Republic of Iraq
Ministry of Higher Education and Scientific Research
University of Babylon
College of Medicine
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The effect of 2-deoxy-D-glucose and metformin on the viability of SW480 colon cancer cells. An *in vitro study*

A thesis tjujryujyuj

Submitted to the Council of the College of Medicine, the University of Babylon, as a Partial Fulfillment of the Partial Requirements for the Degree of Master of Science (M.Sc.) in Pharmacology

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنْكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ﴾

صدق الله العليَ العظيم

سورة المجادلة: (الآية 11)

Dedication

I Dedicate This Thesis To *My Family* .

A special feeling of gratitude to *my loving parents (Salah and Layla),* who encouraged me all the way.

My husband *Ahmed* and my little angle *Yaasoob*

***Sisters* and *brothers* who never left my side were very special. I also dedicate this thesis to my family in low *(uncle Fadhil and aunt fayqa)* and *friends* who have supported me throughout the process, and will always appreciate all they have done.**

Sarah Salah Hassan

Certification

We certify that this thesis entitled (**The effect of 2-deoxy-D-glucose and metformin on the viability of SW480 colon cancer cells. An *in vitro study***) was prepared by (**Sara Salah Hassan Salman**) under our supervision at the department of pharmacology and toxicology, College of Medicine, University of Babylon (Iraq) in partial fulfillment of the requirements for the Master degree of Sciences in Pharmacology .

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We certify that we have read this thesis entitled "(**The effect of 2-deoxy-D-glucose and metformin on the viability of SW480 colon cancer cells. An *in vitro study*)**" and as an examining committee examined the student "**Sarah Salah Hassan** " in its contents and that in our opinion it meets the standard of a thesis for the degree of Master, in Pharmacology .

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Acknowledgments

First, I want to thank God for helping me and giving me the strength, willingness, and patience to finish what I have started. I would like to express my great appreciation to *Prof. Dr. Entisar Jawad* for her supervision, help, continuous advice, efforts in helping me.

I would like to express my deepest gratitude and thanks to *Pro. Dr. Kaiser N. Madlum* for his help and contribution to the laboratory.

I would like to express my deepest gratitude to the dean of Medicine College *Prof. Dr. Safaa Sahib Naji* and head of Pharmacology and department *Assist. Prof. Dr. Selman Mohammed Selman* for their kindness and help. My special thanks also go to *Dr.Hammed N.Obaid* and *Dr.Rana A.Ghaleb* for their help and support. My special thanks also go to *Dr.Najwa Kahtan* and my sister *Dr.Batool Salah* for their help and support .Thanks go to all the staff in the Department of Pharmacology, College of Medicine, University of Babylon...

I owe special thanks to my family, *my father, mother, my husband*, my *brothers* and *sisters*, my lovely child *Yaasoob*, **and** *all my family*. Who supported me and helped me throughout my life, and during this study. Their love, emotion, and moral has been and always be the source of my inspiration, I dedicate this work to them all.

Sarah Salah

Summery

Colon cancer is one of the leading cause of tumor related death in the world, it is considered among the big killers, together with lung, prostate, and breast cancers. In the present study, an attempt has been made to evaluate the effect of 2-deoxy-d-glucose and metformin in combination with 5-fluorouracil and doxorubicin on the viability of colon cancer (SW 480) cell line and normal Vero cell line.

The experimental work was performed in the post-graduate student's research laboratory at the College of Medicine/University of Babylon during the period from October 2020 to August 2021.

The first experiment involved the treatment of SW480 colon cancer cell line with different concentrations of 2-deoxy-d-glucose, metformin, 5-fluorouracil, and doxorubicin to determine the cytotoxic concentrations of these chemical agents on this type of cells. The effect of each drug was evaluated in separated 96 wells plate divided into 7 groups; one control group and six serial dilutions of the agent. These serial dilutions started from 4000μg/ml to 125 μg/ml for 2-deoxy-d- glucose and metformin, from 1000 to 31.25 μg/ml for 5florouracil, and from 250 to 7.25 μg/ml for doxorubicin. All plates were incubated for 24 hours at 37c, then, the MTT cytotoxicity assay was performed.
The same parameters were repeated on Vero cell line to evaluate the effects of these drugs on normal cells.

After the assessment of cytotoxicity for each drug on the cells, normal and colon cancer cells were treated with different drugs combinations involved:

1- Combinations of 2-deoxy-d-glucose with metformin.
2- Combinations of 2-deoxy-d-glucose with 5-fluorouracil
3- Combinations of 2-deoxy-d-glucose with doxorubicin.
4- Combinations metformin with 5-fluorouracil
5- Combinations metformin with doxorubicin.
6- Combinations of 2-deoxy-d-glucose plus metformin plus 5-florouracil .

7- Combinations of 2-deoxy-d-glucose plus metformin plus doxorubicin.

The result showed that 2-deoxy-d-glucose at concentrations (500,1000,2000, and 4000 µg/ml), cause a significant ($P<0.050$) decrease in the viability of normal and colon cancer cells compared to the control group.

Metformin at concentrations of (2000, and 4000 µg/ml) caused a significant ($P<0.050$) decrease in the viability of normal and colon cancer cells.

Result showed that 5-fluorouracil (from 1000 to31.25µg/ml) and Doxorubicin (from 250 to 7.8 µg/ml) caused a significant ($P<0.050$) decrease in the viability of both SW480 colon cancer cells and normal Vero cells at all used concentrations compared to the control group.

Regarding the effect of tested combinations, results showed that the combination of 2-deoxy-d-glucose plus metformin caused a significant ($p<0.05$) decrease in the viability of SW480 colon cancer cells at all applied concentrations, The most effective combination was that contains (2-deoxy-d-glucose 1000 µg/ml+ metformin 4000 µg/ml).

Results showed that the growth of SW480 colon cancer cells was significantly ($p<0.05$) inhibited after the treatment with 5-fluorouracil in combination with 2-deoxy-d-glucose or metformin, and the most effective combination was that containing (2-deoxy-d-glucose 2000 µg/ml+ 5-fluorouracil 125 µg/ml) and (2-deoxy-d-glucose 2000 µg/ml +metformin4000 µg/ml +125 µg/ml 5-fluorouracil).

Results showed that the growth of SW480 colon cancer cells was significantly ($p<0.05$) inhibited after the treatment with doxorubicin in combination with 2-deoxy-d-glucose or metformin, and the most effective combination was that containing (2-deoxy-d-glucose 2000 µg/ml+ doxorubicin 15 µg/ml) and (2-deoxy-d-glucose 2000 µg/ml +metformin4000 µg/ml +15 µg/ml doxorubicin).

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List of Abbreviations

Abbreviations	Meaning
2DG	2-Deoxy-d-glucose
2-DG-6-P	2-deoxy -glucose-6-phosphate
2-DG-P	2-deoxyglucose-phosphate
5FU	5-Fluorouracil
5-FU	5-Fluorouracil
CC	Colon cancer
CH2–THF	5,10-methylenetetrahydrofolate
CRC	Colorectal cancer
CT	computed tomography
DDW	double distilled water
DOX	Doxorubicin
EDTA	Trypsin-ethylenediaminetetraacetic acid
EMT	epithelial-to-mesenchymal transition
ER	endoplasmic reticulum
FA	Folinic acid
FBS	fetal bovine serum
FDA	Food and drug administration
FdUMP	5-fluorodeoxyuridine monophosphate

FdUTP	fluorodeoxyuridine triphosphate
FOLFOX	Oxaliplatin
FUTP	fluorouridine triphosphate
GBC	gall bladder Cells
HK2	hexokinase2
hPBLs	human peripheral blood lymphocytes
IC50	The half maximal inhibitory concentration
JNK	c-Jun N-terminal kinase
lncRNAs	Long noncoding RNAs
MAPK	mitogen-activated protein kinase
MCA	metachronous colorectal adenomas
NCI	National Cancer Institute
PBS	Phosphate buffer saline
RIP	receptor-interacting protein kinase
ROS	reactive oxygen species
RPMI-1640)	Roswell Park Memorial Institute
TS	thymidylate synthase
UCA1	-urothelial carcinoma-associated 1
WHO	World Health Organization

Chapter One

Introduction and Literature review

1.1 Introduction

Cancers are defined by the National Cancer Institute (NCI) as a collection of diseases in which abnormal cells can divide and spread to nearby tissue. Cancer is one of the main causes of death worldwide. Despite the significant development of methods of cancer healing during the past decades, chemotherapy remains the main method for cancer treatment. Depending on the mechanism of action, commonly used chemotherapeutic agents can be divided into several classes (antimetabolites, alkylating agents, mitotic spindle inhibitors, topoisomerase inhibitors, and others) (Bukowski *et al*., 2020).

More than 200 different forms of cancer were being diagnosed, each arising in a specific way; yet, what they all share is the fact that they are all caused in the same way: a shift in a cell's normal internal structure. Common features among different types of this disease are: 1) increased growth signals; 2) failing to respond to anti-growth signals; 3) unscheduled cell death; 4) angiogenesis, and 5) invasion of tissue and metastasis so that cancer cells can spread to other parts of the body via the bloodstream or lymph (Saraei *et al*., 2019).

Colon cancer is the most common type of cancer globally. Therapies for colon cancer include surgery, chemotherapy, targeted therapy, and immune therapy. However, colon cancer is characterized by both aggressive behavior and a poor response to chemotherapy (Guo *et al.,* 2020). Chemotherapeutic agents cause many side effects including digestive problems, leukopenia, and hair loss due to affecting normal cells through damaging DNA of the cell (Munker *et al*., 2018).

Glucose analogs have been found to profoundly inhibit glucose metabolism in cancer cells in vitro and in vivo. 2-Deoxy-d-glucose is a glucose molecule that cannot undergo further glycolysis. The ability of 2-deoxy-d-

glucose(2-DG) to interfere with d-glucose metabolism demonstrates that nutrient and energy deprivation is an efficient tool to suppress cancer cell growth and survival (Fokt *et al* ., 2020).

Metformin a biguanide class of anti-diabetic drugs, possesses anti-cancer properties, metformin can: 1) reduce the incidence of cancers, 2) reduce the mortality from cancers, 3) increase the response to treatment in cancer cells when using radiotherapy and chemotherapy, 4) optimize tumor metastasis and reduce the malignancy, 5) reduce the likelihood of relapse. Therefore, this drug can be used as a complementary therapeutic agent for cancer treatment and prevention (Saraei *et al*., 2019).

1.2 The main aim of the present study were:

1. To study the effects of 2-deoxy-d- glucose, metformin, doxorubicin, and 5-fluorouracil on SW480 colon cancer cell line.
2. To evaluate the safety profile of 2-deoxy-d-glucose, metformin, doxorubicin, and 5-fluorouracil on a normal VERO cell line.
3. Study the effects of 2-deoxy-D-glucose and metformin together or separately on the viability of SW480 colon cancer and normal VERO cell line. And on the activity of anticancer drug (5-fluorouracil and doxorubicin) on viability of SW480 colon cancer and normal VERO cell line.

1.3 Cancer

Cancers are a group of diseases characterized by uncontrolled growth and the spread of abnormal cells. If the spread of cancer cells (this stage is known as metastasis) is not controlled, it can result in death. Cancer cells adopt different strategies allowing them to proliferate and invade other tissues, including avoidance of apoptosis, lack of sensitivity to growth inhibitors, independence from growth signals, unlimited replication potential, angiogenesis, immune escape, genetic instability, induction of chronic inflammation, and finally changes in cell metabolism. Cancer is caused by many external factors (tobacco, chemicals, radiation, and infectious organisms) as well as some internal factors (inherited mutations, hormones, immune conditions, and random mutations). The causes of cancer are diverse complex and only partially understood. Many things are known to increase the risk of cancer, including dietary factors, certain infections, lack of physical activity, obesity, and environmental pollutants((Mathur *et a l*., 2015) ; (Fokt *et al*., 2020)).

1.4 Symptoms of cancer:

Symptoms of cancer depend on the type and location of cancer. For example, lung cancer can cause coughing, shortness of breath, or chest pain. Colon cancer often causes diarrhea, constipation. Some cancers may not have any symptoms at all, for example, pancreatic cancer. In certain cancers, such as pancreatic cancer, symptoms often do not start until the disease has reached an advanced stage.

The following symptoms can occur with most cancers :

Chills, fatigue, fever, loss of appetite, malaise, night sweats, weight loss, cough or hoarseness that does not go away, changes in bowel or bladder habits,

unexplained bleeding or discharge, any sore that does not heal, unusual upset stomach or difficulty in Swallowing (Mathur *et al*., 2015).

1.5 Types of Cancer

Cancer can be classified on basis of the affected tissues or organs as follow:

1.5.1On the basis of the affected tissue:

Carcinomas are characterized by cells that cover internal and external parts of the body such as lung, breast, and colon cancer.

Sarcomas are characterized by cells that are located in bone, cartilage, fat, connective tissue, muscle, and other supportive tissues.

Lymphomas are cancers that begin in the lymph nodes and immune system tissues.

Leukemias are cancers that begin in the bone marrow and often accumulate in the bloodstream.

Adenomas are cancers that arise in the glands such as the thyroid, the pituitary gland, the adrenal gland, and other glandular tissues.

1.5.2 On the basis of the affected organ:

Colorectal cancer, Lung Cancer, Liver Cancer, Stomach Cancer, Cervical Cancer, Bladder Cancer, Esophageal Cancer, Non-Hodgkin Lymphoma, Cancers of the Lip and Oral Cavity, Nasopharyngeal Cancer, Kaposi Sarcoma (Mathur *et al.*, 2015).

1.6 Cancer Metastasis

Metastasis occurs when genetically unstable cancer cells adapt to a tissue microenvironment that is distant from the primary tumor. Metastases account for

the great majority of cancer-associated deaths, yet this complex process remains the least understood aspect of cancer biology. The dissemination of cancer cells from primary tumors and their subsequent seeding of new tumor colonies in distant tissues involves a multi-step process known as the invasion metastasis cascade. This sequence of events involves the local invasion of primary tumor cells into surrounding tissues; intravasation of these cells into the circulatory system and survival during hematogenous transit; arrest and extravasation through vascular walls into the parenchyma of distant tissues; formation of micrometastatic colonies in this parenchyma; and the subsequent proliferation of microscopic colonies into overt, clinically detectable metastatic lesions, this last process being termed colonization. The growth of an overt metastatic colony represents the final and most deadly phase in the malignant progression of a tumor. Still, the vast majority of carcinoma cells in circulation seem ill-prepared for growth in a distant organ environment as in (figure 1.1) (Lambert *et al* ., 2017).

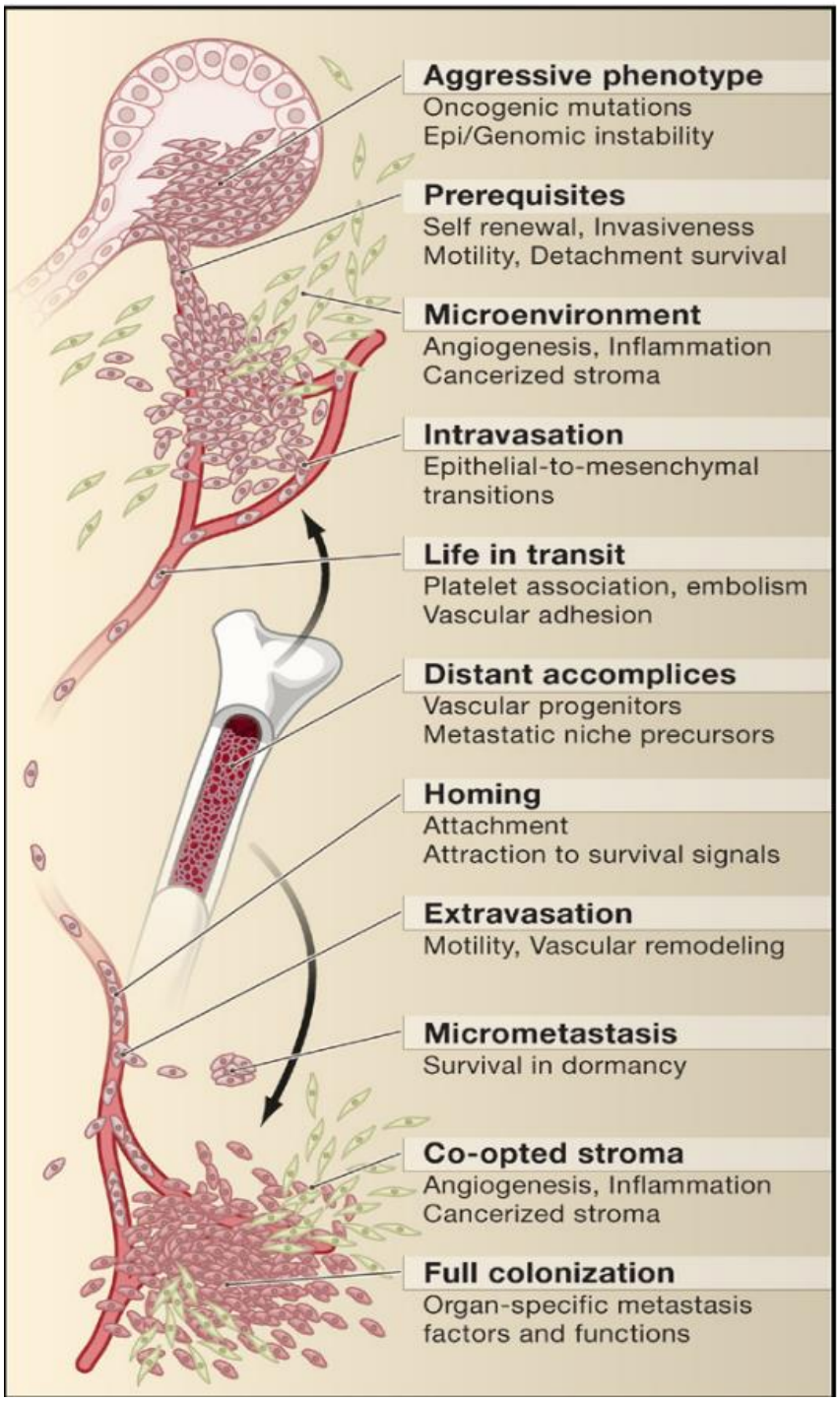

Figure 1.1 Stages of Metastatic Progression.

1.7 Cancer Epidemiology

Global cancer statistics in 2018 assesses that 18.1 million newly diagnosed cases and 9.6 million cancer deaths occurred globally. Over one-half of these cancer deaths occur in Asia, estimated by(57.3%) because of nearly(60%)of the

population located there. Europe reports (20.3%) of cancer deaths which (globally accounts only 9%), followed by(14.4%) of cancer deaths occurred in America. Lung cancer is the first leading cancer death which is about (18.4%) of all cancer deaths followed by (9.2%) for colorectal cancer and (8.2%) for both stomach cancer, and liver cancer. The most incidence rate of cancer has been seen in lung cancer(11.6%) of all newly diagnosed cases in 2018, closely followed by breast cancer (11.6%), colorectal cancer (10.2%), and prostate cancer (7.1%). According to the gender, breast cancer has a higher incidence rate among females, followed by colorectal and lung cancer, while in terms of mortality the breast cancer also is the first and lung cancer is the second cause of death for women followed by colorectal cancer, in contrast, lung cancer has a higher incidence in men (Ferlay *et al*., 2018).

