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An In Vitro Study to Evaluate the Effect of Propolis Extracts on Normal and Cancer Cells

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بسم الله الرحمن الرحيم

وَأَوْحَى رَبُّكَ إِلَى النَّحْلِ أَنِ اتَّخِذِي مِنَ الْجِبَالِ بُيُوتًا وَمِنَ الشَّجَرِ وَمِمَّا يَعْرِشُونَ (٦٨)

ثُمَّ كُلِي مِنْ كُلِّ الثَّمَرَاتِ فَاسْلُكِي سُبُلَ رَبِّكِ ذُلُلًا يَخْرُجُ مِنْ بُطُونِهَا شَرَابٌ

مُخْتَلِفٌ أَلْوَانُهُ فِيهِ شِفَاءٌ لِلنَّاسِ إِنَّ فِي ذَلِكَ لَآيَةً لِقَوْمٍ يَتَفَكَّرُونَ (٦٩)

صدق الله العظيم

سورة النحل الآيات ٦٨-٦٩

Dedication

I dedicate my thesis work to my father, mother, husband and, my family who encourage me and pray for me... for my children especially my little daughter "Mina" who is such a good girl. Also, I dedicate my work to my friends especially " Maha Ali" who support me all the time.

Maha

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Summary

Cancer is a disease manifested by uncontrolled growth of abnormal cells that can potentially spread to other tissue of the body. Prostate cancer begins while the normal cells of the prostate start changing and uncontrollably grow. Burkitt lymphoma is a type of the non-Hodgkin lymphoma a cancer of the lymphatic tissue, occurred while the body forms abnormal B lymphocytes.

For many years, nature plays a promising role for the discovery of different natural compounds that can be used as new drugs for the treatment of many diseases including cancer which has special attention in medicine. Propolis, a natural resinous substance known for centuries, is a complex product obtained by honey bees from substances collected from parts of different plants in different geographic areas.

The primary aim of this study was to evaluate the antiproliferative effect of aqueous and ethanolic extracts of propolis against Raji cell line, LNCaP prostate cancer cell line, Vero cell line and peripheral blood mononuclear cells. The secondary aim was to assess the immunomodulatory effects of these propolis extracts on Raji cell line and peripheral blood mononuclear cells through measuring the levels of specific cytokines (IL-4, IL-10, IL-17 and IFN-γ) produced by these cells.

The peripheral blood mononuclear cells were isolated from a healthy young donor by using a density-gradient medium, the isolated peripheral blood mononuclear cells were then purified and checked for viability. Then cells from cell lines Raji, LNCaP, Vero and peripheral blood mononuclear cells were seeded in tissue culture 96 well plate and treated with different concentrations (1000, 500, 250, 125, 62.5, 31.25 µg/ml) of each aqueous and ethanolic extracts of propolis and incubated for 24 hours. Then the MTT cytotoxicity assay was used to assess the effect of the extracts on the

viability of these cells, and the half-maximal inhibitory concentration (IC50) was measured for the aqueous and ethanolic extracts of propolis. Similarly, the anticancer activity of doxorubicin alone and in combination with propolis ethanolic extract against the LNCaP cell line has been assessed.

In order to evaluate the immunomodulatory effect of the aqueous and ethanolic extracts of propolis on Raji cell line and peripheral blood mononuclear cells, these cells were treated with 1000, 250, and 50 µg/ml of each propolis extracts. The ELISA assay was performed to detect the levels of IL-4, IL-10, IL-17 and IFN-γ.

Results showed that all concentrations of both aqueous and ethanolic extracts of propolis caused a significant ($p \leq 0.001$) decrease in the viability of LNCaP cell line. In regard to the anticancer activity of doxorubicin it caused a significant ($p \leq 0.001$) decrease in the viability of the LNCaP cell line at all concentrations (250, 125, 62, 31, 15, and 7.5 µg/ml). The combination of doxorubicin (30 µg/ml) and ethanolic extract of propolis (1000, 500, 250, 125, 62.5, 31.25 µg/ml) caused a significance ($p \leq 0.001$) decrease in the viability of the LNCaP cell line in all concentrations.

Regarding the effect of propolis extracts on Vero cell line, results revealed that the viability of these cells was significantly ($p \leq 0.05$) decreased in concentrations 1000 and 500 µg/ml of propolis aqueous extract, and there was a significant ($p \leq 0.001$) decrease in the viability of these cells at all the concentrations of ethanolic extract.

Results showed that aqueous extract of propolis in concentrations 1000, 500, and 250 µg/ml caused a significant ($p \leq 0.001$) decrease in the viability of Raji cell line. While only the high concentration of ethanolic extract (1000 µg/ml) caused a significant ($p \leq 0.001$) decrease in the viability of these cells.

The IC50 of propolis aqueous and ethanolic extracts were (1416.43 µg/ml versus 717.36 µg/ml) for the LNCaP cell line, (1773 µg/ml versus 1845 µg/ml) for the Raji cell line, and (2304 µg/ml versus 821 µg/ml) for Vero cell line.

Regarding the peripheral blood mononuclear cells results showed an efficient separation with high lymphocyte count, low platelets, and no red blood cells were found with cell viability of more than 95%. The concentrations 1000 and 500 µg/ml of aqueous extract result in a significant ($p \leq 0.001$) increase in the viability of peripheral blood mononuclear cells, while the concentrations 125, 62.5, and 31.25 µg/ml of the same extract result in a significant ($p \leq 0.05$) decrease in the viability of peripheral blood mononuclear cells. The ethanolic extract of propolis result in a significant ($p \leq 0.001$) increase in the viability of peripheral blood mononuclear cells in the concentrations 1000, 500, 250, and 125 µg/ml.

For the immunomodulatory effect of propolis extracts on the Raji cell line and peripheral blood mononuclear cells, for Raji cell line, the results showed a significant ($p \leq 0.001$) decrease in the level of IL-4 after the treatment with 50, 250, and 1000 µg/ml of the aqueous extract and 1000 µg/ml of the ethanolic extract.

Regarding the peripheral blood mononuclear cells, results showed a significant ($p \leq 0.001$) increase in the level of IL-4 after the treatment with both aqueous and ethanolic extracts in concentrations 50 and 250 µg/ml, while there was a significant ($p \leq 0.001$) decrease in the level of IL-4 after the treatment with 1000 µg/ml of ethanolic extract.

For Raji cell line, the results showed a significant ($p \leq 0.001$) decrease in the level of IL-10 after the treatment with 1000 µg/ml of the ethanolic extract, while there was a significant ($p \leq 0.001$) increase in the level of IL-10 after the treatment with 50 µg/ml of the ethanolic extract. For the peripheral blood mononuclear cells, the results showed a significant ($p \leq$

0.001) increase in the level of IL-10 after the treatment with 250 µg/ml of both aqueous and ethanolic extracts.

Regarding Raji cell line, the results showed a significant ($p \leq 0.001$) decrease in the level of IL-17 in all concentrations used for both extracts.

For the peripheral blood mononuclear cells, the results showed a significant ($p \leq 0.001$) increase in the level of IL-17 after the treatment with 50 and 250 µg/ml of both extracts, while there was a significant ($p \leq 0.05$) decrease in the level of IL-17 after the treatment with 1000 µg/ml of the ethanolic extract.

Regarding the Raji cell line, the results showed a significant ($p \leq 0.001$) decrease in the level of IFN-γ in all concentrations used and for both extracts.

For the peripheral blood mononuclear cells, the results showed a significant ($p \leq 0.001$) increase in the level of IFN-γ after the treatment with 50 and 250 µg/ml of aqueous extract and 1000 µg/ml of ethanolic extract.

In conclusion, propolis extracts have anti-proliferative effects on LNCaP and Raji cancer cell lines, in addition to immunomodulatory effects on Raji cell line and the isolated peripheral blood mononuclear cells.

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List of Abbreviations

Abbreviation	Meaning
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
Arte C	Artepillin C
ATP	Adenosine triphosphate
Bax	Proapoptotic gene
Bcl-2	Antiapoptotic gene
BCR/ABL	gene sequence found in an abnormal chromosome 22

BL	Burkitt's lymphoma
BM	Bone marrow
CAPE	Caffeic acid phenethyl ester
CBC	Complete blood count test
CD	Cluster of differentiation
CD+4	Glycoprotein located on the surface of immune cells
CD+5	Cluster of differentiation expressed on the surface of T cells
CD+8	Transmembrane glycoprotein that serves as a co-receptor for T cells receptor
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CNS	Central nervous system
DCs	Dendritic cells
DDW	deionized distilled water
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid

FBS	Fetal bovine serum
H2O2	Hydrogen peroxide
HeLa	Cancer cell line named after Henrietta lacks
HIV	Human immunodeficiency virus
IC50	Median Inhibitory Concentration
IFN-γ	Interferon –γ
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
LNCaP	Lymph node carcinoma of the prostate
MHC	Major histocompatibility complex
MLL	Mixed-lineage leukemia
MTT assay	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay
Myc	Family of regulator genes
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NK	Natural killer cells
Nm	Nanometer
PBLs	Peripheral blood lymphocytes
PBS	Phosphate buffer saline
PCa	Prostate cancer

PGE2	Prostaglandin E2
Rpm	Revolution per minute
RPMI	Roswell Park Memorial Institute (RPMI) 1640 Medium
Th 17	T-helper 17
Th cells	Helper T cells
Th1	T-helper 1
TKI	tyrosine kinase inhibitor
TNF	Tumor necrosis factor
WHO	World health organization

Chapter One

Introduction

and

Literature Review

1.1 Introduction

Propolis is a multifunctional material used by bees in the protection and maintenance of their hives. It has been used by humans for a long time.

