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College of Medicine
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Effect of Quercetin on Oxidative Stress, Apoptosis, Inflammation, as Add on Agent to Doxorubicin and Carboplatin: *In Vitro* Study on Colorectal SW480 Cells

A Thesis

Submitted to the Council of the College of Medicine, the University of Babylon, as a Partial Fulfilment of the Requirements for the Degree of Master in Pharmacology\ Pharmacology and Toxicology.

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

((يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ ۚ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ))

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We certify that we have read this thesis entitled "**Effect of Quercetin on Oxidative Stress, Apoptosis , Inflammation, as Add on Agent to Doxorubicin and Carboplatin: In Vitro Study on Colorectal SW480 Cells**" and as an examining committee examined the student "**Sada Jawad Kadhum**" in its contents and that in our opinion it meets the standard of a thesis for the degree of Master, in Pharmacology and Toxicology .

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Dedication

I Dedicate This Thesis
To
My Family and My Friends
A Special Feeling of Gratitude To My
Loving Parents,
Whose Words of Encouragement and Push
for Tenacity Ring in My Ears…
My Wonderful Sisters Whose Supported
Me…
My Friends Whose Have Been My Best
Heartening…

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All My Teachers …with Deepest
Appreciation

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Sada Jawad Kadhum 2023

الخلاصة

يعد سرطان القولون والمستقيم ثالث أكثر أسباب الوفيات المرتبطة بالسرطان شيوعًا ، مما يدل على أن العلاجات الحالية لا يمكنها القضاء على بعض الخلايا السرطانية بشكل فعال. الكيرسيتين هو أحد مركبات الفلافونويدات المهمة التي لها خصائص مضادة للأكسدة ومحفزة لموت الخلايا السرطانية المبرمج ويمكنه أن يثبط نمو الأورام السرطانية . الدوكسوروبيسين هو عامل مجزء للحمض النووي ينتمي الى عائلة الانثراسيكلين (مضادات حياتية) يستخدم لعلاج مجموعة متنوعة من أمراض السرطان . المعقدات البلاتينية مثل الكربوبلاتين تمنع تضاعف ونسخ الحمض النووي عن طريق توليد مقاربات الحمض النووي لذلك تستخدم لعلاج الكثير من الامراض السرطانية . في الدراسة الحالية في المختبر ، تم تقييم تأثيرات الكيرسيتين والكاربوبلاتين والدوكسوروبيسين ومزيجها على حيوية الخلايا السرطانية والاجهاد التأكسدي وتحريض موت الخلايا المبرمج والتأثير المضاد للالتهاب على خلايا سرطان القولون والمستقيم بعد فترة حضانة دامت 48 ساعة.

تم تنفيذ الجزء العملي للدراسة البحثية الحالية في مختبر أبحاث طلبة الدراسات العليا / قسم علم الأدوية والسموم / كلية الطب / جامعة بابل خلال الفترة من نوفمبر 2022 إلى ابريل 2023.

تم زرع الخلايا الطبيعية في طبق مؤلف من 96 مكان زرعي بمعدل (2×10^4 خلية / حفرة) وتم الحفاظ عليها عند 37 درجة مئوية، وبعد 24 ساعة تم استبدال الاوساط القديمة، وعولجت الخلايا بـ (200,100,50,25,12.5) ميكر مول \اليترمن مادة الكيرستين وذلك لتحديد التراكيز الأمنه على الخلايا الطبيعية. ايضا تم زرع الخلايا السرطانية في طبق مؤلف من 96 مكان زرعي بمعدل (2×10^4 خلية / حفرة) وتم الحفاظ عليها عند 37 درجة مئوية، وبعد 24 ساعة تم استبدال الاوساط القديمة، وعولجت الخلايا بـ (200,100,50,25,12.5) ميكرو مول \اليتر من الكيرستين و(16,8,4,2,1) ميكرو مول \اليتر من الدوكسوروبوسين و (140,70,35,17.5,8.75) ميكرو مول \اليتر من الكاربوبلاتين لقياس تركيز المادة الموافق لتثبيط 50% من الخلايا السرطانية حيث اظهرت النتائج تأثيرا ملحوظا على تثبيط تكاثر الخلايا السرطانية بمستوى يعتمد على تركيز العلاجات المضافة وكان تركيز المادة الموافق لتثبيط 50% من الخلايا السرطانية (185 ،8 و 70) ميكرو مول \ ليتر لكل من الكيرستين، الدوكسوروبيسين و الكاربوبلاتين على التوالي . تراكيز الكيرستين (25 و50) ميكرو مول\اليتر اظهرت تأثيرا سميا ملحوظا على الخلايا السرطانية دون ان تسبب اي تأثير سمي على الخلايا الطبيعية لذلك تم اختيارها للمزج مع تراكيز العلاجات الكيميائية التي تقتل 50%

من الخلايا السرطانية والتي كانت 8 ميكرو مول\اليتر من الدوكسوروبيسين و 70ميكرو مول\اليتر من الكاربوبلاتين لمدة 48 ساعة وتم سحب السوائل الطافية لفحص الاجهاد التأكسدي ،موت الخلايا المبرمج والخاصية المضادة للالتهاب للعلاجات المنفردة والمزدوجة. حيث اظهر العلاج المركب تأثيرا عكسيا اذ ان هذا المزيج قلل من فعالية العلاج الكيميائي ضد الخلايا السرطانية. اما بالنسبة للكاربوبلاتين فان مزج 70 ميكرو مول /ليتر منه مع التركيز القليل جدا من الكيرستين 25 ميكرو مول \اليتر يقلل من فعاليته ضد الخلايا السرطانية اما مزجه مع تركيز 50 ميكرو مول \اليتر من الكيرستين فلا يؤثر على فعاليته ضد الخلايا السرطانية .

يؤدي الكيرستين الى انخفاضا ملحوظا في مستوى الكلوتاثايون في التراكيز العالية فقط عند مقارنته مع المجموعة الغير معرضة للعلاج وكذلك زيادة نسبية ملحوظة بشكل كبير في تركيز المالون دايالديهايد في التراكيز العالية فقط اما علاجات الدوكسوروبيسين والكاربوبلاتين فتقلل وبشكل ملحوظ من الكلوتاثايون حيث كلما زاد تركيزها زاد النقصان في تركيز الكلوتاثايون اما المالونداياالديهايد فيزداد بشكل ملحوظ مع هذه العلاجات كلما زاد تركيزها. على عكس ذلك اظهرت المعالجة المشتركة للكيرستين مع العلاجات الاخرى تأثيرا على مستوى المؤشرات الحيوية للإجهاد التأكسدي (الكلوتاثايون و المالونداياالديهايد) معاكسا لتأثير الكاربوبلاتين والدوكسوروبوسين عندما تستخدم بشكل منفرد.

أظهرت النتائج زيادة ملحوظة في تركيز الكاسبيز 3 لكل من الكيرسيتين، الدوكسوروبيسين والكاربوبلاتين بشكل يعتمد على التركيز حيث كلما زاد تركيز العلاج زادة مستوى الكاسبيز 3 . اما العلاج المركب فان تركيز 25 ميكرو مول \اليتر من الكيرستين يظهر تأثيرا ضعيفا على مستوى الكاسبيز3 لكلا العقارين اما تركيز 50 ميكرو مول\اليتر من الكيرستين فيظهر تأثيرا عكسيا على مستوى الكاسبيز3 مع الدوكسوروبيسين بينما لا يؤثر على تركيزه مع الكاربوبلاتين.

اظهرت النتائج انخفاضا ملحوظا في تركيز عامل نخر الورم في التراكيز العالية المستخدمة لكل من الكيرستين ، الدوكسوروبوسين والكاربوبلاتين حيث كلما زاد تركيز العلاج زاد تأثيره على مستوى عامل نخر الورم بعكس ذلك فان العلاج المركب اظهر تأثيرا معاكسا بدرجات متفاوتة عند مقارنته مع الدوكسوروبيسين والكاربوبلاتين عندما تستخدم بشكل منفرد.

الكيرستين اعطى مفعولا ثنائي الطور حيث انه في التراكيز العالية سلك كعامل محفز للإجهاد التأكسدي وبالتالي حفز موت الخلايا المبرمج اما في التراكيز القليلة فيسلك كعامل مضاد للإجهاد التأكسدي لذلك ادى الاستخدام المزدوج للتراكيز القليلة للكيرستين مع العلاج الكيميائي الى تقليل فعالية العلاج الكيميائي ضد السرطان. تركيز 50 مايكرومول من الكيرستين فقط مع الكاربوبلاتين يقلل الاجهاد التأكسدي للكاربوبلاتين دون ان يأثر على فعاليته السمية ضد الخلايا السرطانية.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل/ كلية الطب

تأثير الكيرستين على الاجهاد التأكسدي، موت الخلية المبرمج، الالتهاب، كعامل اضافي للدوكسوروبيسين والكاربوبلاتين: دراسة مختبرية على خلايا القولون والمستقيم

SW480

رســـالـــــــة

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1444 هـ **2023 م**

Summary

Colorectal cancer (CRC) is the third most common cause of cancer related deaths, demonstrating that current therapies cannot eradicate some of cancer cells effectively. Quercetin is one of the important flavonoids that have biological benefits, such as antioxidant, anticancer, and anti-inflammatory properties, at an acceptable dosage. Doxorubicin is a DNA intercalating agent that belongs to the anthracycline family of antibiotics used to manage various types of cancer such as leukaemia, breast cancer, and cervical cancer. Platinum complexes like carboplatin hinder DNA replication and transcription by generating DNA adducts. In present in vitro study, effects of quercetin, doxorubicin, carboplatin and their combination on cell viability , oxidative stress, apoptosis induction and inflammation were evaluated on human colorectal cancer SW480 cells after 48 hr incubation period.

The experimental work was performed in the postgraduate student's research laboratory at the College of Medicine\University of Babylon through the period from November 2022 to April 2023.

Vero (normal) cells were seeded in 96-well plates (2×10^4 cells/well) and maintained overnight at 37°C, old media was aspirated, the cells were treated with (12.5, 25, 50, 100, 200) µM of quercetin to determine the effective concentrations of quercetin that have no toxic effect on normal cells. Likewise, SW480 cells were seeded in 96-well plates (2×10^4 cells/well) and maintained overnight at 37°C, old media was aspirated, the cells were treated with (12.5, 25, 50, 100, 200) µM, (1, 2, 4, 8, 16) µM and (8.75, 17.5, 35, 70, 140) µM of quercetin, doxorubicin and carboplatin respectively. Low concentrations of quercetin (25,50) µM exhibited no cytotoxic effect on vero (normal) cells and significant

$p<0.05$ cytotoxic effect on SW480 cancer cells so these concentration were selected to be used in combination with IC50 of doxorubicin and carboplatin (8 µM and 70µM) respectively, in treatment of SW480 cells at 48 hr. Supernatant of each well was withdrawn for assessment of oxidative stress, apoptosis and anti-inflammatory effect.

It has been found that quercetin exhibited significant ($p <0.05$) toxic effect on normal cells only at high concentrations (100, 200) µM. However, following 48 hr, quercetin, doxorubicin and carboplatin significantly ($p <0.05$),($p <0.001$) decreased proliferation of SW480 cancer cells in a concentration-dependent manner , the IC50 were (185, 8, and 70) µM for quercetin, doxorubicin and carboplatin respectively. Importantly, co-treatment of quercetin at concentrations 25µM with IC50 of doxorubicin 8µM and carboplatin 70µM decreased the anti-proliferative effect of each drug when used alone. However, quercetin at 50µM reduced the cytotoxicity of doxorubicin but not carboplatin. Quercetin induced highly significant ($p < 0.001$) decrease in GSH (antioxidants) level and significant ($p <0.05$),($p <0.001$) increase in MDA (oxidative stress) level only in high concentrations (100, 200) µM when compare to the control group, while low concentrations (25,50) µM exhibited no effect on oxidative stress biomarkers (GSH, MDA). Doxorubicin and carboplatin significantly ($p <0.05$),($p <0.001$) decreased GSH level and significantly ($p <0.05$),($p <0.001$) increased in MDA level in concentration-dependant manner. However, the combination treatment showed a significant decrease in oxidative stress biomarker (MDA) and significant increase in antioxidant biomarker (GSH) in compare to the each drug alone. Also, the results showed a significant ($p < 0.05$) increase in apoptotic protein (caspase 3) in concentration - dependant manner of quercetin , doxorubicin, and carboplatin. co-

treatment of quercetin at concentrations 25µM with IC50 of doxorubicin and carboplatin decreased the caspase3 level of each drug when used alone. However, quercetin at 50µM reduced the caspase3 level of doxorubicin but not carboplatin. On the other hand, quercetin , doxorubicin and carboplatin after 48hr induced a significant decrease in TNF- α level in concentration- dependant manner ($p < 0.05$), ($p < 0.001$). The combination treatment exhibited significant increase in TNF-α in various degrees in a compare to the each drug alone.

Quercetin exerted biphasic, at high concentrations behaved as pro oxidant agent because it dramatically induced oxidative stress and consequently, promotes apoptosis in SW480 cells while at low concentrations, quercetin behaved as antioxidant proposing a mechanism through which quercetin attenuated the effects of anti-neoplastic medications. Quercetin at concentrations 50µM reduce the oxidative stress that associated with carboplatin, without change its cytotoxicity.

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Abbreviations	Meaning
Ab	Antibody
Apaf-1	Apoptosis protease activating factor 1
Bax	B cell lymphoma-2 (Bcl-2)-associated X protein (pro apoptotic gene)
Bcl-2	B cell lymphoma-2 (Anti apoptotic gene)
CAT	Catalase
CRC	Colorectal cancer
DDW	Deionized distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene di amine tetra acetic acid
EGFR	Epidermal growth factor receptor
EP	Eppendorf
FAP	Familial Adenomatous Polyposis
FBS	Fetal bovine serum
GSH	Glutathione
HNPCC	Hereditary Non-Polyposis Colorecatal Cancer
HRP	Horseradish Peroxidase
IC50	Median Inhibitory Concentration
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
µM	Micro molar
NF-κB	Nuclear Factor kappa
Nrf2	Nuclear factor (erythroid- 2)-related factor2

P53	Tumour suppressor gene
PBS	Phosphate Buffer Saline
PI3K	Phosphoinositide 3-kinase
ROS	Reactive oxygen species
Rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute
SAD	Superoxide dismutase
TGFB	Transforming growth factor beta
TMB	Tetramethylbenzedine
TNF-α	Tumour Necrosis Factor-alpha
TRIAL	TNF-related apoptosis-including ligand

Chapter One

Introduction and Literature Review

1.1.Introduction

Colorectal cancer (CRC) is a commonly seen gastrointestinal cancer in clinical practice and contributes for over 930,000 fatalities according to the global cancer statistics 2020 that released by the international agency, it ranks the third among all malignancies in terms of overall mortality (Siegel *et al*., 2021). Various factors, including Western diet and lifestyle, insufficient exercise, weight gain, genetic changes, and epigenetic modifications are thought to be linked to colon cancer morbidity and mortality rates. Meanwhile, the accumulation of gene mutations and epigenetic changes may affect the emergence and progression of non-neoplastic to neoplastic adenocarcinoma. (Akimoto *et al*., 2021). Likewise, Aging is also a process of steady decline characterized by the accumulation of gene mutations and cellular-level epigenetic alterations, which may ultimately lead to the development of aging-associated illnesses like colon cancer. (Zabransky *et al*., 2022).

Surgery, radiation, chemotherapy, and targeted therapy are currently the standard treatments for CRC, however the appearance of CRC invasion and metastasis at intermediate and advanced stages led to poor efficacy and became a major cause of death (Modest *et al*., 2019). The progression of CRC and high resistance to chemotherapy and radiotherapy may due to apoptotic dysfunction (Patra *et al*., 2021). Due to improvements in early detection and new medicines, the average survival duration for persons with advanced CRC has doubled in the last ten years . Unfortunately, they typically die within three years since their chances for survival are still low (Day and Velayos , 2015). Chemotherapy is thought to be a promising cancer treatment and the control of reactive oxygen species (ROS) production has been suggested as an excellent therapeutic approach for selectively targeting cancer

cells. Novel medications have been discovered that elevate ROS levels and alter the potential of the mitochondria, exposing tumour cells to death (Hao Jiang *et al*., 2023). Several studies have shown that cancer cells display an adaptive response to oxidative stress by increasing expression of antioxidant enzymes and molecules so, blocking antioxidant defence in tumours decreases their ability to balance oxidative insult and results in cell death (Nakamura and Takada, 2021). There are many chemotherapies cause high amounts of ROS like alkylating drugs, anthracyclines (such as doxorubicin), epipodophyllotoxines, platinum coordinated complexes (such as carboplatin), and camptothecin, contrary to these agents, several others, such as vinca alkaloids (vincristine and vinblastine), taxanes, and antimetabolites (such as antifolates), only produce low amounts of ROS (Conklin, 2004). The primary cause of side effects in chemotherapy patients is the overproduction of ROS caused by the cytotoxic drugs (Wang *et al*., 2021).

The development of side effects is a common drawback of chemotherapy after chemoresistance, whereas chemotherapy drugs destroy cancer cells but they have a negative impact on healthy cells and tissues, side effects highlight that the chemotherapy lacks selectivity (Zhao *et al*., 2015). ROS have been linked to the genesis of side effects like cardiac damage, myelosuppression, hepatic and renal failure which are all frequent side effects that occur during or after chemotherapy treatment (Qi *et al*., 2015). The ability to have the most impact on cancer cells while causing the least amount of harm to nearby normal cells is one of the main requirements for possible anticancer medications. Therefore should use anticancer agents with oxidative capacity does not harm normal cells. A challenge in the current study is to find a safe and

effective combination of drugs that can prevent tumour cell invasion, metastasis and side effects.

Traditional Chinese medicine used in cancer treatment for a long history return to more than 2000 years. Nightshade is the whole grass of *Solanum nigrum L.* in the *Solanaceae*, where modern studies have found that nightshade has different mechanism against cancer compromising blockage of the cell cycle, inhibition of cell proliferation and tumour growth, induction of cell apoptosis, and inhibition of epithelial mesenchymal transformation and tumour metastasis in addition to increase the efficacy of radiotherapy, chemotherapy, targeted therapy, and reversal of drug resistance (Dong *et al*., 2021). Epidemiological evidence has indicated that an increased dietary intake of flavonoids through high consumption of fruits and vegetables may be considerably reduce cancer incidence (Villota *et al*., 2022). Quercetin is a major flavonoid in the nightshade having high anti-proliferative activity (Datta *et al*., 2022). Due to its chemopreventive effects that have been demonstrated in both in vitro and in vivo models, quercetin has high potential in oncology.

Aims of The Study

This study designed to investigate:

1. The effect of quercetin on the viability , oxidative stress ,apoptosis and inflammation in SW480 human colorectal cancer cells
2. The effect of quercetin on the efficacy of chemotherapeutic agents (doxorubicin and carboplatin) against the viability of SW480 human colorectal cancer cells.

1.2. Colorectal Cancer

Colorectal cancer (CRC) also known as colorectal adenocarcinoma, often develop when some cells of the epithelium in large intestine acquire a number of genetic or epigenetic alterations that give them a selective advantage. These hyper-proliferative cells produce benign adenomas, which can later develop into carcinoma and spread throughout the body over many years due to abnormally high replication and survival rates (Rawla *et al*., 2019). CRC can be diagnosed by taking a sample of the colon via a sigmoidoscopy or colonoscopy. Medical imaging is then used to ascertain whether the disease has spread.

1.2.1. Epidemiology

Medical professionals and researchers worldwide are greatly concerned because of the rise in cases of colorectal cancer . Its occurance and mortality rates alter significantly around the world. According to the World Health Organization's GLOBOCAN database, CRC is the second most frequently diagnosed cancer in women and the third most frequently diagnosed cancer in men worldwide (Global Cancer Observatory, 2023). Males have significantly greater rates of incidence and mortality than females. Incidence and death of CRC have been continuously declining in the United States (Cronin *et al*., 2022).

Annually, around 153,000 new cases of large bowel cancer are diagnosed, including roughly 46,000 cases of rectal cancer and nearly 107,000 cases of colon cancer. (Siegel *et al*., 2023). Each year, over 52,500 of people die the United States due to CRC. Globally, there are regional variations in the incidence of CRC; these variations appear to be caused by variations in food and environmental exposures, low socioeconomic level, and lower rates of CRC screening, which are added

to a background of varied susceptibility CRC prevalence. About 4% of patients in the United States with average risk have a lifetime incidence of CRC (Siegel *et al*., 2023). The incidence of CRC is almost 20% higher in Black Americans than White Americans and approximately 33% higher in males than females in the United States. Patients with particular hereditary disorders that increase their risk of developing CRC have a greater incidence of the disease. (Siegel *et al*., 2023).

1.2.2.Pathogenesis

Colorectal cancer almost usually starts as a polyp, a noncancerous growth that appear in the inner lining (mucosal layer) of the colon or rectum. Polyps are frequently , dignosed in about half (compremising serrated polyps) of average-risk people 50 years of age or older who undergo to colonoscopy, with greater prevalence in older age peoples and among men than women. However, less than 10% of polyps are thought to develop into invasive cancer, which typically develops slowly over a period of 10 to 20 years and is more likely that polyps get larger (Rawla *et al*., 2019). Certain dietary and lifestyle choices can exacerbate intestinal inflammation and change the normal flora of the gut, which can cause an immunological reaction and promote the growth of polyps and their progression into cancer (Rawla *et al*., 2019).

The mechanism of cancer initiation is a complicated, multistage process that involves consecutive mutational events that take place as the tumour progresses. There are various biological processes, such as cell proliferation, differentiation, angiogenesis, apoptosis, and survival are thought to be regulated by the Wnt/β-catenin, tumour suppression gen (p53), transforming growth factor beta (TGF-β), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Notch, vascular

endothelial growth factor (VEGF), and janus kinase/ signal transducer and activator of transcription (JAKs/STAT3) pathways (Malki *et al*., 2020). Numerous genes are linked to these signalling pathways that have frequently been deregulated in the CRC as a result of mutations or the altered function of their products, these genes have been identified as being responsible for enhanced proliferation, invasion, progression, or suppression of apoptosis in CRC cells (Liao *et al*., 2018).

Inflammatory bowel disease (IBD) which involve both ulcerative colitis and crohns disease are risk factors for growth of CRC (Lucafò *et al*., 2021). Chronic inflammation is a known risk factor for CRC development, progression, metastasis, and resistance to cancer therapy (Schwager *et al*., 2019). TNF-α an inflammatory mediator which is one of several cytokines present at high levels in solid tumours and the serum of CRC patients (Li *et al*., 2017). It has been demonstrated that the presence of inflammatory cells and inflammatory mediators (e.g. pro-inflammatory cytokine TNF-α) in the tumour microenvironment causes abnormal activation of particular transcription factors, such as (NF-B), which in turn stimulates and promotes angiogenesis, metastasis, and the proliferation and survival of cancerous cells (Xia *et al*., 2018).

1.2.3. Classification of Colorectal Cancer

Colorectal cancer can be categorized into three major groups according to the source of mutations : Sporadic colorectal cancer compromise for approximately 65-70% of the cases as presented in (Fig 1.1). Only 5–10% of newly diagnosed cases of colorectal cancer are linked to hereditary disorders like Lynch syndrome or familial adenomatous polyposis (FAP). 20–25% of CRC cases experience a

familial disposition without linked or known gremlin mutation (Kasi A *et al* ., 2020) .

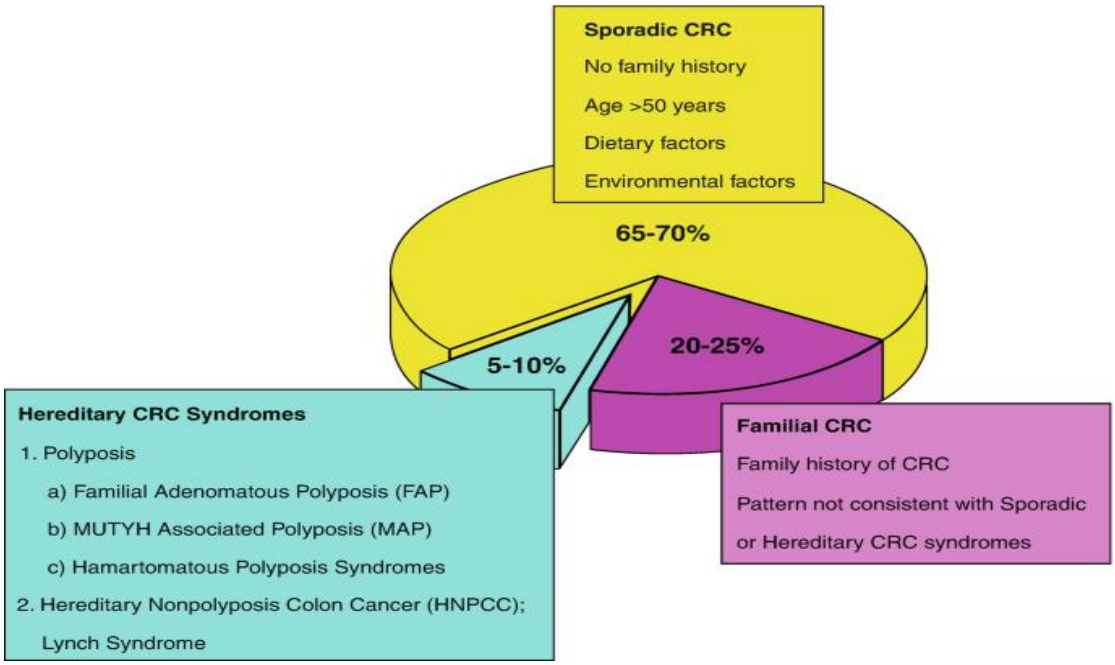


Figure 1.1. Classification of colorectal cancer according to origen (Sandeep *et al* ., 2019).

Hereditary CRC syndromes can be categorised phenotypically into polyposis and non-polyposis syndromes, primarily based on the polyps conditions. Lynch syndromes (LS) and familial adenomatous polyposis (FAP) respectively are the typical representative of hereditary CRC syndromes (Chen L *et al*., 2022).

1. Familial Adenomatous Polyposis (FAP)

Familial adenomatous polyposis which responsible for 1% of all colorectal malignancies, occurs due to mutations in the adenomatous polyposis coli (APC) gene that individual received from parents , APC is a tumour suppressor gene that represent a key role in the Wnt signalling pathway . In this type of hereditary CRC syndrome , hundreds or thousands of polyps grow in the colon and rectum, which typically

begin at around age 10 to 12 years. If these polyps has not removed one or more of them develop in to malignancy within 10 years (Wong *et al*., 2019).

2. Lynch Syndrome (Hereditary Non-Polyposis CRC, or HNPCC)

The most typical kind of hereditary colorectal cancer occurs without association to polyposis, about 2% to 4% of all colorectal cancers are caused by it. Although mutations in other genes can also result in Lynch syndrome, the most common genetic cause of this condition by mutational inactivation of DNA mismatch repair (MMR)-related genes (MSH2, MLH1, and PMS2). Normally, these genes aid in the repairing the DNA that has been damaged (Mármol *et al*., 2017). The cancers associated with this syndrome typically appear in young patients. Depending on which gene is affected, a person with this condition may have a 50% lifetime risk of developing CRC (Mármol *et al*., 2017).