1.8 Colon Cancer

Colon cancer (CC) is one of the leading death tumors in the world and it is considered among the big killers, together with lung, prostate, and breast cancer (Roberto Labianca *et al.,* 2010). Colon cancer is a type of gastrointestinal malignancy originating from the colon. (Mattiuzzi *et al*., 2019). The incidence of CC is increasing because of smoking, lack of physical activity, overweight, and obesity, consuming red and processed meat, and excessive alcohol-consuming (Hamza *et al* ., 2017).

1.8.1 Colon cancer epidemiology :

Colon and rectal cancers altogether (i.e., colorectal cancer) are the second leading overall cause of cancer death as well as the fourth cause of cancer mortality in men and women(Mattiuzzi *et al*., 2019). colorectal cancer is the third most common type of cancer worldwide. Cancer of the colon is more frequent than rectal cancer: in industrialized countries, the ratio of the colon to

rectum cases is 2:1 or more (rather more in females) while in non-industrialized countries rates are generally similar (Labianca *et al*., 2010).

1.8.2 Risk factors of colon cancer :

The risk of developing colon cancer depends on factors that can be classified into lifestyle or behavioral factors (such as smoking, high red meat consumption, obesity, physical inactivity), and genetically determinant factors. According to international guidelines, screening tests are stratified according to the personal risk of disease. Age is considered the major unchangeable risk factor for sporadic colon cancer: nearly (70%) of patients with colon cancer are over 65 years of age.

Individuals with: (i) personal history of adenoma, colon cancer, inflammatory bowel disease (Crohn's disease and ulcerative colitis), (ii) significant family history of colorectal cancer or polyps, (iii) an inherited syndrome (5–10% of all colon cancers) such as familial adenomatous polyposis coli and its variant (1%) (Labianca *et al*., 2013).

1.8.3 Symptoms of colon cancer

Colon cancer develops in the early stages mostly without any symptoms. However, CC patients with symptoms such as bowel perforation, obstruction, or gastrointestinal bleeding present in 15–30 % of cases. These complications occur mostly in older patients (Ferlay *et al*., 2018)

The most common colon cancer symptoms are:

• Change in bowel habits, such as diarrhea, constipation, which lasts longer than a few days.

• Feeling of incomplete evacuation.

• Blood in the feces which makes the stools appear black.

• Pain in the abdomen, and bloating.

• Feeling of abdominal fullness, even after not eating for a while.

• Fatigue or exhaustion.

• Weight loss inexplicable (Mathur *et al*., 2015).

1.8.4 Diagnosis of colon cancer

Endoscopy is the main procedure for diagnosis and can be carried out by either sigmoidoscopy (as >35% of tumors are located in the rectosigmoid) or (preferably) a total colonoscopy. The advantages of endoscopy are many, e.g. determination of the exact localization and biopsy of the lesion, detection of (further) synchronous precancerous or cancerous lesions, and removal of polyps. Before surgery, if a complete colonoscopy cannot be carried out for whatever reason, the rest of the colon should be visualized by combining a limited left-sided colonoscopy with a barium enema to study the proximal colon. Virtual colonoscopy or computed tomography (CT) colonography are not yet standard investigations but are valuable instruments to identify with precision the location of the tumor or to detect synchronous lesions or polyps, and they are potentially helpful for patients eligible for laparoscopic resection. In any case, if not carried out before, a complete colonoscopy should be carried out within 3–6 months after surgery (Labianca *et al*., 2013). According to the National Comprehensive Cancer Network (NCCN) guideline of colonal cancer screening, fecal occult blood tests and colonoscopy are the main screening options for CC. However, these two methods contain the limitation in weak compliance, missing diagnosis, and generating false positives in large-scale RC screening (Wu et al., 2020).

1.8.5 Classification of colon cancer

According to the World Health Organization (WHO) classification presented for colon cancer, it can be classified into:

1. Premalignant lesions
2. Serrated lesions
3. Carcinomas
4. Neuroendocrine neoplasms

The most common type of CC is carcinomas which include adenocarcinoma, adenosquamous carcinoma, spindle cell carcinoma, squamous cell carcinoma, undifferentiated carcinoma (Aust, 2011).

As presented by WHO the adenomas are clonal lesions that showed low-grade dysplasia characterized by enlarged, hyperchromatic, and elongated nuclei arranged in a stratified configuration along basement membrane (Shih *et al*., 2001).

Adenocarcinoma has several histologic variants including cribriform comedo type adenocarcinoma, medullary carcinoma, micropapillary carcinoma, mucinous adenocarcinoma, serrated adenocarcinoma, and signet ring cell carcinoma (Aust, 2011). More than (90%) of colorectal carcinomas are adenocarcinomas originating from epithelial cells of the colorectal mucosa.

Conventional adenocarcinoma has shown glandular formation, which is the basis for histologic tumor grade determination. In well-differentiated adenocarcinoma,>95% of the tumor has a gland. Moderately differentiated adenocarcinoma has 50-95% of the gland. The poorly differentiated adenocarcinoma is mostly solid with <50% of gland formation (Fleming *et al*., 2012). Adenocarcinoma types include:

1.8.5.1 Medullary carcinoma

Medullary carcinoma is very rare, representing about 5-8 cases for every 10,000 patients diagnosed with CRC, it has a mean annual incidence of 3.47 (± 0.75) per 10 million population (Kondo *et al*., 2009). This type of tumor has a feature of sheets of epithelioid neoplastic cells with large vesicular nuclei. This cancer is a distinctive histologic subtype which strongly associated with microsatellite unstable high (MSI-H). medullary carcinoma has a favorable prognosis despite its poorly differentiated or undifferentiated histology ((Alexander *et al*., 2001);(Tani *et al*., 2001)).

1.8.5.2 Mucinous adenocarcinoma

Mucinous adenocarcinoma is characterized by that more than 50% of tumor volume is composed of mucin extracellularly. Cancer with mucinous components less than 50% is termed adenocarcinoma with mucinous features. It typically showed large glandular structures with pools of mucin extracellularly, many individual cancer cells such as signet ring cells might be seen ((Verhulst *et al*., 2012);(Kang *et al*., 2005)).

1.8.5.3 Signet ring cell adenocarcinoma

Signet ring cell adenocarcinoma is rare in the colorectum, constituting <1% of all CRC cases. It is defined as the presence of >50% of tumor cells having signet ring cell features characterized by a prominent intracytoplasmic mucin vacuole that pushes the nucleus to the periphery. Signet ring cells may have an infiltrative growth pattern or are present within the extracellular mucin. It is poorly differentiated (high grade) and carries a worse outcome as compared to conventional adenocarcinoma. The other types of adenocarcinoma are very rare including cribriform comedo type adenocarcinoma, micropapillary carcinoma, and serrated adenocarcinoma ((Kang *et al*., 2005);(Chen *et al*., 2010)).

1.8.6 Pathogenesis of colon cancer

Genetically, colon cancer is a clearly understood type of cancer. The first step in the carcinogenesis of colon cancer is the presence of specific type neoplastic polyps in the colonic mucosa. In terms of histology, a polyp is critical for determining malignancy. The common histologic types are hyperplastic and adenomatous. Hyperplastic polyps histologically contain an increased number of glandular cells with less cytoplasmic mucin and generally lack nuclear hyperchromasia, stratification, and types. Adenomatous nuclei are usually hyperchromatic, enlarged cigar-shaped, and crowded together in a palisading

pattern. Adenomas are classified as a tubular which is composed of branched tubules or villous which contain digitiform villi arranged in a frond. In tubulovillous adenomas, both element are present (Cappell, 2008). Most colon cancers arise from adenomas as determined by the following findings: first: colonic cancer specimens have one or more synchronous adenomas, second, increasing number of adenomatous polyps increase the risk for colon cancer (Schlussel *et al*., 2014), third, adenomatous tissue is present with frank carcinoma, forth, patients have hundreds of adenomatous polyps with familial adenomatous polyps will develop colon cancer, fifth, patients with adenomatous polyps larger than 1 cm may develop colon cancer at a rate of 1 to 1.5 percent per annum (Cappell 2005).

1.8.7 Metastasis

About 50% of colon cancer patients develop distant metastases. Colon tumors show distinct tropisms and frequencies regarding the target organs of metastasis. They spread to about 30–70% into the liver, 20–40% into the lung, 5–10% into the bone, and with lower frequencies into the brain, adrenal gland, and ovary Approximately one in four patients with colorectal cancer initially present with distant metastases (synchronous metastasis) at the time of diagnosis. A similar percentage will develop distant metastases during their disease (metachronous metastasis), which occurs usually within 2 years following initial and successful surgery of their primary tumor, Taken together, the metastatic dissemination of primary tumors is directly linked to patient's survival and accounts for about 90% of all colon cancer deaths (Mattiuzzi *et al*., 2019). Today, the prognosis of a patient's survival and treatment planning is mainly based on histopathological staging. Tumor infiltration depth (T), lymph node metastasis (N), and distant metastasis (M) are the key parameters defining the International Union Against Cancer / American Joint Committee on Cancer (UICC/AJCC) tumor stages. The early tumor stages I and II show neither lymph

node nor distant metastasis (stage I: T1-2 N0 M0; stage II: T3-4 N0 M0). Stage III tumors have developed lymph node metastases (T1-4 N1 M0) and tumors at stage IV have already spread to distant organs**.** (Arlt et al., 2009) **.**

1.8.8 Stages of Colon Cancer

1.8.8.1 Stage 0 Colon Cancer

When abnormal cells are found in the wall, or mucosa, of the colon, it is considered stage 0 colon cancer. This is also called carcinoma in situ.

1.8.8.2 Stage I Colon Cancer

Cancer that has invaded the mucosa and the submucosa is considered stage I colon cancer. The submucosa is the underlining of the large intestine and it lies beneath the mucosa. In stage I colon cancer, malignant cells may have also affected the deeper muscle layer of the colon wall, but have not invaded any areas outside of the colon (Li *et al*., 2016).

1.8.8.3 Stage II Colon Cancer

Cancer has spread through or into the colon or rectum wall, but still has not reached neighboring lymph nodes

1.8.8.4 *Stage III Colon Cancer*

Cancer has invaded the adjacent lymph nodes, but other parts of the body have not yet been affected.

1.8.8.5 Stage IV Colon Cancer

The cancer spreads to other body parts, including other organs such as the liver, the membrane that lines the abdominal cavity, the lung, or the ovaries figure 1.2 (Facts, 2014).

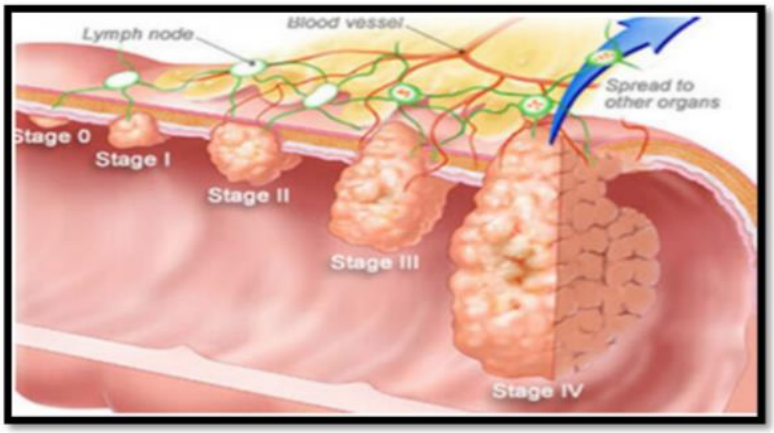

Figure1.2 Colorectal cancer stages (Hamza *et al*., 2017)

1.8.9 Treatment

Endoscopic diagnosis plays an important role in determining therapeutic strategy. Endoscopic diagnosis is intended to predict the oncological characteristics of a lesion based on endoscopic characteristics, such as size, and also to distinguish between malignant and benign tumors (Ishida *et al*., 2019).

Colon cancer treatment options depend on the stage of the tumor that is, how far it has spread, or how deeply it affects the intestinal wall and other tissues, and whether it is located in the colon and rectum or the colon only. Patients with colon cancer generally receive post-operational chemotherapy if the lymph nodes are positive. Treatment is also adjusted to the age, medical history of the patient, overall health, and tolerance of specific medications and therapies (Taner *et al*., 2021).

Treatment for colon cancer mostly include surgery and chemotherapy.

1.8.9.1 Surgery

Most patients with colon cancer are surgically treated, with the postoperative association of chemotherapy and possibly immunotherapy in advanced cases. Surgical treatment is chosen depending on the evolution stage, tumor topography and the existence of complications, colonic surgery being dictated by colonic vascularization. (Mastalier *et al*., 2012) **.** The surgical treatment of colon cancer is evolving. The advent of minimally invasive surgical techniques has given surgeons the option of a laparoscopically assisted approach to colon resection. Laparoscopic-assisted colon resection was first reported in 1913 and is being readily applied for both benign and malignant diseases of the colon(Jackson *et al*., 2007).

1.8.9.2 chemotherapy

Chemotherapy only targets the rapidly dividing cells that form the bulk of the tumor (Nangia-Makker *et al*., 2014). For the treatment of CRC, drugs such as fluorouracil (5-FU), irinotecan, oxaliplatin, and humanized monoclonal antibodies (bevacizumab, cetuximab, and panitumumab) were approved. Also, the introduction of an orally active drug, capecitabine in the chemotherapeutic protocol for the CRC (Ferlay *et al*., 2018).

5-Fluorouracil (5FU), Folinic acid (FA), and Oxaliplatin (FOLFOX) or 5FU, FA, and Irinotecan (FOLFIRI)or Leucovorin (LV) are standard regimens for palliative chemotherapy of metastatic colon cancer (Munker *et al*., 2018). Combined chemotherapy of f-FU/LV/oxaliplatin (FOLFOX) or 5-FU/LV/ irinotecan (FOLFIRI) provides higher response rates (RRs), longer progression-free interval (PFS), and better survival than 5-FU/LV alone ((Douillard *et al*., 2000); (de Gramont *et al*., 2000)).

Intravenous 5-FU/leucovorin regimens are less toxic than bolus regimens and have to be preferably used. The oral capecitabine is an alternative to I.V. 5-FU/LV (Van Cutsem *et al*., 2004).

A Triplet combination of chemotherapeutic treatment with 5-FU, oxaliplatin, and irinotecan (FOLFOXIRI**)** is interesting, where an Italian randomized phase III study showed a better outcome for patients treated with FOLFOXIRI compared to FOLFIRI (Falcone *et al*., 2007).

1.9 Glucose Metabolism

Glucose is the most common source of cellular energy and a substrate for many biochemical processes. Glucose is typically produced from ingested dietary carbohydrates but may also be created within the body by gluconeogenesis (Fokt *et al., 2020)*. Cancer cells switch from mitochondrial oxidative phosphorylation to glycolysis, even in the presence of normal oxygen concentrations. Inhibition of the glycolytic pathway is therefore a critical strategy in cancer therapy as in figure 1.3 (T.Matsuo, 2020).

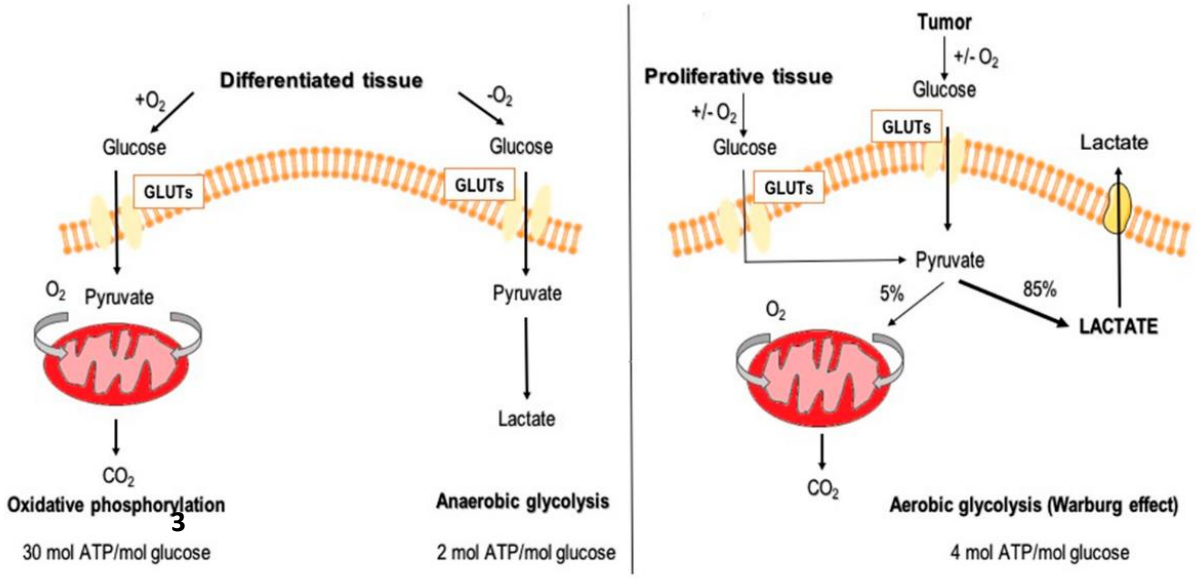

Figure 1.3: Schematic representation of glucose metabolic pathways: oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (glucose transporters, GLUTs).

1.9.1 2-Deoxy-d-glucose

A glucose derivative 2-Deoxyglucose (2-DG), can be phosphorylated by hexokinase, resulting in the formation of 2-deoxyglucose-phosphate (2-DG-P). The product of this reaction is trapped within the cell and cannot be used by subsequent steps of glycolysis, resulting in the accumulation of 2-DG-P and causing inhibition of hexokinase2 (HK2). In vitro studies on 2-deoxyglucose demonstrated inhibition of HK2 as well as subsequent ATP depletion; however, disappointing results have been reported by in vivo studies using xerographs, they revealed that 2-deoxyglucose as a single agent does not inhibit tumor growth (Seo *et al*., 2013). The structure of 2-DG is similar to that of mannose, which is important for N-glycosylation in proteins and normal protein folding in the endoplasmic reticulum (ER). It has been reported that inhibition of N-glycosylation by its inhibitor Tunicamycin (Tm) induced ER stress. Inhibition of N-glycosylation by 2-DG also increases misfolded proteins in the ER and results in ER stress-induced cell death(T.Matsuo, 2020).

1.9.2 Biological Activity of 2-DG in Cancer Cells:

Rather than simply acting as a roadblock in glycolysis, 2-DG interacts with multiple cellular pathways and has a range of biological effects include :

1.9.2.1 Inhibition of Glycolysis

The 2-DG competes with glucose and can competitively inhibit glucose transport into the cell. Oxygen deficiency is more common in the intratumoral environment, which increases the expression of glucose transporters and glycolytic enzymes, and thus increases 2-DG uptake in cancer cells as compared to normal cells in an aerobic environment (Fokt *et al*., 2020).

After entering the cell, 2-DG is phosphorylated by hexokinase II to 2-deoxy -glucose-6-phosphate (2-DG-6-P) but, unlike glucose, 2-DG-6-P cannot be

further metabolized by phosphoglucose isomerase (PGI) to a 5 carbon ring, this leads to the accumulation of 2-DG-6-P within the cell, cell cycle arrest and inhibition of cell growth, and eventually, cell death(Bost *et al*., 2016).

