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An in Vitro Study of the Anticancer and Antioxidant Effects of Ethanolic Leaf Extract of *Anthurium andraeanum*

A Thesis

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

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

وَلَمَّا بَلَغَ أَشُدَّهُ وَاسْتَوَىٰ آتَيْنَاهُ حُكْمًا وَعِلْمًا ۚ وَكَذَٰلِكَ نَجْزِي الْمُحْسِنِينَ ﴿١٤﴾

صدق الله العظيم

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Certification

We certify that this thesis entitled "**An in Vitro Study of the Anticancer and Antioxidant Effects of Ethanolic Leaf Extract of *Anthurium andraeanum***" prepared by (**Noor Mohammed Rasool**) under our supervision at the department of Pharmacology. College of Medicine, University of Babylon (Iraq) in partial fulfillment of the requirements for the master degree of sciences in pharmacology and toxicology.

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Dedication

OUR GOAL IN SCIENCE IS TO DISCOVER UNIVERSAL LAWS OF NATURE

That pursuit fills me with wonder.

I dedicate this research project to my brother Mustafa, whose soul rests in heaven

And indeed, to the prayers of my beloved grandmother, parents for their great support even when things were so tough for their constant kept on encouraging me to work extra hard,

My brothers and friends for

Moral support and encouragement throughout my studies and lastly, I thank and pray to God for my success

Noor M. Rasool

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Summary

Plant chemical constituents are crucial in the treatment of various diseases and disorders due to their impact on cell growth and repair, so the aims of this study were to identify the cytotoxicity of *Anthurium andraeanum* leaf extracts (alcoholic extract) mammalian cell lines; Vero, LNCap prostate cancer cell line, SW480 colon cancer cell line and A549 lung cancer cell line. The second aim was to study the antioxidant effect of alcoholic leaf extract of *A. andraeanum* plant on LNCap and SW480cell lines. The last aim was to evaluate the effect of *A. andraeanum* extract on the anticancer activity of doxorubicin and carboplatin chemotherapeutic drugs.

Vero, LNCaP, SW480, A549 lung cancer cell line was seeded in a tissue culture 96-well plate and treated with alcoholic leaf extracts in different concentrations (1000, 500, 250, 125, 62, 31) µg/ml for each of the extracts and incubated for 24 hours. Then, the (3-(4,5 Dimethylthiazole-2-yl)-2,5-diphenyl-2Htetrazolium bromide) MTT assay was performed.

The cytotoxicity results revealed that leaves extract of *A. andraeanum* caused a significant decrease in the viability for Vero, SW480, and A549 cell lines at higher concentrations (more than 125 µg/ml) and more than (250 µg/ml) for LNCap cell line in comparison to the control groups.

The antioxidant activity was evaluated using Reducing Antioxidant Capacity (CUPRAC) method. Prostate cancer (LNCap) and colon cancer (SW480) cell lines seeded in tissue culture plates were treated with plant extract at concentrations of (1000 ,250 ,31) µg/ml. After incubation for 24 hr, supernatants were examined to determine the antioxidant activity. The results of this experiment show that there was a significant increase in the antioxidants level for both cell lines at concentrations of (1000, 250) µg/ml

for colon cancer (SW480) cell lines and (1000) µg/ml for Prostate cancer (LNCap) in comparison with the control groups.

The third part of this study involved measuring the effect of plant extract on the anticancer activity of doxorubicin and carboplatin using SW480 and LNCap cancer cell lines as models. The effect of plant extract on each drug was evaluated in separated 96 wells plate divided into 8 groups; one negative control group, one positive control group treated with 15 µg/ml doxorubicin, and six serial dilutions (started from 1000 to 31 µg/ml) of plant extract with a constant concentration of doxorubicin (15 µg/ml). All plates were incubated for 24 hours at 37C, and then the MTT(3-(4,5-Dimethylthiazole-2-yl)- 2,5-diphenyl-2Htetrazolium bromide) cytotoxicity assay was performed.

The result for this experiment showed a decrease in the cytotoxic effect of doxorubicin on colon cancer cell at all concentrations used in comparison to positive and negative control groups. The viability of LNCap was drop when treated with plant extract concentration of (1000) µg/ml.

The same experiment was repeated for carboplatin using constant concentration (100 µg/ml) for positive control and for combination groups. The result for this experiment showed an increase in the cytotoxic effect of carboplatin on colon cancer cell and prostate cancer cell at all concentrations used in comparison to positive and negative control groups.

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Abbreviations	Meaning
5-FU 5	5-fluorouracill
a549	Human lung cancer
Apaf-1	Apoptotic peptidase activity factor 1
AR	androgen receptor (AR
Bax	B cell associated X protein
Bcl	B cell lymphoma protein
BM	Bone marrow
BRs	brassinosteroids
BsAbs	bispecific antibodies
BT	Brachytherapy
CAM	complementary and alternative medicine
CAR	chimeric antigen receptor
CRC	Colorectal cancer
DDW	deionized distilled water
DISC	death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
DPPH	1,1diphenyl-2picryl hydrazyl (free radical)
DR	Death receptor
DU-145	prostate cancer cell line DU-145
EBRT	external-beam radiation therapy
EDTA	2 Trypsin
EGCG	Epigallocatechin Gallate

ER	Endoplasmic reticulum
ET	electron transfer
FADD	Fas- associated death domain
HAT	hydrogen atom transfer
Hep G2	Human liver cancer
HIF-1α	Hypoxia inducible factor
ILD	Interstitial lung disease
LNCaP	Type of prostate cancer cell line
LUTS	lower urinary tract symptoms
MCF-7	Human breast cancer cell line
MDR	multi-drug resistance
mmol	Millimole
NH_4Ac	Ammonium acetate buffer
nm	Nanometer
Nrf2	Nuclear factor
NSCLC	non-small cell lung cancer
OXPHOS	Oxidative phosphorylation
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffer Saline
PC	Prostate cancer
PC3	Type of prostate cancer cell line

PKM2	Pyruvate kinase
A549	Lung cancer cell line
PSA	Prostate-specific antigen
PWR-1E	Normal prostate cell line
ROS	Reactive oxygen species
SCLC	Small cell lung cancer
SKQ1	Mitochondria targeted antioxidant
SW 480	Type of Colon cancer cell line
TACAs	Tumor associated carbohydrate antigens
TAS	Total antioxidant status
Tet 1	Ten –eleven translocation enzymes
TNFR1	Tumor necrosis factor receptor 1
TNM	Tumor node metastasis
TOS	Total oxidative status
TRADD	Tumor receptor associated death domain

Chapter one

Introduction

&

Literature review

1. Introduction and literature review

1.1 Introduction

Medicinal plants are useful for healing and curing human diseases because of the presence of hundreds of phytochemical constituents. Phytochemicals are naturally occurring in medicinal plants leaves, stems and roots that have defense mechanism and protect from various diseases. Phytochemicals are primary and secondary compounds. Chlorophyll, proteins and common sugars are included in primary constituents while secondary compounds include terpenoid, alkaloids and phenolic compounds(Wadood, 2013).

Phytochemicals and derivatives present in plants are promising options to improve treatment efficiency in cancer patients and decrease adverse reactions. A number of these phytochemicals are naturally occurring biologically active compounds with significant antitumor potential (Choudhari *et al.*, 2020).

Recent research has shown that the use of herbal remedies for cancer treatment resulted in fewer or diminished side effects compared with conventional chemotherapy or radiotherapy, resulting in a longer survival period for many patients(Yu *et al.*, 2015).The drugs used for the treatment of different disorders is either expensive or not freely available in the market. So, for better therapeutic approach it is necessary to innovate and access low-cost materials with reasonable safety. Bio-based materials with combination of modern chemistry could be used for the better approach in the pharmaceutical industries(Patel et al, 2016). Herbal medicine has also been reported to be able to prevent the progression of colon carcinoma, gastric cancer, and breast cancer, as well as their metastasis to the liver,

lung, and bone. Moreover, hepatocellular carcinoma has been shown to become smaller without severe side effects after treatment with herbal medicine. Reports strongly suggest that herbal medicine remedies would be good candidates for the treatment of several types of cancer(Yu *et al.*, 2015).

1.2 Literature review

1.2.1. Cancer:

Cancer is a major public health problem that has a significant global impact on both developed and developing countries. One of characterizing features of cancer is the quick production of abnormal cells that developed out of their origin, and would then be able to attack bordering portions of the organ and spread to other parts of the body, the last process is called metastasizing, metastases are a significant reason of death from the disease (Nelson *et al*., 2019).

1.2.1.1 Cancer epidemiology:

According to the International Agency for Research on Cancer, an estimated 19.3 million new cancer cases occurred worldwide which are likely to increase to 23.6 million new cases each year by 2030. About 18.1 million excluding nonmelanoma skin cancer and almost 10 million cancer deaths (9.9 million excluding nonmelanoma skin cancer) occurred worldwide in 2020. Female breast cancer has surpassed lung cancer as the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%), followed by lung (11.4%), colorectal (10.0 %), prostate (7.3%), and stomach (5.6%) cancers. Lung cancer remained the leading cause of cancer death, with an estimated 1.8 million deaths (18%), followed by colorectal (9.4%), liver (8.3%), stomach (7.7%), and female breast (6.9%) cancers (Sung *et al.*, 2021)

1.2.1.2 Cancer pathogenesis:

Cancer is a complex genetic disease that is caused by specific changes to the genes in one cell or group of cells. These changes disrupt normal cell function – specifically affecting how a cell grows and divides. In contrast to normal cells, cancer cells don't stop growing and dividing, this uncontrolled cell growth results in the formation of a tumor. Cancer cells have more genetic changes compared to normal cells, however not all changes cause cancer, they may be a result of it (Laura, 2018).

Many factors can cause cancer including external factors such as tobacco smoking, radiation, chemicals, and infections), while internal factors include inherited mutations, hormones, immune conditions, and random mutations(Tyagi *et al.*, 2017).The genetic changes that contribute to cancer usually affect three specific types of gene; proto-oncogenes, tumor suppressor genes and DNA repair genes(Laura, 2018).

Considering the high-profile nature of the disease, its treatment has been a constant struggle with relatively less success. Currently available options for cancer treatment involve surgical removal and radiation treatment of the large accumulated biomass of cancer, typically followed by systemic chemotherapy treatment for maintenance. The primarily available chemotherapeutic agents include antimetabolites (e.g., methotrexate), DNA-interactive agents (e.g., cisplatin, doxorubicin), anti-tubulin agents (taxanes), hormones, and molecular targeting agents. The major disadvantages of chemotherapy are recurrence of cancer, drug resistance, and toxic effects on non-targeted tissues that can restrain the use of anticancer drugs and thus impair patient’s quality of life. To overcome the problems of present

therapy, search for new promising anticancer agents with better efficacy and lesser side effects continues (Choudhari et al, 2020).

1.2.1.3 Difference between normal and cancer cell

There are certain characteristics of healthy, normal cells that distinguish them from cancer cells as in figure (1.2). These include:

a-Cell shape: Normal human cells come in many shapes and sizes – as they differentiate and adopt specialized functions their shape changes accordingly for instance, a red blood cell looks very different to a nerve cell. Different types of cells do not look alike, but, if you analyze cells of the same cell type, they will look extremely similar, maintaining a uniform shape (Laura, 2018).

Under a microscope, normal cells and cancer cells may look quite different. In contrast to normal cells, cancer cells often exhibit much more variability in cell size—some are larger than normal and some are smaller than normal. In addition, cancer cells often have an abnormal shape, both of the cell, and of the nucleus (the "brain" of the cell.) The nucleus appears both larger and darker than normal cells.(Eldridge, 2014).For years researchers have been peering down microscopes, looking for distinct features that can help them determine the difference between a cancer cell and normal cell. Cancer cells are misshapen, and appear as a chaotic collection of cells, in an array of shapes and sizes. Researchers have been investigating the relationship between cancer cell shape and a patients' outlook, and whether cell shape may also help to distinguish between the different types of cancer (Laura, 2018).

b-Nucleus: The rates of ribosome production by a nucleolus and of protein biosynthesis by ribosomes are tightly correlated with the rate of cell growth

and proliferation (Stępiński, 2018). In normal cells, the nucleus has a smooth appearance and maintains a uniform, spheroid shape. Several structural components are involved in the regulation of nuclear morphology. One of these structural components is the nuclear lamina. Cancer cell nuclei are frequently misshapen and bulges known as "blebs" can often be observed in cells' nucleus (Laura Elizabeth Mason, 2018).

c-Chromatin: The fine, evenly distributed chromatin found in normal cells transforms into coarse, chromatin in cancer cells – aggregating into irregular clumps that vary in both size and shape.

d-Nucleolus: Tumor aggressiveness and clinical outcome can both be measured by observing the morphology of a cancer cell's nucleolus.

e-nucleoli: The nucleolus becomes increasingly enlarged and more irregular in cancer cells – cells can have multiple nucleoli within the nucleus.

f-Blood supply: Normal cells undergo a process called angiogenesis only as part of normal growth and development and when new tissue is needed to repair damaged tissue. Cancer cells undergo angiogenesis even when growth is not necessary. One type of cancer treatment involves the use of angiogenesis inhibitors—medications that block angiogenesis in the body in an effort to keep tumors from growing(Eldridge, 2014)**.**

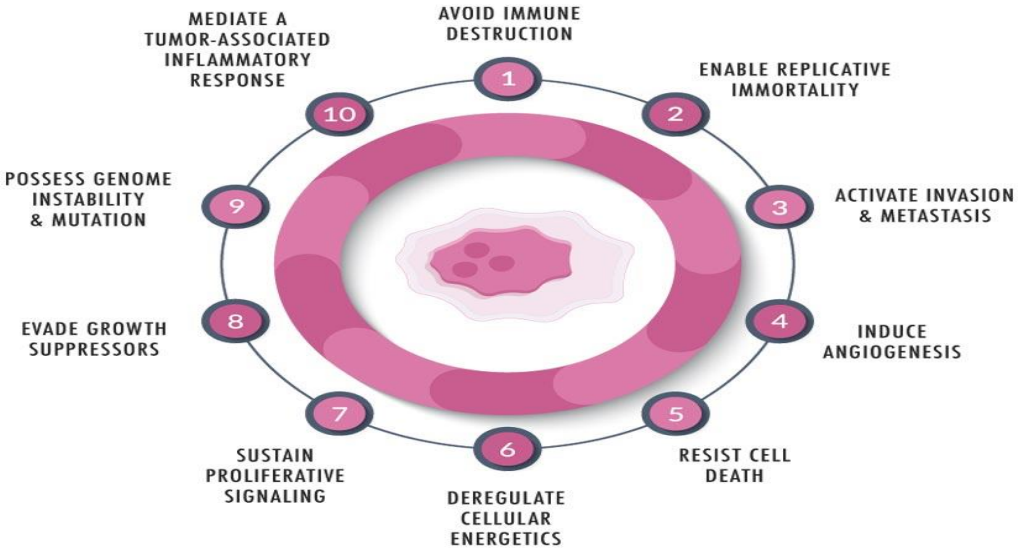

Figure (1.1): Normal cell versus cancer cell – the key differences(December *et al*, 2020)

1.2.1.4 Colorectal cancer CRC:

Colorectal cancer Is the third common cases of cancers in the world with near 5-year survival of 50% Colorectal cancers could be graded due to the size of the tumor and the amount of invasion as well as the rate of tumor node metastasis (TNM) stages (Rostami-Nejad *et al*, 2019).