1.2.4.Colorectal Cancer Grading

Once cancer has been diagnosed, it will go through one stage, these stages indicate the tumour's size, how much of the colon or rectum wall it has penetrated, and whether the cancer has migrated to the lymph nodes or other areas of the body beyond the site where it first appeared. CRC classify in to 5 stages (Fig. 1.2):

Grade 0 is only present in the colon's mucosa, which is the deepest layer in the colon; **Grade I** signifies that the tumour has grown outside the inner lining of the colon but is still contained there and has not reached the lymph nodes, filter-like small organs called lymph nodes are a component of the immune system; **Grade II** extends to the thick outer muscle layer of the colon but still not reach to the lymph nodes; **Grade**

III colon cancer has reached one or more lymph nodes outside of the colon; **Grade IV** cancer can spread to several body organs, including liver, membrane lining the abdomen, lung, or ovaries (Hamza *et al* ., 2017).

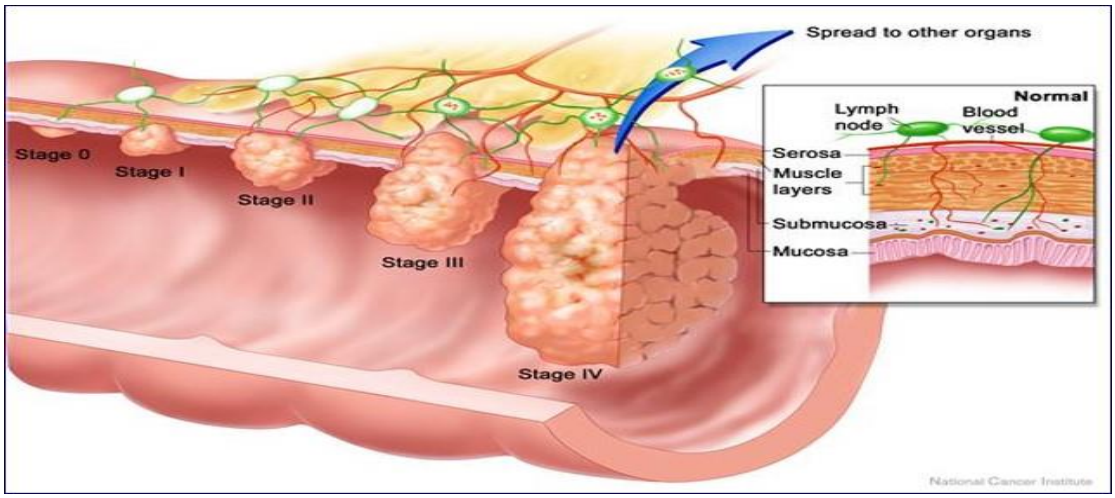


Figure 1.2. Colorectal cancer Grading (Hamza *et al* ., 2017).

1.2.5. Risk Factors

Despite the fact that the etiology of colorectal neoplasms is not fully understood and that the direct causes are still unknown, years of research have allowed us to identify a number of risk factors. The incidence of colorectal cancer is correlated with :

- Changeable factors associated with the environment and lifestyle like diet rich in fat, sugar, alcohol consumption , red meat, overweight, smoking, and a lack of physical exercise (Cronin *et al* , 2018).
- Unchangeable risk factors involving age , family history of adenomatous polyps or colorectal cancer, Past inflammatory bowel disease (Crohn's disease or ulcerative colitis) , risk due to genetic inheritance (FAP and HNPCC) (Muhammad *et al*., 2021).

1.2.6. Symptoms

The majority of the time, CRC develops early without any symptoms, although 15–30% of CRC patients experience symptoms such intestinal bleeding, blockage, or perforation. Most likely, elderly people will experience these types of problems. The most common CRC symptoms include a change in bowel habits that lasts for more than a few days, such as diarrhoea, constipation, or constriction of the stools, blood in the stools, rectal haemorrhage with brilliant red blood, bloating and abdominal pain, exhaustion, and unintentional weight loss. (American Cancer Society, 2022).

1.2.7. Chemotherapy

Today, the chemotherapy encompass both single-agent therapy, which is mostly depend on fluoropyrimidine (5-FU), and multiple-agent regimens, which contain one or more medicines, such as oxaliplatin (OX), irinotecan (IRI), and capecitabine (CAP). Despite the fact that some studies have suggested that first-line single-agent therapy is not inferior to combined regimens in terms of overall survival (OS), the combined therapy regimens (5-FU+OX), (5-FU+IRI) or (CAP+OX), and (CAP+IRI) remain the standard first-line treatment options, while patients with poor performance or at low risk of deterioration are advised by single agent (Koopman *et al*., 2007). Chemotherapy, however, have several drawbacks including systemic toxicity that already exists, an unsatisfactory response rate, unpredictability in innate and acquired resistance, and a lack of tumour-specific selectivity. As a result, significant founding has been committed to the development of innovative strategies to improve or possibly replace current CRC chemotherapy.

1.3. Oxidative Stress-Cancer Relationship

Reactive oxygen species are reactive oxygen-containing molecules thatz take role in cellular electron transport (Harris and Denicola, 2020). Low ROS levels have the ability to regulatory activate signalling pathways, which are necessary for cellular proliferation, differentiation, and metabolic adaptability (Nakamura and Takada, 2021). An equilibrium between the production and removal of ROS is known as redox homeostasis, there are three main sources for the production of ROS : mitochondria, NADPH oxidase, and endoplasmic reticulum (ER). The most common ROS form is the superoxide anion radical (O2•), which quickly changes into hydrogen peroxide (H2O2) by superoxide dismutase (SOD). H2O2 can be converted to the H2O by catalase (CAT), peroxiredoxins (Prx), thioredoxins (Trx), glutaredoxins (Grx), glutathione peroxidases (GPx), and glutathione (GSH) or catalysed in the presence of Fe2+ or Cu^{2+} ions to hydroxyl radical (•OH) via phenton reaction (Juan *et al*., 2021).The amount of ROS in a cells determines their fate as presented in (Fig. 1.3).

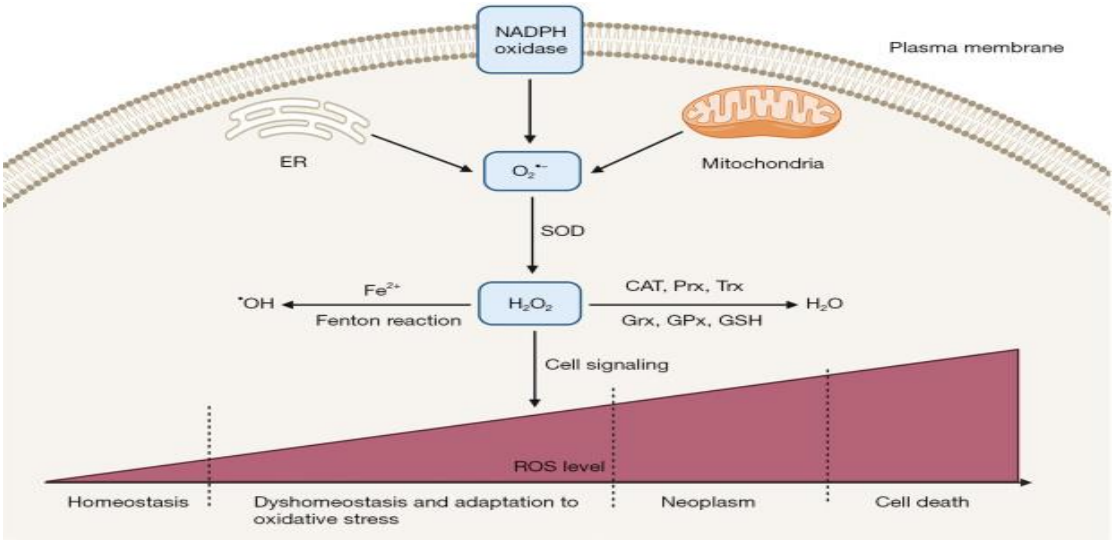


Figure 1.3. The vital role of antioxidants and reactive oxygen species in the development and therapy of cancer (Mareno *et al*., 2016).

The low levels of ROS are required for cell signalling and homeostasis; intermediate levels of ROS cause a loss of homeostasis and increased oxidative stress adaptation, which then causes a cell to develop a neoplasm; and a severe and prolonged increase in ROS results in cell death (Bekhet and Eid, 2021). ROS comprise the radicals hydroxyl (•OH), superoxide anion (O2•), and hydrogen peroxide (H2O2), in previous years, it has been proposed that these species are only pathogenic and toxic (Forman, 2017). However, it has received a lot of attention for its ability to affect a variety of physiological processes, such as cell division and proliferation, signalling, and adaptation to hypoxia (Holmström and Finkel , 2014). In addition to controlling biochemical effectors and aiding in growth factor signal transmission, ROS are essential in the cell's mitochondria-to-nucleus and membrane-to-nucleus signalling pathways (Espinosa-Diez , 2015).

The up-regulation of ROS levels and down-regulation in cellular antioxidant enzymes result in various malignancies via various molecular factors, such as NF-κB and nuclear factor (erythroid-2)-related factor2 (NrF2), which are the two essential transcription factors that regulate response of cells to oxidative stress and inflammation, respectively (Puar *et al*., 2018). NrF2 controls the cellular defence against toxic and oxidative assaults through the regulation of genes included in oxidative stress response and drug (He *et al*., 2020). ROS degrade DNA by nucleobases oxidation like guanine, since the oxidized pair is undergoes to repair, a mistake could occur and result in mutagenesis (Puar *et al*., 2018). The primary redox couples in cells are: peroxiredoxin (Prx)-sulfiredoxin (Srx), thioredoxin (Trx)/thioredoxin disulfide (TrxSS), GSH/glutathione disulfide (GSSG), NADH/NAD+, NADPH/NADP+,

and cysteine (Cys)/cystine (CySS), to regulate the levels of ROS, these enzymes work with thiol redox (Chaiswing , 2018).

Despite the fact that antioxidants have the capacity to neutralize ROS and other free radicals that damage DNA and contribute to cancer, many clinical trials have been unsuccessful in demonstrating an obvious positive effect, on the other hand, numerous epidemiological studies demonstrated a negative relationship between diets high in antioxidants and cancer risk, for instance, a study on 393 pancreatic cancer patients and 353 pancreatic cancer-related deaths examined total antioxidant capacity (TAC), measured through ferric-reducing ability of plasma score, and came to the conclusion that lower TAC increases the risk of developing pancreatic cancer (Zhong *et al* ., 2020).

1.4.Lipid Peroxidation

Lipid peroxidation occur when oxidants like ROS target lipids lead to formation of lipoperoxyl radicals (LOO) and lipid hydroperoxides (LOOH). Cholesterol, glycolipids, and phospholipids are the primary targets of lipid peroxidation. Lipid hydroperoxides break down into reactive aldehydes like malondialdehyde (MDA) and 4-hydroxynoneal (4-HNE) during the lipid peroxidation process. Because they are stable and easily diffuse across cell membranes, lipid-derived aldehydes function as second messengers of free radicals (Su *et al*., 2019).

These aldehydes are important because they can bind to DNA and create adducts, which facilitate mutagenesis effects. Low rates of lipid peroxidation allow cells to activate their antioxidant defence mechanisms or to induce antioxidant transcription factors .When lipid peroxidation rates are high, however, the oxidative damage overwhelms the cell's protective antioxidant processes, resulting in cell damage or cell death

(Ayala *et al*., 2014). Growing evidence suggests that lipid peroxidation products play a role in a range of clinical disorders, including inflammation, atherosclerosis, diabetes, neuropathic pain, and most significantly, carcinogenesis (Su *et al*., 2019).

1.5.Antioxidants

There are two main categories of antioxidants in the body: **endogenous antioxidants** (enzymatic or non-enzymatic), like glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD), among many others, and **exogenous antioxidants**, like vitamins (E and C), polyphenols (such as quercetin), or carotenoids, which can be consumed from food as a main source or supplement in other circumstances (Andre *et al*., 2010). The cellular redox equilibrium is sustained by the synergistic action of both endozsdfdgenous and exogenous antioxidant systems.

Although in vitro studies have demonstrated the cytoprotective effect of dietary antioxidant components like polyphenols or carotenoids against oxidative stress or cell death, they can also show pro oxidant activity, like a number of polyphenols known for their antioxidant activity such as catechin, epicatechin, and quercetin, in high concentrations or in the presence of metal ions (Bouayed , 2010) . The metal ions Cu^{2+} and Fe^{3+} may be affected by antioxidants' strong reducing power, which may increase their capacity to produce hydroxyl radicals in high quantities via the Fenton reaction with peroxides (Bouayed , 2010). The well-known enzymes that directly neutralize ROS include glutathione peroxidases (which reduces H2O2 to 2 molecules of H2O), catalases (which reduce H2O2 to H2O and O2), and SODs (which reduce superoxide to H2O2). GSH, on the other hand, is frequently referred to as the most significant

non-enzymatic antioxidant in cells due to its importance in numerous antioxidant activities (Kennedy *et al*., 2020).

Glutathione , a tripeptide made of glutamate, cysteine, and glycine, present in millimolar concentrations (1–10 mM) throughout cellular compartments. GSH play important role in the maintenance of ROS in 'healthy' levels in numerous ways can't be detoxified enzymatically, which contribute to its detrimental impacts in cells (Kennedy *et al*., 2020). First, GSH has a role in the regeneration of both enzymatic and non-enzymatic antioxidants, like the glutathione peroxidases (GPXs) that detoxify lipid hydroperoxides and H2O2 . GSH is directly utilized in glutathione-S-transferase (GST) reactions in the detoxification of oxidative stress products or xenobiotic (chemical substance present within an organism that are not naturally generated by or thought to be found within that organism like insecticide, heavy metals and drugs) (Koirala *et al*., 2022).

High levels of GSH led to the improvement of the tumour cell and prevention of DNA damage, overall survival, and apoptosis-resistant phenotypes; these conditions can result in resistance to chemotherapies and its depletion is related to the early stages of initiation cell death (Martinez *et al*., 2015). Recent research has emphasized the significance of GSH in crucial signal transduction processes as a regulator of cell differentiation, proliferation, apoptosis, ferroptosis, and immunological function, so molecular alteration in the GSH antioxidant system and disruptions in GSH homeostasis have been linked to initiation, progression, and treatment response of tumours (Kennedy *et al*., 2020). Glutathione, therefore, plays both pathogenic and protective roles as presented in (Fig.1.4) .

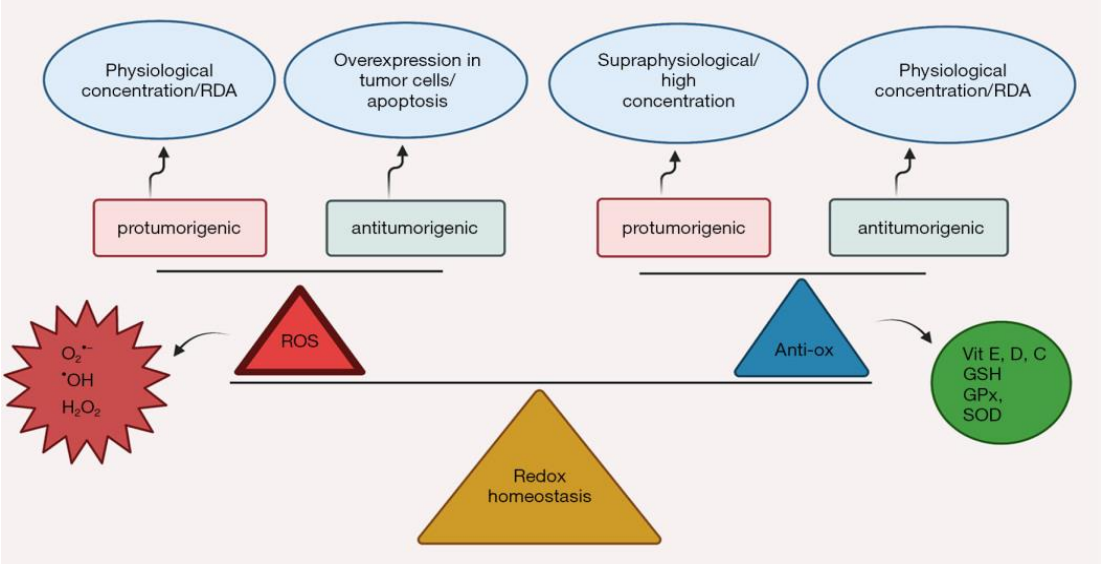


Figure 1.4. The relationship between reactive oxygen species and antioxidants in maintaining redox equilibrium (Bekhet and Eid, 2021). RDA: recommended dilatory allowance.

Maintaining cellular redox homeostasis is vital for healthy biological system, as a result , therapeutic modalities for cancer including antioxidants and ROS management should be used at certain doses to target specific redox pathways involved in cancer progression without disrupting the overall redox balance in normal cells.

1.6. Apoptosis

Apoptosis is a major type of programmed cell death which occurred in multicellular organisms to maintain tissue homeostasis and act as a defensive strategy to remove infected, damaged or mutated cells (Bedoui *et al* ., 2020). Apoptosis can be triggered via two principle pathways, the mitochondrial pathway results from oxidative stress, DNA damage and death receptor-mediated pathway that results from the extracellular signals (death-inducing signals by other cells) **(**Carneiro and El-Deiry, 2020).

This mechanism, which primarily relies on caspase cascades to carry out cell death, results in the proteolytic cleavage of thousands of target proteins inside the cells, including cytoskeletal and nuclear proteins. The apoptotic cells then undergo a series of morphological and biochemical changes that lead to their recognition by macrophages and cell phagocytosis (Neophytou *et al*., 2021). The B-cell lymphoma-2 (Bcl-2) family of proteins is also well known for controlling the cellular program of apoptosis through mitochondrial outer membrane permeabilization, which acts as the crucial decision-point at which cells commit to death, demonstrating their critical role in preventing of cancer (Carneiro and El-Deiry, 2020). Impaired apoptosis can come from deficiencies at any point along the apoptotic pathways as well as dysfunction of the regulatory mechanism, which leads to the development of cancer.

Caspases, which are cysteinyl, aspartate-specific proteases, are crucial for the promotion and execution of both intrinsic and extrinsic apoptotic pathways (Eskandari and Eaves, 2022). They are divided in to initiators and executors caspases. Initiator caspases (casp-2, -8, -9, and -10) are expressed as inactive pro-enzymes which are activated in the early stages of apoptosis and prompt a series of events to trigger the apoptotic process. The next phases of the apoptotic process include the activation of executor caspases (casp-3, -6, and -7), which are responsible of cleaving components of cells. Once initiator caspases cleave downstream caspases, the apoptotic signalling pathway is amplified, starting a proteolytic cascade (Neophytou *et al*., 2021).

In extrinsic pathway, the extrinsic factors such as steroid hormones in addition to various ligands of the tumour necrosis factor (TNF) receptors superfamily (e.g., Fas ligand (FASL), Targeting TNF-related apoptosis-inducing ligand (TRAIL), and TNF-α) (Aram *et al*., 2017).

Extracellular-mediated ligand linked to one of these death receptors lead caspase-8 to link to the Fas-associated through death domain (FADD) adaptor protein, which form a death-inducing signalling complex (DISC). Caspase-8 recruitment to DISC makes it easier for it to be activated through self-cleavage. After that, cleaved caspase-8 triggers the activation of downstream effector caspases, whose actions subsequently cause the last phase of apoptotic death (Julien and Wells, 2017).

The intrinsic or mitochondrial pathway initiates by a variety of stimuli leading to apoptosis involving viral infections, hypoxia, hyperthermia, oxidative stress, and intrinsically detected stress signals brought on by exposure to toxic chemicals or radiation , as happens , for instance , in cancer individuals receiving chemotherapy or radiotherapy (Julien and Wells, 2017). Such pro-apoptotic cellular stresses lead to the permeabilization of the mitochondrial outer membrane and the release of apoptogenic substances like cytochrome c into the cytosol from the mitochondrial intermembrane gapl. Subsequently, formation of an apoptosomal complex (containing cytochrome c/apoptosis protease activating factor 1(Apaf-1)/caspase-9) prompts the stimulation of effector caspases (caspase-3/7) (Julien and Wells, 2017).

1.7. Doxorubicin

Doxorubicin is a DNA intercalating medication that used to manage a variety of cancers, such as leukaemia, breast cancer, and cervical cancer. It is isolated from a mutant strain of *Streptomyces peucetius* and its a member of anthracycline family of antibiotics with similar structure daunorubicin, epirubicin, and idarubicin (van der Zanden, 2021). The anti-cancer mechanism of doxorubicin involves intercalating with DNA and destruction of topoisomerase II enzyme, ultimately

resulting in cell death by stopping the biological process of DNA replication (van der Zanden, 2021). Another established anti-cancer mechanism for doxorubicin involves its capacity to cause oxidative stress through the generation of reactive oxygen species, where intracellular oxidoreductase reacts with doxorubicin metabolites to create semiquinone radical and ROS (Aniogo *et al*., 2017).

Cardiotoxicity being the most well-known and most comprehensively studied adverse effect related to doxorubicin (Sheibani *et al*., 2022). Also, alopecia, nephrotoxicity, and haematological suppression limit the usage of doxorubicin, despite the fact that it is powerful in the treatment of cancer (Kullenberg *et al*., 2021). The failure of treatment with mono-administration of doxorubicin is mostly attributable to the severe adverse effects , which can significantly impact the well-being and life quality of cancer patients, leading to dose modifications and interruption or discontinuation of anti-cancer treatments in severe cases (Hao Jiang *et al*., 2023). Despite the fact that doxorubicin treatment has demonstrated major potential in delaying the progression of disease over the years, clinically utilized doses exhibit insufficient anti-tumour activity, while larger doses frequently cause systemic toxicity in patients (Kullenberg *et al*., 2021).

1.8. Carboplatin

Platinum-based drugs like carboplatin, analogue of cisplatin, continue to be among the most widely used chemotherapeutics because of their broad-spectrum anticancer actions, low cost, and unique mechanism against cancer (Qi L *et al*., 2019). The anticancer effectiveness of platinum medicines is mostly due to covalent binding of the platinum core to DNA . There are four steps of this process, cell

absorption, aquation (hydrolysis), DNA platinum adduct creation, and cell death induction, to be more precise, carboplatin enters the cell by passive diffusion and undergoes hydrolysis to take a form that interacts with nucleophilic purine bases in the DNA strand, resulting in major intra-strand cross linkages and minor inter-strand cross linkages. Subsequently, cross linkages inhibit DNA replication, causing mistakes in transcription, these platinum DNA adducts have the ability to induce apoptosis by stimulation of cellular processing (Raudenska *et al*., 2019).

Here, briefly go over how DNA damage results in apoptosis, the platinum−DNA adducts can be identified by proteins like mismatch repair proteins (MMR) and non-histone chromosomal high-mobility group proteins 1 and 2 (HMG1 and HMG2) . HMG1 and HMG2 can stimulate tumour suppressor p53 and simplify p53 binding to DNA. Following that, many target genes are activated, including p21 (involved with cell cycle progression) and B cell lymphoma-2 (Bcl-2)-associated X protein (Bax; pro apoptotic gene) (Yu C *et al*., 2020). Activation of p21 can cause cell cycle arrest for DNA repair while increased expression of Bax can result in the release of cytochrome c from mitochondria, subsequent cleavage of procaspase 9, activation of caspases 3, 6, and 7, and ultimately apoptotic cell death , so they play a significant part in the management of a number of solid tumours (Yu C *et al*., 2020).

Nepherotoxicity , ototoxicity, and numerous side effects, including emesis, nausea and myelosupression (decrease in bone marrow activity that result in reduced production of blood cells), as well as its low tolerance for long-term use are all frequent side effects that occur during or after carboplatin treatment (Wlodarczyk *et al*., 2018).

1.9. Quercetin

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is the most crucial component in the nightshade found in a variety of fruits and vegetables (Vafadar *et al*., 2020). Nightshade is the entire grass of *Solanum nigrum L.* in the *Solanaceae* family. Several studies indicated that it has an anti-tumour activity and its commonly used to treat different types of cancer (Dong *et al*., 2021). According to literature search findings, nightshade contain multiple active components including solanine, solamargine, solaoiacid, solasonine, solanine, solasodine, degalactotigonin, quercetin, and diosgenin, can cause anti-tumour activity (Nawab *et al*., 2012). Quercetin is consumed as a dietary supplement and has the potential to fight cancer when consumed regularly, studies in vivo and in vitro have suggested that quercetin ingestion could have biological benefits, such as antioxidant, anticancer, and anti-inflammatory properties, at an acceptable dosage (Lotfi *et al* ., 2023). Many studies have been demonstrated that quercetin has valuable antitumor effects against wide range of cancers included breast, lung, gastric, ovarian, colorectal and hepatic cancer (Yang *et al*,. 2019; Hisaka *et al*., 2020). Anticancer properties of quercetin have been attributed to a number of mechanisms including apoptosis, angiogenesis inhibition, P-glycoprotein channel blocking, reduced oncogene expression, and signalling pathways regulation (Zhou *et al*., 2017).

1.9.1. Bioavailability of Quercetin

Quercetin is categorized as flavonol, one of the six subclass of flavonoid compounds which composed of a heterocyclic pyrone ring that connect the two benzene rings to form the core nucleus (Fig.1.5).

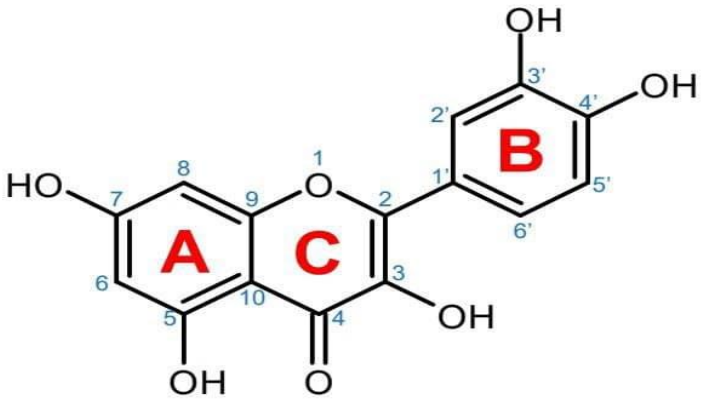


Figure 1.5. Chemical structure of quercetin (Wang *et al*., 2022) .

Quercetin naturally found as either conjugated to the sugar (glycoside) or without conjugation to the sugur (aglycone), the two forms are biologically active (Alrawaiq and Abdullah, 2014). Quercetin has low bioavailability, hydrophobic nature, poor solubility and poor permeability make it difficult for absorption and availability (Azeem *et al*., 2023). Bioavailability of quercetin is greatly increased when it is consumed as an fundamental dietary component in the form of fruits and vegetables. Pharmacokinetic research reveals that the peak concentration of quercetin in blood after food uptake reaches to a peak of around 10 μM, most of the studies which have shown the antitumor activity of quercetin were performed with concentrations ranging from 25 μM to 200 μM (Jeong *et al*., 2009).