1.9.2.2 Induction of Autophagy

Autophagy is a degenerative mechanism present in every living cell. In cancer cells, autophagy has a dual role in tumor development and survival. Autophagy enhances tumor growth in the early growth phase and, in advanced tumors, increases cancer cell survival and promotes metastasis(Cheong, 2015). It has been Demonstrated that 2-DG-mediated glucose deprivation is also able to stimulate the production of reactive oxygen species (ROS) and the autophagy process, which could be blocked by up-regulation of cellular antioxidant potential using a thiol antioxidant, N-acetyl-l-cysteine or catalase (Shutt *et al*., 2010). In addition to the UPR response, it has recently been appreciated that ER stress also induces the well-described and studied process of autophagy which is considered to be a cell survival mechanism previously shown to be activated in response to nutrient deprivation. During this process, an isolated double membrane structure is formed and expands to encompass cytoplasmic contents and/or cellular organelles, and finally comes to a closure to become an autophagosome. This structure then fuses with a lysosome to form the so-called autolysosome, where its contents are degraded and recycled for use by cells under stress. Although autophagy has been shown to be activated by environmental conditions and/or drugs that lower ATP or induce ER stress, it remains unclear which of these processes is responsible for autophagy activation when cells are treated with 2-DG (Xi *et al*., 2011).

1.9.2.3 Induction of Apoptosis:

Prolonged autophagy and extensive self-degradation ultimately lead to apoptotic cell death. It appears that the potent 2-DG induced apoptosis is transmitted via various pathways (autophagy, extrinsic, and intrinsic), depending on the type of the cell (Muñoz-Pinedo *et al*., 2003).

It was found that 2-DG treatment significantly down-regulates antiapoptotic cellular FLICE-like inhibitory protein (cFLIP) and receptor-interacting protein kinase (RIP) protein expression, leading to increased TNF-related apoptosis-inducing ligand (TRAIL)-induced cell death. Similarly, found that 2-DG can sensitize cells to TRAIL-induced apoptosis, at least in part through suppressing c-Jun N-terminal kinase (JNK)-mediated cytoprotective autophagy processes. Further, several reports have demonstrated the ability of 2-DG to increase ROS generation, leading to cell death (Fokt *et al*., 2020).

1.9.3 Metformin

Metformin, (1,1-dimethyl biguanide hydrochloride) is an FDA-approved biguanide anti-diabetic drug, In addition to its function as a gluconeogenesis suppressor, metformin has recently been shown to possess strong anti-cancer properties. metformin can act directly on a tumor while also indirectly lowering insulin levels in the host. In some preclinical studies, metformin reduced proliferation, induced apoptosis, caused cell cycle arrest, and reduced incidence and growth of experimental tumors in vivo ((Nangia-Makker *et al*., 2014);(Rocha *et al*., 2015)).

1.9.3.1 Mechanisms of action of metformin

Metformin has been proven to regulate the expression of Long noncoding RNAs (lncRNAs) through various mechanisms, LncRNAs have important functions in the progression of many cancers. It has been reported that lncRNA-

urothelial carcinoma-associated 1 (UCA1) plays a critical role in tumorigeneses, such as that of laryngeal squamous cell carcinoma, lung adenocarcinoma, and colon cancer., these mechanisms include 1-altering DNA methylation via regulation of the lncRNA-H19, 2-regulating tumor cell migration, and invasion by interfering with the lncRNA-H19 ((Zhong *et al*., 2016);(Guo *et al*., 2020)).

1.10 5-fluorouracil

5-Fluorouracil (5-FU) is an antimetabolite drug that is widely used in the treatment of cancer. Over the past 20 years, increased understanding of the mechanism of action of 5-FU has led to the development of strategies that increase its anticancer activity, Antimetabolite drugs work by inhibiting essential biosynthetic processes, or by being incorporated into macromolecules, such as DNA and RNA, and inhibiting their normal function (Longley *et al*., 2003).

The 5-Fluorouracil (5-FU) is an essential component of systemic chemotherapy for colorectal cancer (CRC) in the palliative and adjuvant settings. it is also widely used to treat other cancers, such as gastric, pancreatic, breast, ovarian, and head and neck cancers (Kurasaka *et al*., 2021). Over the past four decades, several modulation strategies including the implementation of 5-FU-based combination regimens and 5-FU pro-drugs have been developed and tested to increase the anti-tumor activity of 5-FU and to overcome the clinical resistance (Vodenkova *et al*., 2019).

1.10.1 Mechanism of Action of 5-FU

As an analog of the nucleobase uracil, 5-FU can enter cells via the same mechanism of facilitated transport as uracil. Then, 5-FU is converted to 5-fluorodeoxyuridine monophosphate (FdUMP), which is a potent inhibitor of thymidylate synthase (TS). FdUMP forms a covalent complex with TS in the presence of 5,10-methylenetetrahydrofolate (CH2–THF). The inhibition of TS depletes the intracellular dTTP pool and subsequently inhibits DNA synthesis.

Another effect by which 5-FU can exert its cytotoxic action is its incorporation as fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) into DNA and RNA (Figure 1.4). By this action, 5- FU can fight cancerous cells ((Entezar-Almahdi *et al*., 2020);(Kurasaka *et al*., 2021)).

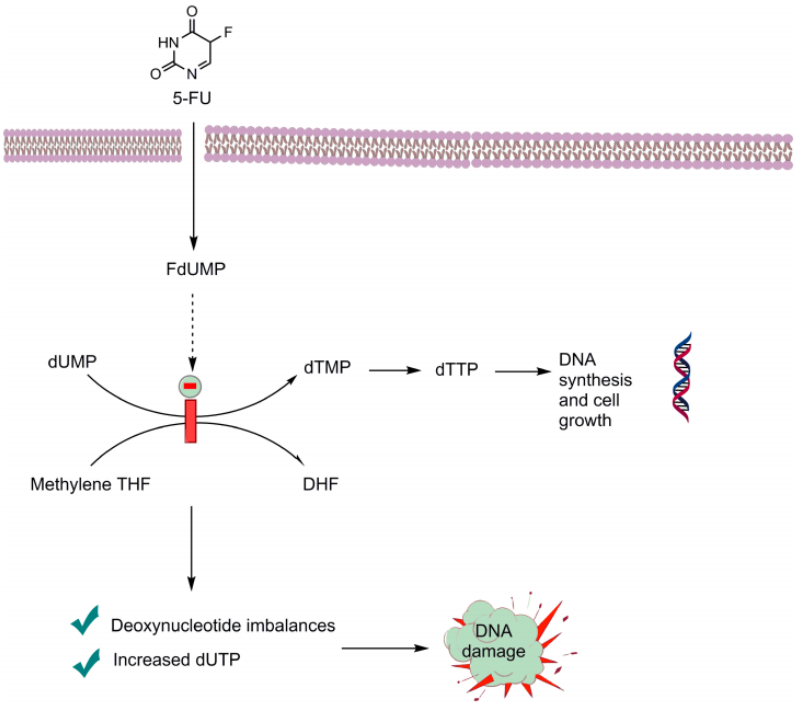

Figure1.4: Mechanism of action of 5-FU.

Abbreviations: FdUMP, fluorodeoxyuridine monophosphate; dUTP, deoxyuridine triphosphate; dTMP, deoxythymidine monophosphate; dTTP, deoxythymidine triphosphate methylene; THF, methylenetetrahydrofolate; DHF, dihydrofolate.

1.10.2 Cancer cells resistance to 5-FU

Studies show that cancer cells develop resistance to 5-FU through complex mechanisms. Of note, the TS enzyme and other enzymes involved in 5-FU anabolism or catabolism are often altered in expression or function to promote 5-FU resistance. In addition, altered cell death and autophagy, expression/functional changes in drug transporters and epigenetic changes

represent putative 5-FU-resistant mechanisms (Kurasaka *et al.*, 2021). The other mechanism of chemoresistance is the tumor microenvironment potentially acts as a principal factor. Oxygen deprivation (hypoxia) is a hallmark of solid tumors, which is caused by the consumption of oxygen by rapidly proliferating cells that exceed the oxygen supply. Hypoxia can promote tumorigenesis, the epithelial-to-mesenchymal transition (EMT), tumor metastasis, as well as affect tumor cell responsiveness to anti-tumor agents (Xu *et al*., 2020).

1.11 Doxorubicin

Doxorubicin (DOX) is a widely used anthracycline-based antitumor agent for both solid and liquid tumors. DOX, are designed to kill cancer cells by generating oxidative stress. However, the administration of DOX frequently results in the development of drug resistance, a critical hurdle in cancer treatment (Locatelli *et al*., 2021). Dox has been used, in combination with other drugs, to treat many different types of cancers, such as breast, lung, ovarian, and bladder cancers, as well as neuroblastoma, leukemia, Hodgkin's, and non-Hodgkin's lymphoma (Pan *et al*., 2021). DOX has marked toxic side effects after intravenous infusions, such as bone marrow inhibition and cardiotoxicity. Large doses may result in irreversible heart failure. Therefore, the use of DOX in tumor treatment is limited (Miao *et al*., 2014).

1.11.1 Mechanism of Action of Doxorubicin

The primary mechanism of action of DOX involves the drug’s ability to intercalate within DNA base pairs, causing breakage of DNA strands and inhibition of both DNA and RNA synthesis. DOX inhibits the enzyme topoisomerase II, causing DNA damage and induction of apoptosis. When combined with iron, It also causes free radical-mediated oxidative damage to DNA, further limiting DNA synthesis. Iron chelators, such as dexrazoxane, may

prevent free radical formation by limiting the binding of DOX with iron (Johnson-arbor *et al*., 2020).

1.12 Cell culture

Cell culture is the process by which human, animal, or insect cells are grown in a favorable artificial environment. Animal cell culture is now one of the main techniques used in the life sciences in areas of research because many animal cells can be induced to grow outside of their organs or tissue of origin under specified conditions when supplemented with a nutrient-containing and growing medium. For *in vitro* growth of cells, the culture conditions must mimic *in vivo* conditions concerning temperature, pH, carbon dioxide (CO_2), oxygen (O_2), osmolality, and nutrition (Capes-Davis *et al*., 2010).

1.12.1 Primary Cultures

Primary culture refers to the stage of cultivation after the cells are isolated from the tissue and proliferated under appropriate conditions until they occupy all the available substratum (i.e., reach confluence). The cells must be sub-cultured (i.e. passaged) at this stage by transferring them to a new vessel with a fresh growth medium to provide more room for continued growth. Primary cultures have the advantage of being derived directly from the tissue of interest and not having undergone any genetic or epigenetic modifications. In general, they exhibit the primary tissue phenotype from which they were isolated((Butler 2004); (Freudenrich *et al.,* 2020))

1.12.2 Cell Line

Once a primary culture is sub-cultured or passaged it represents a cell line. The number of passages is the number of subcultures through which the cells have passed. It should be recorded and it should not be getting too high. This is designed to prevent the use of genetically modified cells and other variations. A cell line experiencing indefinite cell growth during subsequent subculture is called a continuous cell line whereas finite cell lines experience cell death after sundry subcultures. Typically, continuous cell lines are derived from tumors or normal embryonic tissues (Phelan *et al.,* 2017).

1.12.3 Cell Strain

If a subpopulation of a cell line is positively selected from the culture by cloning or some other method, this cell line becomes a cell strain. A cell strain often acquires additional genetic changes after the initiation of the parent line (Barile 2017).

1.12.4 Finite and Continuous Cell Line

Normally, regular cells are divided only for a small number of times before they lose their capacity to proliferate, which is a genetically defined phenomenon known as senescence; these cell lines are considered finite. However, some cell lines become immortal through a mechanism called transformation which may occur naturally or can be caused either chemically or virally. When a finite line or strain of cells undergoes transformation and acquires the ability to divide indefinitely, it becomes a continuous cell line (Roy *et al*., 2011).

1.12.5 Culture Morphology

In terms of growth mode, cell cultures take one of two forms, growing either in suspension (as single cells or small free-floating clumps) or as a monolayer that is attached to the tissue culture flask. The form taken by a cell line reflects the tissue from which it was derived e.g. cell lines derived from blood (leukemia, lymphoma) tend to grow in suspension whereas cells derived from solid tissue (lungs, kidney) tend to grow as monolayers. Attached cell lines can be classified as endothelial such as BAE-1, epithelial such as HeLa, neuronal such as SH-SY5Y, or fibroblasts such as MRC-5, and their morphology reflects the area within the tissue of origin. (Zimmerman *et al*., 2012).

1.12.6 Applications of cell culture:

The production of animal cells has been used in numerous areas, from basic to advanced science. It provides a model system for a variety of research efforts including:

1. In pharmaceutical research during the phase of drug screening and development. Cell-based experiments are very predictive of toxicity and permeability assessments of preclinical drugs (T. Şahin *et al.,* 2017).
2. Tissue engineering for example the production of tissue in vitro such as skin or cartilage for the treatment of burns (Volume *et al.,* 2014).
3. Tests of the effects of chemical compounds on particular cell types.
4. Produce biological reagents (e.g. recombinant proteins, antibodies, and vaccines).
5. To study carcinogenesis and mutagenesis.
6. Excellent modeling systems for studying normal cell physiology and biochemistry (e.g., metabolic studies, aging).
7. Production of vaccines, monoclonal antibodies, interferons, clotting factors, and hormones (Hudu *et al*., 2016).

1.12.7 Advantages of cell culture:

The main advantages of the cell culture technique are (Capes-Davis *et al*., 2010):

1. Control of the environment by altering pH, temperature, O_2/ CO_2 ratio, and osmotic pressure of the culture media to study their effects on the cell culture.
2. Provides a good tool to study cell metabolism and investigate the physiology and biochemistry of cells.
3. Investigate the cytotoxic effect of various compounds or drugs on specific types of cells such as liver cells.
4. Enable the study of biology and origin of the cells obtained from homogenous cell culture.
5. Specific proteins can be synthesized in large quantities from genetically modified cells in large-scale cultures.
6. Consistency and reproducibility of the results can be obtained by the use of a single clonal population.
7. Specific cell types can be detected by the presence of markers such as molecules or by karyotyping.

1.12.8 Disadvantage of cell culture

The main disadvantages of using cell culture in basic and advanced research are ((Capes-Davis *et al*., 2010);(Philippeos *et al*., 2012)) :

1. Expenditure and expertise: cell culture is a specialized technique that requires aseptic conditions, trained personnel, and costly equipment.
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. Low amount of product: The minuscule amount of monoclonal antibody and recombinant protein produced followed by downstream processing for extracting pure products increases expenses tremendously.
4. Contamination: mycoplasma and viral infection are difficult to be detected and are highly contagious.
5. Instability: Aneuploidy chromosomal constitution in continuous cell lines leads to instability.

Chapter Two

Materials and Methods

2. Materials and Methods

The present experimental work was performed in the cancer research laboratory at College of Medicine\ University of Babylon during the period (October 2020-August 2021).

2.1 Materials

2.1.1 Chemicals

The chemicals used in this study are listed in **(Table 2.1)** with their suppliers.

Table 2.1 Chemicals used in the study

Chemicals	Company	Country
Alcohol spray (ethanol 70%)	Ameta Fze	UAE
Dimethyl sulfoxide (DMSO)	Roth	Germany
Fetal bovine serum (FBS)	Gibco	UK
Gentamycin (80 mg vial)	The Arab pharm.	Jordan
MTT(3-(4,5-Dimethylthiazole-2-yl)-,5-diphenyl-2H-tetrazolium romide) dye powder	Roth	Germany
Phosphate buffer saline tablet	Gibco	UK
Roswell Park Memorial Institute-640 (RPMI-1640) powder medium	Gibco	UK

Sodium bicarbonate powder	Ludeco	Belgium
Trypsin- Ethylenediaminetetraacetic cid (EDTA) powder	US biological	USA
Doxorubicin HCL (50 mg/25ml)	Pfizer	USA
5-fluorouracil	EBEWE pharma	AUSTARIA
Metformin powder	MERCK	USA
2-deoxy-d-glucose powder	Zydus	India

2.1.2 Instruments and Tools:

The instruments and tools used in the study are listed in **(Table 2.2)** with their suppliers.

Table 2. 2 List of Instruments and Tools Used in the Study

instrument or tool	**Company**	**Country**
Autoclave	Biotech	Korea
Automatic micropipette (different sizes)	Human	Germany
Cell culture flask (25m	SPL	Korea
Cell culture plate (96-wells)	SPL	Korea
Distiller	ROWA	Germany

Double distillation wat stills	GFL	Germany
Electric oven	Memmert	Germany
ELISA Plate Reader	Human	Germany
(Sterile freezing vial (1.5 ml	Biofilm	Australia
Incubator	Memmert	Germany
Inverted microscope	T.C Meiji techno	Japan
Laminar airflow cabin	Labtech	Korea
Liquid nitrogen container GT38	Air Liquide	France
Magnetic stirrer	Labinco	Netherland
Microcentrifuge	Memmert	Germany
Millipore filter (0.45, 0.22µm)	Biofil	Australia
Whatman filter paper	Merck	Germany
pH Meter	WTW	Germany
Refrigerator	Arcelik	Turkey
Sensitive Balance	Labtech	Korea
Vortex	Kottermann	Germany
Water bath	Memmert	Germany

2.1.3 Cell lines

1- Colon cancer cell line (SW 480)

The cell line SW480 is a human colorectal cancer cell line. This cell line was cultured in monolayer in Roswell Park Memorial Institute (RPMI-1640) medium containing 10% heat-inactivated fetal bovine serum (FBS) and 1% Gentamycin at 37 °C with 5% CO2 .

2- Normal cell line (VERO)

The VERO cell line is a human kidney epithelial cells (VERO) used for research. The growth of VERO cells is anchorage-dependent and VERO cells can only proliferate when provided with a suitable surface. Adherent VERO cells a serum-free developed in T-flasks in a humidified incubator with 5% CO_2 and at 37 °C. The culture was diluted and passaged twice a week (Xi *et al*., 2011).

2.2 Methods

2.2.1 Preparation of Reagents and Solutions

2.2.1.1 Phosphate Buffer Saline (PBS):

According to the Gibco manufacturer's manual, the PBS was prepared by dissolving one tablet of PBS in 500 ml of double-distilled water (DDW) with continuous stirring at room temperature on a magnetic stirrer, the pH will be 7.4, and no adjustment is required. It was then sterilized at 120˚C for 20 minutes by autoclave and kept sterile in a bottle until it was used (Trusted *et al.*, 2008).

2.2.1.2 Gentamicin Stock Solution:

Gentamycin vial of 40 mg/ml solution was considered as a stock solution and stored at 4˚C for use. According to (Khokhlova *et al*., 2019) the working concentration of gentamicin in the medium is 50 μg/ml, so 1.25 ml of gentamycin solution was added to 1L of RPMI-1640 medium.

2.2.1.3 Trypsin-EDTA Stock Solution:

As indicated by US biological headings, a weight of 10.1 g of trypsin-EDTA powder was dissolved in 0.9 liters of DDW with continuous mixing at room temperature. 7.2 of PH value should be reached and then the volume was completed to 1 Liter by DDW, the solution was sterilized by using millipore filters of 0.45 and 0.22 μm respectively, after that, the solution was kept at (-20C˚) temperature.

