group (M 300)	92.89 ± 1.56 (A, a)	225.41 ± 19.26 (D, b)	151.61 ± 2.11 (C, b)	139.31 ± 2.42 (B, b)	**0.000****
***P* -value**	0.437	**0.000****	**0.000****	**0.000****	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group ≥ 0.05.
- ✓ Analysis by one way –ANOVA, Post –Hoc Duncan′s test.
- ✓ Vertical Colum different superscript small letter black color significant difference, same letter non-significant.
- ✓ Horizontally mean superscript capital letter blue color same letter significant difference while different letter non - significant
- ✓ * Significant differences at p<0.05, ** Highly significant level <0.01

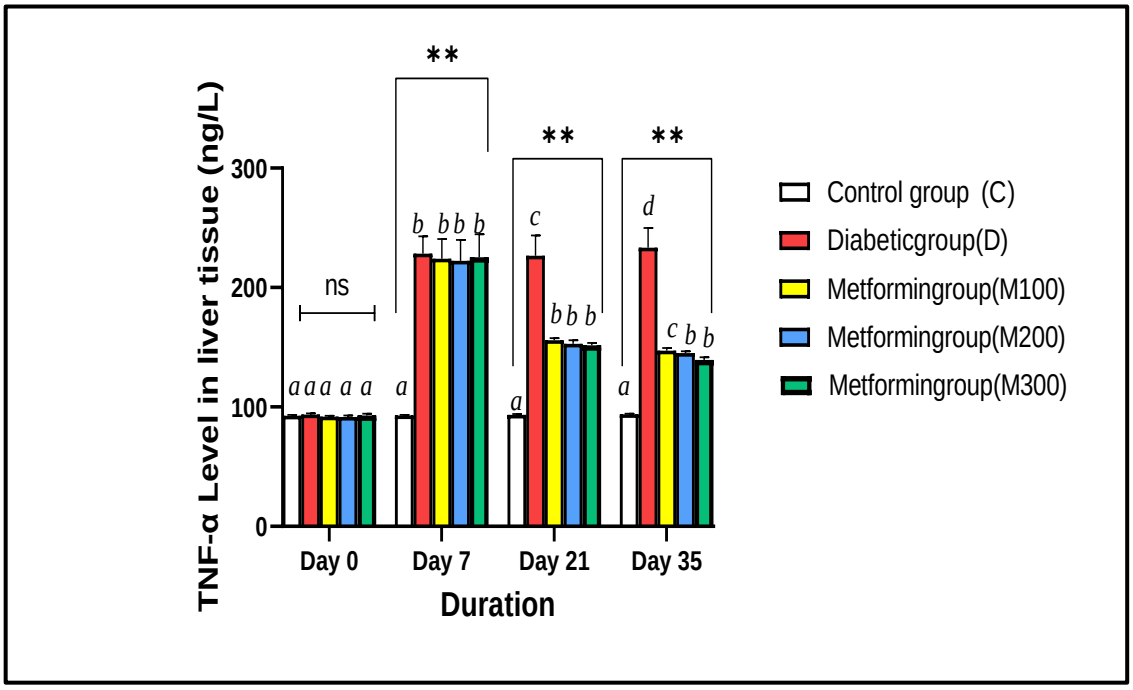


Figure (3.8): Effect of Metformin on Level TNF-α (ng/L) Liver Tissue at Same Periods of Different Groups (Effect Related to Dose).

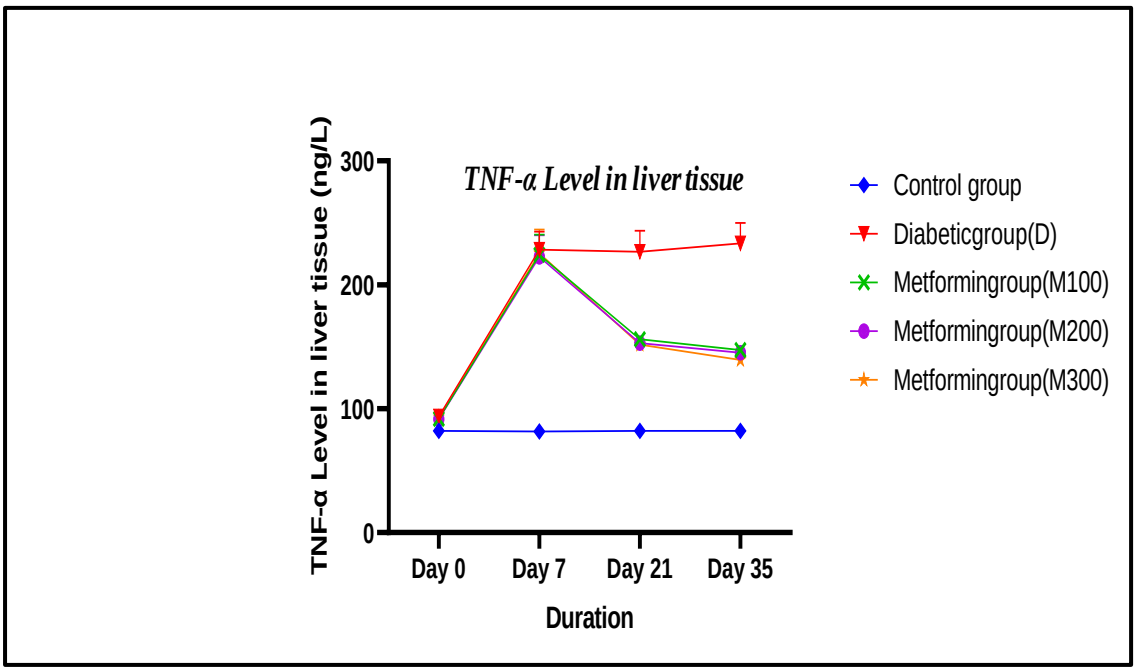


Figure (3.9): Effect of Metformin Drug on Level of TNF-α (ng/L) in Liver Tissue Different Periods for Each Groups (Effect Related to Time).

3.1.5.2: Biochemical Assessment Rat Interleukin -1beta

3.1.5.2. A: Results Serum Rat Interleukin -1beta (IL-1*β*)

Table (3.7), figure (3.10) and figure (3.11) show effect metformin on serum level IL-1*β*.

1. Comparison of IL-1*β* values of five groups at day 0 showed non-significant difference (*p*=0.974).
2. Comparison of between five groups at day 0, day 7, day 21 and day 35, showed non - significant difference (*p*=0.379) for control group
3. A significant increase difference of IL-1*β* level for the diabetic groups at day 7, day 21 and day35 rather than day 0 but no difference of values at day 7, day 21and day 35.
4. Regarding metformin groups, there are a significant difference between serum IL-1*β* level of three groups at day 7, day 21and day 35 as compared with the day 0, but there is a reduction of values of IL-1*β* level at day 21, day 35 as compared with day 7 indicating a reduction effect of metformin on serum IL-1*β*. When compared between different dose showed is significantly decreased M300 rather than M200 and M100 in day 21, in day 35 different significant in three dose**.**

Table (3.7): Effect Metformin on Serum IL-1 β Level (pg /L) Related to Dose and Time.

Groups \ Duration	Serum IL-1β Level (pg/L) Mean ± SEM				
	(Day 0)	**(Day 7)**	**(Day 21)**	**(Day 35)**	***P*- Value**
Control group (C)	14.35±0.32 (A , a)	13.85 ± 0.25 (A , a)	14.53 ±0.28 (A , a)	14.13 ±0.27 (A , a)	0.379
Diabetic group (D)	14.13 ± 0.31 (A , a)	33.99± 0.62 (B , b)	33.89 ±0.64 (B , d)	32.89 ±0.66 (B , e)	**0.000****
Metformin group (M 100)	14.15 ±0.29 (A , a)	34.25± 0.48 (D , b)	29.15±0.36 (C , c)	24.11 ±0.70 (B , d)	**0.000****
Metformin group (M 200)	14.03 ±0.47 (A , a)	33.69 ±0.54 (D , b)	27.43 ±0.51 (C , b)	22.25 ±0.62 (B , c)	**0.000****
Metformin group (M 300)	14.15± 0.20 (A, a)	33.29± 0.58 (D, b)	26.24 ±0.520 (C , b)	18.94 ±0.33 (B , b)	**0.000****
***P* -value**	0.974	**0.000****	**0.000****	**0.000****	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group ≥ 0.05.
- ✓ Analysis by one way –ANOVA, Post –Hoc Duncan′s test.
- ✓ Vertical Colum different superscript small letter black color significant difference, same letter non-significant.
- ✓ Horizontally mean superscript capital letter blue color same letter significant difference while different letter non - significant
- ✓ * significant differences at p<0.05, ** Highly significant level <0.01

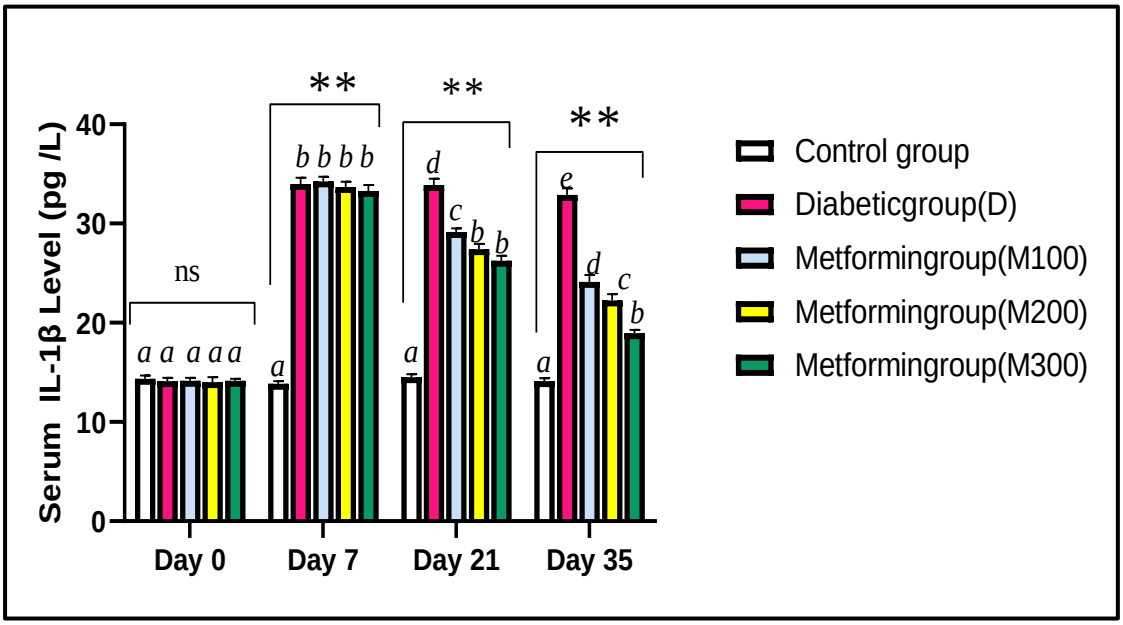


Figure (3.10): Effect Metformin Drug on Serum Level IL-1β (pg /L) at Same Periods of Different Groups (Effect Related to Time)

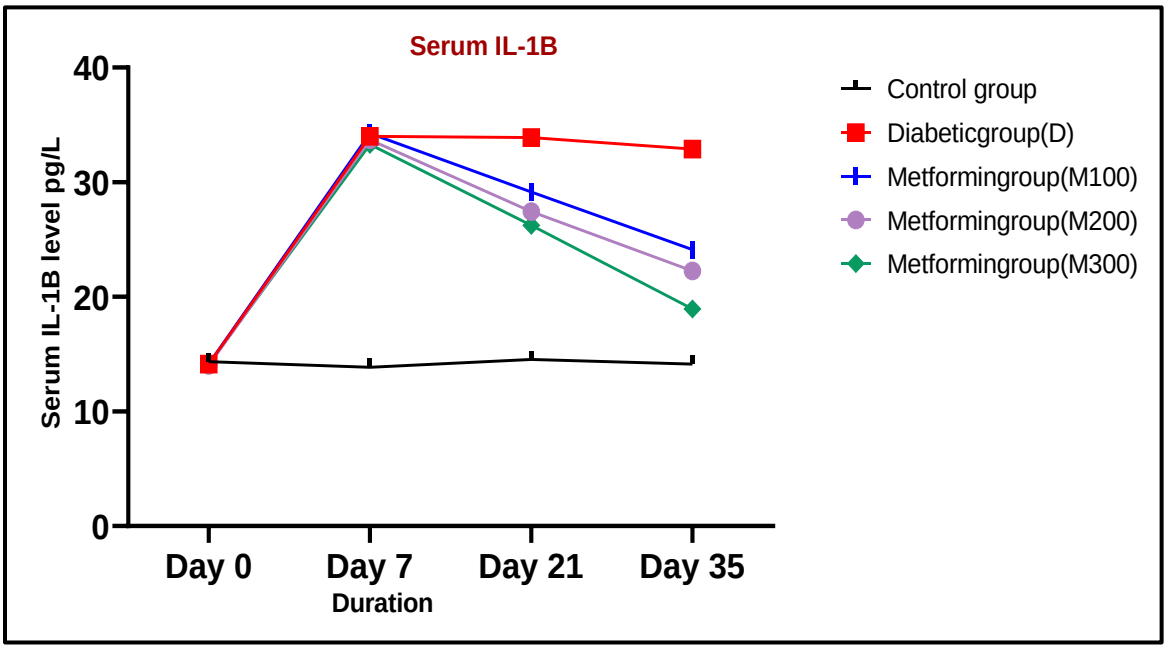


Figure (3.11): Effect Metformin Drug on Serum IL-1β Level at Different Periods for Each Groups (Effect Related to Time).

3.1.5.2. B: Results Rat (IL-1β) on Liver Tissue

In table (3.8), figure (3.12).and (3.13) show metformin effect on liver tissue IL-1β.

1. Comparison of IL-1β values of five groups at day 0 showed non-significant difference (p= 0.855)
2. Comparison of between day 0, day 7, day 21and day 35 of control groups, showed non-significant difference for control group (p=0.873).
3. A significant increase difference of IL-1β level for the diabetic groups at day 7, day 21, day 35 rather than day 0, but no difference of values at day 7, day 21and day 35.
4. Regarding metformin groups, there are a significant difference between liver tissue IL-1β level of three groups at day 7, day 21and day 35 as compared with the day 0, but there is a reduction of values of IL-1β of day 21, day 35 as compared with day 7 indicating a reduction effect of metformin on IL-1β.

Table (3.8): Effect Metformin on liver tissue IL-1*β* Level (pg /L) Related to Dose and Time.

Duration / Groups	IL-1*β* Level in liver tissue (pg. /L) Mean ± SEM				
	(Day 0)	(Day 7)	(Day 21)	(Day 35)	*P*-Value
Control group (C)	24.37 ± 0.70 (A, a)	25.01 ± 0.65 (A, a)	25.02 ± 0.46 (A, a)	24.91 ± 0.68 (A, a)	0.873
Diabetic group (D)	24.31 ± 1.50 (A , a)	43.15 ± 0.46 (B , b)	42.49 ± 0.83 (B , b)	42.52 ± 0.80 (B , b)	**0.000****
Metformin group (M 100)	23.28 ± 1.22 (A , a)	42.97 ± 0.50 (C, b)	32.19 ± 0.44 (B , b)	31.42 ± 0.42 (B , b)	**0.000****
Metformin group (M 200)	24.12 ± 0.56 (A , a)	43.26 ± 0.67 (C, b)	31.51 ± 0.36 (B , b)	31.03 ± 0.74 (B , b)	**0.000****
Metformin group (M 300)	24.87± 0.60 (A , a)	42.81 ± 0.59 (C , b)	31.18± 0.43 (B , b)	30.01 ± 0.21 (B , b)	**0.000****
***P* -value**	0.855	0.000**	0.000**	0.000**	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group ≥ 0.05.
- ✓ Analysis by one way –ANOVA, Post –Hoc Duncan′s test.
- ✓ Vertical Colum different superscript small letter black color significant difference, same letter non-significant.
- ✓ Horizontally mean superscript capital letter blue color same letter significant difference while different letter non - significant

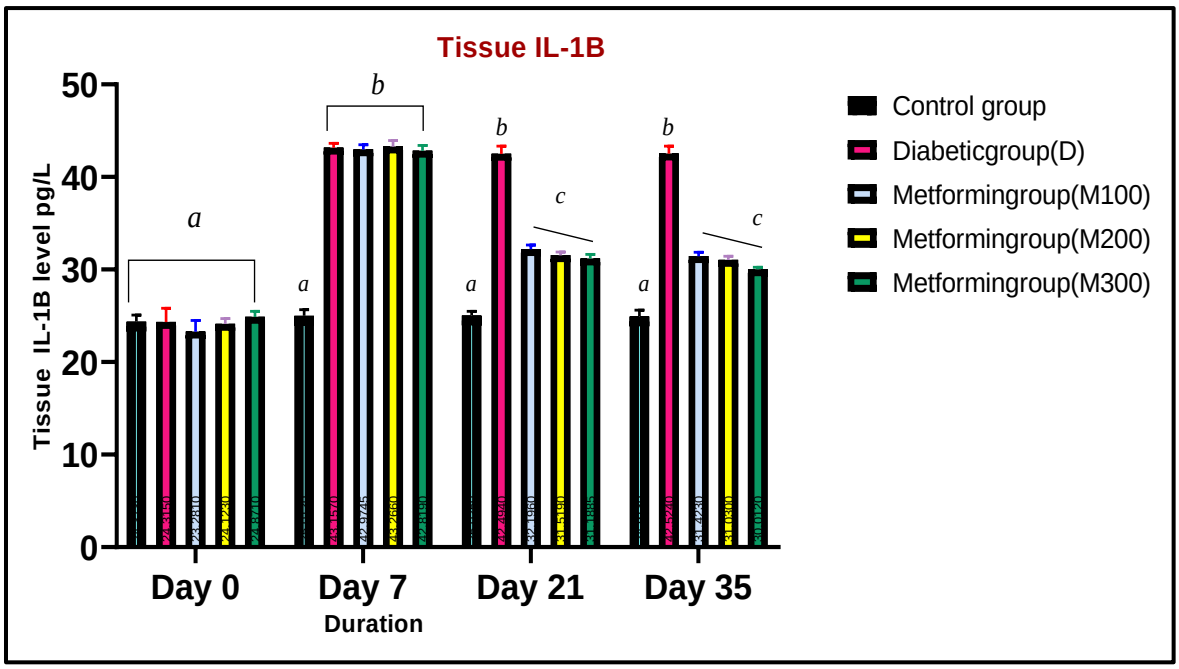


Figure (3.12): Effect Metformin Drug on Level IL-1β Level(pg/L) Liver Tissue at Same Periods of Different Groups (Effect Related to Dose).

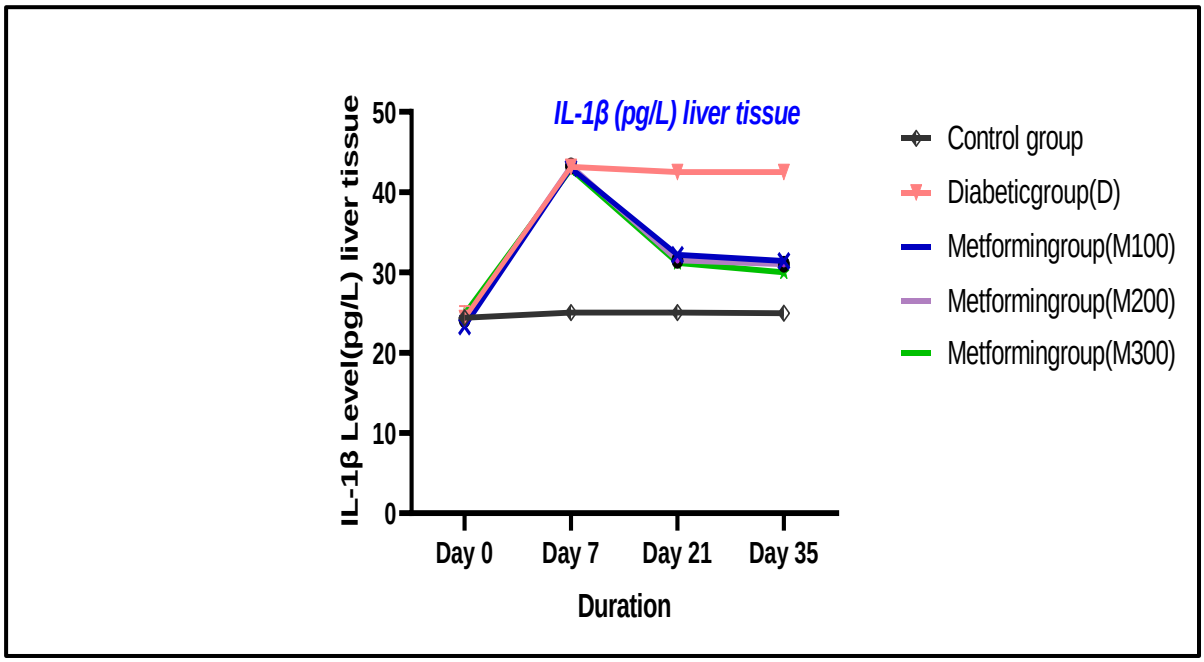


Figure (3.13): Effect of Metformin Drug on Level of IL-1β Level(pg./L) in Liver Tissue Different Periods for Each Groups (Effect Related to Time)

3.1.5.3 A: Results Serum Rat Total antioxidant status (TAOS).

Table (3.9) figure (3.14).and figure (3.15) show metformin effect on serum level TAOS.

1. Comparison of TAOS value of five groups at day 0 showed non-significant difference (p= 0.991).
2. Comparison of between day 0, day 7, day 21and day 35 of five groups, showed non-significant difference for control group (p=0.985).
3. A significant decrease difference of TAOS level for the diabetic groups at day 7, day 21, day 35 rather than day 0, but no difference of values at day 7, day 21and day 35.
4. Regarding metformin groups, there are a significant difference between serum TAOS level of three groups at day 7, day 21and day 35 as compared with the day 0, but there are increase of values TAOS of day 21, day 35 as compared with day 7 indicating increment effect of metformin on TAOS. When compared between different dose showed non-significant difference between dose at day21, significant difference at day 35.

Table (3.9): Effect Metformin on Serum Level TAOS (U/ml) at Different Period (Effect Treatment to Time).

Duration / Groups	Serum TAOS Level (U/ml) Mean ± SEM				
	(Day 0)	**(Day 7)**	**(Day 21)**	**(Day 35)**	***P*- Value**
Control group (C)	5.27 ±0.46 (A, a)	5.22 ± 0.34 (A ,b)	5.38 ± 0.34 (A , c)	5.16 ± 0.49 (A ,c)	0.985
Diabetic group (D)	5.35 ± 0.39 (B, a)	1.88 ± 0.10 (A ,a)	1.77 ± 0.160 (A , a)	1.64 ± 0.15 (A , a)	**0.000****
Metformin group (M 100)	5.13 ± 0.33 (C , a)	1.53 ± 0.19 (A, a)	3.25 ± 0.34 (B , b)	3.80 ± 0.55 (B ,b)	**0.000****
Metformin group (M 200)	5.11 ± 0.34 (C , a)	1.78 ± 0.08 (A , a)	3.74 ± 0.26 (B , b)	4.15± 0.42 (B, bc)	**0.000****
Metformin group (M 300)	5.31 ± 0.47 (C , a)	1.61 ± 0.20 (A, a)	4.027 ± 0.14 (B , b)	4.74 ± 0.36 (BC, c)	**0.000****
***P* -value**	0.991	**0.000****	**0.000****	**0.000****	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group ≥ 0.05.
- ✓ Analysis by one way –ANOVA, Post –Hoc Duncan′s test.
- ✓ Vertical Colum different superscript small letter black color significant difference, same letter non-significant.
- ✓ Horizontally mean superscript capital letter blue color same letter significant difference while different letter non - significant
- ✓ * Significant differences at p<0.05, ** Highly significant level <0.01

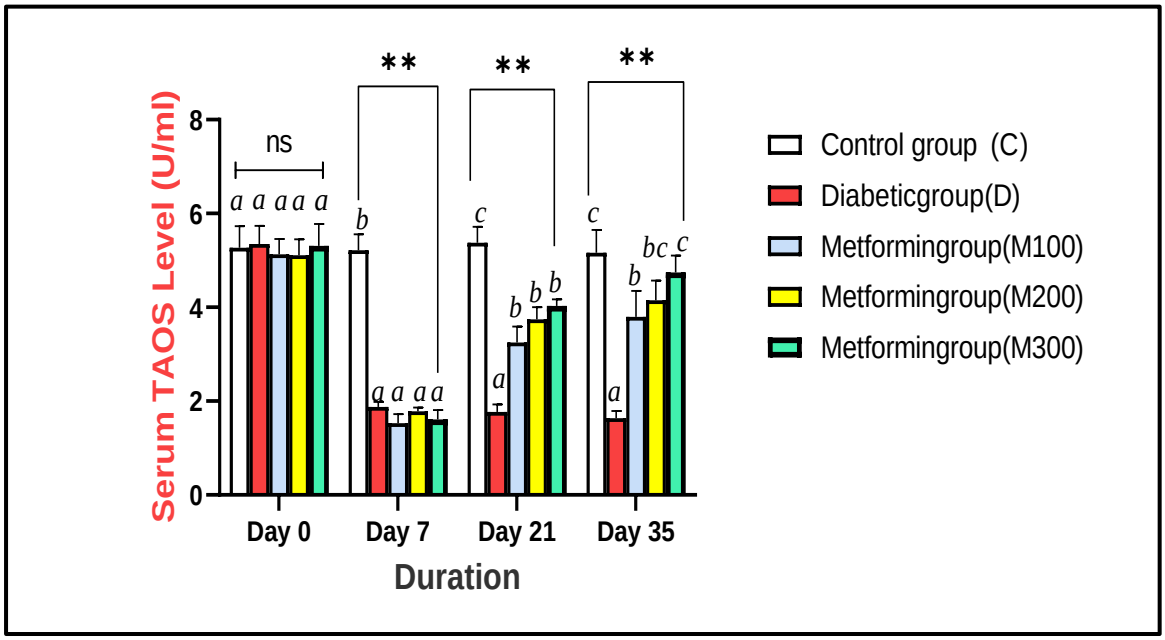


Figure (3.14): Effect Metformin Drug on Serum Level TAOS (U/ml) at Same Periods of Different Groups (Effect Related to Dose).

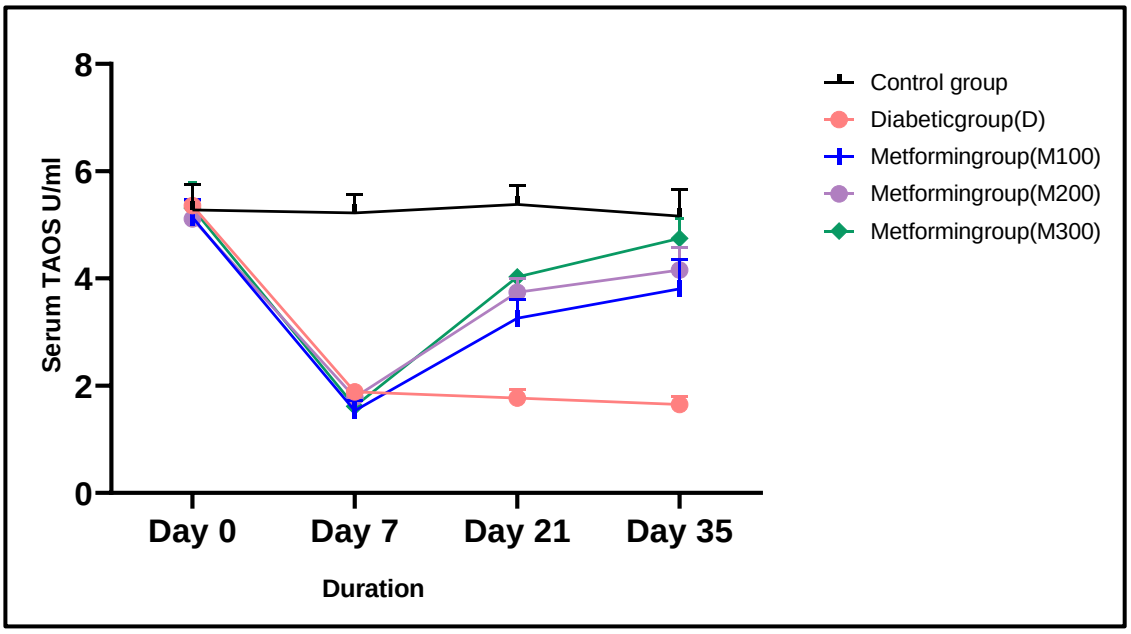


Figure (3.15): Effect Metformin Drug on Serum Level TAOS(U/ml) Different Periods for Each Groups (Effect Related to Time).

3.1.5.3 B: Results Pancreatic Rat Total Antioxidant Status (TAOS)

Table (3.10), figure (3.16) and figure (3.17) show the effect of metformin on pancreatic TAOS levels.

1. A Comparison of TAOS value of five groups at day 0 showed non-significant difference (p= 0.395).
2. Comparison of day 0, day 7, day 21and day 35 of five groups, showed non-significant difference for the control group (p= 0. 630).
3. A significant decrease difference of TAOS level for the diabetic groups at day 7, day 21, day 35 rather than day 0, but no difference of values at day 7, day 21and day 35.
4. Regarding metformin groups, there are a significant difference between the pancreatic TAOS level of three groups at day 7, day 21and day 35 as compared with the day 0, but there are increase of values TAOS of day 21, day 35 as compared with day 7 indicating increment effect of metformin on TAOS. When compared between different dose showed non-significant difference between groups in day 21, while day 35 different significantly in M300 than M200and M100.

Table (3.10): Effect Metformin on Pancreatic Tissue Level TAOS (U/ml) Related to Dose and Time.

Duration / Groups	Total antioxidant status (TAOS U/ml) pancreatic tissue Mean + S.E				
	(Day 0)	**(Day 7)**	**(Day 21)**	**(Day 35)**	**p-value**
Control group (C)	6.53 ±0.22 (A, a)	6.70 ± 0.17 (A , b)	6.73± 0.10 (A ,c)	6.37± 0.34 (A ,c)	0.395
Diabetic group (D)	6.51 ± 0.23 (B, a)	1.07 ±0.20 (A , a)	1.21± 0.27 (A, a)	1.19± 0.10 (A ,a)	**0.000****
Metformin group (M 100)	6.24 ±0.26 (A , a)	1.24± 0.11 (A , a)	4.13± 0.48 (B, b)	4.77± 0.78 (B, b)	**0.000****
Metformin group (M 200)	6.65 ± 0.03 (A , a)	1.21± 0.34 (A , a)	4.50± 0.11 (B , b)	5.07 ± 0.56 (B ,b)	**0.000****
Metformin group (M 300)	6.73 ± 0.56 (A , a)	1.40 ±0.43 (A, a)	5.14± 0.54 (B ,b)	5.84 ± 0.25 (B ,c)	**0.000****
P-Value	0. 630	**0.000****	**0.000****	**0.001****	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group ≥ 0.05.
- ✓ Analysis by one way –ANOVA, Post –Hoc Duncan′s test.
- ✓ Vertical Colum different superscript small letter black color significant difference, same letter non-significant.
- ✓ Horizontally mean superscript capital letter blue color same letter significant difference while different letter non - significant
- ✓ * Significant differences at p<0.05, ** Highly significant level <0.01.