Specific transferase enzymes in enterocytes subject quercetin to a number of enzymatic processes, including methylation, hydrolysis, sulfonylation, and glucuronidation (Brito *et al*., 2015). The primary quercetin-derived circulating molecules in plasma, quercetin-3-glucuronide and quercetin-3-sulfate, are generated via further conjugation events after being transported into the intestinal lumen and then the liver (Alrawaiq and Abdullah, 2014).

1.9.2. Quercetin and Oxidative Stress

Cancer cells frequently produce higher than normal levels of ROS as a result of defect in intracellular signalling networks, which causes oxidative stress and make the cells sensitive to pro-oxidant agents that disrupt redox homeostasis (Gao *et al*, 2017). Quercetin is widely known for its antioxidant and cell-protective properties, however, in B16F10 melanoma cells and many other cancer cells, quercetin also exhibits strong pro-oxidant actions and raises the cellular levels of ROS to lethal levels (Rafiq *et al*., 2015); as a result, quercetin should be utilized therapeutically and to kill cancer cells selectively.

Quercetin have antioxidant and pro-oxidant behaviours, so the switching between them is determined by the amount of intracellular reduced glutathione (GSH) as presented in (Fig. 1.6).

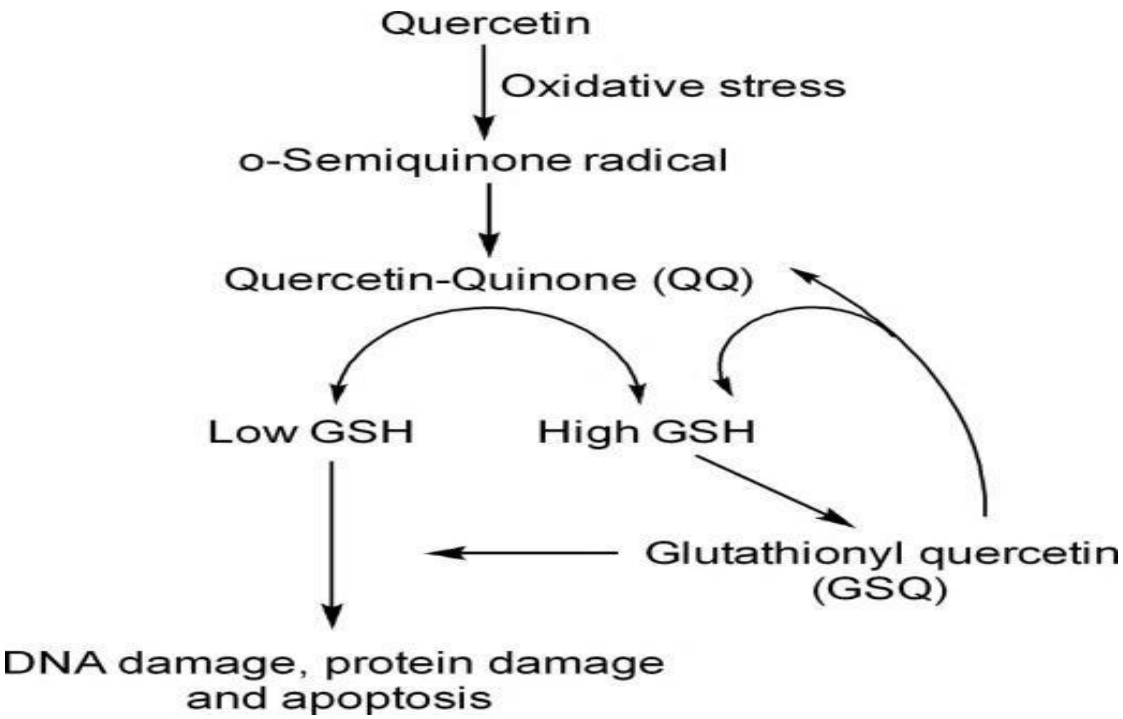


Figure 1.6. The molecular mechanism of switching between pro-oxidant and antioxidant effects of quercetin (Rather and Bhagat, 2020).

Quercetin reacts with hydrogen peroxide (H2O2) in the presence of enzyme peroxidase during oxidative stress to produce o-semiquinone radical which are instantly oxidized to form quercetin–quinone products (QQ). Quercetin-quinone products are cytotoxic and prompt cell death during their interaction with protein thiols and DNA (Biswas *et al*., 2022). When GSH levels are high, QQ preferentially combines with GSH to create glutathionylquercetin (GSQ) adducts such as 8-GSQ and 6-GSQ, which prevent cell death. This reaction is reversible so permit dissociation of GSQ into GSH and QQ (Xu *et al*., 2019) .

However, when GSH levels are low, QQ reacts with protein thiols, which results in cellular damage and apoptosis. Similar to this, prolonged exposure to high quercetin concentrations results in a significant drop in GSH levels, reducing quercetin's capacity to scavenge ROS, quercetin's pro-oxidant action outweighs its antioxidant activity , causing DNA damage and cell death (Biswas *et al*., 2022).

1.9.3. Direct Pro- apoptotic Effects of Quercetin

One of the most important characteristic of quercetin is its pro-apoptotic action , which is brought about by up regulating pro-apoptotic molecules including P53, BAX, caspase-3, and caspase-9 or activating the mitochondrial apoptosis pathway, or down regulating anti apoptotic proteins (Bcl-2 family proteins) (Asgharian *et al*., 2022). Quercetin can prompt activation intrinsic and extrinsic pathways of apoptosis. In intrinsic pathway , quercetin causes depolarization of mitochondrial membrane potential by elevating the intracellular levels of ROS and Ca^{+2} lead to release of cytochrome c and activation of caspase-3 and -9 (Teekaraman *et al*., 2019). Alternatively, quercetin cause depolarization of mitochondrial membrane potential and subsequently induction of

apoptosis by translocation of Bax from cytosol into mitochondria membrane and concurrently obstructing the phosphatidylinositol 3-kinase PI3K/ protein kinase B (Akt) and extracellular signal-regulated kinases (ERK) signals (intracellular signal transduction pathways that promote metabolism, proliferation, cell survival, growth and angiogenesis in response to extracellular signals), normally Bcl-2 and Bcl-XL proteins inhibit Bax penetration into mitochondrial membrane (Asgharian *et al*., 2022) (Fig.1.7). Quercetin induce extrinsic pathway by stimulation of TRIAL via promoting destruction of survivin (an inhibitor apoptosis protein) and activation of caspase 8 (Rafiq *et al*., 2015).

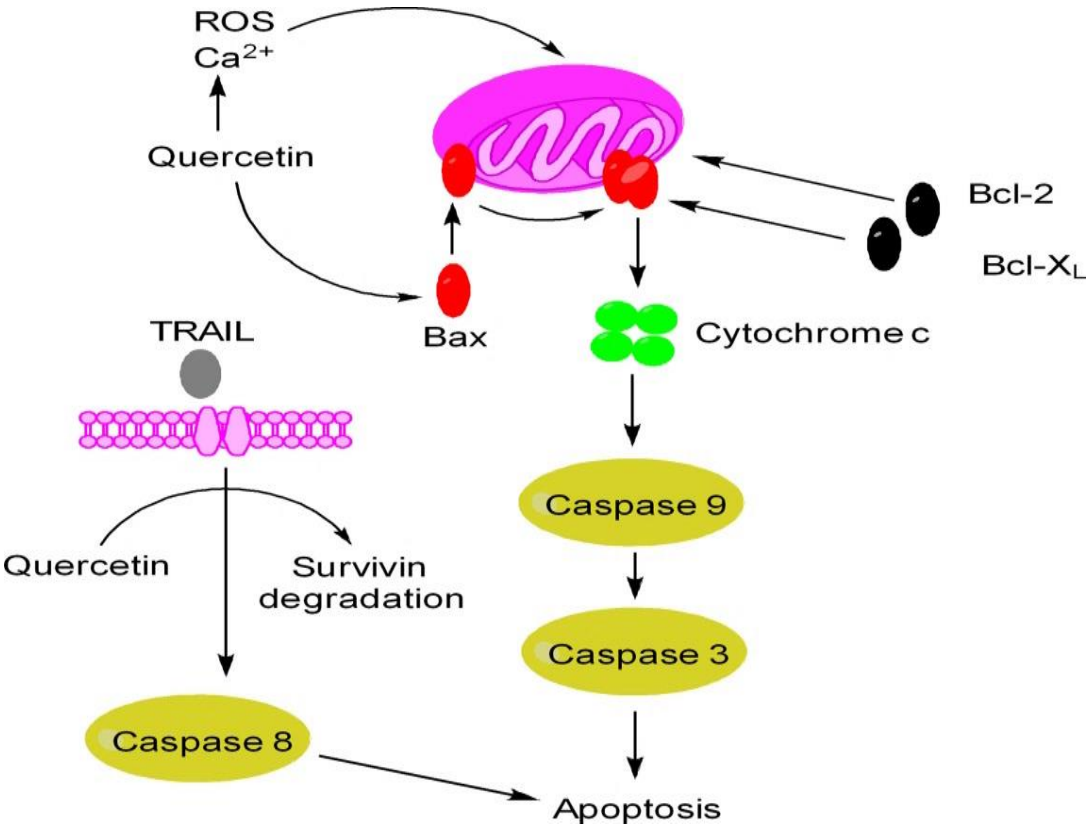


Figure 1.7. Molecular mechanism of quercetin-induced apoptosis (Rather and Bhagat, 2020).

1.9.4. Anti-inflammatory Effect of Quercetin

According to research, quercetin has a potent anti-inflammatory properties that work primarily by reducing cytokine production, cyclooxygenase and lipoxygenase expression, and preserving the stability of mast cells (Carullo *et al*., 2017). Additionally, it can lessen the synthesis of pro-inflammatory cytokines like tumour necrosis factor (TNF), interleukin (IL)-1, and IL-6. TNF-α ligand of the TNF family, as mentioned above, primarily generated by macrophages and tumour cells. Because its activity considered one of the key forces influencing the development of CRC, quercetin's ability to block TNF-α is crucial in the fight against CRC (Li *et al*., 2019).

1.10.Cell Culture

Cell culture is a complicated process that include removing cells from their in vivo (natural environment) habitats and allowing them to grow in an artificial, controlled environment (in vitro). The cells may be isolated from a previously identified cell line or strain or they may be directly extracted from the tissue and disaggregated before culturing using enzymatic or mechanical techniques (Hudu *et al*., 2016) .

1.10.2. Types of Cell Cultures

Depending on how often cells divided, Primary cell culture and cell lines are two different types of cell culture:

1. Primary cells

Primary cells are isolated directly from tissue and cultivated in vitro under ideal conditions with the proper temperature, medium, CO2 level, and static or rocking method. Since the cells are directly derivate from tissue, they are the most accurate representations of the original tissues

and have a high degree of physiologic resemblance to in vivo conditions (Chongbei Zhao, 2023). It has been a popular method for researching aging, metabolic signalling, and carrying out pharmacological investigations in the biochemistry field because they often exhibit the primary tissue phenotype from whence they were taken. Primary cells have various uses in cellular biology research, including cancer research, drug screening, gene engineering, vaccinations, gene therapy, and virology. It is important to note that primary cells have a finite lifespan before they undergo replicative senescence, which can be a significant drawback in a variety of circumstances (Chongbei Zhao, 2023).

2. Secondary Cells (cell line)

Cell lines can be broadly categorised into three groups: (i) Finite cell lines, (ii) Continuous cell lines, also called as immortalized or indefinite cell lines and (iii) Stem cell lines (Pamies *et al*., 2022). **Finite cell lines** often come from primary cultures and develop slowly. As a result, they can only be grown in culture for a certain number of cell generations before they eventually experience aging and senescence, which is a process that characterized by the loss of the usual cell shape and accumulation of cytoplasmic lipids. It's significant to note that, finite cell lines are contact-inhibited and they are arrested in the G0, G1, or G2 phase after formation monolayers (Weiskirchen *et al*., 2023).

Controversy, **continuous cell lines** are always isolated from transformed or malignant cells and divide quickly and reach significantly higher cell densities in culture than finite cell lines. Sometimes, continuous cell lines show heteroploidy (abnormal number of chromosomes in the cells). They frequently proliferate at lower serum concentrations, do not exhibit contact -inhibited, and may form several

layers. **Stem cells** are an undifferentiated or only partially differentiated pluripotent cells, derived from multicellular organisms. These cells can be multiplied indefinitely to create more cells of the same kind, or they can be stimulated under the correct circumstances to create new cells with specialized functions. (Weiskirchen *et al*., 2023).

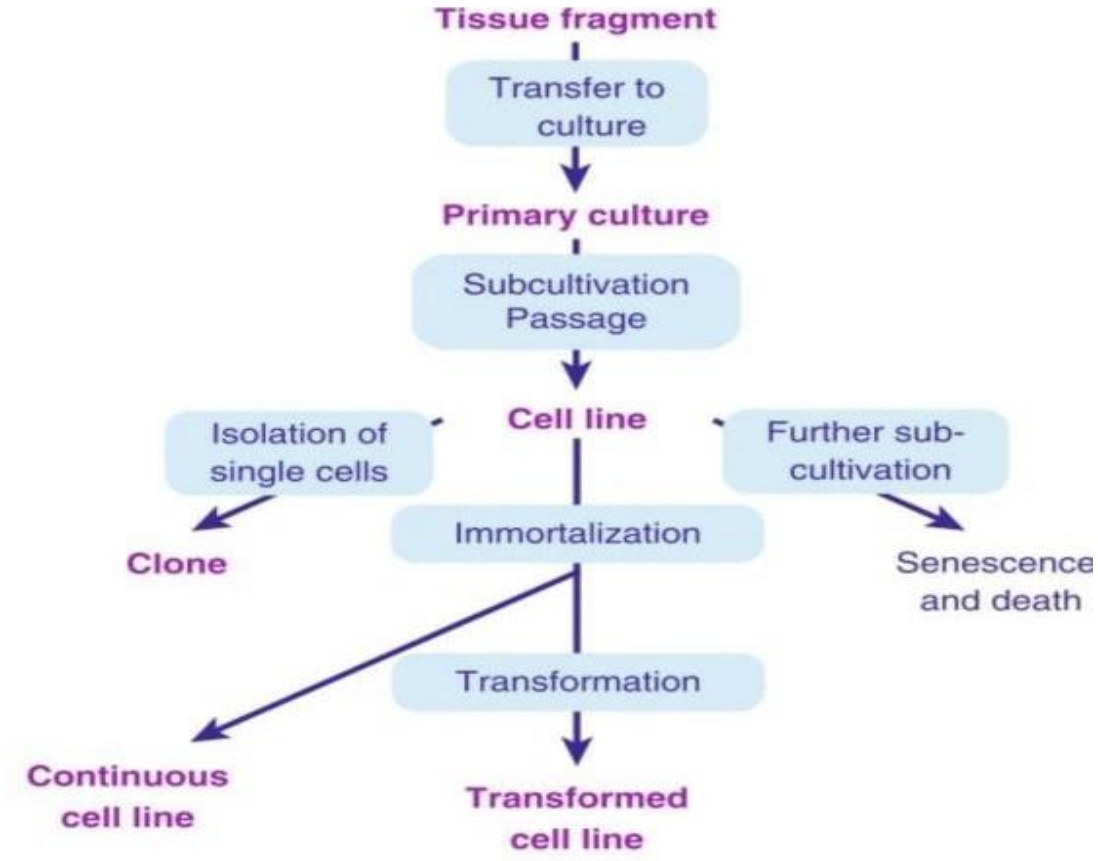


Figure 1.8. Relation between primary cells, transformed, immortalized cell lines, and clones **(Verhoeckx *et al*., 2015).**

1.10.3. Cells Morphology

Regularly evaluating the morphology (form and appearance) of the cells in culture is essential for the successful experiment of cell culture. Checking the cells each time under a microscope and by eyes , they are handled, make it easy to recognise and prevent any early indications of contamination before it transferred to other cultures, in addition to

confirm the cells health status. Granularity around the nucleus and cell detachment from the substrate are signs of cell deterioration. These can be happen due to a various of causes, like contamination of medium, cell line senescence, or toxic components present in the medium, they can also easily indicate that the culture need to change the medium (Cell Culture Basics Handbook, 2020).

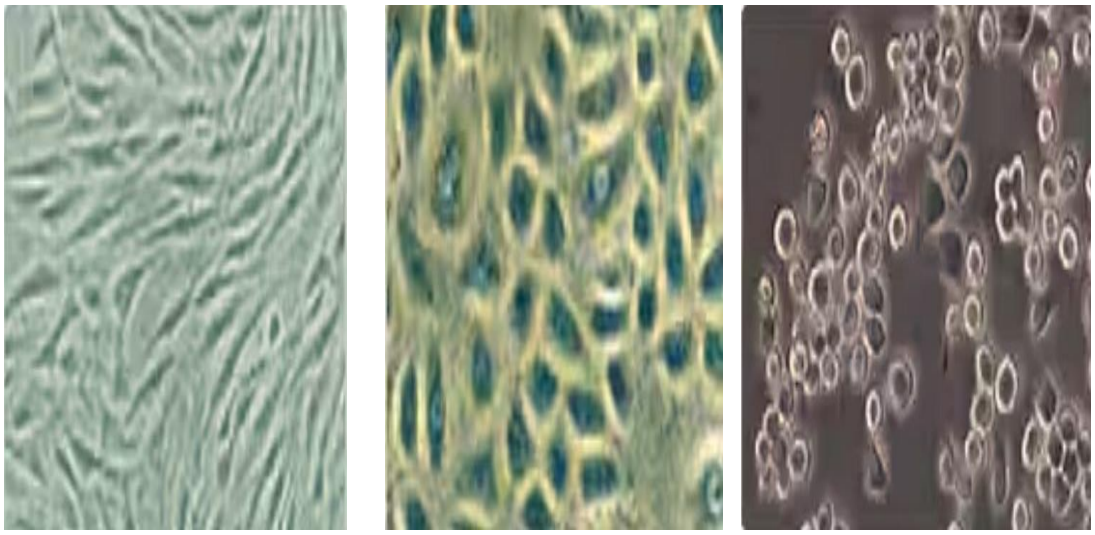
1.10.4. Shapes of Cells in Culture

Cells in culture divided in to the three main classifications depending on their structure and appearance (Picture 1.4) .

1. Fibroblastic (or fibroblast-like) cells appear bipolar or multipolar with elongated shapes, and grow connected to a substrate.

2. Epithelial-like cells have a polygonal shape with more regular dimensions, and they develop in distinct patches attached to a substrate.

3. Lymphoblast-like cells have spherical shape, typically develop in suspension without adhering to a surface (Oyeleye *et al*., 2016).



Fibroblastic-like cell Epithelial-like cells lymphoblast-like cell

Figure 1.9. Shapes of cells in culture (Oyeleye *et al*., 2016).

1.10.5. Applications of Cell Line

From basic to advanced research, animal cell culture has been used in a variety of contexts. It has served as a model system for a variety of research projects:

1. Very good modelling systems for studying normal cell physiology and biochemistry (e.g. metabolic studies, aging).
2. Test of the toxicity to study how new drugs affect.
3. Gene therapy by using cells that carry functional gene in replacement of non-functional gene .
4. The characteristics of cancer cells and how they are affected by radiation, viruses, and different substances.
5. Production of pharmaceutical drugs, monoclonal antibodies (mABs), and vaccines .
6. Cultivation of viruses that utilized in vaccine manufacturing (e.g., chicken pox, polio, rabies, hepatitis B, and measles. Today, mammalian cell culture is necessary for production biological treatments such as hormones, antibodies, clotting factors, and vaccines. (Verma *et al*., 2020).

1.10.6. Advantages and Disadvantages of Cell Culture

❖ Advantages

1. Physiochemical and physiological state: The role and impact of pH, temperature, O2/CO2 concentration, and osmotic pressure of the culture media can be varied to evaluate their impacts on the cell culture .
2. Cytotoxic assay: It is possible to study how different substances or medications affect particular cell types, such as liver cells.

3. Homogenous cultures: These cultures assist in the study of cell biology and cellular genesis.
4. Important biological information from large-scale cell cultures: In large-scale cultures, genetically engineered cells can produce large amounts of a particular proteins
5. Consistency in results: The ability to get consistent results while using a single type or clonal population.
6. Cell type identification is possible through the use of karyotyping or by looking for markers like molecules.
7. Ethics: It is possible to avoid moral, legal, and ethical complications while using animals in research. (Verma *et al.*, 2020).

❖ Disadvantages

1. Cost and skill: This is a specialist method that needs aseptic settings, skilled workers, and costly equipment.
2. Dedifferentiation: Cell features can alter following a period of continuous cell development in cultures, resulting in differentiated qualities compared to the original strain.
3. Low production: The extremely low production levels of mAB (monoclonal antibodies) and recombinant protein, along with the subsequent downstream processing necessary to recover pure products, greatly raise costs.
4. Contamination: Viral infections and mycoplasma are extremely infectious and difficult to detect.
5. Instability: In continuous cell lines, aneuploidy chromosomal composition results in instability. Additionally, this system cannot take the place of a complex live animal for studying the effects of

vaccines, poisons, or the reactions of chemicals. (Verma *et al*., 2020).

1.11. SW 480 Cell Line

The colonic cancer SW480 cell line originates from primary tumour of an adenocarcinoma of the colon in a 50 year old male (Siekmann *et al*., 2019).

1.12. Vero Cells

The establishment of Vero cells as an experimental tool for studies ranging from virology to toxicology started in 1962 with the extraction of the cells from the Female African Green Monkey kidney by Yasumura and Kawakita. It was later discovered the Vero cell line is a continuous cell line, meaning that it can be passaged over a significant period of time without losing its growth characteristics. Importantly, this is maintained without acquisition of tumorigenic functions as compared to other primary cell lines with limited passage numbers, allowing for several sub lines to be derived and cell banking (Marie-Angélique *et al*., 2022).

Chapter Two

Materials and Methods

2. Materials and Methods

The study periods was performed from December 2022 to April 2023 in the tissue culture laboratory at the College of Medicine\ University of Babylon.

2.1. Materials

2.1.1. Chemical Materials

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

Table 2.1. List of chemical materials

Chemicals	Company	Country
Alcohol (ethanol 70%)	Aljoud	Iraq
Carboplatin	Aprazer	India
De-ionized water	Promega	USA
Dimethyl sulfoxide (DMSO)	Roth	Germany
Doxorubicin	Pfizer	USA
Fetal bovine serum (FBS)	Gibco	UK
Liquid nitrogen	Clever	USA
Memorial Institute- 1640 (RPMI-1640) powder medium	Gibco	UK
Penicillin-Streptomycin solution	Sigma Aldrich	Germany
Phosphate buffer saline(PBS) tablet	Gibco	UK
Quercetin	Sigma Aldrich	Germany
Sodium bicarbonate	BDH	England

powder		
Trypsin-Ethylenediaminetetraacetic acid (EDTA) powder	Gibco	UK
MTT(3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) dye powder	Roth	Germany

2.1.2. Equipment

The equipment that used in the current study are listed in (Table 2.2) with their suppliers.

Table 2.2. List of Equipment

Equipment	Company	Country
Autoclave	Jeiotech	Korea
Automatic micropipettes (different sizes)	Human	Germany
Cell culture flask (25ml)	SPL	Korea
Cell culture plate (96-wells)	SPL	Korea
Deep freezer -80°C	Labtech	Korea
Disposable face masks	Ky	China
Disposable gloves	Fe	Malaysia

Distiller	ROWA	Germany
Double distillation water stills	GFL	Germany
Electric oven	Memmert	Germany
ELISA Reader	Human	Germany
Eppendorf tube	Eppendorf	Germany
Freezer -20 °C	Mettler	Switzerland
Incubator	Memmert	Germany
Inverted microscope	T.C Meiji tech	Japan
Laminar air flow cabinet	Labtech	Korea
Liquid nitrogen container GT38	Air Liquide	France
Magnatic stirrer	Labinco	Netherland
Microcenterifuge	Hettich	Germany
Millipore filter(0.45,0.22)	BiofilJET	Australia
Paraffin film	Analab	Ileland
Refrigerator	Arcelic	Turkey
Sensitive balance	Labtech	Korea
Sterile freezing vial (1.5 ml)	Biofil	Australia
Ultrasonic	Binder	Germany
Water bath	Minilyotrap	England

2.1.3. Assay kits

The assay kits that used in this study are in the table (2.3)

Table 2.3. List of kits used in the study.

Kits	Company	Country
ELISA kit GSH	Elabscience	USA
MDA kit	Bilişim Destek Hizmetleri	Turkey
ELISA kit Caspase 3	Elabscience	USA
ELISA kit TNF-α	Elabscience	USA

2.1.4. Preparation of Chemicals

2.1.4.1. Phosphate Buffer Saline (PBS)

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

2.1.4.2. Trypsin-(EDTA) Solution

According to the Gibco manufacturer's manual, 1.24 gm of trypsin-EDTA powder was dissolved in 90 ml of DDW and mixed by stirring at room temperature. The solution's pH was then adjusted to 7.2 and the volume was then completed to 100 ml. The solution is sterilized by utilizing filters with a pore size of 0.45 and 0.22 μmillipore, respectively. The material was kept at (-20˚C) while it used.

2.1.5. Preparation of Tissue Culture Medium

2.1.5.1. Preparation of Serum-Free Medium

This medium was created by Roswell Park Memorial Institute, hence the name RPMI. Leukocytes, both healthy and malignant, have been cultured in RPMI-1640 media, which also aided in the growth of numerous other cell types. According to the Gibco product manual, liquid RPMI-1640 medium was prepared from powdered RPMI-1640 medium as follows: 10.43 g of the powdered RPMI-1640 medium was dissolved in over 900 ml of DDW in a volumetric flask. Added 1% penicillin-Strep solution and 2 gm of sodium bicarbonate powder as needed while stirring continuously, the volume was brought to one litter

by adding DDW, and the medium's pH was then adjusted to 7.4. Filters with a pore size of 0.45 and 0.22 μmillipore were used /or sterilizing. The medium should be distributed aseptically into sterile containers and kept at (2–8 ˚C) until use (Mesut and Ozsoy, 2017).

2.1.5.2. Preparation of Serum-Medium

Preparation of Serum-medium was prepared as mentioned in (2.1.5.1) with the addition of 10% FBS.

2.1.5.3.Preparation of Freezing Medium

The following ingredients were mixed to make 10 ml of freezing media: six ml of serum-free medium, three ml of FBS, and one ml of DMSO were gradually added to the mixture. The solution was kept at about (-20 ˚C) until used (Barrette, 2016).

2.1.6. Preparation of SW480 and Vero Cell Lines

SW480 colorectal cancer and Vero (normal) cell lines in the frozen vial were obtained from the Tissue Culture Laboratory in the College of Medicine/ University of Babylon. Its growth was maintained in a 25 ml culture flask, with a complete growth medium containing 10% FBS and incubated at 37˚C

2.1.6.1. Thawing of Cells

1. The datasheet on the cell line was read to determine the specific requirements for our cell line, then we labelled the cell culture flask with the name of the cell line.
2. A vials of cells were brought on, which have been stored in liquid nitrogen.