The use of products containing propolis has resulted in extensive contact and it is now increasingly being used as a dietary supplement. Unlike many natural remedies, there are much data on the biological activity and toxicity of propolis indicating it may have many antibiotic, antifungal, antiviral, and antitumor properties (Burdock, 1998).

Propolis is a natural treatment that has been used since old times. Egyptians knew the anti-putrefactive properties of propolis and used it to embalm cadavers. Propolis was recognized for its medicinal properties by Greek and Roman physicians. Propolis was employed as an antiseptic and healing power in wound treatment, and as a mouth disinfectant with these uses being carried out in the Middle Ages and among Arab physicians. Propolis was also recognized by other people: Incas used propolis as an antipyretic, and the London pharmacopeias of the seventeenth century considered propolis as an official drug. Between the seventeenth and twentieth centuries, propolis became very popular in Europe on account of its antibacterial activity. Present-day herbalists suggest it for its anti-bacterial, anti-viral, anti-fungal, and anti-inflammatory properties, to increase immunity and treat gastro-duodenal ulcers. When propolis is used as an ointment, it alleviates many kinds of dermatitis caused by bacteria and fungi. Propolis is provided as capsules (either in pure form or combined with aloe gel and rosa canina), as an extract (hydroalcoholic or glycolic), as a mouthwash (with melissa, sage, mallow, and rosemary), in throat lozenges, creams, and in powder form (to be used in gargles or for internal use once dissolved in water) (Castaldo and Capasso, 2002).

Aims of the study

The main objectives of the present study are:

1. To determine the in vitro effect of propolis extracts (aqueous and ethanolic) on the viability of:
    - Cancer and normal cell lines (LNCaP, Raji, and Vero cell lines).
    - Peripheral blood mononuclear cells.
2. Evaluation of the immunomodulatory effect of propolis extracts on Raji cell line and peripheral blood mononuclear cells by measuring the level of some cytokines in these cells.

1.2 Cancer

Cancer is the uncontrolled growth of abnormal cells anywhere in the body. These abnormal cells are named cancer cells, malignant cells, or tumor cells. cancer cells can invade normal body tissues. The abnormal cells that make up the malignant tissue are recognized by the name of the tissue that they arise from (for example, breast cancer). When damaged cells do not die they show uncontrolled division and growth - a mass of cancer cells will develop. Frequently, cancer cells can break away from this original mass of cells, travel through the blood and lymph systems, and lodge in other organs where they can again repeat the uncontrolled growth cycle. This process through which the cancer cells leave their original area and grow in another body area is termed metastatic spread or metastasis. For example, if breast cancer cells spread to a bone, it means that the individual has metastatic breast cancer to the bone (Becker, 2013).

1.2.1 Cancer metastasis

Cancer metastasis is a process in which cancer cells disseminate from the cancerous primary tumor, where they settle and grow at a site other than the primary cancerous tumor site, about 90% of cancer deaths are due to cancer metastasis rather than primary cancer. Cancer research has mainly concentrated on the evolution of methods or agents that can discover the tumor at the early stage, and on agents that prevent tumor growth. Most solid cancers are now manageable or curable if they are diagnosed and treated before metastasis. Once tumors metastasize, they are almost untreatable and lethal. Because the mechanisms of the underlying metastatic process are not understandable, finite success has been made in the prevention and inhibition of tumors metastasis. Metastasis is a complex process that includes many sequential, and interrelated steps and with much to be explained. Metastasis is facilitated by four basic steps: detachment,

migration, invasion, and adhesion. tumor cells first detach from the primary tumor, undergo migration, invasion, and travel to many sites through blood and lymphatic vessels, then settle and grow. Metastasis is controlled by different signaling pathways and is affected by the nearby extracellular matrix. Metastasis genes are stress-response genes that physiologically play a part in inflammation, wound healing, and stress-induced angiogenesis (Guan, 2015).

1.2.2 Types of cancer

Cancers are divided into many types:

a. Carcinomas: It begins in the skin or the tissue which coats the glands and internal organs. It builds a solid tumor such as breast cancer.

b. Sarcomas: It begins in the tissues which connect and support the body. It can be constituted in nerves, tendons, joints, fat, blood vessels, bone, and lymph vessels.

c. Leukemia: Leukemia is a cancer of the blood. It starts when healthy blood cells grow uncontrollably and change. It is split into 4 types, that are acute myeloid leukemia, acute lymphocytic leukemia, chronic myeloid leukemia, and chronic lymphocytic leukemia.

d. Lymphomas: Lymphoma is a tumor that starts in the lymphatic system and it is a network of glands and vessels that helps to prevent infection. Hodgkin lymphoma and NonHodgkin lymphoma.

e. Central Nervous System Cancers (CNS): tumors that begin in brain tissues and spinal cord called "brain and spinal cord tumors", and others primary CNS lymphomas, gliomas, pituitary adenomas, meningiomas.

f. Multiple Myeloma: Multiple myelomas are infrequent tumors that start in plasma cells, a type of immune cell. The myeloma cells which are plasma cells are rises from BM and make tumors in bones.

g. Melanoma: It begins in cells that develop into melanocytes. These cells are specific cells that produce melanin, i.e., the pigment that gives color to the skin. Melanomas grow on the skin, but they can also grow in other pigmented tissue like an eye.

h. Other Types of Tumors: such as Germ Cell Tumors, a type of cancer that begins in the cells which develop into eggs or sperms it can be either malignant or benign. Neuroendocrine tumors develop from cells that secret hormones into the blood in response to a signal from the nervous system. These tumors, which can produce an elevated quantity of hormones, will cause various symptoms. It may be either benign or malignant (Saini *et al.*, 2020).

1.2.3 Causes and risk factors of cancer

The cause of some cancer remains unknown but many cancers have environmental or lifestyle causes or may develop from more than one cause. Some are influenced by a person's genetic makeup. Many patients develop tumors due to heredity, ionizing radiation, chemical substances, dietary factors, viruses, stress, and age (Becker, 2013).

1.2.4 Mortality for malignant diseases

According to World Health Organization (WHO) update published in 2019, cancer is the second cause of death (8.97 million deaths) worldwide. Notably, the rank of cancer among the leading causes of mortality differs in the various regions, starting first in the Western Pacific and America regions, second in the South-East Asia, European,

and Eastern Mediterranean regions, but only fourth in the African region (Mattiuzzi and Lippi, 2019).

1.3 Prostate cancer (PCa)

Prostate cancer (PCa) is a big health concern, especially in developed countries with a greater proportion of older men in the general population. PCa is the most common cancer in elderly males in Europe. The three well-established risk factors for PCa are increasing age, ethnic origin, and genetic predisposition. There is no evidence to suggest that dietary interventions would reduce the risk of PCa.

Screening early by prostate-specific antigen (PSA) testing could be used to detect men at risk and in need of further follow-up (Heidenreich *et al.*, 2008).

Patients with prostate cancer usually present with symptoms like erectile dysfunction, blood in the semen, pain in the lower back, hips, and/or upper thighs, urinary problems, or enlargement of the prostate. Enlargement of the prostate can lead to obstruction with reduced flow, hesitancy, post-micturition dribbling, or even retention, bleeding, and/or infection (James, 2014).

1.3.1 Risk factors

The factors that determine the risk of developing clinical PCa are not well known, even though there are three well-known risk factors for PCa: increasing age, ethnicity, and inheritance. If one of the first-degree relatives has PCa, the chance is at least doubled. The risk increases by 5-11 times if two or more first-degree relatives are affected. True hereditary PCa affects a tiny percentage of men with PCa (approximately 9%). Three or more affected relatives, or at least two relatives who have developed the disease

before the age of 55, are considered early-onset. Patients with genetic PCa frequently get the disease six to seven years earlier than those who develop it spontaneously. Exogenous factors such as the foods consumed, the pattern of sexual behavior, alcohol consumption, exposure to ultraviolet radiation, chronic inflammation, and occupational exposure may have an important impact on the risk of progression from so-called latent PCa to clinical PCa. Several 5-alpha-reductase inhibitors (5-ARIs) have been researched to see how effective they are at lowering the risk of PCa. Although 5-ARIs appear to have a potential benefit in avoiding or delaying the development of PCa, this must be balanced against treatment-related adverse effects (Mottet *et al.*, 2015).

1.3.2 Epidemiology

Prostate cancer is the third most commonly diagnosed malignancy. It is a heterogeneous disease with incidence rates that vary across the world. Age-standardized incidence rates are the highest in Northern Europe and lowest in South-Central Asia. Men of African origin are more prone to the disease compared with other ethnicities. Mortality rates differ significantly from incidence rates, with the highest in the Caribbean, Sub-Saharan Africa, and Micronesia/Polynesia (Bott, 2015).

1.3.3 Treatment

The common chemotherapeutic drugs used in the treatments of advanced prostate cancer include mitoxantrone, DOX, vinblastine, paclitaxel, and docetaxel. Mitoxantrone is an anthracenedione antineoplastic agent. Mitoxantrone plus prednisone reduces pain and improves the quality of life in patients with advanced PCa but does not improve the survival rate. Docetaxel is well-established antimitotic chemotherapeutic medication. This drug interferes with the cell cycle by

binding with the microtubules. The monotherapy with anthracyclines DOX, epirubicin, or their combination with other agents has been used extensively in the treatment of PCa (Chen and Zhao, 2013).