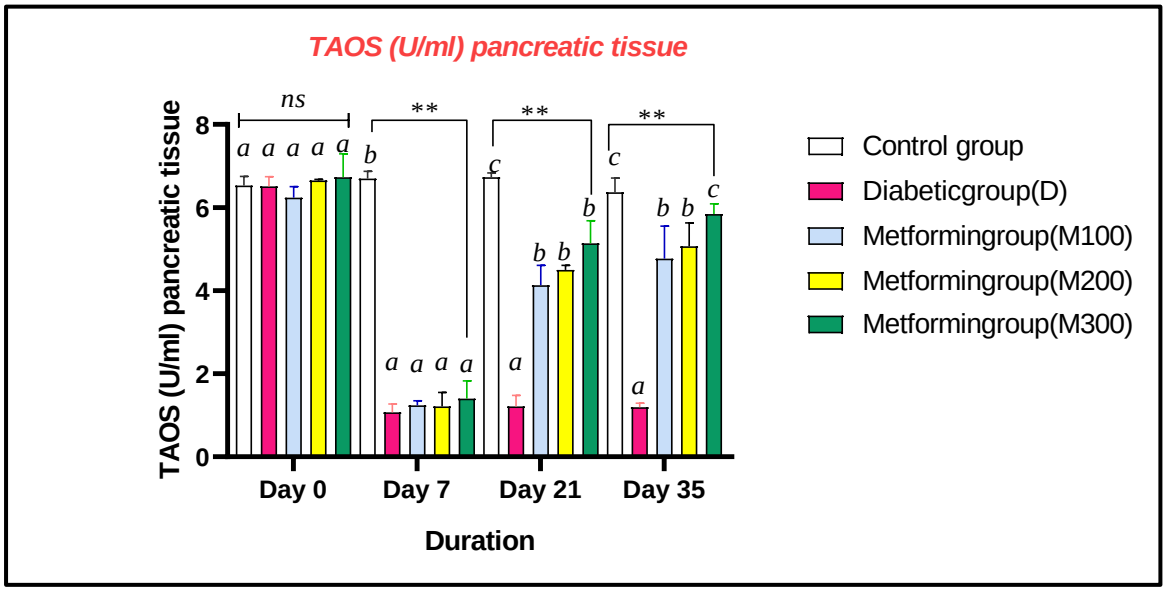


Figure (3.16): Effect Metformin on TAOS (U/ml) Rat Pancreatic at Same Periods of Different Groups (Effect Related to Dose).

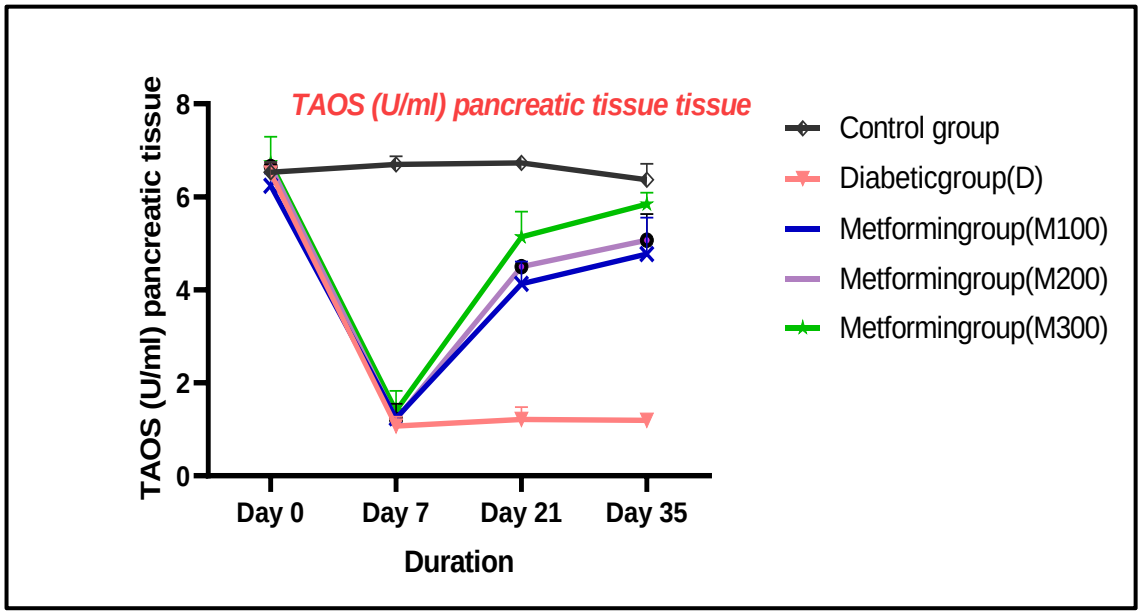


Figure (3.17): Effect Metformin on TAOS (U/ml) Rat Pancreatic Different Period of Each Group (Related to Time).

3.1.5.3.C: Results Serum Rat Malondialdehyde (ng/mL) Level

Table (3.11) figure (3.18).and figure (3.19) show effect metformin of serum level MDA.

1. Comparison of MDA value of five groups at day 0 showed non-significant difference (p=0.742)
2. Comparison of between day 0, day 7, day 21and day 35 of five groups, showed anon significant difference for control group (p=0.496).
3. A significant increase difference of MDA level for the diabetic groups at day 7, day 21, day 35 rather than day 0, but no difference of values at day 7, day 21and day 35.
5. Regarding metformin groups, there are a significant difference between serum MDA level of three groups at day 7, day 21and day 35 as compared with the day 0, but there is a reduction of values of MDA of day 21, day 35 as compared with day 7 indicating reduction effect of metformin on serum MDA. When compared between different dose showed significant difference between groups in same periods.

Table (3.11): Effect Metformin on Serum MDA (ng /mL) Related to Dose and Time.

Duration / Groups	Serum MDA Level (ng /mL) Mean ± SEM				
	(Day 0)	**(Day 7)**	**(Day 21)**	**(Day 35)**	***P*- Value**
Control group (C)	59.72± 2.43 (A , a)	61.52 ± 0.26 (A ,a)	60.72+ 3.15 (A , a)	61.01±2.25 (A ,a)	0.496
Diabetic group (D)	54.98 ± 2.38 (A, a)	119.65± 2.87 (B ,b)	118.1± 1.13 (B , d)	112.61± 3.25 (B , c)	**0.000****
Metformin group (M 100)	59.72± 2.43 (A , a)	107.9.1± 3.28 (C, b)	89.51± 2.17 (B , c)	85.42± 1.51 (B ,b)	**0.000****
Metformin group (M 200)	57.02± 2.88 (A , a)	115.1± 4.49 (C, b)	77.63± 3.43 (B , bc)	71.48± 3.07 (B , b)	**0.000****
Metformin group (M 300)	58.89±3.86 (A , a)	116.82 ± 4.12 (C, b)	71.48± 3.07 (B , b)	61.01± 1.53 (A , a)	**0.000****
P -value	0.742	**0.000****	**0.000****	**0.000****	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group ≥ 0.05.
- ✓ Analysis by one way –ANOVA, Post –Hoc Duncan′s test.
- ✓ Vertical Colum different superscript small letter black color significant difference, same letter non-significant.
- ✓ Horizontally mean superscript capital letter blue color same letter significant difference while different letter non - significant
- ✓ significant differences at p<0.05, ** Highly significant level <0.01.

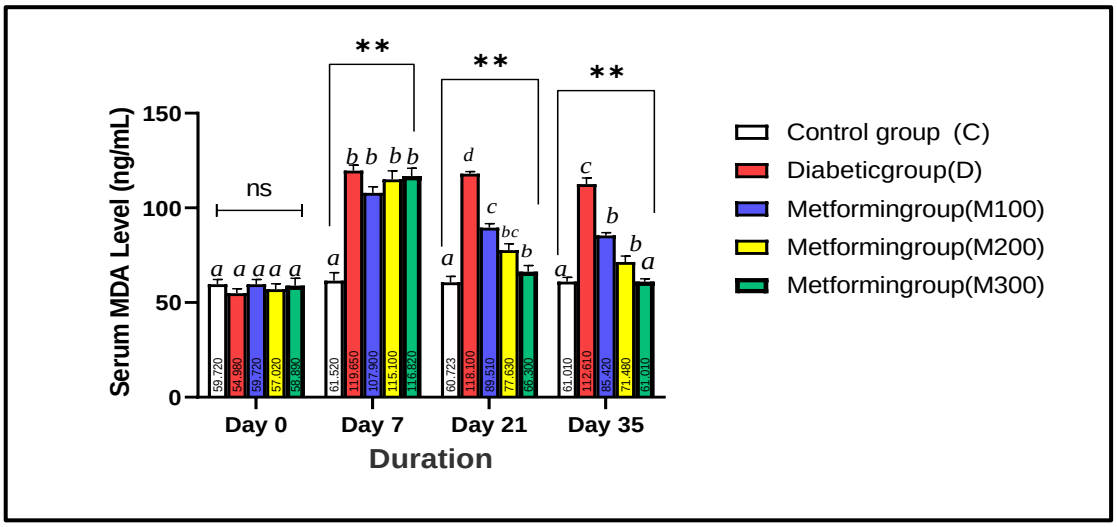


Figure (3.18): Effect Metformin Drug MDA Level (ng/mL) Different Periods for Different Groups (Effect Related to Dose)

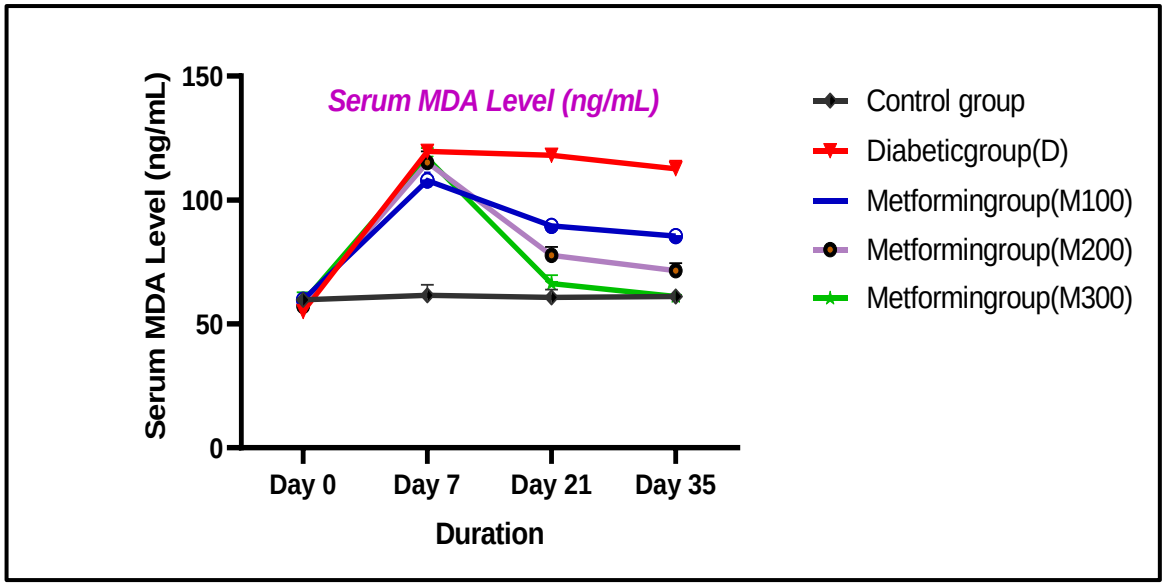


Figure (3.19): Effect Metformin Drug MDA Level (ng/mL) Different Periods for Each Groups (Effect Related to Time)

3.1.6: In Vitro Antioxidant Activity Screening of metformin

Table (3.12) show that the assay for scavenging DPPH radicals results showed a significant difference at 10 min when the comparison between ascorbic acid and metformin concentrations was 250, 500, or 1000 g/ml, and a significant

difference at 30 and 60 min when the comparison between ascorbic acid and metformin concentrations was found to be concentration dependent with interval tim

Table (3.12) Comparison DPPH Scavenging Power of Free Radical between Metformin and Ascorbic Acid in Different Time.

Concentration	10 min			30 min			60 min		
	M	**A.A**	***p*-value**	**M**	**A.A**	***p*-value**	**M**	**A.A**	***p*-value**
250 µg/ml	2.64 ± 0.15	44.81 ± 0.53	**0.000****	3.96± 0.42	52.38 ±0.26	**0.000****	11.87 ± 0.74	51.45 ± 0.664	**0.000****
500 µg/ml	3.34 ± 0.37	48.76 ± 1.04	**0.000****	9.43± 0.34	52.68 ± 0.91	**0.000****	13.82 + 0.95	53.25± 0.263	**0.000****
1000 µg /ml	11.87± 0.74	51.85 ±0.508	**0.000****	13.84± 0.95	53.25 ± 0.46	**0.000****	14.72+ 0.56	52.65 + 0.28	**0.000****

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk data in all group
- ✓ Independent T-test of two means was used (M= Metformin, A. A=ascorbic acid
- ✓ *Significant differences at p<0.05, ** Highly significant level <0.01.

Table (3.13) showed when comparing the different concentrations of metformin, non-significant difference at 60 min (*p*=0.061) and non- significant difference between ascorbic acid (250 500,1000 µg/ml). The IC50 value of metformin and the ascorbic acid (498.0, 29.62 µg/ml) respectively at 60 min in different concentration was shown as figure (3.20).

Table (3.13) Comparison DPPH Scavenging Power of Free Radical Between Metformin and Ascorbic Acid in Different Concentrations at 60 min

Concentration	60 min	
	Metformin	**Ascorbic Acid**
250 µg/ml	11.874 ± 0.749 a	51.451 ± 0.664 a
500 µg/ml	13.827 ± 0.956 a	53.259 ± 0.263 a
1000 µg /ml	14.723± 0.564 a	52.652 ± 0.281 a
***P* -value**	**0.061**	**0.121**

- ✓ ll data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk data in all group.
- ✓ ANOVA test for each group, Post –Hoc Duncan′s test.
- ✓ Similar letter mean non –significant difference.

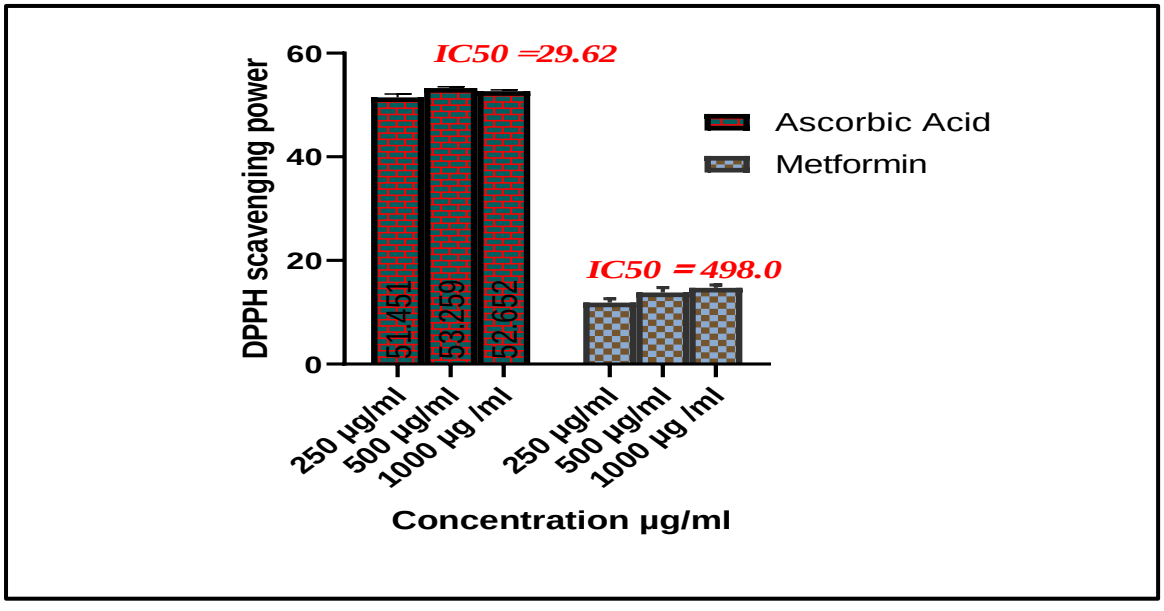


Figure (3.20): IC50 Value of Metformin and Ascorbic Acid.

3.1.7: Histological Evaluation:

In the current study, the histological assessment included descriptive and determination scores, histological slides were prepared and then examined under light microscopic by three histopathologists for inflammation and pathological scoring separately; the mean of three readings was calculated and, after that, settled for statistical analysis.

3.1.7.1: **Microscopic Observation**

3.1.7.1.A: **Histomorphological findings on pancreatic tissue**

Control Group: Microscopic observation of stained pancreas of the control rats presented a normal as descriptive photomicrograph of pancreas of healthy control group that normal architecture of pancreatic tissue representing by pancreatic acini (A), islets of Langerhans (B) and interlobular duct (C) figure (3.21).

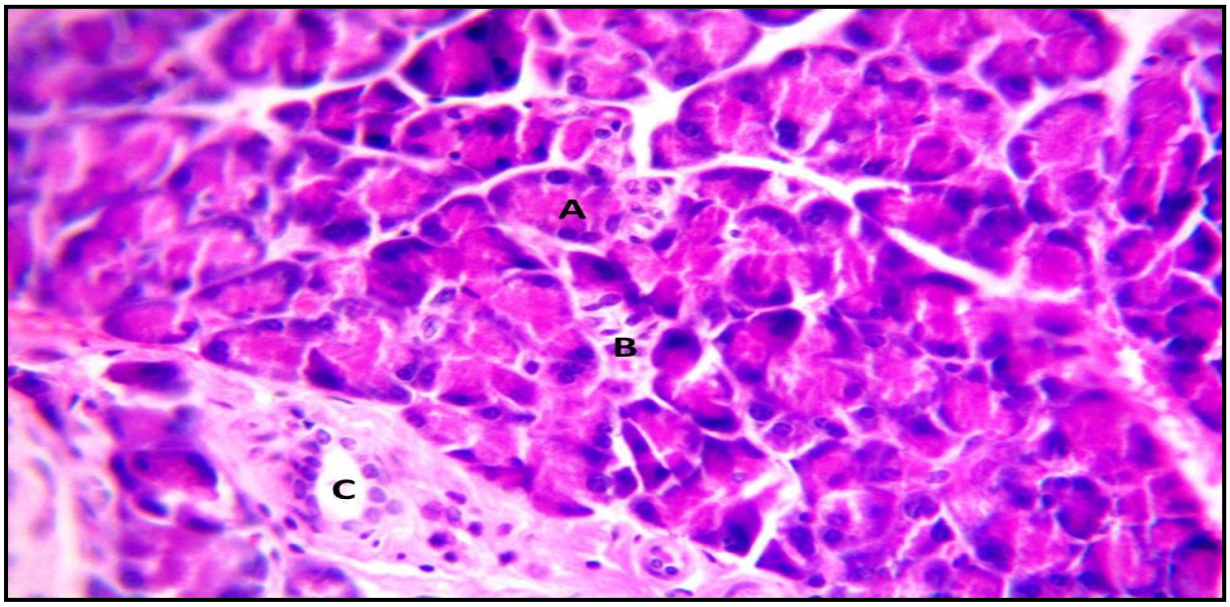


Figure (3.21): Photomicrograph of Pancreas of Control Group Rats 400X.

Diabetic Group: Though a histological section of alloxan-diabetic pancreatic tissue degenerative and excessive necrotic tissue lesions the pancreas, showing a breakdown in the areas of internal and external secretion with deposition reduction in pancreatic tissue, in addition to atrophy of the islets of Langerhans. Also notice the irregularity of pancreatic vacuities associated with

histological destruction, necrosis, reduction of some cells, as well as atrophy of some cells. This is mainly due to the ability of alloxan to be highly toxic to the cell through the formation of free radicals ROS as pancreatic beta cells have a defensive capacity as shown figure (3.22).

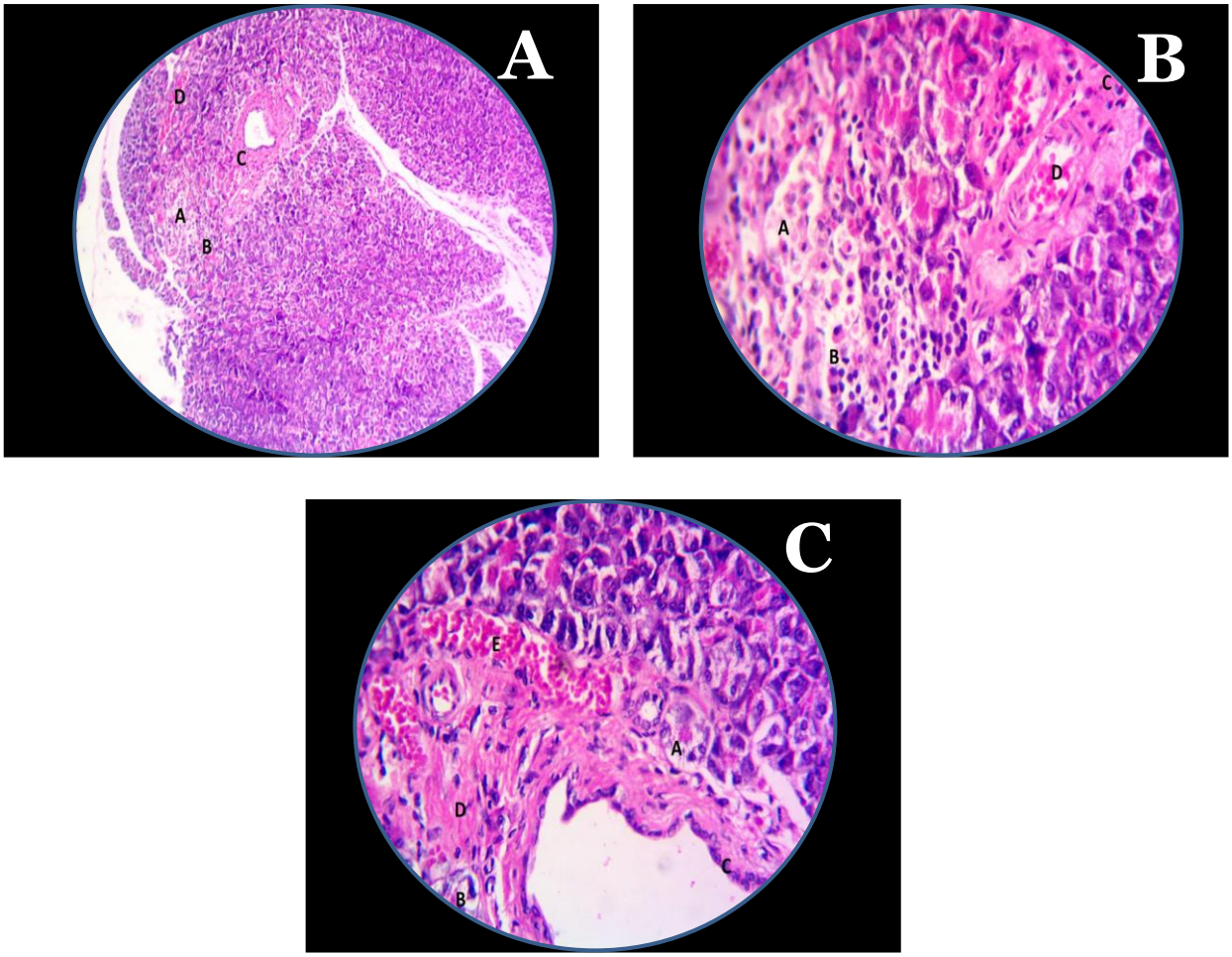
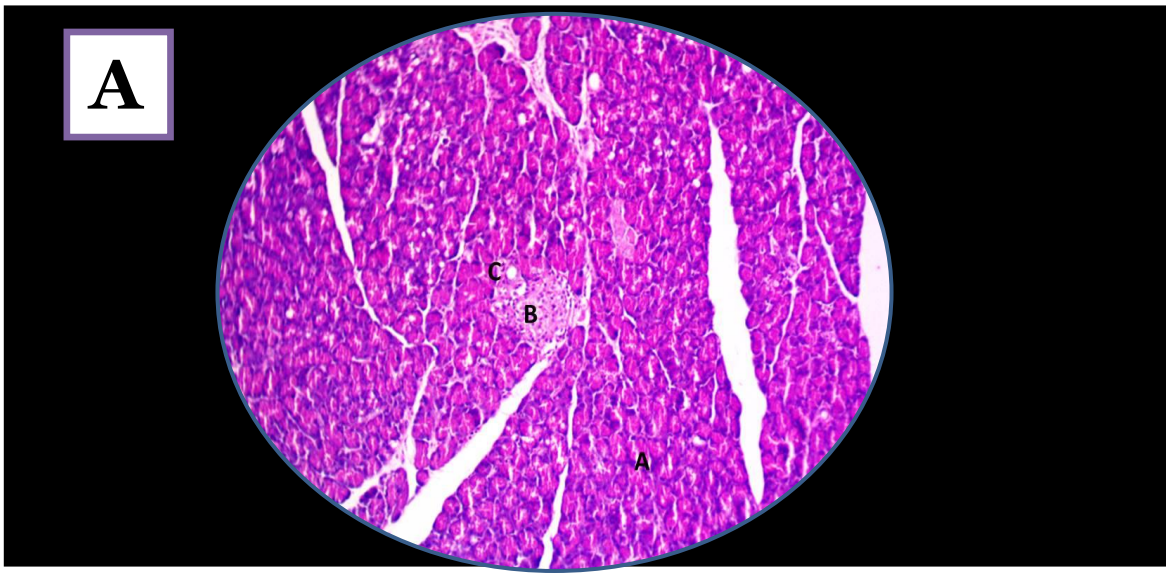
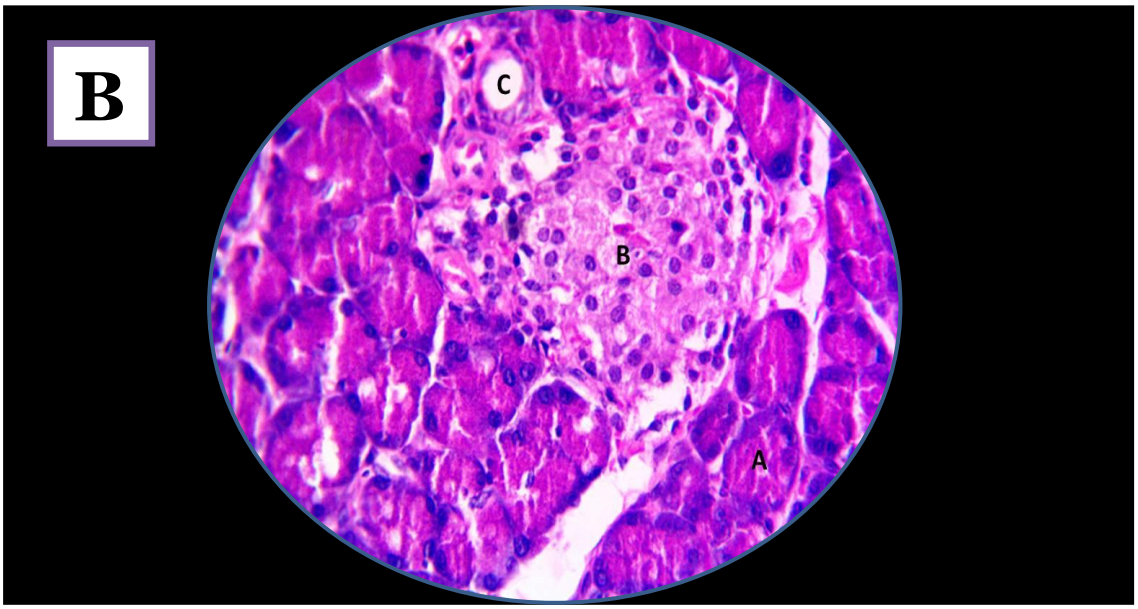


Figure (3.22): Photomicrograph of Pancreas of Diabetic Group, shows pancreatitis representing.
(A) Necrosis of pancreatic acini cells. **(B)** Inflammatory infiltration. **(C)** Fibrosis surrounding intralobular duct. **(D)** Congestion of blood vessels, H&E stain, 100X.in A& H&E stain, 400X.in B &C.

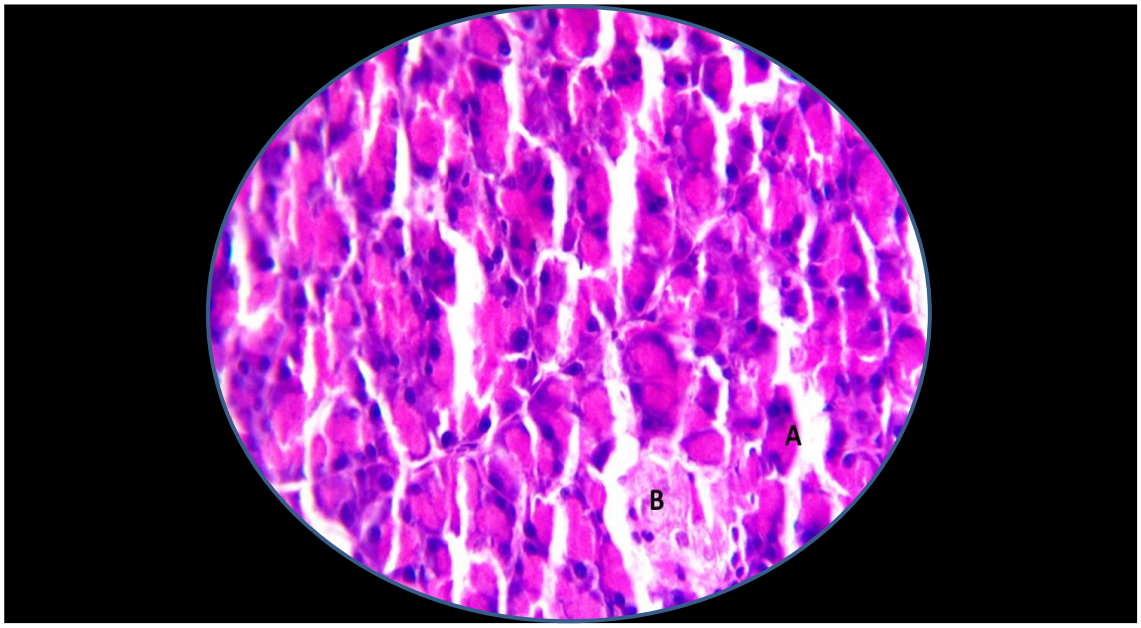
Metformin Group (M_{100}): Showing moderate necrotic changes of the pancreas associated with a mild reduction in the size and number of the islets of Langerhans, figure 3.23 A and B at 21 days while figure 3.24 at 35 day.