3. Hold a tissue soaked in 70% alcohol around the cap of the frozen vial in a microbiological safety cabinet and turn the cap a quarter turn to release any residual liquid nitrogen that may be trapped, and tighten the cap. 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 (2-3 minutes).
4. Tissue soaked with 70% of alcohol was used to wipe the vial with the prior opening.
5. The entire content of the vial pipette into a tissue culture flask to achieve cell seeding, then slowly a 5ml pre-warmed medium was added, which has already been supplemented with the appropriate components.
6. Incubated to the recommended temperature and CO2 level on the datasheet.
7. After 24 hours examined the cells microscopically, the media changed as needed. (Mesut and Ozsoy, 2017).

2.1.6.2. Harvesting and Sub-Culturing of Cells

Harvesting is a technique using the proteolytic enzyme (trypsin) to detach and disaggregate the adherent monolayer cells from the culture flask base, it is done when the cells need to be harvested for cell counting and cell line subculture. That procedure was carried out as follows:

1. When the cells growth reaches a monolayer (in the exponential phase), the medium was aspirated and discarded.
2. Then the cells were washed with 3ml of warm PBS solution.
3. About 1-2 ml of warm trypsin EDTA solution was added to cover the monolayer and with gentle shaking of the flask culture 4-5 times to immerse the cells.

4. The flask was returned to the incubator at 37˚C to allow the cells to detach from the inside surface of the flask (the length of time depends on the cell line, but usually this will occur within 2–10 minutes). To avoid over trypsinization which can severely damage the cells, it is essential to check them every few minutes.
5. The cells were examined using an inverted microscope to ensure that all the cells are detached and in suspension as soon as the cells have detached (the flask may require some gentle taps), some culture media with 5-10 % FBS added to the flask (FBS will inactivate the trypsin).
6. The cell suspension were transferred into two new flasks, these flasks should then be supplemented to the required volume with the culture media. The cells left for recovery and settling in the incubator at 37°C overnight. This process was repeated as demanded by the growth characteristics of the cell line (Phelan and May 2017).

2.1.6.3. Freezing of Cells

Cryopreservation is a method by which cells are frozen, keeping their viability until defrosted, months, or years later. Cells are cryopreserved to minimize the genetic change and avoid loss by contamination. Cryopreserve cells are best when they are at their optimum growth rate. Cell lines source were kept frozen at (-180 ˚C to -196˚C) in a nitrogen tank by the following protocol:

1. Labelled cryovials with date, researcher name, number of passages, and type of cell.
2. Then the cell culture media removed from the tissue culture flask, washed the cells in PBS, added enough trypsin to cover the cells, and incubate in a 37˚C incubator for about 2 min, and re suspended

in the cell culture media and transferred to 15 ml sterile plastic centrifuge tube. Centrifugation was done at room temperatures of 1000 rpm for 5 minutes. Then, prepared freezing media, as shown in (2.1.5.3).

3. The supernatant was decanted and the cell pellet was re-suspended with 1 ml of the freezing media and transferred into 1.5 ml sterile freezing vial and secure the lids. Cells should not be in freezing media for more than 10 minutes at room temperature.
4. These vials were placed in the vapour phase of a liquid nitrogen container, which is equivalent to a temperature of -80˚C for a minimum of three hours (or overnight). Nearly after 24 h we removed the cryovials from the vapour phase of the liquid nitrogen container and transfer them to the liquid phase for long term storage (Whaley *et al* ., 2021).

2.1.6.4. Maintenance of Cell Culture

Cells were routinely checked under an inverted microscope for any contamination and the cells were given new medium (RPMI) every 2 to 3 days based on colour changes. The cells were maintained in supplemented medium with 10 % serum and kept at 37 ºC in an incubator. After the cells have achieved more than 80 % confluence, they were sub cultured.

2.2. Methods

2.2.1. Experimental Design

SW480 cancer cells were divided into the following six groups:

Group I: Control group untreated cells.

Group II: Cells were treated with quercetin.

Group III: Cells were treated with doxorubicin.

Group IV: Cells were treated with carboplatin;

Group V: Cells were treated with combination of quercetin, at low concentrations, with IC50 value of doxorubicin.

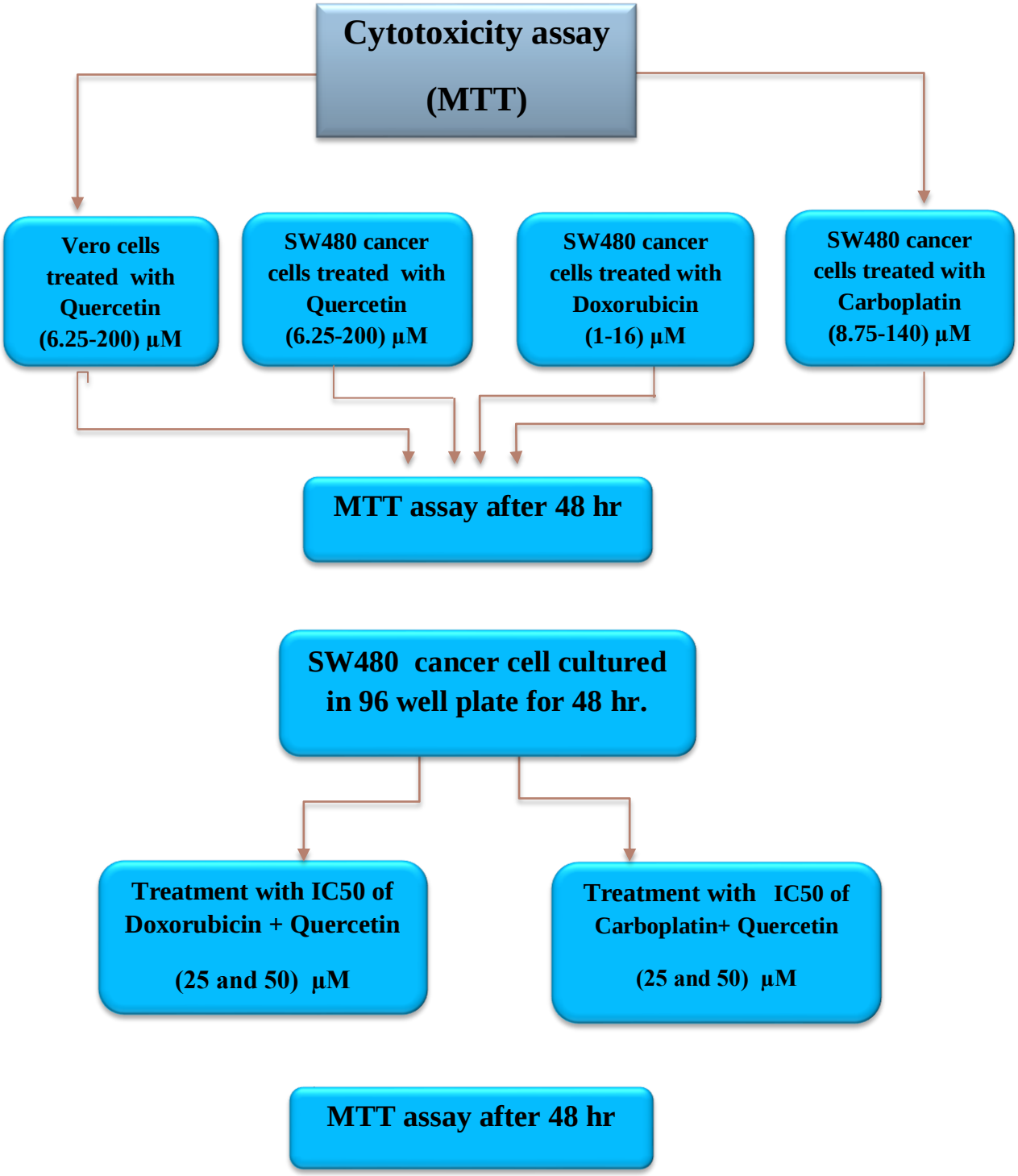
Group VI: Cells were treated with combination of quercetin, at low concentrations, with IC50 value of carboplatin.

Group VII: Vero cells were treated with quercetin.

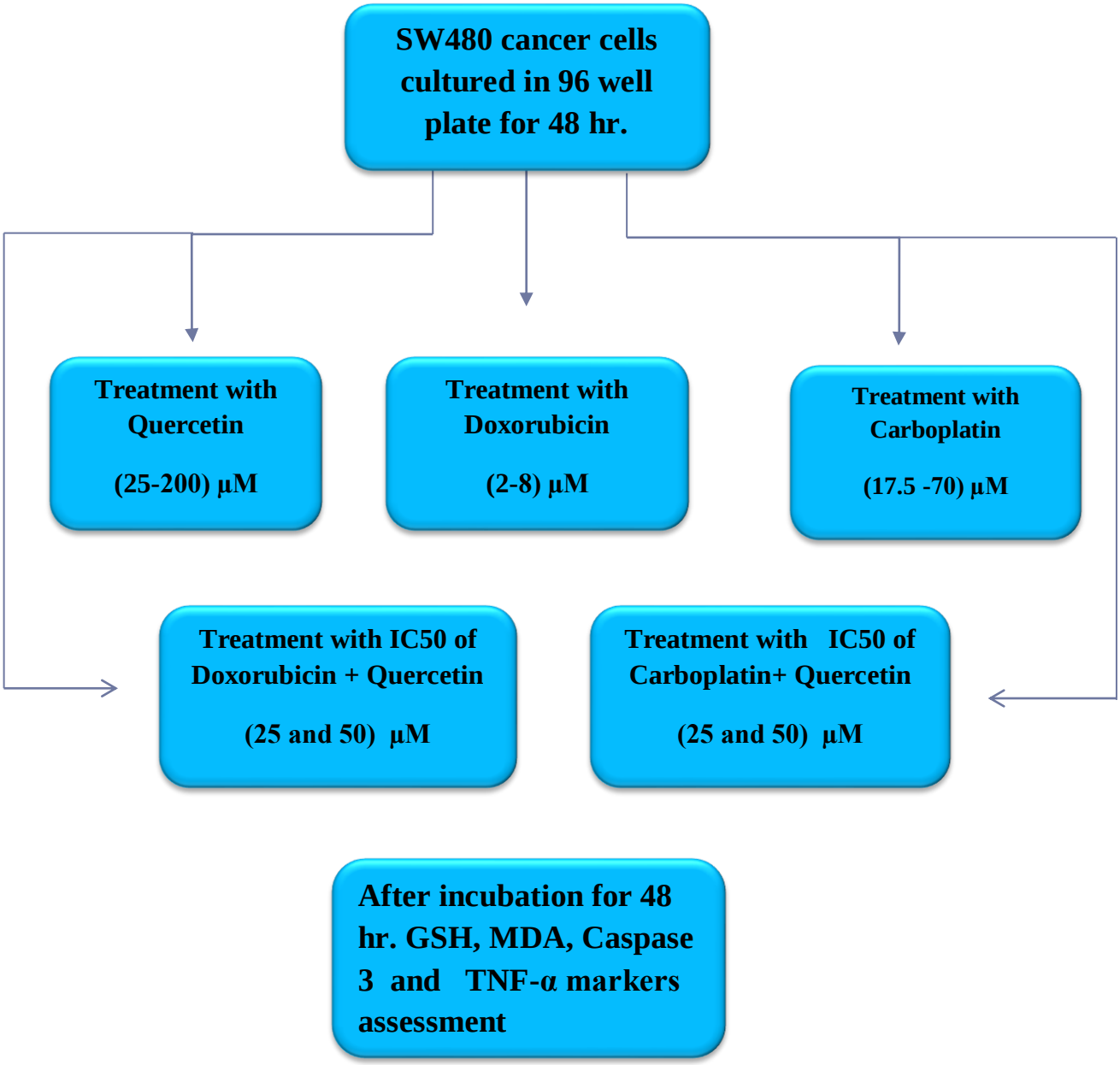
Experiments were performed in triplicate in 3 independent experiments. All treatments were done using cells at 70–80% confluence. The SW480 cells were incubated in a CO_2 incubator for 48 hours, trypsinized, and immediately prepared for cytotoxicity assay and molecular analysis. Supernatants were kept at $-20^{\circ}C$ for detection the antioxidant, pro-oxidant , apoptotic and anti-inflammatory effect by ELISA. Vero cells were prepared only for cytotoxicity assay of quercetin.

2.2.2. Study plan

Part 1: Cytotoxicity Assay



Part 2: Biochemical Assessment



2.2.3. Trypan Blue Exclusion Method for Cell Counts

Trypan Blue Solution, 0.4%, is frequently used as a cell stain to determine the cell counting using the dye exclusion test. This test is frequently carried out while counting cells with a haemocytometer during routine sub culturing, but it can be executed whenever a quick and precise assessment of cell viability is required. The dye exclusion test is depend upon the idea that dead cells are permeable and absorb the dye, but living cells do not absorb impermeable dyes (like Trypan Blue).

When utilizing a glass haemocytometer with a coverslip, the device was cleaned with alcohol before used, wetted the coverslip with water, and then attached it to the haemocytometer. Newton's refraction rings indicating appropriate adhesion can be seen underneath the coverslip. Cell suspension was prepared by gently shaking the flask to spread the cells equally. Using a sterile pipette with a capacity of 5 mL and withdrew 0.5 mL of the cell solution and inserted it in an Eppendorf tube before the cells have a chance to settle. Then transferred 100 µL of cells and 400 µL of 0.4% Trypan Blue (final concentration: 0.32%) into a fresh Eppendorf tube and mixed slowly. Then applied 100µ L of the trypan Blue-treated cell suspension to the haemocytometer using a pipette. By using a glass haemocytometer, filled both chambers gently and placed a coverslip on top to allow capillary action to draw the cell suspension out. Used a pipette to transfer the cell suspension into the counting chamber of a disposable haemocytometer, allowed capillary action to bring the suspension within.

Utilizing a microscope, zoomed in with a 10X objective to focus on the haemocytometer’s grid lines. In one set of 16 squares, counted the living, unstained cells with a hand tally counter (living cells do not absorb Trypan Blue). Used a technique where cells are only tallied when they are

arranged within a square or on the bottom or right boundary line while counting. Dead cells stained with Trypan Blue can also be counted for a viability estimate using the same procedures, if necessary. The counting continue until all four sets of 16 corner squares have been counted by moving the haemocytometer to the subsequent set of 16 corner squares. To determine the number of viable cells per millilitre , the average number of cells was calculated in each set of 16 corner squares and multiplied by 10,000 (10^4) and multiplied by 5 to account for the Trypan Blue addition's 1:5 dilution. (Strober *et al*., 2015).

2.2.4. Preparation of test agents

Quercetin was initially dissolved in 100% DMSO (fresh, sterile) at a concentration of 40mM per ml, and kept at 4°C and protected from the light. Vortexing should be used to ensure solubilisations of the compound. Noteworthy, the final working concentration of DMSO should generally be 0.5% if started with 200µM concentration of quercetin. Low concentrations of DMSO (i.e. 0.1%) may lead the compound to precipitate out of solution. However, the amount of DMSO needed to keep the compound in solution is dependent on the concentration of the compound. For example, in 0.5% DMSO, drug concentrations above 200 µM also lead to precipitation (depending on which aqueous buffer is used and the temperature). Starting with 200µM (withdrawn 5µl of stock), different concentrations of quercetin were freshly prepared in complete culture medium before use and added to the cells in different experiments. In all experiments, DMSO solvent never exceeded 0.5%, which has no effect on SW480 cells. Doxorubicin (2mg/ ml) and carboplatin (10mg/ml) were diluted in complete culture medium freshly before use and added to the cells at different concentrations. For

doxorubicin starting with 16µM (withdrawn 5µl of stock) and for carboplatin starting with 140 µM (withdrawn 5µl of stock).

2.2.5. MTT (Cytotoxicity) Assay

The MTT assay, which was first introduced by Tim Mosmann in 1983, is a viability assay that is most frequently utilized globally. This colorimetric assay measures cellular metabolic activity as a representative for cell viability by reducing a yellow tetrazolium salt called 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The MTT reagent is converted to formazan, an insoluble crystalline product with a deep purple colour, by the presence of NAD(P)H-dependent oxidoreductase enzymes in living cells. A solubilizing solution (DMSO) is then used to dissolve the formazan crystals, and a plate-reader is used to detect the absorbance at 500–600 nanometres .When solution be darker , the number of viable (metabolically active) cells be greater (Meerloo *et al*., 2011).

Procedures

1. Cells were seeded in 96-well plates (2×10^4 cells/well) and kept overnight at 37°C, old media was discarded, the cells were treated with different concentrations of drugs for 48 hr.

2. To achieve final concentration of 0.5 mg/mL, 1 ml of MTT solution (5 mg/ ml) was added to 10 ml of medium. Then poured 100 µl of the resultant solution into each well and incubate for 4 hours at 37 °C, or until intracellular purple formazan crystals can be seen under a microscope

4. Then MTT was removed and substituted solubilizing solution (100 microliter DMSO) and triturated. Continued incubating for 30 minutes at 37 °C or at room temperature, or until the purple crystals disintegrate and

calculated the absorbance at 570 nanometers. Subtracted the absorbance of the blank from each samples. The percentage of cell viability or proliferation is then calculated by dividing the absorbance readings from the test samples by those from the control samples and multiplying the result by 100 (see the calculation below). The lower absorbance values than control indicate cell death or cell growth inhibition, while values higher than the control indicate cell proliferation (Meerloo *et al*., 2011).

$$\% \text{ viable cells} = \frac{(abs_{sample} - abs_{blank})}{(abs_{control} - abs_{blank})} \times 100$$

Where:

abs sample: Absorbance of treated cells (drug)

abs blank : Absorbance of blank (only media).

abs control : Absorbance of control (untreated cells)

% Inhibition = 100 – % viability.

2.2.5.1. Evaluation The Cytotoxic Effect of Quercetin on Vero (normal) Cells

Assessment the cytotoxic effect of quercetin on normal cells aimed to determine the effective concentrations of quercetin that kill cancer cells without exhibit cytotoxic effect on normal cells. Then, these concentrations were selected for subsequent experiments in combination with IC50 of chemotherapies (doxorubicin and carboplatin) in SW480 cancer cells. Vero cells were seeded in 96-well plates (2×10^4 cells/well) and maintained overnight at 37°C, old media was aspirated, the cells

were treated with (12.5, 25, 50, 100, 200) µM of quercetin for 48 hr. Then MTT assay was achieved as mentioned above.

2.2.5.2. Evaluation The Cytotoxic Effect of Quercetin, Doxorubicin, Carboplatin and Their Combinations on SW480 Cancer Cells by MTT Assay

Cells were seeded in 96-well plates (2×10^4 cells/well) and maintained overnight at 37°C, old media was aspirated, the cells were treated with (12.5, 25, 50, 100, 200) µM, (1, 2, 4, 8, 16) µM and (8.75, 17.5, 35, 70, 140) µM of quercetin, doxorubicin and carboplatin respectively for 48 hr. Depending on IC50 of each drug and the concentrations of quercetin that have cytotoxic effect on SW480 CRC cancer cells but safe effect on normal cells, various combination of quercetin (25,50) µM with IC50 of each of doxorubicin and carboplatin 8µM and 70µM respectively were prepared at 48 hr. Then, MTT assay was achieved as mentioned above.

2.2.6. Biochemical Assessment

This assessment used to study the antioxidant , apoptotic and anti-inflammatory effect of quercetin and other drugs alone and in combination on SW480 CRC cancer cells. Cells were seeded in 96-well plates (1×10^4 cells/well) and maintained overnight at 37°C, old media was aspirated, the cells were treated by (25, 50, 100, 200) µM, (2, 4, 8) µM and (17.5, 35, 70) µM of quercetin, doxorubicin and carboplatin respectively and the combination of quercetin (25,50) µM with IC50 of doxorubicin and carboplatin at 48 hr. The supernatants of each well were withdrawn by micropipette and taken for enzyme-linked immunosorbent

(ELISA) assay using glutathione (GSH) , malondialdehyde (MDA) , caspase 3 and tumour necrosis factor (TNF- α) kits as described below:

2.2.6.1. Glutathione ELISA Kit

This ELISA kit operates using the Competitive-ELISA theory. This kit includes a micro ELISA plate that has been pre-coated with GSH. During the procedure, a predetermined amount of GSH on the solid phase supporter and GSH in the samples or the standard compete for GSH-specific sites on the biotinylated detection antibody. The excess conjugate and unbound sample or standard are removed from the plate, and then each microplate well is loaded with Avidin-conjugated to Horseradish Peroxidase (HRP) and incubated. Then, added a tetramethylbenzidine (TMB) substrate solution to each well. Added stop solution for termination an enzyme-substrate reaction and measured the colour change spectrophotometrically at a wavelength of 450±2 nm. The OD of the samples is then compared to the standard curve to determine the concentration of GSH in the samples.

Reagent preparation

1. All reagents were brought to room temperature (18-25℃) before used, took out the important strips and reagents for present experiment, and the remaining strips were stored and reagents at required condition.

2. Wash Buffer: To prepare 750 mL of wash buffer, 30 mL of concentrated wash buffer was diluted with 720 mL of distilled water deionized , if crystals have developed in the concentrate, warm it with water bath in a 40℃ and mix it gently until the crystals have fully dissolve.

3. Standard working solution: The standard was centrifuged at 10,000×g for 1 minute. Then, 1.0 mL of reference standard and sample

diluent were added, the mixture was left to stand for 10 minutes, and then it was gently inverted several times. Use a pipette to properly combine it when it has completely dissolved . This reconstitution creates a working solution with a concentration of 100 g/mL (or you can add 1 mL of reference standard and sample diluent, let it sit for 1-2 minutes, and then thoroughly mix it with a low-speed vortex meter). By centrifuging at a low speed, bubbles created during the vortex might be eliminated .Then prepared serial dilutions as required. The suggested gradient for dilution is 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0 g/mL

Dilution method: Took 7 EP tubes and added 500uL of Reference Standard & Sample Diluent to each one. Then, pipetted 500uL of the 100 μg/mL working solution to the former tube and mixed up to prepare a 50 μg/mL working solution. Then, 500uL of the solution was pipetted from the first tube into the last one according to this step.

4. Biotinylated detection antibody working solution: The necessary amount was computed before the experiment (50μL/well). Prepared little more than calculated . Biotinylated detection antibody at 800×g for 1 min, then diluted the 100× concentrated biotinylated detection Antibody to 1× working solution with Diluent biotinylated detection Antibody (concentrated biotinylated: diluent= 1: 99).

5. HRP conjugate working solution: Before the experiment, calculated the required amount (100 μL/well). Also prepared slightly more than the calculated amount. Concentrated HRP conjugate centrifuged at 800×g for 1 min, then diluted the 100× HRP conjugate to 1× working solution with conjugate diluent HRP (concentrated HRP conjugate: HRP conjugate diluent= 1: 99).

Procedure

1. Wells were determined for diluted standard, blank and sample. Added 50 μL each dilution of standard, blank and sample into the suitable wells.

Added 50 µL of biotinylated detection Ab working solution to each well immediately. The plate was covered with the sealer that supplied in the kit. Incubated for 45 min at 37℃. The solutions was added to the bottom of the micro ELISA plate well, avoid touching the inside wall and foaming formation also should be avoided as much as possible.

2. The solution was aspirated from each well and added 350 µL of wash buffer to each one. After soaking for one minute, decanted or aspirated the solution from each well, then patted it dry with fresh, absorbent paper. Three times, repeated the washing step.

3. Added 100 µL HRP conjugate working solution to each well, and covered the plate with a new sealer, then incubated at 37°C for 30 min.

4. The solution aspirated from each well, the wash process repeated for 5 times as described in step 2.

5. Substrate reagent 90 µL was added to each well, the plate sealed with a new sealer and incubated at 37°C for roughly 15 min. Shielded the plate from light, the reaction time can be shortened or prolonged depending on the actual colour change, but not for longer than 30 minutes. The microplate reader was preheated for about 15 min before measure the OD .

6. Each well received 50 µL of the Stop Solution, which should be put in the same order as the substrate solution.

7. Using a microplate reader with the 450 nm setting, optical density (OD value) was calculated for each well at once.

2.2.6.2. Lipid Peroxidation kit

Thiobarbituric acid reactive substances (TBARS) were used to measure lipid peroxidation. The TBARS test provides a fundamental, repeatable, and standardized tool for calculating serum lipid peroxidation. The malondialdehyde-thiobarbituric acid (MDA-TBA) adduct is measured calorimetrically at 530-540 nm or fluorometrically at an

excitation wavelength of 515 nm and an emission wavelength of 555 nm. This reaction has a significantly higher sensitivity when fluorometrically measured.

Reagent preparation The working solution was prepared by dissolving 0.514 g of thiobarbituric acid (TBA) in D.W and adding 25 g of trichloroacetic acid (TCA), followed by adding 0.5 mL of hydrochloric acid, and then using D.W, completing the volume to 190 ml. The volume was then completed to 200 ml and mixed well (See Table 2.3).

Table 2.4: The lipid peroxidation test method.

Reagents	Test	Blank
Supernatant	100 µl	----
Distilled water	----	100 µl
Working solution	2 ml	2ml
The sample was vortexed, heated for 50 minutes in a 90° C water bath, and then let to cool		
The sample was then centrifuged for 5 min at 5000 rpm then spectrophotometrically measured the absorbance of the supernatant at 532 nm wave length against a reagent blank. By the same procedure above except change the sample with DW, the reagent of blank was prepared.		

2.2.6.3. Caspase 3 ELISA Kit

This kit performs an ELISA, or enzyme-linked immunosorbent assay. Human CASP3 antibody has been pre-coated on the plate. When CASP3 from the sample is added, it binds to the antibodies that have been coated on the wells. After that, biotinylated human CASP3 antibody was added, and it binds to CASP3 in the sample. The biotinylated CASP3 antibody is

then bound by the addition of streptavidin-HRP. Unbound Streptavidin-HRP is removed during a washing step after incubation. The amount of Human CASP3 is then correlated with the development of colour in the substrate solution. By adding an acidic stop solution, the process is stopped, and absorbance is measured at 450 nm.

Specimen Collection: The supernatant of cell culture was centrifuged at 2000-3000 rpm for 20 minutes. Then, collected the supernatant and executed the assay.

Reagent Preparation

- Before use, all reagents was brought at room temperature.
- To create a 12ng/ml standard stock solution, reconstituted 120ul of the standard (24ng/ml) with 120ul of the standard diluent. Before producing dilutions, let the standard sited for 15 minutes with gentle agitation. The standard stock solution (12ng/ml) was serially diluted (1:2) with the standard diluent to create solutions at 6ng/ml, 3ng/ml, 1.5ng/ml, and 0.75ng/ml. Any solution that is left over needs to be frozen at -20°C and utilized within a month (table 2.5).

Table 2.5: Dilution of standard solutions of caspase 3 assay.