Colorectal cancer consists of four stages depending on the location of the tumor (figure 1.3). In stage 0, the cancer found only in the inner lining of the colon or rectum. In stage I, the tumor has grown into the inner wall of the colon or rectum. In stage II, the tumor extends more deeply through the wall of the colon or rectum. It may have invaded nearby tissue but does not extend to the lymph nodes. In stage III, the cancer has spread to nearby lymph nodes, but not to other parts of the body. In stage IV, the cancer has spread to other parts of the body, such as the liver (Koch *et al*, 2015).

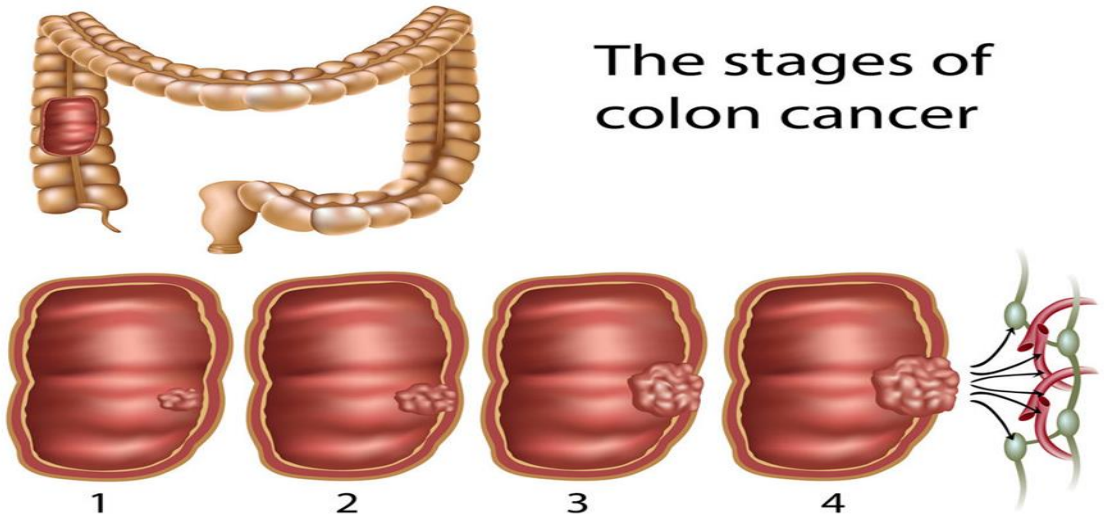

Figure (1.2) Stages of Colorectal Cancer (Fong, 2018)

1.2.1.4.1 Symptoms of colorectal cancer:

Many cases of carcinoma without clear symptoms. The symptoms Will indicate colon cancer subsequence, the symptom may be:

- Pain and tenderness in the lower abdomen
- Bloody stool
- Change in bowel habits may be Diarrhea, constipation, or other
- Weight loss with no known reason (Al-Humadi, 2009)

1.2.1.5 Prostate cancer

The prostate has various functions. These include producing the fluid that nourishes and transports sperm, secreting prostate-specific antigen (PSA), a protein that helps semen retain its liquid state, and helping aid urine control(Leslie *et al.*, 2022).Other than skin cancer, prostate cancer is the most common trusted source cancer affecting males in the United States. Around 1 in 8 males receive a diagnosis of prostate cancer at some point in their life. However, only 1 in 41 of these will die as a result. This is because treatment is effective, especially in the early stages. Routine screening

enables doctors to detect many cases of prostate cancer before they spread (Seunggu, 2019).

The growth of prostatic carcinoma cells is androgen-dependent and can be effectively treated by hormone depletion either using surgical or pharmacological methods. However, the hormone depletion therapy only causes a temporary regression of prostate tumors; with some tumor cells become androgen-independent in 6–18months.Several factors have been demonstrated to be involved in the development of androgen-independent growth in prostate cancer. For instance mutation amplification, and over expression of the androgen receptor (AR) gene have been observed in androgen-independent prostate cancer (Lu *et al*, 1999).

1.2.1.5.1 Symptoms of prostate cancer

The widespread use of PSA as a screening test for prostate cancer in some countries has led to increasing diagnoses being made in asymptomatic men. Men may present to their doctor complaining of lower urinary tract symptoms (LUTS) or other Genito-urinary symptoms, and are thus investigated for prostate cancer. It also is suspected that there are a significant number of men who go through life and die with undiagnosed prostate cancer; this suspicion is based on the findings of autopsy studies showing that up to three quarters of men over the age of 85 had neoplastic changes in the prostate, not all of whom had been diagnosed prior to their death (Merriel *et al,* 2018)**.**

1.2.1.6 Lung cancer:

Lung cancer is the leading cause of cancer deaths worldwide, with an estimated 2 million new cases and over 1.8 million deaths in 2018 Tobacco

smoking is responsible for most cases of lung cancer (approximately 90 percent for men and 70 to 85 percent for women) (Cohen *et al.,* 2006) .Lung cancer is divided into two main categories: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is further classified into squamous cell carcinoma, adenocarcinoma, NSCLC represents 80% of all lung cancers, with adenocarcinoma accounting for 40% of all cases of lung cancer. Squamous cell carcinoma occurs most frequently in the central zone of the lung whereas adenocarcinoma tumors are peripheral in origin, arising from the alveolar surface epithelium or bronchial mucosal glands. The second major type of lung cancer is SCLC, in which there are also several histologic groupings: pure small cell, mixed small cell, and large cell carcinoma, as well as combined small cell. SCLC is usually more aggressive than NSCLC and presents as a central lesion with hilar and mediastinal invasion along with regional adenopathy (Yoder, 2006) .

1.2.1.6.1 Diagnosis of lung cancer

Guidelines for the diagnostic workup of people with known or suspected lung cancer include obtaining a thorough history, physical examination, and appropriate laboratory tests to screen for metastatic disease. Confirmation of the type of lung cancer by cytology is of utmost importance before treatment can be determined(Cohen & Khuri, 2006) .

1.2.7 Treatment of cancer

If the cancer is small and localized (Early-stage prostate cancer), a doctor may recommend:

a-Surgery :A primary treatment for resectable and operable early stage disease (Stage I and II) is surgery which provides the best option for long-term survival (Boni *et al.,* 2020)

b-Radiation therapy

This uses radiation to kill cancer cells or prevent them from growing. Options for early stage of cancer may include the use of external radiation therapy, this method uses a machine outside the body to send radiation toward the cancer cells. Conformal radiation therapy is a type of external radiation that uses a computer to help guide and target a specific area, minimizing the risk to healthy tissue and allowing a high dose of radiation to reach the prostate tumor (Seunggu , 2019).Nowadays a few complete treatment modalities, including, brachy therapy (BT), BT joined with external-beam radiation therapy (Fuerst, 2019).

c-Chemotherapy: This option uses drugs to help stop the growth of cancer cells. While it can kill cancer cells around the body, it may cause adverse effects.(Komura *et al.*, 2018) The primarily available chemotherapeutic agents include antimetabolites (e.g., methotrexate), DNA-interactive agents (e.g., cisplatin, doxorubicin), anti-tubulin agents (taxanes) (Choudhari et al, 2020).

d-Hormonal therapy: are male. The main androgens are testosterone and dihydrotestosterone. Blocking or reducing hormones such as Androgens and estrogen appears to stop or delay the growth of cancer cells. (Seunggu, 2019).

e- Immunotherapy: Immunotherapy is a type of cancer treatment that helps immune system fight cancer. It is made up of white blood cells and organs and tissues of the lymph system , Several types of immunotherapy are used to treat cancer, these include immune checkpoint inhibitors, which are drugs that block immune checkpoint(nivolumab) and Monoclonal antibodies (bevacizumab) (Fay *et al*, 2020). Only trials with a combination of

chemotherapeutic agents and a biological targeted treatment consistently reported a median survival exceeding 24 months. The antibody Bevacizumab binds to the circulating VEGF-A, increases the activity of any chemotherapeutic regimen (Tebbutt et al, 2010).

1.2.2 Oxidation and antioxidant system

1.2.2.1 Oxidative Stress:

Oxidative stress is an important risk factor in the pathogenesis of numerous chronic diseases. Free radicals and other reactive oxygen species are recognized as agent involved in pathogenesis of sickness such as inflammatory arthropathy, asthma, diabetes, Parkinson and Alzheimer disease, cancer as well as atherosclerosis. Reactive oxygen species are also said to be responsible for the human aging(Mahdi-Pour *et al*, 2012).

In the normal healthy situation, the scavenging system of cells control reactive oxygen species levels by the balance between the reactive oxygen generation and elimination .in oxidative stress conditions, the increase of reactive oxygen species can destroy the cellular component like proteins, lipids and DNA, which will lead to lethal injury and damage in cells that participate to carcinogenesis (Nimse *et al.,* 2015).Under the physiological conditions, the balance between division and necrobiosis support the homeostasis of tissue . The Mechanisms correlating with this equilibrium include checkpoints of the cell cycle, repairing and recombination of DNA, and the necrobiosis. The term cellular redox state is classical won't designate the balance of GSH/GSSG,NAD+/NADH,NADP+/NADPH, and its cognition to a different combination of metabolites and therefore its control the cell metabolism (Kidd, 1997) .The (Redox balance) term refer to the balance between oxidants and antioxidants, which is

important in keeping a healthy cellular environment. Any alteration in this balance leads to ROS production and creation of oxidative stress So the qualification of the cell antioxidant as a defense system (Phaniendra *et al.*, 2015) .Cells have several mechanisms to rework and eliminate ROS to avoid their harmful effects. The synergistic action of both antioxidant proteins and enzymes and exogenous antioxidants neutralize free radicals and modulate cell(Watson *et al*, 2011).

1.2.2.2 Free radical

Free radicals Is an unstable, short lived and highly reactive molecules containing one or more unpaired electrons naturally formed in stress, exercise and when the body convert food into energy, also may be acquired from an environmental source, when the body exposed to free radicals like cigarette smoke, pollution, and sunlight forming highly unstable molecules (Magammana, 2018).

There are many types of free radicals:

•Superoxide Ion Radical (O 2-).

•Hydroxyl Radical (OH·)

•Peroxyl Radical (ROO·)

•Hydrogen Peroxide (H_2O_2)

•Singlet Oxygen (1O2)

•Ozone (O3)

•Hypochlorous Acid (HOCl)

•Nitric Oxide or Nitrogen Monoxide

1.2.2.3 Antioxidants

Antioxidant are biologically active compounds that protect the body from damage caused by active oxygen species, active nitrogen, and active

chlorine, which cause disease (Zargoosh et al, 2019). Antioxidant is any substance that, when present at low concentrations, compared with those of the oxidizable substrate, considerably delays or inhibits oxidation of the substrate. Antioxidants are manufactured or natural substances that block or alter the cell damage caused by ROS. Vegetables and fruits, consider as good source of antioxidants, are found to be healthful; however, research has shown that antioxidant supplements does not have advantageous effect in preventing diseases(Brar *et al.*, 2014)Antioxidant play an important role in food preservation by inhibiting oxidation processes and contributing to health promotion rendered by Many dietary supplements, nutraceuticals and functional food ingredients. Its activity can be monitored by a variety of assays with different mechanisms, including hydrogen atom transfer (HAT), single electron transfer (ET), reducing power, and metal chelation, among others.(Shahidi & Zhong, 2015),

Biological reactive oxygen and nitrogen species such as hydrogen peroxide, superoxide and nitric oxide, as well as endogenous antioxidant systems, are important modulators of cell survival and death in diverse organisms and ell types. Thiol redox modifications are a major mechanism by which oxidants and antioxidants influence specific regulated cell death pathways in mammalian cells. Growing evidence indicates that redox modifications of cysteine residues in proteins are involved in the regulation of multiple cell death modalities, including apoptosis, necroptosis and pyroptosis (Benhar, 2020).

There are many types of antioxidants; natural antioxidant which also divided into two class, i.e., enzymatic antioxidants and non-enzymatic antioxidants. This classification depend on either are synthesized in the body through

normal metabolism process or are external supplement from other natural sources, and the activity of these antioxidant extremely depends on their physical and chemical properties and their mechanism of action (Brar *et al.*, 2014).

Enzymatic antioxidants work by breaking down and removing free radicals. The antioxidant enzymes convert dangerous oxidative products to peroxide (H_2O_2) then to water, during a multi-step process in presence of cofactors like copper, zinc, manganese, and iron (Nimse *et al*, 2015)

Non-enzymatic antioxidants are a classification of the antioxidants that required to be supplemented for the right metabolism because they aren't found within the body naturally act interrupting radical chain reactions. Few examples of the non-enzymatic antioxidants are, vitamin E, vitamin C, carotenoids, glutathione and plant polyphenol (Vaisi *et al*, 2007).

1.2.3 Description of *A. andraeanum*

A. andraeanum is a genus of popular ornamental plants that produce highly-colored modified leaf structures known as spathes. Studies have shown that anthocyanins are the primary pigments responsible for the striking red colors of A. species (Clark *et al.*, 2012).

A. andraeanum is a flowering plant belongs to the family Araceae. It is a perennial herbaceous plant cultivated for its continuing and attractive heart shaped inflorescence. *A. andraeanum* a leaf, bearing numerous small botanical flowers on a pencil like protrusion and has a vase life of 14-28 days(Abima Shazhni *et al.*, 2016). Cut *A. andraeanum* quality is defined as postharvest longevity or vase life, as well by physical attributes such as color, spathe diameter/length and stem length, all of which vary by species and cultivars (Favero *et al.*, 2020).

Figure (1.3): *A. andraeanum* from Al-Ataba Al- Hussainiya plant nursery

1.2.3.1 Phytochemical components

The phytochemical components of *A. andraeanum* (root, stem, leaf, flower and seed) include carbohydrates, proteins, tannins, alkaloids, flavonoids, phlobatannins, steroids, phenols and saponins. These phytochemicals have antioxidant, analgesic, anti-inflammatory and anti-cancer properties (Gilble *et al*, 2017).

1.2.3.1.1 Phenols

Epidemiological studies indicate that populations consuming high levels of plant derived foods have low incidence rates of various cancers. Recent findings implicate a variety of phytochemicals, including phenolics, that have these anticancer properties (Wahle *et al.*, 2010)**.** Both monophenolic and polyphenolic compounds from a large variety of plant foods, spices and beverages have been shown to inhibit or attenuate the initiation, progression and spread of cancers in cells both *in vitro* and *in vivo* .The inhibitory effect of natural phenolic in carcinogenesis and tumor growth may be through two

main mechanisms: 1) modifying the redox status and, 2) interfering with basic cellular functions (cell cycle, apoptosis, inflammation, angiogenesis, invasion and metastasis(Dai *et al*, 2010) , the cellular mechanisms that phenolic modulate to elicit these anticancer effects are multifaceted and include regulation of growth factor-receptor interactions, and cell signaling cascades including kinases and transcription factors, that determine the expression of genes involved in cell cycle arrest, cell survival and apoptosis or programmed cell death (Wahle *et al.*, 2010).