2.2.1.4 MTT Solution:

MTT powder (0.5 gm) was dissolved in PBS (100 ml) to achieve 5 mg/ml concentration. A 0.2 μm millipore filter was utilized to sterilize the MTT solution and stored in a sterile and light-protected bottle. The solution was stored at 4°C of temperature for multiple uses or at -20°C of temperature for long storage (Meerloo *et al.,* 2011).

2.2.2 Preparation of Tissue Culture Media:

2.2.2.1 Preparation of Serum-Free Medium:

Liquid RPMI-1640 medium was prepared from powdered RPMI-1640 medium according to the Gibco product manually as the following: from the RPMI-1640 powdered medium, 10.43 g was dissolved in nearly 900 ml of DDW in a volumetric flask. The other components include: 2 g of sodium

bicarbonate powder or as needed and 1.25 ml of gentamicin stock solution had been added with continuous stirring, then the volume was completed by adding DDW to complete the volume to one liter and the medium pH adjusted to 7.4. The sterilization was performed by 0.45 and 0.22 μm filters. Distribute the medium aseptically into sterile containers, then store it at 2-8ºC until use (Tekarslan *et al*., 2017).

2.2.2.2 Preparation of Serum-Medium :

Medium with serum was prepared as described in the preparation of serum-free medium in (2.2.2.1) with the addition of 10% of fetal bovine serum (FBS).

2.2.2.3 Preparation of Freezing Medium:

Ten ml of freezing medium were prepared from the following constituents: six ml serum-free medium, three ml of FBS, and one ml of DMSO were added drop by drop with the mixture. The solution was stored in between uses around (- 20˚C) (Herring *et al*., 2016).

2.2.3 Culturing of Colon SW480 and Normal VERO Cell Lines

Colonic SW480 cell line and VERO cell line in frozen vials were obtained from the laboratory of tissue culture in the college of medicine/University of Babylon (Meleady *et al*., 2006).

2.2.3.1 Thawing of Colon SW480 and Normal VERO cell line:

1. The vials of cells that have been stored in liquid nitrogen has been evolved from the freezing container.

2. Alcohol (70%) spray was used to decontaminate the vial. Then the vial was quickly transferred to a 37˚C water bath until there remained only one or two small ice crystals, it took about (1-2 minutes).

3. A 70 % alcohol-soaked tissue was used to wipe the vial before opening inside the laminar flow hood.

4. The entire content of the vial has been pipetted into a tissue culture flask, then a 5ml pr-warmed medium, which has already been supplemented with the appropriate components was added slowly.

5. Incubate the flask at 37 C for 24 hours .

6. Next day, the cells were examined microscopically, changed the media as needed (T. Şahin *et al*., 2017).

2.2.3.2 Harvesting and sub-culturing of Colon SW480 and normal VERO cell lines:

Harvesting is a procedure that uses proteolytic enzymes such as trypsin to detach and disaggregate the monolayer of adherent cells from the bottom of the flask of culturing. It was performed at whatever point the cells should be collected to be harvested for cell counting and sub-culturing of the cell line. This methodology was done according to the following:

1. The inverted microscope was utilized to analyze that the cells are healthy and sub-confluent without contamination.
2. The spent medium had been emptied by a pipette and added sufficient amount of pre-warmed trypsin solution to wash the monolayer to guarantee the removal of all medium from the cell culture flask. This step could be repeated if the cells were still adherent strongly.

3. The convenient volume of trypsin solution that was used to wash cell monolayer utilizing (1-2 ml) per 25-cm^2 flask. Flask was rotated to completely cover the monolayer with trypsin.
4. To detach the cells from the surface of the flask, the flask was returned to the incubator at 37°C. Usually, the cell's detaching period depends on a cell line which could take 2 to 10 minutes.
5. An inverted microscope could be used to evaluate the cells whether they are detached and floating or not. The flask might be tapped gently on its side to detach any remaining cells.
6. An equal volume of serum-containing medium was added to deactivate trypsin in the flask.
7. If there are enough cells in the flask, an aliquot of cells could be transferred to another flask labeled with cell line name with a pre-warmed serum-containing medium (5–7ml for a 25-cm^2 flask). RPMI-1640 medium used for colon SW480 cell line and normal VERO cell line.
8. The flask was incubated at 37C° temperature.
9. This process has been repeated according to the characteristics of the growth for each cell line.

2.2.3.3 Freezing of Colon (SW480) and normal (VERO)cell lines:

The cell lines source were kept frozen at (-196) ºC in a nitrogen tank according to the following protocol:

1. Tissue culture flask with a monolayer near the exponential phase was taken and washed twice with 5 ml of PBS, then 3 ml of warm trypsin was added. Halve of the trypsin volume was decant.
2. The flask was incubated at 37C° until the cell layer detached and the cells were aided to disaggregate into single cells by gentle rocking on the flask sides.

3. The flask content was transferred into a 15 ml sterile plastic centrifuge tube. Centrifugation was done at 800 rpm for 10 minutes.
4. The supernatant was decanted and the cell pellet was re-suspended with 1 ml of the freezing media and transferred into a 1.5 ml sterile freezing vial.
5. The vial was kept for 10 minutes at room temperature and transferred to – 80C° deep freezer for 24 h and then stored for a long time in the liquid nitrogen tank after one minute (Kielberg 2010).

2.2.4 Preparation of the drugs

2.2.4.1 Preparation of 2-deoxy-D-glucose

The 2-deoxy-D-glucose (20 mg) was solubilized in RPMI-1640 (5 ml) to obtain a stock solution of (4000 µg\ml) and from this stock, the serial dilutions ware made.

2.2.4.2 Preparation of metformin

Metformin (20 mg) was solubilized in RPMI-1640 (5 ml) to obtain a stock solution of (4000 µg\ml) and from this stock, the serial dilutions ware made.

2.2.4.3 Preparation of 5-Fluorouracil

Solution of 5-Fluorouracil (50mg/ml) in sealed vial was used to prepare the stock solution by diluting with RPMI-1640 to obtain a stock solution of (1000 µg\ml) and from this stock, a serial dilution was made.

2.2.4.4 Preparation of Doxorubicin

Solution of Doxorubicin (250mg/25ml) in sealed vial was used to prepare the stock solution by diluting with RPMI-1640 to obtain a stock solution of (1000 μg\ml) and from this stock.

2.2.5 Cytotoxicity assay:

2.2.5.1 Principle of MTT Assay :

The general use of the MTT assay is to quantify viable cells in moderately high throughput (96-well plates) without the requirement for elaborate cell counting. Therefore the most widely recognized use is to evaluate the cytotoxicity of several drugs at various concentrations. The principle of the MTT assay is that for most mitochondrial activity of viable cells is consistent and thereby any change in the number of viable cells is directly proportional to the mitochondrial activity. The mitochondrial activity of the cells is reflected by the transformation of the pale yellow colored tetrazolium salt (MTT dye) into dark purple formazan crystals by NADH (Figure 2.1) which can be solubilized for homogenous measurement. Thus, any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected by optical density (absorbance) using a plate reader at 570 nm. The darker the solution, the greater the number of viable and metabolically active cells (Meerloo *et al*., 2011).

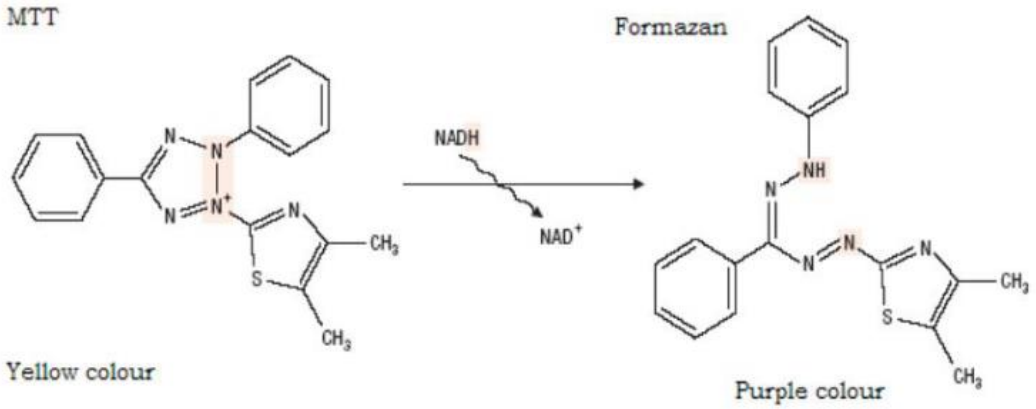

Figure 2.1: Principle of MTT Assay (Sukhramani *et al.*, 2016)

2.2.5.2 Procedure :

At the end of the drugs exposure period, the medium was removed from the wells, and afterward, the cells were washed with PBS. A blank control was carried to evaluate unspecific formazan transformation.

A volume of 1.2 ml of MTT solution (5 mg/ ml) was added to 10.8 ml medium to get final concentration of 0.5 mg/ml. Then, 200 µl of the final solution was added to each well.

The 96-well plate was incubated for 3 hours at 37°C until intracellular purple formazan crystals were obvious under the inverted microscope.

The supernatant was extracted and 100 µl DMSO was added for each well to dissolve the produced formazan crystals.

The 96-well plate was incubated at room temperature for 30 minutes until the cells have lysed and purple crystals have broken up.

A microplate reader at 570 nm was used to evaluate the absorbance.

The blank and control readings of absorbance must be subtracted from each sample. Absorbance readings from samples should then be divided by those of the control and multiplied by 100 to get percentage cell proliferation or

viability. Greater absorbance values compared to the control demonstrate cell proliferation, while lower values propose cell death or inhibition of proliferation. Percentage of cell viability or percentage of inhibition was calculated by the following formula (Yüksel *et al.*, 2017) :

$$\% \text{ viability} = (Abs_{sample} - Abs_{Blank}) / (Abs_{control} - Abs_{Blank}) \times 100\%$$

$$\% \text{ Inhibition} = 100 - \% \text{ viability}$$

2.2.6 Experimental Design:

According to (Capes-Davis *et a*l., 2010) the cytotoxicity assays were applied for the determination of the effect of 2DG and metformin on the anticancer activities of 5-FU or Dox on Colon SW480, and normal VERO cell lines. Different concentrations of these agents were tested for defined time durations. At the point when the growth in the flask became a monolayer (80-90% of growth), the cell monolayer was harvested and re-suspended with a serum-free medium in a concentration of $5*10^5$ cells/ml and seeded in a 96-well cell culture plate. Where the cell development arrives 80%, the wells were treated with serial dilutions of each type of agent as in the following experiments:

2.2.6.1 The Effect of 2-deoxy-D-glucose on SW480 and VERO Cell Lines

Cells were seeded in 96-well plate 24 hours before the treatment with the drugs, when the cell monolayer reach a confluence of more than 80%, the plate was treated with the drugs. The plate was divided into seven column, each column contain four replicates, the first one is the control group, and the others were treated with serial dilutions of each drug separately. Serial dilutions of 2-DG (4000,2000,1000, 500, 250, 125 μg/ml) was applied. Then the plate was covered with a self-plastic lid and incubated for 24 hours. After

the end of the exposure time, the wells were washed with 200 µl of sterile PBS. The effect of 2-DG on sw480 line growth was assessed by MTT assay. This experiment is also repeated to assess the effect of 2-DG on the VERO cell line in the same way.

2.2.6.2 The effect of metformin on SW480 and VERO cell lines

The same procedure was used to assess the effect of metformin on cancer and normal cells. Serial dilutions started from 4000 to 125 µg/ml were used. Finally, MTT assay was used to evaluate the cytotoxicity effects of metformin.

2.2.6.3 The effect of 5-fluorouracil on SW480 and VERO cell lines at 24 hours incubation time.

The tissue culture plates of SW 480 and vero cell lines were prepared as mention above and treated with 5-FU in serial dilutions of (1000, 500, 250, 125, 62.5, 31.25 µg/ml).

2.2.6.4 The effect of Doxorubicin on SW480 and VERO cell lines at 24 hours incubation time.

The tissue culture plates of SW 480 and vero cell lines were prepared as mention previously and treated with serial dilutions of Doxorubicin (250, 125, 62.5, 31.25, 15.62,7.8 µg/ml).

2.2.6.5 The Effect of (2-deoxy-D-glucose – Metformin) combination on SW480 and normal VERO Cell Lines

Plates were seeded with SW480 and vero cells as mention before, and devided into five groups in each plate. The first group was the untreated (control) group. The treatment groups were treated with (2-DG 2000 µg/ml+

metformin 4000 µg/ml) , (2-DG 1000 µg/ml+ metformin 4000 µg/ml) , (2-DG 2000 µg/ml+ metformin 2000 µg/ml) and (2-DG 1000 µg/ml+ metformin 2000 µg/ml). Then the plate was covered with a self-plastic lid and incubated once for 24 hours. After the end of the exposure, the wells were washed with 200 µl of sterile PBS. The effect of these drugs combinations on sw480 and vero cell lines growth was assessed by MTT assay.

2.2.6.6 The effect of (2-deoxy-D-glucose - 5-fluorouracil) ,(metformin - 5-fluorouracil) and (2-deoxy-D-glucose - metformin - 5-fluorouracil) combinations on SW480 and VERO cell lines

Plates were seeded with SW480 and vero cells as mention before, and divided into five groups in each plate. The first group was the untreated (control) group. The treatment groups were exposed to (2-DG 2000 µg/ml+ 5-FU 125 µg/ml) , (2-DG 1000 µg/ml+ 5-FU 125 µg/ml) , (metformin 4000 µg/ml+5-FU 125 µg/ml) , (metformin 2000 µg/ml+ 5-FU 125 µg/ml) , (2-DG 2000 µg/ml+ metformin 4000 µg/ml+5-FU 125 µg/ml). Then the plate was covered with a self-plastic lid and incubated once for 24 hours. After the end of the exposure, the wells were washed with 200 µl of sterile PBS. The effect of 2-DG and metformin on the cytotoxicity of 5-FU on SW480 and vero cell lines growth was assessed by MTT assay.

2.2.6.7 The effect of (2-deoxy-D-glucose – doxorubicin), (metformin – doxorubicin) and (2-deoxy-D-glucose - metformin - doxorubicin) combinations on SW480 and VERO cell lines

Plates were seeded with SW480 and vero cells as mention before, and divided into five groups in each plate. The first group was the untreated

(control) group. The treatment groups were exposed to (2-DG 2000 µg/ml+ DOX 15 µg/ml) , (2-DG 1000 µg/ml+ DOX 15 µg/ml) , (metformin 4000 µg/ml+ DOX 15 µg/ml) , (metformin 2000 µg/ml+ DOX 15 µg/ml) , (2-DG 2000 µg/ml+ metformin 4000 µg/ml+ DOX 15 µg/ml). Then the plate was covered with a self-plastic lid and incubated once for 24 hours. After the end of the exposure, the wells were washed with 200 µl of sterile PBS. The effect of 2-DG and metformin on the cytotoxicity of doxorubicin on SW480 and vero cell lines was assessed by MTT assay.

2.3 Statistical analysis

All data were collected and analyzed by Microsoft Office Excel 2010 and Sigmaplot version 12 software. The differences between the means of the tested concentrations were analyzed by using a one-way Anova test. P-value ≤ 0.05 and ≤ 0.001 were considered to be statistically significant and highly significant, respectively.

Chapter Three

RESULTS

3. Results

3.1 Effect of 2-deoxy-d-glucose on the viability of SW480 colon cancer and normal Vero cell lines

Results showed that 2-DG at concentrations from (500 to 4000 µg/ml) causes a significant (P<0.050) decrease in the viability of SW480 colon cancer as shown in figure 3.1.

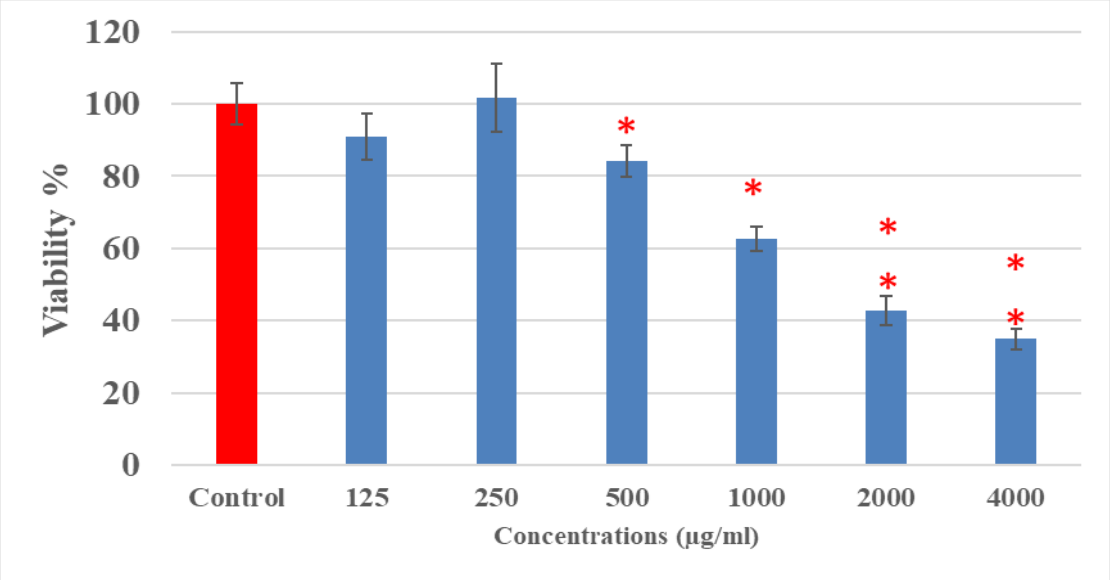

Figure 3.1: Effect of 2-deoxy-d-glucose on the viability of SW480 colon cancer cells. *mean significant , * *mean highly significant

The half-maximal inhibitory concentration (IC50) value for 2-DG was (2538 µg/ml) (figure 3.2) .

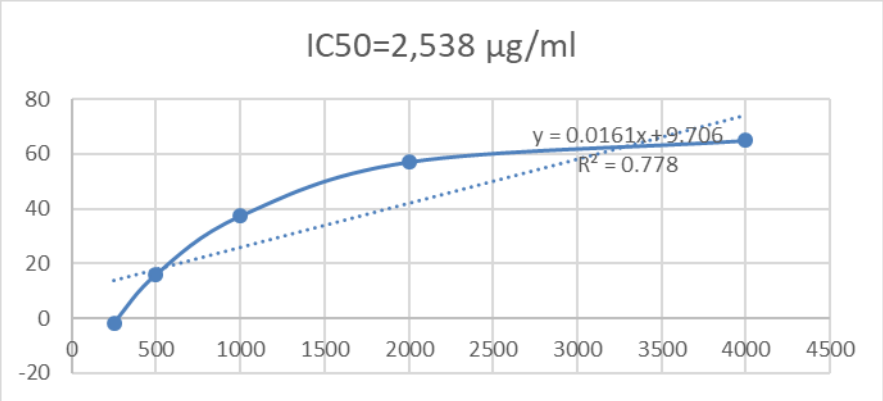

Figure 3.2: IC50 value of 2-deoxy-d-glucose on SW480 cancer cells

Results shown in figure 3.3 revealed that 2-DG at concentrations from (500 to 4000 µg/ml) cause a significant (P<0.050) decrease in the viability of Vero normal cells while the concentrations 250, 125 µg/ml causes no significant effect compared to control group.