1.3.4 Doxorubicin

Doxorubicin (DOX) is an anthracycline antibiotic that has been used as an effective treatment against several cancers. When used as primary treatment, it has shown positive results in adult and childhood cancers, including both solid tumors and hematological malignancies. It is used mostly for breast cancers, multiple myelomas, lung cancers, and ALL. Doxorubicin 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 and Baron, 2020).

1. **Mechanism of action of doxorubicin**

The exact mechanism of action of DOX is complex and still unclear. Doxorubicin interacts with the DNA by intercalation thus, inhibiting macromolecular biosynthesis. This further inhibits the progression of the enzyme topoisomerase II and relaxes supercoils in DNA for transcription. Doxorubicin stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication. Another mechanism of DOX is its ability to generate free radicals that induce DNA and cell membrane damage (Rivankar, 2014).

2. Pharmacokinetics of doxorubicin

Doxorubicin is rapidly moved from plasma and concentrated in tissues after intravenous treatment. The plasma half-life is triphasic, with an early phase of about 10 minutes, indicating quick distribution, the second phase of 1–3 hours, and the third phase of around 30 hours, indicating slow distribution. Doxorubicinol is formed as a result of hepatic metabolism. The main route of elimination is biliary excretion, with 10–20 percent of the injected dose recovered in feces within 24 hours and roughly 50% within 7 days. Because DOX clearance accounts for around 60% of hepatic blood flow, impaired liver function can lead to delayed excretion and higher plasma and tissue concentrations (Agrawal, 2007).

3. Adverse effects of doxorubicin

Cardiotoxicity is the major side effect associated with DOX treatment, which is often developed at cumulative dosages of >550 mg/m2. Among the symptoms 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, particularly leukopenia, local skin and deep tissue damage following extravasation, alopecia, stomatitis, and darkening of the skin, particularly the nail beds, are among the other side effects (Agrawal, 2007).

1.4 The lymphatic system

With the significant exclusions of the brain and spinal cord, the lymphatic system can be considered as a second circulatory system that runs in parallel and in combination with the cardiovascular system. It spreads into every major region of the body. The lymphatic system's conduits are lymphatic vessels, which carry a watery fluid called lymph; the term 'lymph' originates from the Latin word lympha, which means water. The lymphatic system differs from the circulatory system as it is not a closed system with a separate pump like the heart. Lymphatic vessels, on the other hand, are physically similar to veins in that they have thin walls and convey lymph under low pressure. As a result, most of the bigger lymphatic channels like veins are fitted with valves to prevent lymph from flowing backward due to gravity. Tissue drainage, fat transfer, and immunological responses are the three main activities of the lymphatic system. Lymph is a fluid that runs through many lymphoid organs, its composition is continually monitored. There are two main types of lymphoid organs:

1. Red BM and the thymus gland consider primary lymphoid organs, where lymphocytes develop from immature progenitor cells.
2. The spleen and lymph nodes are secondary lymphoid organs, where lymphocytes dwell and are positioned to initiate immunological responses (Practice, 2020).

1.5 Leukemia

Hematopoiesis is the process by which all lineages of blood cells are given rise from immature cells present in the BM and released into circulating blood and peripheral organs for further maturation steps and/or effector function. At the apex of this hierarchy are hematopoietic stem

cells (HSCs), which are the only self-renewing cells capable of the life-long production of all lineages of blood cells (Warr and Pietras, 2011).

Leukocytosis or arising in white blood cell count is a natural reaction to infection but when it becomes chronic or increases without apparent cause it could be a sign of cancer. The word leukemia means "white blood" because it is a neoplastic proliferation of one type of blood cell, typically a leukocyte or white blood cell. The BM, lymphatic system and spleen are all affected by leukemia. In the United States, around 24,690 men and 21,840 women were diagnosed with leukemia in 2010, with 21,840 dying from the disease. Leukemia is frequently thought of as a childhood disease because it causes 33 percent of cancers in children and 1340 deaths in children each year. The highest rate of leukemia in children is between the ages of one and four, and the highest death rate is between the ages of ten and nineteen (Org and Lockwood, 2021).

1.5.1 Diagnosis

The median age for diagnosis is 66 years, and the median age of death from leukemia is 74 years. Despite much research on leukemia, the cause is often elusive. Leukemia appears to result from a combination of factors, which can include genetic predisposition, chromosomal changes, chemical agents such as benzene, chemotherapeutic agents, radiation, immunocompromised state, and viruses. The malignant cells in the BM irrespective of the type of leukemia, suppress the creation of other cells resulting in a variety of negative consequences.:

• Anemia occurs due to a fall in the production of the erythrocyte.

• Risk of infection occurs as neutrophil count decreases.

• Risk of bleeding due to reduction in the production of clotting factors and platelets (thrombocytopenia).

• Risk of physiological fracture is increased as a result of the weakness of the periosteum due to the proliferation of BM cells.

• As a result of the infiltration of malignant cells, hypertrophy and fibrosis can occur in other organs such as the liver, spleen, and lymph nodes.

• Infiltration of malignant cells into the central nervous system can cause increased intracranial pressure, ventricular dilatation, and irritation of the meninges, resulting in headache, vomiting, coma, and death.

• Loss of appetite, weight loss, overall weariness, and muscle atrophy are all symptoms of a hypermetabolic condition, which deprives cells of nourishment (Org and Lockwood, 2021).

1.5.2 Classification

Hematopoiesis is the process through which blood cells are created, and it entails the synthesis of the required cells from stem cell precursors based on the body's requirements. An abnormality in the myeloid or lymphoid stem cell causes leukemia. The uncontrolled multiplication of leukocytes in the bone marrow is a common hallmark of all kinds of leukemia. Depending on the type of damaged stem cell, leukemia is classed as lymphoid or myeloid.

Leukemia is categorized as blast cells or stem cells refer to lymphoid abnormality. It is also subdivided into acute and chronic. The four basic types of leukemia are acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML) chronic myelogenous leukemia (CML), and chronic lymphocytic leukemia (CLL) which are further subdivided.

Leukemia is known by a variety of names depending on the cell type involved, which can be perplexing at times. Leukemia is categorized into acute and chronic types according to the cell maturity and the beginning of the disease:

• Acute types of leukemia are fast-growing diseases with a rapid onset (typically within weeks) and a rapid death rate (commonly between weeks to months) if left untreated. The generation of normal cells is reduced by aberrant cell proliferation. Cells can grow in the liver and spleen, and they can infiltrate other organs such as the meninges, gums, lymph nodes, and skin. Because leukocyte development often stops at the blast phase, the majority of afflicted leukocytes are undifferentiated or blasts, unable to perform their normal tasks. Because the cells are arrested at the blast stage in acute leukemia, the white blood count may remain low. Adults and children both can develop acute forms.

• Patients with chronic types of leukemia typically proceed more slowly and have a higher number of mature cells. These mature cells can, generally, perform some of their usual activities. The onset is typically more gradual, taking months or even years. Normal cell production may continue for a long time, but abnormal cells interfere with normal cell creation in the late stages of chronic disease. The vast majority of leukocytes are fully developed. In children, chronic types of leukemia are uncommon (Org and Lockwood, 2021).

With myeloid leukemia, a cancerous change begins in a marrow cell that normally forms certain blood cells—that is, red cells, some types of white cells, and platelets. With lymphocytic (lymphoblastic) leukemia, the cancerous change begins in a marrow cell that normally forms lymphocytes (another type of white cell) (Tallman *et al.*, 2011). Leukemic cells, like

other cancers, must acquire a set of aberrant properties during their genesis. Among these are the ability to survive and proliferate, overcome negative growth signals, and spread to different tissues within and outside the hematolymphoid system. In addition, it is now recognized that leukemia (and perhaps all cancers) are organized as a hierarchy and that a small less differentiated population of leukemia-initiating cells or leukemia "stem" cells with the aberrant capacity to self-renew is responsible for the maintenance of the disease. In normal hematopoiesis, cytokine and growth factors are essential for various functions and act by binding to their cell-surface receptors and triggering complex cascades of intracellular signaling. Acute lymphoid and myeloid leukemia require additional mutations to block the process of differentiation and, in many instances, this is a consequence of aberrant or mutant transcription factor expression (Floden, A, Combs, 2012).

1.6 Burkitt's lymphoma

Burkitt's lymphoma (BL) is a fast-growing non-Hodgkin's Lymphoma that occurs primarily in young males. The causes of BL include chromosome rearrangement and virus infection, but accurate and complete reasons remain to be discovered. The available medications for BL are chemotherapy and radiation therapy. It remains to be the highly aggressive and incurable B-cell lymphoma despite the current therapy *(Li et al.,* 2016).

In regions where malaria is holoendemic, such as equatorial Africa, Brazil, and Papua New Guinea, BL is the most frequent childhood malignancy. Sir Albert Cook, a missionary doctor in Uganda, and other medical staff operating in west, east, and central Africa reported the high prevalence of jaw tumors and children's lymphomas in the early twentieth century. Denis Burkitt, an Irish surgeon working in Uganda, described cases of children with quickly developing jaw or belly tumors in 1958.