Photomicrograph of the pancreas of the treated group (dose 100 for day 21) pancreatic tissue represented by pancreatic acini (A), islets of Langerhans (B) intralobular duct (C). H&E 100X.



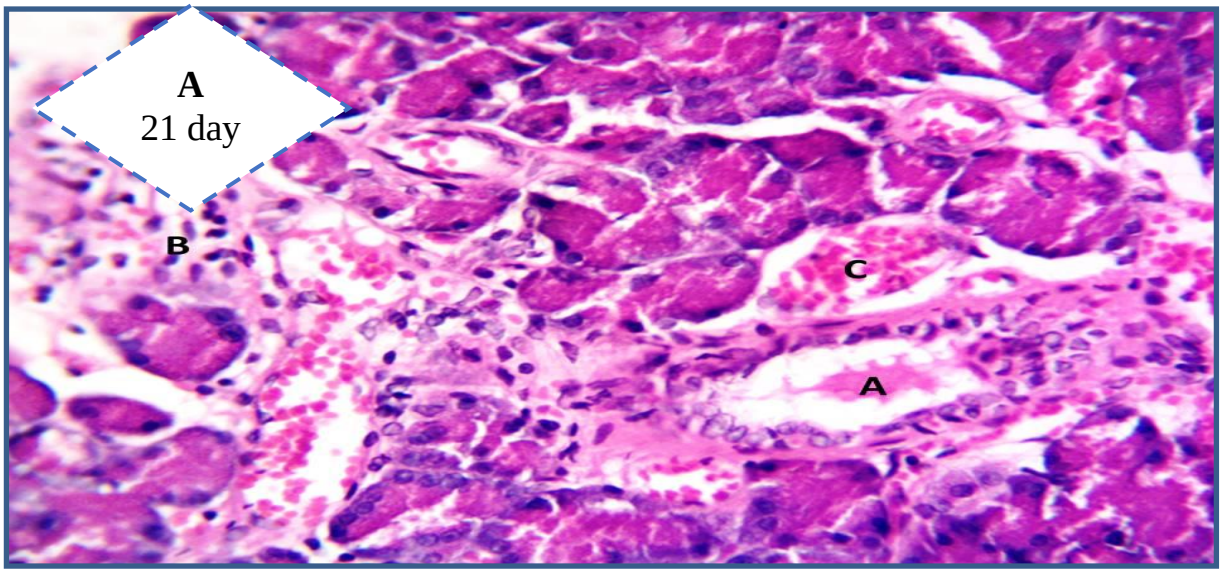
Photomicrograph of histological section of the pancreas of rats treated M100 shows the degenerative changes, Abnormal shape of some pancreatic lobules and some dystrophy and irregularity in histological structure of pancreatic tissue .H&E ×400.



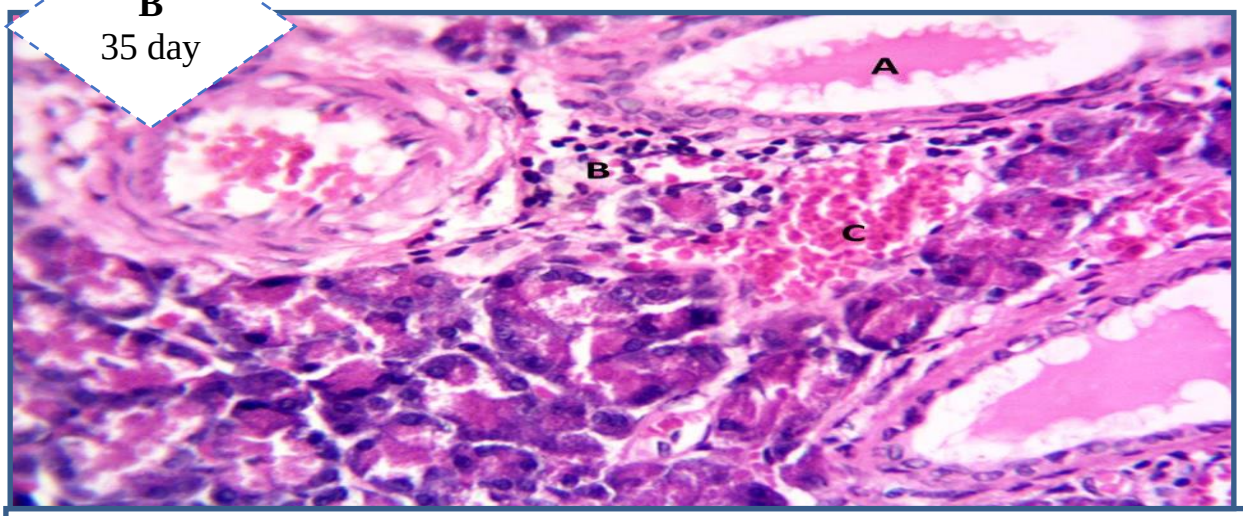
Photomicrograph of the pancreas of the treated group (dose 100 for day 35), shows atrophy of pancreatic acini (A) and islets of Langerhans (B). 400X.

Figure (3.23): The Pancreas of the Diabetic Rats Which Treated with 100 mg /kg /day of Metformin at Day 21.

Metformin Group (M200): Showing mild reduction of necrotic changes of the pancreas and normal architecture of pancreatic acini (figure 3.24) after 21-day treatment (A) and 35-day treatment (B).



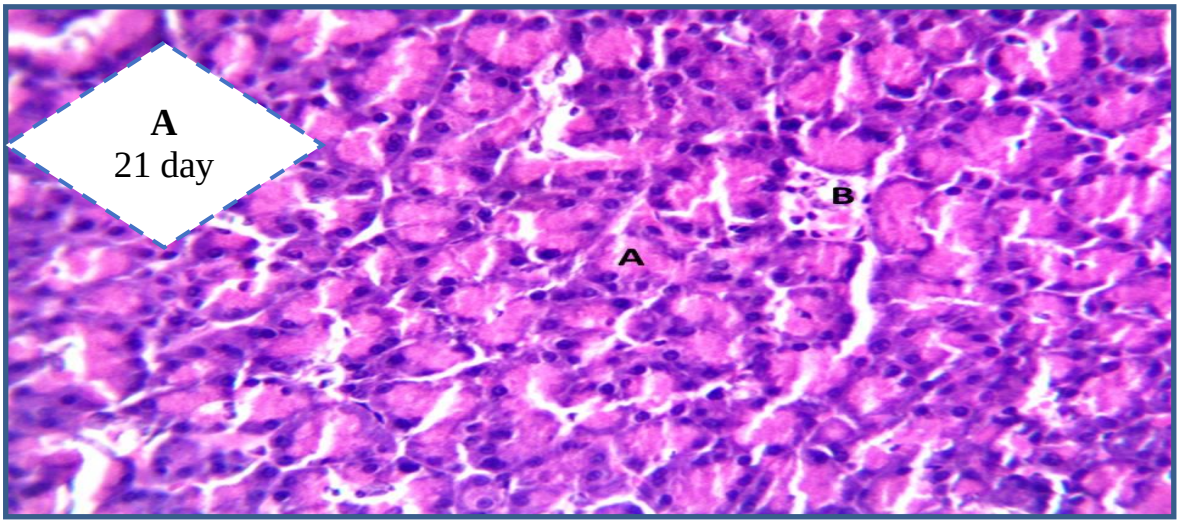
Photomicrograph of the pancreas of the treated group (dose 200 for day 21) shows normal architecture of pancreatic acini (A), mild infiltration of inflam[illegible]atory cells (B) congestion of blood vessels (C).400X.



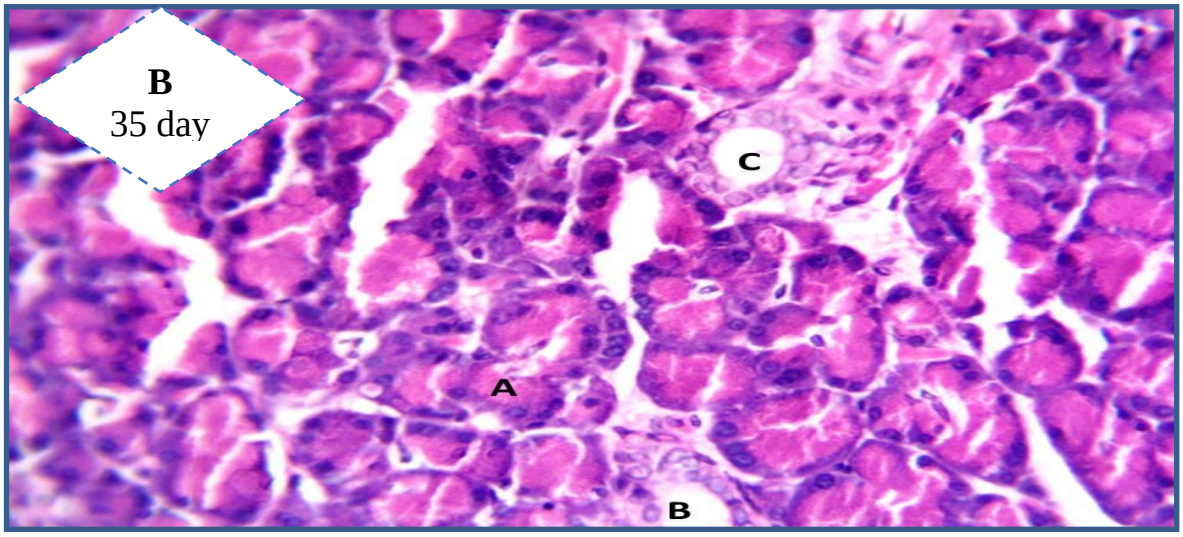
Photomicrograph of the pancreas of the treated group (dose 200 for 35day) shows normal architecture of pancreatic acini (A), mild infiltration of inflammatory cells (B), and congestion of blood vessels (C). H&E stain, 400X.

Figure (3.25): The Pancreas of the Diabetic Rats which Treatment with 200mg /kg /day of Metformin.

Metformin Group (M_{300}): Showing normal architecture of pancreatic tissue representing by pancreatic acini, intralobular duct As figure (3.26) after 21-day (A) and 35 day (B)



Photomicrograph of the pancreas of the treated group (dose 300 day21), shows the normal architecture of pancreatic tissue represented by pancreatic acini (A), and intralobular duct (B). H&E stain, 400X.



Photomicrograph of the pancreas of the treated group (dose 300 day 35), shows the normal architecture of pancreatic tissue represented by pancreatic acini (A), intralobular duct (B), and interlobular duct (C). H&E stain, 400X.

Figure (3.26): The Pancreas of the Diabetic Rats that Treatment with 300mg /kg / day of Metformin

3.1.7.1.B: Histopathological Findings on Liver Tissue

Control group: Observation of the hepatic tissue of the control group rats showed histological features like radially arranged hepatocytes around the central vein. The cells have an acidophilic cytoplasm and rounded central vesicular euchromatic nuclei with well-defined nucleoli. The hepatocyte plates are separated by thin-walled blood sinusoids lined by flat endothelial cells (figure 3.27).

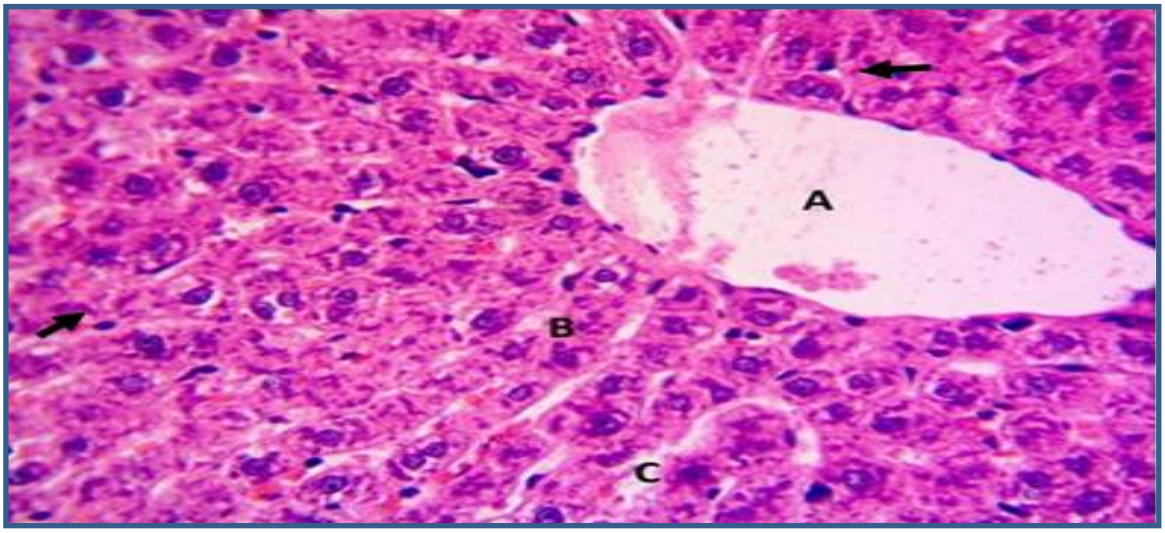
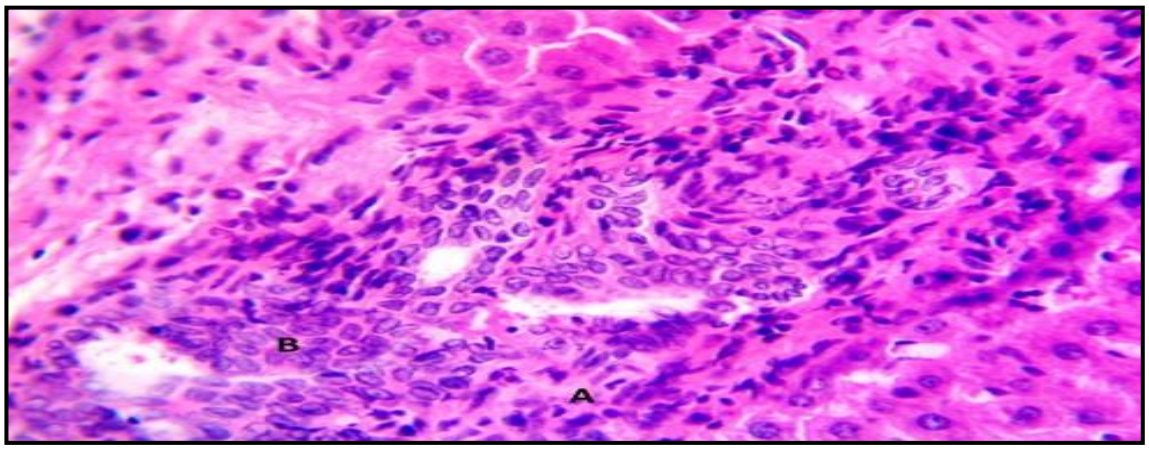
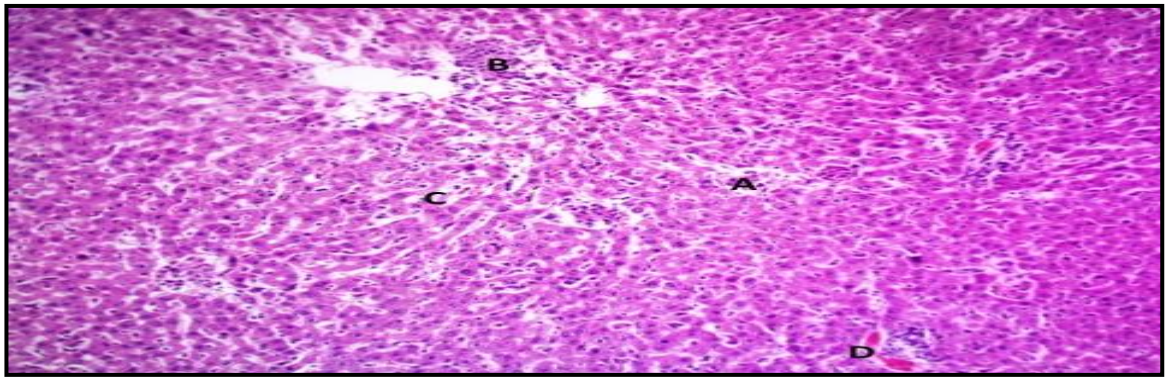


Figure (3.27): Photomicrograph of liver of healthy control group A, shows the normal architecture of hepatic tissue representing by central vein (A), portal area (B) and hepatocytes (C).
H&E stain, 400X

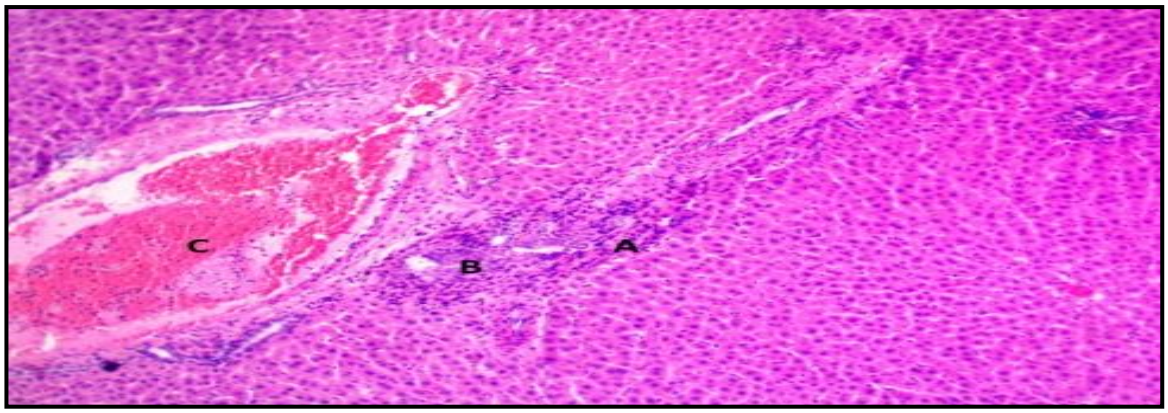
Diabetic group: The section of the hepatic tissue of untreated diabetic control rats showed an increase in apoptotic hepatocytes (shrunken and dark-stained cells with small degenerated nuclei) as figure (3.28).



(A) Photomicrograph of liver of diabetic group shows **(A)** inflammatory infiltration in the portal area (**B)** Hyperplasia of bile duct cells H&E stain, 400X



(B) Photomicrograph of liver of diabetic group shows (A)necrotic foci of hepatocytes (B)Inflammatory infiltration in surrounding (C) Dilation of sinusoids (D) congestion of blood vessels .H&E stain, 100X.

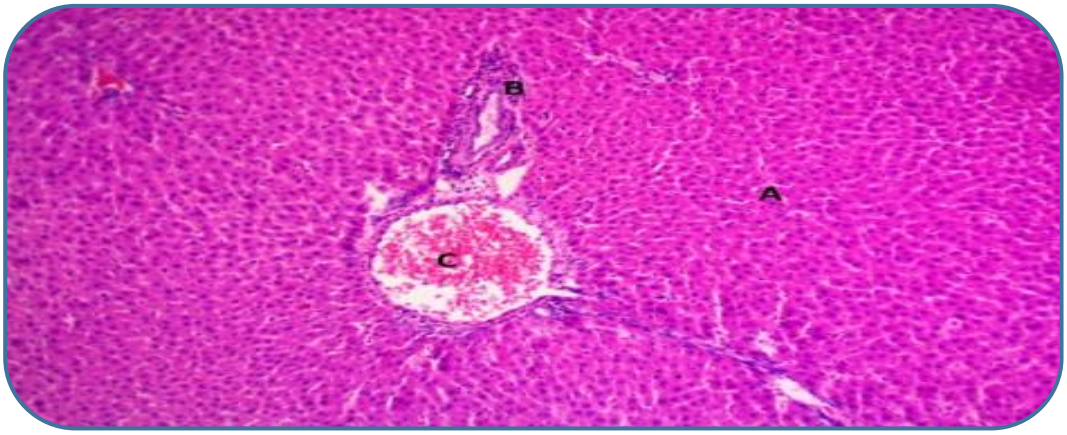


(C) Photomicrograph of liver of diabetic group shows necrotic foci of hepatocytes (A) Inflammatory infiltration in surrounding (B) Dilation of sinusoids (C) Congestion of blood vessels (D) H&E stain, 100X

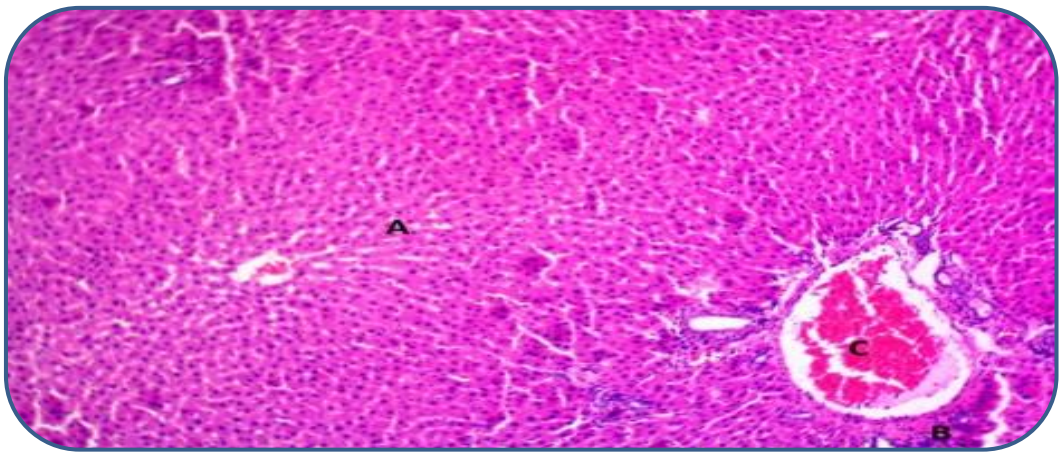
Figure (3.28): Photomicrograph of Liver Diabetic rats group A , B and C.

Metformin Group (M_{100}): Showing a moderate focal infiltration of inflammatory cells (figure 3.29).

Metformin Group (M_{100})



Photomicrograph of liver of treated group day 21, shows the normal architecture of hepatocytes (A), mild infiltration of inflammatory cells in the portal area (B) and congestion of blood vessels (C). H&E stain, 100X.

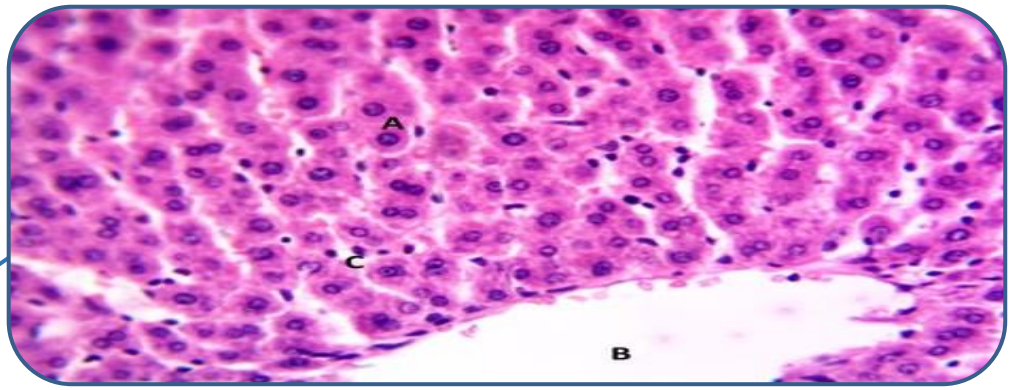


Photomicrograph of liver of treated group (dose 100 day 35) shows normal architecture of hepatocytes (A) mild infiltration of inflammatory cells in portal area (B) congestion of blood vessels (C). H&E stain, 100X.

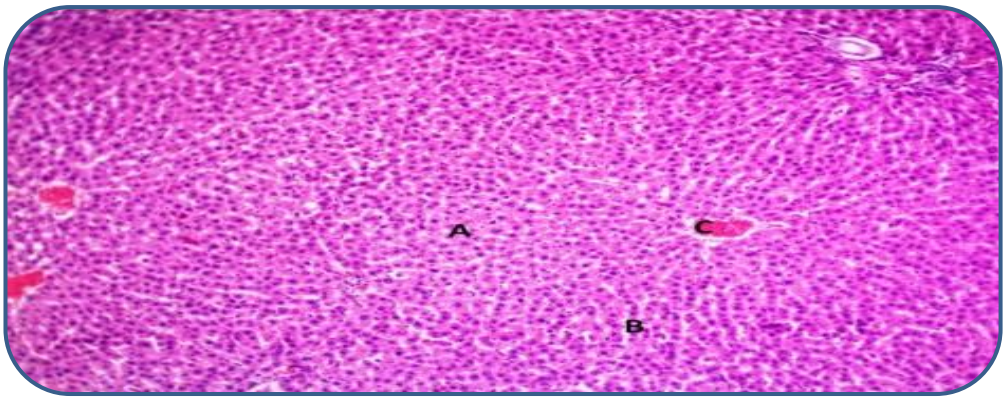
Figure (3.29): The Liver of the Diabetic Rats that Treatment with 100 mg /kg /day of Metformin

Metformin Group (M_{200}): Showing a mild focal infiltration of inflammatory cells Illustration figure (3.30).

Metformin Group (M_{200})



Photomicrograph of liver of treated group (dose 200 at day21) shows the normal architecture of hepatocytes (A), central vein (B) increased number of Kupffer cells (C). H&E stain, 100X.



Photomicrograph of liver of treated group (dose 200 at day 35) shows normal architecture of hepatocytes (A), sinusoids (B) and congestion of central veins (C). H&E stain, 100X.

Figure (3.30): The Liver of The Diabetic Rats That Treatment with 200 mg /kg /day of Metformin.

Metformin Group (M_{300}): Shows normal architecture of hepatocytes demonstrated figure (3.31)

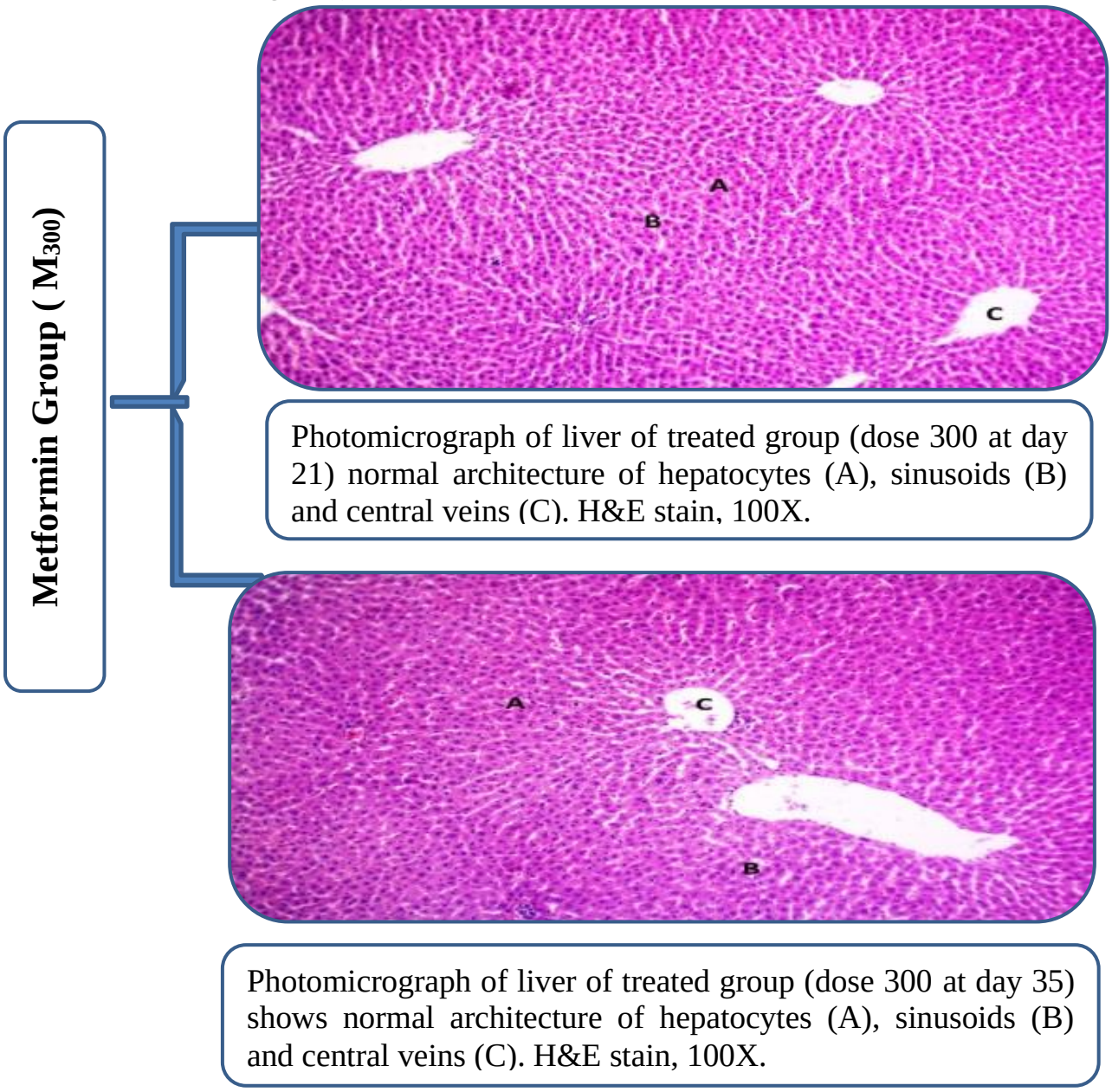


Figure (3.31): The Liver of The Diabetic Rats That Treatment with 200 mg /kg /day of Metformin

3.1.8.2: Histological analysis results:

3.1.8.2.A: Pathological Change In Pancreas

The pathological score of the pancreas, which was reported, showed a statistical difference between groups on days 21 and 35 of the experiments, as shown in table (3.14) and figure (3.31).

Table (3.14): Demonstrate The Pathological Score of Pancreas.