Several phenolic acids caffeic and gallic acid derivatives were synthesized and screened for their potential antiproliferative and cytotoxic properties, on different human cancer cell lines: mammary gland and cervix adenocarcinomas and lymphoblastic leukemia (Gomes *et al.*, 2003).

Plant species are one of the important sources of antioxidants which protect the body from cancers. Plants that are rich in antioxidant compounds can protect cells from oxidative damage. Natural antioxidants increase the strength of the antioxidants in the plasma and decrease the incidence of some diseases, such as cancer, heart disease, and stroke (Zargoosh *et al.*, 2019)**.**

It is generally appreciated that the toxicity associated with some phenolic compounds is mediated by their oxidative activity, which can accelerate oxidative damage in vitro, either to DNA or to proteins and carbohydrates. Another possible, although insufficiently investigated, mechanism of phenol cytotoxicity may be associated with their pro-oxidant properties. Phenolic acid derivatives structurally varying in the number of OH ring substituents and in the length and/or degree of saturation of the carbon chain between the

phenyl and the terminal carboxylate groups, which are known to exhibit antioxidant activity (Gomes *et al.*, 2003).

1.2.3.1.2 Alkaloids

Alkaloids that possess an isoquinoline moiety are one of the largest groups of natural substances. Isoquinoline is a heterocyclic compound consisting of a benzene and pyridine ring fused at C3/C4 of the pyridine ring. The anti-cancer activity of isoquinoline alkaloids is noteworthy(Yun *et al.*, 2021). However, adverse reactions to conventional treatment and drug resistance have led some to use complementary and alternative medicine (CAM) in conjunction with conventional medical treatments. As interest in complementary therapies increases, so has the value of natural remedies.

Isoquinoline alkaloids, a group of plant-derived bioactive compounds, have traditionally been used as alternative treatments for their anti-inflammatory, antimicrobial, and analgesic effect. Recently, biomedical and pharmacological developments have begun to uncover the anticancer effects

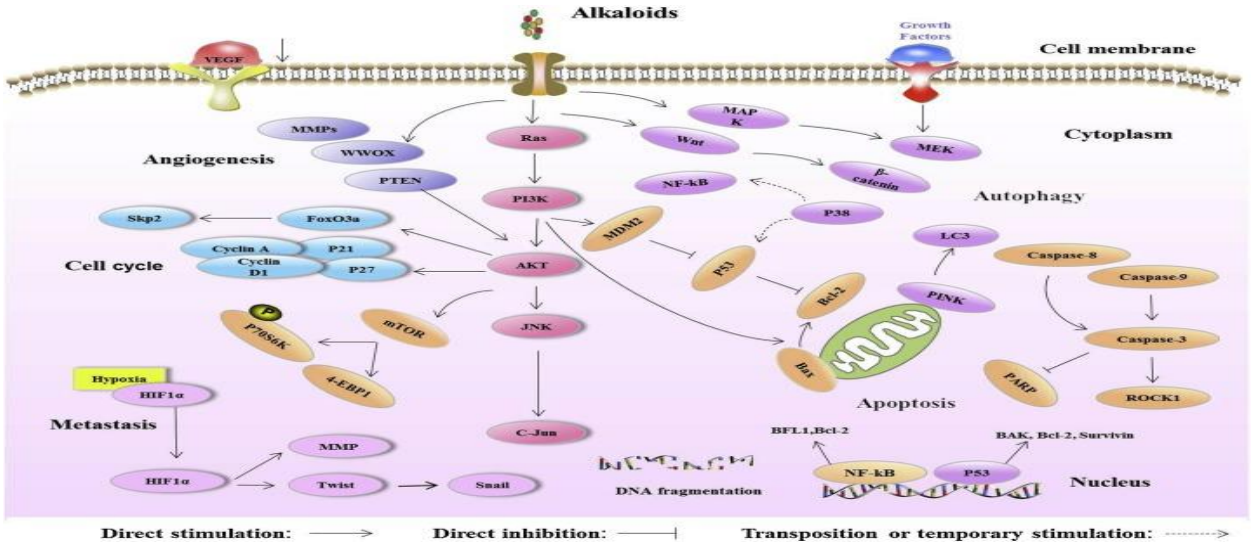

Figure (1.4): Mechanism of Alkaloid apoptosis (Caiyan Liu *et al, 2019)*

Apoptosis-Mediated Cell Death is a promising target for anticancer therapy as show in figure (1.4). Apoptosis is triggered by the extrinsic and intrinsic pathways. The extrinsic pathway is triggered by external stimuli. Ligand and death receptor

(DR) binding interacts with the Fas- associated death domain (FADD) and tumor necrosis factor receptor 1 (TNFR1)-associated death domain (TRADD). A death-inducing signaling complex (DISC) is then formed andcaspase-8 is recruited to DISC. This leads to the activation of caspase-8, which cleaves and activates caspase-3/6/7, initiating apoptosis. The intrinsic pathway is triggered by exogenous and endogenous stimuli, including DNA damage and oxidative stress. The Bcl family members, Bax and Bcl-2, act as pro- or anti-apoptotic regulatory proteins through binding to the mitochondrial membrane. The release of cytochrome C in the cytoplasm recruits Apaf–1 and procaspase-9 to form the apoptosome, which triggers downstream caspase-9/3 cascades (Yun *et al.*, 2021).

1.2.3.1.3 Tannins

Potent anticancer activities have been observed in tannins (especially Epigallocatechin Gallate EGCG). Epidemiological studies have shown that tannin could benefit human health, owing to their antioxidant property. It can fight cancer via multiple mechanisms, such as apoptosis, cell cycle arrest, and inhibition of invasion and metastases. However, the applications of tannins have been hindered due to their poor liposolubility, low bioavailability, off-taste, and shorter half-life time in human body, such as Epigallocatechin Gallate EGCG, gallic acid, and ellagic acid(Cai *et al.*, 2017)

1.2.3.1.4 Steroids

Steroids, a widespread class of natural organic compounds occurring in animals, plants and fungi, have shown great therapeutic value for a broad array of pathologies. Plant hormone brassinosteroids (BRs) has been found that they also affect cell growth inhibition, cell cycle arrest and induction of apoptosis in human tumor cell lines , breast and prostate cancer cell lines with no effect on normal cells(Huskova *et al.*, 2020)

Plant steroids are renowned to be necessary for their cardiotonic, insecticidal and anti-microbial properties. They are conjointly utilized in nutrition, herbal medicines, cosmetics and they are habitually utilized in drugs due to their profound biological activities. Steroids present in plants are cardiotonic in nature and are reported to have antidiabetic and anti-fungal properties. They are stocked in plant cells as inactive precursors but are readily converted into biological active antibiotics by enzymes in response to microorganism attack(Beltagi *et al.*, 2019)

The effect of BRs on the nuclear steroid receptors was manifested by changes in their expression and localization, in DU- 145 prostate cancer a significant block in G0/G1 phase after the BR treatment was observed. BRs demonstrated their significant effect on prostate cancer cells and the compounds have potential used in anticancer drug research and cancer treatment (Franěk *et al.*, 2003)

1.2.3.2 Pharmacological activity of *A. andraeanum*

1.2.3.2.1 Antimicrobial activity

The phytochemical constituents of *A. andraeanum* such as alkaloids, flavonoids, phenolic compounds, steroids, tannins, saponins and various other aromatic compounds are secondary metabolites of plants that serve a

defense mechanism against prediction by many microorganisms, worms and other herbivores(Abima Shazhni *et al.,* 2016).

Flavonoids are hydroxylated phenolic substance synthesized by plants in response to microbial infection. Phenols are largest group of plant metabolites, which have many biological properties such as antiapoptosis, antiaging, anticarcinogen, anti-inflammation and cell proliferating activities Tannins bind to proline rich proteins and interfere with the protein synthesis (Sultana *et al.*, 2012).

The antimicrobial activities of this plant documented against different types of bacteria and fungi like *B. cereus*, *E. coli*, *K. pneumoniae*, *A. fumigatus* and *P. chrysogenu*. Among the plant parts used, flower, stem and root extracts were significantly affecting the pathogenic organisms than leaf extracts. The flower extracts showed superior zone of inhibition against the fungal pathogen A. fumigatus, also the bacterial pathogen *E. coli*. The zone of inhibition was varied with different solvents used, ethanol extracts were showed fine activity against the pathogens followed by chloroform and acetone extracts.(Abima *et al.*, 2016).

1.2.3.2.2 Anticancer activity

Studies regarding anticancer activity of *A. andraeanum* showed that different plant part have different anticancer capacity. These differences between plant parts are mainly due to the fact that the chemical composition of these extracts are not the same quantitively and qualitatively.(Gilble *et al*, 2017)

Cytotoxicity effect is one of the important properties of anticancer molecules .*A. andraeanum* was effective against the cell line, Hep G2 human liver cancer cell line,A549 lung cancer cell line and MCF-7

respectively , the purified compound was very effective against the cell line MCF-7 .this result in accordance the observations made previously (Solowey *et al.*, 2014).

1.3 Carboplatin

Carboplatin is an analog of cisplatin. Like cisplatin, it contains a platinum atom surrounded in a plane by two ammonia groups and two other ligands in the cis position and classified as alkylating agent. The exact mechanism of action of carboplatin is not known. Carboplatin undergoes intracellular activation to form reactive platinum complexes which are believed to inhibit DNA synthesis by forming interstream and intrastrain cross-linking of DNA molecules. Carboplatin is a radiation-sensitizing agent. It is cell cycle-phase nonspecific.(Beckwith *et al*, 2002).

Primary use of carboplatin is in the Brain tumors, Endometrial cancer, Breast cancer, Germ cell tumors, Cervical cancer, Head and neck cancer and prostate cancer. It is mutagenic in both in vitro and in vivo studies, it also may cause gonadal suppression (amenorrhea, azoospermia) which is generally related to dose and length of therapy and may be irreversible. FDA classified it as Category D for pregnancy. There is positive evidence of human fetal risk, but the benefits from use in pregnant women may be acceptable despite the risk (Go *et al*, 1999)

1.4 Doxorubicin

Doxorubicin (Adriamycin) is one of the primary chemotherapeutic agents classified as anthracycline antineoplastic antibiotic, utilized for the treatment of breast cancer, prostate cancer bladder carcinoma, and gastric cancer. (Gewirtz *et al*,1994). DOX has shown great efficacy in killing rapidly dividing cells and delaying the progression of solid and liquid tumors, drug

resistance and several side effects end up developing throughout the DOX treatment, making it a major limitation as an effective cancer treatment(Micallef *et al*, 2020).

The exact mechanism of action of DOX is complex and still unclear. In addition to its capacity to intercalate into DNA and to inhibit macromolecular biosynthesis, doxorubicin has been reported to interact directly with the cell membrane and to inhibit the enzyme helicase, which unwinds DNA for replication. (Gewirtz *et al*, 1994) .

The electrocardiographic abnormalities and arrhythmias, which are normally reversible, as well as dose-related and slowly progressive irreversible cardiomyopathy culminating in congestive heart failure, BM suppression are the major side effect of it. Doxorubicin is clastogenic in mammalian in vitro and in vivo chromosome. Treatment with doxorubicin may produce gonadal suppression, resulting in amenorrhea or azoospermia. It is also classed as FDA Pregnancy Category D(Yadav *et al*, 2007) .

1.5 Cell culture

Cell culture is the process by which human, animal, or insect cells are grown in a favorable artificial environment. Because many animal cells may be made to grow outside of their organs or tissue of origin under specific conditions when supplemented with a nutrient-containing and growing media, animal cell culture is currently one of the primary techniques utilized in the biological sciences in areas of inquiry. The culture settings must replicate in vivo parameters with regard to temperature, pH, carbon dioxide (CO2), oxygen (O2), osmolality, and nutrition for cells to grow in vitro (Capes *et al*, 2010).

1.5.1 Primary culture

Primary cell culture is the term that used to describe the cells that are obtained directly from tissues and organs of the patient by mechanical or chemical disintegration, or by enzymatic digestion then by using a complex media, these cells are induced to grow in a suitablei glass or plastic containers with a complex medium. The preparation of primary cultures is intensive labor and they can be maintained in vitro only for a limited period. During their relatively limited lifespan, primary cells usually retain many of the differentiated characteristics of the cell in vivo. Primary cultures by definition have not been passaged; as soon as they are passaged, they become a cell line and are no longer primary. The primary cell culture, when sub-cultured, it becomes a cell line or cell strain that can be finite or continuous, depending on its life span in culture (Philippeos *et al*, 2012).

1.5.2 Cell line

The term cell line refers to the propagation of cell culture after the first subculture. In other words, a cell line is a permanently established cell culture that will proliferate through given appropriate fresh medium and space. A given cell line contains several cell lineages of either similar or distinct phenotypes. Cloning or physical cell separation or any other technique could be used to select particular cell linage. This cell line that obtained by cloning or selection is referred to as strain. Cell strains do not have infinite life, as they die after some divisions(Chaudhary *et al*, 2019).

Cell lines also provide a pure population of cells, which is valuable since it provides a consistent sample and reproducible results. However, despite being a powerful tool, one must be careful when using cell lines in place of

primary cells. Cell lines should display and maintain functional features as close to primary cells as possible (Ulrich *et al*, 2013).

There are two kind of cell lines; finite and continuous. In Finite Cell Lines**,** **t**he cells in culture divide only a limited number of times, before their growth rate declines and they eventually die. The cell lines with limited culture life spans are referred to as finite cell. While in Continuous Cell Lines, cells are capable of growing faster and has unlimited life. They are designated as continuous cell lines(Chaudhary *et al*, 2019).

Serial passage of cell lines can further cause genotypic and phenotypic Variation over an extended period of time and genetic drift can also cause heterogeneity in cultures at a single point in time. Therefore, cell lines may not adequately represent primary cells and may provide different results. The other major problems associated with cell lines are contamination with other cell lines and mycoplasma(Ulrich *et al*, 2013)

1.6 Cell models:

1.6.1 Vero cell:

Derived from the kidney of an African green monkey (Cercopithecus aethiops) in the 1960s, Vero cells are a common mammalian continuous cell line used in research. Vero cells have been licensed in the United States for production of both live (rotavirus, smallpox) and inactivated (poliovirus) viral vaccines, and throughout the world Vero cells have been used for the production of a number of other viruses, including Rabies virus, Reo virus, and Japanese encephalitis virus. For long-term storage, Vero cells are kept either in liquid nitrogen or at −80◦C. This protocol describes how to start growing vero cells obtained from frozen stock. After recovery from frozen stock, Vero cells usually take 2 to 3 passages to reach their regular growth

rate, and this should be taken into account if planning to use the cells for experiments, infections, etc.(Ammerman *et al*, 2008).

1.6.2 SW 480 colon cancer cell line

Colorectal cancer (CRC) is one of the most lethal and prevalent cancers throughout the world (Shanehbandi *et al.*, 2021). The colonic cancer SW480 cell line originates from primary tumor of an adenocarcinoma of the colon in a 50 year old male (Siekmann *et al*, 2019).

1.6.3 LNCap cell line

LNCap cell line is a type of cancerous cell line that originated from a metastatic lymph node lesion of human prostate cancer which is androgen receptor (AR) positive, exhibits androgen sensitive growth (Castanares *et al.*, 2016).

Androgens are essential for growth of LNCaP cells, unless they have become transformed to androgen-independent clones. Androgen-dependent and androgen-independent LNCaP cell lines have previously been used for studying differentially expressed genes in the respective stages of prostate carcinoma (Vaarala *et al.*, 2000).