These tumors appeared to be round-cell sarcomas. In 1960, pathologist George O'Connor established that the tumor was of lymphoma lineage. The Epstein-Barr virus was named after three virologists, Michael Anthony Epstein, Yvonne Barr, and Bert Achong, who discovered viral particles in tumor cells in 1964 which is (EBV). Burkitt traveled through eastern and central Africa to chart the tumor's spread, and he discovered records of the infected children in all of these regions' malarial areas. These links between malaria and EBV have sparked studies all around the world.

Burkitt's lymphoma plays a significant role in carcinogenesis research. It was the first human tumor to be linked to a virus, one of the first tumors to be found with a chromosomal rearrangement activating an oncogene, and the first lymphoma to be linked to human immunodeficiency virus infection. With a cell doubling rate of 24–48 hours, BL is the fastest growing human tumor, and it was the first childhood malignancy to respond to chemotherapy alone (Molyneux *et al.*, 2012).

1.6.1 Classification of Burkitt's lymphoma

Burkitt's lymphoma is classified by the World Health Organization (WHO) into three clinical variants: endemic, sporadic (the most common kind observed in non-malarial countries), and immunodeficiency-related. Malaria endemicity is linked to the endemic variation, and EBV is present in almost all cases. The sporadic form is seen mostly in the rest of the world (namely in North America and Europe) and is only occasionally linked to EBV infection. In Europe and North America, sporadic-type BL accounts for 1–2% of adult lymphomas and 30–40% of children's non-Hodgkin lymphomas. The immunodeficiency-related form is more common among HIV patients, with EBV accounting for less than 40% of infections in the United States and Europe. BL caused by immunodeficiency is more common when the CD4 T-cell count is greater

than 200 per μl. (early in the progression of HIV infection). In the endemic form of Burkitt's lymphoma, the link between HIV and BL is less obvious (Molyneux *et al.*, 2012).

1.6.2 Pathogenesis and histologic features of Burkitt's lymphoma

Germinal center B cells are the source of Burkitt's lymphoma. B cells at various phases of development are believed to give rise to the three subtypes of BL. The MYC gene, which encodes for the c-Myc protein transcription factor, is found on chromosome 8q24 and controls cell proliferation, differentiation, and apoptosis, which is required for BL development. BL is defined by abnormally high amounts of c-myc, which can be caused by a variety of causes, the most prevalent of which is the translocation of chromosome 8's long arm (which contains the MYC gene) and chromosome 14's Ig heavy chain gene. Overexpression of c-Myc causes rapid B cell proliferation, which accounts for the BL tumor cells' rapid doubling time (between 24 and 48 h). BL has a "starry sky" look histologically, with histiocytes containing copious, transparent cytoplasm distributed among homogenous, basophilic tumor cells. Both growth and apoptotic cell death occur at alarmingly high rates in most cases (Kalisz *et al.*,2019).

1.6.3 Diagnosis of Burkitt's lymphoma

The majority of adult Burkitt's lymphoma cases have all of the criteria for the diagnosis which are high mitotic rate, together with an Ig-positive MYC translocation (Linch, 2012). Diagnostic criteria for Burkitt's have been refined in the recent WHO lymphoma classification system. Several ancillary diagnostic techniques are currently employed in the diagnosis of Burkitt's lymphoma like flow cytometry (Troxell *et al.*, 2005).

1.6.4 Risk factors of Burkitt's lymphoma (Ferry, 2006)

- **Epstein-Barr virus**
- **Malaria**
- **HIV infection**

1.6.5 Treatment of Burkitt's lymphoma

Burkitt's lymphoma is a highly aggressive B-cell non-Hodgkin lymphoma and is the fastest growing human tumor so a very intensive, highly effective, the non-cross-resistant regimen should start immediately this regimen was developed by Magrath *et al*. is CODOX-M/ IVAC regimen.

CODX-M →C=cyclophosphamide, O=oncovin/vincristine, Dox=Doxorubicin and M for High dose methotrexate

IVAC → Ifosfamide, Etoposide (VP-16), Cytosar (Ara-C) + intrathecal therapy. The combination chemotherapy is said to give a high cure rate. However associated toxicities include frequent myelosuppression, severe mucositis, nausea and vomiting, neuropathy, and treatment-related deaths (Olaniyi JA, 2012).

1.7 Human immunity

Immunity refers to the ability of the host to resist the invasion of foreign objects that would otherwise destroy it. Immunity can be divided into adaptive immunity (acquired immunity) and innate immunity (natural immunity or innate resistance) (Hoebe *et al.*, 2004).

A healthy individual is protected from potentially harmful microorganisms in the environment by several effective mechanisms, present from birth, that do not depend upon prior exposure to any particular microorganism. The innate defense mechanisms show broad specificity in the sense that they are effective against a wide range of potentially infectious agents (Stewart, 2012). The simplest way to avoid infection is to prevent the microorganisms from gaining access to the body. The major line of defense is the skin which is impermeable to most infectious agents. Mucus, secreted by the membranes lining the inner surfaces of the body acts as a protective barrier to block the adherence of bacteria to epithelial cells. Among other mechanical factors which help protect the epithelial surfaces is the washing action of tears, saliva, and urine. Many of the secreted body fluids contain bactericidal components such as acid in gastric juice, spermine and zinc in semen, lactoperoxidase in milk, and lysozyme in tears, nasal secretions, and saliva (Coligan and Vogel, 2008).

The first line of immune defense is based on the detection of pathogen-associated molecular patterns (PAMPs) that evoke a toxic and inflammatory response (Hansson *et al.*, 2014). Phagocytic cells have evolved a system of receptors capable of recognizing PAMPs which are shared by a large group of infectious agents and distinguishable from self patterns. Engagement of the pattern recognition receptor generates a signal pathway that alerts the cell to danger and initiates the phagocytic process (Coligan and Vogel, 2008).

Adaptive immunity recognizes specific molecular structures and depends on the generation of large numbers of antigen receptors. Once T cells recognize foreign antigens presented to them, they initiate adaptive immune responses against precisely these antigens. These responses include direct attack of antigen-bearing cells by cytotoxic T lymphocytes, stimulation of B cells to produce antibodies against the antigens, and induction of inflammation, with enhanced innate responses, in the area where the antigen is present. All these responses cooperate during host defenses to eliminate the foreign particle or microorganism. When expressed inappropriately, they can give rise to autoimmune diseases or allograft rejection (Hansson *et al.*, 2014).

1.8 Cytokines

Cytokines are a cell-signaling group of low molecular weight extracellular polypeptides/ glycoproteins synthesized by different immune cells mainly by T cells, neutrophils, and macrophages, which are responsible to promote and regulate immune response (activity, differentiation, proliferation, and production of cells and other cytokines). These polypeptides act on signaling molecules and cells, stimulating them toward sites of inflammation, infections, and traumas, acting on primary lymphocyte growth factors and other biological functions (Borba *et al.*, 2018). Cytokines are immune system intercellular messengers that connect the functions of several cell types in different body compartments to form a coordinated immunological response (Joshi, 2017).

1.8.1 Interleukins

Interleukins are types of cytokines that were first seen to be expressed by leucocytes. They are glycoproteins involved in the signaling of many types of immune system functions (Khadka, 2014). They act as

immunological regulators in the body also involved in autoimmune illnesses and have been linked to many diseases. They are useful in the diagnosis and prognosis of a variety of diseases (O'Neill and Bowie, 2001).

1. **Interleukin-4**

Interleukin-4 (IL-4) is a pleiotropic cytokine produced by T cells, basophils, and mast cells (Chandramohan *et al.*, 2017). It is capable to suppress proinflammatory cytokines and thus is considered an anti-inflammatory cytokine. IL-4 aids in the polarization of antigen-stimulated naïve Th cells into Th2 effector cells as well as propagates Th2 responses by binding to its receptor, IL-4 not only mediates Th2 cell function but also plays a part in the regulation of T regulatory cells (Chatterjee *et al.*, 2014).

2. **Interleukin-10**

Interleukin-10 is an anti-inflammatory cytokine. During infection, it inhibits the activity of Th1 cells, NK cells, and macrophages, all of which are required for optimal pathogen clearance but also contribute to tissue damage. In consequence, IL-10 can both impede pathogen clearance and ameliorate immunopathology. Many different types of cells can produce IL-10, with the major source of IL-10 varying in different tissues or during acute or chronic stages of the same infection (Couper *et al.*, 2014).

3. **Interleukin-17**

Interleukin-17 plays the main role in inflammation and the immune response. It is produced by Th17 cells, a special helper T-cell subset that varies from Th1 and Th2 cells. Natural killer T (NKT) cells produce IL-17 in response to innate stimuli. Interleukin-17 acts as a proinflammatory cytokine that can promote the release of certain cytokines from other cells (Xu and Cao, 2010). By stimulating cells to produce chemokines, IL-17A attracts neutrophils to mediate defenses against different pathogens. IL-17A and TH17 cells are involved in several inflammatory disorders such as

rheumatoid arthritis. High levels of IL-17A have also been found in psoriatic patients, inflammatory bowel disease, allergic asthma, and atopic dermatitis (Zenobia and Hajishengallis, 2015).

Evidence indicates that IL-17 and IL-17-producing cells are involved in the pathogenesis of many diseases such as allergies, allograft transplantation, and malignancy. They may also play protective roles in host defense against infectious diseases and promote induction of cytotoxic T lymphocyte (CTL), responses against cancer (Xu and Cao, 2010).