Days	Group	Mean ± S.E.M
Day 21	Control Group	**0.340 ± 0.037**
	Diabetic Group	**4.332 ± 0.092**
	Metformin group (M 100)	**2.452 ± 0.150**
	Metformin group (M 200)	**1.832 ± 0.393**
	Metformin group (M 300)	**1.432 ± 0.349**
P-Value	**0.001****	
Day 35	Control Group	**0.34 ± 0.021**
	Diabetic Group	**4.492 ± 0.181**
	Metformin group (M 100)	**2.152 ± 0.170**
	Metformin group (M 200)	**1.632 ± 0.296**
	Metformin group (M 300)	**1.032 ± 0.470**
P-Value	**0.000****	

(*): significant difference *p*-value < 0.05, highly significant (**): data not fit with normal distribution, statistical analysis by kurskal-wallis non-parametric with descriptive mean and

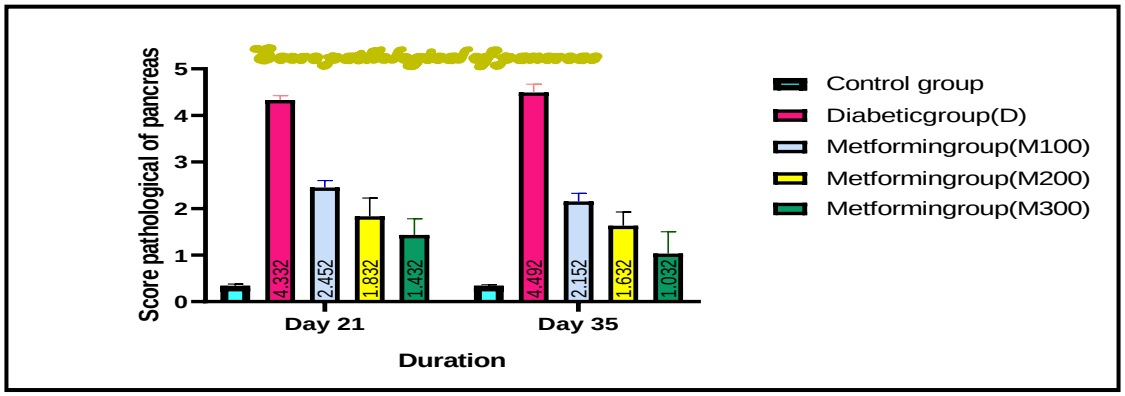


Figure (3. 32): Shows The Pathological Score of Pancreas.

slandered error of mean..

3.1.8.2.B: Inflammation Findings:Histopathologists reported inflammation scored blindly, the mean was calculated and settled as a final score for statistical analysis by kurskal-wallis data not fit with normal distribution was showed the static difference between five groups at day 21 and day 35 as a table (3.15) and figure (3.33).

.15): Demonstrated Inflammation Score of Liver. **3Table (**

Days	Group	Mean ± S.E
Day 21	Control Group	0.380 ± 0.096
	Diabetic Group	3.972 ±0.170
	Metformin group (M 100)	2.712± 0.124
	Metformin group (M 200)	2.312± 0.351
	Metformin group (M 300)	1.632 ±0.323
P-Value	**0.001****	
Day35	Control Group	0.300 ± 0.077
	Diabetic Group	4.000 ± 0.308
	Metformin group (M 100)	2.112 ± 0.193
	Metformin group (M 200)	1.712 ± 0.476
	Metformin group (M 300)	1.232 ± 0.398
P-Value	**0.001****	

(*): Significant difference *p*-value < 0.05, ** Highly significant *p*-value < 0.01.

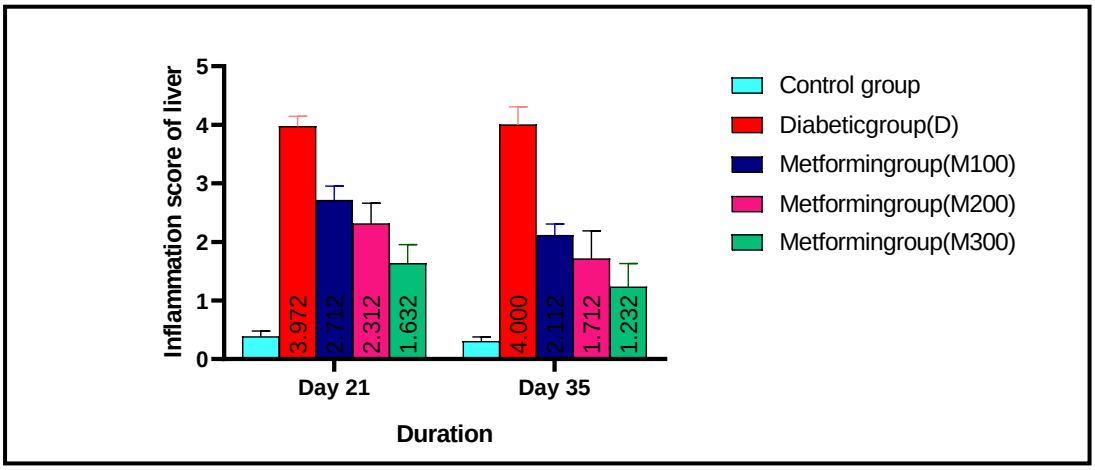


Figure (3.33): Demonstrate Inflammation Score of Liver.

3.2. Part Two Related Effect of Metformin on Cancer cells line.

3.2.1 Evaluation of Cytotoxicity of Metformin on Human Cervical Cancer Cell Lines (Hela) After 48and 72hr.

The result of the current study showed Metformin's cytotoxic effect on the human cervical cell line for 48 hours determined by MTT testing as shown as table (3.16). The statistical designation of three replicates observed a significant difference as actual points. Results demonstrated the decline in the cell survival rate (86.093, 49.206, 26.466, 25.097, 24.869, 24.185, 23.044%) with the increment concentrations of metformin in HeLa cell line (5,10,25,35,45,65,130 μM) that different significantly to 0 concentration (before add metformin).

Table (3.16): Cytotoxic Effect of Metformin on Viability of Cervical Cancer Cells Lines **(**Hela) after 48 hrs.

Cytotoxic effect of Metformin On Human Cervical Cancer Cells Lines (Hela) After 48 hrs.									
Met. Conc. (μM)	**0**	**5**	**10**	**25**	**35**	**45**	**65**	**130**	***P*-Value**
Mean Viability	97.517 D	86.093 C	49.206 B	26.466 A	25.097 A	24.869 A	24.185 A	23.044 A	**0.000****
SEM	0.324	1.089	1.747	0.235	0.235	0.294	0.256	0.176	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group more than 0.05.
- ✓ Analysis by one way –ANOVA, Post –Hoc Duncan′s test.
- ✓ Significant differences at p<0.05, ** Highly significant level <0.01.
- ✓ Differentletter = significantly difference, same letter= non –statically difference.

After 72 hours results the cell survival rate declines with the increasing concentrations of metformin in HeLa cell line reach to 73.132, 43.057, 22.452,

15.649, 15.305, 15.279, 15.081 µM) significantly. The decline was significant at higher concentrations as shown in table (3.17).

Table (3.17): Cytotoxic effect of Metformin on Viability of Cervical cancer Cells Line (Hela) after 72 hrs.

Cytotoxic Effect of Metformin on Human Cervical Cancer Cell Line (Hela) After 72 hrs.									
Met. Conc. µM	**0**	**5**	**10**	**25**	**53**	**45**	**65**	**130**	**P-Value**
Mean Viability	97.517 D	73.132 C	43.057 BC	22.452 B	15.649 A	15.305 A	15.279 A	15.081 A	**0.000****
SEM	0.324	1.372	1.602	0.754	0.272	0.135	0.251	0.1677	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group more than 0.05.
- ✓ Analysis by one way –ANOVA, Post –Hoc Duncan′s test.
- ✓ * Significant differences at p<0.05, ** Highly significant level <0.01.
- ✓ Different letter = significantly difference, same letter= non –statically difference

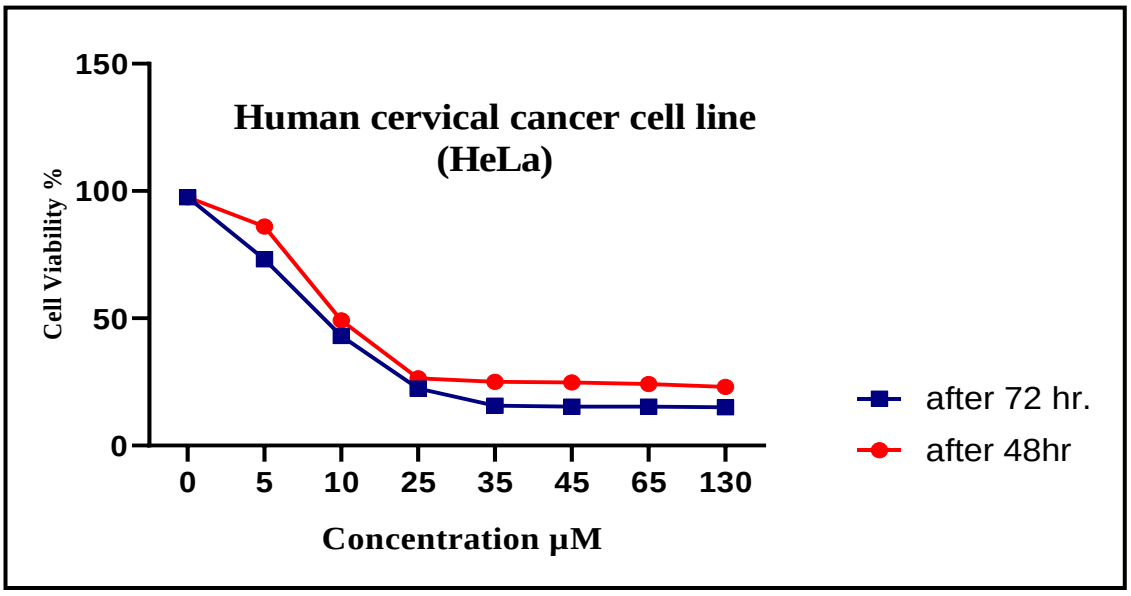


Figure (3.34): Cytotoxic Effect of Metformin on Human Cervical Cancer Cell Line (Hela) cell lines after 48 and72 hrs.

In Figure (3.34), metformin reduced cell viability in the cervical cancer cell line (Hela) in a time and concentration-dependent manner. Cell viability was

determined by the MTT assay in Hela (incubated for 48 and 72 hours with (0-130 μM) concentrations of metformin.

3.2.2 Evaluation of Cytotoxicity of Metformin on Breast Cancer Cell Line (AMJ3) After 48 and 72Hrs:

The viability of AMJ3 cells was assessed by MTT. As shown in Table (3.18), AMJ3 viability was clearly reduced in a dose-dependent manner since the viability decreased significantly to 31.130% when the concentration was increased to 130 μM after 48 hrs.

Table (3.18): Cytotoxic Effect of Metformin on Viability of Breast Cancer Cell Line (AMJ3) After 48 Hrs.

Cytotoxic Effect of Metformin On Breast Cancer Cell Line AMJ3 After 48 hrs.									
Conc. μM	**0**	**5**	**10**	**25**	**35**	**45**	**65**	**130**	***P*-Value**
Mean Viability	97.835 E	95.547 DE	95.373 DE	90.205 C	84.657 C	47.219 B	45.102 B	31.130 A	**0.000****
SEM	0.885	1.886	1.937	0.753	2.075	1.787	2.144	1.1425	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group more than 0.05.
- ✓ Analysis by One Way ANOVA, Post –Hoc Duncan′s test.
- ✓ * Significant differences at $p<0.05$, ** Highly significant level <0.01.
- ✓ Different letter = significantly difference, same letter= non –statically difference

After 72 hours, the cell viability declines with the increasing concentrations of metformin as table (3.19).

Table (3.19): Cytotoxic Effect of Metformin on Viability of Breast Cancer Cell Line (AMJ3) After 72 Hrs.

Cytotoxic Effect of Metformin on Breast Cancer Cell Line AMJ3 After 72 hrs.									
Conc. µM	**0**	**5**	**10**	**25**	**35**	**45**	**65**	**130**	***P*-Value**
Mean Viability	97.835 E	93.967 E	33.022 De	22.400 Cd	22.295 C	22.084 B	21.979 A	21.453 A	**0.000****
SEM	0.885	2.971	0.297	0.128	0.196	0.223	0.074	0.128	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group more than 0.05.
- ✓ Analysis by One Way ANOVA, Post –Hoc Duncan′s test.
- ✓ * Significant differences at p<0.05, ** Highly significant level <0.01.
- ✓ Different letter = significantly difference, same letter= non –statically difference.

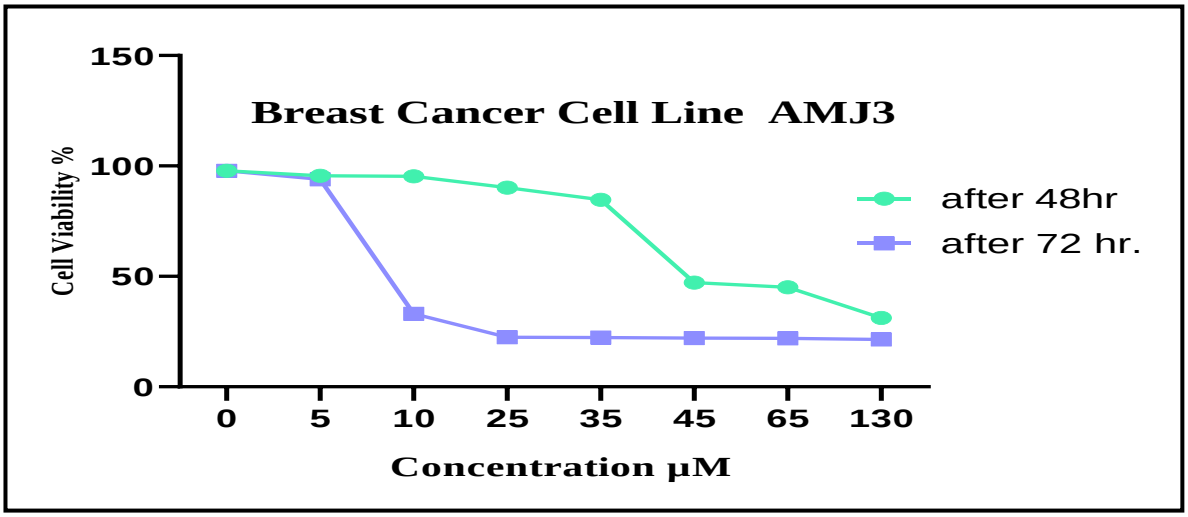


Figure (3.35): Cytotoxic Efect of Metformin on Breast Cancer Cell line (AMJ3) After 48 And 72 Hrs.

Figure (3.35) we showed the cytotoxic effect of Metformin reduced in a dose-time dependent manner with viability reducing to 31.130% at 48 while at 72 hrs., the viability reached 21.453 at a dose of 130 μM..

3.2.3: Evaluation of Cytotoxicity of Metformin on the HCAM Cell Line After 48&72Hrs.

At 48 hrs. of incubation, the viability was reduced to (44.466 %) at high metformin concentration, which is statistically significant as compared with the viability at zero concentration (before adding metformin (99.320%), table 3.20).

Table (3.20): Cytotoxic Effect of Metformin on Liver Cancer Cell Line (HCAM) After 48 Hrs.

Cytotoxic effect of metformin on liver cancer cell line HCAM after 48 hours.									
Conc. μM	**0**	**5**	**10**	**25**	**35**	**45**	**65**	**130**	***P*-Value**
Mean Viability	99.3201 E	98.004 E	92.941 E	78.463 D	73.503 CD	69.655 C	56.089 B	44.446 A	**0.000****
SEM	0.490	0.874	2.257	2.482	1.388	2.825	3.061	2.170	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group more than 0.05.
- ✓ Analysis by One Way ANOVA, Post –Hoc Duncan′s test.
- ✓ * Significant differences at p<0.05, ** Highly significant level <0.01.

HCAM viability was significantly reduced after 72 hours of incubation in a dose-dependent manner because it declined to 36.838 % when the concentration was increased to 130 μM, table (3.21)

Table (3.21): Cytotoxic Effect of Metformin on Liver Cancer Cell Line (HCAM) After 72 Hrs.

Cytotoxic effect of metformin on liver cancer cell line HCAM after 72 hrs.									
Conc. µM	**0**	**5**	**10**	**25**	**35**	**45**	**65**	**130**	***P*-Value**
Mean Viability	99.320 D	71.890 C	70.814 C	55.058 B	54.513 B	53.641 B	52.224 B	36.838 A	**0.000****
SEM	0.490	0.295	1.988	1.582	0.708	1.918	0.712	1.462	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group more than 0.05.
- ✓ Analysis by One WayANOVA, Post –Hoc Duncan′s test.
- ✓ * Significant differences at p<0.05, ** Highly significant level <0.01.

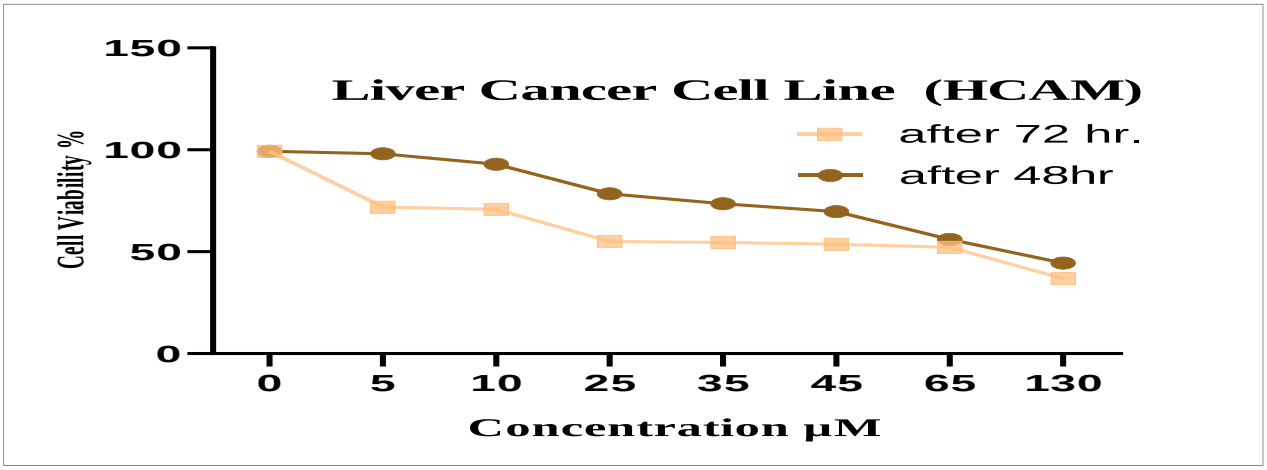


Figure (3.36): Cytotoxic Effect of Metformin on Liver Cancer Cell Line (HCAM) After 48and 72 Hrs.

Figure (3.36) showed the viability of metformin on HCAM for 48 and 72 hrs. of incubation. HCAM cell viability was clearly reduced in a time–dependent manner. In 48 hrs. high concentration reduced viability to 44.466%, significantly less than 99.320% at concentration before adding the metformin to a plate

containing an HCAM cell line. In 72 hrs, HCAM viability was significantly decreased to 36.838 when the concentration was increased to 130 µM.

3.2.4: Evaluation Cytotoxicity of Metformin on Glioblastoma Cancer Cell A172 After 48&72hrs.

As shown as table (3.22) reduced significantly to 74.258 % when the concentration was increased to 130 µM after 48 hrs. While, table (3.23) showed a statistically significant reduction of viability to 47.820 % after 72 hrs.

Table (3.22): Cytotoxic Effect of Metformin on Glioblastoma Cancer Cell A172 After 48 Hrs**.**

Cytotoxic Effect of Metformin on A172 Cell Line After 48 Hrs.									
Conc. µM	**0**	**5**	**10**	**25**	**35**	**45**	**65**	**130**	***P*-Value**
Mean Viability	97.761 E	96.892 E	93.175 D	91.345 D	87.301 C	86.437 BC	83.988 B	74.258 A	**0.000****
SEM	1.022	0.727	0.664	0.305	1.197	0.404	1.001	1.054	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group more than 0.05.
- ✓ Analysis by One Way ANOVA, Post –Hoc Duncan′s test.
- ✓ * Significant differences at p<0.05, ** Highly significant level <0.01.

Table (3.23): Cytotoxic effect of metformin on Glioblastoma Cancer Cell A172 After 72 Hrs.

Cytotoxic Effect of Metformin on A172 Cancer Cell Line After 72 Hrs.									
Conc. µM	**0**	**5**	**10**	**25**	**35**	**45**	**65**	**130**	***P*-Value**
Mean Viability	98.375 F	95.873 F	86.006 E	75.031 D	75.645 D	64.500 C	55.305 B	47.820 A	**0.000****
SEM	0.911	0.865	1.401	0.361	1.366	1.855	1.195	0.618	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group more than 0.05.
- ✓ Analysis by One WayANOVA, Post –Hoc Duncan′s test.
- ✓ * Significant differences at p<0.05, ** Highly significant level <0.01.

Figure (3.37) demonstrated viability effect of metformin on A172 cell was decreased more significantly with greater metformin doses and longer treatment durations, a significant decrease in cell viability (*P* = 0.000) at a 48-hour reach to (74.258 µM), while higher concentrations of Metformin demonstrated highly significant decreases in viability (47.820) at 72 hrs.

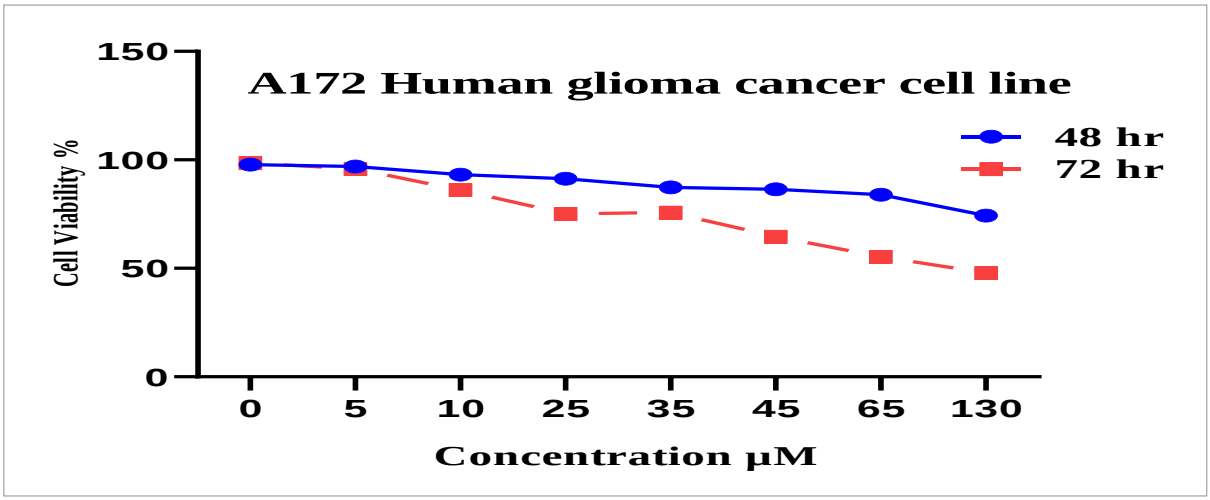


Figure (3.37): Cytotoxic Effect of Metformin on A172 Cancer Cell Line After 48 &72 Hours.

3.2.5: Evaluation Cytotoxicity of Metformin on HBL100 Normal Breast Cell.

Results showed that metformin at concentrations130 μM causes a significant decrease in the viability of normal cells, while the other concentrations didn't cause any significant effect compared to the 0 concertation as shown in table (3.24).

Table (3.24): Cytotoxic Effect of Metformin On Normal Breast Cell HBL100 After 72 Hrs.

Cytotoxic Effect of Metformin on HBL100 After 72 Hrs.									
Conc. μM	**0**	**5**	**10**	**15**	**35**	**45**	**65**	**130**	***P*-Value**
Mean Viability	99.636 B	98.848 B	98.219 B	95.547 B	94.329 AB	94.132 AB	93.688 AB	86.518 A	**0.022***
SEM	0.086	3.721	4.539	1.886	1.735	2.322	1.373	2.498	

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk data in all group more than 0.05.
- ✓ Analysis by One Way ANOVA, Post –Hoc Duncan′s test.
- ✓ * Significant differences at $p<0.05$, ** Highly significant level <0.01.

Table (3.25) demonstrated cytotoxic effect of metformin on HeLa, AMJ3, HCAM, A172 cancer cell line, and HBL100 normal cell lines at 72hrs.The results appeared the significant reduction in viability of HeLa was greater than that of other lines at130 μM.

Table (3.25): Effect of metformin on HeLa, AMJ3, HCAM, A172 cancers cells lines and HBL100 normal cell lines after 72 hours of exposure to metformin

Cytotoxic effect of metformin on different cell line after 72 hrs.						
Met. Conc. µM	**HBL100**	**HeLa**	**AMJ3**	**HCAM**	**A172**	***P*-Value**
0	99.636±0.0869 A	97.563±0.3703 A	97.835 ±0.885 A	99.070 ±0.233 A	98.375±0.911 A	0.273
5	98.848± 3.721 B	73.132±1.372 A	93.967 ± 2.971 B	71.876 ±0.295 A	95.873 ±0.865 B	**0.000****
10	98.219 ±4.539 E	43.057± 1.602 C	33.022 ± 0.297 A	70.814±1.988 B	86.006±1.401 D	**0.000****
25	95.547 ±1.886 D	22.452±0.754 A	22.400 ±0.128 A	55.058±1.582 B	75.645± 1.366 C	**0.000****
35	94.329 ±1.735 E	15.649±0.272 A	22.295 ± 0.196 B	54.513±0.708 C	75.031±0.361 D	**0.000****
45	94.132 ±2.322 E	15.305 ±0.135 A	22.084± 0.223 B	53.641±1.918 C	64.500±1.855 D	**0.000****
65	93.688 ±1.373 D	15.279 ±0.251 A	21.979 ± 0.074 B	52.224±0.712 C	55.305±1.195 C	**0.000****
130	86.518±2.498 E	15.081 ±0.167 A	21.453±0.128 B	36.838±1.462 C	47.820±0.618 D	**0.000****

- ✓ All data before enter to analysis to fit with normal distribution by tests of normality Kolmogorov-Smirnov, Shapiro-Wilk) data in all group more than 0.05.
- ✓ Analysis by One Way ANOVA, Post –Hoc Duncan′s test.
- ✓ *Significant differences at p<0.05, ** Highly significant level <0.01

3.2.6 Determination of IC50 for Metformin on all cancer cell lines

In table (3.26) and figure (3.38), (3.39), (3.40), (3.41) and (3.42). Results showed IC50 of Hela cells was highly significantly (p=0.000) among cell line Hela < AMJ 13<HCAM< A172< HBL100.

Table (3.26): Evaluation of IC50 for Metformin Among Cancer Cell Lines.

IC50(µM) of Metformin in Cancer Cell Lines.						
Type Cancer cell	**Hela**	**AMJ 13**	**HCAM**	**A172**	**HBL100**	***P* -value**
Mean	7.447 A	9.168 B	10.150 B	28.299 C	48.910 D	**0.000****
SEM	0.281	0.244	0.405	0.298	0.589	

- ✓ Analysis by One Way ANOVA, Post –Hoc Duncan′s test.
- ✓ *Significant differences at p<0.05 , ** Highly significant level <0.01

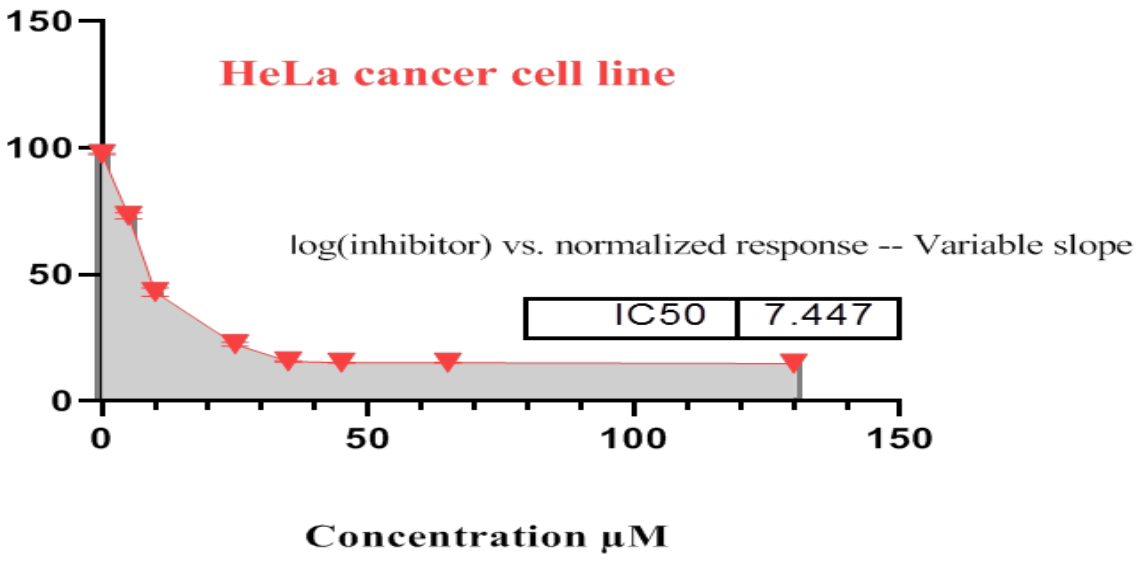


Figure (3.38): Evaluation of IC50 Value for Metformin with Hela Cells.

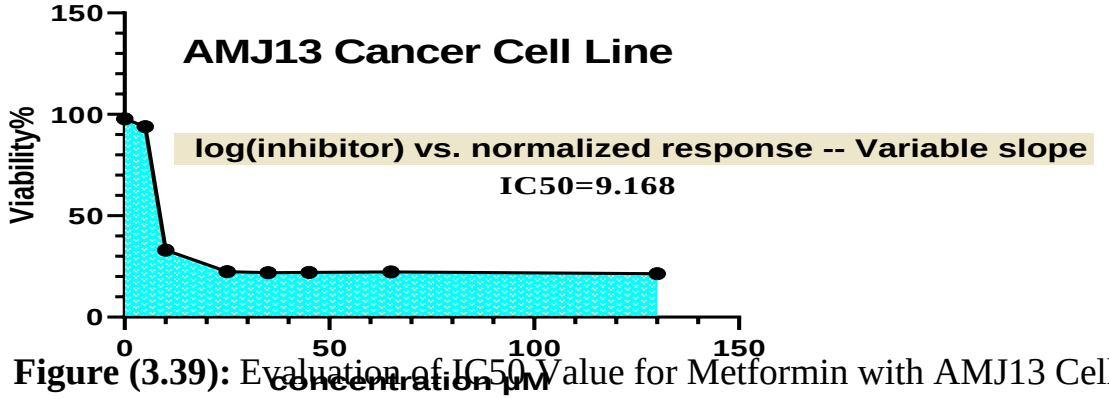


Figure (3.39): Evaluation of IC50 Value for Metformin with AMJ13 Cells.