12ng/ml Standard No.5	Standard No.5	120ul Original standard + 120ul Standard diluent
6ng/ml	Standard No.4	120ul Standard No.5 + 120ul Standard diluent
3ng/ml	Standard No.3	120ul Standard No.4 + 120ul

		Standard diluent
1.5ng/ml	Standard No.2	120ul Standard No.3 + 120ul Standard diluent
0.75ng/ml	Standard No.1	120ul Standard No.2 + 120ul Standard diluent

- Wash Buffer: Diluted 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to prepare 500 ml of 1x Wash Buffer. If crystals have developed in the concentrate, stir slowly until they are fully dissolved.

Procedure

1. Constructed all reagents, standard solutions, and samples in accordance with the directions. All reagents was at room temperature before used. At room temperature, the assay was carried out.

2. Determined the strips number needed for the assay. Putted the strips in the frames in order to use and stored the unused strips at 2-8°C.

3. Added 50ul standard to standard well. Because the standard solution already contains biotinylated antibody, avoid adding it to the standard well.

4. Added 40ul sample to sample wells and then added 10ul Human CASP3 antibody to sample wells, then added 50ul streptavidin-HRP to sample wells and standard wells and not the blank control well. Mixed well, sealed the plate with a sealer and incubated 60 minutes at 37°C.

5. Removed the sealant and used a wash buffer to wash the plate five times. For each wash, soaked wells in 300ul of wash buffer for 30 to 60 seconds. Aspirated or decanted each well for automatic washing, then

used wash buffer five times. Placed paper towels or another absorbent material nearby to blot the plate.

6. Added 50ul substrate solution A to each well and then added 50ul substrate solution B to each well and incubated plate sealed with a new sealer in the dark for 10 minutes at 37°C

7. Added 50ul Stop Solution to each well, immediately the blue colour turned into yellow

8. Within 10 minutes of adding the stop solution, measured the optical density (OD value) of each well using a microplate reader set to 450 nm.

2.2.6.4. Tumour Necrosis Factor ELISA kit

This ELISA kit depend on the Sandwich-ELISA principle. The micro ELISA plate supplied in this kit has been pre-coated with an antibody that is specific to Human TNF-α. Standards or samples are poured to the micro ELISA plate wells and mixed with the specific antibody. Then successively a biotinylated detection antibody specific for Human TNF-α and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Unbounded components are removed by washing and substrate solution is poured to each well, the wells that contain Human TNF-α, biotinylated detection antibody and Avidin-HRP conjugate will show blue colour. Addition of stop solution terminate the enzyme-substrate reaction and the colour changes to yellow. Then, spectrophotometrically optical density (OD) is calculated at a wavelength of 450 nm ± 2 nm, the concentration of Human TNF-α is proportional to the OD value. By comparing the OD of the samples to the standard curve , the amount of Human TNF- α in the samples was determined .

Sample collection : Cell culture supernatant was centrifuged for 20 min at 1000×g at 2~ 8℃. Then, the supernatant was collected and executed the assay.

Reagent preparation

1. All reagents were brought to room temperature (18~25℃) before used and followed the Microplate reader manual for set-up and preheated it for 15 min before OD measurement.
2. **Wash Buffer**: To make 750 mL of Wash Buffer, combined 30 mL of concentrated Wash Buffer with 720 mL of deionized or distilled water. Note: If crystals have developed in the concentrate, gently stir it while warming it in a water bath at 40°C until all of the crystals have vanished.
3. **Standard working solution:** Centrifuged the standard for 1 minute at 10,000 g. Inverted it gently several times after adding 1.0 mL of Reference Standard & Sample Diluent and let it stand for 10 minutes. Used a pipette to properly combined it when it had completely dissolved. The working solution after reconstitution was 500pg/mL. Then, as necessary, performed serial dilutions. The recommended dilution gradient was as follows: 500、250、125、62.5、31.25、15.63、7.81、0 pg/mL.

Dilution method: Took 7 EP tubes and filled each one with 500uL of the reference standard and sample diluent. To create a 250 pg/mL working solution, pipetted 500 µL of the 500 pg/mL working solution into the first tube. In accordance with this procedure, pipetted 500uL of the solution from the first tube into the second. Recalled that the final tube was treated as a blank. Don't pipette solution from the old tube into it.

4. **Biotinylated Detection Ab working solution:** Before the experiment, calculated the required amount (100 μL/well). During preparation, prepared slightly more than calculated. The stock tube was centrifuged before utilized, diluted the 100× Concentrated Biotinylated Detection Ab to 1×working solution with Biotinylated Detection Ab Diluent.

5. **Concentrated HRP Conjugate working solution:** Before the experiment, calculated the required amount (100 μL/well). During preparation, prepared slightly more than calculated. Diluted the 100× Concentrated HRP Conjugate to 1× working solution with concentrated HRP Conjugate Diluent.

Procedure

1.The **Standard working solution** was poured to the first two columns: added each concentration of the solution in duplicate, to one well each, side by side (100 uL for each well). Added the samples to the other wells (100 uL for each well). Sealed the plate with the sealer supplied in the kit. Incubation at 37℃ for 90 min Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible. Remove the liquid of each well; do not wash.

2.Added 100 μL of **Biotinylated Detection Ab working solution** to each well right away and applied plate sealer on top to seal. Gently stirred and incubated at 37 °C for 1hr.

3.The solution from each well was aspirated or decanted and then, added 350 uL of **wash buffer** to each well. After soaking for one and a half minutes, aspirated or decanted the solution from each well, then patted it dry with fresh, absorbent paper. This wash step was repeated 3 times.

4.Added 100 μL of **HRP Conjugate working solution** to each well and sealed by the Plate sealer and incubated at 37°C for 30 min.

5.The solution was aspirated or decanted from each well, the wash process was repeated for 5 times as described in step 3.

6.Added 90 μL of **Substrate Reagent** to each well. Sealed with another plate sealer and incubated at 37°C for about 15 min. The plate was protected from light. Take note that the reaction time can be cut or extended depending on the actual colour change, but not beyond 30 minutes.

7.Stop Solution 50 μL was added to each well. Note: addition of the stop solution was done in the same manner as the substrate solution.

8. Utilized a microplate reader set to 450 nm to simultaneously calculate the optical density (OD value) of each well

2.3. Statistical Analysis

All experiments were performed in three technical replicates. Results were reported as mean ± standard deviation (SD). Statistical significance was determined by one-way ANOVA with Dennett s correction for comparisons with control group (*p <0.05),(**p <0.001) using SPSS version 22.0.

Chapter Three

Results

3.1. Evaluation The Effect of Quercetin on The Viability of Vero Cells

The results demonstrated that high concentrations of quercetin (100, 200)µM had significant $p<0.05$ cytotoxic effects on normal cells (inhibiting 18.73% and 25% of vero cells respectively) in a compare to the control group after incubation for 48 hr when SW480 cancer cells treated with various concentrations (12.5, 25, 50, 100, 200)µM of quercetin for 48hr at 37°C (Fig.3.1), whereas low concentrations of quercetin 25µM and 50µM had no significant effect $p>0.05$ on normal cells so these concentrations were selected in subsequent experiments in combination with IC50 of chemotherapies (doxorubicin and carboplatin) in the treatment of SW480 cancer cells.

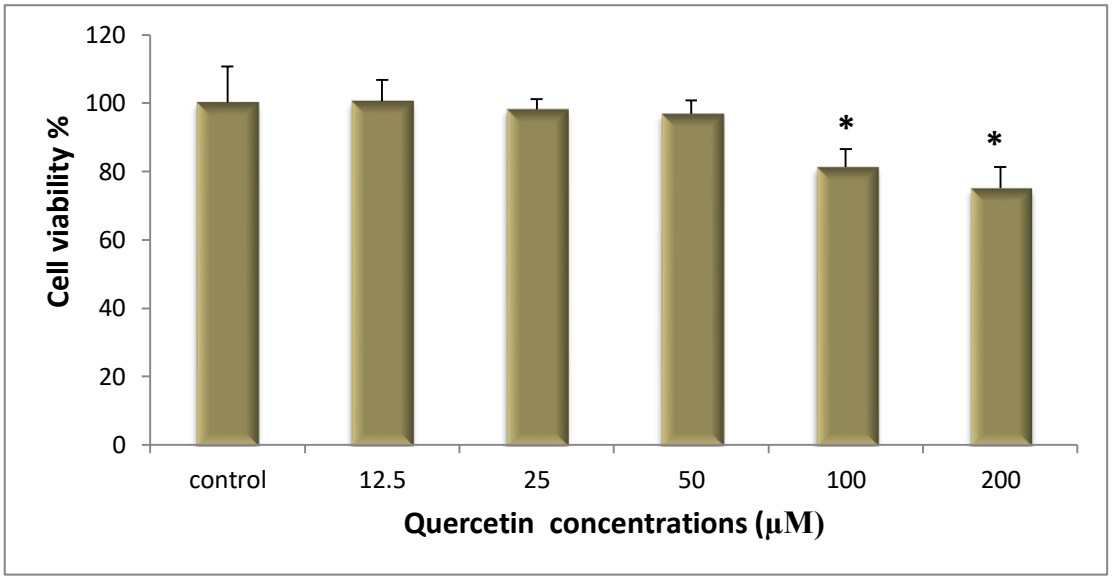


Figure 3.1. Effect of quercetin at various concentrations on the viability of vero cells. (*, $p< 0.05$). control: untreated cells (RPMI).

3.2. Evaluation The Effect of Quercetin on The Viability of SW480 Cells

The results showed a highly significant $p < 0.001$ decrease in cells viability in high concentrations (100, 200) µM and significant $p < 0.05$ decrease in low concentrations (25, 50) µM when SW480 cancer cells treated with various concentrations (12.5, 25, 50, 100, 200) µM of quercetin in a compare to the control group after incubation for 48 hr at 37 ^{o}C (Fig.3.2a). Depending on MTT results, the IC50 of quercetin was estimated by using the excel software and was 185 µM as shown in (Fig 3.2 b).

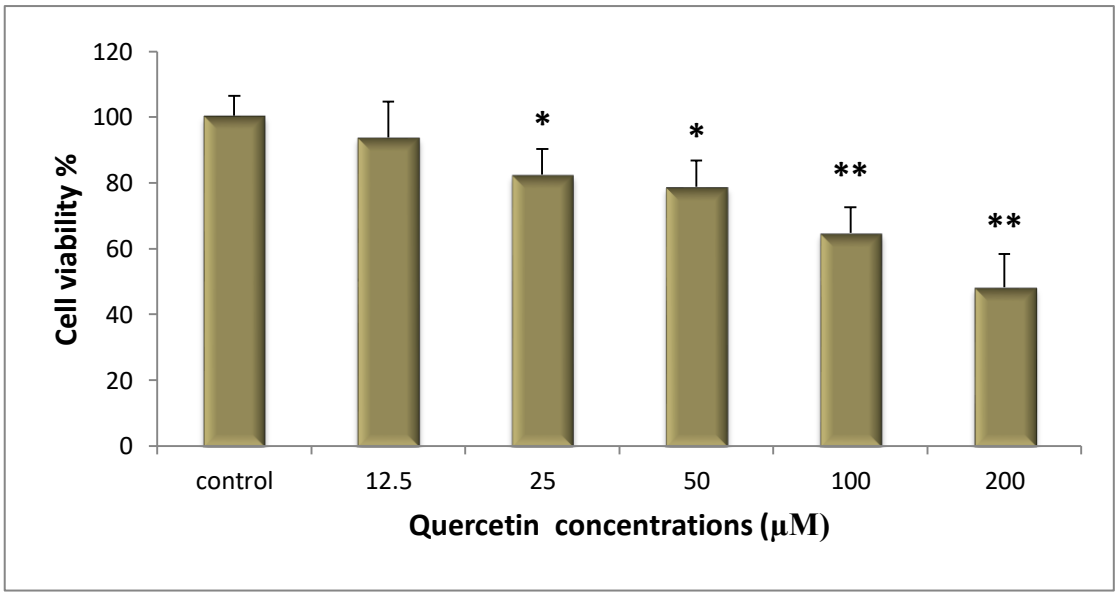


Figure 3.2a Effect of quercetin at various concentrations on the viability of SW480 cells. (*$p < 0.05$),(**$p < 0.001$). control: untreated cells (RPMI).

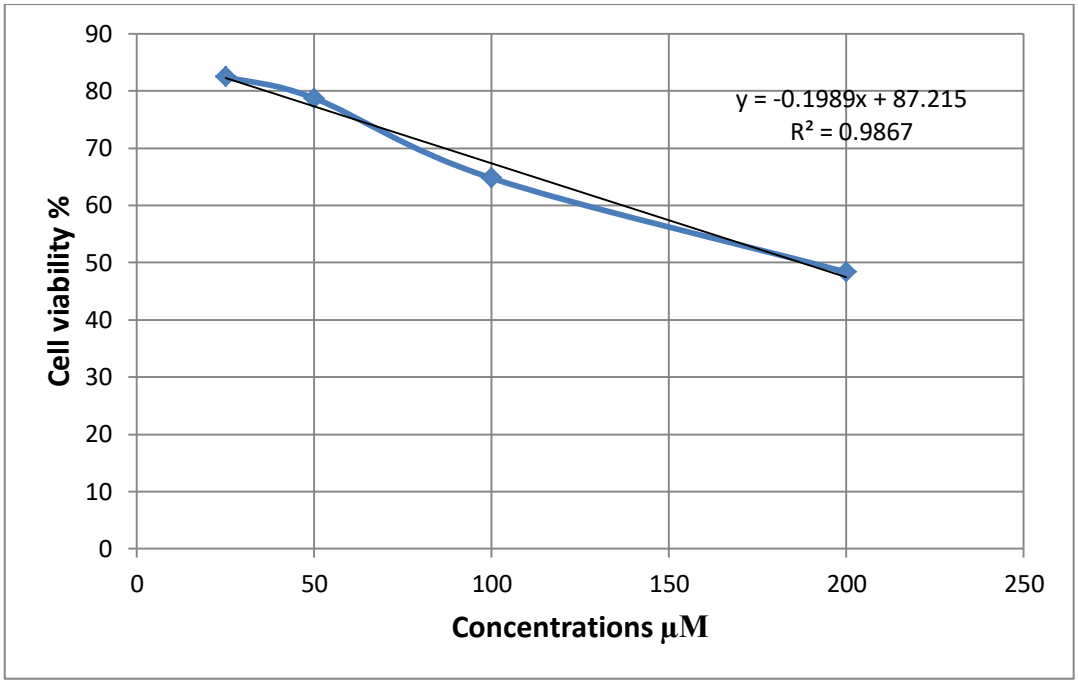


Figure 3.2b. IC50 calculation of quercetin in SW480 cell line. Cells were exposed to different concentrations of quercetin (12.5, 25, 50,100,200) µM for 48hr, then the cytotoxic effects of quercetin were assessed by MTT assay and IC50 was calculated deepening on the results.

3.3. Evaluation The Effect of Doxorubicin on The Viability of SW480 Cells

The results demonstrated a significant ($p < 0.05$), ($p < 0.001$) decrease in cells viability in concentration-dependant manner when SW480 cancer cells treated with various concentrations (1, 2, 4, 8, 16)µM of doxorubicin after incubation for 48 hr at 37°C when compare to the control group (Fig.3.3). Depending on MTT results, the IC50 of doxorubicin was estimated by using the excel software and was 8 µM as shown in (Fig 3.3 b).

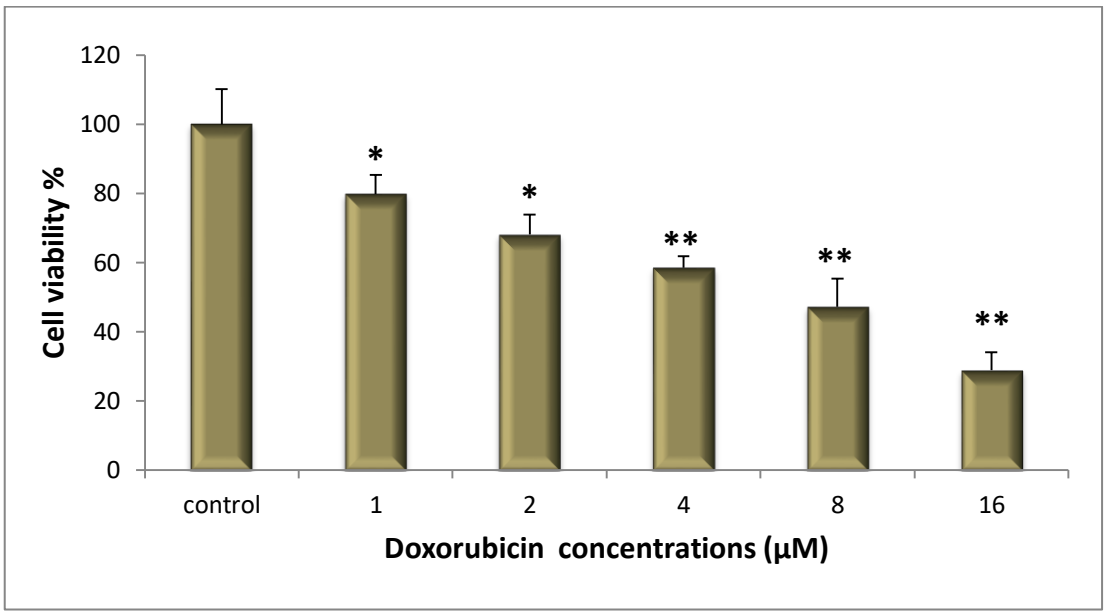


Figure 3.3 a. Effect of doxorubicin at various concentrations on the viability of SW480 cells. (*$p < 0.05$),(**$p < 0.001$). control: untreated cells (RPMI).

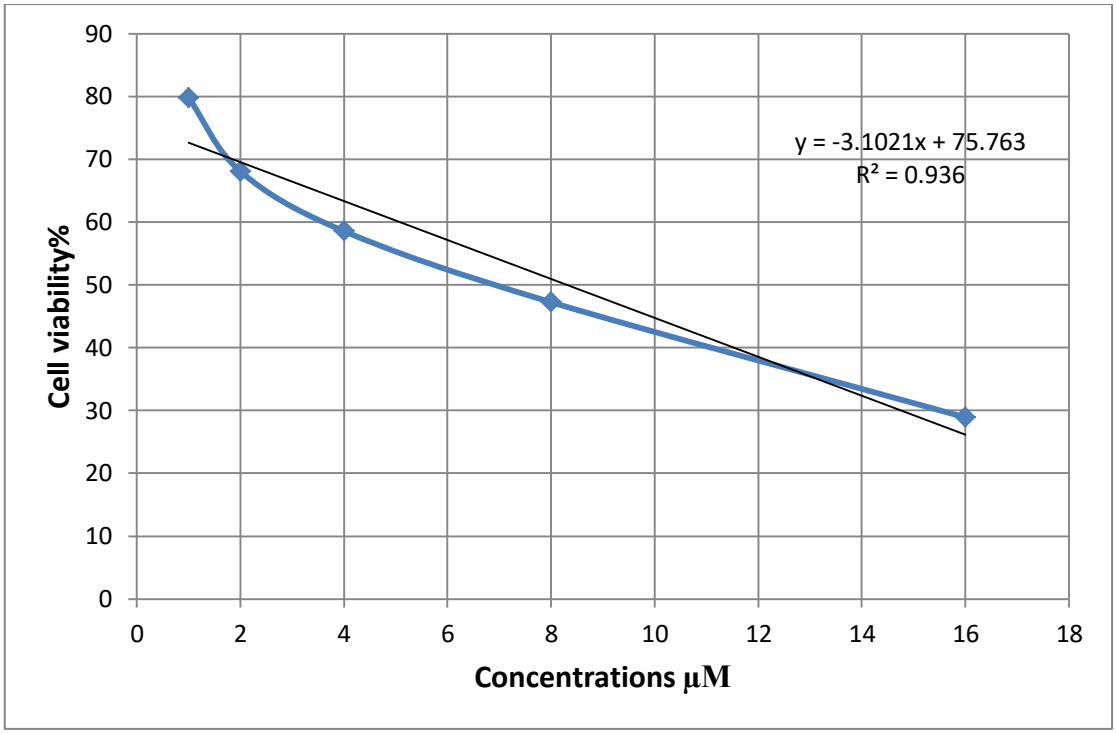


Figure 3.3b. IC50 calculation of doxorubicin in SW480 cell line. Cells were exposed to different concentrations of doxorubicin (1,2,4,8,16) µM for 48hr, then the cytotoxic effects of doxorubicin were assessed by MTT assay and IC50 was calculated deepening on the results.

3.4. Evaluation The Effect of Carboplatin on The Viability of SW480 Cells

The results demonstrated a significant ($p < 0.05$),($p < 0.001$) decrease in cells viability in concentration-dependant manner when SW480 cancer cells treated with various concentrations (8.75, 17.5, 35, 70, 140) µM of carboplatin after incubation for 48 hr at 37 ^{o}C in a compare to the control group (Fig. 3.4a). Depending on MTT results, the IC50 of carboplatin was estimated by using the excel software and was 70 µM as shown in (Fig 3.4 b).

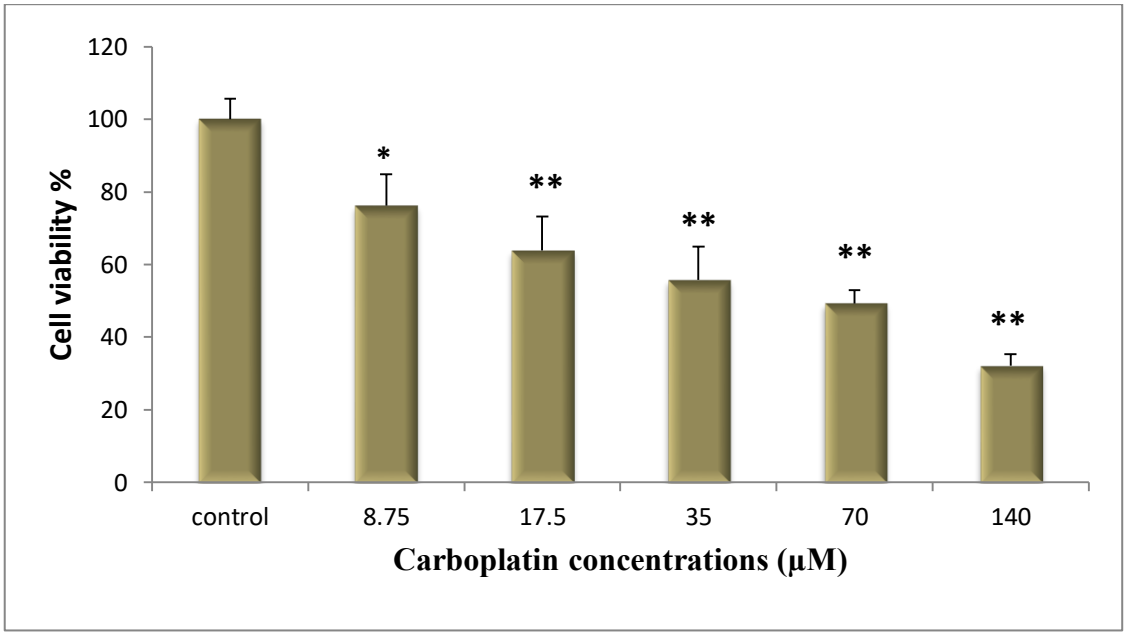


Figure 3.4 a. Effect of carboplatin at various concentrations on the viability of SW480 cells. (*$p < 0.05$),(**$p < 0.001$). control: untreated cells (RPMI).

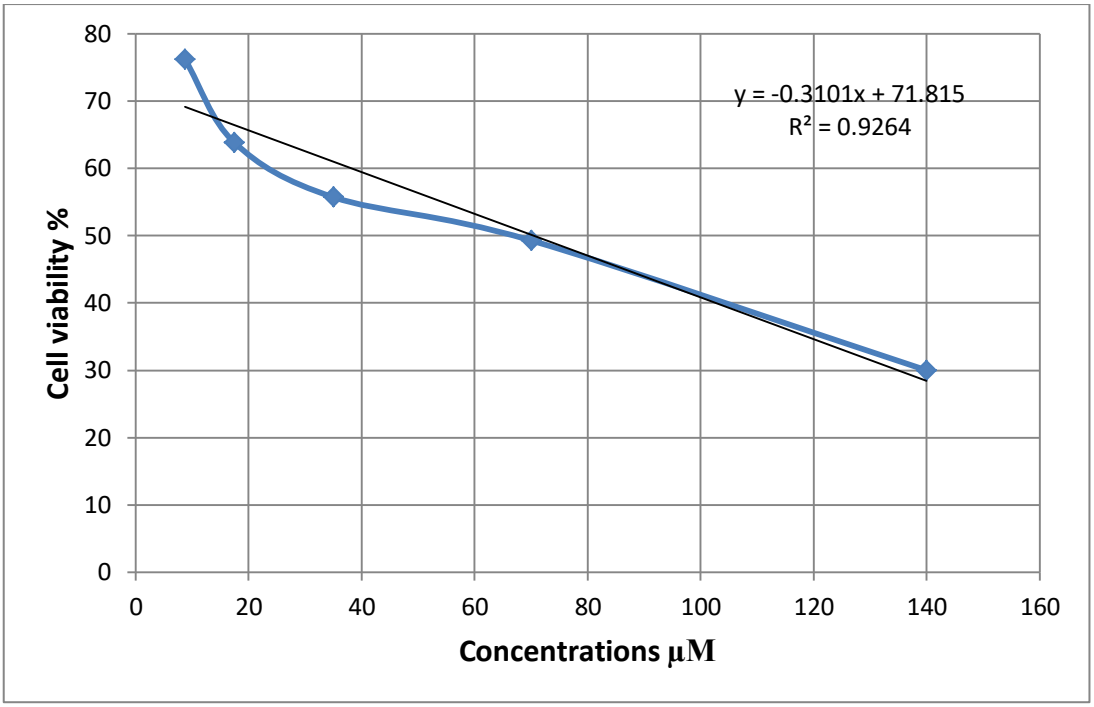


Figure 3.4 b. IC50 calculation of carboplatin in SW480 cell line. Cells were exposed to different concentrations of carboplatin (8.75, 17.5, 35, 70, 140) µM for 48hr, then the cytotoxic effects of carboplatin were assessed by MTT assay and IC50 was calculated deepening on the results.

3.5. Evaluation The Combined Effect of Doxorubicin and Quercetin on The Viability of SW480 Cells

Doxorubicin alone exhibited significant $p<0.001$ decrease in cell viability in a compare to the control group. Combination of low concentrations of quercetin (25, 50) µM with IC50 of doxorubicin (8)µM resulted in significant $p<0.05$ increase in viability of these cells in a compare to the doxorubicin alone (Fig. 3.5). Quercetin at low concentrations significantly reduced the efficacy of doxorubicin on the viability of SW480 cancer cells in a compare to the doxorubicin alone.