1.8.2 Interferon-gamma

The Interferon family constitutes a widely expressed group of cytokines. IFNs have a powerful antiviral effect as they inhibit viral replication, increase the lysis capacity of natural killer (NK) cells and the expression of MHC class I molecules on virus-infected cells, and stimulate the development of Th1 cells. IFN-γ plays an important role in macrophage activation in both innate and adaptive immune responses (Borba *et al.*, 2018). IFN-γ is produced primarily by T cells, and NK cells and it has a wide range of actions in both host defense and immunological modulation including antiviral, antibacterial, and anticancer activities (Miller *et al.*, 2009).

1.9 Propolis

Propolis is a natural substance collected by honey bees from various plants. Originally, it was an antiseptic meant for preventing bee-hive from microbial infections along with preventing decomposition of intruders. Traditionally, propolis has been used in folk medicine for centuries. The biological characteristics of propolis depend upon its chemical composition, plant sources, geographical zone, and seasons. More than 300 compounds have been identified in propolis such as phenolic compounds, aromatic acids, essential oils, waxes, and amino acids (Ishtiaq *et al.,* 2018).

Propolis has several biological effects such as immunomodulatory, anti-inflammatory, antioxidant, antibacterial, antiviral, antifungal, and antiparasite. Studies documented that Propolis and its separated compounds have antitumoral effects in vivo and in vitro, which is occurred due to immunomodulatory action, basically through stimulation of innate immunity via macrophage activation, which in turn could secrete soluble factors which interfere directly with the tumor cells or in the function of other immune cells (Sawicka *et al.*, 2012).

1.9.1 Propolis and human health

Propolis has been used worldwide as a dietary supplement to maintain and improve human health. It is also used in many medical formulas to treat infections, allergies, inflammatory diseases, and many human diseases. Propolis is also present in many topical products including ointment, cream, lotion, mouth rinses, and cosmetics like soaps and liquid foundation (Farooqui, 2012). Propolis has multiple and varied biological functions such as antimicrobial, anticancer, anti-inflammatory

activities, and antiulcer properties. These diverse effects promote propolis as a key player in wound healing (Abdelrazeg *et al.*, 2020).

1.9.2 Chemical composition of propolis

The chemical composition of propolis greatly affects its biological activities. Bees utilize multiple natural vegetations for propolis production which causes variations in its chemical compounds, based on the specificity of plant sources, geographical area, and collection season. The biological functions of propolis are closely related to the presence of active compounds like flavonoids, phenolic acid, and terpenoids (Abdelrazeg *et al.*, 2020).

Polyphenols (phenolic compounds) are a group of metabolites that are widely distributed in plants as well as in honey and propolis. Flavonoids and phenolic acids are the two most studied groups of propolis' contents regarding their antileukemic effect. Flavonoids represent a chemical group of polyphenols with different structures and biological properties. About nine types of flavonoids were obtained from the ethanolic extract of propolis (Abubakar *et al.*, 2014).

1.10 Cell culture

Cell culture refers to laboratory methods that enable the growth of eukaryotic cells in physiological conditions. Its origin can be found in the early 20th century when it was introduced to study tissue growth and maturation, virus biology and vaccine development, the role of genes in disease and health, and the use of large-scale hybrid cell lines to generate biopharmaceuticals. The experimental applications of cultured cells are as diverse as the cell types that can be grown in vitro. Cell culture is most commonly linked to creating model systems that study basic cell biology,

replicate disease mechanisms, or investigate the toxicity of novel drug compounds. One of the advantages of using cell culture for these applications is the feasibility to manipulate genes and molecular pathways. Furthermore, the homogeneity of clonal cell populations or specific cell types and well-defined culture systems removes interfering genetic or environmental variables and therefore allows for data generation of high reproducibility and consistency that cannot be warranted when studying whole organ systems (Segeritz and Vallier, 2017).

1.10.1 Primary cultures

The initial in vitro culture of harvested cells and tissues taken directly from animals and humans is called primary culture. In many cases, such cultures also exhibit key characteristics similar to those seen in vivo, so they are widely used for basic research and for several in vitro applications. Although cells in some primary cultures can proliferate and can be subcultured (as early passage cultures), they generally have a limited lifespan and with time they are known to change their differentiated characteristics (Coecke *et al.*, 2004).

1.10.2 Suspension cultures

Although most primary cells and cell lines are adherents, some cell types are non-adherent. Primary immune cells like B and T lymphocytes and lymphoid cells, natural killer cells, and granulocytes grow in suspension. It is easier to passage cells growing in suspension than adherent cells since trypsinization or detachment from the tissue culture plastic is not necessary. When the cells reach a density of around 2×10^6 /ml, they should be split back to $2–3 \times 10^5$ cells/ml. An optimal cell concentration will usually be around 10^6 /ml (Verhoeckx *et al.*, 2015).

1.10.3 The advantages of cell culture:

The main advantages of the cell culture technique are:

1. Control of the environment by altering pH, temperature, O_2/CO_2 ratio, and osmotic pressure of the culture media to study their effects on the cell culture.
2. Provides a good tool to examine cell metabolism and study the physiology and biochemistry of cells.
3. Investigate the cytotoxic action of different compounds or drugs on certain types of cells such as lung cells.
4. Enable the study of biology and origin of the cells obtained from homogenous cell culture.
5. Large-scale cultures can produce a large number of specific proteins from genetically modified cells.
6. Consistency and reproducibility of the results can be got by using a single clonal population.
7. particular types of cells can be noticed by the presence of markers such as molecules or by karyotyping (Freshney, 2010).

1.10.4 The disadvantage of cell culture:

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

1. costs and expertise: cell culture is a specialized technique that needs aseptic conditions, exercised personnel, and expensive apparatus.
2. Dedifferentiation: cell properties are changed after a time of constant growth of cells in cultures, which leads to differentiated properties compared to the initial cell strain.
3. Little quantity of product: The tiny quantity of monoclonal antibodies and recombinant protein made followed by downstream

processing for extracting pure products increases the cost to a very great extent.

4. Contamination: mycoplasma and viral infection are hard to be detected and are very contagious.
5. Instability: Aneuploidy chromosomal constitution in cell lines leads to instability (Freshney, 2010).

Chapter Two

Materials

and

Methods

2. Materials and methods

The present work was performed in the postgraduate lab/department of pharmacology and toxicology/ college of medicine\ university of Babylon, during the period from October 2020 to June 2021.

2.1 Materials

2.1.1 Chemicals

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

Table 2. 1 Chemical Used in The Study.

Chemical	Company	Country
Dimethyl sulfoxide	Sigma Aldrich	Germany
ethanol 70 %	Aljoud	Iraq
Ethanol 99.9%	France Alcools	France
Fetal bovine serum (FBS)	Capricorn	Germany
Lymphocyte separation medium 1.077 g/ml gradient	Capricorn	Germany
MTT(3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) dye powder	Roth	Germany

Penicillin- streptomycin solution	Capricorn	Germany
Phosphate buffer saline packets	BioPLUS chemicals	USA
RPMI 1640 medium w/L-glutamine, 25mM HEPES (powder)	US Biological life science	USA
Sodium bicarbonate powder	Ludeco	Belgium
Trypan blue solution	Sigma-Aldrich	Germany
Trypsin-Ethylenediaminetetraacetic acid (EDTA) powder	US biological	USA

2.1.2 Instruments and tools

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

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

instrument or tool	**Company**	**Country**
Autoclave	Jeiotech	Korea
Blood cells analyzer	Swelab Alfa analyzer	Switzerland
Cell culture flask	SPL	Korea

(25ml)		
Cell culture plate (96-wells)	SPL	Korea
Centrifuge	Rotanta	Germany
Distiller	ROWA	Germany
Double distillation water stills	GFL	Germany
Electric oven	Memmert	Germany
ELISA Reader	Human	Germany
Eppendorf centrifuge 5702 RH	Eppendorf	Germany
Incubator	Memmert	Germany
Inverted microscope	T.C Meiji techno	Japan
Laminar airflow cabinet	Labtech	Korea
Lithium Heparin tubes	Arth alrafidain	China
Magnetic stirrer	Scotech	Germany
Micropipettes (different sizes)	Dragon-Med	India
Millipore filter (0.45, 0.22μm)	Biofil	Australia
Neubauer Hematocytometer cell counter	HBG	Germany

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

2.1.3 Assay kits

The assay kits used in this study are listed in Table 2.3:

Table 2.3 List of ELISA Assay Kits Used in The Present Study

ELISA kit Interleukin 4	Elabscience	USA
ELISA kit Interleukin 10	Elabscience	USA
ELISA kit Interleukin 17	Elabscience	USA
ELISA kit Interferon gamma	Elabscience	USA

Kit contents are listed in table 2.4:

Table 2.4 List of Contents of The ELISA Assay Kit

Micro ELISA Plate (Dismountable)	96T: 8 wells ×12 strips 48T: 8 wells ×6 strips
Reference Standard	96T: 2 vials

	48T: 1 vial
Concentrated Biotinylated Detection Ab (100×)	96T: 1 vial, 120 µL 48T: 1 vial, 60 Ml
Concentrated HRP Conjugate (100×)	96T: 1 vial, 120 µL 48T: 1 vial, 60 µL
Reference Standard and Sample Diluent	1 vial, 20 Ml
Biotinylated Detection Ab Diluent	1 vial, 14 mL
HRP Conjugate Diluent	1 vial, 14 mL
Concentrated Wash Buffer (25×)	1 vial, 30 mL
Substrate Reagent	1 vial, 10 mL
Stop Solution	1 vial, 10 mL
Plate Sealer	5 pieces
Manual	1 copy
Certificate of Analysis	1 copy

2.1.4 Cell lines

Frozen vials of prostate cancer cells (LNCaP), Burkitt's lymphoma cells (Raji), and normal cells (Vero) cell lines were received from the cell culture technique laboratory in the college of medicine/university of Babylon.