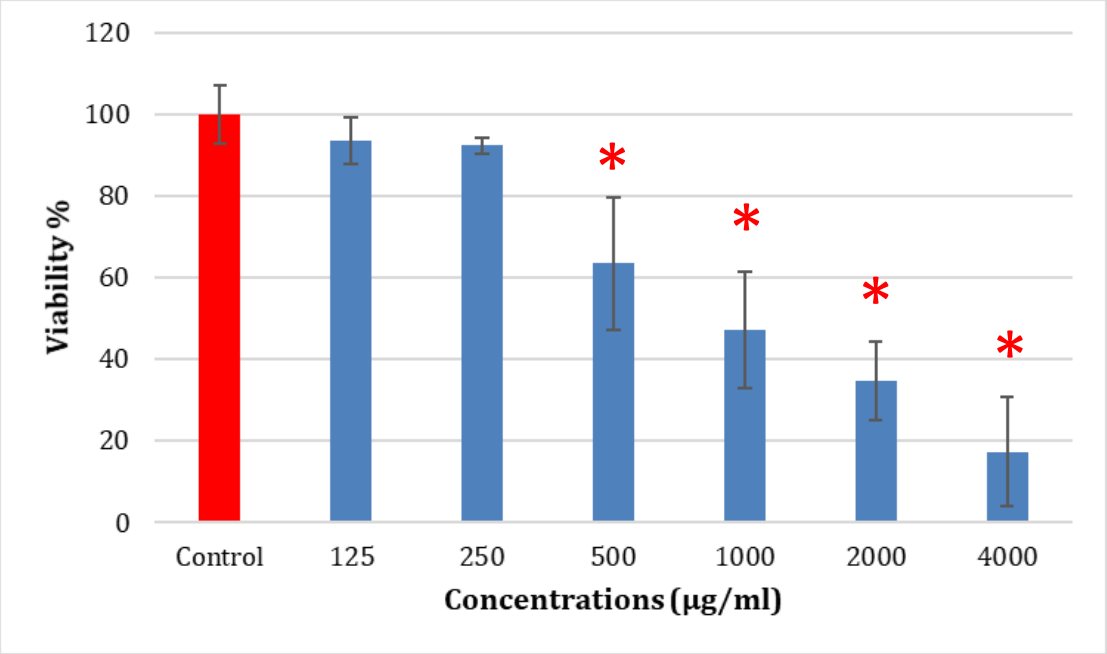

Figure 3.3: Effect of 2-deoxy-d-glucose on the viability of Vero cells.

The IC50 of 2-DG on Vero cells was 1992 µg/ml (figure 3.4)

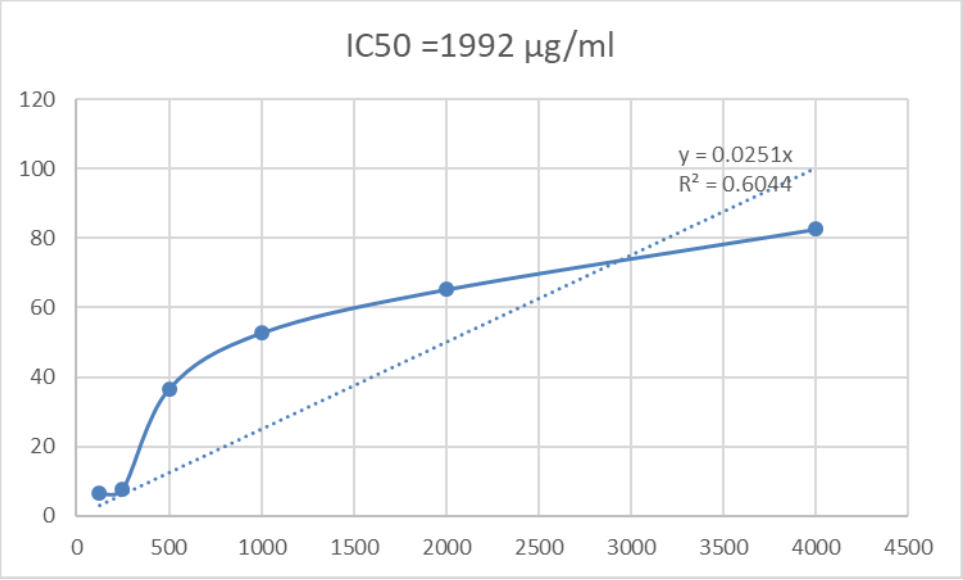

Figure 3.4: IC50 value of 2-deoxy-d-glucose on Vero cells.

3.2 Effect of Metformin on the viability of SW480 colon cancer and normal VERO cell lines

The results showed that metformin at higher concentrations (2000, and 4000 µg/ml) causes a highly significant (P<0.050) decrease in the viability of SW480 colon cancer cells while lower concentrations didn't affect cell viability as shown in figure 3.5.

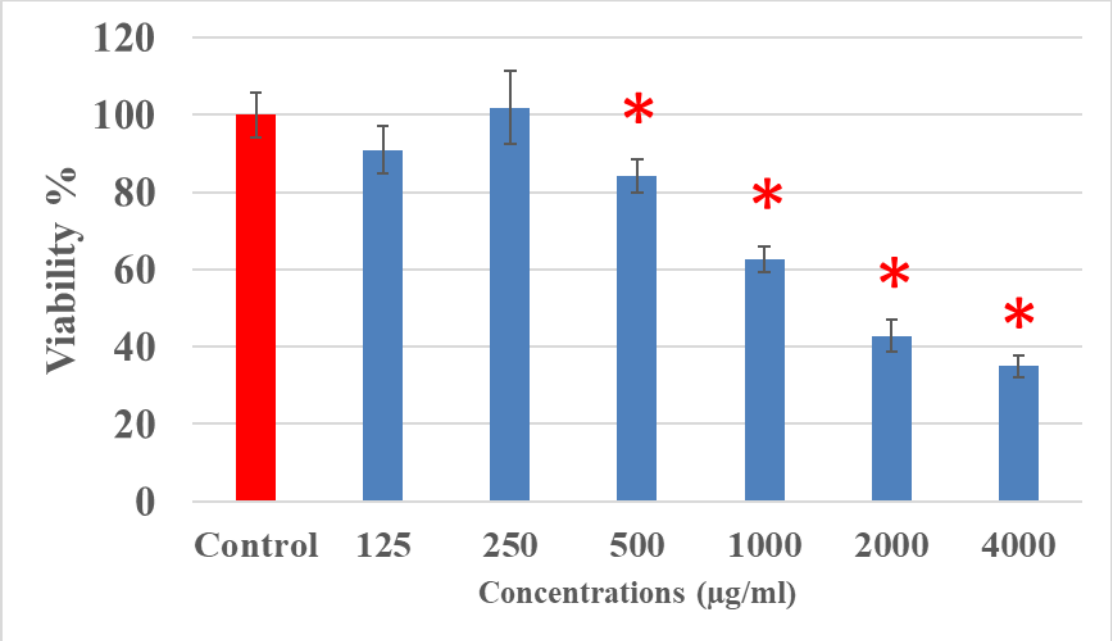

Figure 3.5: Effect of Metformin on the viability of SW480 colon cancer cells.

The IC50 value for metformin was (2050 µg/ml) (figure 3.6)

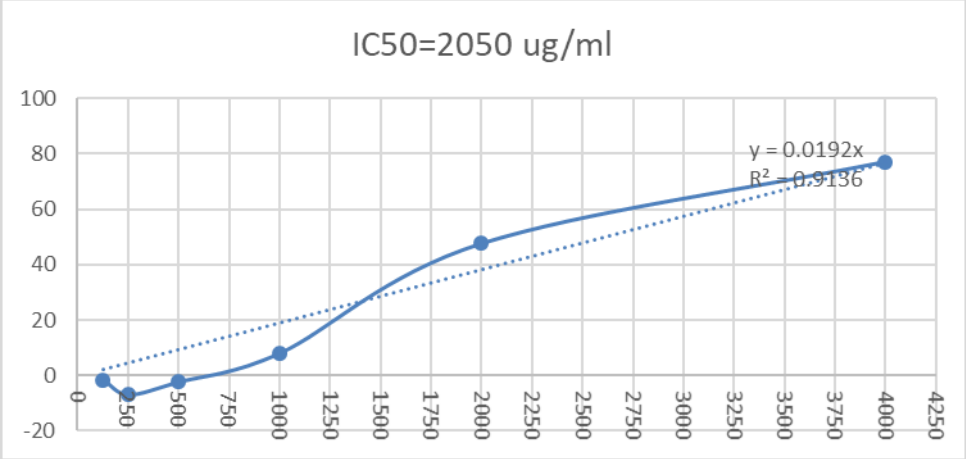

Figure 3.6 IC50 value for metformin on SW480 colon cancer cells

Results showed that metformin at concentrations (2000, and 4000 µg/ml) causes a highly significant (P<0.050) decrease in the viability of normal Vero cells while the other concentrations didn't cause any significant effect on Vero cells viability compared to the control group as shown in (figure 3.7).

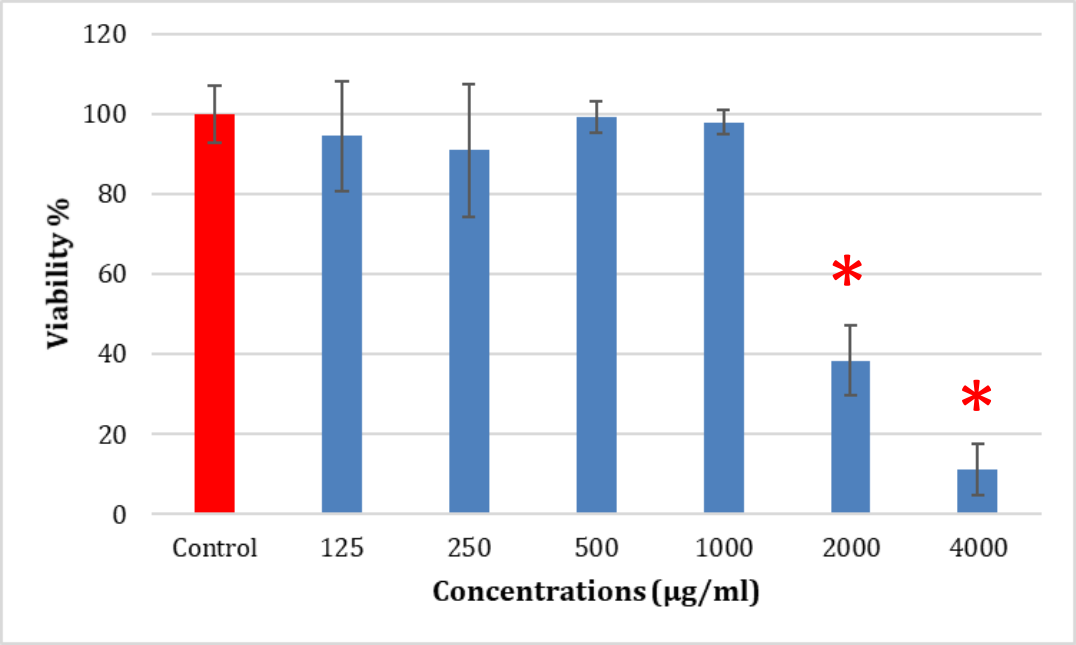

Figure 3.7: Effect of metformin on the viability of VERO cells.

The IC50 value for metformin on normal Vero cells was (1992 µg/ml) (figure 3.8)

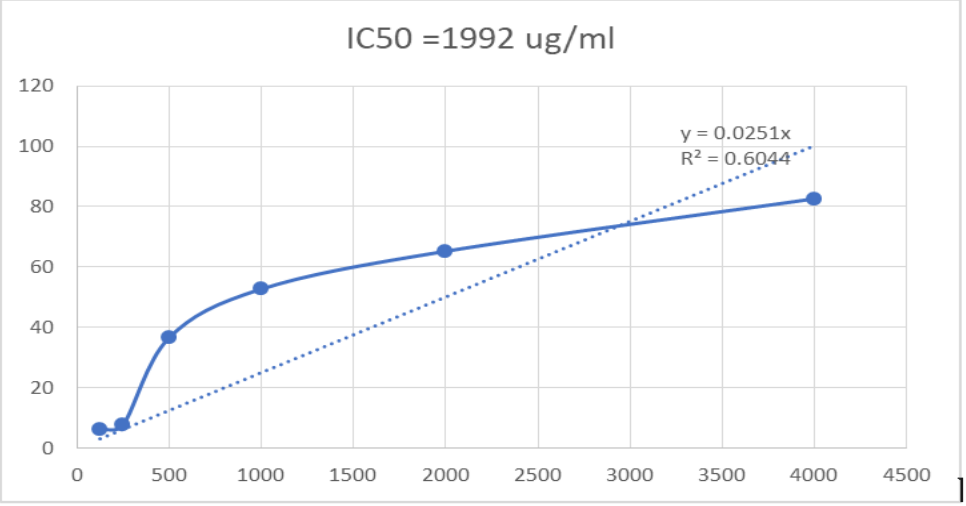

Figure 3.8 IC50 value for metformin on normal Vero cells.

3.3 Effect of 5-fluorouracil on the viability of SW480 colon cancer and normal VERO cell lines

The result showed that 5-FU at all concentrations used from (1000-31.25µg/ml) caused a highly significant (P<0.050) decrease in the viability of SW480 colon cancer cells compared to the control group as shown in figure 3.9.

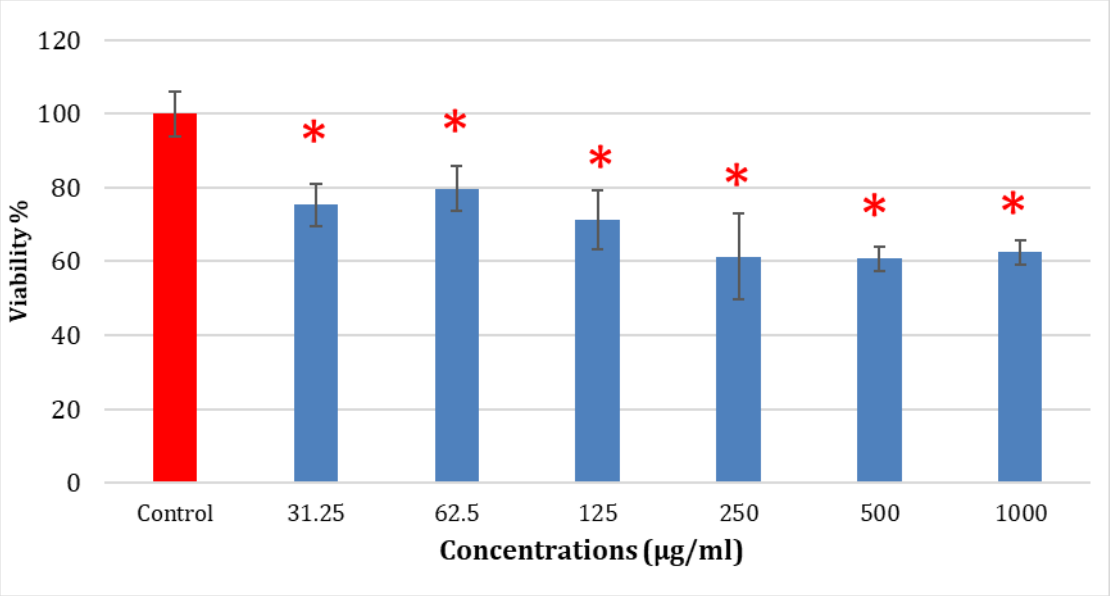

Figure 3.9: Effect of 5-fluorouracil on the viability of SW480 colon cancer cells.

The IC50 value for 5- FU on SW480 colon cancer cell was (1551.6 µg/ml) (figure 3.10)

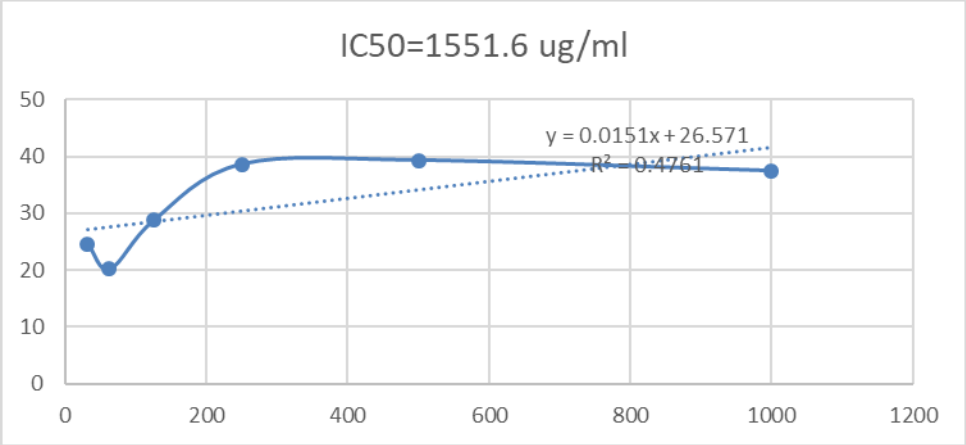

Figure 3.10 IC50 value for 5-FU on SW480 colon cancer cells.

Results showed that 5-FU at all concentrations used from (1000-31.25µg/ml) cause a significant (P<0.050) decrease in the viability of normal Vero cells compared with the control group as shown in figure 3.11.

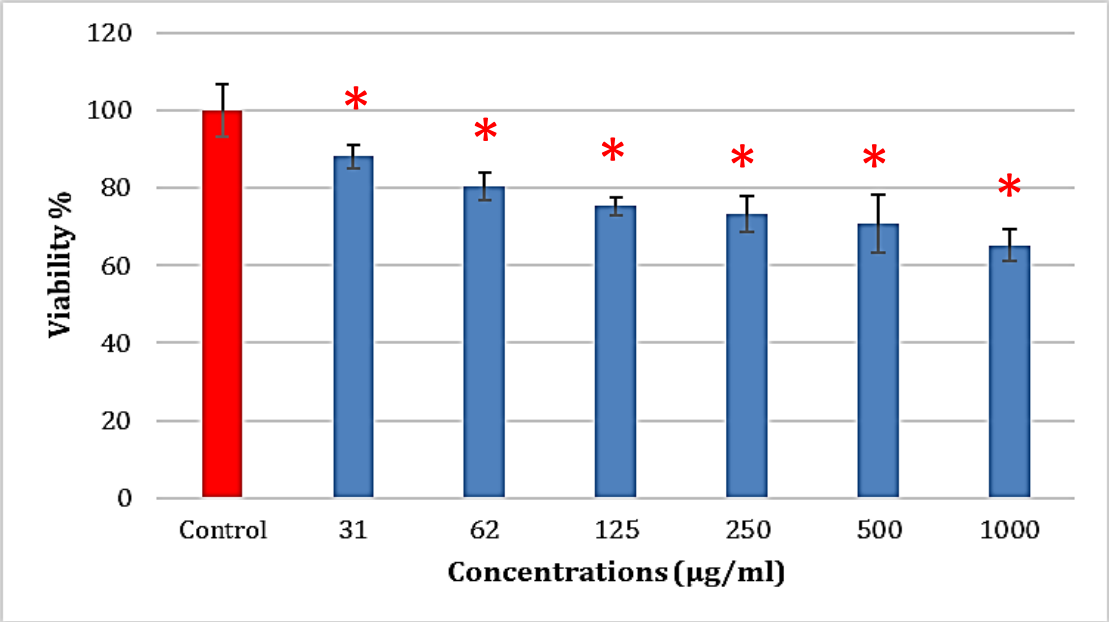

Figure 3.11: Effect of 5-FU on the viability of normal VERO cells.