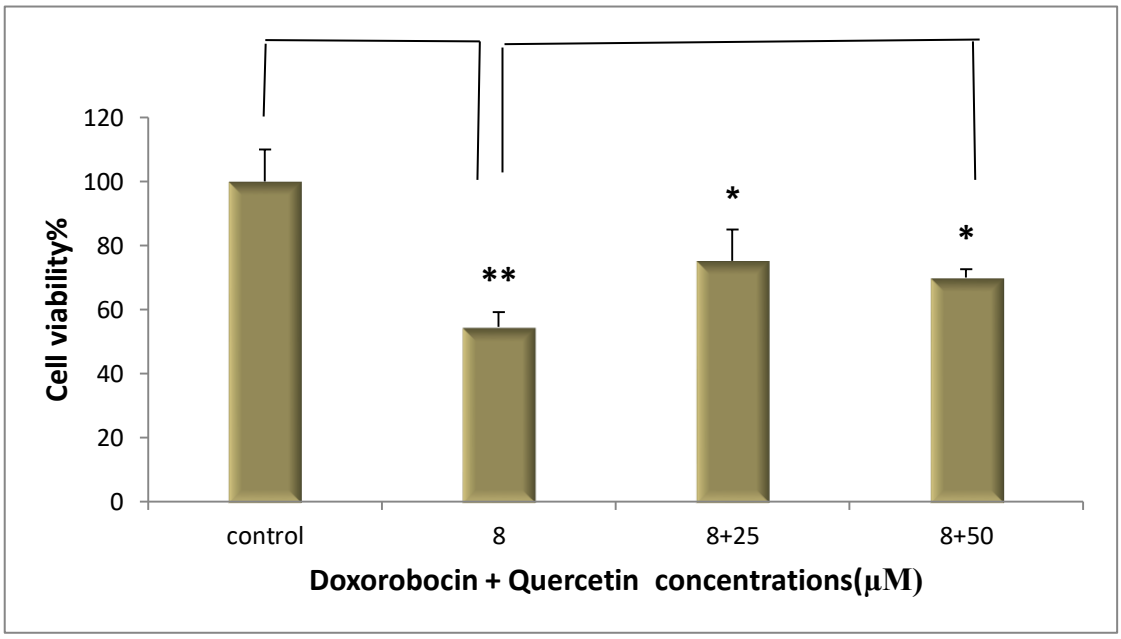


Figure 3.5. The combined effect of doxorubicin and low concentrations of quercetin on the viability of SW480 cells. (*p <0.05),(**p <0.001). control: untreated cells (RPMI).

3.6. Evaluation The Combined Effect of Carboplatin and Quercetin on The Viability of SW480 cells

Carboplatin alone exhibited significant $p<0.001$ decrease in cell viability in a compare to the control group. Combination of low concentration of quercetin 25 µM with IC50 of carboplatin (70µM) resulted in remarkable increase in viability of these cells in a compare to the carboplatin alone (Fig. 3.6). However, there has no apparent effect on the cytotoxicity of carboplatin when combined with 50µM of quercetin in a compare to the carboplatin alone. Quercetin at concentration 25 µM reduced the efficacy of carboplatin on the viability of SW480 cells.

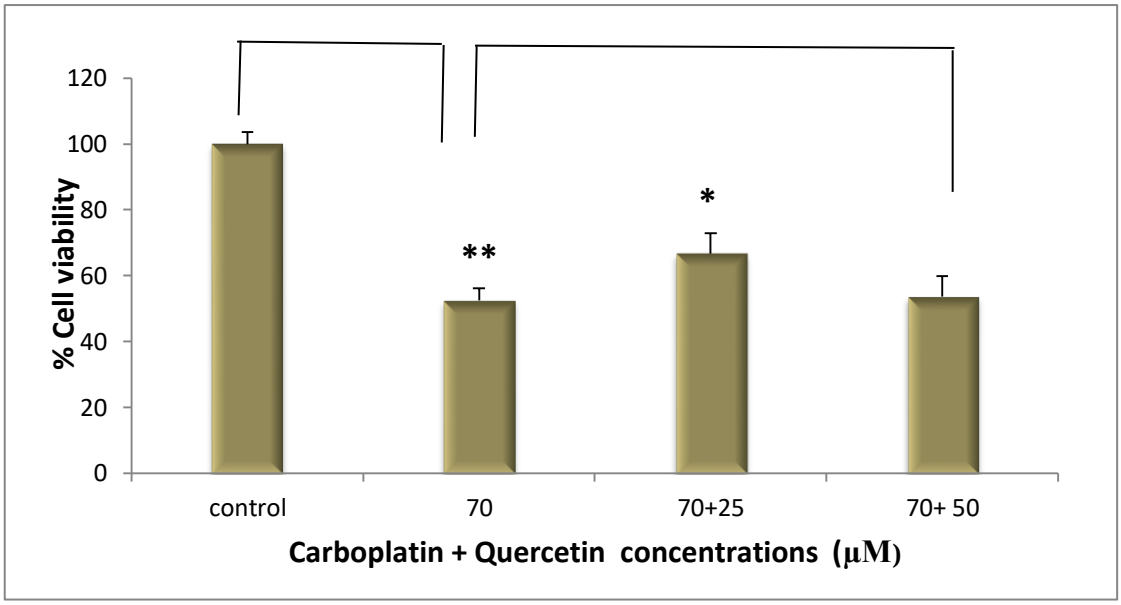


Figure 3.6. The combined effect of carboplatin and low concentrations of quercetin on the viability of SW480 cells. (*p <0.05),(**p <0.001). control: untreated cells (RPMI).

3.7. The Effect on Glutathione (GSH)

3.7.1. Evaluation The Effect of Quercetin on GSH Level in SW480 Cells

The high concentrations (100µM and 200µM) of quercetin showed a significant decrease ($p < 0.05$), ($p < 0.001$) respectively in GSH level, but low concentrations 25 µM and 50 µM of quercetin exhibited no effect on GSH level when SW480 cells treated with various concentrations (25, 50, 100, 200) µM of quercetin in a compare to the control group after incubation for 48 hr at 37 ^{o}C (Fig 3.7.1).

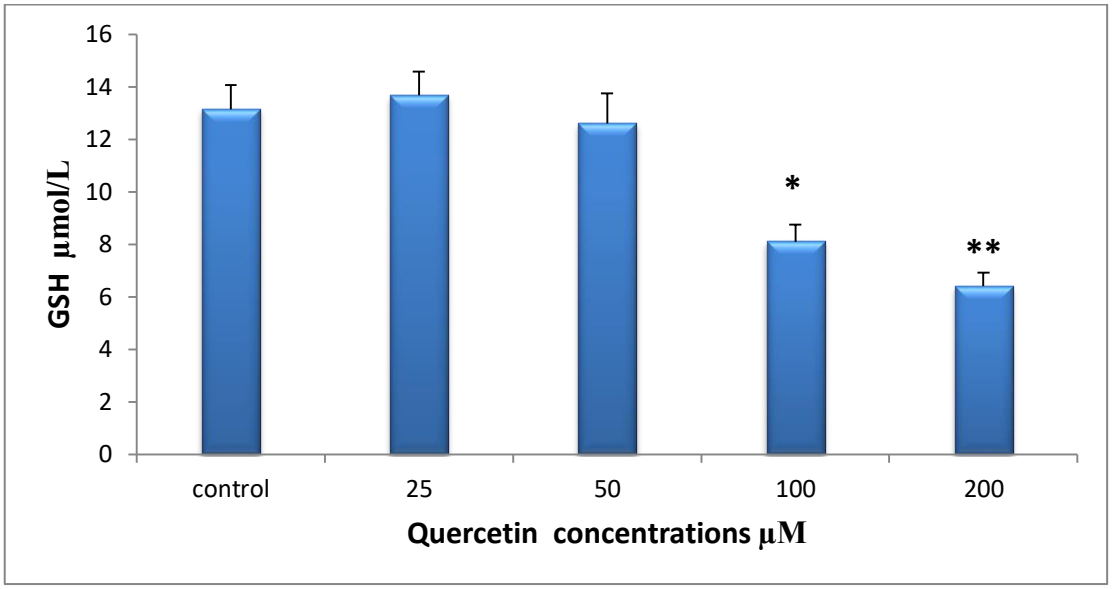


Figure 3.7.1. Effect of quercetin at various concentrations on the GSH level in SW480 cells. (*$p < 0.05$),(**$p < 0.001$). control: untreated cells (RPMI).

3.7.2 Evaluation The Effect of Doxorubicin on GSH Level in SW480 Cells

The results showed a significant decrease ($p < 0.05$),($p < 0.001$) in GSH level in a concentrations- dependant manner when SW480 cancer cells treated with various concentrations of doxorubicin (2, 4, 8) µM in a compare to the control group after incubation for 48 hr (Fig. 3.7.2).

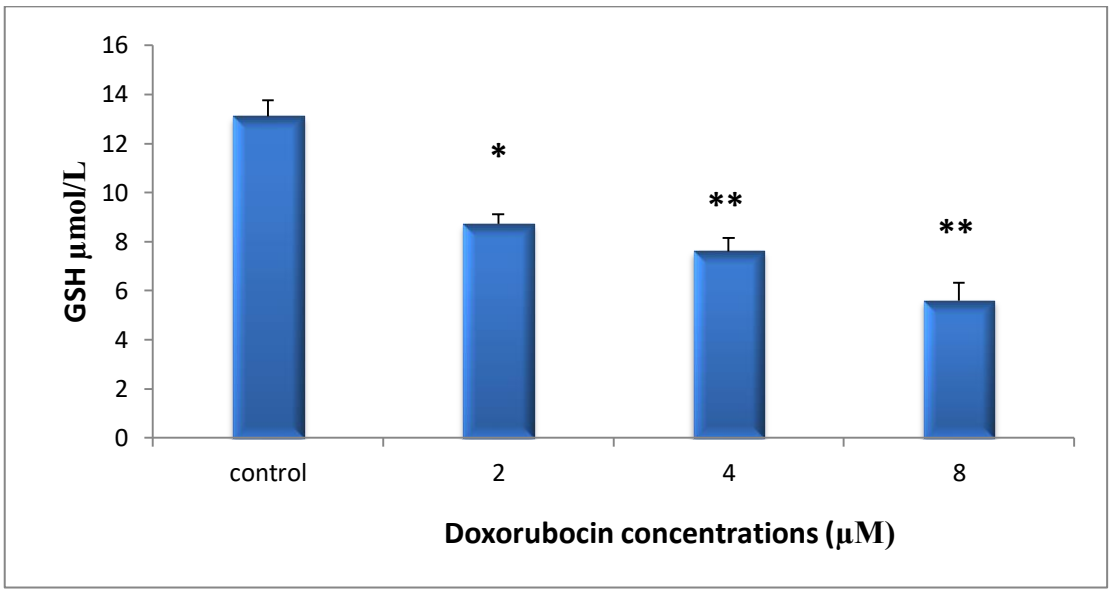


Figure 3.7.2. Effect of doxorubicin at various concentrations on the GSH level in SW480 cancer cells. (*p <0.05),(**p <0.001). control: untreated cells (RPMI).

3.7.3. Evaluation The Effect of Carboplatin on GSH Level in SW480 Cells

The results showed a significant ($p < 0.05$), ($p < 0.001$) decrease in GSH level in concentrations dependant manner when SW480 cancer cells treated with various concentrations (17.5, 35, 70)µM of carboplatin in a compare to the control group after incubation for 48 hr at 37 ^{o}C (Fig. 3.7.3).

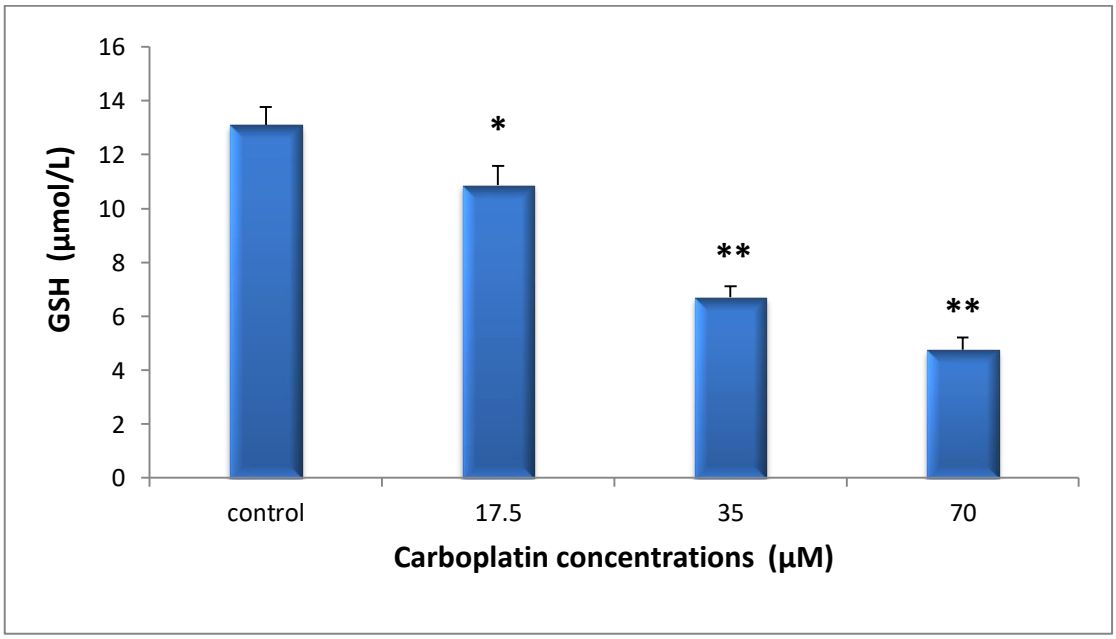


Figure 3.7.3. The effect of carboplatin at various concentrations on the GSH level in SW480 cells. (*p <0.05),(**p <0.001). control: untreated cells (RPMI).

3.7.4. Evaluation The Combined Effect of Doxorubicin and Quercetin on GSH Level in SW480 Cells

Doxorubicin alone exhibited significant $p<0.001$ decrease in GSH level in a compare to the control group. Combination of low concentrations of quercetin 25µM with IC50 (8µM) of doxorubicin resulted in highly significant $p<0.001$ increase in GSH level in a compare to the doxorubicin alone and significant $p<0.05$ increase in GSH level when SW480 cancer cells treated with 50µM of quercetin and 8µM of doxorubicin (Fig. 3.7.4). Quercetin at low concentrations significantly reduced the efficacy of doxorubicin at various degrees on GSH level in a compare to the doxorubicin alone.

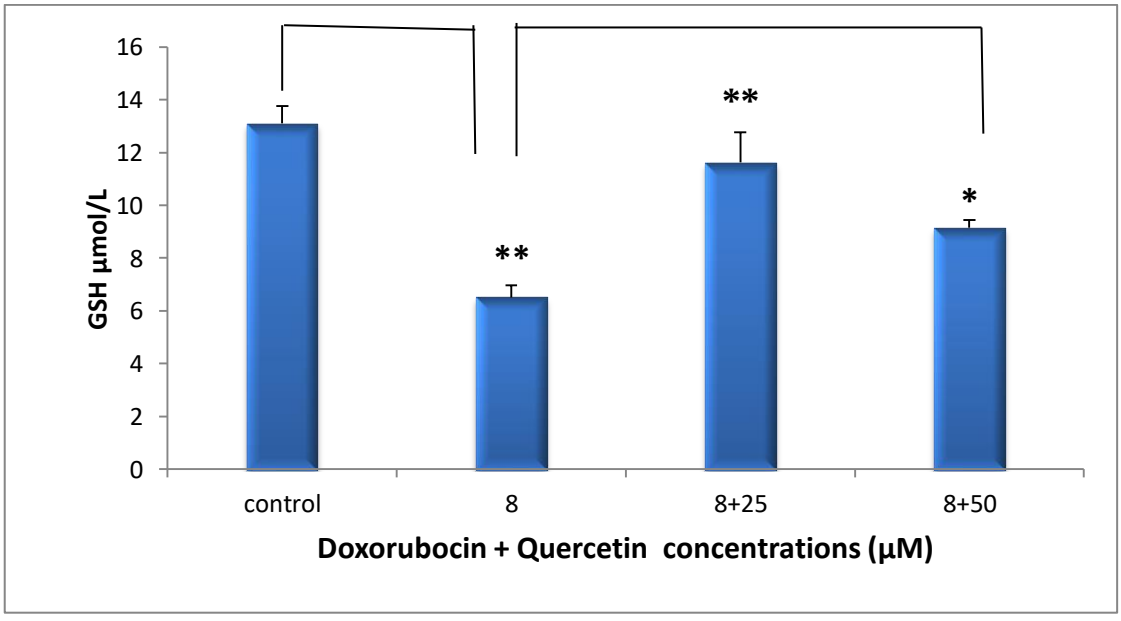


Figure 3.7.4. The combined effect of doxorubicin and low concentrations of quercetin on the GSH level in SW480 cells. (*p <0.05),(**p <0.001). control: untreated cells (RPMI).

3.7.5. Evaluation The Combined Effect of Carboplatin and Quercetin on GSH Level in SW480 Cells

Carboplatin alone exhibited significant $p<0.001$ decrease in GSH level in a compare to the control group. Combination of low concentrations of quercetin 25µM with IC50 (70µM) of carboplatin resulted in highly significant $p<0.001$ increase in GSH level in a compare to the carboplatin alone and significant $p<0.05$ increase in GSH level when SW480 cancer cells treated with 50µM of quercetin and 70µM of carboplatin (Fig. 3.7.5). Quercetin at low concentrations significantly reduced the efficacy of carboplatin at various degrees on GSH level in a compare to the carboplatin alone.

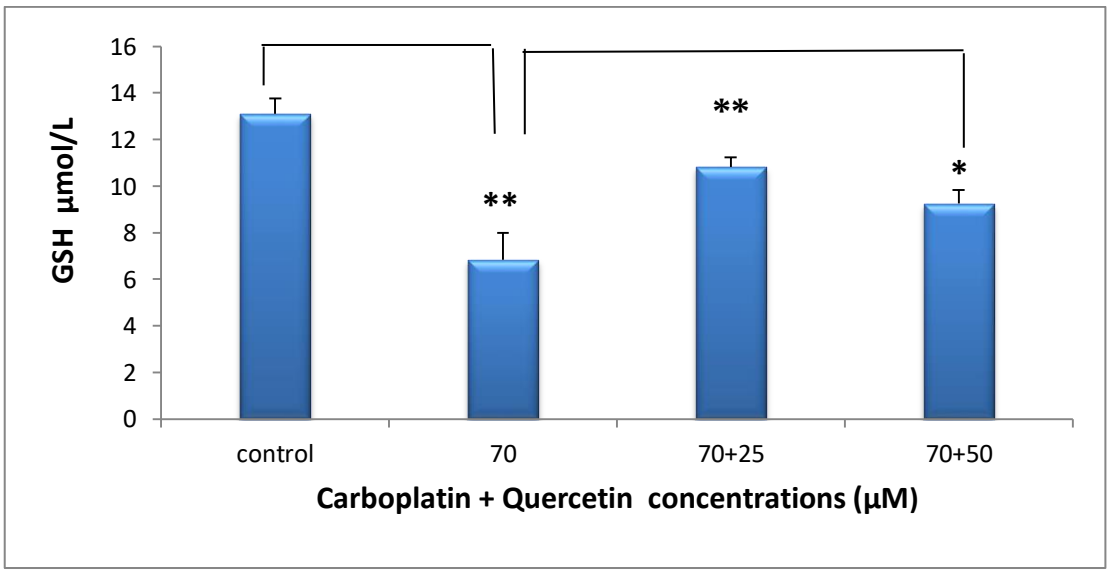


Figure 3.7.5. The combined effect of carboplatin and low concentrations of quercetin on the GSH level in SW480 cells. (*p <0.05),(**p <0.001). control: untreated cells (RPMI).

3.8. Oxidative Stress (MDA) Assessment

3.8.1. Evaluation The Effect of Quercetin on MDA Level in SW480 Cells .

The high concentrations (100µM and 200µM) of quercetin showed a significant increase ($p < 0.05$), ($p < 0.001$) respectively in MDA level, but low concentrations (25 µM and 50 µM) exhibited no effect on MDA level when SW480 cells treated with various concentration (25, 50, 100, 200) µM of quercetin in a compare to the control group after incubation for 48 hr at 37 ^{o}C (Fig 3.8.1)

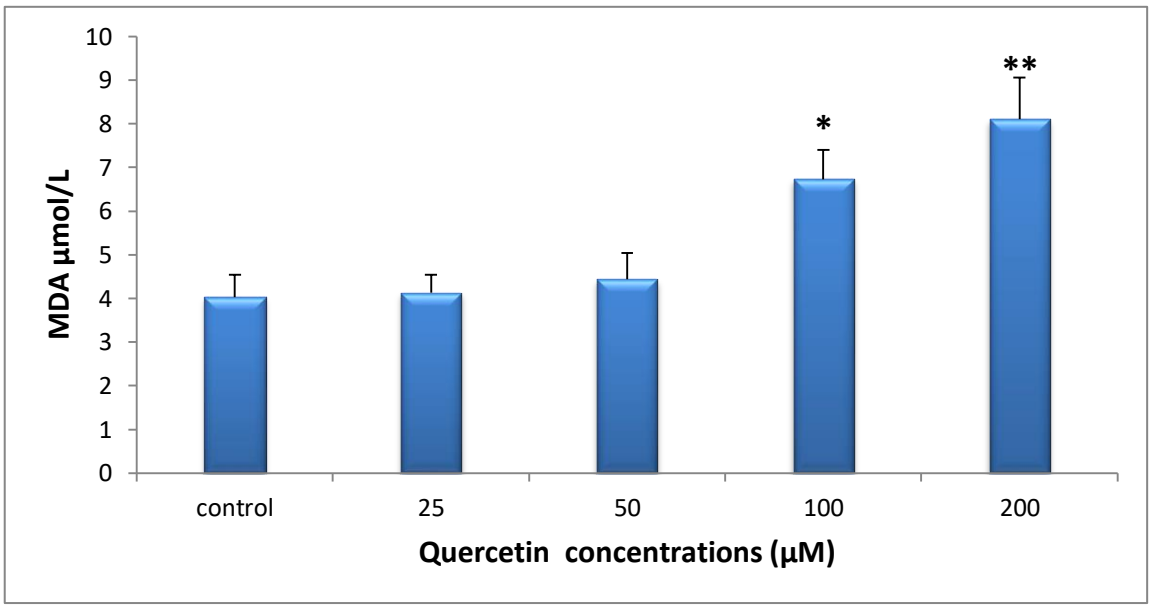


Figure 3.8.1. Effect of quercetin at various concentrations on the MDA level in SW480 cells. (*$p < 0.05$),(**$p < 0.001$). control: untreated cells (RPMI).

3.8.2. Evaluation The Effect of Doxorubicin on MDA Level in SW480 Cells

The results showed a significant ($p < 0.05$), ($p < 0.001$) increase in MDA level in concentration-dependant manner when SW480 cancer cells treated with various concentrations (2, 4, 8) µM of doxorubicin in a compare to the control group after incubation for 48 hr at 37 ^{o}C (Fig 3.8.2).

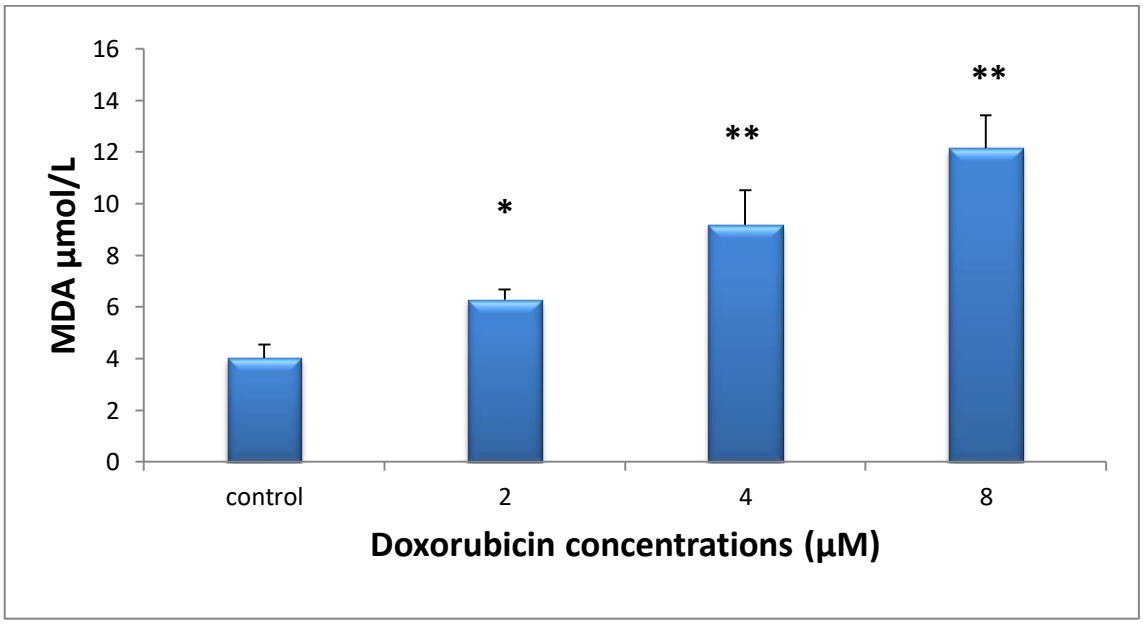


Figure 3.8.2. Effect of doxorubicin at various concentrations on the MDA level in SW480 cells. (*$p < 0.05$), (**$p < 0.001$). control: untreated cells (RPMI).

3.8.3 Evaluation The Effect of Carboplatin on MDA Level in SW480 Cells

The results showed a significant ($p < 0.05$),($p < 0.001$) increase in MDA level in concentration dependant manner when SW480 cancer cells treated with various concentrations of carboplatin (17.5, 35, 70) µM in a compare to the control group after incubation for 48 hr at 37 ^{o}C (Fig.3.8.3).

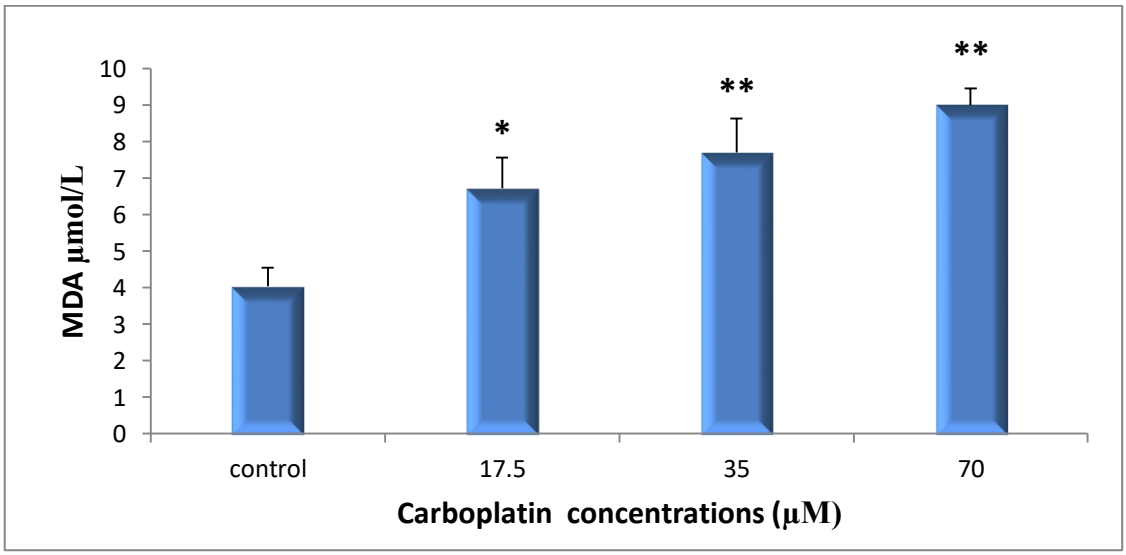


Figure 3.8.3. Effect of carboplatin at various concentrations on the MDA level in SW480 cells. (*$p < 0.05$), (**$p < 0.001$). control: untreated cells (RPMI).