1.6.4 A549 cell line

A549 Lung cancer cell line was originated from primary lung cancer cells. cell lines have previously been used for studying differentially expressed genes in the respective stages of lung cancer (Torky *et al*, 2005).

1.7 Aim of the study

This study aimed to study:

1. The cytotoxicity of ethanoic leaves extract of this plant
2. The antioxidant activities of *A. andraeanum* ethanolic leaves extract

3. The possible anticancer activity of the *A. andraeanum* ethanoic leaves extract

4. The effect of *A. andraeanum* ethanolic leaves extract on carboplatin and doxorubicin activity

Chapter two

Materials and Methods

2. Study design

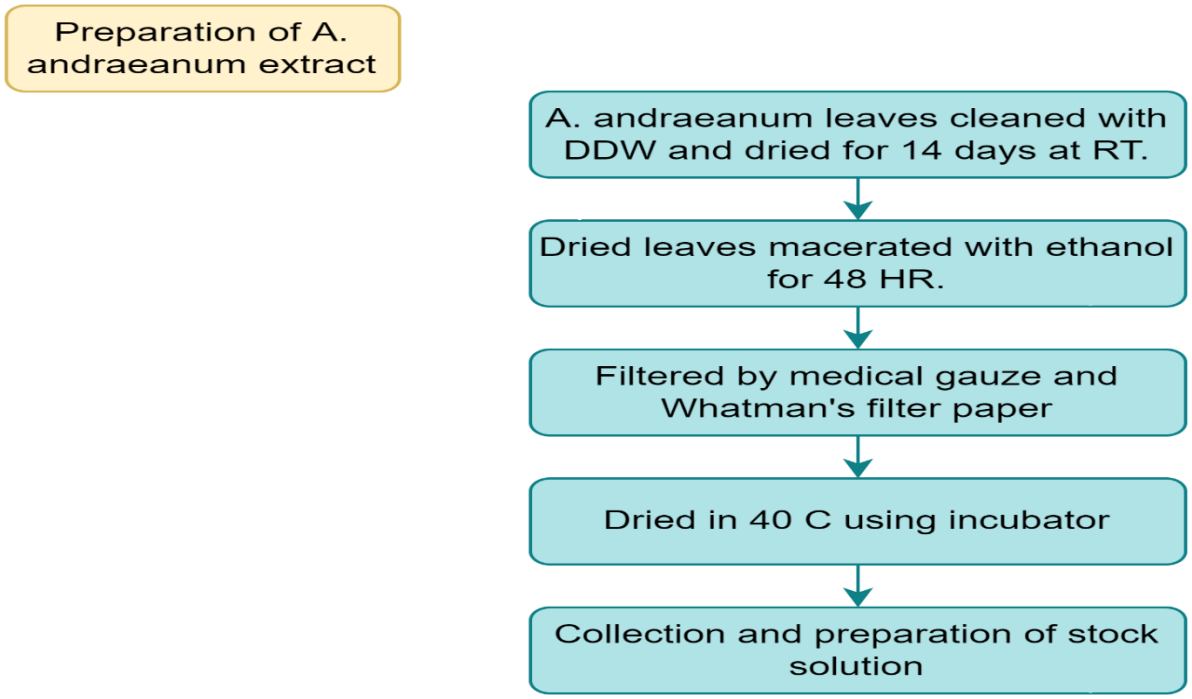

Figure (2.1) preparation of *A. andraeanum* extracts

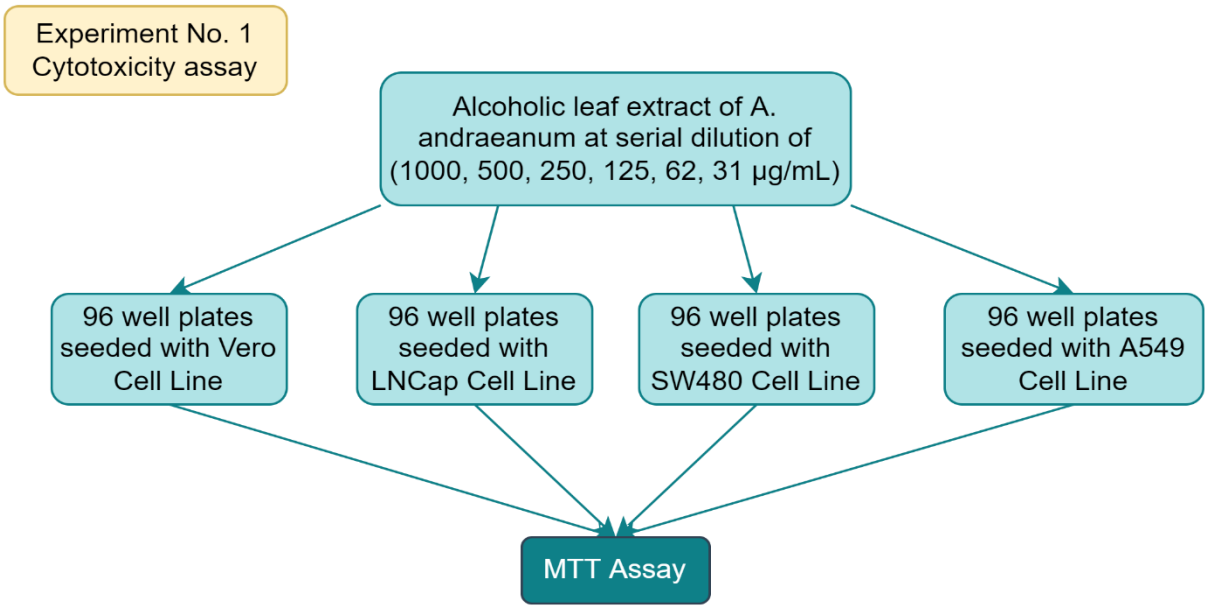

Figure (2.2) Cytotoxicity assay

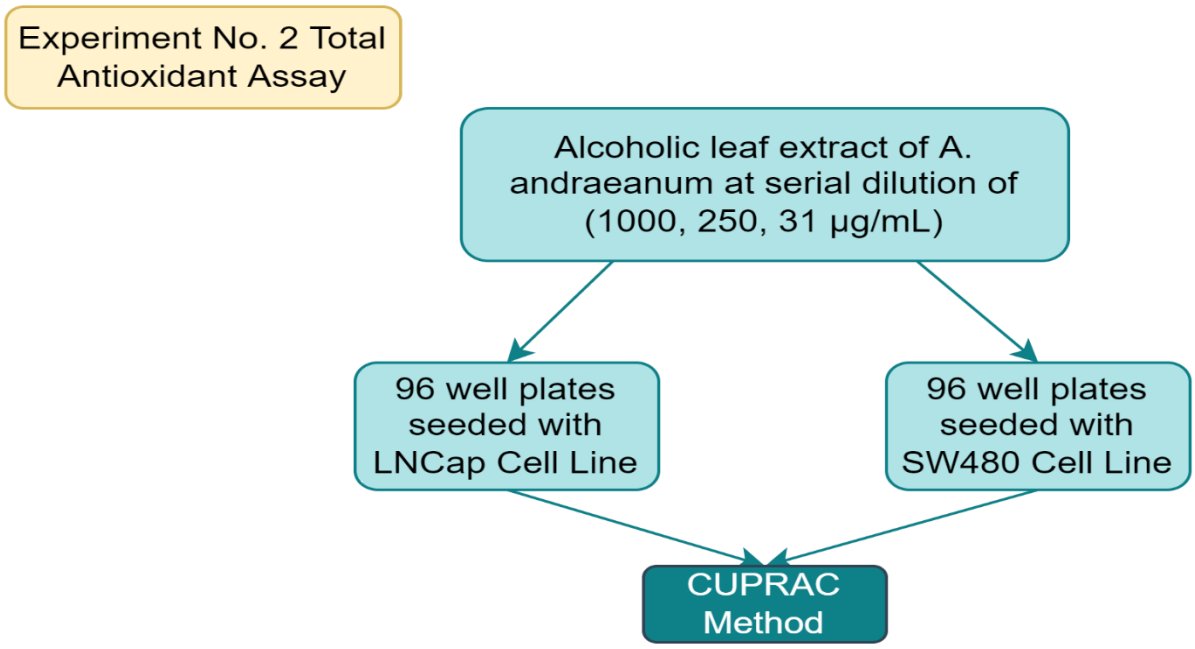

Figure (2.3) Antioxidant assay

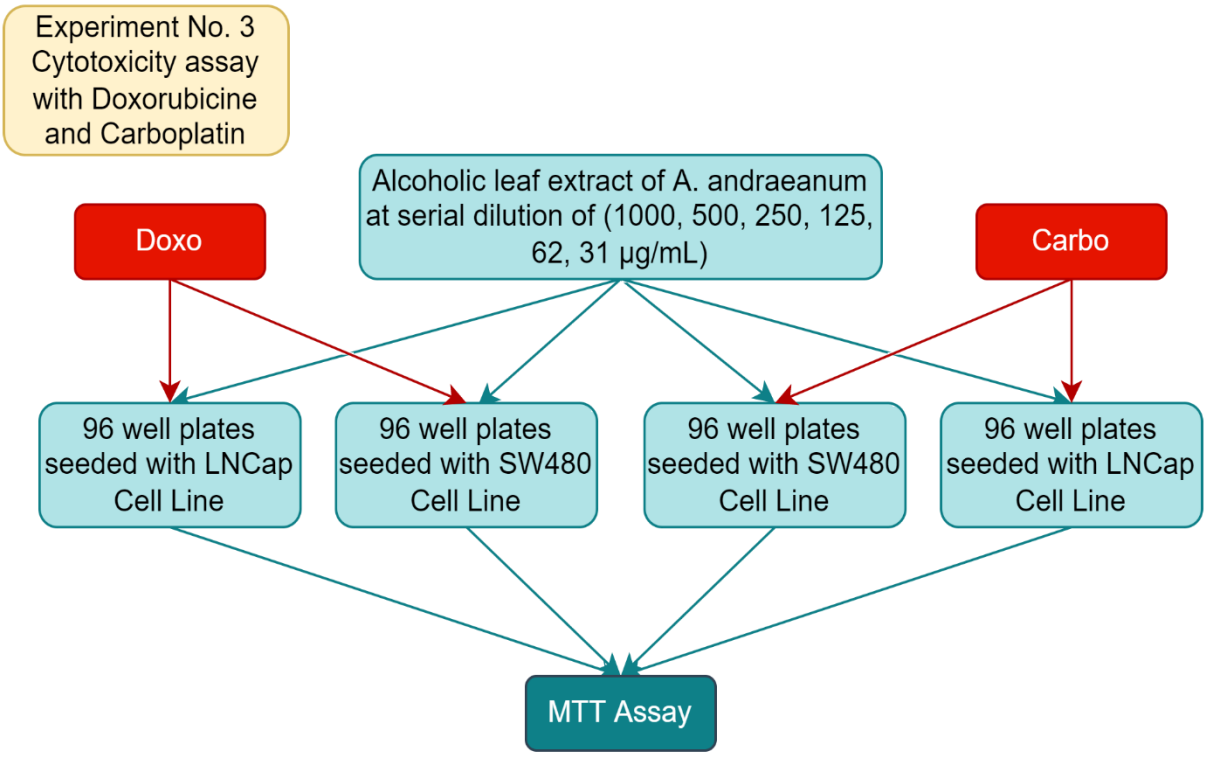

Figure (2.4) Cytotoxicity assay after addition of carboplatin and doxorubicin

2. Method and materials

2.1 Introduction

The experimental work was performed in the Tissue Culture laboratory/ Pharmacology Department /Collage of Medicine / University of Babylon during the period from October 2021 to April 2022.

2.2Materials

2.2.1 Chemicals

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

Table (2.1): Chemicals used in the study

Chemical	Company	Country
Alcohol spray (ethanol 70%)	Aljoud	Iraq
Dimethyl sulfoxide	Sigma Aldrich	Germany
Ethanol 99.9%	France Alcohols	France
Fetal bovine serum (FBS)	Capricorn	German
MTT(3-(4,5-Dimethylthiazole-2-yl)- 2,5-diphenyl-2Htetrazolium bromide) dye powder	Roth	German
Phosphate buffer saline Packets	BioPLUS chemicals	USA
Penicillin- streptomycin Solution 1%	Capricorn	Germany
RPMI 1640 medium w/L-glutamine, 25mMHEPES (powder)	US Biological life Science	USA

Sodium bicarbonate powder	Ludeco	Belgium
Trypsin Ethylene diamino-tetra-acetic acid (EDTA) powder	US biological	USA

2.2.2 The drug used in the study were listed in (Table 2.2) with their suppliers.

Table 2.2 list of drugs used in the study

Drug	Company	Country
Doxorubicin 50 mg/25 mL vial	Pfizer	USA
Carboplatin 450 mg/45 mL vial	Vianex	Greece

2.2.3 Instruments and Tools:

The instruments, glassware, and tools used in the study are listed in table (2.3) with their suppliers.

Table 2.3 List of Instruments and Tools Used in the Study

Instrument or tool	Company	Country
Autoclave	Jeiotech	Korea
Cell culture flask (25ml)	SPL	Korea
Cell culture plate (96-wells)	SPL	Korea
Centrifuge	Rotanta	Germany
Distiller	ROWA	Germany
Double distillation	GFL	Germany

water stills		
Electric oven	Memmert	Germany
Eppendorf centrifuge 5702 RH	Eppendorf	Germany
Incubator	Memmert	Germany
Inverted microscope	T.C Meiji techno	Japan
Laminar air flow Cabinet	Labtech	Korea
Magnetic stirrer	Scotech	Germany
Micropipettes (different sizes)	Dragon-Med	India
Millipore filter (0.45,0.22μm)	Biofil	Australia
pH Meter	WTW	Germany
Refrigerator	Arcelik	Turkey
Sensitive Balance	Labtech	Korea
Syringe 5 ml	MED	China
Water bath	Memmert	Germany
Whatman filter paper	Merck	Germany

2.2.4 Cell lines

Frozen vials of human prostate cancer LNCaP, Colon cancer SW480, lung cancer A549, and Vero cell lines were obtained from the Tissue Culture laboratory/ Pharmacology Department /Collage of Medicine.

2.3 Methods

2.3.1 Preparation of Reagents and Solution

2.3.1.1 Phosphate Buffer Saline (PBS):

According to the BioWorld manufacturer manual, the PBS was prepared through dissolving only one packet in 500 ml of deionized distilled water (DDW) with continuous stirring by a magnetic stirrer at room temperature resulting in PH value of (7.45). Autoclaving is required for complete sterilization and stored in a closed bottle until use to keep sterile.

2.3.1.2 Trypsin-(EDTA) Solution:

As indicated by US Biological headings, a weight of a 10.1 gm of trypsin-EDTA powder and dissolving in 0.9 Liter of double distilled water (DDW) with continuous mixing at room temperature. 7.2 of PH value should be reached and complete the volume to 1 Liter by DDW, the solution was sterilized through using Millipore filters of 0.45 and 0.22 μm respectively, after that, the solution was kept at (- 20C˚) of temperature.