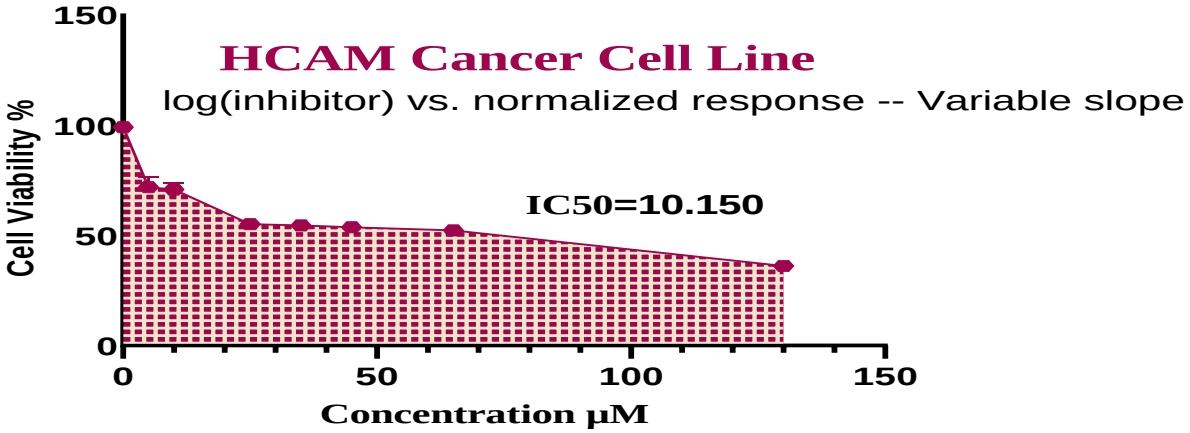


Figure (3.40): Evaluation IC50 Value for Metformin with HCAM Cells.

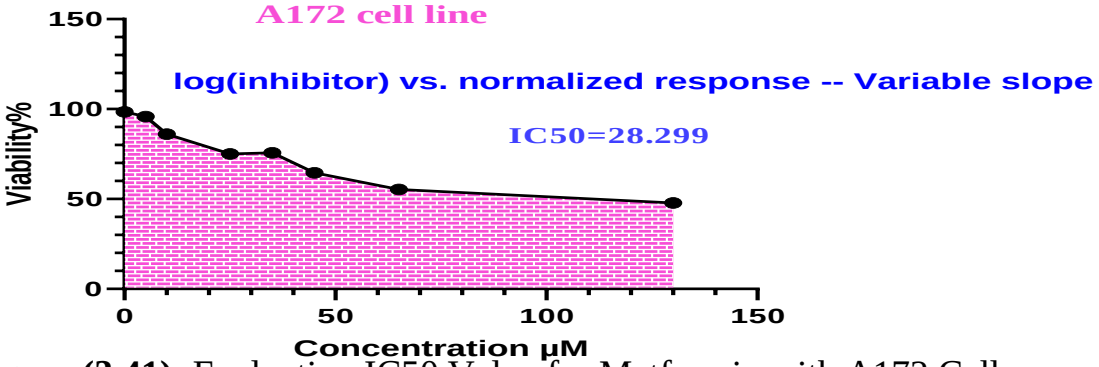


Figure (3.41): Evaluation IC50 Value for Metformin with A172 Cells.

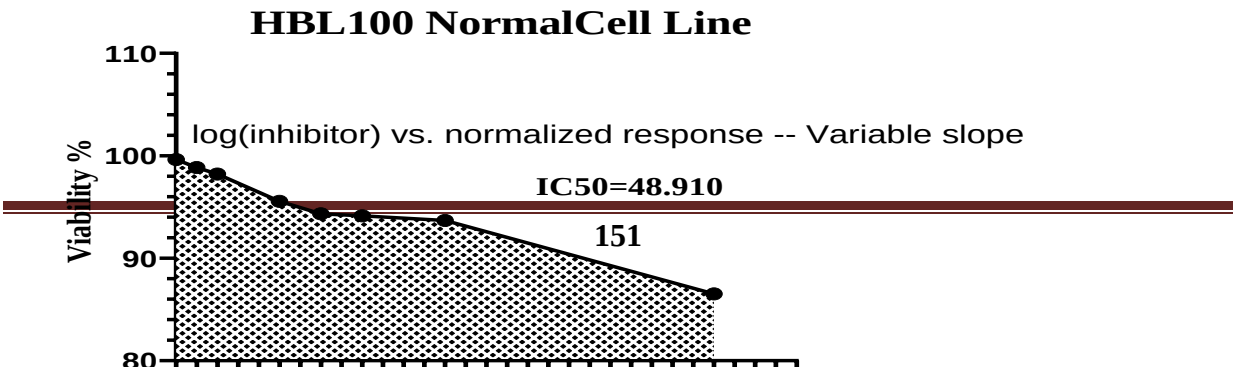


Figure (3.42): Evaluation IC50 Value for Metformin with HBL100 cells.

3.2.7 Comparison The Effect of Metformin with The Same Dose of Paclitaxel (Anti-Tumor) on Cervical Cancer Cells Lines (Hela) After 72Hrs.

Result showed a significant difference at concentration 5,10 and 15 and non-significant difference among other concentration as shown as table (3.27) and figure (3.43).

Table (3.27): Comparison Effect of Metformin with the Same Dose of Paclitaxel on Cervical Cancer Cells lines (Hela) After 72Hsr.

Concentration (µM)	Metformin		Paclitaxel		*P*- value
	Mean	SEM	Mean	SEM	
0	97.563 A	0.3703	98.334 A	0.489	0.184 NS
5	73.132 B	1.372	56.341 A	1.733	**0.000****
10	43.057 B	1.602	28.385 A	2.036	**0.000****
15	22.452 B	0.754	17.442 A	1.131	**0.000****
35	15.649 A	0.272	15.763 A	0.621	0.847 NS
45	15.305 A	0.135	15.059 A	0.537	0.716 NS
65	15.279 A	0.251	14.883 A	0.048	0.275 NS
130	15.081 A	0.167	14.531 A	0.102	0.060. NS

- ✓ **Independent Samples T- test**
- ✓ ***Significant level <0.05, ** Highly significant level <0.01, NS: Non-significant.**

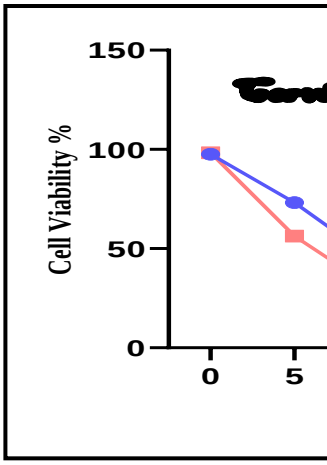


Figure (3.43): Co
Paclitaxel C

8 Com paris on The Effect of Metformin With The Same Dose of Paclitaxel on HBL100 Normal Cell After 72 Hrs.

Table (3.28) showed a comparison of the impact of metformin with the same dose of paclitaxel on normal breast cells after 72 hrs. significant in all concentrations.

Table (3.28): Comparison The Effect of Metformin With The Same Dose of Paclitaxel on HBL100 Normal Breast Cell After 72 Hrs.

Concentration (Mm)	Metformin		Paclitaxel		*P*- value
	Mean	SEM	Mean	SEM	
0	99.636 A	0.086	98.775 A	0.229	0.025 *
5	98.848 B	3.721	66.593 A	3.743	**0.000 ****
10	98.219 B	4.539	45.851 A	0.773	**0.000 ****
15	95.547 B	1.886	32.872 A	0.320	**0.000 ****
35	94.329 B	1.735	32.811 A	0.962	**0.000 ****
45	94.132 B	2.322	32.569 A	0.468	**0.000 ****
65	93.688 B	1.373	31.901 A	0.696	**0.000 ****
130	86.518 B	2.498	31.356 A	0.457	**0.004****

- ✓ **Independent Samples t Test .**
- ✓ ***Significant level <0.05, ** Highly significant level <0.01, NS: Non-significant.**

Figure (3.44) A showed the effect paclitaxel and metformin on HBL100 after 72 hours showed the cytotoxic effect of normal breast cell of paclitaxel rather than metformin.

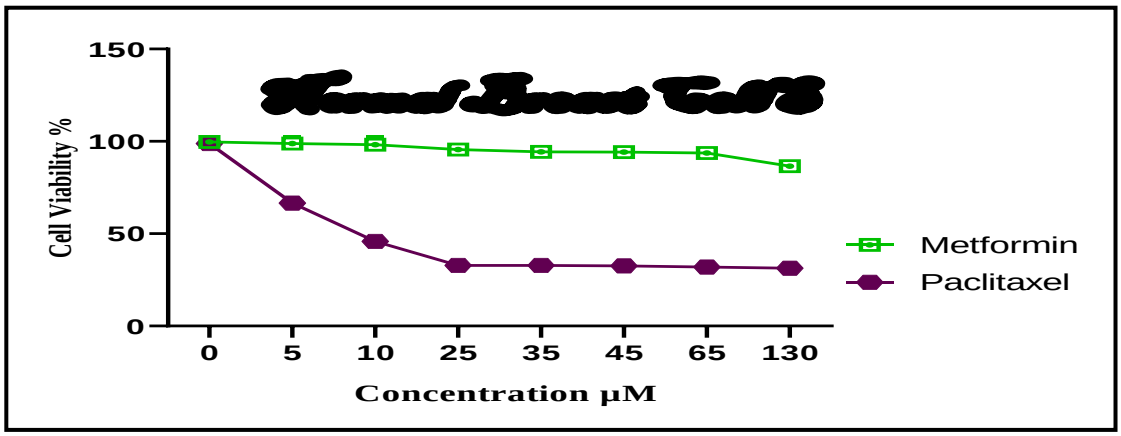


Figure (3.44): Comparison The Effect of Metformin with The Same Dose of Paclitaxel on Normal Cells Lines (HBL100) After 72Hrs..

3.2.9 Morphological Analysis.

Figure (3.45) showed untreated HBL100 cells were grown in monolayer and figure (3.46) demonstrated the Hela untreated.

The microscopic examination by staining with H and E revealed different cytomorphological changes observed on the cells after 72 hrs of metformin on cervical cancer cell lines (Hela) as showed in figure (3.47) the atrophy, spherical and irregular shapes observed in cell lines is one of the hallmarks of the cytopathic effect for cells to keep their shape, the cytoskeleton plays a key role in this, so any change in the organization causes a change in the shape of the cell. if the organization were to change then it would affect the cell's structure. Large spaces appeared in cell cultures as a result of cell atrophy, shrinking, and decomposition. Karyopyknosis is one of the characteristics of apoptotic cells as shown as in. figure (3.48), (3.49) and (3.50).

Other cells were suffering from hyperchromy. In addition to nuclei atrophy in other cells, most cells also suffer from vacuolativ degeneration (cytoplasmic vacuolation). Nucleic fragmentation and the development of apoptotic bodies are both common apoptotic features. There were some cells that looked to be multinucleated, many of the cells of Hela had cytomorphological alterations as shown in figure (3.51).

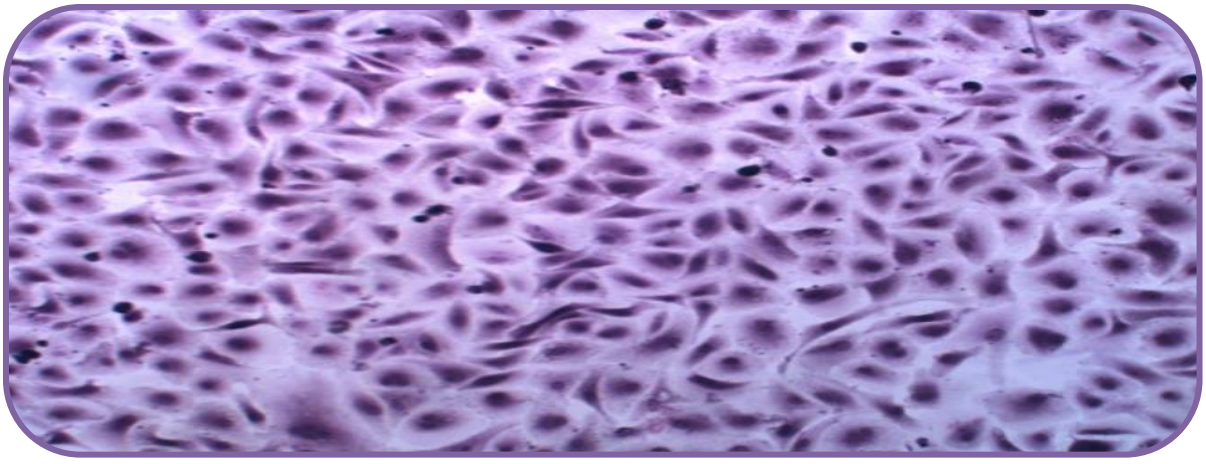


Figure (3.45): Untreated HBL100 cells were grown in monolayer with normal cell lines HBL100 morphology 10 X.

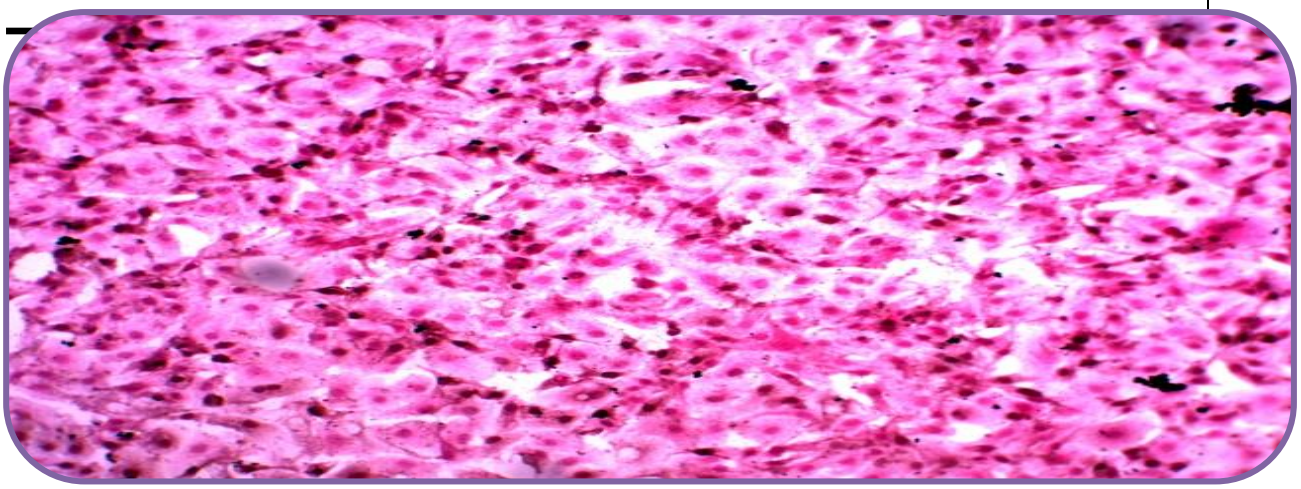


Figure (3.46): Untreated cells were grown in monolayer with Cervical Cancer Cells Lines (Hela) morphology 10 X.

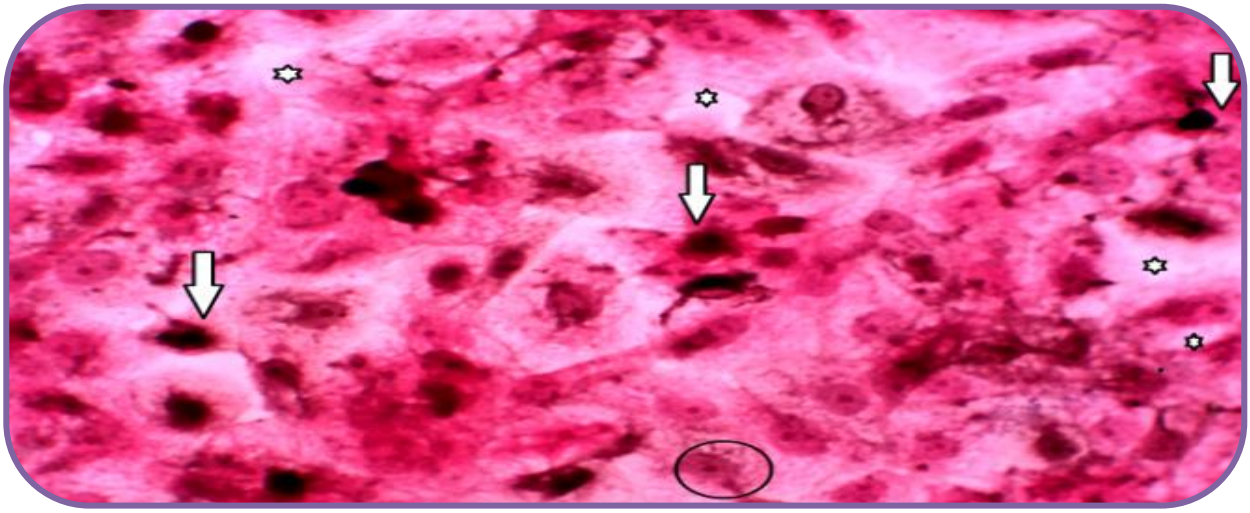


Figure (3.47): Treated cells of metformin on Hela for72hrs, showing change the cells shape (white arrow) decaying some cells (circle) and large spaces between cells (stars). 40X.

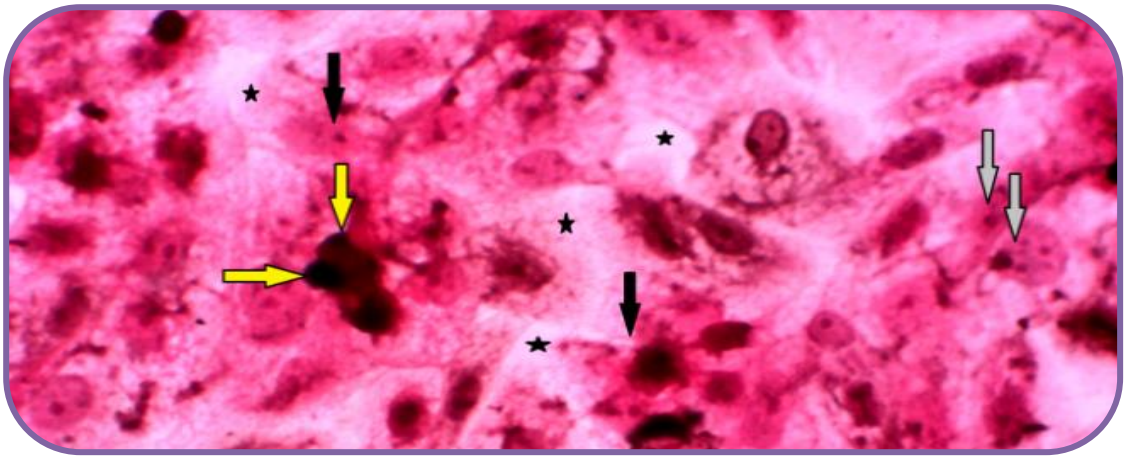


Figure (3.48): Treated cell of metformin on Hela for72hrs, showing change the cells shape decaying some cells (gray arrow) necrosis (black arrow), karyopyknosis (yellow arrow) large spaces between cells. (stars) 40X..

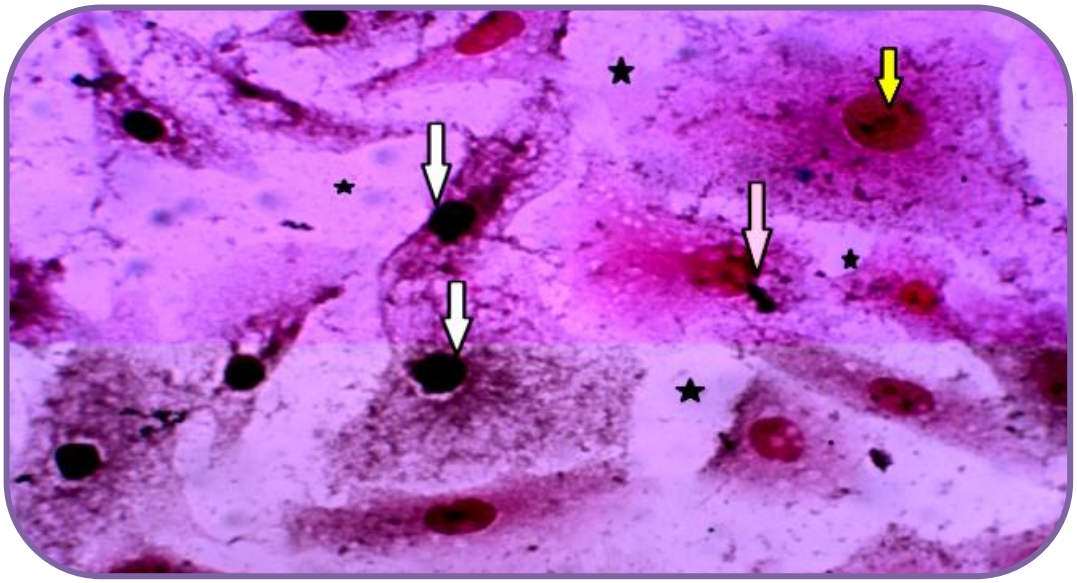


Figure (3.49): Treated cells of metformin on Hela for72hrs (pink head), karyopyknosis (white arrow) show converting cells to spherical-shape yellow arrow) and large spaces between cells (stars), 40 X.

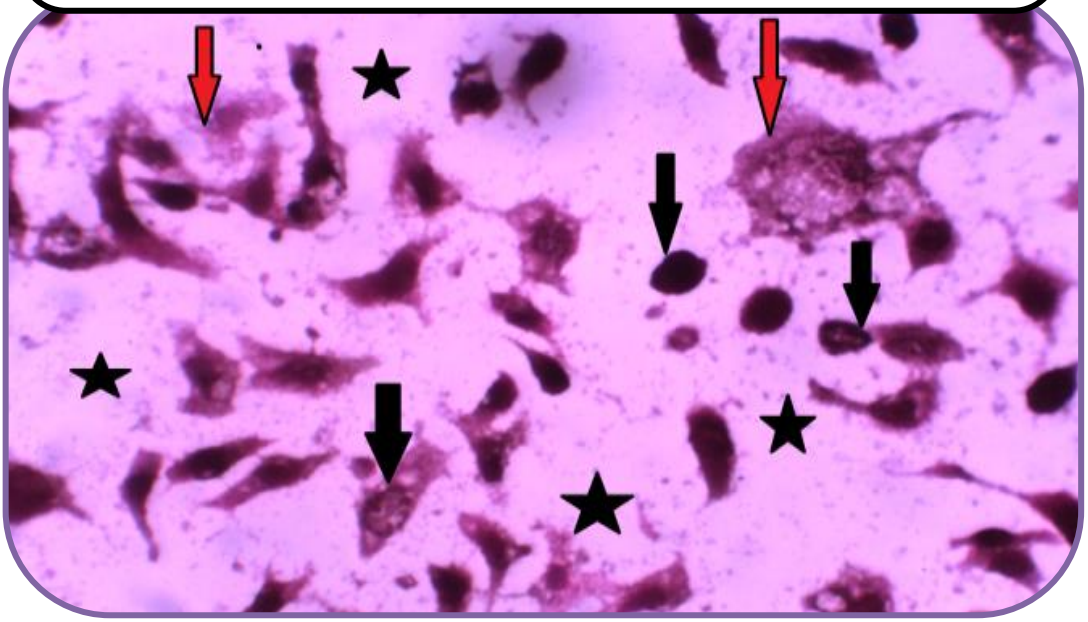
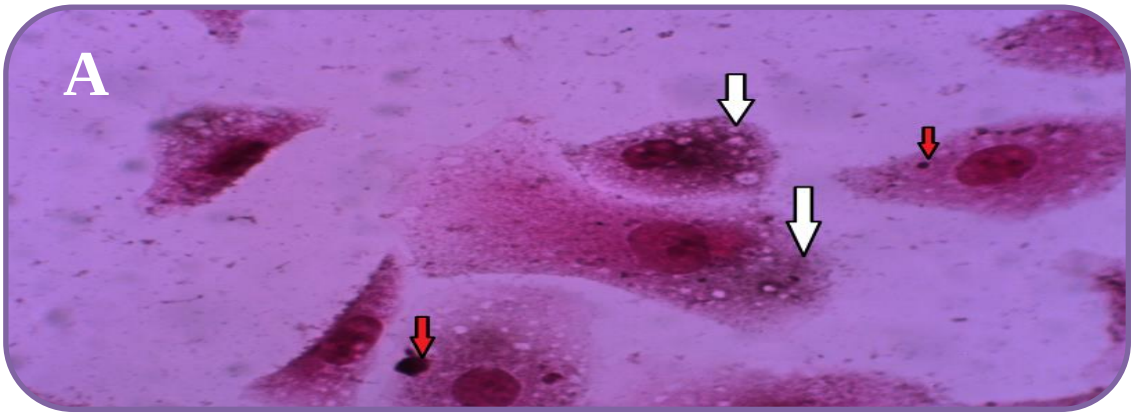
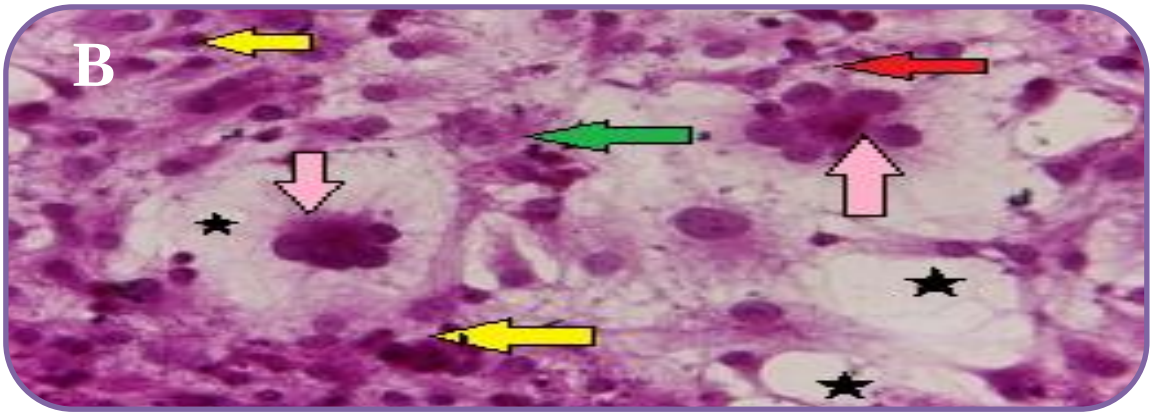


Figure (3.50): Treated cells of metformin on Hela for72hrs. converting cells to spherical-shape (black arrow) formation of apoptotic bodies (red arrow) and large spaces between cells (stars), 40X.



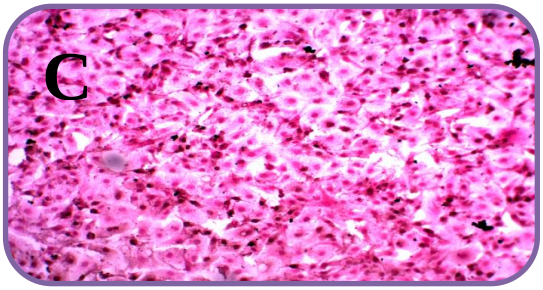
Hela Cells Vacuolation

(A) Treated cells vacuolated degeneration (white arrows change the cell shape (black arrows) necrosis (red arrows) cells decomposition karyopyknosis nuclei degradation, large spaces between cells was observed and cells decomposition formation of apoptotic bodies100X.



Hela Necrotic & Degradation

(B) Treated cells showing karyopyknosis (yellow arrows) multinucleated (pink arrows) and necrosis (green arrow), showing degradation (red arrow) large spaces between cells (stars) 40X.



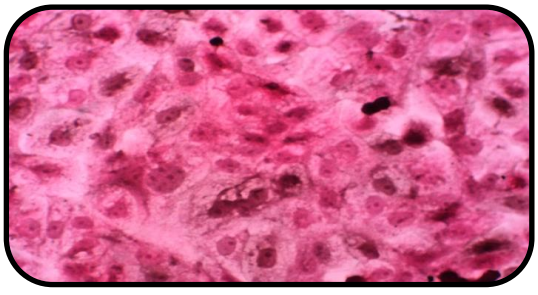


Figure (3.51): Morphological of the untreated cells. HeLa **(A).** Hela Cells Vacuolation**(B)** Hela Necrotic and Degradation, **(C)** Hela Before and After Treatment Metformin on Hela for72Hrs.

3.2.10 Cytomorphological Death Regarding to AO/EB Apoptosis Assay

The microscopic examination of Hela, HBL-100 cell lines stained with AO/EB dye showed that the untreated cells were stained with the green color of AO which indicates intact cellsHBL-100 (Figure.3.52). The cells treated Metformin were stained with the yellow or red color of EB dye, which indicates cell death as (figure 3.54 and figure 3.55)

AO/EB assay is used to observe the differential uptake of fluorescent DNA-binding stain AO/EB. The cytomorphology that was processed by the AO/EB protocol is at least shown to, if not ensure, the mechanism of cytotoxic impact on cell lines, as well as confirms necrosis in all cell lines that had an incidence. The staining with red indicates that the necrotic and apoptotic cells suffered from a defect in permeability, which caused the penetration of EB through the plasma membrane and nuclear envelope, then connecting with the nuclear material of the cell. The yellow cells are ongoing to death as pro-apoptotic or pro-necrotic cells. Untreated cells were stained with the green color of AO stain, an indication of the integrity of their membranes related to HB100and Hela as shown as figure 3.51and 3.57.

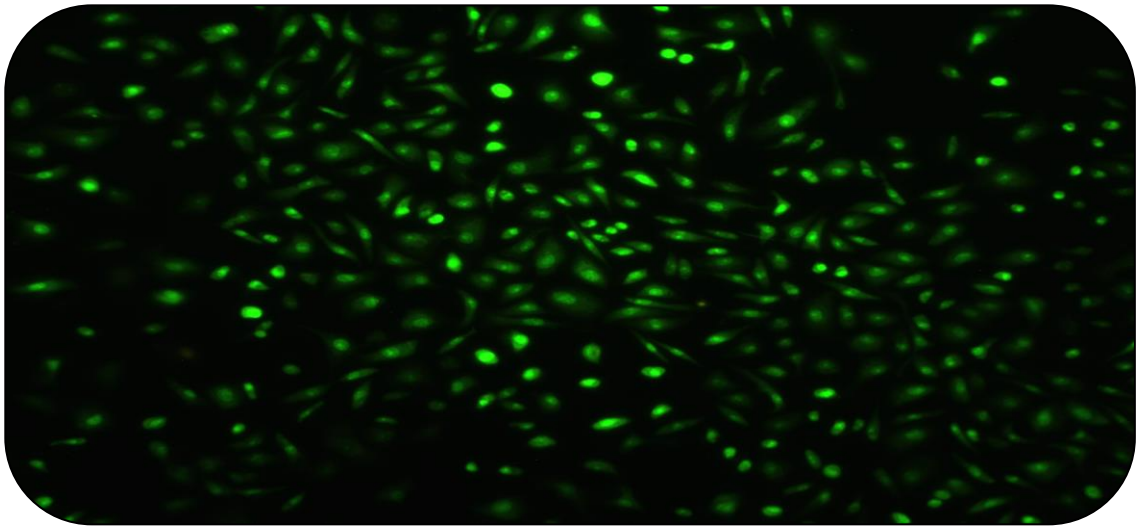


Figure (3.52): Untreated HBL-100 cells 10 X.