3.8.4. Evaluation The Combined Effect of Doxorubicin and Quercetin on MDA Level in SW480 Cells

Doxorubicin alone exhibited significant $p<0.001$ increase in MDA level in a compare to the control group. Combination of low concentrations of quercetin (25µM) with IC50 (8µM) of doxorubicin resulted in highly significant $p<0.001$ decrease in MDA level in a compare to the doxorubicin alone and significant $p<0.05$ decrease in MDA level when SW480 cancer cells treated with 50µM of quercetin and 8µM of doxorubicin (Fig. 3.8.4). Quercetin at low concentrations significantly reduced the efficacy of doxorubicin at various degrees on MDA level in a compare to doxorubicin alone.

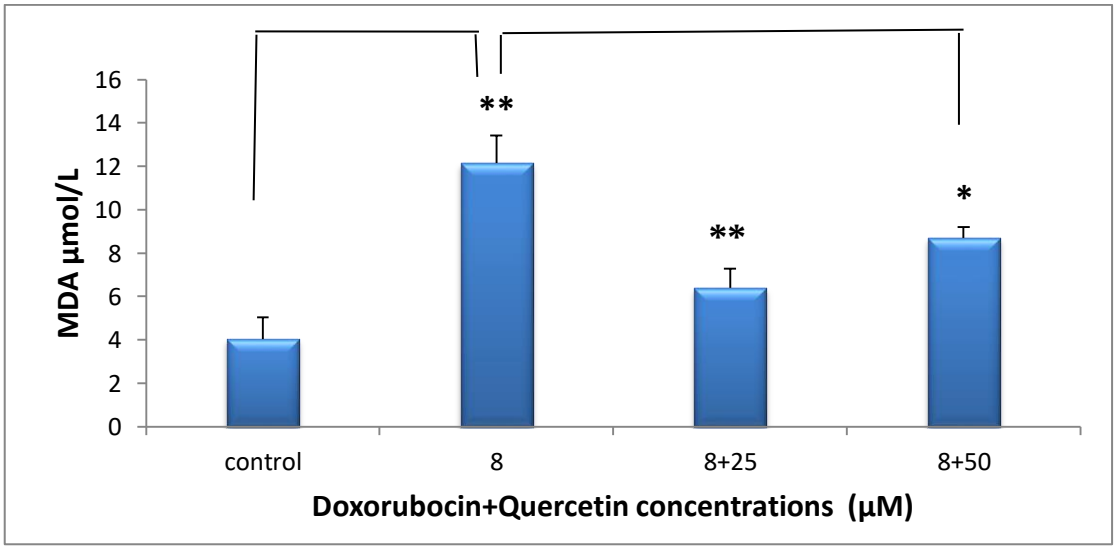


Figure 3.8.4. The combined effect of doxorubicin and low concentrations of quercetin on the MDA level in SW480 cancer cells. (*p <0.05),(**p <0.001). control: untreated cells (RPMI).

3.8.5. Evaluation The Combined Effect of Carboplatin and Quercetin on MDA Level in SW480 Cancer Cells

Carboplatin alone exhibited significant $p<0.001$ increase in MDA level in a compare to the control group. Combination of low concentrations of quercetin 25µM with IC50 (70µM) of carboplatin resulted in highly significant $p<0.001$ decrease in MDA level in a compare to the carboplatin alone and significant $p<0.05$ decrease in MDA level when SW480 cancer cells treated with 50µM of quercetin and 70µM of carboplatin (Fig. 3.8.5). Quercetin at low concentrations significantly reduced the efficacy of carboplatin at various degrees on MDA level in a compare to carboplatin alone.

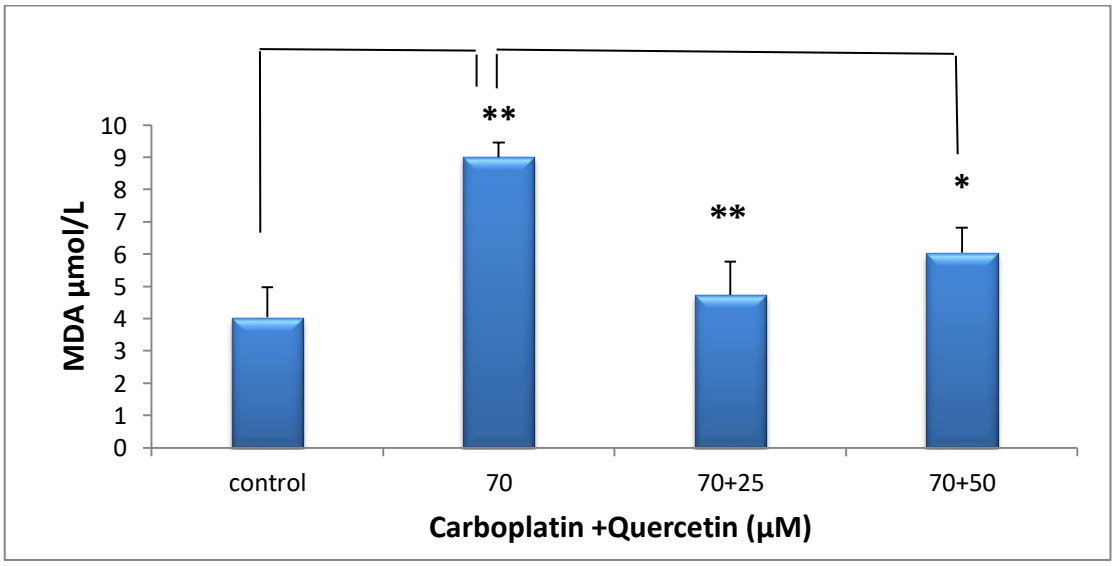


Figure 3.8.5. The combined effect of carboplatin and low concentrations of quercetin on the MDA level in SW480 cells. (*p <0.05), (**p <0.001). control: untreated cells (RPMI).

3.9. Apoptotic Effect (Caspase3)

3.9.1. Evaluation The Effect of Quercetin on Caspase3 Level in SW480 Cells

The results showed a significant ($p < 0.05$) increase in caspase 3 levels at high concentrations (100µM and 200 µM) when SW480 cancer cells treated with various concentrations (25, 50, 100, 200) µM of quercetin in a compare to the control group after incubation for 48 hr at 37 ^{o}C (Fig.3.9.1).

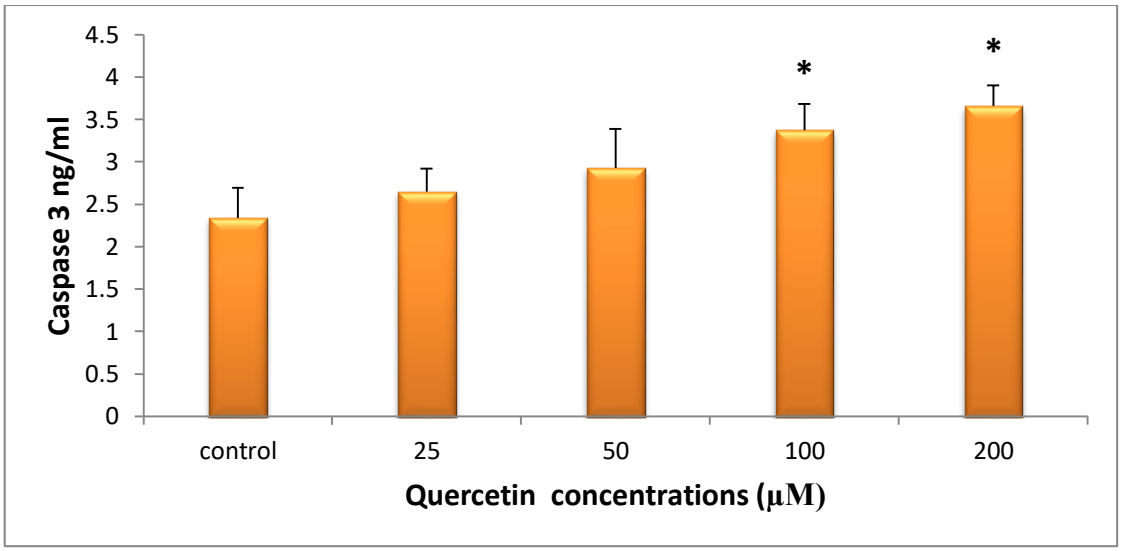


Figure 3.9.1. Effect of quercetin at various concentrations on the caspase3 level in SW480 cells. (*$p < 0.05$). control: untreated cells (RPMI).

3.9.2. Evaluation The Effect of Doxorubicin on Caspase3 Level in SW480 Cells

The results showed a significant ($p < 0.05$) increase in caspase3 level in concentrations-dependant manner when SW480 cancer cells treated with various concentrations (2, 4, 8) µM of doxorubicin in a compare to the control group after incubation for 48 hr at 27 ^{o}C. (Fig.3.9.2).

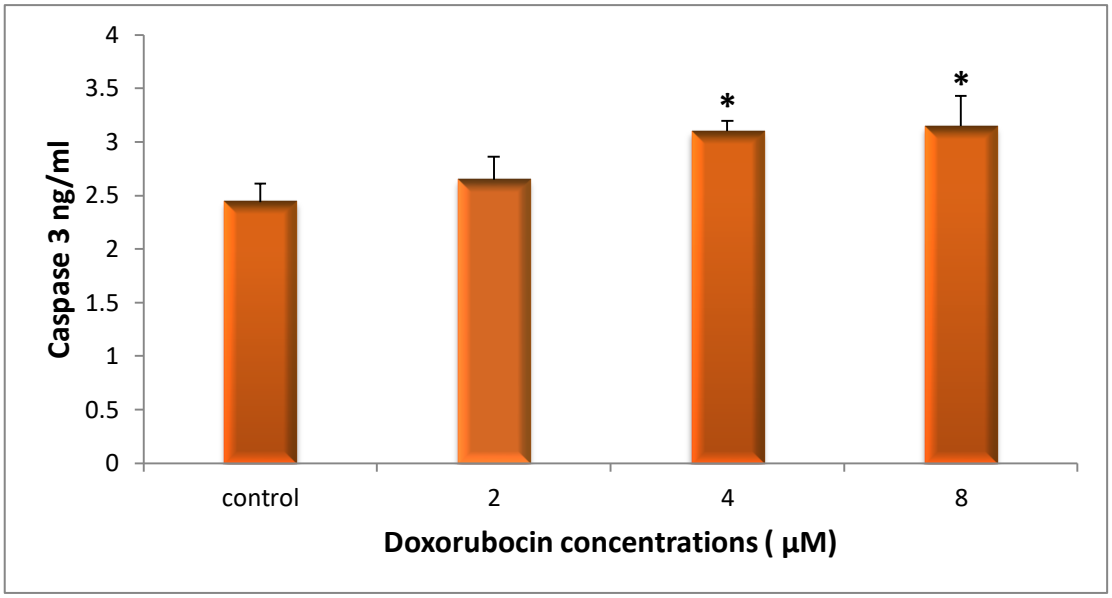


Figure 3.9.2. Effect of doxorubicin at various concentrations on the caspase3 level in SW480 cells. (*$p < 0.05$). control: untreated cells (RPMI).

3.9.3. Evaluation The Effect of Carboplatin on Caspase3 Level in SW480 Cells

The results showed a significant ($p < 0.05$) increase in caspase3 level in concentration-dependant manner when SW480 cells treated with various concentrations of carboplatin (17.5, 35, 70) µM in a compare to the control group after incubation for 48 hr at 37 ^{o}C(Fig 3.9.3).

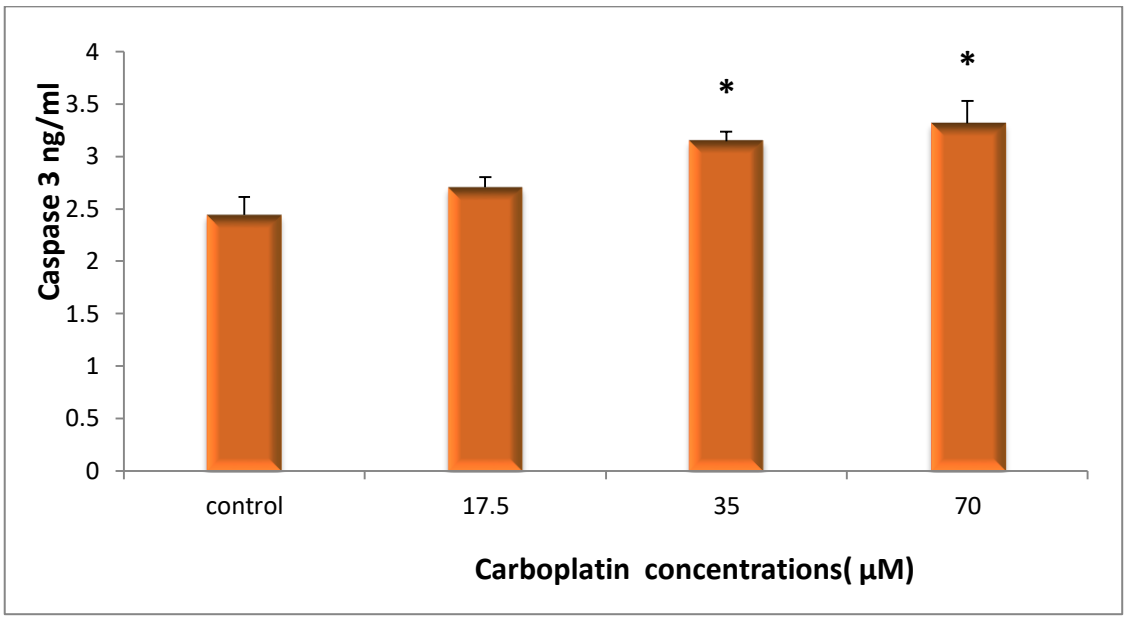


Figure 3.9.3. Effect of carboplatin at various concentrations on the caspase3 level in SW480 cells. (*$p < 0.05$). control: untreated cells (RPMI).

3.9.4. Evaluation The Combined Effect of Doxorubicin and Quercetin on Caspase3 Level in SW480 Cells

Doxorubicin alone exhibited significant $p<0.05$ increase in caspase3 level in a compare to the control group. Combination of low concentrations of quercetin (25, 50)µM with IC50 of doxorubicin (8µM) resulted in a significant $p<0.05$ decrease in caspase3 level in a compare to the doxorubicin alone (Fig. 3.9.4). Quercetin at low concentrations significantly reduced the efficacy of doxorubicin on caspase3 level in a compare to doxorubicin alone after incubation for 48 hr .

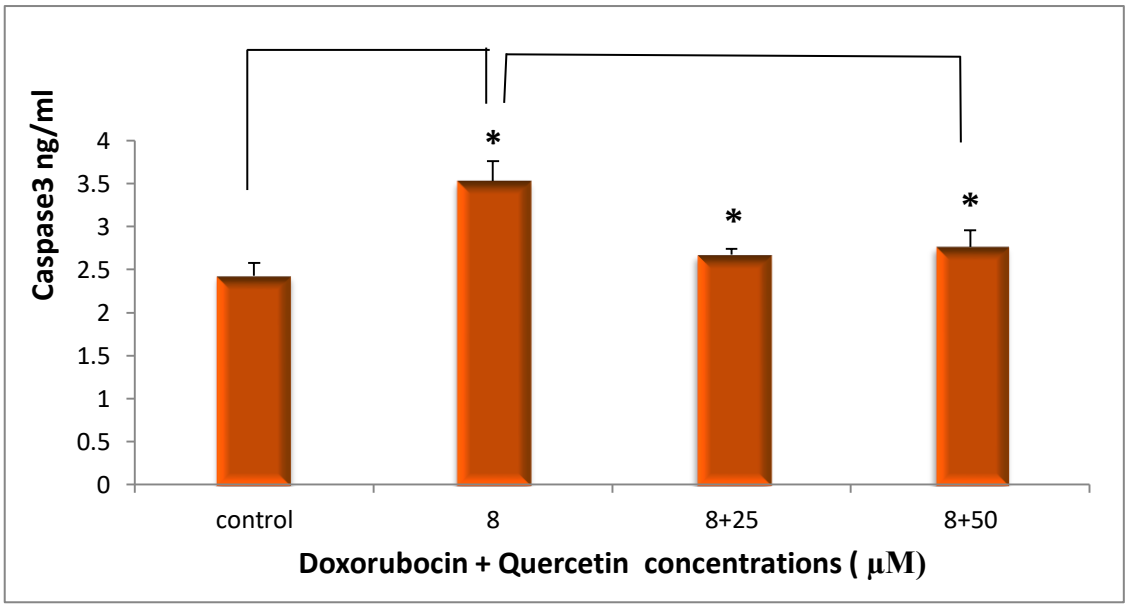


Figure 3.9.4. The combined effect of doxorubicin and low concentrations of quercetin on the caspase3 level in SW480 cells. (*p <0.05). control: untreated cells (RPMI).

3.9.5. Evaluation The Combined Effect of Carboplatin and Quercetin on Caspase3 Level in SW480 Cells

Carboplatin alone exhibited significant $p<0.05$ increase in caspase3 level in a compare to the control group. Combination of low concentration of quercetin (25µM) with IC50 of carboplatin (70µM) resulted in remarkable decrease in caspase3 level in a compare to the carboplatin alone (Fig. 3.9.5). However, there has no apparent effect on caspase3 level of carboplatin when combined with 50µM of quercetin in a compare to the carboplatin alone. Quercetin at 25µM concentration reduced the efficacy of carboplatin on caspase3 level.

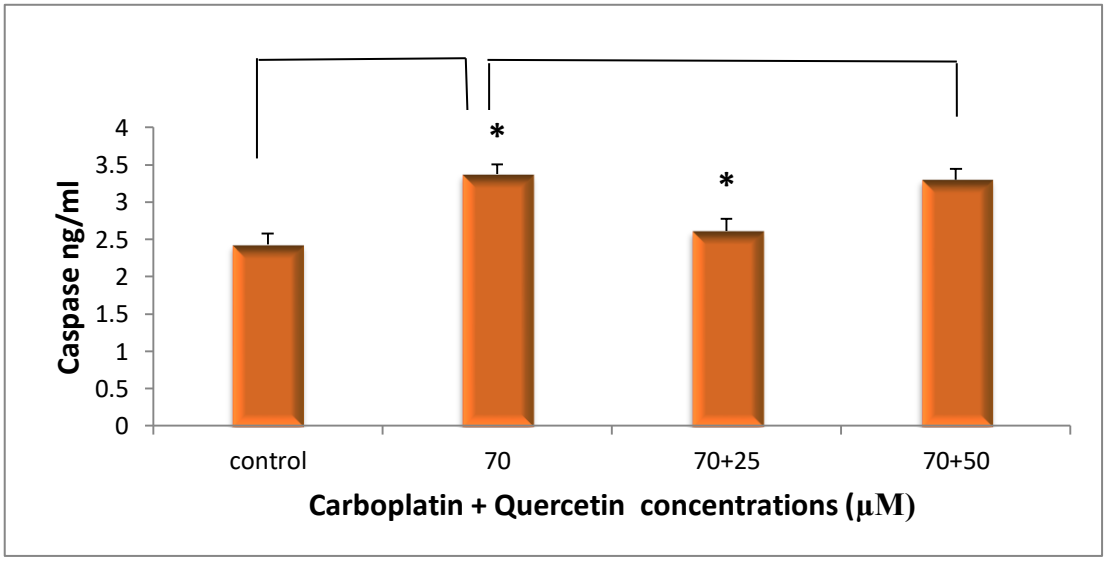


Figure 3.9.5. The combined effect of carboplatin and low concentrations of quercetin on the caspase3 level in SW480 cells. (*p <0.05). control: untreated cells (RPMI).

3.10. Anti-inflammatory Effect

3.10.1. Evaluation The Effect of Quercetin on TNF-α Level in SW480 Cancer Cells

The high concentrations (100µM and 200µM) of quercetin showed a significant increase ($p < 0.05$), ($p < 0.001$) respectively in TNF-α level, but low concentrations (25µM and 50µM) exhibited no effect on TNF-α level when SW480 cancer cells treated with various concentration (25, 50, 100, 200) µM of quercetin in a compare to the control group after incubation for 48 hr at 37°C(Fig.3.10.1).

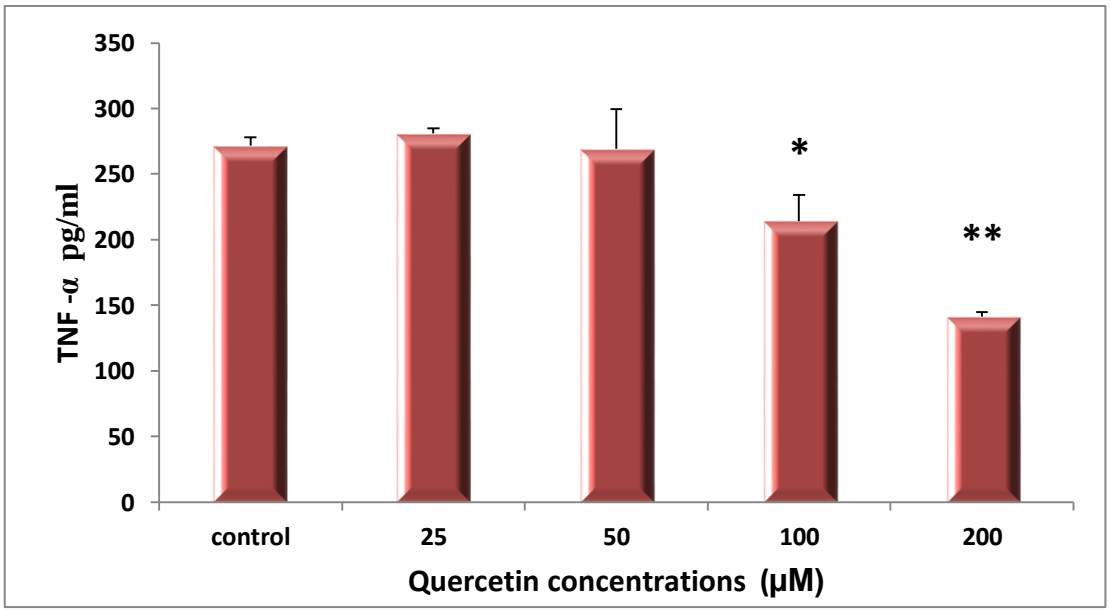


Figure 3.10.1. The effect of quercetin at various concentrations on the TNF-α level in SW480 cells. (*$p < 0.05$), (**$p < 0.001$). control: untreated cells (RPMI).

3.10.2. Evaluation The Effect of Doxorubicin on TNF-α Level in SW480 Cells

The results showed a significant ($p < 0.05$),($p < 0.001$) increase in TNF-α level in concentration-dependant manner when SW480 cancer cells treated with various concentrations of doxorubicin (2, 4, 8) µM in a compare to the control group after incubation for 48 hr at 37°C(Fig.3.10.2).

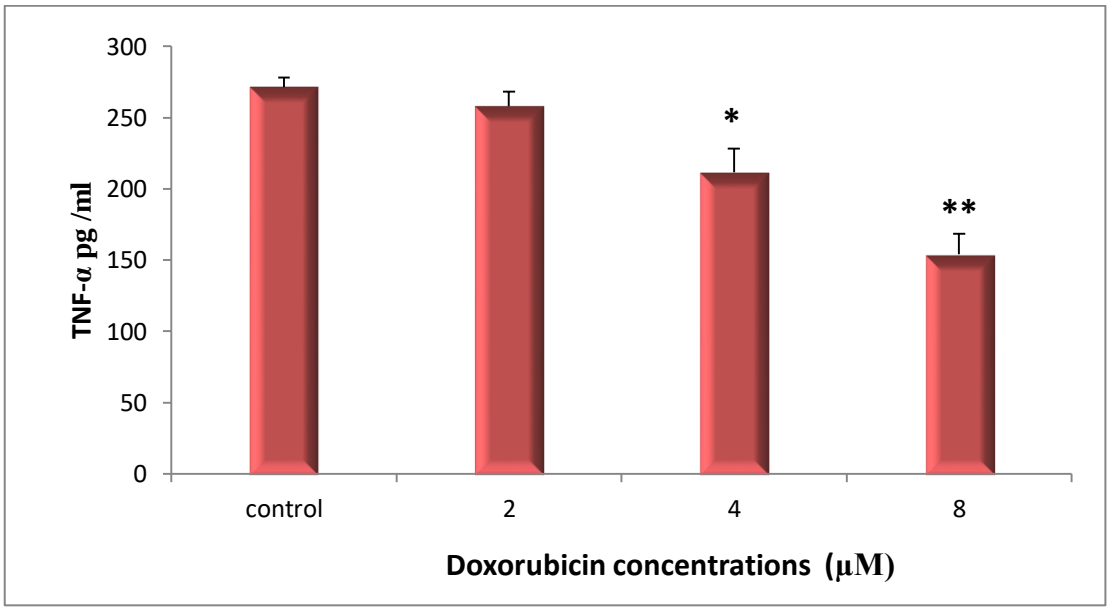


Figure 3.10.2. The effect of doxorubicin at various concentrations on the TNF-α level in SW480 cells. (*$p < 0.05$),(**$p < 0.001$). control: untreated cells (RPMI).

3.10.3.Evaluation The Effect of Carboplatin on TNF-α Level in SW480 Cells

The results showed a significant ($p < 0.05$), ($p < 0.001$) increase in TNF-α level in concentration dependant manner when SW480 cancer cells treated with various concentrations (17.5, 35, 70) µM of carboplatin in a compare to the control group after incubation for 48 hr at 37 ^{o}C(Fig.3.10.3).