The IC50 value of 5-FU on Vero cells was 1096 µg/ml (figure 3.12)

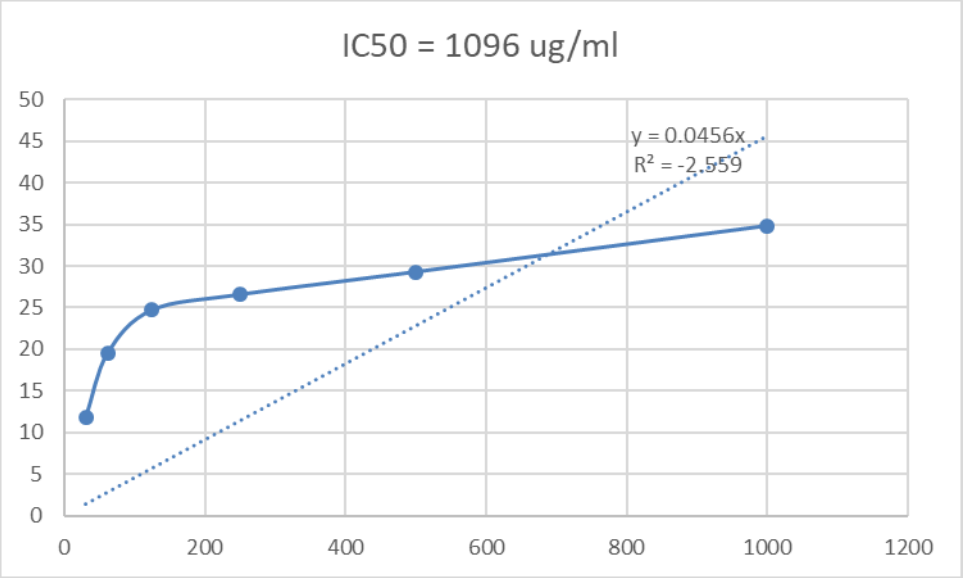

Figure 3.12: IC50 value of 5-fluorouracil on Vero cells.

3.4 Effect of doxorubicin on the viability of SW480 colon cancer and normal VERO cell lines

Results showed that DOX at all concentrations used (Form 250-7.8 µg/ml) causes a significant (P<0.050) decrease in the viability of SW480 colon cancer cells compared to the control group as shown in figure 3.13

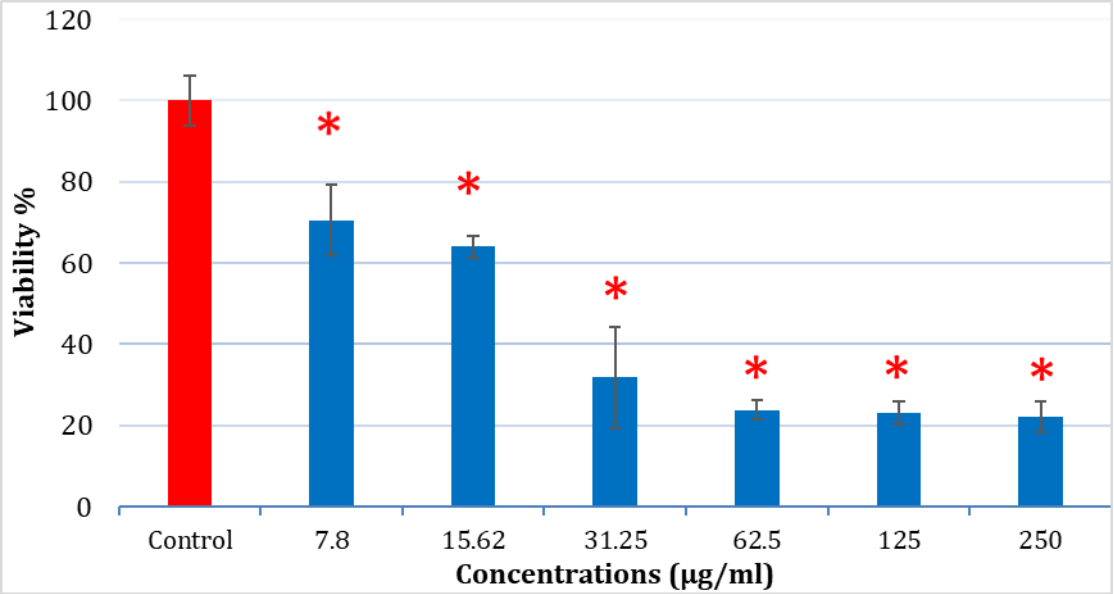

Figure 3.13: Effect of doxorubicin on SW480 colon cancer cells proliferation.

The IC50 value for DOX on SW480 colon cancer cells was (20.7 µg/ml) (figure 3.14)

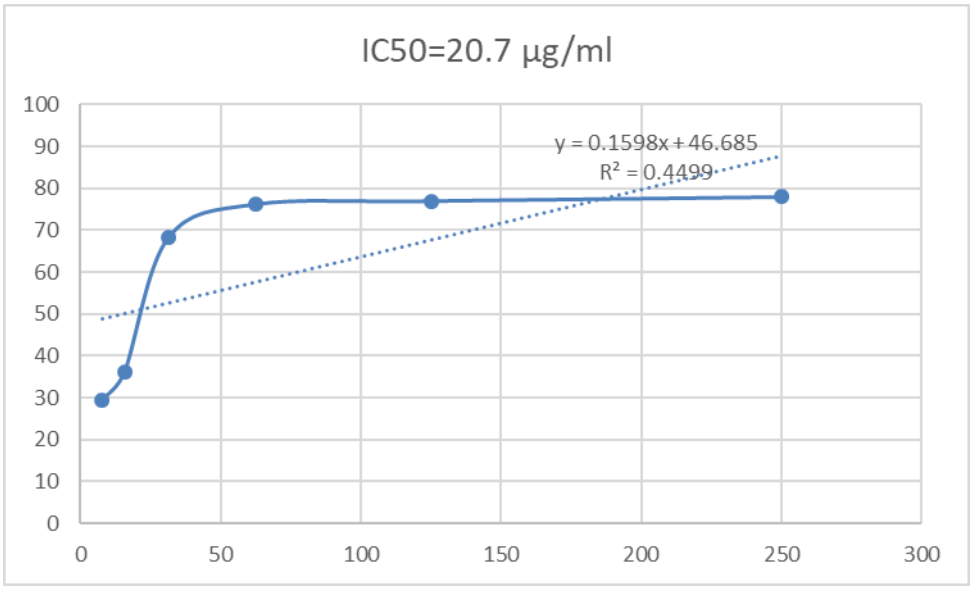

Figure 3.14 IC50 value for doxorubicin on SW480 colon cancer cells.

Results showed that DOX at all concentrations used from (250-7.25µg/ml) causes a significant (P<0.050) decrease in the viability of normal Vero cells compared to the control group as shown in figure 3.15.

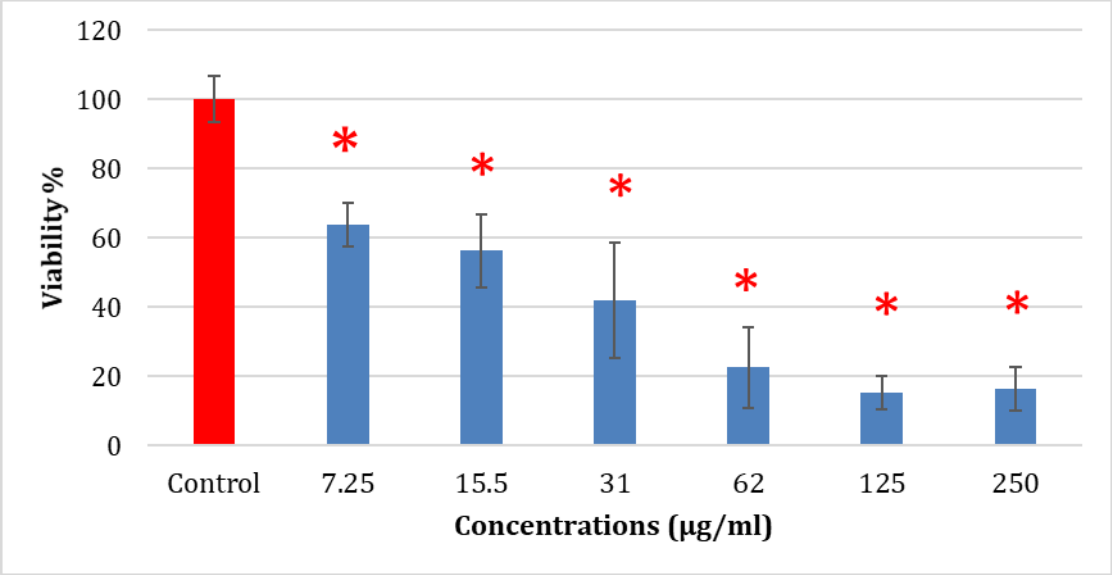

Figure 3.15: Effect of doxorubicin on the viability of normal Vero cells.

The IC50 value was 25.1 µg/ml for doxorubicin with Vero normal cells (figure 3.16)

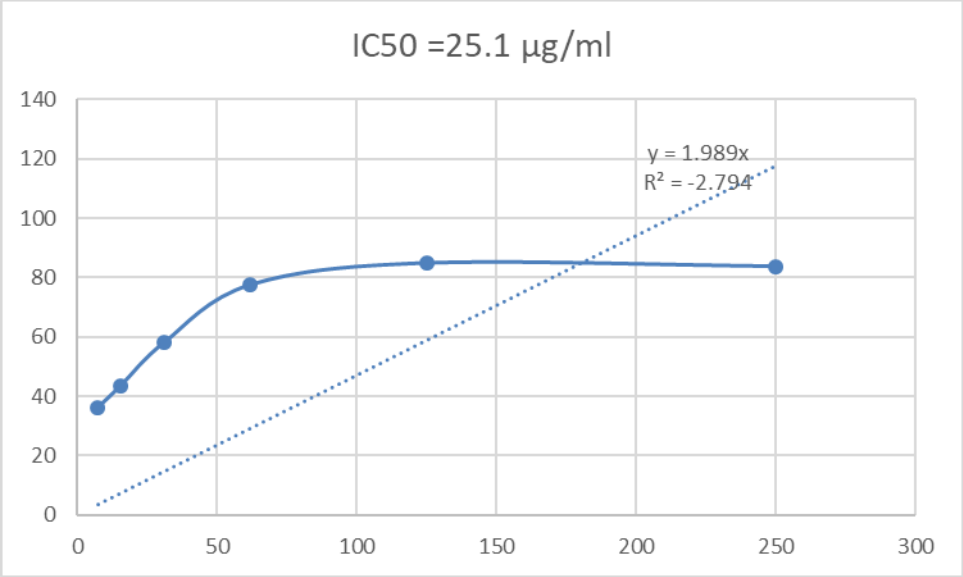

Figure 3.16 IC50 value for doxorubicin with Vero normal cells

3.5 Effect of (2-deoxy-D-glucose – metformin) combination on the viability of SW480 colon cancer and normal VERO cell lines

Results showed that when a combination of 2-DG with metformin was used to treat SW480 colon cancer cells there was a significant ($p<0.05$) inhibition of cell growth at all concentrations, The most effective combination was that contain (2-DG 1000 µg/ml+ metformin 4000 µg/ml) as shown in figure 3.17

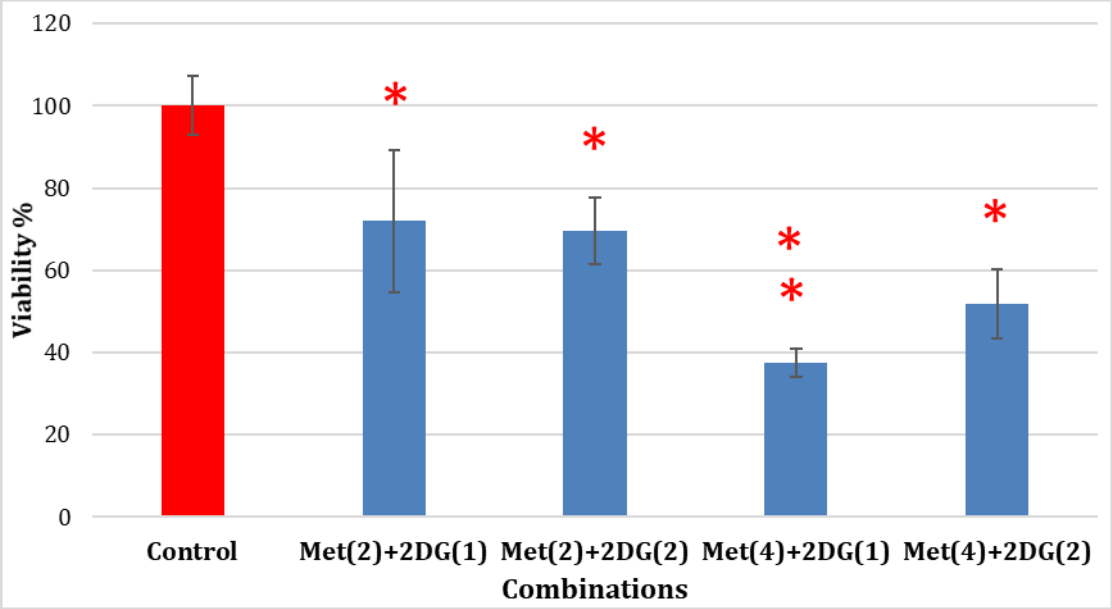

Figure 3.17: Effect of 2-deoxy-d-glucose plus metformin combination on SW480 colon cancer cells.(2-DG= 2-deoxy-d-glucose , met=metformin)

Results showed that when a combination of 2-DG with metformin was added to normal Vero cells, there was a significant ($p<0.05$) decrease in cell growth at all concentrations. The most effective combination was that contain (2-DG 2000 µg/ml+ metformin 4000 µg/ml) as showed in figure3.18

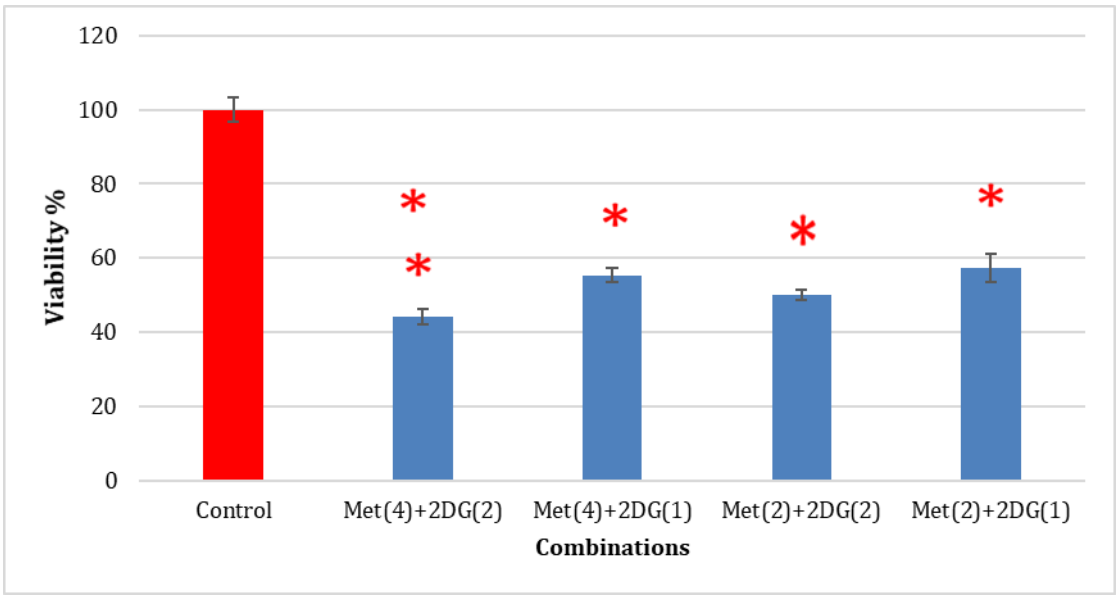

Figure 3.18: Effect of 2-deoxy-d-glucose plus metformin on normal VERO cells.

3.6 Effect of (2-deoxy-D-glucose-5-fluorouracil), (metformin-5-fluorouracil) and (2-deoxy-D-glucose - metformin-5-fluorouracil) combinations on SW480 colon cancer and normal Vero cell lines viability

Results showed that the growth of SW480 colon cancer cells was significantly (P<0.050) inhibited after the treatment with 5-FU in combination with 2-DG or metformin, the most effective combination was that containing (2-DG 2000 µg/ml+ metformin 4000 µg/ml + 5-FU 125 µg/ml) and (2-DG 1000 µg/ml+ 5-FU 125 µg/ml) as shown in (figure 3.19).

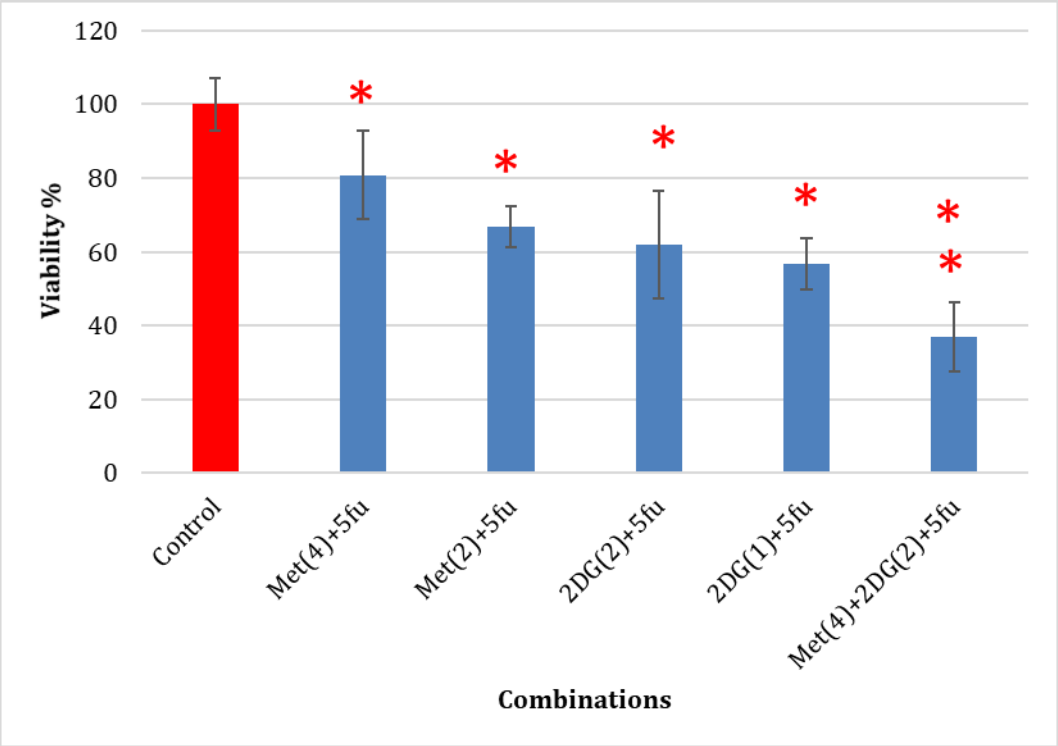

Figure 3.19 : Effect of 5-fluorouracil, 2-deoxy-d-glucose and metformin combinations on SW480 colon cancer cells viability . (4)=4000 µg/ml, (2)=2000 µg/ml ,(1)=1000 µg/ml. 5-FU=125 µg/ml, met=metformin, 2DG= 2-deoxy-d-glucose,5-FU= 5-fluorouracil

Results showed that the growth of normal Vero cells was significantly (P<0.050) inhibited after the treatment with 5-FU in combination with 2-DG or metformin, the most effective combination

was that containing (2-DG 2000 µg/ml + metformin 4000 µg/ml +125 µg/ml 5-FU) and (2-DG 2000 µg/ml+ 5-FU 125 µg/ml) as shown in (figure 3.20).