2.3.1.3 Preparing MTT assay solution

A weight of 0.5 g of MTT powder was dissolved in 100 ml PBS to achieve a concentration of 5 mg/ml. Then the MTT solution was sterilized by filtration through a 0.2 μm Millipore filter into a sterile and light protected container and stored at 4°C for frequent use or at (-20)°C for long term storage (Kaspers, 2011) .

• **Procedure**(Kaspers, 2011)

1- At the end of the drug exposure period, the medium was removed from the wells and then the cells were washed with PBS. A blank control was carried to assess unspecific formazan conversion.

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

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

4- The supernatant was removed and 100 µl DMSO was added in each well to dissolve the resultant formazan crystals.

5- The plate was incubated at room temperature for 30 minutes until the cells have lysed and purple crystals have dissolved.

6- Absorbance was measured by a microplate reader at 570 nm.

The absorbance reading of the blank must be subtracted from all samples. Absorbance readings from test samples must then be divided by those of the control and multiplied by 100 to give percentage cell viability or proliferation. Absorbance values greater than the control indicate cell proliferation, while lower values suggest cell death or inhibition of proliferation. Percent of cell viability or percent of inhibition was calculated by the following formula:

% viability = (AT – AB) / (AC – AB) × 100%

Where, AT = Absorbance of treated cells (drug).

AB = Absorbance of blank (only medium).

AC = Absorbance of control (untreated).

% Inhibition = 100 – % viability

2.3.2 Preparation of Tissue Culture Medium

Liquid RPMI-1640 medium was prepared according to US Biologics from RPMI-1640 medium powder as the following: Weighing RPMI-1640 powder to obtain 10.4 grams and dissolved in 900ml of deionized H_2O

without heating, 2 g of sodium bicarbonate can be added if required with gentle stirring to adjust pH additional water was added to obtain 1 liter then filtered using 0.22-micron membrane. Penicillin-Streptomycin at 1% was added and 10% fetal bovine serum also added, then filter-sterilized using 0.22-micron membrane filter. The mixture contains heat labile compounds that can be damaged with autoclaving. The prepared media should be kept at 4°C and used within a short period of time.

2.3.3 Preparation of cell line

2.3.3.1 Thawing of Vero, LNCaP, SW480, and A549 cell lines

The frozen cell line vial was removed from liquid nitrogen container with caution and directly placed into a beaker containing pre-warmed (37°C) sterile DDW. The vial was removed from the water before the ice floccule dissolved completely, then it was wiped with 70% ethanol. Without delay, the cell suspension content of the vial was pipetted under laminar flow cabinet into a 15 ml sterile plastic centrifuge tube containing 10 ml of pre-warmed serum-free medium. Centrifugation was done at 1000 rpm for 5 minutes and the supernatant was aspirated and decanted. The cells pellet was re-suspended into 5ml warm (37C˚) serum-medium and transferred into 25 ml size cell culture flask, incubated at 37C˚ and the serum medium replaced on the next day (Phelan *et al*, 2017).

2.3.3.2 Sub-culturing of cell culture

The cells are checked and examined using the inverted microscope with phase contrast capabilities to ensure that the cells are healthy and sub confluent and free of contamination.

- The laminar flow is sanitized by wiping off the surface of working area with 70% ethanol.

- The growth medium is removed from the flask using a pipette and wash the monolayer with a sufficient volume of PBS to ensure the removal of all media from the flask.
- an appropriate volume of the trypsin /EDTA solution is added to the flask and incubate at 37 c° to allow the cells to detach from the inside surface of the flask (within 2-10 min).
- The cells were examined using an inverted microscope to ensure that all the cells are detached and in suspension. Gently tap the flask with the palm of the hand a couple of times to release any remaining detached cells.
- the trypsin is inactivated by adding an equal volume of serum-containing media to the flask.
- then the cell suspension is divided into two flask and label each flask with cell line name, passage number, and date.- The cell line was incubated at 37C°for 24 hr.(Meleady *et al* 2006).

2.3.3.3 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 color 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 subculture (Freshney, 2002).

2.3.3.4 Harvesting of cell culture

Harvesting is a technique that uses proteolytic enzymes to detach adherent cells from the surface of a cell culture flask. First, the growth medium in the vessel was aspirated and discarded. PBS was used to wash the cells twice. Afterward, the enzymatic harvesting solution was added to

the vessel. After 15 minutes, the proteolytic reaction was neutralized by adding the serum-containing culture medium. The cells in the tissue culture flasks were harvested by using different enzymatic solutions composed of different concentrations of trypsin and Ethylene diaminetetraacetic acid (EDTA) (Viazzi *et al.*, 2015).

2.3.4 Preparation of *A. andraeanum* leaf extracts

Fresh leaves of *A. andraeanum* were collected from the third District of al-ataba alhusainya -karbala- Iraq. The plant leaves were washed with deionized water thoroughly and left to dry for 14 days in dark place at room temperature, then grinded to fine powder and stored in tight dry container for further use. Then100 gm of leaf powder were soaked and macerated in 1 liter of alcohol (ethanol 99.9%) at room temperature for 48 hours.

Alcoholic mixture was filtered using Whitman filter paper no.1 and the final alcoholic liquid extract is then placed in oven at 40 c until drying then collected and stored at 4 C^0 as show in figure (2.1) (Abima Shazhni *et al.*, 2016).

Preparation of stock solution of the ethanoic extracts of *A.andraeanum* was made by weighing 10 mg of extract powder and dissolved in 5 ml of serum free RPMI 1640 to obtain final concentration of 2,000 µg/ml then the solution was filtered using Millipore filter syringe (0.22 um)for sterilization and to discard any impurities (Ojha, 2017).

2.3.5 Preparation of doxorubicin stock solution

The concentration of doxorubicin in the vial was 50 mg/ 25 ml (2 mg/ml), a serial dilution was made in complete medium.

2.3.6 Preparation of carboplatin stock solution

The concentration of carboplatin in the vial was 450 mg/ 45 ml (2

mg/ml), a serial dilution was made in complete medium.

2.3.7 Study the cytotoxic effect of alcoholic extracts of *A. andraeanum* leaves on (Vero, LNCaP, SW480 and A549) cell lines.

Figure (2.2) show Cell lines; Vero, (LNCaP), (SW480), and (A549) cell lines were seeded and labeled in 96 tissue culture plates. All cells were treated with serial dilutions of plant extracts ranged from 1000 to 31 .25 µg\ml (four replicates were used for each concentration of the extract for each type of cells) along with four replicates as a control group for each cell type. Then the plate was covered with a self-plastic lid and incubated for 24 hours, at the end of the exposure period, the cell lines growth was assessed by MTT cytotoxicity assay.

The concentration required for a 50% inhibition of viability (IC50) was determined by using an excel sheet and fitted by blotting graphically of relative cell inhibition percentage in the Y ax is versus the concentration of each compound used in the X axis. Calculation of cell viability percentage is done by dividing absorbance measured for each group by the absorbance of the control group multiplied by100 according to (Chiang et al., 2003) .

2.3.8 Effect of (Doxorubicin-*A. andraeanum* alcoholic extract) combination on the viability of prostate cancer cells

In 96 tissue culture plate, sixty wells were seeded with prostate cancer (LNCaP) cell line. Cells were treated with serial dilutions (1000, 500, 250, 125, 62, and 31 µg/ml) of ethanolic extract of *A. andraeanum* in the presence of a constant concentration (15 µg/ml) of doxorubicin in each well. Positive control contains (15 µg/ml) of doxorubicin. The plate was then

covered with a self-plastic lid and incubated for 24 hours, after which the cell line's growth was measured using the MTT cytotoxicity assay as show in figure (2.4).

2.3.9 Effect of (Carboplatin-*A. andraeanum* alcoholic extract) combination on the viability of prostate cancer cells

In 96 tissue culture plate, sixty wells were seeded with prostate cancer (LNCaP) cell line. Cells were treated with serial dilutions (1000, 500, 250, 125, 62, and 31 µg/ml) of ethanolic extract of *A. andraeanum* in the presence of a constant concentration (100 µg/ml) of carboplatin in each well. Positive control contains (100 µg/ml) of carboplatin. The plate was then covered with a self-plastic lid and incubated for 24 hours, after which the cell line's growth was measured using the MTT cytotoxicity assay as show in figure(2.4).

2.3.10 Total Antioxidants Capacity Assay: The CUPRAC Method

A- Principle:

The CUPRAC method is comprised of mixing the antioxidant solution (directly or after acid hydrolysis) with a copper (II) chloride solution, a neocuproine (2,9-dimethyl-1,10-phenanthroline) alcoholic solution, and an ammonium acetate aqueous buffer at pH 7, and subsequently measuring the developed absorbance at 450 nm after 30 min (normal measurement). Since the color development is fast for compounds like ascorbic acid, gallic acid and quercetin but slow for naringin and naringenin, the latter compounds were assayed after incubation at 50 C on a water bath for 20 min after Cu (II)- Nc reagent addition) so as to enable complete oxidation (incubated

measurement). The flavonoid glycosides were hydrolyzed to their corresponding aglycones by refluxing in 1.2 M HCl-containing 50% MeOH so as to exert maximal reducing power towards Cu (II)-Nc (hydrolyzed measurement). Thus, the total CUPRAC antioxidant capacity of a mixture containing various antioxidants should be finally measured after a suitable combination of hydrolysis and incubation procedures as show in figure(2.3), so as to obtain maximum absorbance at 450 nm **(**Apak *et al.*, 2007**).**

Total antioxidants + Cu^{+2} Cu^{+} ⟶

Cu^{+} + 2,9-dimethyl-1,10-phenanthroline ⟶ complex (λ_{max} at 450 nm)

B- Reagents preparation

1. Copper (II) chloride solution at a concentration of 10^{-2}M was prepared from $CuCl_2 \cdot 2.H_2O$ weighing 0.4262 g, dissolving in H_2O and diluting to 250 ml with water.
2. Ammonium acetate (NH_4Ac) buffer pH = 7.0 was prepared by dissolving 19.27 g of NH_4Ac in water and completed the volume to 250 ml.
3. Neocuproine (Nc){2,9-dimethyl-1,10-phenanthroline} solution at a concentration of 7.5 * 10^{-3}M was prepared by dissolving 0.039 g Nc in 96% EtOH, the volume was completed to 25 ml with ethanol.
4. The standard solutions of sample antioxidants were prepared at 1.0 * 10^{-3}M Torolox.

2.3.10.1 Reagents mixtures

Table (2.4) reagents used in this study

Reagents	Test	STD	Blank
Copper (II) chloride solution	1ml	1ml	1ml
Sample	50 µl	------	-------

Working standard solution	-------	50 µl	-------
D.W	-------	-------	50 µl
Neocuproine (Nc) solution	1ml	1ml	1ml
Ammonium acetate (NH_4Ac) buffer	1ml	1ml	1ml
Test tubes was mixed by vortex and incubated for 30 minutes at 37°C, after that the absorbance was read on a spectrophotometer at 450 nm.			

2.3.10.2 calculation

$$\text{Total antioxidan ts levels} = \frac{\text{A.test}}{\text{A.STD}} * Conc.of \quad STD \ \text{(mmol/l)}$$

(Apak *et al.*, 2007).

2.3.11 Statistical analysis

Results were expressed as means ± standard deviations. Between-group Comparisons were performed using an analysis of variance (one way ANOVA). For all tests, $P < 0.05$, $P \leq 0.001$ was considered as statistically significant. For the statistical analysis, Microsoft excels 2016 and sigma plot software (version12.0) were used.

Chapter three

Results

3. Results

3.1 Cytotoxicity evaluation of *A. andraeanum* ethanolic leaf extracts on Vero cell line:

As shown in figure (3.1), there was a significant (P≤ 0.001) decrease in the viability of Vero cell line at all concentrations above 125 µg/ml in comparison to the control group after an incubation period of 24 hours.

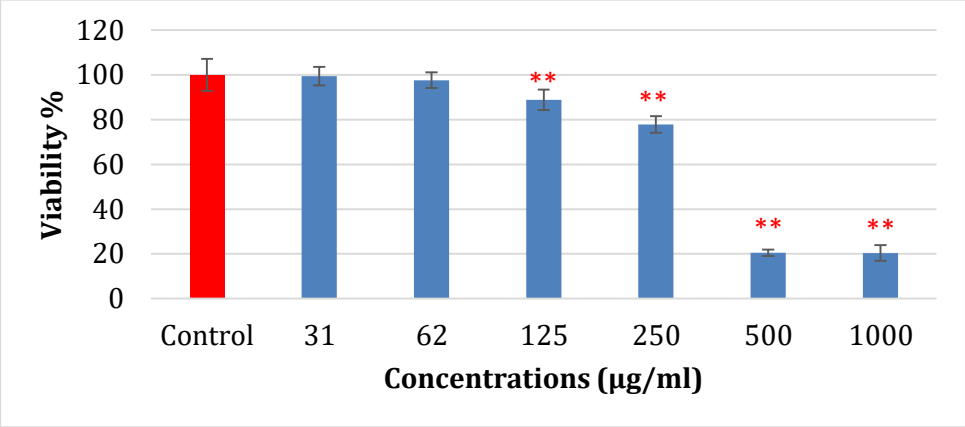

Figure 3.1: Effects of ethanolic leaf extracts of *A. andraeanum* on the viability of Vero cell line measured by MTT assay.

The IC50 value for this extract on the Vero cell line was equal to 520.34 µg/ml as estimated using excel software and illustrated in figure (3.2).

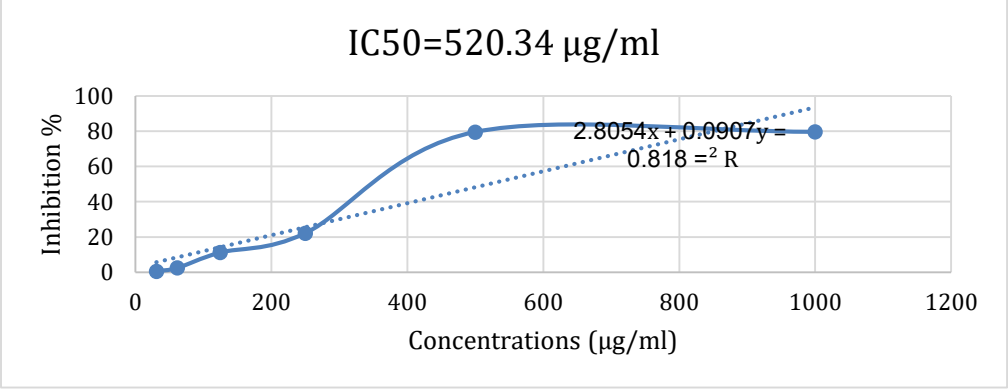

Figure (3.2): Estimated IC50 value of ethanolic leaf extracts of *A. andraeanum* on the Vero cell line.

3.2 Cytotoxicity evaluation of *A. andraeanum* ethanolic leaf extracts on SW480 cell line

It is apparent from figure (3.3) that the ethanolic extract of *A. andraeanum* caused a significant decrease in the viability of SW480cell line at all concentrations used (31-1000 µg/ml) in comparison to the control group after an incubation period of 24 hours.