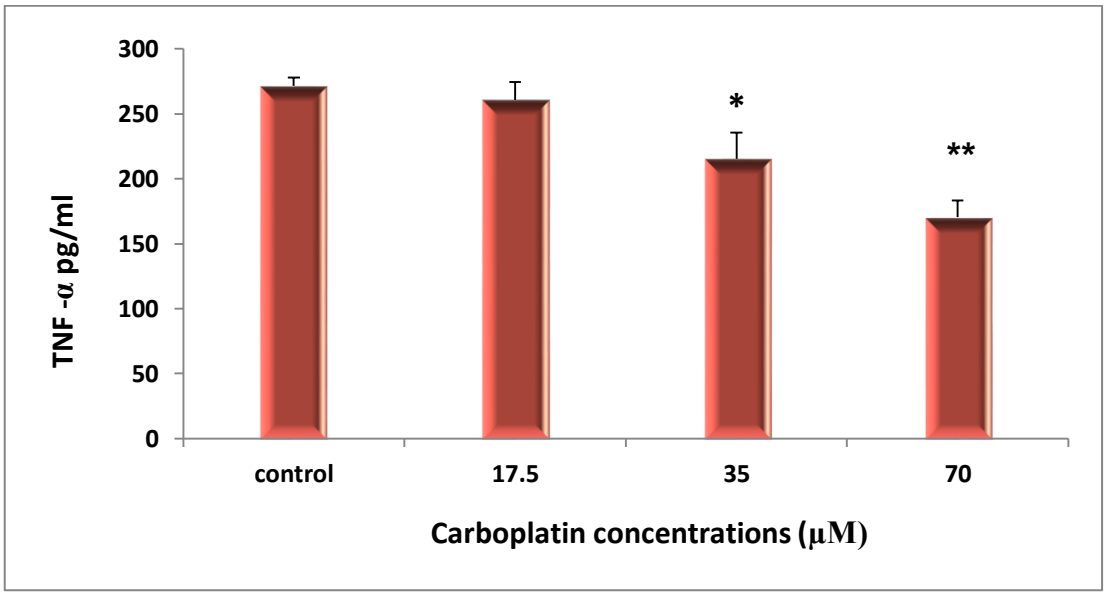


Figure 3.10.3. The effect of carboplatin at various concentrations on the TNF-α level in SW480 cells. (*$p < 0.05$),(**$p < 0.001$). control: untreated cells (RPMI).

3.10.4. Evaluation The Combined Effect of Doxorubicin and Quercetin on TNF-α Level in SW480 Cells

Doxorubicin alone exhibited significant $p<0.001$ decrease in TNF-α level in a compare to the control group. Combination of low concentrations of quercetin (25µM) with IC50 of doxorubicin (8µM) resulted in highly significant $p<0.001$ increase in TNF-α level in a compare to the doxorubicin alone and significant $p<0.05$ increase in TNF-α level when SW480 cancer cells treated with 50µM of quercetin and 8µM of doxorubicin (Fig. 3.10.4). Quercetin at low concentrations significantly reduced the efficacy of doxorubicin at various degrees on TNF-α level in a compare to doxorubicin alone.

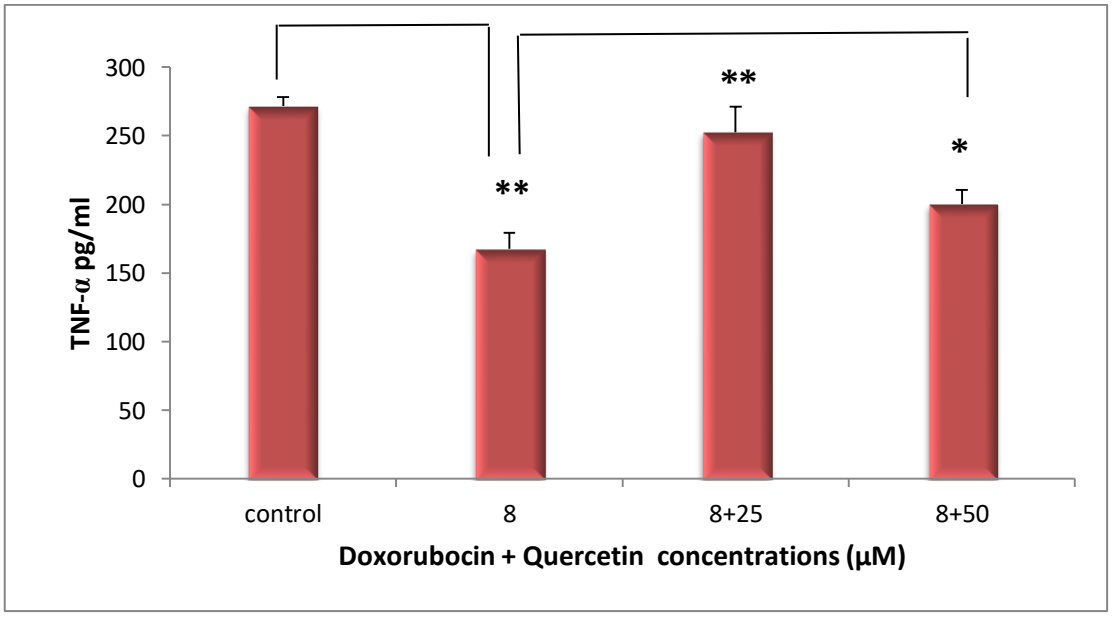


Figure 3.10.4. The combined effect of doxorubicin and low concentrations of quercetin on the TNF-α level in SW480 cells. (*p <0.05), (**p <0.001). control: untreated cells (RPMI).

3.10.5. Evaluation The Combined Effect of Carboplatin and Quercetin on TNF-α Level in SW480 Cells

Carboplatin alone exhibited significant $p<0.001$ decrease in TNF-α level in a compare to the control group. Combination of low concentrations of quercetin 25µM with IC50 of carboplatin (70µM) resulted in highly significant $p<0.001$ increase in TNF-α level in a compare to the carboplatin alone and significant $p<0.05$ increase in TNF-α level when SW480 cancer cells treated with 50µM of quercetin and 70µM of carboplatin (Fig. 3.10.5). Quercetin at low concentrations significantly reduced the efficacy of carboplatin at various degrees on TNF-α level in a compare to carboplatin alone.

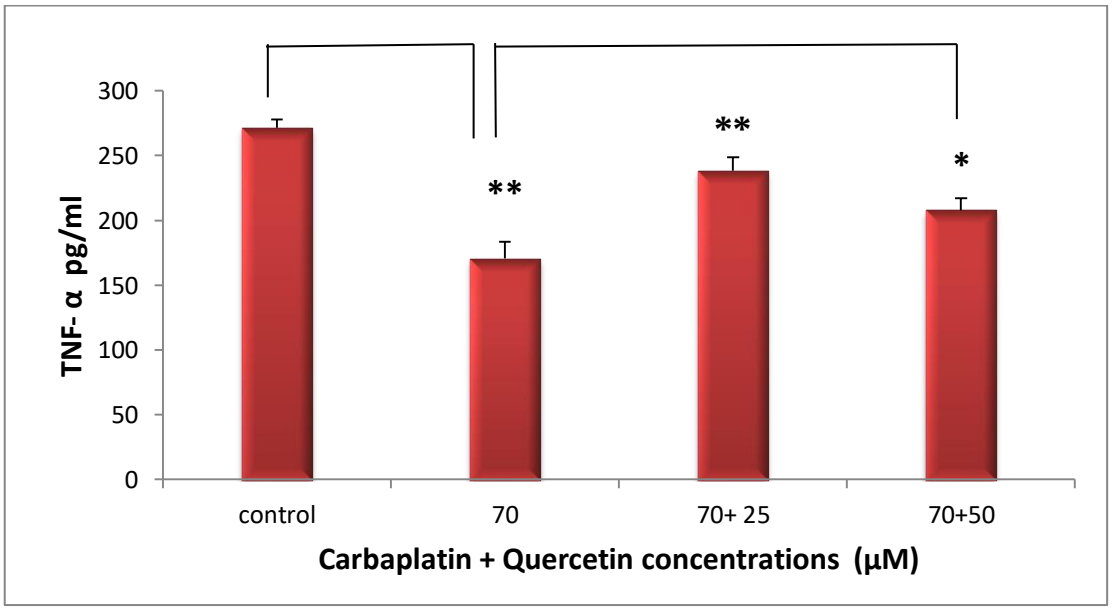


Figure 3.10.5. The combined effect of carboplatin and low concentrations of quercetin on the TNF-α level in SW480 cells. (*p <0.05),(**p <0.001). control: untreated cells (RPMI).

Chapter Four

Discussion

4. Discussion

Colorectal cancer is a third most prevalence cause of cancer related fatalities (Xi and Xu, 2021). Doxorubicin and carboplatin are most commonly used anticancer agents. It's worth mentioning that doxorubicin and carboplatin-induced side effects are mostly attributed to the formation of ROS inside the tumour and normal cells /tissues, the cause that limit their use. Quercetin is a well-known flavonoid with a powerful anti-oxidant capacity that protects several organs/tissues from the damage from oxidative stress. On the other hand, many studies have been demonstrated that quercetin have valuable antitumor effects against wide range of cancers included breast, lung, gastric, ovarian, colorectal and hepatic cancer (Hisaka *et al*., 2020). Herein, we further studied the anticancer impact of quercetin and the effect of quercetin ROS scavenging capacity on carboplatin- and doxorubicin- induced cytotoxicity against SW480 colorectal cancer cells.

4.1. Cytotoxic Effect of Quercetin, Doxorubicin, Carboplatin and their combinations on SW480 Cells

The anti-proliferative effects of quercetin were initially assessed on normal cells for 48 hr to determine the effective concentrations of quercetin that kill cancer cells without producing cytotoxic effect on normal cells. Consequently, these concentrations were selected to be used in the combination with IC50 of doxorubicin and carboplatin in treatment of SW480 cancer cells. Results showed that quercetin produced significant cytotoxic effect on normal cells only at high concentrations 100µM and 200µM (Fig.3.1). On the other hand, quercetin significantly decreases proliferation of SW480 cancer cells in concentrations-dependant manner after incubation of 48 hr (Fig.3.2a). However,

quercetin at concentrations 25µM and 50µM exhibited significant decrease in cancer cells without induced apparent cytotoxic effect on normal cells ; as a result, these concentrations were used in subsequent experiments in combination with chemotherapies in SW480 cancer cells. These findings were in agreement with Bhatiya *et al* (2023) study who demonstrated a strong cytotoxic effect of quercetin in the normal (epithelial) cells and colon cancer cells proliferation in vitro study.

Yang *et al* (2016) demonstrated that quercetin significantly inhibit HT-19 colon cancer cells proliferation in concentration-dependant manner.

Doxorubicin is an effective and mostly utilized anthracycline chemotherapeutic agent in many human cancers. The major anticancer activities of doxorubicin are DNA intercalation, topoisomerase II inhibition and free radical formation leading to cell death or growth inhibition (Kalyanaraman, 2020). Present results showed that doxorubicin reduced viability of cells in dose- dependent manner (Fig 3.3a). This study augmented previous study indicated that doxorubicin can decrease proliferation of HT29 colon cancer cells in a concentration-dependent manner (Atashpour *et al*., 2015).

Platinum anticancer agents, involving cisplatin and carboplatin, have been extremely used as chemotherapy to treat different cancer types. The molecular mechanisms behind carboplatin anticancer actions depend upon binding to DNA in the nucleus, which blocks synthesis of DNA, resulting in apoptosis or necrosis (Ho GY *et al* ., 2016). Findings in this study demonstrated that carboplatin decreased cell viability in dose dependant manner (Fig.3.4a).

Importantly, combination of low concentration of quercetin 25µM with IC50 of doxorubicin and carboplatin offered a decrease in the cytotoxicity effect of doxorubicin but less extent in the cytotoxicity of carboplatin. Additionally, quercetin at 50 µM decreased the cytotoxicity of doxorubicin but exhibited no apparent effect on the cytotoxicity of carboplatin (Fig 3.5 and 3.6). This means that combination of quercetin and doxorubicin might not be suitable in the treatment of colorectal cancer cells. Quercetin at low concentration displayed a significant decrease in anti-proliferative effect of doxorubicin and carboplatin on SW480 CRC cells in a compare to each drug alone.

Controversy, Chen *et al* (2021) demonstrated that quercetin has been used with a variety of chemotherapeutic drugs, including doxorubicin and Platinum anticancer agents. Due to its lipophilic nature, quercetin can cross cell membranes and produce synergistic effect with doxorubicin and other chemotherapeutic drugs .

4.2. The Effect on Oxidative Stress Biomarkers (GSH and MDA)

Findings of the present study showed that quercetin induced significant decrease in GSH level in high concentrations when compare to the control group (Fig.3.7.1) and significant increase in MDA level in high concentrations (Fig.3.8.1). This data explain that quercetin at high concentration induce oxidative stress and subsequently produce highly significant cytotoxic effect. Moreover, present findings confirmed the idea that programmed cell death comes from ROS over production and antioxidant defences suppression, which is thought to be one of the key mechanisms underlying quercetin antitumor action (Di Bella *et al*., 2013) .

Reactive oxygen species is a crucial regulator of cellular signal transduction. According to previous studies, ROS exert dual function in the cellular process in a concentration-dependent manner since in moderate levels they play a role in cancer onset and development through inflammation and DNA mutation, while in higher levels they act as an anticancer agent by induction of apoptosis (Lin *et al*, 2018) .

Free radicals can attack unsaturated lipids in a cell and result in a chain reactions of free radicals formation , these reactions are terminated by the production of lipid breakdown products, lipid alcohols, aldehydes and malondialdehyde (MDA). Therefore, measurement of MDA concentration is a common method for determination of primary toxic effect caused by free radicals in experiments (Mohideen *et al*., 2021).

Glutathione is an essential intracellular antioxidant and regulator of cellular redox status, protects cells from xenobiotic, ROS , and lipid peroxides (Kennedy *et al*., 2020). Elevated GSH levels in tumour cells are linked to tumour progression and increased resistance to chemotherapeutic drugs, despite the fact that in healthy cells they are essential for the elimination and detoxification of carcinogens (Kim *et al*., 2019). Recently, several innovative drugs have been designed directly target the GSH antioxidant system in tumours to enhance response and lower treatment resistance, this has led to the conclusion that ROS modulators may be used as primary cancer inhibitors to develop therapy strategies (Srinivas *et al*, 2019).

Based on the results of previous studies, anticancer properties of doxorubicin, at least in part, mediated through the induction of oxidative DNA damage (Wallace *et al*., 2020), which is in line with those of the present study. Findings of the present study indicated that doxorubicin

significantly decrease GSH level in dose- dependent manner (Fig.3.7.2), and that MDA level significantly increased in SW480 cells after exposure for 48 hr to doxorubicin in dose- dependent manner (Fig 3.8.2).

Doxorubicin causes oxidative damage in cancer cells and it relies on redox cycling associated with the release of iron from cells, where doxorubicin inhibit nucleic acid synthesis and induce the accumulation of Fe^{2+} in cells, which can generate large amounts of O2− and OH− through the fenton reaction and cause various forms of oxidative stress damage to the cells (Martins-Teixeira and Carvalho, 2020). In cancer cells, the oxidative damage pathway has been regarded as a key anticancer strategy.

Combination of low concentrations of quercetin with IC50 of doxorubicin exerted less substantial cellular damage in SW480 cells than doxorubicin alone. Importantly, this combination led to significant increase in antioxidant level (GSH) (Fig.3.7.4 and significant decrease in MDA level of doxorubicin (Fig.3.8.4), which mean that quercetin at low concentrations reduced the oxidative effect of doxorubicin.

Similarly, Henidi *et al* (2020) demonstrated that quercetin elucidated vascular protective effects (due to its anti-oxidant activity) but ameliorated doxorubicin-induced anti breast cancer properties against MCF-7 and MDA-MB-231 cell lines with profound and moderate antagonistic interaction, respectively.

Doxorubicin and anthraquinones in general have a unique chemical structure, which is thought to be responsible for the intracellular ROS release phenomena (Aniogo *et al*., 2017). The capacity of quercetin to quickly (immediately) scavenge ROS is a potential explanation for the antagonistic interaction between doxorubicin and

quercetin in current work. Quercetin may have an intracellular ROS-scavenging activity that reduces the intracellular active form of doxorubicin by scavenging intracellular ROS caused by doxorubicin.

Controversy , Staedler *et al* (2011) demonstrated that doxorubicin and quercetin synergistically suppressed cell proliferation and promoted apoptosis by lowering GSH in breast cancer cell lines.

Data in this study indicated there were significantly decrease in GSH and increase in MDA level in dose- dependent manner when SW480 cells incubated with carboplatin for 48 hr (Fig.3.7.3) and (Fig.3.8.3) respectively. The results explain that carboplatin may causes oxidative stress and exert cytotoxic effect. Cheng *et al* (2008) reported that carboplatin could induce oxidative stress to produce ROS, which in turn promoted apoptosis in cardiomyocytes.

Additionally, He JP *et al* (2018) demonstrated that carboplatin exerts promising antitumor effects in laryngeal squamous cell carcinoma (LSCC), inhibits cell proliferation and migration, and promotes apoptosis through the excessive expression of ROS in LSCC cells.

Combination treatment of quercetin at low concentration with IC50 of carboplatin exhibited a significant reduction in oxidative stress that associated with carboplatin (increase in GSH and decrease in MDA level) in compare to the carboplatin alone (Fig.3.7.5) and (Fig.3.8.5) respectively. Previous studies indicated that cancer cells could use endogenous GSH to chelate platinum complex (Pt) chemotherapies and create inactive GSH-Pt adducts, which can be selectively excreted out by membrane transport proteins and decrease its toxic impact on cancer cells (Zhao *et al*., 2015) ; hence, an increase in the level of GSH due to

low concentration of quercetin hindered the cytotoxic effect of carboplatin on SW480 cancer cells in the current study.

At low concentrations, quercetin behaved as antioxidant agent, where it has been noticed to reduce the intracellular ROS level that associated with the chemotherapies and excess the endogenous expression of antioxidants , proposing a mechanism through which quercetin attenuate the effects of anti-neoplastic medications so, quercetin supplementation while receiving chemotherapy can result in resistance to chemotherapies.

Jana *et al* (2018) demonstrated that quercetin exhibit biphasic and concentration-dependent impact, when it applies at low concentrations , it works as an antioxidant and exhibits chemopreventive effects. However, when quercetin present in large concentrations, it behaves as a pro-oxidant and may exhibit chemotherapeutic benefits.

4.3. Apoptotic Effect

The results exhibited a significant increase in apoptotic protein caspase3 excretion when SW480 cells were treated with high concentrations of quercetin (Fig.3.9.1). Present study is in line with Na S *et al* (2022) study who demonstrated that quercetin therapy accelerates the apoptosis of SW480 cancer cells because it increase the expression of the pro apoptotic protein caspase-3. Other previous study demonstrated that quercetin induces pro-apoptotic signalling pathways, which lead to cell death (Ma YS *et al*., 2018).

Caspase-3 is an essential factor in the execution of apoptosis and cleaved caspase-3 is an activated form of caspase-3 (Pfeffer and Singh, 2018). As previous mentioned, apoptosis is known to involve a number

of different potential pathways; extrinsic apoptotic pathway and intrinsic apoptotic pathway which is interested in this study, however, the two pathways result in caspase-3 activation, then endonucleases finally cause intranucleosomal DNA fragmentation, and the last stages of apoptosis take place (Hounsell and Fan, 2021).

Teekaraman *et al* (2019) demonstrated that quercetin at high concentration prompt the intrinsic pathway by elevating the level of intracellular ROS and Ca^{2+} which causes depolarization of mitochondrial membrane potential that lead to release of cytochrome c and activation of caspase-3 .

Doxorubicin offered a significant increase in caspase3 level in high concentrations (Fig.3.9.2). Pilco-Ferreto and Calaf (2016) demonstrated that doxorubicin enhance apoptosis by down regulation of Bcl-2 protein expression (anti-apoptotic protein) and up regulating of Bax, caspase-8 and caspase-3 (pro apoptotic protein) in breast cancer cell line.

Rawat *et al* (2021) demonstrated that doxorubicin induces apoptosis through activation of caspase-3, suggesting that apoptosis has an important role in the progression of cardiomyopathy due to doxorubicin.

Previous study demonstrated that doxorubicin finally enhance oxidative stress. It result in an opening of mitochondrial membrane permeability transition pore of the mitochondrial membrane and exert of pro apoptotic proteins involving cytochrome c from the mitochondrial matrix. Since the membrane potential could not be adequately managed in such a situation, this release of cytochrome c is understood to be brought on by mitochondrial impairment. Cytochrome c triggers caspase-3 and caspase-9, which causes apoptosis (Wenningmann *et al*., 2019).

However, the combination treatment of quercetin with IC50 of doxorubicin exhibited significant decrease in caspase3 level in a compare

to the doxorubicin alone (Fig. 3.9.4). Quercetin at low concentrations decreased the level of oxidative stress ; as a result, cytochrome c release was reduced that led to less caspase-3 activation and subsequently less apoptosis occurred.

In present results, carboplatin significantly promoted caspase3 level in dose - dependent manner (Fig.3.9.3) . It is currently accepted that the anticancer effectiveness of platinum medicines (carboplatin) is mostly due to covalent binding of the platinum core to DNA. These platinum DNA adducts have the ability to trigger apoptosis by inducing cellular processing (Johnstone *et al*., 2016).

Shen *et al* (2018) research demonstrated that when cells expose to carboplatin, excessive amounts of cytosolic Ca^{2+} has been first produced in HN 3 Laryngeal carcinomas cells. This has been followed by the development of mitochondrial depolarization and oxidative stress, which result in release of apoptosis triggering factor later on.

Al-Khayal *et al* (2020) demonstrated that platinum complex significantly increase ROS generation and depletion of glutathione in colorectal cancer cells. Furthermore, platinum complex increase cytochrome c release into cytosol and enhanced cleavage of poly (ADP-ribose) polymerase (PARP) (a protein that included in several cellular processes like genomic stability , DNA repair and programed cell death) leading to activation of intrinsic apoptotic pathway. Cytochrome c release into cytosol leads to caspase-9 activation which in turn activates caspase3. In this study Caspase 3 was detected in carboplatin -treated colorectal cancer cells, it found that carboplatin induced caspase-3 activation in concentration dependent manner.

Combination treatment of quercetin at 50µM with IC50 of carboplatin drug exerted no change in caspase3 level in compare to the carboplatin alone (Fig 3.9.5). This result explain that quercetin at 50 µm

reduce the oxidative stress that associated with chemotherapy and exhibit no change in apoptotic activity of the drug. This findings in line with previous study demonstrated that a natural flavonoid quercetin had been shown to afford nephroprotection by reducing cisplatin-associated oxidative stress without affecting its antitumor activity (Sánchez-González *et al*., 2017). Because carboplatin is an analogue of cisplatin, it exhibited a similar effect on cancer cells when combined with quercetin in current study.

Quercetin at 25 µM concentration displayed a significant decrease in caspase 3 level and subsequently decrease in cell viability in a compare to the carboplatin alone.

Li N *et al* in 2014 demonstrated that in contrast to the pro-apoptotic impact of high concentration (40 µM–100 µM) of quercetin, low concentrations (5 µM–30 µM) of quercetin result in different degrees of mitigation of cisplatin- induced cytotoxicity. The same of anti-apoptotic impact were noticed when quercetin was combined with other anti-neoplastic drugs: Taxol (plant alkaloid), Pirarubicin (analogue of doxorubicin) and 5-Fu in ovarian cancer cells.

The combination treatment of quercetin at concentrations 50µM with 70µM of carboplatin reduce the oxidative stress that associated with carboplatin, and subsequently the side effects, without change its cytotoxicity, and thereby might promise a therapeutic regimen in promoting the clinical efficacy of the treatment of patients with colorectal cancer.

4.4. Anti-Inflammatory Effect

Data that present in (Fig. 3.10.1) showed the effects of different concentrations of quercetin on TNF-α in SW480 cancer cells at 48 hr of

incubation as quantitated by elabsance TNF-α ELISA kit . Quercetin at 48 hr produced a significant decrease in TNF-α in high concentrations.

These data suggest that quercetin significantly modulated TNF-α concentration. We hypothesize that quercetin exerted anti-inflammatory effects by inhibiting the endogenous production of the pro-inflammatory cytokine TNF-α and that mediated through the regulation of NF-κβ.

Nair *et al* (2006) prepared direct evidence that quercetin induce anti-inflammatory impact on human peripheral blood mononuclear cells (PBMC), which are mediated by the inhibition of the proinflammatory cytokine TNF-α via modulation of NF-κβ. The key inflammatory pathway, NF-κB is triggered by linking of TNF-α to TNF receptor (TNFR1) which induce tumour cell proliferation , apoptosis inhibition, and induces the tumour angiogenesis ability and the potential of invasion and metastasis (Tang *et al*., 2017). Consequently, the chemotherapeutic agents that target these factors of inflammatory and pathways will act as a means to manage or prevent development of cancer.

Boesch-Saadatmandi *et al* (2011) proved that quercetin significantly inhibited the increase of TNF-α in Raw264.7 cells (monocyte /macrophage-like cell line) that triggered by lipopolysaccharide, which is an essential trigger of inflammatory response, demonstrating that it has a perfect anti-inflammatory impact in vitro.

Doxorubicin and carboplatin produce significant decrease in TNF-α in high concentrations (Fig. 3.10.2 and 3.10.3). The combination treatment show significant increase in TNF-α level, in various degrees, in compare to each drug alone (Fig.3.10.4 and 3.10.5). TNF-α is one of the key pro inflammatory cytokines included in the pathogenesis of chronic inflammatory diseases and is modulated via oxidative stress (calamine,

2003). As previous mentioned, based on the level of ROS, various redox-sensitive transcription factors are triggered and coordinate diverse biological responses. A low level of sustained oxidative stress predominantly activate the antioxidant transcription factor NrF2 (He *et al*., 2020), whereas an intermediate level of ROS stimulate an inflammatory response via the activation of NF-κB by TNF-α. Finally, high amount of oxidative stress induce the interruption of electron transfer and biomolecular damage eventually lead to apoptosis or necrosis (Fabiola *et al*., 2012).

Heiko *et al* (2016) demonstrated that deregulation of TNFR1 signalling pathway is linked to inflammatory diseases, low concentrations of ROS have essential roles in regulating pathways such as TNFR1 signalling that result in cell survival, whereas excessive concentrations of ROS ultimately cause bimolecular damage and death of cell, that explains why co-treatment of quercetin and chemotherapies in the current study led to increase in TNF-α level of each doxorubicin and carboplatin.

Present findings suggest that quercetin can inhibit the cytokine TNF-α, which may be of clinical importance in host defence mechanisms against several infections. A reduce in production of endogenous TNF-α because of quercetin proves that flavonoids have the ability to modulate the immune response and have prospective anti-inflammatory activity (Nair *et al*., 2006).

Conclusions and Recommendations

Conclusions

1. Quercetin, at high concentrations, behaved as pro oxidant agent because it dramatically induced oxidative stress and consequently, promotes apoptosis in SW480 cells.
2. Low concentrations of quercetin attenuated the effects of anti-neoplastic agents on SW480 cells.
3. Quercetin at concentrations 50µM reduce the oxidative stress that associated with carboplatin, without change its cytotoxicity.
4. Quercetin had the capacity to modulate the inflammatory pathway negatively.

Recommendations

1. In vivo and clinical research must be done to ascertain the short- and long-term effects of quercetin, alone and in combination, on the effectiveness of cancer chemotherapy and the development of side effects.
2. Explore another combinations and another tumour suppression pathways brought on by quercetin and other chemotherapies.

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