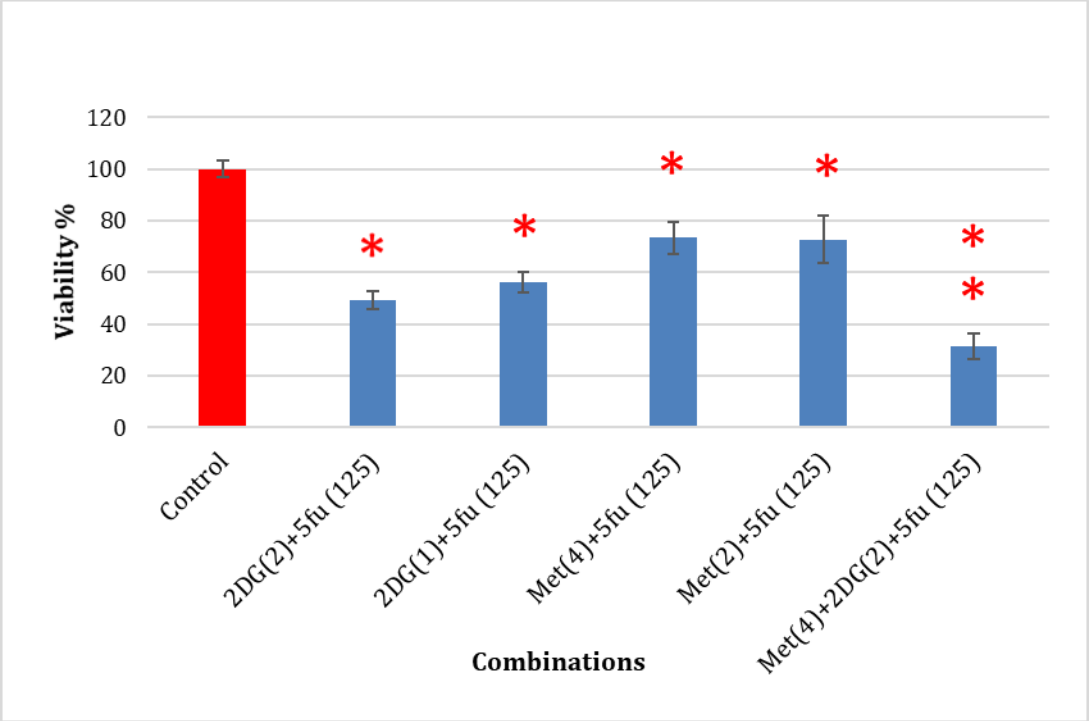

Figure 3.20 : Effect of 5-fluorouracil, 2-deoxy-d-glucose and metformin combinations on normal Vero cells viability . (4)=4000 µg/ml ,(2)=2000 µg/ml ,(1)=1000 µg/ml. 5-FU=125 µg/ml, met=metformin ,2DG= 2-deoxy-d-glucose,5-FU= 5-fluorouracil

3.7 Effect of (2-deoxy-D-glucose-doxorubicin), (doxorubicin-metformin) and (2-deoxy-D-glucose - metformin- doxorubicin) combinations on SW480 colon cancer and normal VERO cell lines viability

Results showed that the growth of SW480 colon cancer cells was significantly ($p<0.05$) inhibited after the treatment with DOX in combination with 2-DG or metformin, the most effective combinations were that containing (metformin 4000 µg/ml + 2-DG 2000µg/ml +

DOX15 µg/ml) and (DOX 15 µg/ml + metformin 4000 µg/ml) as shown in (figure 3.21).

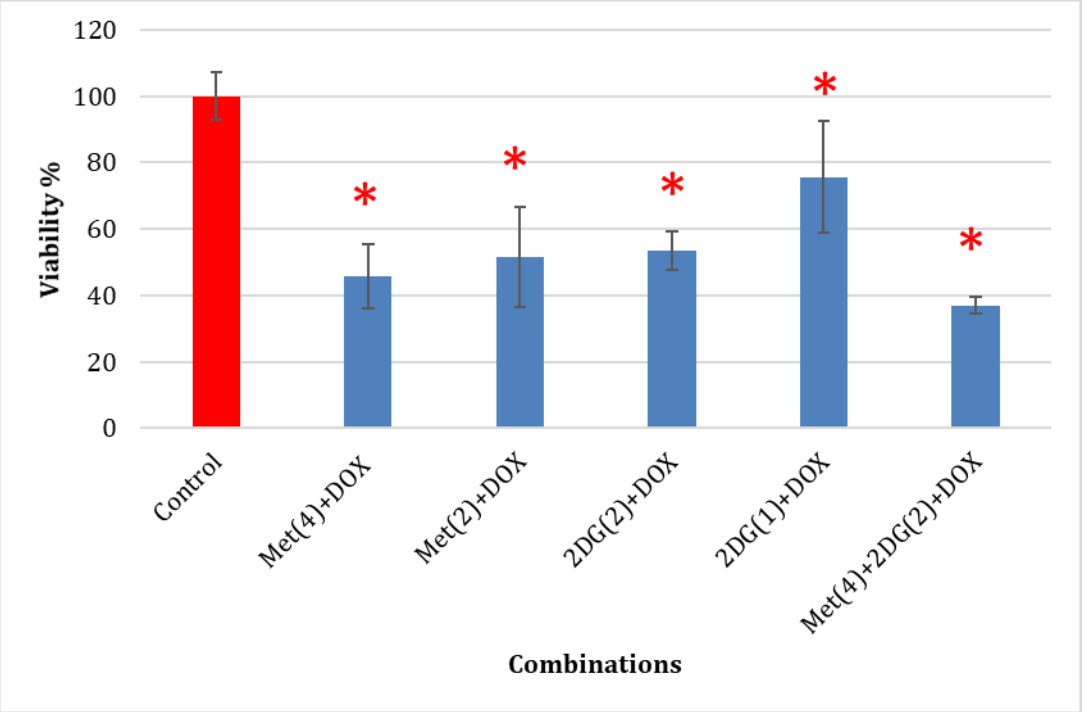

Figure 3.21 : Effect of 2-deoxy-d-glucose and metformin in combination with doxorubicin on SW480 colon cancer cells proliferation (4=4000µg/ml, 2=2000 µg/ml , 1=1000 µg/ml), DOX=15 µg/ml ,DOX= doxorubicin, met=metformin ,2DG= 2-deoxy-d-glucose

Results showed that the growth of normal Vero cells was significantly ($p<0.05$) inhibited after the treatment with DOX in combination with 2-DG or metformin or both, the most effective combinations were that containing (2-DG 2000µg/ml +metformin 4000 µg/ml + DOX15 µg/ml) (2-DG 2000 µg/ml +DOX 15 µg/ml) as shown in figure 3.22

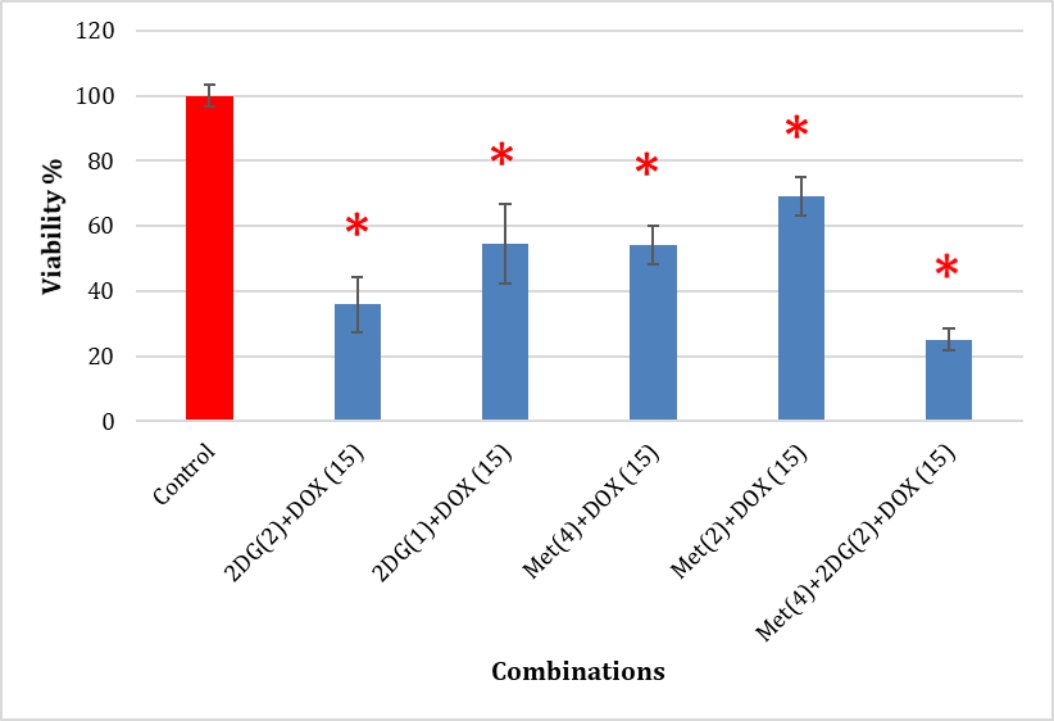

Figure 3.22 : Effect of 2-DG and metformin in combination with DOX on Vero cells proliferation . (4=4000µg/ml, 2=2000 µg/ml , 1=1000 µg/ml) ,DOX= doxorubicin =15 µg/ml , met=metformin ,2DG= 2-deoxy-d-glucose.

Chapter Four

Discussion

4. Discussion

In this in vitro study, the anticancer effect of different types of agents were evaluated. These agents including metformin, 2DG, DOX, and 5-FU were used in this study to find their effect on normal cells and cancer cells, also to find the effect of metformin and 2DG on cytotoxicity of 5-FU and DOX. The effect of these agents on the cancer cell line of the colon SW480 was investigated over a period of 24 hours incubation. A normal VERO cell line was used to evaluate whether these agents affect the growth of normal cells or not. MTT assay method was used for determining cell viability.

4.1 Effect of 2-deoxy-D-glucose on the viability of colon cancer SW480 and normal VERO cell lines

In the present study, the result revealed that there was a significant decrease in the viability of colon cancer SW480 cell line at the high concentrations of 2-DG while low concentrations do not affect cell viability compared with the control group as shown in(figure 3.1) which agrees with the result of (Ahadova *et al*., 2015) study, and this effect may be related to glucose deprivation of cancer cell as have been reported by many studies which have demonstrated that the glucose metabolism inhibitor 2-DG has a pronounced growth-inhibitory effect on cancer cells (Kurtoglu *et al.*,2014) The vast majority of these studies focused on the metabolism-related effects of 2-DG, specifically inhibition of glycolysis, and only a few studies addressed the question of altered glycosylation or other metabolism-independent effects of 2-DG(Lehrman *et al.*, 2007). However, an effect of 2-DG on N-linked glycosylation seems to play an important role in mediating toxicity in tumor cells under certain conditions (Stein *et al.*, 2010)

The 2-DG can enhance certain types of protein glycosylation, which may provide opportunities for novel diagnostic or therapeutic intervention approaches based on the recognition of 2-DG induced glycoprotein structures in cancer cells (Jiang *et al.*, 2014). It has been demonstrated that 2-DG induces a substantial growth suppression of several cancer cell lines in vitro include: WIDR, LOVO, HT-29, SW620 and, COLO cell lines (Tuccillo *et al.*, 2014). A study using HeLa cells shows that the competitive inhibitor of glucose metabolism, 2-DG, is both cytotoxic and capable of inducing radiosensitization in human cancer cells in vitro (Raez *et al*., 2013) Moreover, 2-DG prevents the phosphorylation of other available sugars such as glucose. Early work on rat tumors suggested that 2-DG treatment blocks glycolysis at the hexokinase step, supported by the fact that 2-DG inhibits glycolysis more than fructolysis (given that hexokinase activity in the brain and yeast has a better affinity towards glucose than towards fructose (Kurtoglu *et al.*, 2014)

Generally, 2DG only leads to growth inhibition of most cancer cells. To achieve more efficacious anticancer treatment, different strategies must be combined with 2DG to overcome the defects of monotherapy (Zhang *et al*., 2014).

In this figure the lower concentration 125µg/ml showed more inhibitory effect than the higher concentration 250µg/ml on cancer cells this may result from the effect of dilution or because of contamination ,this effect showed in many figures .

Regarding the viability of normal VERO cells in (figure3.3), there was an insignificant decrease in the viability only at the low concentrations of 2-DG while the high concentration could be toxic to normal cells due to a high decrease in the viability of cells over 24 hours

period compared with the control group. (Farooque *et al*., 2009) agree with this study as it showed that in vitro study by longer exposures to 2-DG inhibited the growth of Vero cells in a dose-dependent manner. A study done by Yang *et al*. (2020) agrees with part and disagree with the other of this study as he found that glucose analog 2-DG, an inhibitor of glycolytic ATP production has been shown to enhance radiation- and chemotherapeutic drug-induced damage in some cancers cells under in vitro and in vivo conditions while sparing or protecting normal cells, which disagree with our study or it may be due to the use of only low doses of 2- DG in his study.

Recent in vitro studies have shown that 2-DG is toxic to the glioblastoma cells but not to the normal astrocytes under similar conditions of exposure, which appears to be linked to the maintenance of redox balance with no significant decrease in ATP levels of astrocytes (Jelluma *et al.,* 2006)

4.2 Effect of metformin on the viability of SW480 colon cancer and normal VERO cell lines :

Apoptosis is well known as an essential homeostatic mechanism that acts to equalize cell division and cell death to sustain the appropriate cell number in the whole body. Through the process of apoptosis, the cells will be shrinkage, blebbing of the plasma membrane, and chromatin condensation without any lysis of the cells (Rashid *et al*., 2019). Metformin is an anti-diabetic drug that now is drawing much attention as an anti-tumor. Especially, after several groups of researchers approved its activity as anti-cancer by increasing apoptosis (Lesan *et al.*, 2014). The results of the current study revealed that Metformin is significantly

inhibited SW480 colon cell proliferation particularly at higher concentrations (2000,4000µg/ml).

A study by (Saini *et al*., 2017) agrees with the present study as it showed that metformin inhibits cell proliferation by inducing cell cycle arrest in various cell line models of breast, renal, pancreatic, colon, and prostate cancers. Also, another study performed by (Lesan *et al*., 2014) revealed that Metformin caused arrest in the cell cycle at phase G0/G1, resulting in accumulation of cell population at this phase which in turn led to induction of apoptosis. Furthermore, the inhibition in cell proliferation of SW480colon cancer cells after treatment with Metformin could be attributed to the re-expression of specific genes as had been reported by an experimental study on pancreatic cancer cells (Rocha *et al*., 2011). Moreover, in vitro study demonstrated that Metformin may activate ERK/MAPK pathways. ERK is a signaling pathway that has been well documented to have a major role in regulating proliferation and apoptosis (Rashid *et al*., 2019). Different cell lines or different concentrations would be the main reason for different respond of colon cancer cells to Metformin in the present study compared to the previous study. A clinical study found that the use of low doses of metformin in one year reduced the likelihood of occurrence of metachronous colorectal adenomas and polyps in patients who had polypectomy(Higurashi *et al*., 2016). Other researchers have also shown that the use of metformin along with chemotherapy reduces not only the size of the tumor but also the risk of malignity and tumor displacement to other organs in the body (Saraei *et al*., 2019).

Regarding the effect of metformin viability of normal VERO cells in (figure 3.6), the present study revealed that metformin does not inhibit the growth of normal cells at clinically achievable doses, while the high

concentrations could be toxic to normal cells due to a significant decrease in the viability of cells over 24 hours period compared with the control group.

These results from Metformin exposure disagree with another study by (Mortezaee *et al*., 2019) that used normal human gall bladder Cells (GBC), These finding declared that Metformin alone suppresses cell proliferation and encourage cell apoptosis, metformin has shown the ability to target cancer cells without any toxicity to normal cells. Another study by (Cheng *et al*., 2016) 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.

4.3 Effect of 5-fluorouracil on the viability of SW480 colon cancer and normal VERO cell lines

Results showed that 5-FU at all used concentrations cause a significant decrease in the viability of SW480 colon cancer cells compared with the control group as shown in (figure 3.7)

The 5-FU has become one of the most widely employed antimetabolite chemotherapeutic agents in recent decades. It has been used as a first-line antineoplastic agent in the treatment of several cancers, such as colorectal, breast, head and neck, pancreas, and stomach cancers (Sun *et al*., 2019). Research has indicated that 5-FU exerts its anticancer effects mainly through inhibition of TS, for which the pathways have not been fully interpreted (Sotelo *et al*., 2006).

A current study by Attoub *et al.,* (2018) made on three human colon cancer cell lines, namely, HT-29, HCT-116, and HCT8/S11, demonstrate that 5-FU causes a concentration and time-dependent reduction in the number of colon cancer cells which agree with the present study. Another *in vitro* study also agree with this study by (Álvarez-Sala *et al*., 2018) used 5-FU treatments on two types of colon cancer cells (Caco-2 and HT-29) the result showed the highest effect reaching over 50% of cell growth inhibition in Caco-2 cells and over 40% in cells in a time-dependent manner, although no clear dose-dependent effect was observed between 25 and 50 µM, showing similar effects.

Also, another study by (Angelis *et al.,* 2006) revealed that treatment of the HCT116 cell lines with 5-FU (in low and moderate concentrations) for 24 hours resulted in S phase arrests, p53 accumulation, alteration in cell cycle-regulatory and apoptosis-regulatory pathways, and apoptosis induction in the HCT116 cell lines.

Clinical research was conducted by (Hulme *et al*., 2006) suggests that resistance to 5-FU could be overcome through better control of its intratumoral activation and the use of an encapsulated formulation.

In CRC cells It is established that 5-FU exerts its cytotoxic effects in tumor cells primarily through inhibition of DNA synthesis. (Chen *et al*., 2019) also agree with our study by approving that it is evident in the significant arrests of G1/S and S phases of the cell cycle, as well as reduced G2/M phase population in colon cancer cells post-5-FU treatment.

Regarding the effect of 5-FU on the viability of normal VERO cells in (figure 3.9), the present study revealed that 5-FU caused only little inhibition to the growth of normal cells at clinically achievable

doses, while the high concentrations could be toxic to normal cells as it cause a significant decrease in the viability of cells over 24 hours period compared with the control group.