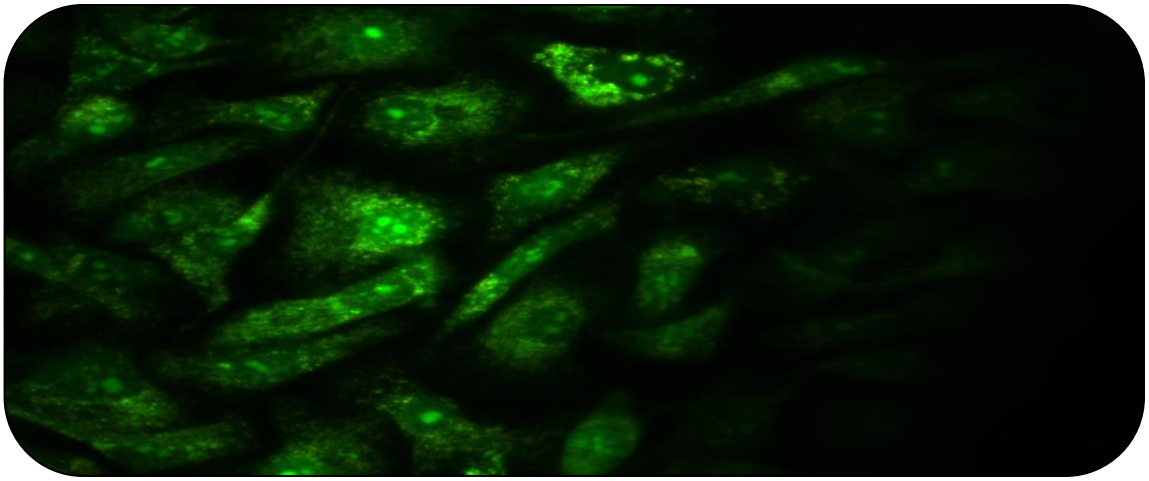


Figure (3.53): Untreated HBL-100 cells green color of cell live cell

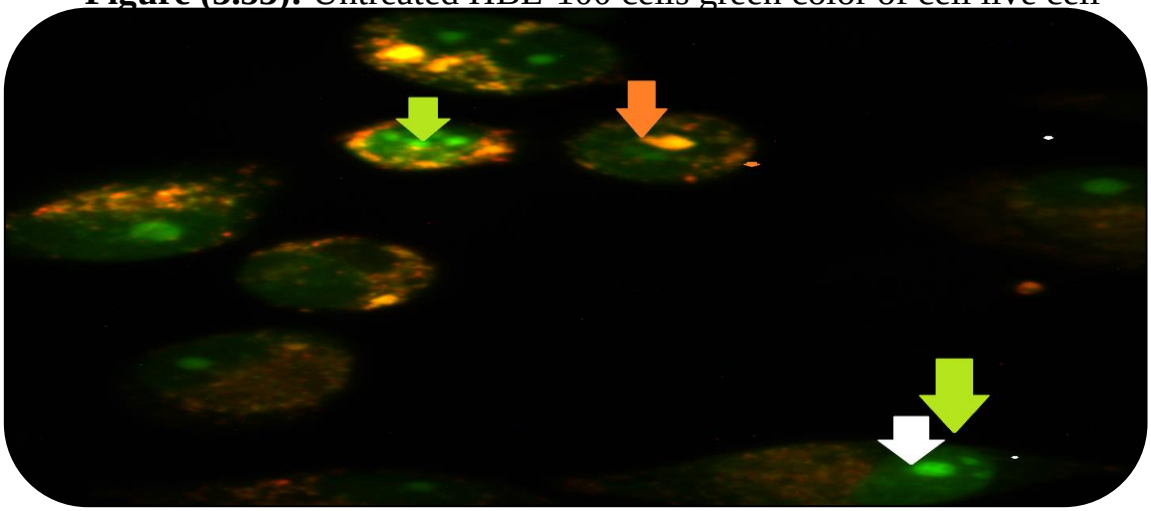


Figure (3.54): Treated HBL-100 cells with metformin green arrow of cell live cell, red arrow dead cell, white arrow related to nucleus 40X.

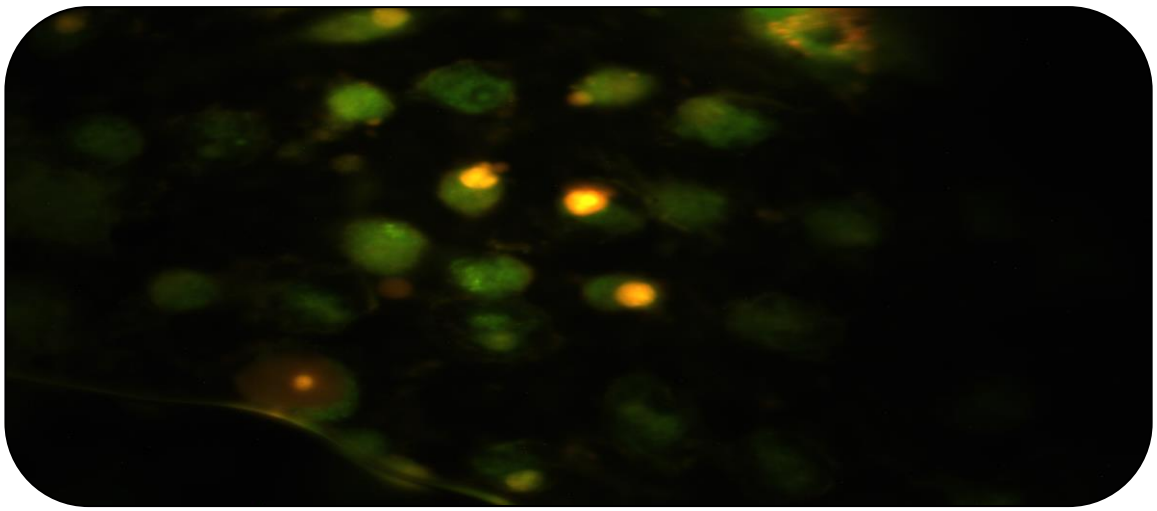


Figure (3.55): Treated HBL-100 cells with Metformin 40X.

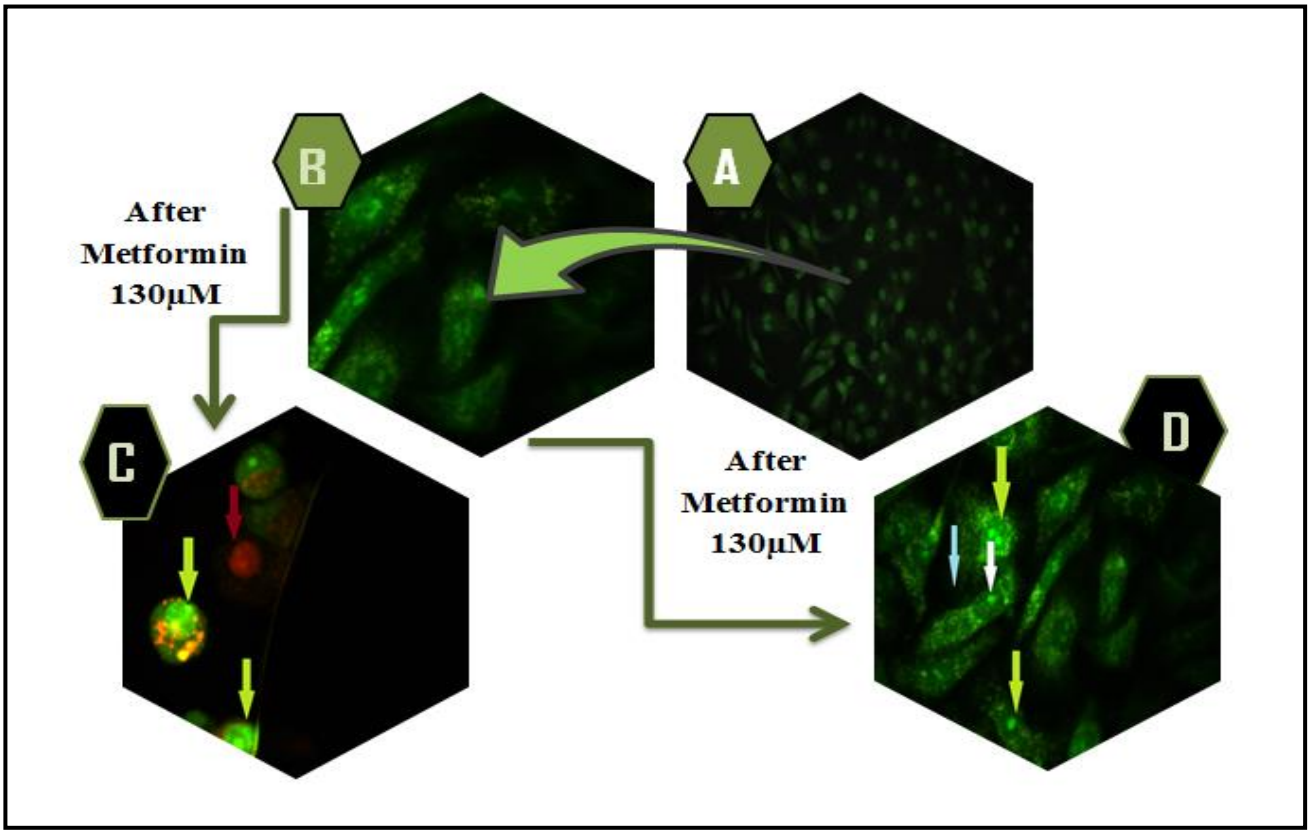


Figure (3.56): (A) Untreated HBL-100 cells 10X**, (B)** Untreated HBL-100 cells 40X, **(C)** cells treated with metformin green arrows referred to living cell, red arrow referred to dead cell **(D)** cells treated the white arrow referred to the nucleus, the blue arrow refers to the cytoplasm, the green arrow refers to the living cell, the red arrow refers to the dead cell, AO/EB staining cells 40 X magnification .no notable cytotoxic effect of metformin in normal cervical cells that evaluation by pathologist

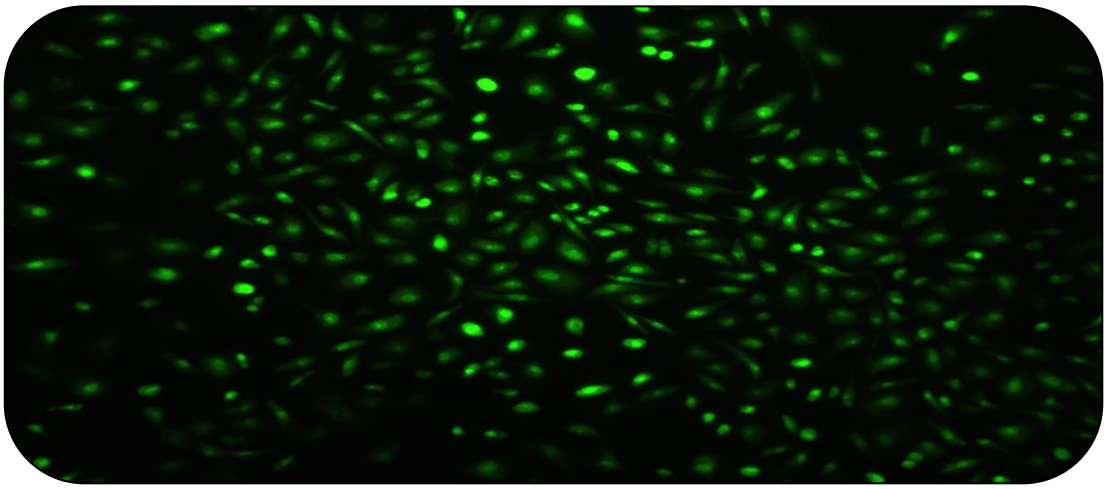


Figure (3.57): Untreated Hela 100 cells 10X, the green cell means live cell.

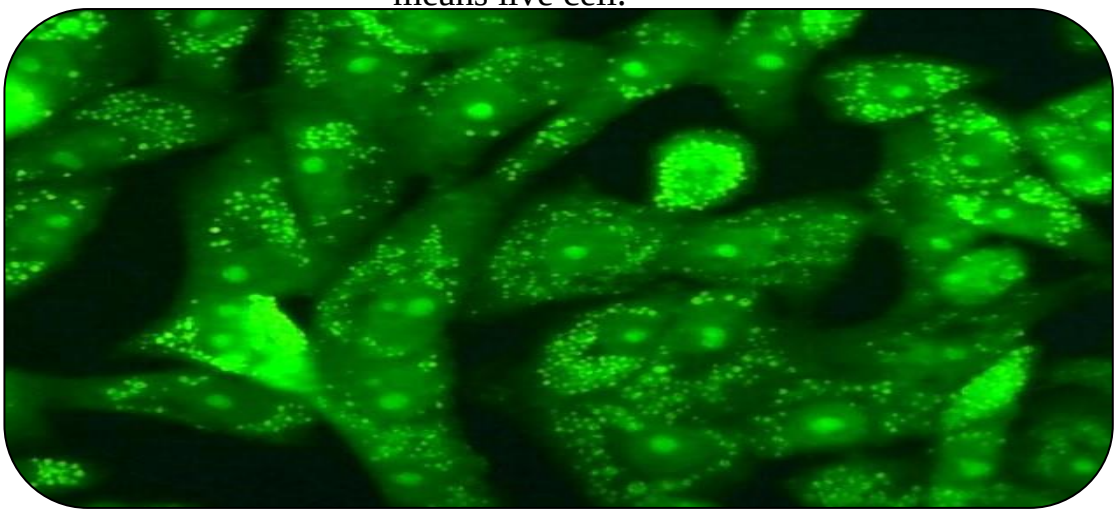


Figure (3.58): Untreated Hela 40X, the green cell means live cells.

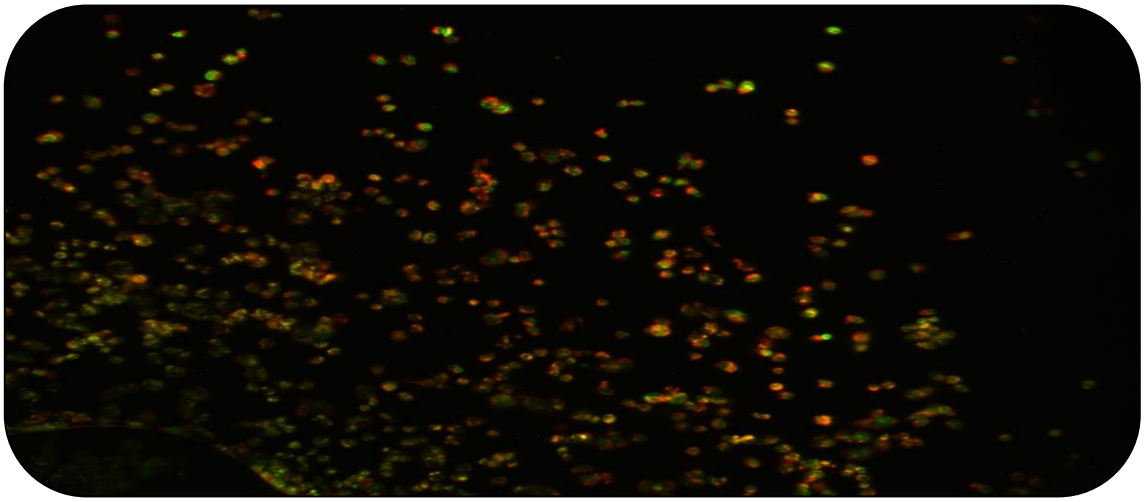


Figure (3.59): Hela Cells Treated with Metformin Green Cell Referred to Living Cells, Red Arrow Referred to Dead Cells10X.

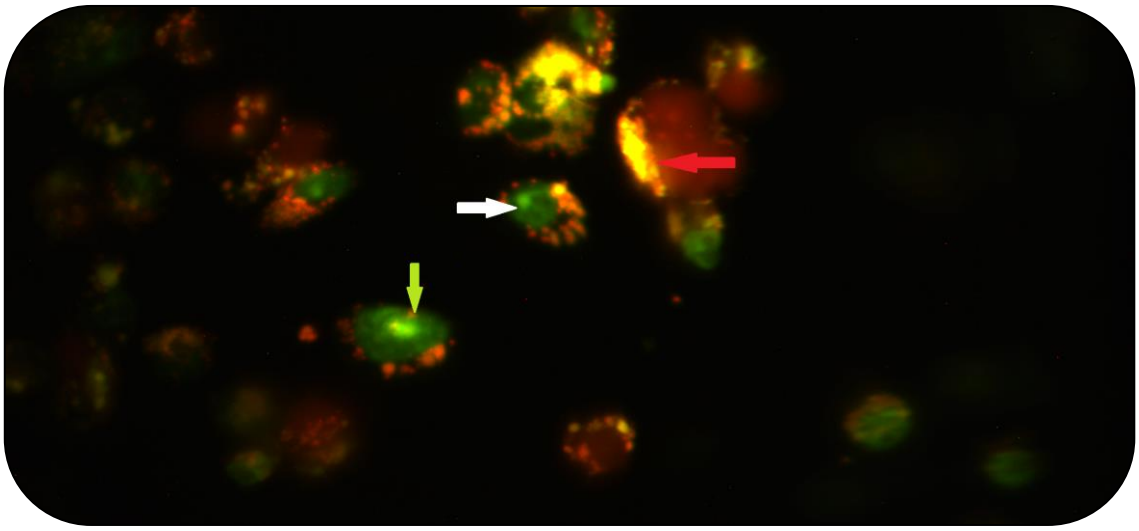


Figure (3.60): Hela Cells Treated with Metformin Green Arrow Referred to Living Cell, Red Arrow Referred to Dead Cell, White Arrow Referred to Nucleus 40X.

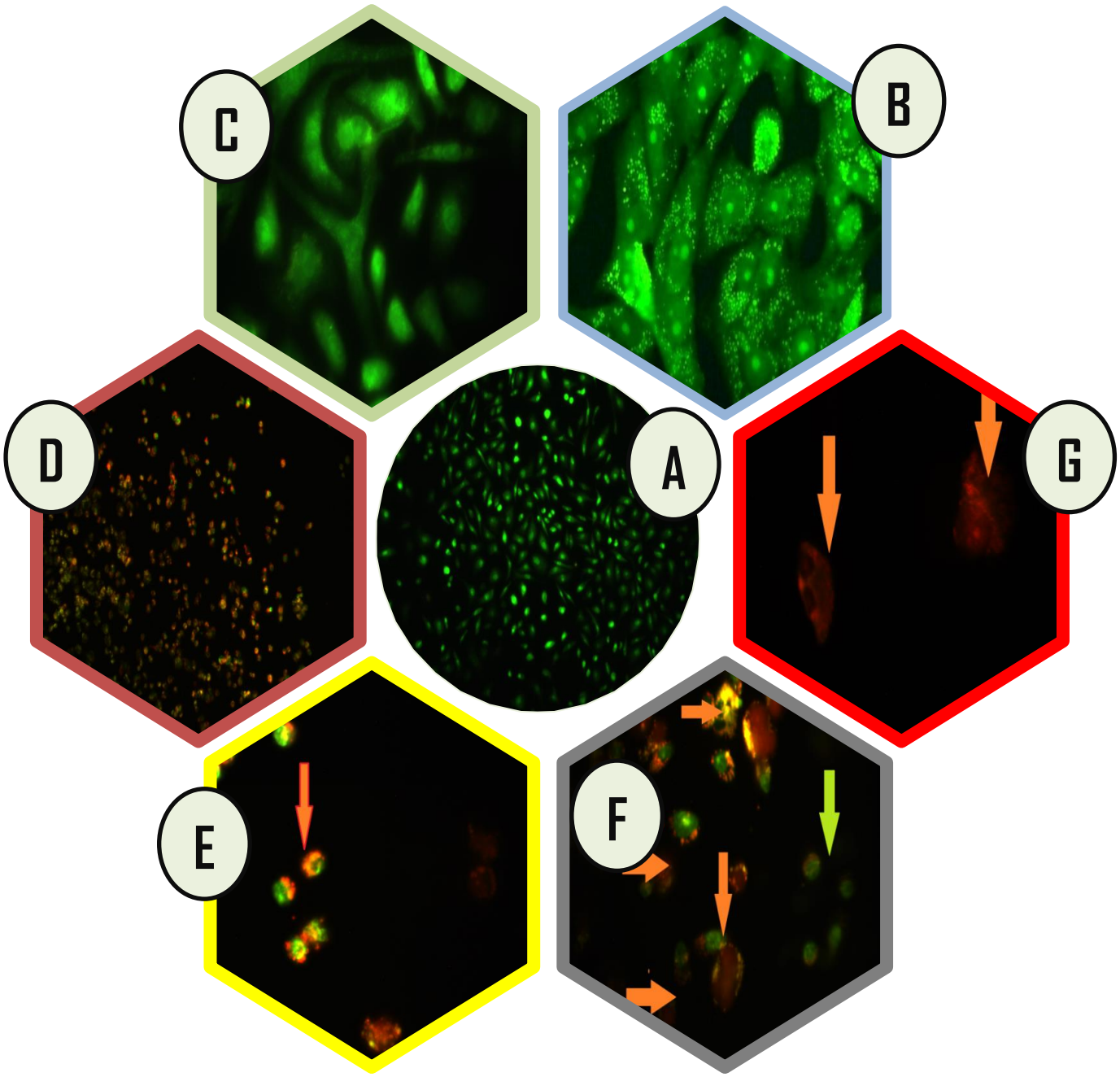


Figure (3.61): (A) Untreated Hela cells 10X, **(B)** Untreated Hela cells 40X, **(C)** Untreated Hela cells 10X **(D)** cells treated with metformin green cell referred to living cell, red arrow referred to dead cell 10X **(E)**cells treated the red arrow referred to the nucleus green cell refers to the living cell show the changing of cell the red arrow refers to the dead cell **(F)** after 72 hrs more dead cell red arrow refers to the dead cell green arrow referred to living cell **(G)** dead arrows mean dead cell (AO/EB) staining cells.

3.2.11: Reactive oxygen species (ROS) Evaluation.

Table (3.29) and figure (3.62) showed highly significant difference reduction ROS at concentration 130µM on Hela cervical cells *p*= 0.000.

Table (3.29): Effect of Metformin on ROS production on Cervical Cancer Cells Lines (Hela) after 72Hrs.

Concentration (µM)	ROS (ng/ml)		*p*- value
	Mean	SEM	
Control	0.593	0.027	**0.000****
Metformin 130µm	0.370	0.010	
*** (P≤0.05) Significant, ** (P<0.01) highly significant Independent sample t –test**			

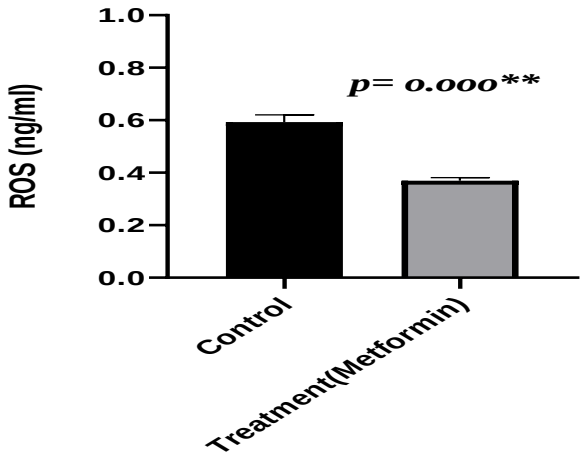


Figure (3.62): Effect of Metformin on ROS production on Cervical Cancer Cells Line (Hela) After 72Hrs.

3.2.12 Result Of Gene Expression Related to Impact of Metformin on PIK3CA/ AKT1 /mTOR Pathway.

The expression of the PIK3CA gene was slightly increased, but this was not significant. Unlike the PIK3CA gene, the expression of the mTOR gene was significantly down-regulated whereas the expression of AKT1 was significantly up-regulated. This indicates that treatment of metformin had effects on both mTOR and AKT1 genes but not PIK3CA. as shown in figure (3.63) & (3.64).

The data consist of the mean and standard error of the mean. The normalized transcript abundances for the PIK3CA, mTOR and AKT1 genes were calculated using the formula $2^{-\Delta Ct}$ where ΔCt is the Ct value for the housekeeping gene (GAPDH) subtracted from the Ct value for the target gene (PIK3CA, mTOR or AKT1).

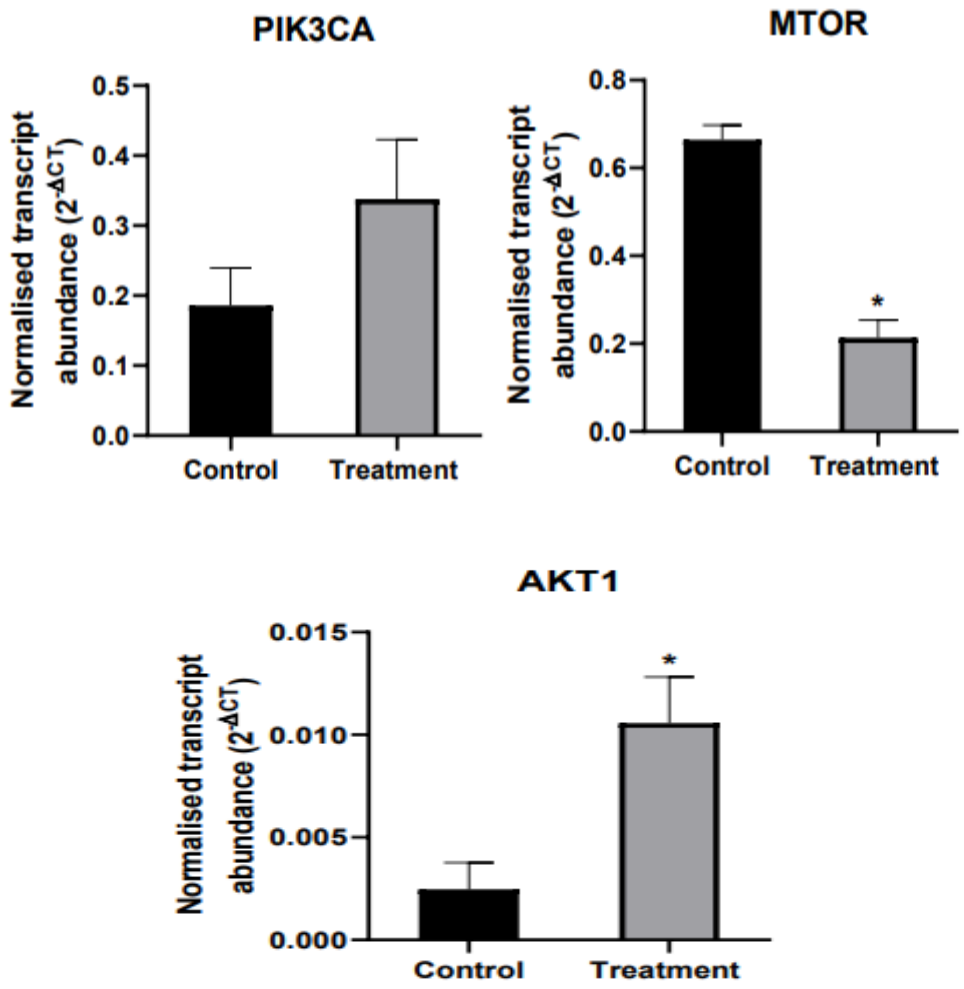


Figure (3.63): The Expression of PIK3CA, mTOR and AKT1 genes in HeLa cells treated with metformin.

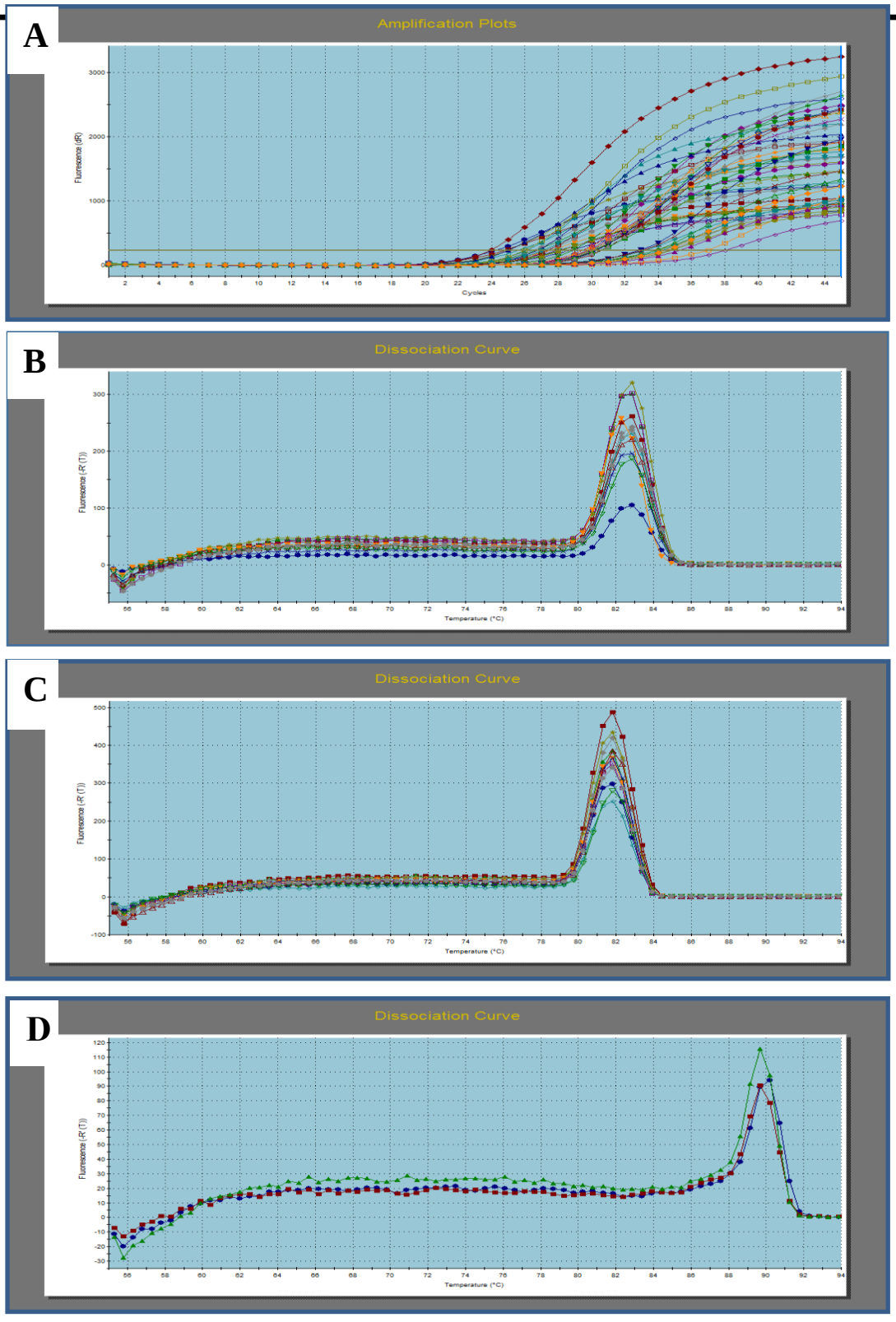


Figure (3.64): (A) Amplication plots &The Dissociation curve **(B)**PIK3CA, **(C)**mTOR and**(D)** AKT1 genes in HeLa cells treated with Metformin.