2.2 Methods

2.2.1 Preparation of reagents and solutions

2.2.1.1 Phosphate buffer saline (PBS):

According to the BioWorld manufacturer manual, the PBS was prepared by dissolving only one packet in 500 ml of deionized distilled water (DDW) with continuous stirring by a magnetic stirrer at room temperature resulting in a PH value of 7.45 without need for adjustment. Autoclaving is required for complete sterilization and then the PBS was stored in a closed bottle until used to keep it sterile.

2.2.1.2 Trypsin-ethylenediaminetetraacetic acid (EDTA) solution:

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

2.2.1.3 Preparing MTT assay solution

The general purpose of the MTT assay is to measure the viability of the cells in relatively high throughput (96-well plates) with no need for cell counting. Therefore, the most usual use of MTT assay is to find out the cytotoxicity of many drugs at various concentrations. The concept of the MTT assay is to detect the cellular mitochondrial activity of the viable and thereby a rise or

decline in the number of viable cells is related to the mitochondrial activity. The mitochondrial activity of the cells is indicated by the changing of the pale-yellow tetrazolium salt (MTT dye) into dark purple formazan crystals by NADH which can be solubilized for homogenous measurement. Thus, any increment or decline in the number of viable cells can be noticed by measuring formazan concentration which is indicated by the measurements of optical density (absorbance) using a plate reader at 570 nm. The darker the solution, the greater the number of viable and metabolically active cells. The following MTT Procedure was performed according to (Meerloo *et al.*, 2011).

1- At the end of the drug exposure period, the media were withdrawn from the wells and rinsed with PBS. To measure unspecific formazan conversion, a blank control was used.

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 to each well.

3- The plate was incubated for three hours at 37°C till intracellular purple formazan crystals were detectable by the inverted microscope.

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

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

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

The absorbance of the blank must be subtracted from all samples. The absorbance of the test samples must then be divided by those of the control and multiplied by 100 to give a percentage of cell

viability or proliferation. Absorbance values greater than the control indicate cell proliferation, while lower values indicate cell death or inhibition of proliferation. The percentage of cells viability or percentage 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

2.2.2 Preparation of tissue culture medium

Liquid RPMI-1640 medium was prepared according to US Biologics from RPMI-1640 medium powder as follows:

An amount of 16.353 grams was dissolved in 900ml of DDW 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 a 0.22 μm syringe filter.

A 1% penicillin-Streptomycin and 10% fetal bovine serum was added then filter-sterilized using a 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.

2.2.3 Preparation of propolis extracts and stock solutions

The Propolis sample was obtained from a local apiary in AL-Hilla city and identified by a botanist at Al-Qassim green university/college of agriculture.

An 11.5 g of propolis was soaked in 200 ml of 99% ethanol and the other 11.5 g was soaked in 200 ml of DW and left at room temperature for 48 hours.

The aqueous extract was filtered many times using medical gauze and the extract was then filtered using Whitman filter paper no.1. The aqueous extract was placed in the oven at 40 C^0 until drying then collected and stored.

The ethanolic extract was filtered using Whitman filter paper no.1. The filtrate was cooled and filtered to remove any dissolved wax. The final alcoholic liquid extract is then placed in the oven at 40C^0 until drying then collected and stored.

Stock solutions were prepared as follows;

For aqueous extract, 8.5 mg of aqueous extract was dissolved in 4.25 ml complete RPMI 1640 medium to reach a final concentration of 2000 µg/ml, the stock solution was then filtered through a millipore syringe filter to remove any contaminants.

For ethanolic extract, 11.7 mg of ethanolic extract was dissolved in 1 ml of 99 % ethanol then 4.850 ml of complete RPMI 1640 medium to reach a final concentration of 2000 µg/ml, then filtered with a Millipore filter syringe to remove any contaminants (Alwaeely *et al.*, 2021).

2.2.4 Preparation of cell line

2.2.4.1 Thawing of attached cell lines

The frozen cell line vials were removed from the liquid nitrogen container with caution and directly placed into a beaker containing pre-warmed (37°C) sterile DDW. The vials were removed from the water before the ice floccule dissolved completely, then they were wiped with 70% ethanol. Without delay, the cell suspension content of each vial was pipetted under a 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 resuspended into a 5 ml warm (37°C) serum medium and transferred into a 25 ml size cell culture flask, incubated at 37°C and the serum medium replaced on the next day.

2.2.4.2 Sub-culturing of cell culture

- The cells were checked and analyzed using an inverted microscope to ensure that they were healthy, sub-confluent, and free of contamination.
- Using a pipette, the growth medium was removed from the flask, and the monolayer was washed with enough volume of PBS to remove all of the media from the flask.
- To allow the cells to detach from the flask's interior surface, a suitable volume of trypsin/EDTA solution was applied to the flask and incubated at 37°C (for 2-10 min).
- An inverted microscope was used to inspect the cells to confirm that they were all separated and suspended. To release any

remaining detached cells, the flask was lightly taped with the palm a couple of times.

- Trypsin was inactivated in the flask by adding an equal volume of the serum-containing medium.
- The cell suspension was then split into two flasks, each with a cell line name, passage number, and date labeled on it.
- The cell line was incubated at 37°C for 24 hours (Meleady and O'Connor, 2006).

2.2.4.3 Thawing of Raji cell line:

Raji cell line stock vial was taken from liquid nitrogen and thawed in a 37 C^0 water bath. the thawed cells were suspended in 5 ml growth media. Then centrifuged at 1000 rpm for 3 minutes, and the media was discarded. The cells pellet was resuspended in 15ml growth media and transferred into a tissue culture flask. Cells were grown in a 37ºC incubator at 5% CO2 (Meleady and O'Connor, 2006).

2.2.4.4 Subculturing of Raji cells:

The medium was changed every 2 days, and the cultures were divided using the following procedure.

1. The cell suspension (in growth medium) was transferred to centrifuge tubes and spun at 1000 rpm for 3minutes.
2. The supernatant was aspirated and 10 ml of fresh growth media was added.
3. Tissue culture flasks were prepared, and an appropriate volume of pre-warmed growth media was added to each flask, followed by adding an appropriate volume of the cells suspension to each flask and incubating at 37°C (Meleady and O'Connor, 2006).

2.2.4.5 Maintenance of cell culture

Cells were routinely checked under an inverted microscope for any contamination and the cells were given a 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 subcultured.

2.2.4.6 Harvesting of cell culture

Harvesting is a technique that uses proteolytic enzymes to separate adherent cells from the surface of the 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 (containing trypsin) 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 EDTA (Viazzi *et al.*, 2015).

2.2.5 Preparation of doxorubicin stock solution

The concentration of doxorubicin in the vial was 2 mg/ml, a serial dilution was made.

2.2.6 Isolation of peripheral blood mononuclear cells

1. A healthy donor's blood (5 ml) was withdrawn and left to cool at room temperature for 30 minutes.

2. Five ml of lymphocyte separation medium 1.077 g/ml were placed in a round bottom polystyrene tube, then carefully 5 ml of the whole blood was added carefully and slowly to avoid mixing the two layers.
3. Centrifuge the tube at 500 g for 30 minutes at room temperature.
4. The upper layer above PBMC was carefully removed and transferred into another polystyrene tube.
5. PBMC layer shows as the first hazy band below the separation medium layer.
6. PBMCs were washed twice in phosphate buffer saline and centrifuged for 10 minutes at 500 g. The supernatant was then piped away from the pellets without contacting them.
7. Viability testing was done by using 0.4 % trypan blue and hemocytometer to confirm that the viability percentage of the yield cells is more than 95%.
8. The pellet was resuspended in 10 ml of complete RPMI medium and 1 ml was sent for cell counting using a blood count analyzer, the remaining volume was transferred to a T25 tissue culture flask and incubated for 24 hours at 37°C and 5% CO2, in this step the monocytes will attach to the surface and the lymphocytes stay in suspension (Lefort and Kim, 2010).

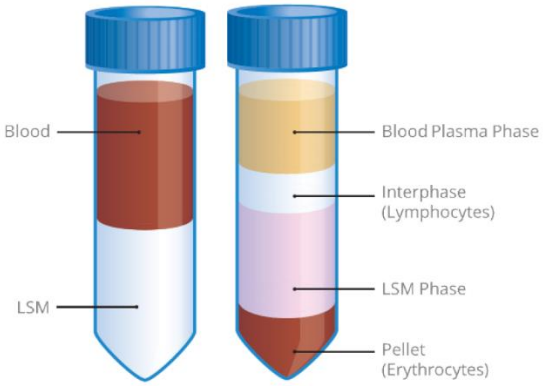


Figure (2.1) Blood separation tube before (left) and after centrifugation (right)

2.2.7 Cytotoxicity measurement of propolis extracts on cancer and normal cells and determination of IC50 value.

Cells were cultured in 96 tissue culture plates. Cells were treated with different concentrations of propolis extracts (ethanolic and aqueous) ranging from 1000 to 31.25 µg\ml serial dilutions (four replicates were used for each concentration) along with four replicates of untreated cells (control group). The plate was then incubated for 24 hours, after which the cell growth was measured using the MTT cytotoxicity assay.