The 5-FU inhibited cell growth dose-dependently and this growth inhibition was accompanied by cell cycle accumulation in the early S phase and increased expression of cyclin A protein that synthesized and localized into the nucleus at the onset of the S phase in nontransformed mammalian fibroblasts (Sereno *et al*., 2008) which agree with the recent study. Some 5-FU metabolites are also associated with toxicity, in particular, alpha-fluoro-beta-alanine has been associated with neuro- and cardiotoxicity (Muneoka *et al*., 2005). also, the (Filgueiras *et al*., 2013) study reported cell cycle block, apoptosis, and alterations of contractility of smooth muscle cells treated with 5-FU *in vitro*.

A recent study conducted to investigate the effects of various 5-FU concentrations, exposure times, and application techniques on in vitro-cultured human corneal cells, shows that the cytostatic effect was time and dose-dependent for HCECs and HCKs cell lines (Midena *et al*., 2013).

4.4 Effect of doxorubicin on the viability of SW480 colon cancer and normal VERO cell lines

The result showed that DOX at all used concentrations (Form 250-7.8 µg/ml) causes a significant decrease in the viability of SW480 colon cancer cells compared to the control group as shown in (figure 3.13).

The DOX is a well-known chemotherapeutic agent which is used in the treatment of a wide variety of cancers inducing intracellular ROS accumulation, cell cycle arrest, and apoptosis (Putri *et al.*, 2016)

A study by Lüpertz *et al*., (2010) investigate the effects of DOX in Hct-116 human colon carcinoma cells to clarify if a time/concentration range for optimal DOX-induced apoptosis exists, the study compared a bolus DOX treatment schedule for cells were incubated for 3 hours with doxorubicin followed by 24 hours in drug-free medium, with a continuous doxorubicin treatment schedule for 24 hours. found that bolus treatment with DOX resulted in a dose-dependent decrease of viable cells and concomitant increase of apoptosis, In contrast, continuous treatment with DOX reduced the number of living cells with no parallel raise in the number of dead cells.

Chemosensitivity of DOX have been investigated in several models of breast cancer cell such as T47D, WiDr colon cancer cells, MCF-7, MCF-7 and showed various cytotoxic effects ((Febriansah *et al*., 2014); (Meiyanto *et al*., 2011); (Putri *et al*., 2016)).

Doxorubicin inhibited tumor growth in the SW480 CRC xenograft model (Emami *et al*.,2019) and stopped cell cycle progression in CT-26 murine CRC cells (Lan *et al.*, 2016)

The results of this study showed that DOX at all used concentrations caused a significant ($P<0.050$) decrease in the viability of normal VERO cells as shown in (figure 3.12)

A key DOX mechanism in the response of cancer cells to chemotherapeutic drugs involves the activation of apoptotic pathway (Gautier *et al.*, 2012) However, the clinical use of DOX is limited by its side effects, the most dangerous being cumulative dose-dependent cardiotoxicity (Lai *et al*., 2010)

The anticancer action of DOX is known to induce DNA damage in cancer cells by multiple mechanisms including intercalation into DNA

and inhibition of topoisomerase II (Minotti *et al*., 2004). DOX-induced toxicity has also been attributed to DOX redox cycling on the mitochondria to generate superoxide anion or other ROS and oxidative stress (Berthiaume *et al*., 2007). However, the definitive mechanism of cardiotoxicity induced by DOX remains unclear.

DOX-induced apoptosis in cultured neonatal cardiomyocytes was also found to parallel an increase inactivation of p38 mitogen-activated protein kinase (MAPK) in concentration- and time-dependent manners (Kang *et al*., 2000).

The cellular mechanisms of DOX-induced cardiotoxicity have also been investigated using isolated adults. Studies using oxidative-sensitive fluorescent dyes have demonstrated the persistent generation of ROS in myocytes from rats treated with DOX (Zhou *et al.*, 2001)

4.5 Effect of 2-Deoxy-D-glucose plus metformin on SW480 colon cancer cell and normal Vero cell lines viability

Results showed that when a combination of 2-DG with metformin was used to treat SW480 colon cancer cells there was a significant inhibition on cell growth at all concentrations (figure 3.17)

The result revealed that the combined effect of 2-DG and metformin significantly reduced cell viability. Further, the 2-DG and metformin combination resulted in a marked increase in cell death compared to either agent alone. Based on these results, we predicted that inhibition of glycolysis alone would not be sufficient to promote bioenergetic stress. inhibition of glycolysis with 2-DG at clinically achievable doses was not sufficient to induce a significant degree of cell death, even with prolonged incubation (Cheong *et al*., 2011). The

combination of metformin and 2-DG induced time-dependent detachment of viable MDA-MB-231 cells *in vitro*. Therefore, extended study to prostate cancer PC-3 cells and breast cancer MCF-7 cells, Combined treatment with metformin and 2-DG did not increase the percentage of dead PC-3 cells; however, it also did not induce their detachment (Bizjak *et al.,* 2017).

The results showed that the combination of both 2DG and metformin caused a decrease in the viability of normal Vero cell, study by Xue *et al*.,(2017) agree with these results (figure 3.18).

The combinational use of low-dose 2-DG and Metformin can markedly inhibit cell proliferation via simultaneously inhibiting glycolysis and oxidative phosphorylation metabolism, reducing intracellular ATP production, activating AMPK activity, and thereby inhibiting the activation of the mTOR proliferation signaling pathway in kidney epithelial cells (Wilson *et al*., 2017).

In addition that 2DG inhibits glycolysis due to the formation and intracellular accumulation of 2-deoxy-d-glucose-6-phosphate (2-DG6P), inhibiting the function of hexokinase and glucose-6-phosphate isomerase, and inducing cell death. (Blum *et al*., 2014) by these mechanisms, both metformin and 2DG may negatively affect cell viability.

4.6 Effect of 5-fluorouracil in combination with 2-deoxy-d-glucose or metformin or both on the viability of SW480 colon cancer cell and normal Vero cell lines

Results showed that the growth of SW480 colon cancer cells was significantly inhibited after the treatment with 5-FU in combination with 2-DG or metformin as shown in (figure 3.19).

The 5-FU has been widely used in combination with other agents for treating cancer progression and metastasis (Miranda *et al*., 2016)

Combining natural products as co-adjuvants in 5-fluorouracil (5-FU) chemotherapy might enhance the effectiveness of 5-FU by avoiding a high dosage and/or reducing treatment times. Metformin and 2-DG are two of the most commonly used AMPK activators. Metformin, an oral antidiabetic drug, is being evaluated in multiple clinical trials as an adjuvant drug to chemotherapy (Haffty *et al*., 2006) The 5-FU as a monotherapy or in combination therapy, has the major drawback of extensive tissue toxicity. The major side effect of 5-FU is myelosuppression, which can lead to many other health complications (DiPiro *et al*., 2011) For this reason, developing anticancer agents with less toxicity to normal tissues would be desirable. The 2-DG was also assessed in several clinical studies as an anticancer agent (Raez *et al.*, 2013) and is commonly used in vitro to mimic glucose starvation. Inhibition of glycolysis is likely its main mechanism of action (Urakami *et al*., 2013). An example of a drug with such an ideal safety profile is 2-DG. It is known to block glycolysis by inhibiting the key glycolytic enzyme hexokinase (Scatena *et al.*, 2008)

Results of a study made by CHENG *et al*.,(2014) demonstrated that the effect of 5-Fu-based chemotherapy on pancreatic cancer is significantly reduced by high glucose, although this effect can be reversed by 2-DG. It is therefore crucial for pancreatic cancer patients to control blood sugar levels to fully benefit from chemotherapy.

Therefore, metformin and 2-DG generate an energy crisis, which increases concentrations of AMP and activates AMPK43. AMPK

activation is augmented, when cancer cells are treated with both compounds simultaneously (Levesley *et al*., 2013)

A study by Olinger *et al*., (2013) investigate the efficiency of 5-FU in combination with 2-DG in colorectal cancer cell lines was found that the IC50 of 5-FU was reduced by the addition of 0.25 mM of 2-DG in SW480 and SW620 . Also, the use of metformin in combination with 5-FU induces cell cycle arrest and increases the cytotoxicity of 5-FU. Metformin and 5-FU were applied to colon 26 (C26) cell lines for improvement of anticancer activity and to overcome side effects. The combination treatment of 5-FU and metformin could induce cell cycle arrest and apoptosis, while subsequently increasing cytotoxicity both in vitro and in vivo.(Entezar-Almahdi *et al*., 2020) . These combinations indicate their good cytocompatibility.

Results showed that when a combination of 5-FU with 2-DG or metformin or both was used to treat normal cells there was also a significant decrease in cells viability at all concentrations (figure 3.20)

4.7 Effect of doxorubicin in combination with 2-deoxy-d-glucose or metformin or both on the viability of SW480 colon cancer and normal Vero cell lines

Because DOX is toxic and causes serious side effects in cancer patients (Johnson *et al* ., 2020)a major challenge is to lower its side effects without decreasing its effectiveness. Results showed that the growth of SW480 colon cancer cells was significantly inhibited after the treatment with DOX in combination with 2-DG or metformin or both, the

most effective combination was that containing (DOX + metformin + 2-DG) as shown in (figure 3.21).

The 2-DG is efficient to inhibit the glycolysis of cancer cells and block the intratumoral energy supply, which works in synergy with the co-loaded chemotherapeutic drug Dox to promote superoxide anion generation and mitochondrial depolarization, finally leading cancer cells to pro-apoptotic pathways. Comparatively, the starvation effect of 2DG can neutralize the toxicity of Dox in normal cells, thus mitigating the side effect of chemotherapy (Yang *et al*., 2020)

A recent study observed that 2-DG enhances the effects of two agents which are known to act on DNA, DOX, and 5FU. Doxorubicin, a member of the anthracycline family, is known to cause the generation of intracellular superoxide and hydrogen peroxide, which can mediate mitochondrial damage and apoptosis in a p. 53-independent manner. The study also found that 2-DG treatment results in increased production of reactive oxygen species (Zhang *et al*., 2019).

Salgado *et al*., (2021) examined the effect of the Combination of Metformin and DOX on Colorectal Cancer Cells the study found that the combination therapy significantly reduced proliferation by promoting apoptosis and autophagy in CRC-derived cells compared with DOX alone. Another study also observed that induction of autophagy by combination therapy had an anti-tumoral role, suggesting that the success of therapy is due to repressing DNA replication by DOX and targeting aberrant metabolism with metformin It has been reported that metformin and DOX arrest cell cycle and induce cell death through negative regulation of Phosphatidylinositol-3-kinase (PI3K) PI3K/AKT pathway (Babichev *et al*., 2016)

Metformin and DOX mono-treatments exhibited opposing action regarding phosphorylated adenosine monophosphate protein kinase. Co-treatment markedly decreased tumor volume, increased survival rate, and improved other parameters compared to DOX treatment alone (El-Ashmawy *et al.*, 2017).

Our results showed that when a normal Vero cells treated with a combination of DOX with 2-DG or metformin or both there was also a significant decrease in cells viability at all concentrations (figure 3.22)

Comparatively, the starvation effect of 2DG can neutralize the toxicity of Dox in normal cells, thus mitigating the side effect of chemotherapy. In a recent study two normal cell lines were observed of increased viabilities after incubation with liposomal Lip-(2DG + Dox) for 24 h compared with those incubated with Lip-Dox, demonstrating the protective effect of starvation triggered by 2DG that reduces the toxicity of Dox in normal cells. It is noted that the viabilities of two normal cell lines after single Lip-Dox or Lip-2DG treatment are slightly higher than those of cancer cell lines, which can be attributed to the differential resistances of different cell lines against 2DG or Dox(Yang *et al*., 2020).

Also, a recent study, observed that 2-DG enhances the effects of two agents which are known to act on DNA, doxorubicin and 5FU (Zhang *et al*., 2019), which mean that it is possible to reduce the dose of DOX and 5-FU by using 2-DG with maintain their effectiveness and so that reduce their side effects

Clinical use of doxorubicin (DOX) is limited by its cardiotoxic side effects. Recent studies established that metformin, an oral antidiabetic drug, possesses antioxidant activity. However, whether it can protect

against DOX-induced energy starvation and mitochondrial damage has not been reported (Álvarez-Sala *et al*., 2018)

Conclusion

&

Recommendation

Conclusions

1. Higher concentrations of both 2-deoxy-d-glucose and Metformin had a great cytotoxic effect on the SW480 colon cancer cell line.
2. Metformin showed a higher cytotoxic influence than 2-DG on cancer cells.
3. Combination of 2-DG with metformin significantly reduces cells viability, particularly at higher metformin concentrations.
4. Metformin and 2-DG increase the cytotoxic effect of 5-fluorouracil and doxorubicin especially when they are used together at higher concentrations with these drugs.

Recommendations

1. Investigate the effects of 2-deoxy-d-glucose and metformin on other types of cancer cell lines.
2. Study the effect of 2-deoxy-d-glucose - metformin combination at different concentrations on animal models.
3. Study the molecular changes of the cell after treatment with 2-deoxy-d-glucose or metformin to determine the exact mechanisms of their cytotoxic activities.
4. Study the drug interaction between 2-DG and metformin with other anticancer drugs in particular antimetabolite anticancer drugs .

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الخلاصة

يعتبر سرطان القولون واحد من اكثر الأورام المسببة للموت في العالم ، ويعتبر من الأمراض الفتاكة إلى جانب سرطان الرئة والبروستات والثدي. في هذه الدراسة ، تم إجراء محاولة لتحديد تأثير كلوكوز ثنائي منقوص الاوكسجين مع المتفورمين عند خلطهم مع 5-فلورويوراسيل و دوكسوروبيسين على فعالية خلايا القولون السرطانية وخط خلايا الطبيعية

تم تنفيذ العمل التجريبي في المختبر البحثي لطلبة الدراسات العليا في كلية الطب / جامعة بابل خلال الفترة من تشرين الأول 2020 إلى آب تم تنفيذ العمل التجريبي في معمل أبحاث طلاب الدراسات العليا في كلية الطب / جامعة بابل خلال الفترة من تشرين الاول 2020 إلى اب 2021.

تتضمن التجربة الأولى: تعريض خط خلية SW 480 لتراكيز مختلفة من كلوكوز ثنائي منقوص الاوكسجين والمتفورمين و5-فلورويوراسيل و دوكسوروبيسين لتحديد التراكيز السمية لهذه المواد الكيميائية على هذا النوع من الخلايا . تاثير كل مادة تم تحديده باستخدام اطباق تحتوي 96 حفرة مقسمة إلى 7 مجاميع :واحدة مجموعة تحكم سلبية وسته . بدأت هذه التخفيفات التسلسلية من 4000 ميكروغرام / مل إلى 125 ميكروغرام / مل لـ 2-ديوكسي-د-جلوكوز والميتفورمين ، من 1000 إلى 31.25 ميكروغرام / مل لـ 5 فلورويوراسيل ، ومن 250 إلى 7.25 ميكروغرام / مل لدوكسوروبيسين. تم تحضين جميع الأطباق لمدة 24 ساعة عند 37 درجة مئوية ، ثم تم إجراء فحص السمية الخلوية .

تم تكرار نفس المعلمات على خط الخلية . Veroلتقييم تأثير هذه الأدوية على الخلايا الطبيعية بعد تقييم السمية الخلوية لكل دواء على الخلايا السرطانية والطبيعية ، عولجت خلايا القولون السرطانية والخلايا الطبيعية بمجاميع مختلفة من العقاقير:

1- تراكيب من 2-ديوكسي-د-جلوكوز مع الميتفورمين.

2- تراكيب من 2-ديوكسي-د-جلوكوز مع 5-فلورويوراسيل

3- تراكيب من 2-ديوكسي-د-جلوكوز مع دوكسوروبيسين.

4- تراكيب ميتفورمين مع 5 يوراسيل

5- تراكيب ميتفورمين مع دوكسوروبيسين.

6- تراكيب من 2-ديوكسي-د-جلوكوز مع الميتفورمين مع 5-فلورويوراسيل

7- تراكيب من 2-ديوكسي-د-جلوكوز مع الميتفورمين مع دوكسوروبيسين.

أظهرت النتائج أن 2-ديوكسي-د-جلوكوز بتراكيز (2000،1000،500 و 4000 ميكروغرام / مل) تسبب انخفاضًا معنويًا (P <0.050) في قابلية بقاء الخلايا السرطانية الطبيعية وسرطان القولون مقارنة بمجموعة التحكم .

تسبب الميتفورمين بتراكيز (2000 و 4000 ميكروجرام / مل) في انخفاض معنوي (P <0.050) في قابلية الخلايا الطبيعية وسرطان القولون للحياة.

أظهرت النتائج أن 5-فلورويوراسيل (من 1000 إلى 31.25 ميكروجرام / مل) والدوكسوروبيسين (من 250 إلى 7.8 ميكروجرام / مل) تسبب في انخفاض معنوي (P <0.050) في قابلية الحياة لكل من خلايا سرطان القولون SW480 وخلايا فيرو الطبيعية بجميع التراكيز المستخدمة مقارنة بمجموعة التحكم.

فيما يتعلق بتأثير التراكيب المختبرة ، أوضحت النتائج أن الجمع بين 2-ديوكسي-د- جلوكوز والمتفورمين تسبب في انخفاض كبير (p <0.05) في قابلية خلايا سرطان القولون SW480 في جميع التراكيز المطبقة ، وكانت التركيبة الأكثر فعالية هي أن يحتوي على (2-دج 1000 ميكروجرام / مل + ميتفورمين 4000 ميكروجرام / مل).

أظهرت النتائج أن نمو خلايا سرطان القولون SW480 قد تم تثبيطه بشكل كبير (p <0.05) بعد العلاج بـ 5-فلورويوراسيل مع -ديوكسي-د- جلوكوز او المتفورمين ، وكانت التركيبة الأكثر فاعلية هي تلك التي تحتوي على (2-ديوكسي-د- جلوكوز 2000ميكروغرام / مل + 5-فلورويوراسيل 125 ميكروجرام / مل) و (2-ديوكسي-د-جلوكوز 2000 ميكروجرام / مل + ميتفورمين 4000 ميكرو جرام / مل + 125 ميكروجرام / مل 5-فلورويوراسيل).

أظهرت النتائج أن نمو خلايا سرطان القولون SW480 قد تم تثبيطه معنويا (p <0.05) بعد العلاج بدوكسوروبيسين مع -ديوكسي-د- جلوكوز او المتفورمين ، وكانت التركيبة الأكثر فعالية هي تلك التي تحتوي على 2-ديوكسي-د-جلوكوز 2000 ميكروجرام / مل + دوكسوروبيسين 15 ميكروجرام / مل) و (2-ديوكسي-د-جلوكوز 2000 ميكروجرام / مل + ميتفورمين 4000 ميكروجرام / مل +15 ميكروجرام / مل دوكسوروبيسين) .

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل/ كلية الطب

فرع الادوية

دراسة تأثير السكر الثنائي منقوص الاوكسجين و عقار المتفورمين على فعالية الخلايا السرطانية. دراسة في الزجاج

رسالة تقدمت بها

الى مجلس كلية الطب في جامعة بابل وهي جزء من متطلبات

درجة الماجستير في الادوية / الادوية

سارة صلاح حسن الكفيشي

بكالوريوس صيدلة

(2013-2014)

اشراف

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