4 Discussion

The current study focuses on two parts. First one is related to effect of metformin as an anti-oxidant ,anti-inflammatory in specific dose and time manner. The second part is related to the possibility that metformin affects cancer lines and study possibility of its effect.

4.1 Impact of Metformin on Diabetes.

4.1.1 Impact of Metformin on Weight and Blood Glucose and Fluid Food intake

The current part associated with diabetes is characterized by an increased in the level of sugar resulting from specifically the destruction of β-cells of pancreatic animal models by using the diabetogenic agent "alloxan" [177].

Diabetes induction is dependent on the type of animal, how it is managed and its nutritional status. We focused on rodent animal's rats which are excellent research subjects because animals and humans are biologically similar, they are susceptible to many of the same diseases, and easily control the environment around animals (food, temperature, and lighting) which is difficult for humans to do in novel drug or new strategy [201].

In the present study we used of alloxan was used for induction of diabetes, alloxan (5,5-dihydroxyl pyrimidine-2,4,6-trione) is an organic compound [202]. Alloxan best to induce diabetic by two distinct pathological effect these modes chemically induction of diabetes due to therapeutic intervention via with partial destruction of pancreatic islet beta cells resulting in selectively reduction in

quantity and quality of insulin inhibit insulin secretion, second effect ability to generate ROS [203] .

Alloxan has two distinct pathological effects:

1. It selectively inhibits glucose-induced insulin secretion through specific inhibition of glucokinase, the glucose sensor of the beta cell, and it causes a state of insulin-dependent diabetes through its ability to induce ROS formation, resulting in the selective necrosis of beta cells, alloxan inhibits glucose-induced insulin release by inhibiting glucosidase in the beta cell pancreatic glucose sensor [176;203].

2. Causing the formation of ROS resulting in a redox cycle which generates superoxide radical [204],which generate hydrogen peroxide side reactions can also produce hydroxyl radicals. These highly ROS may damage cell DNA causing apoptosis [205].

Regarding table 3.1 showed the general appearance of five groups of rats in the experiment after monitoring the general appearance, we notable the increase urine output, increase food and water intake induction of diabetes in the rats. these result in agreement with [206].

Table (3.2) and Figure (3.1) shows the effect of metformin on the weight of the rats, it is obvious increase of body weight of the control rats which is a physiological normal increase, in contrast, a highly reduction of body weight in diabetic groups, these finding are in agreement with other experimental diabetes studies [207; 208].

Body weights of metformin groups were increased as diabetic groups with all doses 100,200,300mg/kg/day these were agree with [206].

The explanation about the increase of body weight in control group is that this increase is regarded as physiological affect while the decrease in body weight in diabetic rats is related to an increase in blood glucose with insulin inhibition which result in a decrease in tissue proteins and an increase in muscle

wasting. Proteolysis and gluconeogenic amino acids are used by the liver to make glucose. The degradation of proteins and lipids caused the induction of negative nitrogen balance [207]. But disagree with [209], which showed no change in body weight in spite, metformin lowers serum leptin levels in normal-weight males without altering body weight or body composition. In turn reduce adiposity in obese T2DM patients. Metformin is frequently associated with mild weight loss because it causes the shift of fat from visceral depots to subcutaneous depots [210].

Table (3.3) and Figure (3.3) show the fluid and food intake of the rats in each of the five groups. The diabetic groups showed higher food intake as compared with the control group. Diabetic group consume fluid more than the other groups. The control group has the lowest drink of fluid, compared to the diabetic theses finding agree with [206].

Treatment of diabetic rats with metformin significantly increased the body weight with a concomitant decrease in food intake and water consumption this is may be due to the impact of metformin on glucose metabolism by improving glucose utilization in insulin target tissues and by decreasing the activities of the gluconeogenic enzymes by preventing muscle wasting [206].

Metformin decreases blood glucose levels thought variety of mechanisms including enhancement of glucose utilization by tissues from blood and intercellular glycolysis, decreased gluconeogenesis in the liver and kidneys, decreased glucose absorption from the gut by activating enterocytes to convert glucose to lactate and lowering plasma glucagon levels [211;212]

Oral administration of metformin in different doses on day 35 resulted in a considerable decrease in blood glucose than in day 21 in a dose-time dependent manner, the findings are consonance with [213].

4.1.2 Impact of Metformin of Rat Pro-Inflammatory Cytokine

Cytokines are pleiotropic polypeptides that trigger cells to regulate inflammatory and immunological responses. They have a crucial role in the pathophysiology of a variety of diseases, including diabetes [134].

Alloxan stimulation large amount of production of IL-1β and TNF-α both of which have been suggested to induce islet inflammation and lead to β-cell dysfunction. The role of inflammation development of diabetes has also been elucidated thanks to the advances in basic and experimental science [214].

A current study as shown as in table 3.5 and 3.6 using metformin with different dose 100, 200 and 300 mg/kg/day at different period of treatment demonstrated decrease TNF-α both on serum and liver tissue according to dose and time in contrast to diabetes rats that showed higher significant. TNF-α ,these results reflected the effectiveness of metformin depending on dose and time manner these findings of study supported by [215;216;217], these studies observed on higher doses of metformin had significantly low levels of TNF-α compared to those on other dosages.

Explanation of these studies presented that metformin diminishes the secretion of TNF-α by lowering expression of protein and mRNA of TNF-α in both macrophages, this result also suggests that increasing metformin dosage may result in direct and indirect anti-inflammatory effects in diabetes patients, metformin reduced the production of pro-inflammatory cytokines (IL-1β and TNF-α) by inhibiting protein and mRNA expression in a dose-dependent manner or in outer mean the protein expression of anti-inflammatory cytokines was up-regulated or maintained by metformin [217].

Current findings of liver tissue assessments that the diabetes group showed highly significant level of TNF-α as compared with control group and treated group these result corroborated in study by [218],which demonstrated metformin's pleiotropic properties suggest that it impacts multiple tissues via

multiple underlying mechanisms rather than a single organ via a single mode of action[218].

Numerous inflammatory cytokines, such as TNF- α which mediate inflammation and repair in physiological conditions are greatly increased during the pathological phase of liver injury, pharmacological and molecular demonstrated metformin anti-inflammatory effects beginning the research in hepatocytes metformin accumulates in the liver at considerably higher levels, metformin therapy reduced the expression of TNF- α a hepatocytotoxic mediator in the liver similar to our findings [219].

Other clarify have revealed that hyperglycemia causes the accumulation of AGE in diabetes patients' tissues which bind to the cellular receptor, the interaction triggers a signaling cascade that results in an increase in nuclear transcription factors, as a result, oxidative stress and the production of pro-inflammatory cytokines increase much more [136].

Controlling the inflammatory response is a potential treatment strategy, metformin has been shown to activate the AMPK/PI3K/Akt signaling pathway in human vascular smooth muscle cells, resulting in anti-inflammatory effects by blocking nuclear transcription factor and lowering pro-inflammatory cytokine generation [220].

But current study which disagreement with [221].Metformin increase level TNF- α in blood in non-obese, non- diabetes patient with coronary heart disease [221].

4.1.3 Impact of Metformin of Oxidative Stress

The present study showed a significant elevation of TAOS in metformin-treated groups at 21 and 35 days compared to a diabetes group that matched the

result histological pathological pancreas as shown in table 3.9 & table 3.10 in serum and tissue respectively.

Current result of serum MDA regularly used for determination oxidant/antioxidant balance in diabetic patients [222]. MDA that indicator of ROS is a marker of lipid peroxidation.

Diabetes is associated with hyperglycemia accomplished by increased oxidative stress that rise by allowance increase in free radical production [220]. The results of the present study demonstrated that serum MDA level was significantly higher in the diabetic group as compared to the control group, these findings were in agreement with numerous studies that observed serum MDA levels were higher, metformin ameliorates reduction of the level of MDA that indicator of ROS is the marker of lipid peroxidation that increases diabetes results were in agreement [214; 223; 224].

In the present study as shown in table 3.11 metformin groups showed a reduction in the MDA and a considerable increase in TAOS.

Establishing that metformin had a critical role as antioxidant protection as anti-diabetic drugs due to protecting the pancreas from oxidative stress-induced diabetes these corroborated by [225].

Regarding metformin, it decreases oxidative stress and protective effects from free radicals that induce oxidative damage by stimulating autophagy via the activation of the AMPK pathway by inhibiting the complex I of the electron transport chain and diminishing mitochondrial ROS [225;226;227].

The results of the current study about DPPH radicals show a significant difference between Vitamin C and metformin at all periods of time. IC_{50} of DPPH the amount of antioxidants required to reduce the original DPPH concentration by 50% is used to calculate antioxidant effect of test samples [228;229].

The IC_{50} value of metformin and the ascorbic acid (498.0, 29.62 µg/ml) respectively at 60 min in different concentration showed that weak antioxidant according to table (4.1)

Table (4.1): Antioxidant Activity according to [230].

IC50 (µg/mL)	Mark
Strong Antioxidant Activity	10-50 µg/mL
Intermediate Antioxidant Activity	50-100 µg/mL
Weak Antioxidant Activity	>100 µg/mL

DPPH radical scavenging assay the easy, rapid, simple and more sensitive method that used to screen is the antioxidant potential of various molecules It has stable colored in the presence of antioxidant, DPPH got reduced to more stable and colorless molecule. This discoloration is measured spectrophotometric ally and gives the measure of antioxidant potential of test compounds [231].

4.1.4 Histological Evaluation

The present results demonstrated histological descriptive and determination scores. The diabetic group of pancreatic tissue presented considerable degenerative and excessive necrotic of tissue lesions in addition to atrophy of the islets of Langerhans with abnormal regular of pancreatic vacuities related to histological destruction explanation of these result mainly due toxic effects of alloxan on the pancreatic cells by formation of free radicals these results were in agreement with results [232;233;234]. In contrast metformin treated groups showed mild to moderate necrotic change of the pancreases with reduction in the size and number of islets of Langerhans [235].Observation of the hepatic tissue of the untreated diabetic showed an increase in necrotic hepatocytes with inflammation, in contrast to treatment metformin dose 100 that

showed moderate focal infiltration of inflammatory cells, metformin dose 200 mild focal infiltration of inflammatory cells, metformin dose 300 showed normal architecture of hepatocytes [236].

To summarized the findings of part one effect of metformin on diabetes decreases the blood glucose level, decrease the inflammatory marker on serum and tissue (IL-1β and TNF-α), decrease MDA level and increase the TAOS. Through the result obtained from this part, that it protects the liver and pancreas from the effects of oxidative stress and that the action of metformin depends on the dose and time which is a primary step to moving to the primary stage in the current research.

4.2 Effect of Metformin on Cancer Cells Lines.

Cancer has become one of the world causes of death and serious morbidity as result treatment of cancer need to continues changes chemotherapeutic drugs cannot successfully control the growth of the tumor that one of problem in

treatment therapy strategies, other problem that resistance to chemotherapy is common, as a result, the tendency toward novel approaches is growing ,the therapeutic benefits of metformin in cancer with diabetes mellitus is quickly taking abig area of interest in both and endocrinology and clinical oncology [237].

New therapeutic approaches are required to improve the overall survival rate of cancer patients ,according to preliminary evidence, metformin seems to lessen the incidence of cancer and improve survival in diabetic patients for this purpose, both in vitro and in vivo studies in various types of cancer associated with metformin have become common [84;238;239].

4.2.1 Cytotoxicity of Metformin on Human Cervical Cancer Cell Lines.

Current findings as shown in figure 3.34 revealed that metformin reduced cell viability of Hela in manner time and concentration-dependent by the MTT assay after incubated for 48 and 72 hours with (0-130 μM) results in agreement with results of [36;240;241;242;243]. All these studies demonstrated the cytotoxicity of metformin on human cervical cells with different mechanisms of inhibition.

Cervical cancer is the fourth most commonly diagnosed malignancy in women and the fourth major cause of cancer death and the second most common malignant neoplasm in women in developing countries, in 2040, it will be the cause of approximately 460 000 deaths and 530 000 cases of cervical cancer will appear worldwide annually [244].

Metformin action on cervical cancer is complicated, it suppresses oxidative stress by lowering hyperglycemia that decrease the circulating insulin levels which has a strong mutagenic effect of insulin like growth factor 1 receptor [241].Critical role of metformin in cancer prevention and treatment [245].

Mechanism of Metformin in Cervical Cancer can be pointed to most important points summarizes by the following:

- ✓ Most usually suggested to explain the link between diabetes and cancer is Insulin resistance which results in secondary hyperinsulinemia is the mechanism most usually suggested to explain the link between diabetes and cancer, additionally, insulin may have mutagenic effects through the insulin-like growth factor 1 (IGF-1) receptor then hyperglycemia may deteriorate carcinogenesis through the trigger of oxidative stress [246].
- ✓ Metformin may lower the level of circulating insulin and stimulate the immune system, intracellular metformin may activate the liver kinase B1 (LKB1)/AMP-activated protein kinase (AMPK) pathway, inhibit protein synthesis, cause cell-cycle arrest and apoptosis, and decrease IGF-1 and insulin-mediated signaling [247].Metformin can suppress cancer cells via various other mechanisms, such as mTOR pathway inhibition and autophagy induction [248].The direct anticancer effects of metformin are essentially mediated by AMPK-dependent by decreasing mTOR, NFκB and increasing p53phosphorylation while AMPK-independent by decreasing ROS, increasing mTORC1, decreasing cyclin D1, increasing autophagy and apoptosis of cancer cells mechanisms [249].
- ✓ Other study reported that metformin therapy increased the levels of p21 and p27 (AMPK-dependent cell cycle inhibitors) which led to an increase in cell cycle arrest and death in HeLa cells relative to untreated cells [244].
- ✓ Metformin's pleiotropic antitumor effect is mediated by a variety of signaling pathways, STAT3 (signal transducer and activator of transcription), hypoxia-inducible factor and insulin-like growth factor in addition, metformin destroys cancer stem cells which are linked to cancer development, metastasis, and treatment resistance [239;215;250]

- ✓ The therapeutic benefits of metformin cancer with diabetes mellitus is quickly big area of interest in both and endocrinology and clinical oncology, its possible use of metformin as an adjuvant in clinical practice, it is reported that metformin has considerable promise role on cancer therapy [244].

Drug development strategies have focused on a specific protein or signaling system. medicines that target several proteins and disease-related signaling pathways are required metformin a biguanide blood sugar lowering drug, because of its safety and efficacy profile, as well as its low cost, it has along in clinical practice. Metformin has been proven in studies to reduce tumor cell proliferation, invasion, and migration by targeting one or more signaling pathways [162].

We demonstrated that hyperglycemia play important role in cancer progression and treatment, how contribution to a more malignant phenotype in cancer cells, as well as medication resistance,as a result, managing hyperglycemia in cancer patients could have significant therapeutic consequences [244].

4.2.2 Cytotoxicity of Metformin on Breast Cancer Cell Line (AMJ3) After 48 and 72Hrs:

Regarding current results the viability of AMJ3 was reduced in a dose-time dependent manner with viability reducing to 31.130% at a concentration of 130 μM after 48 hours which is statistically significant as compared to the initial reading without the addition of any treatment. After 72 hrs. The viability reached 21.453 at a dose of 130 μM. After 72 hours, the cell viability declines with the increasing concentrations of metformin reaching 93.967, 33.022, 22.400, 22.084, 21.979, and 21.453 μM significantly. The decline was significant at higher concentrations. The results were in agreement with the results [4;250; 251;252;253].

The new breast cancer cell line (AMJ13) has been established as unique for the Iraqi population, that is, an Iraqi breast cancer patient and it has been useful in breast cancer research and important for Iraqi researchers, it was derived from an Iraqi patient with poorly differentiated infiltrative ductal carcinoma that was histologically diagnosed and is the first Arabian and Middle Eastern breast cancer cell line and useful resource in breast cancer research and therefore could aid in the development of new medicines [254].

Observed substantial evidence to support the use of metformin as a breast cancer drug as monotherapy or in combination with chemotherapy drugs with known anti-cancer potential with other drugs or therapeutic modalities is critical to achieve therapeutic efficiency in the treatment with minimal side effects when used orally in the treatment of diabetes [4].

Breast cancer is the most dangerous type of female cancer. It is an etiologically complex disease that caused by a multitude of cellular pathways that promote the spread of cancer linked to cellular glucose metabolism [255].

Metformin has an indirect effect by lowering serum insulin, which results in a decrease in cell proliferation by blocking downstream signaling via the

PI3K/Akt and Ras-MAPK pathway and resulting in a decrease in cell proliferation [256].

The beneficial effect of metformin has been noted in breast cancer patients who have hormone receptor-positive, human epidermal growth factor receptor-2 overexpression and high insulin like growth factor-1 receptor expression on the tumor surface. Therefore, metformin has also been demonstrated to reduce metastatic occurrences and increase the levels of metabolic and insulin-related biomarkers linked to cancer [258]. The results were in consistence with that metformin did not have impact on breast cancer–free interval [253;257]. Findings from these studies could not approve or reject the efficacy of metformin for patients with breast cancer [259].

4.2.3 Cytotoxicity of Metformin on the HCAM cell line after 48&72hrs.

After 48 and 72 hrs of treatment with metformin on HCAM cell viability was clearly reduced in a time-dependent manner. In 48 hours high concentration reduced viability to 44.466% significantly less than the 99.320% at concentration before adding the metformin to the plate. In 72 hrs, HCAM viability was significantly decreased to 36.838 when the concentration was increased to 130 μM.

HCAM cell lines were created from the original tumor of a white Swiss albino male mouse with hepatocellular carcinoma that developed spontaneously, anew liver cancer cell line (HCAM) has been identified and is being viewed as a useful tool in the study of liver cancer [260].

The cytotoxic effect of metformin on HCAM is less than Hela and AMJ13, cytotoxic effect novel for our study first study on HCAM cancer cell line and compared with other cells, demonstrated the studies that related to cytotoxic effect metformin related to liver cancer

- ✓ Metformin contributed to the inhibition of tumor growth demonstrate that metformin inhibits the growth of hepatocellular carcinoma by inducing G1 cell cycle arrest via the changes of microRNAs [261].
- ✓ Metformin not reduced the risk of hepatocellular carcinoma in diabetic patients [262].
- ✓ A meta-analysis excluding studies with time-related biases stated that metformin did not significantly decrease the risk of hepatocellular carcinoma [263].
- ✓ Metformin treatment has been independently associated with decreasing the occurrence of HCC and liver-related deaths there is a strong association between the use of metformin and reduced risk of HCC [264].

4.2.4 Cytotoxicity of Metformin on Glioblastoma Cancer Cell A172 After 48&72hrs.

The present results reported an inhibitory effect of metformin on A172 human glioblastoma cells and cell viability was decreased more significantly with increasing dose. At higher Metformin doses and longer treatment durations, a significant decrease in cell viability ($P = 0.000$) at a 48hrs reach to 74.258 μM while higher concentrations of Metformin demonstrated highly significant decreases in viability 47.820 at 72 hrs.

The most established type of malignant brain tumor is glioblastoma, which accounts for 40% of intracranial tumors. Glioblastoma typically treated with surgery, radiation and chemotherapy a major threat to patient lives and health because of its high level of malignancy, post-operative relapse and low 5-year survival rate [265]. Glioblastoma is a prevalent type of intracranial malignant tumor that is increasing exponentially in prevalence posing a major threat to human health [266].

These studies were in agreement with [267;268;269]. Current study has certain limitations in that the related cytotoxic effect of metformin is less than

studies with different concentration of metformin in addition demonstrated an effect when adding to other drugs to enhance the effect of metformin, while our study only used metformin alone ,using metformin at levels greater than those typically used for anti-diabetic therapy may be able to modification the metabolism of cancer cells opening up new potentials for synthetic lethality through good medication combinations, metformin has been used to target cells that are sensitive to energy stress [32], and is now establishing its own place as an adjuvant in the treatment of cancer [270]. Clinical trials about re-purposed Metformin aimed at the treatment of brain cancers (NCT Number **NCT02780024**. Name of study: Metformin, Neo-adjuvant Temozolomide and HypoAccelerated Radiotherapy Followed by Adjuvant TMZ in Patients with GBM, clinical Phase2).

4.2.5 Cytotoxicity of Metformin on HBL100 Normal Breast Cell.

Regarding results showed that metformin at concentrations130 μM causes a significant decrease in the viability of normal cells while the other concentrations didn't cause any significant effect on compared to the 0 concertation as shown in table (3.7). Our results were in agreement with [271;272;273], but other studies were disagreeing with [274].

Mortezaee *et al*.,2019 [275] demonstrated Metformin exposure that used normal human gall bladder cells (GBC), these findings declare that metformin alone suppresses cell proliferation and encourages cell apoptosis. Metformin has shown the ability to target cancer cells without any toxicity to normal cells. Another study [273] showed that metformin, through inhibition of mitochondrial complex I activity, increased the production of superoxide in pancreatic cancer cells, while it does not cause ROS production in normal cells.

Metformin treatment's effect on cancer cells exceeds its effect on normal cells, suggesting that it is a cancer-targeting agent by effect on specific proteins

that are implicated in cancer prognosis and have a great potential to be key targets for cancer therapy [276].

Regarding to our result after 72 hours of exposure to metformin to cells lines, the viability and cytotoxic effect of metformin on HeLa, AMJ3, HCAM, A172 cancer cell line and HBL100 normal cell lines.

Table (3.23) demonstrated at130 μM the concentrations the highly significantly difference cell viability of HeLa than those other lines.

Supported by studies [24; 277; 278] the impact of metformin on HeLa cells the in vitro findings of our study revealed that metformin increased in cytotoxicity and anti-proliferation effects against cervical cancer cell lines better than those of other lines according to dose –time dependent Metformin development of multi-target inhibitors for cervical cancer, metformin targeting both the PI3K/Akt and p53 pathways and exerting antitumor effects in the body [240].Half maximal inhibitory concentration for metformin for evaluate the effect of metformin on cell proliferation, IC_{50} value was measured in normal and cancer cell line low survival cell value indicates cytotoxic effect if survival cell values of less than 50%, IC_{50} values are medication concentrations that suppress tumor cell colony formation by 50%. [279]. Previous studies IC_{50} metformin levels appear to be due to differences in cell types, cytotoxicity tests and treatment periods [280].

These drugs were chosen for further dose-response experiments in cancer cell lines, the activity of the metformin was determined by its IC_{50} values, compounds with IC50 values $\leq$10 μM were selected as active, in this study IC_{50} for metformin on Hela 7.447 showed highly significant difference than other cell lines in accordance [250;281] but different IC_{50} metformin reported in preceding studies appear to be due to differences in cell types, cytotoxicity tests, and treatment times.

Furthermore, the RTCA (Real-time cell analysis system) was able to calculate the IC_{50} value in real-time for at least 72 hours following the addition of anticancer chemicals with no restriction on the length of the experiment. Our findings showed that RTCA systems are useful for cytotoxicity testing and might be employed in the future development of chemotherapeutic drugs employing cancer cells as well as the evaluation of their adverse effects in normal cell RTCA system could be used to measure the cytotoxicity of four anticancer drugs in carcinoma cells [279].

4.2.6 Comparing The Impact of Metformin with The Same Dose of Paclitaxel (Anti-Tumor).

After the focusing in our findings to metformin impact on Hela in order to compare the effect of metformin with the same dose of paclitaxel showed a significant difference at concentration 5, 10 and 15 and non-significant difference at other concentration regarding to our result the effect of metformin and paclitaxel same effect on Hela but in normal cell showed the significant cytotoxic effect of normal cell of paclitaxel rather than metformin. Metformin reduced the viability of cervical cancer cells while having no cytotoxic effects on non-cancerous cells which are in agreement with our result [282]. Metformin is able to inhibit HeLa cell proliferation in dose dependent manner [283].

Metformin used in gynaecological cancer cells through activation of AMP-activated proteinkinase also modulates energy metabolism and inhibition of mammalian target of rapamycin (mTOR) leads to suppression of protein synthesis, leading to apoptosis and autophagy in the presence of tumor suppressor LKB1 [284].

Paclitaxel targets microtubules. At high concentration, that causes mitotic arrest at G2/M phase, whereas at low concentration, apoptosis is induced at G0

and G1/S phase depending on the dose concentration. That had an important role in ovarian cancer [285].

4.2.7 Cytomorphological Changes Related to Hela and HBL100 cells

Several cytomorphological changes were seen in cervical cancer cell lines under a microscope after adding metformin to Hela cells and HB100. Treated HBL100 cells with metformin 130 μM after48hrs.were grown in monolayer in contrast to Hela cells with the same dose of created phenotypic changes such as apoptosis, vacuole degeneration, change in shape of some cells to rod shape, and necrotic cells enlarged the irregular, the atrophy, spherical and irregular shapes observed in cell lines. Necrosis is other cell injury was observed in all cell lines, necrosis may be attributed to free radicals which harm cell systems due to oxidation of plasma membrane lipids. There were some cells that looked to be multinucleated, many of the cells of Hela had cytomorphological alterations as descriptive figure (3.60) and figure (3.61).

In cell cultures related to Hela, we noted a huge gaps developed due to shrinking and decomposition of cells, these ongoing morphological changes brought about by exposure to metformin a karyopyknosis and hyperchromy of the nuclei one of the hallmarks of apoptotic cells is karyopyknosis. Morphological fluctuations when metformin employed on Hela cells. accompanied the Hela cells suffer of hyperchromy addition to nuclei atrophy in other cells also most cells suffered from vacuolative degeneration, nucleic fragmentation and development of apoptotic bodies are both common apoptotic features [286]. Current study the microscopic examination of Hela and HBL-100 cell lines stained with AO/EB dye showed that the untreated cells were stained with the green color (referred to living cell) of AO which indicates intact cells HBL-100the cells treated Metformin were stained with the yellow or red color of EB dye which indicates cell death more in Hela than HBL-100 that reflect differential uptake of fluorescent.

The cytomorphology by AO/EB protocol not only clarify mechanism of cytotoxic effect on cell lines also confirms necrosis in all cell lines these assay for observe the differential up take of fluorescent DNA binding [278]. Mitochondria play an essential role in cellular autophagy [289].

When cells are stimulated mitochondria are damaged then fused with lysosomes to degrade the damaged mitochondria referred to as mitochondrial mitophagy [289].

Defect mitochondrial mitophagy lead to cell death and increase activation of autophagy cell damage leading to mitophagic cell death, mitochondria supply energy as an intracellular and can induce apoptosis through mitochondrial signaling [290].

Metformin suppress the proliferative activity of cervical cancer time-concentration-dependent manner accompanied by a significant increase in apoptosis representing that metformin can inhibit cell proliferation by inducing apoptosis in HeLa cells made it a hot topic of antitumor research in recent years, there is still no conclusive evidence regarding the antitumor properties and mechanism of metformin thus limiting its use in clinical tumor therapy [251].

4.2.8 Evaluation of Reactive Oxygen Species

The current study showed the effect of metformin at concentration 130 µM on the production of ROS on Hela cervical cells reduction difference significantly in ROS after adding metformin, in contrast to control Hela without treatment, these results were in agreement with [289;290].

Explanation to reduce ROS by inhibiting complex 1 directly of mitochondria electron chain that inhibiting directly not unclear [20]. Complex 1 is first that comprise the electron transport chain that in the inner mitochondrial membrane of four complexes after series reaction redox reaction electron sequentially transmitted to final acceptor electron molecular oxygen energy released by this action is used to drive protons across the inner mitochondrial

membrane creating an electrochemical gradient. produces a proton motive force, which is used by ATP synthase to manufacture ATP, the electron transport chain has an interesting trait in that it can also travel backwards,electrons can flow backwards from complex II (succinate dehydrogenase) to complex producing reactive oxygen species [291],

but present study disagrees with [292;293]. Metformin increase ROS by combining with apigenin via AMPK-FOXO3a-MnSOD pathway in pancreatic cancer cell line. Metformin can increase ROS causing a mild leakage of electron transport and resulting in ROS production [293].

Another study related to ROS and metformin is a double-edged sword when metformin act on muscle can cause inhibiting the complex I of the electron transport chain and suppress reactive oxygen species of mitochondria because that the inhibition of complex I can decrease oxidative process in muscles of hypoinsulinemic rats [294].

Metformin reduces paraquat-induced elevated in ROS , as well as related DNA damage and mutations, but has no effect on H_20_2-induced changes, indicating a reduction in endogenous ROS generation, metformin reduced Ras-induced ROS generation and DNA damage ,findings of this study showed that metformin has inhibitory effects on ROS generation and somatic cell changes that introduce a unique mechanism for the cancer risk reduction associated with this drug's use [295].

4.2.9 Result of Gene Expression via Effect of Metformin on PIK3CA/ AKT1 /mTOR Pathway

Our findings related to gene expression that non-significant expression of PIK3CA gene, unlike the PIK3CA gene, the expression of mTOR gene was significantly down-regulated whereas the expression of AKT1 was significantly up-regulated, this indicates that treatment of metformin had either effects on both mTOR and AKT1 genes but not PIK3CA, these part of study have a novelty

because using the different concertation than other studies and unique pathway on cervical cancer.

When inspect of pathogenesis of cancers is very complex therefore it is important to development new drugs with target multiple proteins and disease-related signaling pathways new design strategies drug general target a single protein or as signaling pathway [296]. Metformin can target one or more signaling pathways to suppress tumor cell proliferation, invasion, and migration [162].

In PI3K/Akt/mTOR pathway, PI3K consist of a regulatory subunit of many lipid kinases, Akt that as protein kinase B, is an important downstream molecule of PI3K. Akt immediately attained its activated state by phosphorylation p-Akt and activated Akt. mTOR that play an important role in regulating cell growth, proliferation, survival and glucose metabolism [297;298].

The molecular mechanisms of metformin inhibited cervical cancer cell proliferation targeting mTOR [250], mTOR exists in two different complexes (mTORC1 and mTORC2), mTOR inhibitors that development previously particular mTORC1 targeting agents, the mTORC2 complex functionsas3-phosphoinositide-dependent protein kinase phosphorylates of AKT an obligatory event for full activation of AKT, while mTORC1 phosphorylates the downstream effector p70S6k which then induces the degradation of insulin receptor substrate 1 (IRS-1), thus decreasing insulin-driven Akt activity, Inhibition of mTORC1, therefore, may result in feedback activation of AKT, it is anticipated that a kinase inhibitor of mTOR that can target both mTORC1 and mTORC2 would block the activation of the PI3K pathway more effectively than one that blocks mTORC1 only, and this could potentially overcome the problem of feedback were able to inhibit cell growth and proliferation more effectively this our finding observed that real-time PCR assay was used to investigate the expression of PI3K, AKT, mTOR [299].