The concentration required to decrease cells viability to 50 % (IC50) was determined using an excel sheet and fitted by blotting graphically of relative cell inhibition percentage on the Y-axis versus the concentration of each compound used on the X-axis.

2.2.8 Effect of doxorubicin on prostate cancer (LNCaP) cell line

In 96 microtiter plates, prostate cancer (LNCaP) cell line were seeded and incubated for 24 hours. Cells were exposed to 200

µl of serial dilutions (250, 125, 62., 31, 15, 7.5 µg/ml) of doxorubicin (four wells for each concentration). The plate was then covered with a self-plastic lid and incubated for 24 hours, after which the growth of the cells was measured using the MTT cytotoxicity assay. Calculations of cell viability were done using the same method mentioned before.

2.2.9 Effect of (doxorubicin-propolis) combination on the viability of prostate cancer cells

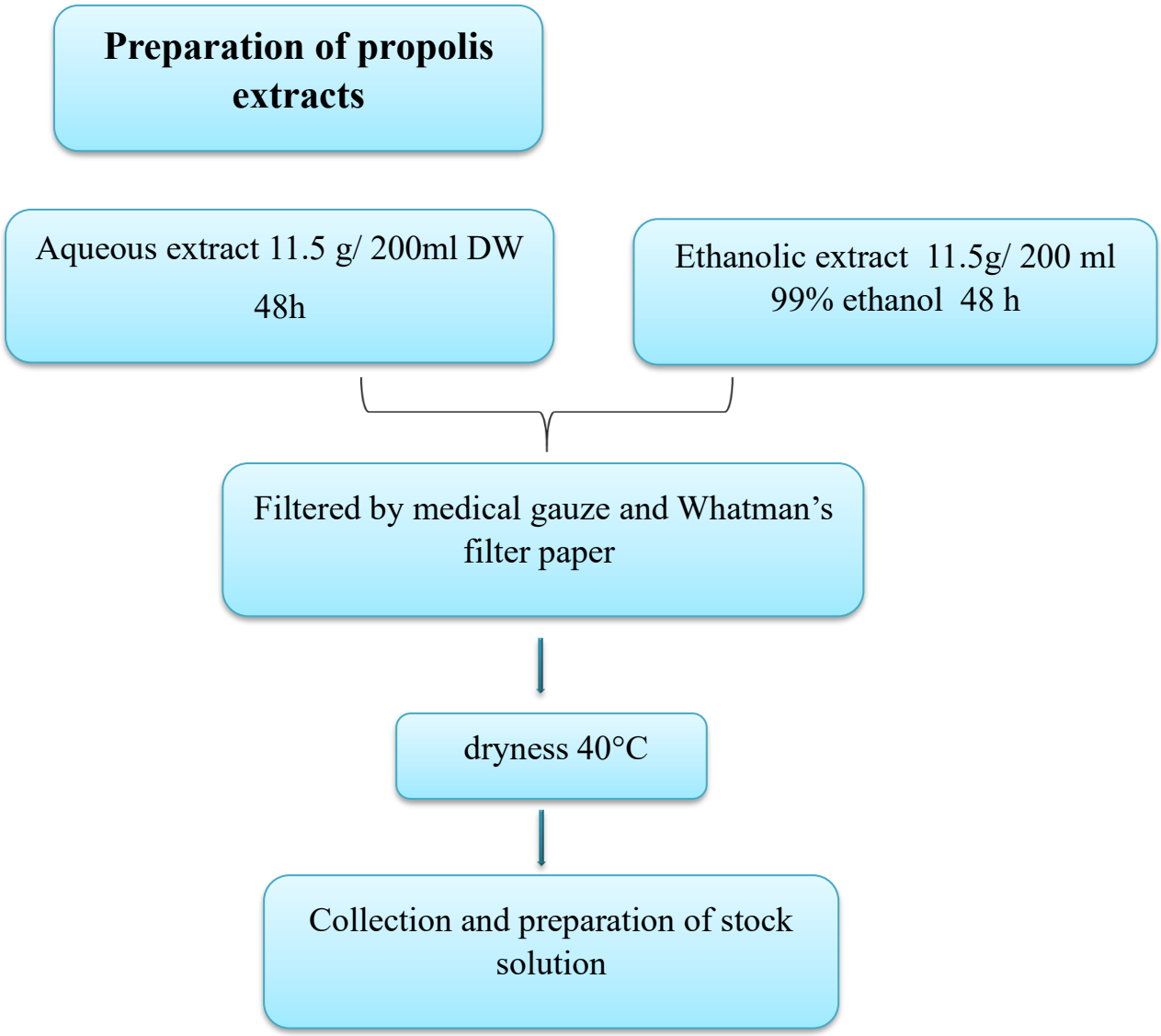
In 96 microtiter plate, sixty wells were seeded with prostate cancer (LNCaP) cell line. Cells were treated with serial dilutions (1000, 500, 250, 125, 62.5, and 31.25 µg/ml) of ethanolic extract of propolis in the presence of a constant concentration (30 µg/ml) of doxorubicin in each well. The plate 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.

2.2.10 Immunomodulatory effect of propolis extracts on Raji cell line and the isolated peripheral blood mononuclear cells

Raji cells and the isolated human peripheral blood mononuclear cells were seeded in a 48 tissue culture plate. Wells were treated with 1000, 250, and 50 µg/ml of propolis aqueous or ethanolic extracts. After 24 hours of the incubation period, each well was withdrawn by a micropipette and centrifuge, and the supernatant of each well was sent for immunoassay by ELISA method to measure the concentrations of IL4, IL10, IL17, and IFN-

gamma (assay procedure is described in detail at page 102 in appendix).

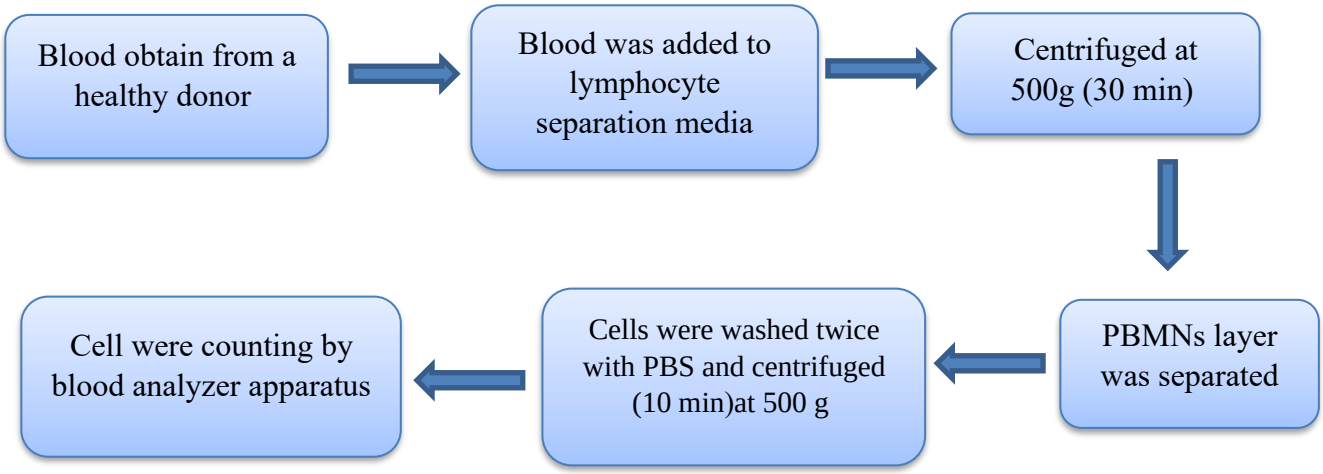
2.2.11 study plan



Part 1

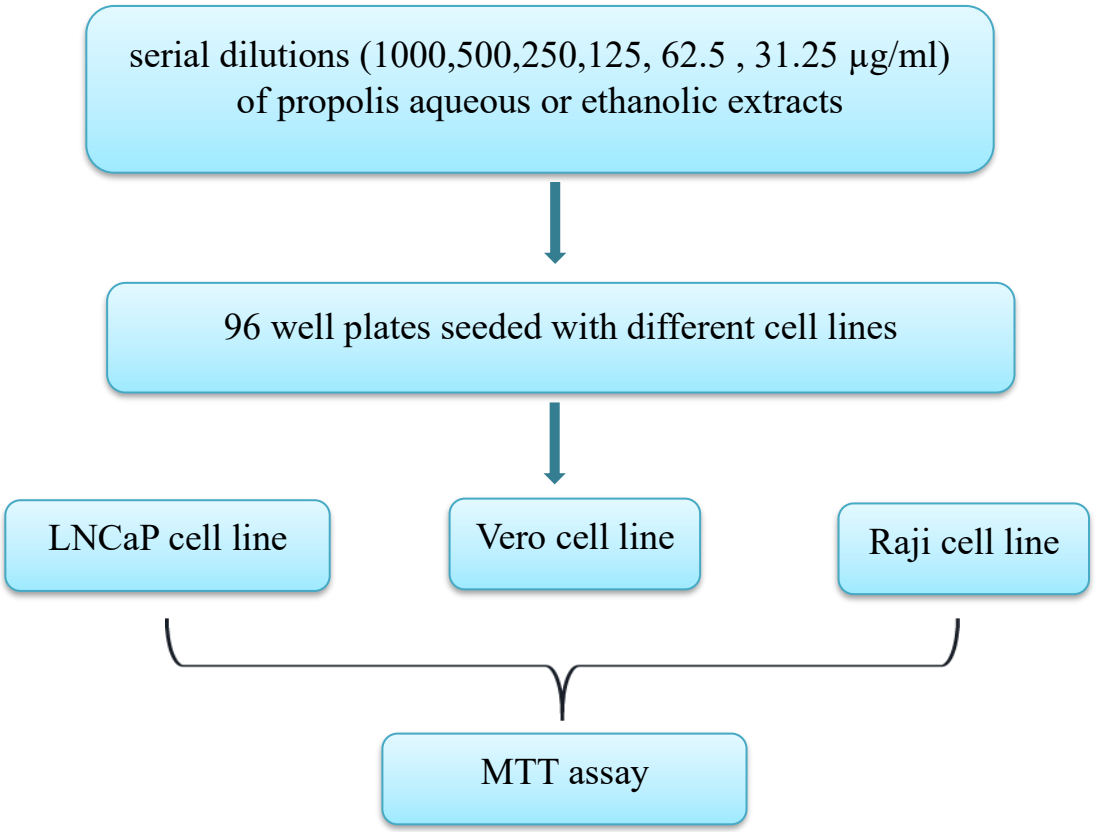
Effect of propolis extracts on the viability of cell lines