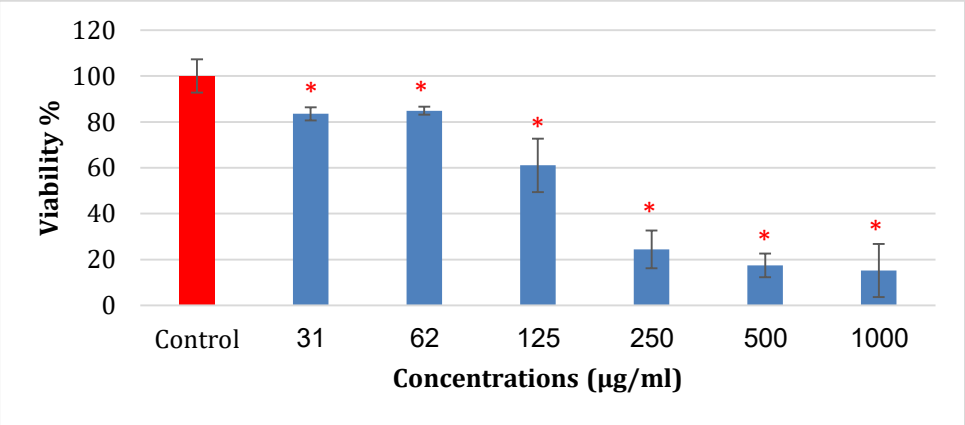

Figure 3.3 Effects of ethanolic of extracts of *A. andraeanum* on the viability of SW480cell line measured by MTT assay.

The value of IC50 was estimated to be about 295.8 µg/ml, as illustrated in figure (3.4).

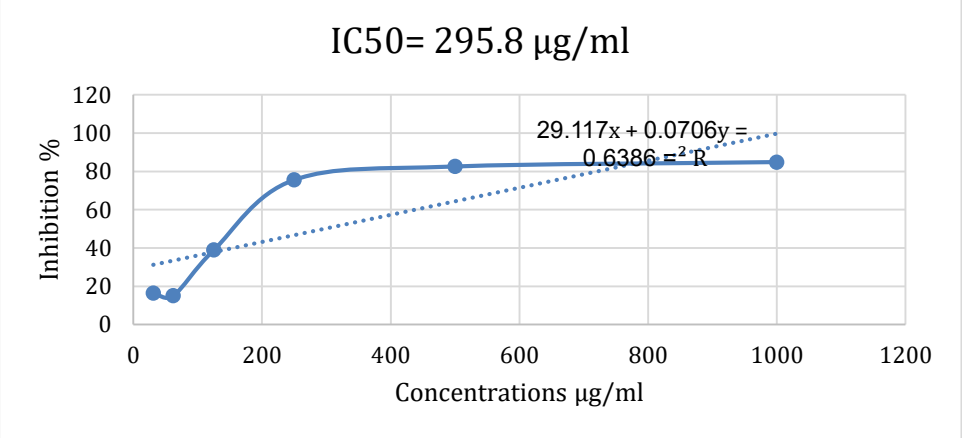

Figure (3.4): The IC50 value of the ethanolic leaf extracts of *A. andraeanum* on SW480 cell line.

3.3 Cytotoxicity evaluation of *A. andraeanum* ethanolic leaf extracts on LNCaP cell line

A. andraeanum ethanolic leaf extracts showed a significant decrease on viability of LNCap cell line at concentrations of (250-1000µg/ml) in comparison the control group after an incubation period of 24 hours as

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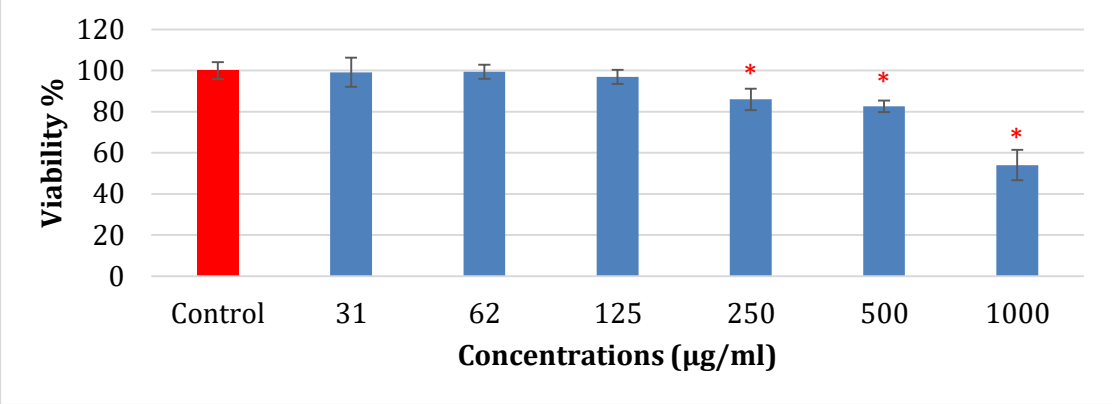

n

(Fig 3.5).**Figure 3.5: Effects of ethanolic of extracts of *A. andraeanum* on the viability of LNCaP cell line measured by MTT assay.**

The estimated IC50 value was (1049 µg/ml), as illustrated in figure (3.6)

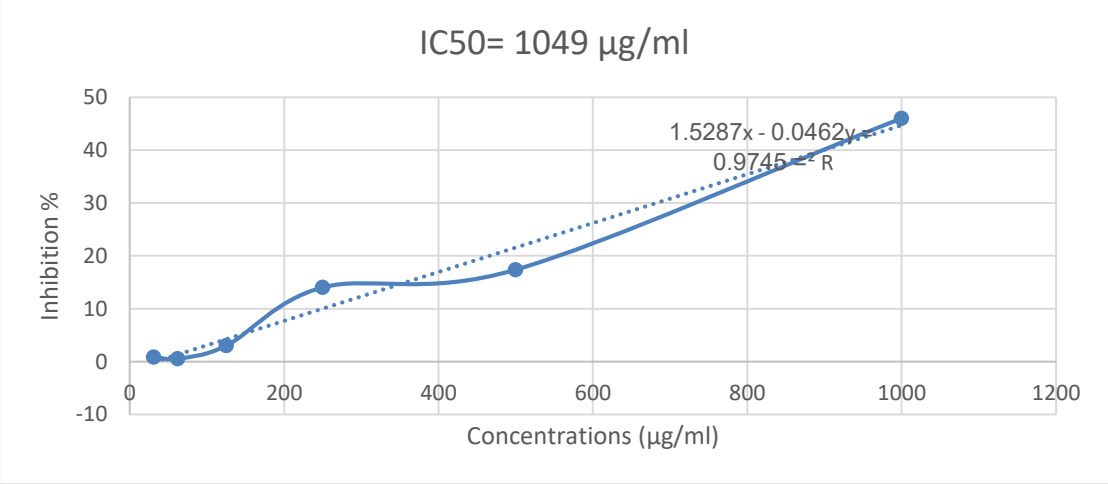

Figure (3.6): Estimated IC50 value of ethanolic leaf extracts of *A. andraeanum* on the LNCap cell line.

3.4 Cytotoxicity evaluation of *A. andraeanum* ethanolic leaf extracts on A549 lung cancer cell line

Figure (3.7) showed the effect of *A. andraeanum* on the viability of the A549 cell line. A significant (P≤ 0.001) decrease in the viability of the cells was observed at concentrations above 125 µg/ml in comparison to the control group after an incubation period of 24 hours. The estimated IC50 value was equal to 349.8µg/ml, as show in figure (3.7).

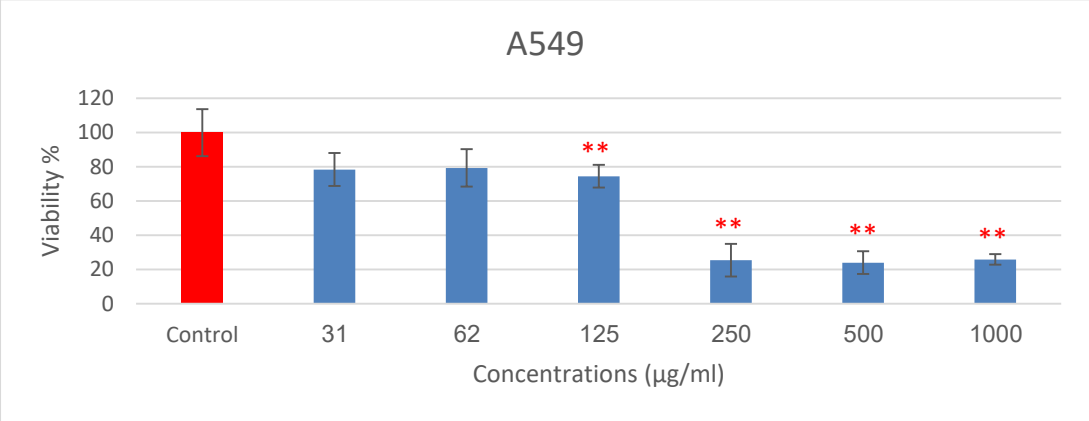

Figure (3.7): Effects of *A. andraeanum* ethanolic leaf extracts of on the viability of A549 cell line measured by MTT assay.

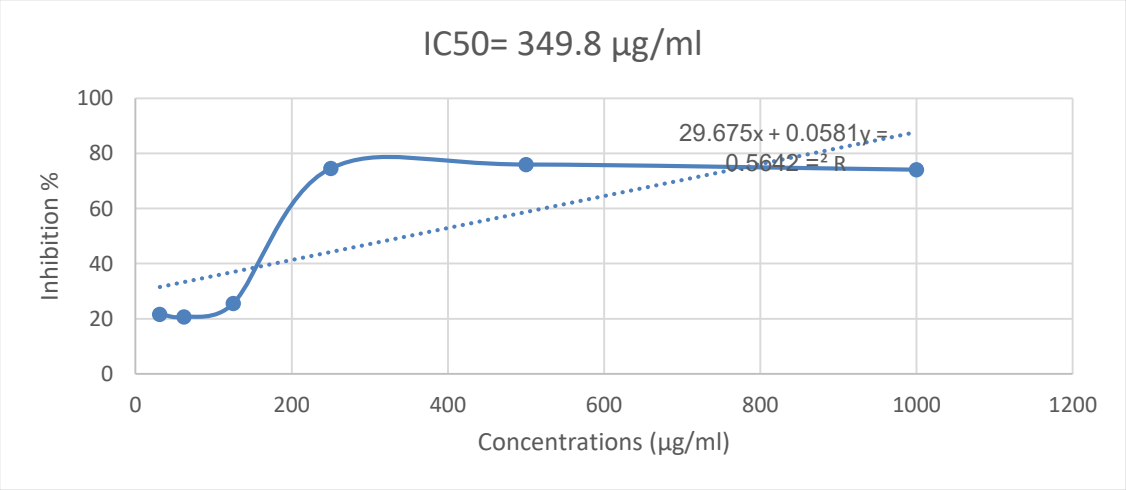

Figure (3.8): estimated IC50 value of ethanolic leaf extracts of A. andraeanum on the A549 cell line

3.5 Antioxidant activity of *A. andraeanum* leaf extract

On SW 480 colon cancer cell line, there was a significant increase in the antioxidant level of total antioxidants at concentrations of (1000,250µg/ml) in comparison to the control group after an incubation period of 24 hours (Fig 3.9).

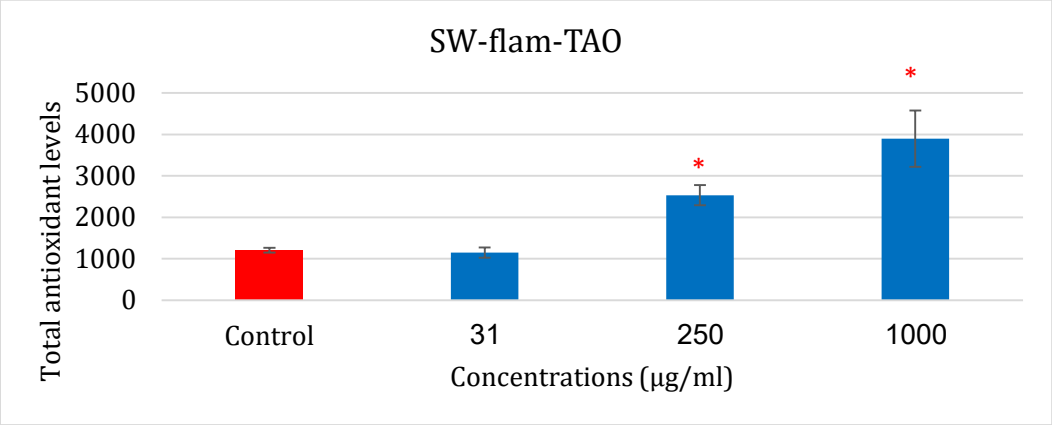

Figure (3.9): Antioxidant effect of *A. andraeanum* ethanolic leaf extracts on SW480 cell line.

While on LNCap cell line, there was a significant increase in the antioxidant level at the concentration of (1000µg/ml) in comparison to the control group. Total antioxidant activity in the culture supernatant was measured after 24 hours of treatment (Fig. 3.10).

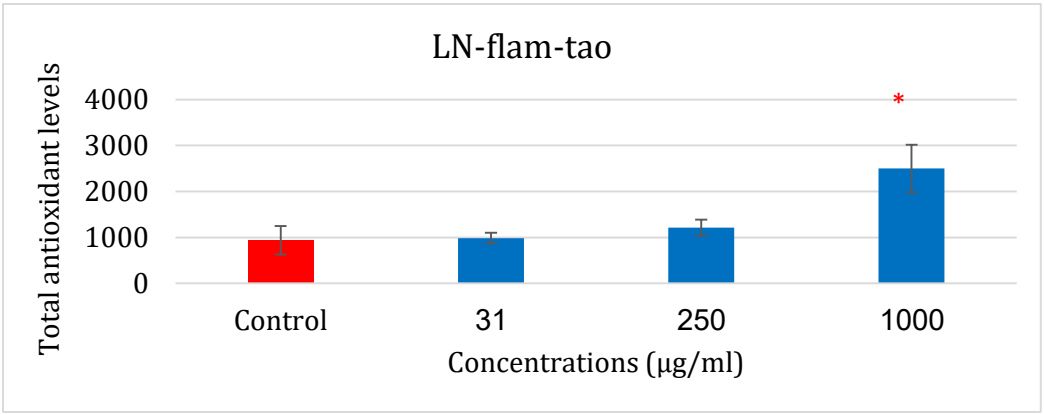

Figure 3.10: Antioxidant effect of A. andraeanum ethanolic leaf extracts of on LNCap cell line.

3.6 Effects of *A. andraeanum* leaf extracts on the anticancer activity of Doxorubicin and Carboplatin against SW480 cell line

The result show that the addition of *A. andraeanum* extract decreased cytotoxicity of Doxorubicin on colon cancer cell line in all concentration of plant in comparison to control group and Doxorubicin alone as seen in figure (3.11).