Metformin evaluated for the treatment Hela cancer cell at physiological pH values, metformin occurs largely as a hydrophilic cationic molecule with little lipid solubility and extremely limited passive diffusion through cell membranes [300].

The PI3K/AKT/mTOR pathway is an intracellular signaling pathway important in regulating the cell cycle, therefore, it is directly related to cellular quiescence, proliferation, cancer, and longevity, PI3K activation phosphorylates and activates AKT, localizing it in the plasma membrane mechanism of action as cytotoxic of metformin reviewed by [301; 302].

The possible cytotoxic mechanism of action of metformin mitochondrial play critical role in the drug's anti-tumorigenic activity by affecting via to complex 1 inhibition has been reported in a different cancer cells lines .by inhibition of mTOR , AMPK-mediated activated of catabolic pathways and suppression of anabolic processes usually results in decreased mitochondrial and ATP depletion ultimately resulting in AMPK-mediated activation of catabolic pathways and inhibition of anabolic processes (mTORC1), while some AMPK- and mTORC1-independent mechanisms can also co-exist [36].

Metformin affecting on cellular metabolism and oncogenic signaling pathways via receptor tyrosine kinase, PI3K/Akt, and mTOR pathways metformin interest due to its cytotoxic effects on cancer cells [303].

The mechanism of action of metformin AMPK-dependent and AMPK independent when metformin cause increment activation of AMPK noted Intracellular energy sensor is activated by elevating the ratio of AMP/ATP, AMPK level causing restores cellular energy levels by suppress anabolic processes and promoting catabolic processes as glycolysis and fatty acid oxidation [304].

Metformin activate AMPK, that has anti-cancer properties [305]. AMPK-independent effects of metformin meaning anticancer effect of metformin was

independent of the AMPK pathway. They used AMPK, RNA to inhibit the two catalytic subunits of AMPK, but AMPK suppression did not block the G0/G1 cell cycle arrest via by metformin. that negative regulator of mammalian target of rapamycin (mTOR) by metformin regulated the development DNA damage 1 mediated the effects of metformin on the cell cycle arrest and cyclin D1 changes [306].

Other study about metformin-mediated inhibition chronic inflammatory responses via with suppression of production TNF-α production in human monocytes, an event that was most likely independent of AMPK activation, chronic inflammation may due to cancer progression, but there was no obvious change in phosphor-AMPKα observed after metformin treatment [306].

Regulating cellular processes including protein synthesis and autophagy by inhibition, mTOR plays important role in controlling cellular energy homeostasis mTOR, signaling commonly activated in malignancies and exerts considerable positive regulation of cell proliferation and carcinogenesis in a different types of tumors, resistance to chemotherapy malignant tumor and targeted molecular treatments all linked to activation of mTOR activation [307]. mTOR important point to overcome chemotherapy resistance that reflected important adding of metformin to other anticancer.

Summarized part two metformin dose dependent and time dependent in its action of variety of cancer cells lines their cytotoxic effect more in cervical cancer cell line (Hela). When compared to anticancer paclitaxel the same effect in same dose but metformin less effect on normal cells. after this focus how act metformin inside Hela and study cytomorphalogy of changes after metformin then described that result show that metformin reduced ROS inside the Hela cancers cells line. And gene expression showed the effect of metformin inhibit mTOR that describe the mechanism of action as cytotoxic effect on Hela cancer cell lines.

5 Conclusion & Recommendations

5.1 CONCLUSION

The present work was concluded regarding to:

Part one related to impact of metformin on albino diabetic rat's models different dose with different period:

1. Protective effect of metformin by decreases the deleterious effect of inflammation also metformin had anti-inflammatory depend on dose - time manner.
2. The antioxidant effect by increasing TAOS and decreasing MDA in diabetes rats, while in vitro DPPH showed weak antioxidant conclude metformin antioxidant in vivo
3. Conclude form part one antioxidant activities with anti-inflammatory of metformin depend on time –concertation in diabetes the fact that diabetes is a state with increased oxidative stress, inflammation all of which can in turn promote carcinogenesis.

Part two Related cytotoxic effect of metformin on variety of cancer cells

1. Metformin has a highly significant cytotoxic effect on HeLa, and lesser effect on AMJ3, HCAM, and A172 cells than on normal cells
2. Metformin showed the apoptotic induction in Hela rather than in normal cells when compared to paclitaxel with metformin.
3. Metformin showed a decrease the ROS in Hela that is good for combination with anticancer drug.

4. Metformin demonstrated cytotoxicity on Hela cells by influencing the PIK3CA/AKT1/mTOR pathway of metformin on gene expression by inhibiting mTOR, which is important in chemotherapy resistance.
5. The benefit of metformin on diabetes mellitus by lowering blood glucose, oxidative stress and inflammation additionally had a protective effect on tissue against diabetes-induced cancer. In other words, metformin has a cytotoxic effect on a variety of cancer cells. The advantageous addition of metformin to the anticancer regimen lowers the deleterious effects of chemotherapy through its impact on mTOR. This point summarizes the novelty of the current study.

5.2 Recommendations

1. Study other markers related to oxidative stress and inflammation.
2. Study treatment of metformin in vivo using mouse tumor models or solid tumor. .
3. Study other parameters such as glycolysis parameters, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) enzyme in the cancerous cells.
4. Studying the effect on levels of glycolysis products of metformin such as hexokinase, pyruvate, ATP and acidity (that represent lactic acid).
5. Study the effect of metformin in combination to chemotherapy .
6. Increase the efficacy of the treatment by combining with more anti-cancers.
7. Trying another dose of metformin on cancer cells with the goal is increasing the outcome effect.
8. Using metformin with another cell cancer lines such as pancreatic cancer, prostatic, skin.etc.

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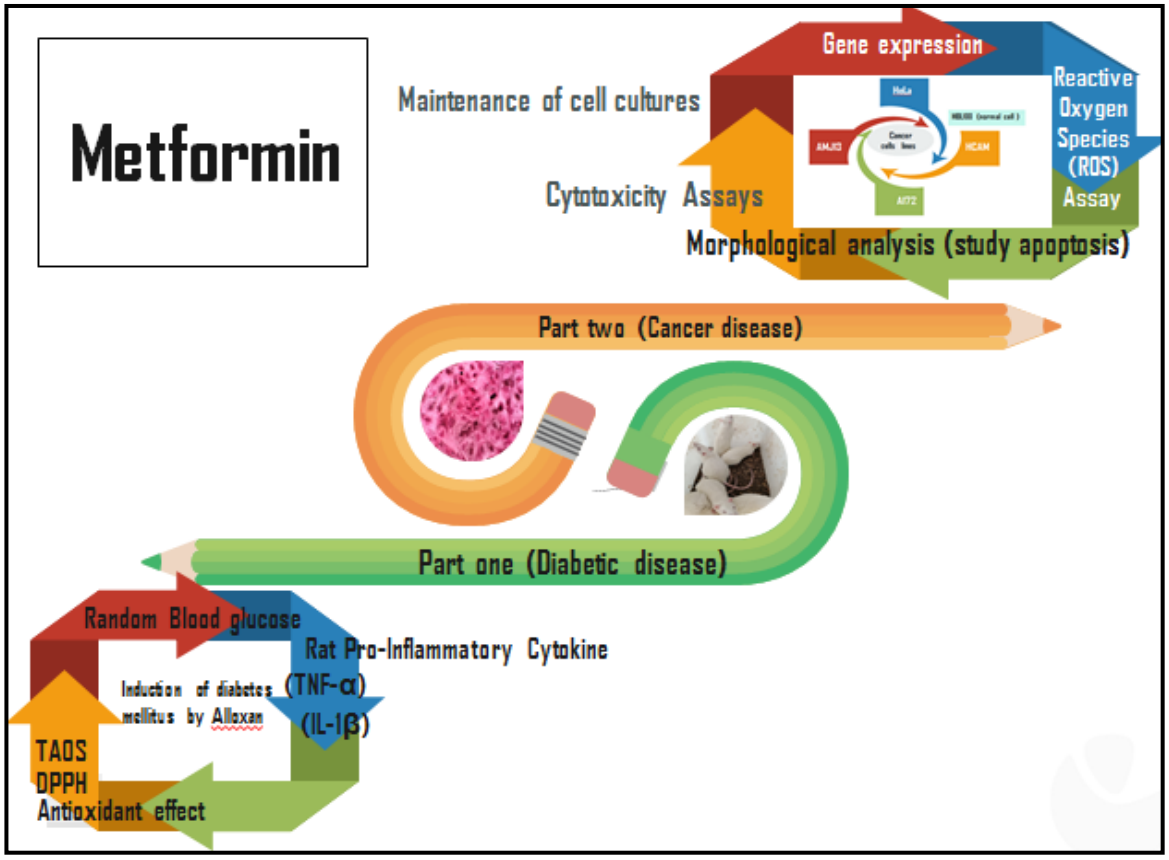
Primer sequences of PI3K/AKT/mTOR pathway genes

Gene	Sequence 5′ - 3	Fragment size (pb)
PIK3CA	Fw: GGTTGTCTGTCAATCGGTGACTGT	108
	Rv: GAACTGCAGTGCACCTTTCAAGC	
PIK3CB	Fw: TTGTCTGTCACACTTCTGTAGTT	166
	Rv: AACAGTTCCCATTGGATTCAACA	
PTEN	Fw: GGTTGCCACAAAGTGCCTCGTTTA	129
	Rv: CAGGTAGAAGGCAACTCTGCCAAA	
AKT1	Fw: TTCTGCAGCTATGCGCAATGTG	181
	Rv: TGGCCAGCATACCATAGTGAGGTT	
MTOR	Fw: GCTTGATTTGGTTCCCAGGACAGT	194
	Rv: GTGCTGAGTTTGCTGTACCCATGT	
EIF4EBP1	Fw: GCATCAGCTTTTAGGTGCAAAGGA	188
	Rv: GGCAACTGCCAAAAGTGATTCAGC	
EIF4E	Fw: GAACGAACCCTTCCTTCCGAATGA	143
	Rv: AGGGCGAAGGTGGCTTTTATTTCC	
RPS6KB1	Fw: ACTGTAGTGTTGACTGCCTGACCA	105
	Rv: TAGCCAGCCAATCACAGTGCTCAT	
TSC1	Fw: GCAGCGTGACACTATGGTAACCAA	144
	Rv: AGTTCTATCCGCAGCTCCGCAAT	
GAPDH	Fw: TGCACCACCAACTGCTTAGC	130
	Rv: GGCATGGACTGTGGTCATGAG	
ACTB	Fw: GACAGGATGCAGAAGGAGATTACT	117
	Rv: TGATCCACATCTGCTGGAAGGT	

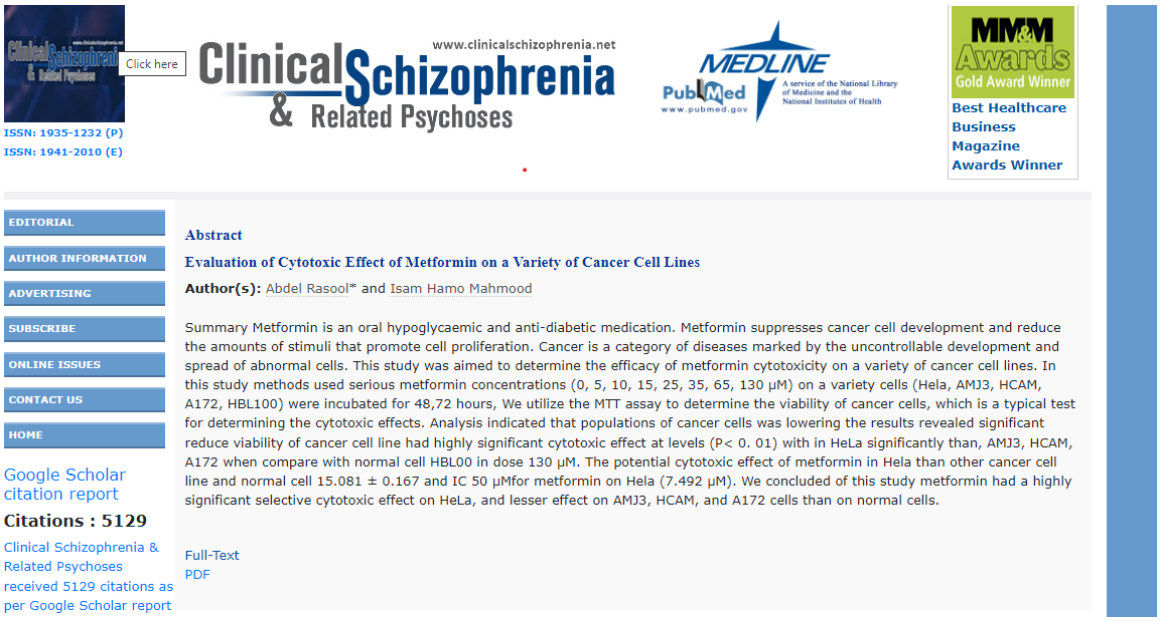
Appendix (1): Primer sequence of PI3K/AKT/mROT pathway genes



Appendix (2): Summary of Main Line of Study.

Publication Article

1. Evaluation of Cytotoxic Effect of Metformin on a Variety of Cancer Cell Lines scoup https://www.clinicalschizophrenia.net/articles/evaluation-of-cytotoxic-effect-of-metformin-on-a-variety-of-cancer-cell-lines-73916.html.



2. Impact of Metformin on Pro-inflammatory Cytokines and Histological Evaluation of the Liver Induced Diabetes on Albino Male Rats https://ijddt.com/volume11issue4/.

3. " Metformin's Antioxidant Activity and Protective Nature"

31. Impact of Metformin on Pro-inflammatory Cytokines and Histological Evaluation of the Liver Induced Diabetes on Albino Male Rats

Abeer M. A. Rasool, Isam H. Mahmood

Abstract

This study aimed to assess metformin effect with different concentration and duration on pro-inflammatory factors serum and tissue of liver and its impact upon the liver of diabetic albino rats. Metformin is administered in multi doses at various times to diabetic rats. Alloxan injected to induced diabetes in the rats, and then metformin was administered by gavage; then blood and tissue collected to analyze the biochemical parameters (a pro-inflammatory cytokine), serum blood glucose, body weight blood, and tissue were collected. Liver tissues were collected for histologic analysis at the beginning of experiments day 0, days 7, days 21, and days 35 (end of experiments) and sacrificed three animals for each period. The findings metformin-treated diabetic rat showed significant changes observed in terms of body weight and food intake behavior. Metformin treatment dropped serum blood glucose levels in diabetic rats. Following metformin treatment, an ELISA assay indicated dropped levels of pro-inflammatory markers in the liver tissues and serum. Further, metformin treatment reduced the intensity of liver inflammation measured by pathologists blindly. **Conclusion:** The results of reducing pro-inflammatory cytokines in the liver tissues and serum recommend the anti-inflammatory effects of metformin to improve inflammation.

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IN HONOUR FOR PARTICIPATING IN THE INVALUABLE CONTRIBUTION
Metformin's Antioxidant Activity and Protective Nature
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A.PROF. DR. ODAY A. AL-OWAEDI
CONFERENCE CHAIR

Date: October 28 2021
Paper ID: IICPS460

LETTER OF ACCEPTANCE

Dear Authors,

On behalf of the IICPS -21 Committee, and based on the reviewers' evaluation after double blind peer review and Guest editors' approval we are pleased to inform you that your paper entitled

" Metformin's Antioxidant Activity and Protective Nature"

Written

Abdel Rasool.A.M , Isam Hamo Mahmood

Has been accepted and will be processed to publication in the IOP Journal of Physics (Online ISSN: 1742-6596, Print ISSN: 1742-6588, IICPS-2021). It is our pleasure to invite you to attend the Iraqi Academics Syndicate 2nd International Conference for Pure and Applied Sciences (IICPS), Babylon Branch, Babylon, Iraq at 14-15 November 2021 to present your paper. We congratulate you for your achievement, the technical details about the publication will be informed later. The publication of the accepted paper will be provided by the end of January 2022.

We Will encourage more quality submissions from you and your colleagues in future

Regards,

Prof. Dr. Shubham Sh. Sharma
Special Issue Guest Editor of IICPS

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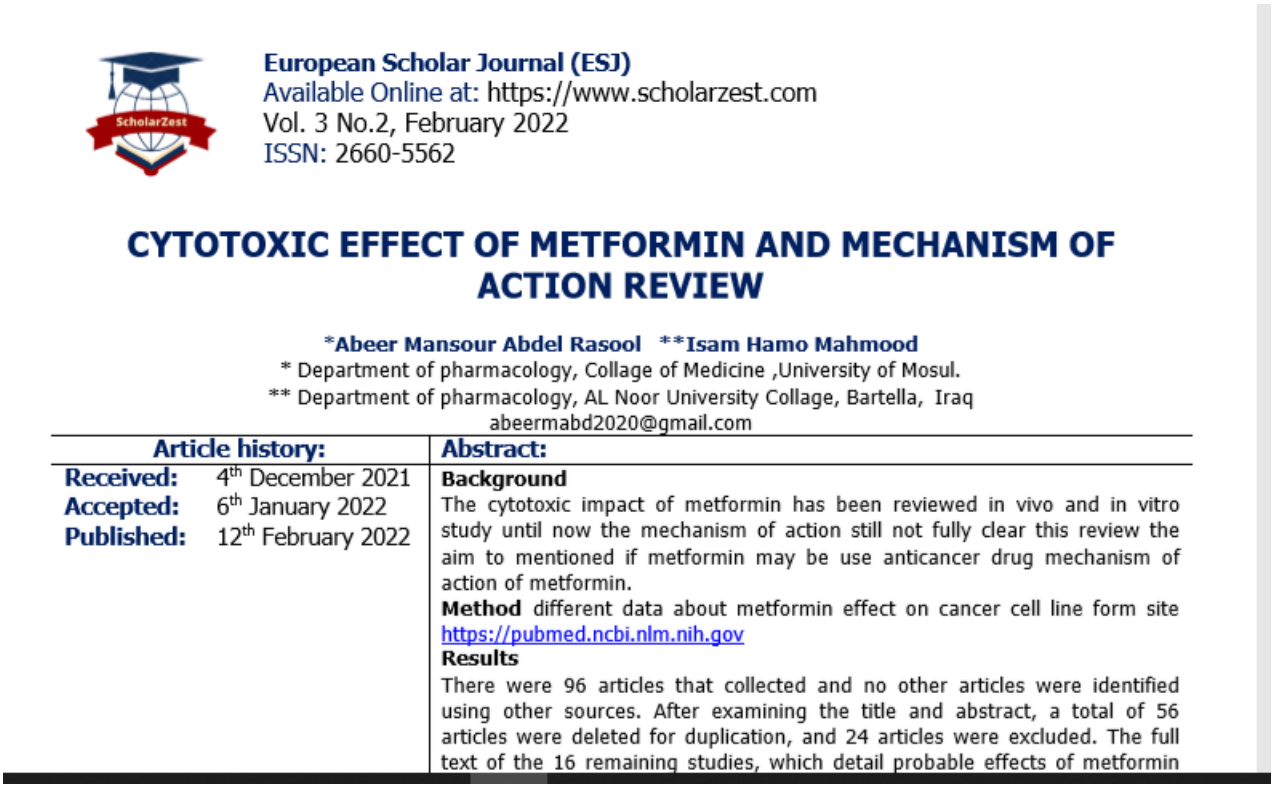
Caution: This Acceptance Letter Made by IICPS Conference Guest Editors and All Approval Inquiries Should Addressed to Editorial Board Committee of IICPS. .

4.Cytotoxic Effect of Metformin And Mechanism Of Action Review



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CYTOTOXIC EFFECT OF METFORMIN AND MECHANISM OF ACTION REVIEW

*Abeer Mansour Abdel Rasool **Isam Hamo Mahmood
* Department of pharmacology, Collage of Medicine ,University of Mosul.
** Department of pharmacology, AL Noor University Collage, Bartella, Iraq
abeermabd2020@gmail.com

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Received: 4th December 2021
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Abstract:

Background
The cytotoxic impact of metformin has been reviewed in vivo and in vitro study until now the mechanism of action still not fully clear this review the aim to mentioned if metformin may be use anticancer drug mechanism of action of metformin.
Method different data about metformin effect on cancer cell line form site https://pubmed.ncbi.nlm.nih.gov
Results
There were 96 articles that collected and no other articles were identified using other sources. After examining the title and abstract, a total of 56 articles were deleted for duplication, and 24 articles were excluded. The full text of the 16 remaining studies, which detail probable effects of metformin

5-Comparison Cytotoxicity effects of metformin and paclitaxel on cervical cells and normal cell.

Date: July 01, 2022

Certificate of Acceptance

Title of Paper: Comparison Cytotoxicity effects of metformin and paclitaxel on cervical cells and normal cell.

Authors: Abeer Mansour Abdel Rasool * and Isam Hamo Mahmood**

***Department of Pharmacology, University of Mosul, Mosul, Iraq.**
****Department of Pharmacology, University of Al Noor, Bartella, Iraq.**

Dear Authors,

The Editorial Board of **Bulletin of National Institute of Health Sciences (ISSN:13434292)** hereby states with great pleasure that following the reviewing process, your above indicated manuscript has been accepted for publication in next issue of 2022.

We would like to thank you for your submitted paper.

Regards,

Editor
Prof. Juro Sakai

الملخص

الميتفورمين هو دواء يستخدم على نطاق واسع لعلاج مرض السكري الذي يتميز بفرط سكر الدم وهو من الأدوية التي يتم وصفها عن طريق الفم من مجموعة البيجوانيدات حظيت التأثيرات المتعددة للميتفورمين مؤخرًا باهتمام متزايد بسبب تأثيره السام للخلايا المتنوعة السرطانية وتأثيره على نمو وتكاثر الخلايا السرطانية حيث تناولت العديد من الأبحاث في هذا المجال حول وجود علاقة بين داء السكري وخطر الإصابة ببعض أنواع السرطان، ويرجع ذلك أساسًا إلى حقيقة أن مرض السكري هو حالة من زيادة الإجهاد التأكسدي والالتهاب والتي بدورها يمكن أن تعزز الإصابة بالسرطان الذي يعرف أنه انتشار ونمو غير طبيعي للخلايا. تهدف الدراسة إلى تقييم تأثير الميتفورمين (بجرع مختلفة وفترات زمنية مختلفة) على مستويات الالتهاب إنترلوكين 1 بيتا (IL- 1β) وعامل نخر الورم (TNF- α) والإجهاد التأكسدي السعة الكلية لمضادات الاكسدة(TAOS) ومالونديالديهيد (MDA) بالإضافة الى تقييم الهدف الرئيسي من الدراسة الحالية هو دراسة الفعالية السمية للميتفورمين على الخلايا السرطانية

المواد وطرق العمل للقيام بذلك تم تقسيم طريقة إلى جزأين الجزء الأول دراسة النموذج المختبري / التداخلي المتعلق باستحداث مرض السكري في ذكور الجرذان البيضاء عن طريق استخدام الوكسان بجرعة 200 مجم / كجم تم تقسيم اجمالي العدد 75(15جرذان / مجموعة) ثم قسمت إلى خمس مجموعات مجموعة الضابطة، ومجموعة مرض السكري و مجاميع العلاج الميتفورمين بجرع 100، 200، 300 مجم / كجم / يوم بعد 21 و35 يوما لتقييم التأثيرات المضاد للالتهابات والأكسدة لجرعات مختلفة من الميتفورمين

تبعًا للهدف الرئيسي للدراسة الحالية، بتسليط الميتفورمين على خلايا الخطوط الخلوية السرطانية (HeLa, HCA, HBL100, A172, AMJ13) باستخدام اختبار MTT الي تم تجهيزها في المختبر العراقي IRAQ Biotech Cell Bank حيث ان جميع الخلايا محفوظة في RPMI-1640 مع(Fetal bovine %10) و (streptomycin 100 g/mL) تم Trypsin-EDTA. وحضنت عند 37 درجة مئوية و 5٪ من ثاني أكسيد الكربون ثم دراسة نسبة السمية الخلوية عن طريق مقايسة MTTوثم حساب IC50 لجميع خطوط السرطان مع دراسة فحص موت الخلايا المبرمج للموت الخلوي ، والاجهاد التاكسدي للخلايا السرطانية والدراسة الخلوية للتعبير الجيني .

أظهرت النتائج من الجزء الأول أن الفئران المصابة بداء السكري التي عولجت بالميتفورمين أظهرت تغيرات كبيرة من حيث وزن الجسم وسلوك الأكل. خفض علاج الميتفورمين مستويات السكرفي الدم لدى الفئران المصابة بداء السكري في اليوم 35 مقارنة باليوم 7 للمجموعات الثلاث P = 0.000

وبالنسبة لمستويات TNF-α في مصل الدم وانسجة الكبد فرق معنوي بين المجموعات الثلاث في اليوم 7 و21 و35 مع اختلاف الجرع ودراسة مستويات

IL-1β المتعلقة بالمصل والكبد أظهرت فرقا معنويا للمجاميع العلاجية بالجرع المختلفة. فيما يتعلق بالإجهاد التأكسدي، أظهرت الدراسة الحالية ارتفاعا ملحوظا في TAOS في المجموعات المعالجة بالميتفورمين في اليومين 21 و35 مقارنة بمجموعة السكري التي تطابقت مع درجة البنكرياس المرضية النسيجية. مع انخفاض MDA بشكل ملحوظ بعد العلاج بالميتفورمين أشارت النتائج إلى دواء المتيفورمين كانت له فعالية ضعيفة في اختزال وكانت الفعالية تدريجية اعتمادا على زيادة تركيز الدواء IC 50 للميتفورمين (498.0 ميكروغرام / مل) بينما IC50 حمض الأسكوربيك (29.62 ميكروغرام / مل) على التوالي عند 60 وعند فحص تأثير الفعالية السمية للميتفورمين وجد انخفاضًا كبيرًا ملحوظا في قابلية الخطوط السرطانية Hela مقارنة بـ AMJ3 وHCAM وA172 علاوة على ذلك، كان هذا العلاج آمن ا نسبي للخلايا الطبيعة HB100ولاحظ موت الخلايا المبرمج مع تغيير الشكل المورفولوجي وأظهر ايضا انخفاضًا كبيرًا في إنتاج ROS بعد إضافة الميتفورمين بالنسبة لدراسة مسلك عمل الميفورمين على الخط السرطاني Hela لوحظ زيادة غير معنوية في التعبير الجيني PIK3CA على عكس جين التعبير عن جين mTOR AKT1لوحظ تغير معنوي بشكل كبير. يشير هذا إلى أن علاج الميتفورمين كان له تأثيرات على كل من جينات mTOR وAKT1 ولكن ليس على PIK3CA

الاستنتاج لوحظ أن لميتفورمين تأثير مفيد على مرض السكري من خلال خفض نسبة السكر في الدم والإجهاد التأكسدي والالتهابات بالإضافة إلى تأثيره الوقائي للأنسجة إلى جانب حمايته من التاثير المسرطن الناجم عن مرض السكري، وبالجانب الاخر كان للميتفورمين فعالية سمية للخلايا على مجموعة متنوعة من الخلايا السرطانية. إضافة الميتفورمين مفيد لتقليل الآثار الجانبية للعلاج الكيميائي معظم الآثار الجانبية لمرض السكري بعد العلاج الكيميائي هي تعديل مقاومة العلاج الكيميائي عن طريق التأثير على mTOR

يعتبر علاجا مستقبليا للخلايا السرطانية وطريقة استراتيجية جديدة لاستخدامه كعلاج فعال عند إضافته مع الادوية المضادة للسرطان.

الملخص

الميتفورمين هو دواء يستخدم على نطاق واسع لعلاج مرض السكري الذي يتميز بفرط سكر الدم وهو من الأدوية التي يتم وصفها عن طريق الفم من مجموعة البيجوانيدات حظيت التأثيرات المتعددة للميتفورمين مؤخرًا باهتمام متزايد بسبب تأثيره السام للخلايا المتنوعة السرطانية وتأثيره على نمو وتكاثر الخلايا السرطانية حيث تناولت العديد من الأبحاث في هذا المجال حول وجود علاقة بين داء السكري وخطر الإصابة ببعض أنواع السرطان، ويرجع ذلك أساسًا إلى حقيقة أن مرض السكري هو حالة من زيادة الإجهاد التأكسدي والالتهاب والتي بدورها يمكن أن تعزز الإصابة بالسرطان الذي يعرف أنه انتشار ونمو غير طبيعي للخلايا. تهدف الدراسة إلى تقييم تأثير الميتفورمين (بجرع مختلفة وفترات زمنية مختلفة) على مستويات الالتهاب إنترلوكين 1 بيتا (IL- 1β) وعامل نخر الورم (TNF- α) والإجهاد التأكسدي السعة الكلية لمضادات الاكسدة(TAOS) ومالونديالديهيد (MDA) بالإضافة الى تقييم الهدف الرئيسي من الدراسة الحالية هو دراسة الفعالية السمية للميتفورمين على الخلايا السرطانية

المواد وطرق العمل للقيام بذلك تم تقسيم طريقة إلى جزأين الجزء الأول دراسة النموذج المختبري / التداخلي المتعلق باستحداث مرض السكري في ذكور الجرذان البيضاء عن طريق استخدام الوكسان بجرعة 200 مجم / كجم تم تقسيم اجمالي العدد 75(15جرذان / مجموعة) ثم قسمت إلى خمس مجموعات مجموعة الضابطة، ومجموعة مرض السكري و مجاميع العلاج الميتفورمين بجرع 100، 200، 300 مجم / كجم / يوم بعد 21 و35 يوما لتقييم التأثيرات المضاد للالتهابات والأكسدة لجرعات مختلفة من الميتفورمين

تبعًا للهدف الرئيسي للدراسة الحالية، بتسليط الميتفورمين على خلايا الخطوط الخلوية السرطانية (HeLa, HCA, HBL100, A172, AMJ13) باستخدام اختبار MTT الي تم تجهيزها في المختبر العراقي IRAQ Biotech Cell Bank حيث ان جميع الخلايا محفوظة في RPMI-1640 مع(Fetal bovine %10) و (streptomycin 100 g/mL) تم Trypsin-EDTA. وحضنت عند 37 درجة مئوية و 5٪ من ثاني أكسيد الكربون ثم دراسة نسبة السمية الخلوية عن طريق مقايسة MTTوثم حساب IC50 لجميع خطوط السرطان مع دراسة فحص موت الخلايا المبرمج للموت الخلوي ، والاجهاد التاكسدي للخلايا السرطانية والدراسة الخلوية للتعبير الجيني .

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