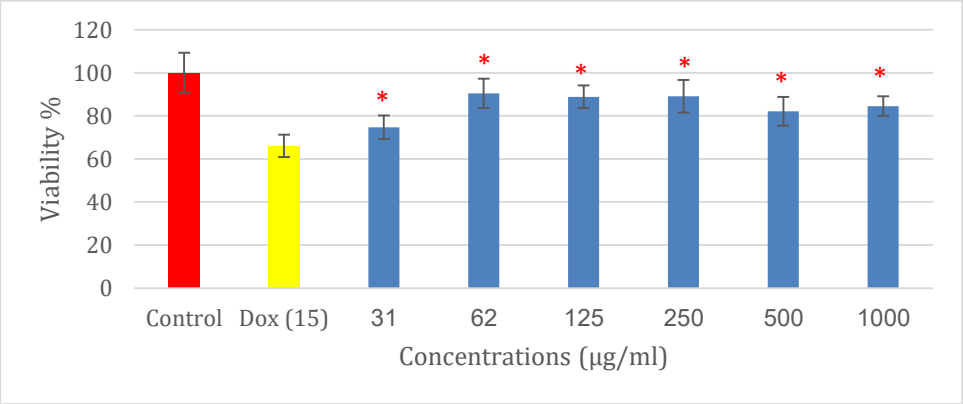

Figure (3.11): Effects of *A. andraeanum* leaf extracts on the anticancer activity of doxorubicin against SW 480 colon cancer cell line. Dox (15); Doxorubicin (15 µg/ml).

The addition of *A. andraeanum* extract with carboplatin increased cytotoxicity of carboplatin on colon cancer cell line in all concentration in comparison to control group and carboplatin alone as shown in figure (3.12).

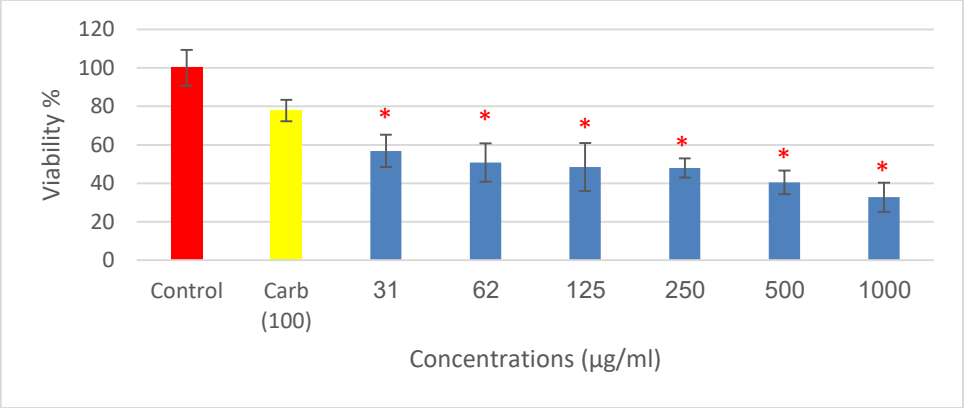

Figure (3.12): Effects of *A. andraeanum* leaf extracts on the anticancer activity of carboplatin against SW 480 colon cancer cell line. Carb (100); carboplatin (100 µg/m).

3.7 Effects of *A. andraeanum* leaf extracts on the anticancer activity of Doxorubicin and Carboplatin against LNCaP cell line

The viability of LNCap was drop when we add *A. andraeanum* extract in concentration (1000) to doxorubicin in comparison to other concentration and control groups as show in figure (3.13).

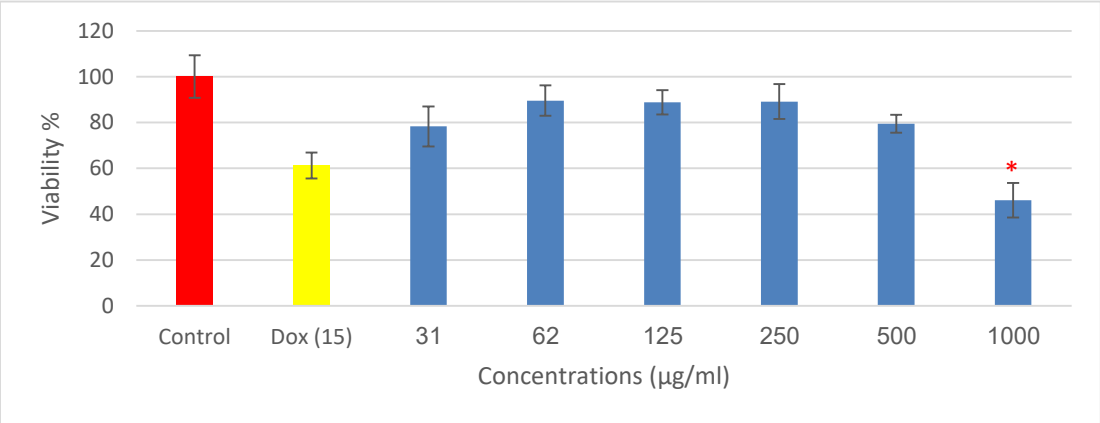

Figure (3.13): Effects of *A. andraeanum* leaf extracts on the anticancer activity of doxorubicin against LNCap cell line. Dox (15); Doxorubicin (15 µg/ml).

While when we added *A. andraeanum* extract with carboplatin the viability of prostate cancer cell was decrease in all concentration of plant as shown in figure (3.14) .

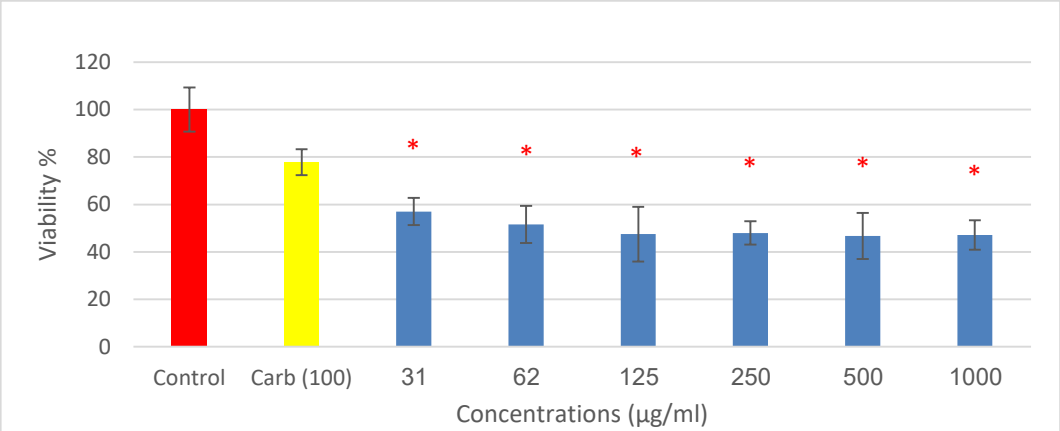

Figure (3.14): Effects of *A. andraeanum* leaf extracts on the anticancer activity of carboplatin against LNCap cell line. Carb (100); carboplatin (100 µg/m).

Chapter four

Discussion

4. Discussion

4.1 Introduction

Recent studies showed that the biological effects of *A. andraeanum* leaf extract might be variable according to the type of cell line or cancer cell. The purified compound shows anticancer activity against various cell lines (Abima Shazhni *et al.*, 2016).

A. species were shown to contain antioxidants and phenolic constituents such as flavone glucosides, which could be responsible for the anti-inflammatory potential. Since antioxidants, like other radical scavengers, protect cells from oxidative DNA damage, possess anti-inflammatory properties and are cancer preventive (T. *et al.*, 2009). The phytochemical components of the plant *A. andraeanum* such as carbohydrates, proteins, tannins, alkaloids, flavonoids, phlobatannins, steroids, phenols and saponins were observed in this plant (Bai *et al.*, 2020).

In this study, the observed effects are thought to be a result of the presence of one or more of these components.

4.2 Cytotoxicity evaluation of *A. andraeanum* alcoholic leaf extracts on the viability Vero cell line:

The IC50 of *A. andraeanum* ethanolic leaf extract on vero cell was (520.34 µg/ml), as shown in figure (3.1). There was a significant ($P \leq 0.001$) decrease in the viability of Vero cell line at all concentrations above 125 µg/ml in comparison to the control group after an incubation period of 24 hours.

This is due to the antiproliferative activity of the extracted phytochemicals which contain phenolic compounds and tannin. Some flavonoids are cytotoxic at higher concentrations to human normal cells.

Further, it is suggested that they are incorporated into cells, increase intracellular ROS levels, and then exert cytotoxicity (Matsuo *et al.*, 2005).

4.3 Cytotoxicity of *A. andraeanum* alcoholic leaf extract on colon cancer cell line

The IC50 of *A. andraeanum* ethanolic leaf extract on SW colon cancer cells was (295.8 µg/ml). It is apparent from figure (3.3) that the ethanolic extract of *A. andraeanum* caused a significant decrease in the viability of the SW480cell line at all concentrations used (31-1000 µg/ml) in comparison to the control group.

The anticancer potential of the alcoholic extract is possibly caused by the presence of tannin, flavonoid, and saponin. Tannins inhibit pyruvate kinase PKM2 activity and subsequently suppress cell proliferation (Baer *et al*, 2020).

Alkaloids inhibit different pathways such aspg-p, PI3k, Fas, and TNRfs, which are, in turn, linked with different mechanistic pathways exhibiting anticancer effects. Inhibition of Bax and Bcl2 leads to activation of caspase-3,8, and 9 pathways, thus leading to apoptosis (Khan *et al.*, 2022).

This study agreed with Mengome study which showed that some Araceae plants' ethanolic extracts that contain flavonoids have strongly inhibited the growth of the human colonic cancer cell line CaCo-2 in vitro (Line-Edwige *et al.*, 2009).

flavonoids can be subdivided into six major subclasses: Flavones, flavanols, flavanones, catechins or flavanols, isoflavones, and anthocyanidins. Intake of flavones was shown to be inversely associated with the risk of colorectal cancer in the Italian population with a 22-23%

reduction in the incidence rate of both colon and rectal neoplasms (Sak, 2017).

Luteolin is one of the very important flavonoids with a lot of health benefits and it strongly prevents cancers via the induction of cell cycle arrest and apoptosis in various human cancer cells. Studies have shown that apigenin, a trihydroxy flavonoid with potential health benefits exhibited strong growth inhibitory activity in various human colon carcinoma cells. It resulted in cell growth inhibition and G2/M cell cycle arrest. It can significantly reduce cell number and induce apoptosis in PWR-1E, LNCaP, PC-3, and DU145 cells (Perveen *et al*, 2017).

Besides the studies that documented the anticancer effects of the phytochemicals found in *A. andraeanum* ethanolic extracts, there is a study that opposed these results and suggested that the intake of stigmasta-5 22-dien-3-ol (phytosterol) increase colorectal cancer in certain cases (Huang *et al.*, 2017).

4.4 Cytotoxicity of *A. andraeanum* alcoholic leaf extracts on prostate cancer cell line:

A. andraeanum ethanolic extracts showed a significant cytotoxic effect on LNCap cell line at concentrations of (250-1000µg/ml) as shown in (Fig 3.5). The IC50 of *A. andraeanum* ethanolic leaf extract on LNCaP was (1049 µg/ml).

The mechanism by which *A. andraeanum* ethanolic extracts inhibits the growth of prostate cancer cells has been explained in other studies by arresting cell cycle in G1 phase leading to apoptosis by tannic acid. The down regulation of pro-survival proteins (Bcl-2 and Bcl-xL), and up regulation of pro-apoptotic markers (Bak, Bim, cleaved caspases 3) and

cleaved poly (ADP-ribose) polymerase were revealed. Additionally, the study results deciphered the anti-metastic activity of tannic acid and its intriguing role in inhibiting EMT progression in prostate cancer cells. Overall, this study provides strong evidence that tannic acid induces ER stress-mediated apoptosis and cell cycle arrest (Nagesh *et al*, 2018).

Paeonol is a phenolic compound and has been reported to effectively inhibit several types of cancer lines. It inhibits prostate cancer by growth inhibition and apoptosis induction. The apoptotic alterations can be explained by the activation of extrinsic and intrinsic apoptotic pathways, which are modulated by balanced Bax/Bcl-2 and blockade of the PI3K/Akt/mTOR cascade (Xu *et al*, 2017).

4.5 Cytotoxicity of *A. andraeanum* ethanolic leaf extracts on lung cancer cell line

Figure (3.7) showed the effect of *A. andraeanum* on the viability of the A549 lung cancer cell line. A significant ($P \leq 0.001$) decrease in the viability of the cells was observed at concentrations above 125 µg/ml in comparison to the control group after an incubation period of 24 hours. The estimated IC50 value was equal to 349.8µg/ml. Yuan Dong study reported that stigmasterol (a phytosterol compound found in *A. andraeanum*) was shown to inhibit the proliferation and promoted the apoptosis of lung cancer cells. Yuan Dong study revealed that retinoic acid-related orphan receptor C (RORC) is directly targeted by stigmasterol in lung cancer (Dong *et al*, 2021).

Plants from the same Araceae family contain compounds with both antioxidant activity and cytotoxicity to cancer cells, and had a high level of cytotoxicity against both drug sensitive and resistant small cell lung

carcinoma cells without serious toxicity to normal Peripheral blood mononuclear cells (PBMC)(Okonogi *et al*, 2013).

Compelling findings generated from mouse studies suggest that antioxidants can facilitate the progression of lung cancer and promote the metastasis of melanoma in mice models Therefore, antioxidants, including flavonoids, should not be considered tumor-preventing agents in absolute terms and across all contexts. Rather, they should be utilized only in the context in which they have undergone rigorous in vitro and in vivo testing and proven to be of tangible benefit (Slika *et al*, 2022).

4.6 Antioxidant effect of *A. andraeanum* alcoholic leaf extracts on prostate and colon cancer cell lines

On SW 480 colon cancer cell line, there was a significant increase in the total antioxidant level at higher concentrations in comparison to the control group (Fig 3.9, 3.10). It is thought that the phenolic structure of anthocyanins is responsible for their antioxidant activity i.e., the ability to scavenge reactive species (ROS) such as superoxide, singlet oxygen, hydrogen peroxide, and hydroxyl radical. The antioxidant effects of anthocyanins in vitro have been demonstrated in several colon and prostate cell lines. In these culture systems, anthocyanins exhibited antitoxic and anti-carcinogenic effects such as: directly scavenging reactive oxygen species (ROS), increasing the oxygen-radical absorbing capacity of cells, stimulating the expression of enzymes, reducing the formation of oxidative adducts in DNA, decreasing lipid peroxidation, inhibiting mutagenesis by environmental toxins and carcinogens, and reducing cellular proliferation by modulating signal transduction pathways (Maldonado-Celis *et al.*, 2014). Since reactive oxygen species (ROS) are known to be triggers of various

human cancers, and antioxidants or scavengers are used to counteract these dangerous species (Sammar *et al*, 2019).

The antioxidant compounds of flavonoids are contributed to transfer an electron or to donate a hydrogen atom to the free radical stability and to chelated metal. Saponin compounds are radical scavengers by forming hydrogen peroxide as a transitional that donates hydrogen to a proton DPPH free radical that finishes radical chain reactions (Nurjanah *et al.*, 2015). There are over 9000 flavonoids that have been described. The core structure of flavonoids is a diphenylpropane skeleton (C6-C3-C6), which contains two phenyl rings and one heterocyclic ring. Their antioxidant capacity has largely been attributed to the presence of multiple 3- and 5-hydroxyl groups as well as 3′- and 4′-catechol groups (Li *et al.*, 2018) .

Luteolin possesses multiple biological effects such as anticancer, anti-allergy, and anti-inflammation. Thus, luteolin behaves as either antioxidant or prooxidant biochemically. These biological effects can be related to each other, for example, the anti-inflammatory properties are associated with its anticancer activity. Its anticancer properties are related to the induction of apoptosis, including DNA damage, redox regulation and protein kinases, inhibition of cell metastasis, proliferation, and angiogenesis. Furthermore, luteolin may be sensitive diversity of cancer cells to therapeutically induced cytotoxicity through damping cell survival pathways and stimulating apoptosis pathways (Perveen *et al*, 2017).

Tannins have abilities to catch free radicals. These compounds are easier to release electron and hydrogen atom and metal chelation activities because there were hydroxyl groups and conjugated double bonds that admit to removing electrons (Sariwati *et al.*, 2019). Gallotannins inhibited the growth

of HCT116 p53+/+ and HT-29p53–/– colon cancer cells in a p53-independent manner but exerted potent apoptosis induction in HCT116 p53– cells associated with an increase of Bax/Bcl-2 protein levels, and also induced S-phase cell cycle arrest in both cell lines(Cai *et al.*, 2017). Tannins do not function solely as primary antioxidants (i.e., they donate hydrogen atoms or electrons), they also function as secondary antioxidants. Tannins can chelate metal ions such as Fe (II) and interfere with one of the reaction steps in the Fenton reaction and thereby retard oxidation. The inhibition of lipid peroxidation by tannin constituents can act via the inhibition of cyclooxygenase (Amarowicz, 2007).

The serum levels of total oxidant status (TOS) and oxidative stress index (OSI) were higher in patients with benign and malignant tumors than in healthy controls; however, TAS levels were significantly lower in patients with benign and malignant tumors than in healthy controls. (Wu *et al*, 2017)

One major contributor of malfunctioning mitochondria to cancer development is ROS overproduction, which is most frequently caused by impaired OXPHOS. The oncogenic mitochondrial ROS complete the vicious cycle between mitochondria, genomic instability, and cancer development. These central roles of mitochondria in cancer make them a promising target in anticancer therapy. Several strategies have been developed for selective drug delivery to mitochondria. Among them, mitochondria-targeted antioxidants are capable of specifically accumulating in mitochondria and efficiently reducing mitochondrial oxidative damage. In consistent with the oncogenic role of ROS, mitochondria-targeted antioxidants, for example SkQ1, are found to be effective in cancer prevention and anticancer therapy. A better understanding of the role of mitochondria in cancer development

and the difference between mitochondria in cancer cells and normal cells are of great value for developing novel therapeutic targets, and for improving the activity and selectivity of mitochondria targeted anticancer agents (Yang *et al*, 2016).

4.7 Effects of *A. andraeanum* leaf extracts on the anticancer activity of Doxorubicin and Carboplatin against SW480 cell line

The result shows that the plant extract in combination with doxorubicin caused a decrease in the cytotoxicity of doxorubicin when compare with the effect of doxorubicin alone as seen in figure (3.11). In contrast, the combination of the plant extract with carboplatin had increased the activity of carboplatin on colon cancer cell line in all concentrations of plant in comparison to the control group and carboplatin alone, as illustrated previously in figure (3.12).

The higher risk of colorectal cancer associated with higher intakes of campesterol and stigmasterol observed among men in this study do not necessarily mean that these sterols alone have an unfavorable effect on large bowel cancer, but that the effect may also depend on intakes of other plant sterols or bioactive substances in the diet that co-exist with the ones being studied. An alternative explanation is that plant sterols inhibit cholesterol absorption. A high intake of cholesterol has been suggested to be associated with an increased risk of colorectal cancer. Changes in membrane fluidity induced by DOX are associated with drug penetration into the cell membrane. This could have important implications for cancer therapy where passive diffusion of cytotoxic agents into cancer cells can be hampered by rigid and tightly packed plasma membranes and the incorporation of specific

plant sterols, which one would predict to have saturated side-chains, could improve drug uptake. Improved understanding of how plant sterols alter bilayer fluidity is key to linking therapeutic potential with a solid robust understanding of their molecular mechanisms. It is plausible that if phytosterols are specifically able to increased membrane fluidity they may be therapeutically applicable during systemic chemotherapy treatment, where they could antagonize the rigid membrane barrier that prevents passive drug diffusion. However, the same process may exacerbate cell motility by increasing fluidity and the potential for cells to traverse basement membranes and junctions so different sterols may be applicable at different stages of treatment. (Fakih *et al*, 2018).

Phytochemicals with chemo preventive and chemotherapeutic potential are possible chemo sensitizing properties when combined with established cancer treatments in which some modes of action could already be identified Phytochemicals apparently enhances antitumor therapies by modulating cell signaling pathways and cell cycle regulators and by inducing apoptotic pathways. They seem to further counteract mechanisms of multidrug resistance by regulating the expression levels of membrane efflux pumps and members of the cytochrome E450 superfamily of enzymes thereby directly modulating intracellular drug metabolism and bioavailability. We could observe a significant increase of Platin antitumor efficacy even at low concentrations when cells were pretreated with phytochemicals (Kaminski *et al*, 2014).

Synergistic interactions are essential in phytomedicine and explain the effectiveness of extremely low doses of active constituents in herbal formulations. Traditional medicine works on the idea that a whole or partly

purified plant extract offers improvements over a single isolated ingredient. Synergism also leads to toxicity reduction and minimization of resistance (Mohan, 2021).

4.8 Effects of *A. andraeanum* leaf extracts on the anticancer activity of Doxorubicin and Carboplatin against LNCap cell line

A. andraeanum extract at the concentration of (1000µg/ml) in combination with doxorubicin (15µg/ml) significantly decreased the viability of LNCap in comparison to other concentration and control groups as shown in figure (3.13), while the combination of the plant extract with carboplatin significantly decreased cell viability at all concentrations used as shown in figure (3.14).

Doxorubicin is an anthracycline, which intercalates into DNA thereby activating DNA repair pathways and elevating levels of p53. PC3 cells are p53-/-, which may explain why doxorubicin as a single agent was relatively ineffective against tumor growth inhibition (El-Zawahry *et al*, 2005).

To delay the occurrence of doxorubicin resistance, combination treatments are routinely used to offset the aforementioned drug-resistance mechanisms, and resensitize tumor cells to doxorubicin. (Shu *et al*, 2018).

Carboplatin is a ‘second generation’ platinum compound, with a different and usually improved toxicity profile over that of cisplatin, and similar efficacy (Kover, 2012).

Studies have shown that prostate cancer is relatively resistant to carboplatin and suggest two causes of resistance: bypassing the cell cycle checkpoints which serve as points of entry into apoptosis, and incomplete execution of the effector mechanisms of apoptosis (Yee *et al*, 1998).

In prostate cancer, oxidative stress and the subsequent Nrf2 activation promote the survival of cancer cells and acquired chemoresistance. The Nrf2 links prostate cancer to endoplasmic reticulum stress, an event that triggers the unfolded protein response, aiming to restore cellular homeostasis as well as an adaptive survival mechanism (Bellezza *et al.*, 2017). Another study showed that in a dose-dependent manner stigmasta-5-22-dien-3-ol isolated from *A. andraeanum* suppresses Nrf2. It was shown that the suppression of Nrf2 sensitizes ovarian cancer cells to doxorubicin and cisplatin (Liao *et al*, 2020).

Studies also reported that blocking Nrf2 transcriptional activity enhanced the efficacy of carboplatin and other chemotherapeutic drugs in resistant cancer cells (Singh *et al.*, 2016). Since Nrf2 involves in chemoresistance, targeting this molecule maybe enhance the response to chemotherapy. Thus, down regulating Nrf2 protein level or repressing its transcription activation is a possible way to inhibit Nrf2 and Nrf2-dirven chemoresistance (Liao *et al.*, 2020). Stigmasterol inhibited Nrf2 protein expression in human endometrial cancer with a dose manner, stigmasterol inhibited Tet1 expression and Tet1-induced hydroxymethylation, following in turn inhibit Nrf2 protein expression. So, the decline of Nrf2 protein by stigmasterol also attributes to decrease activity of transcription. Additionally, stigmasterol inhibits prostate cancer by inducing the expression of p53 protein and simultaneously inhibiting the expression of p21 and P27 proteins (Dong *et al*, 2021).

Phytochemicals are currently gaining great importance in both cancer prevention and treatment due to their antioxidant, antiproliferative,

antiangiogenic, proapoptotic, and anticancer properties (Abotaleb *et al.*, 2020).

Conclusions

&

Recommendations

Conclusions:

1. Alcoholic leaves extract of *A. andraeanum* showed significant cytotoxic effects on normal, lung, prostate, and colon cancer cell lines at higher concentrations above(125 µg/ml).
2. According to our results, plant extract appears to have no cytotoxic effects at lower concentrations below (125 µg/ml), thus, it may be safe for medical uses.
3. Alcoholic leaves extract showed significant increase in antioxidant level on prostate cancer cell line (LNCaP), and colon cancer cell line (SW480) at higher doses, above (125 µg/ml).
4. Alcoholic leaves extract of the plant increased the anticancer activity of carboplatin and doxorubicin.

Recommendations

1- Study the toxicity of the plant extract on the rates models.

2- Study the immunological effects of the plant extract in vitro and in vivo.

3- Study the cytotoxic and anticancer activity of different plant parts on another cell lines.

4- Study the interaction of plant extract with other anticancer drugs.

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الخلاصة

تم تنفيذ الجزء العملي للدراسة البحثية الحالية بمختبر أبحاث طلبة الدراسات العليا / قسم الأدوية والسموم/ كلية الطب جامعة بابل خلال الفترة من أكتوبر 2021 الى أبريل 2022

المكونات الكيميائية للنبات تلعب دورا مهما في علاج الامراض لما لها من دور اساسي في نمو الخلية، ولهذا فأن أهداف هذه الدراسة تشمل:

اولا معرفة مدى سمية المستخلص الكحولي لأوراق نبات الأنثريوم (الفلامينكو) على عدة أنواع من الخلايا تشمل خطوط الخلايا الطبيعية وخطوط الخلايا السرطانية للقولون وخطوط الخلايا السرطانية للبروستات وكذلك خطوط الخلايا السرطانية للرئة. الهدف الثاني هو معرفة مدى قابلية المستخلص الكحولي لأوراق نبات الأنثريوم (الفلامينكو) للأكسدة واجري هذا الاختبار على نوعين من الخلايا السرطانية وهي خط الخلايا السرطانية للقولون SW480وخط الخلايا السرطانية للبروستات LNCap. اما الهدف الأخير فهو معرفة مدى تأثير المستخلص الكحولي على فعالية كل من دواء Doxorubicin وcarboplatin المضاد للسرطان.

الجزء الاول يشمل زراعة خط الخلايا الطبيعية، خط خلايا سرطان البروستات، خط خلايا سرطان القولون وخط خلايا سرطان الرئة في طبق زرعي يتكون من 96 مكان زرعي وتمت معاملتها مع المستخلص الكحولي وبسلسلة تراكيز تخفيفية (1000,500,250,125,62,31) ميكروغرام /مل وتم حضنها لمدة 24 ساعة وبعد ذلك تم الفحص باستخدام التحليل اللوني للفعالية الحيوية MTT.

أضهرت النتائج تثبيط كل من الخلايا الطبيعية وخلايا سرطان الرئة عند استعمال التراكيز العالية (1000,500,250 ,125) مايكرو غرام/مل قياسا بالتراكيز الواطئة (31,62) مايكروغرام/ مل والخلايا التي لم يتم تعريضها للمستخلص (السيطرة السلبية) اما بالنسبة للخلايا السرطانية للبروستات فقد سببت التراكيز العالية أنخفاض ملحوظ بفعالية الخلايا السرطانية عند التراكيز (1000,500,250) مايكرو غرام/ مل قياسا بالتراكيز الواطئة (31,62,125) مايكروغرام/ مل والخلايا التي لم يتم تعريضها للمستخلص (السيطرة السلبية). اما بالنسبة لخلايا سرطان القولون فقد لوحظ انخفاض عدد الخلايا عند استعمال كل تراكيز المستخلص الكحولي لأوراق النبات مقارنة بالخلايا التي لم يتم تعريضها للمستخلص.

اما الجزء الثاني فقد اشتمل دراسة تأثير مضاد الأكسدة على مستخلص الأوراق الكحولية من نبات أنثوريوم بأستخدام طريقة (CUPRAC) على خط خلايا سرطان القولون وخط خلايا سرطان البروستات من خلال

زرعها بطبق زرعي ومعاملتها مع المستخلص الكحولي لأوراق النبات بثلاثة تراكيز (1000، 250 ،31) ميكروغرام / مل،

فكانت نتائج هذه التجربة أن هناك زيادة كبيرة في مستوى مضادات الأكسدة لكلا الخطين من الخلايا السرطانية عند التراكيز (1000,250) ميكروغرام /مل بالنسبة لخلايا القولون و(1000) ميكروغرام/ مل بالنسبة لخلايا البروستات قياسا بالتركيز الواطئ (31) مايكروغرام/ مل والخلايا التي لم يتم تعريضها للمستخلص.

الجزء الثالث من التجربة اشتمل على قياس تأثير كل تركيز من المستخلص الكحولي للنبات على فعالية كل من دواء Doxorubicinو carboplatin في تثبيط الخلايا السرطانية للقولون والخلايا السرطانية للبروستات. تم تقييم تأثير كل تركيز من المستخلص على الدواء بالاعتماد على زراعة الخلايا في طبق زرعي يتكون من 96 مكان زرعي مقسم إلى 8 مجموعات؛ مجموعة تحكم واحدة، وأخرى من Doxorubicinوحده، وستة تخفيفات متسلسلة من المستخلص الكحولي من (1000 إلى 31) ميكروجرام / مل مع تركيز ثابت من Doxorubicin(15 ميكروجرام / مل). تم تحضين جميع الأطباق لمدة 24 ساعة عند 37 درجة مئوية وبعد ذلك تم الفحص باستخدام التحليل اللوني للفعالية الحيوية MTT، كانت نتيجة هذه العملية زيادة التأثير السام على خلية القولون عند إضافة المستخلص الكحولي في جميع التراكيز مقارنة باستخدام Doxorubicin وحده ومجموعة التحكم، بينما انخفضت قابلية الخلايا لسرطان البروستات LNCap عند إضافة مستخلص أنثوريوم أندريانوم بتركيز (1000) إلى Doxorubicin مقارنة باستخدام Doxorubicin لوحده ومجموعة التحكم السلبية الأخرى .

أعيدت نفس التجربة لقياس فعالية carboplatin بتركيز ثابت 100 ميكروغرام / مل وكانت نتيجة اضافة المستخلص الكحولي للنبات مع carboplatin انخفاض عدد خلايا سرطان البروستات LNCap وخلايا سرطان القولون في كل تراكيز النبات قياسا بمجموعة التحكم أوcarboplatin وحده.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل
كلية الطب

دراسة في المختبر للتاثير المضاد للسرطان والمضاد للاكسدة للمستخلص الكحولي لنبات الانثريوم اندرينيوم

رسالة

مقدمة الى مجلس كلية الطب / جامعة بابل كجزء من متطلبات نيل درجة الماجستير في علم الادوية /الادوية والسموم

من قبل

نور محمد رسول علي

(بكلوريوس صيدلة, 2014-2015)

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