Experiment No 1. Isolation of PBMNs



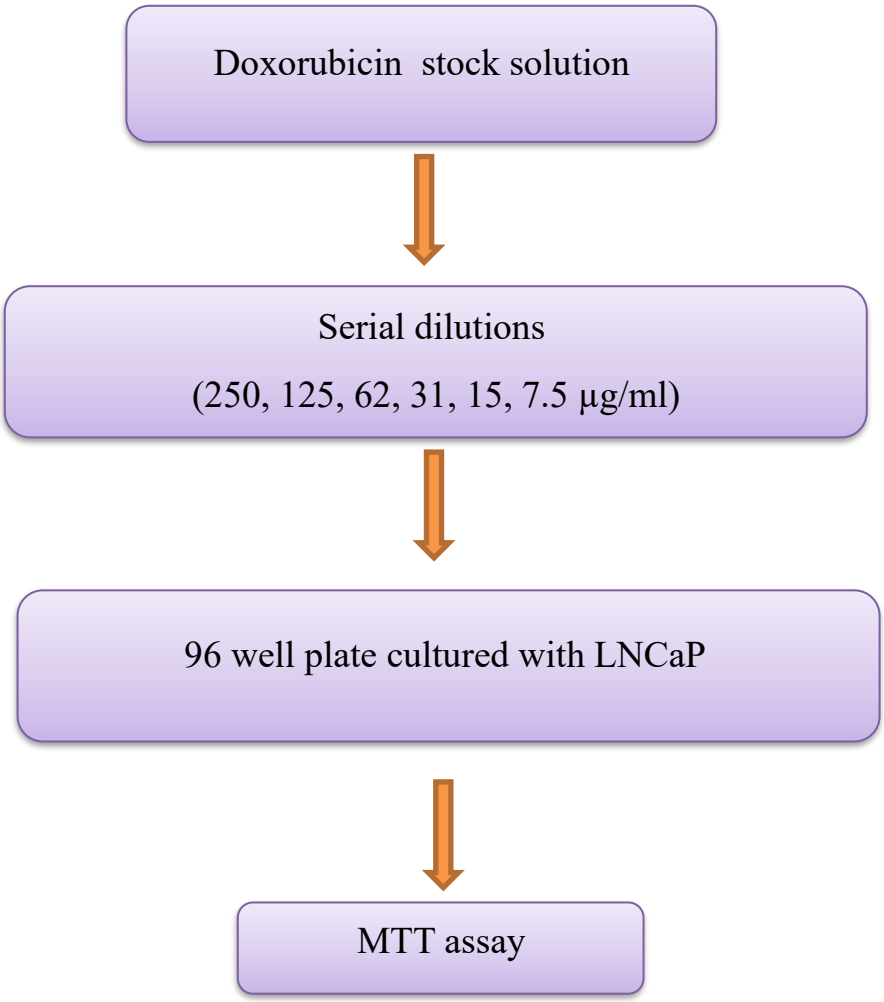
Experiments No. 2

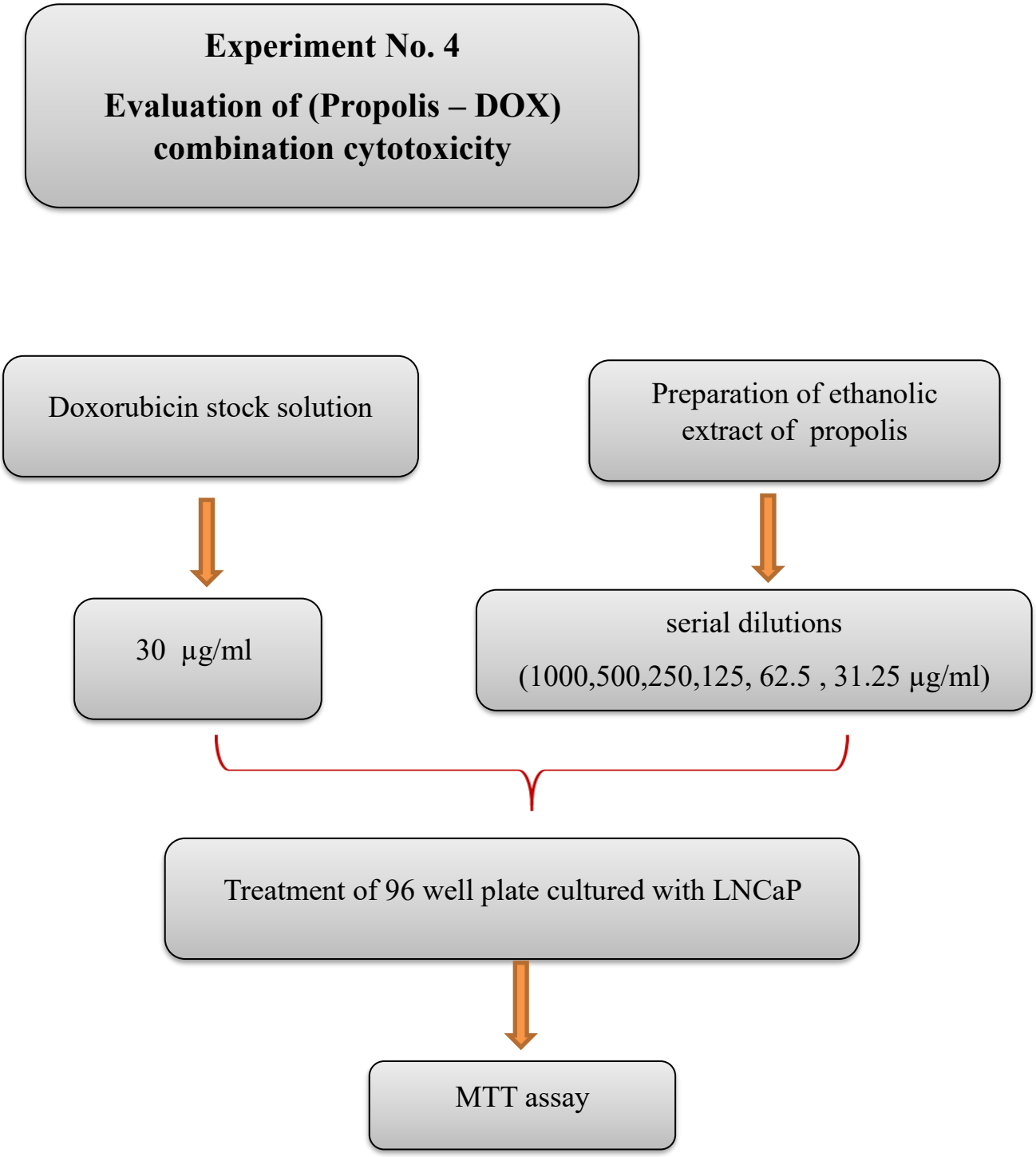
cytotoxicity assays



Experiment No. 3

Doxorubicin cytotoxicity assay




Experiment No. 4
Evaluation of (Propolis – DOX) combination cytotoxicity
Doxorubicin stock solution
Preparation of ethanolic extract of propolis
30 µg/ml
serial dilutions
(1000,500,250,125, 62.5 , 31.25 µg/ml)
Treatment of 96 well plate cultured with LNCaP
MTT assay

Part 2

Immunomodulatory effect of propolis extracts on Raji cell line and PBMNs

Extracts at concentrations (1000, 250, 50 µg/ml) were prepared

↓

Treatment of 48 well plate cultured with Raji cell line and PBMNs

↓

The level of IL-4, IL-10, IL-17, INFγ was measured by ELISA test

2.2.12 Statistical analysis

Microsoft Office Excel 2016 and Sigma plot version 12.5 software were used to analyze the data. The ANOVA one-way test was employed to determine whether there were significant differences between the data means. The p-values ($p \leq 0.001$ and $p \leq 0.05$, respectively) were declared statistically highly significant, and significant.

Chapter Three

Results

3. Results

3.1 Effects of propolis extracts on the viability of prostate cancer cell line (LNCaP)

There was a significant (P≤0.001) decrease in the viability of LNCaP cell line caused by both ethanolic and aqueous extracts of propolis at all concentrations compared with the control group (figure 3.1).

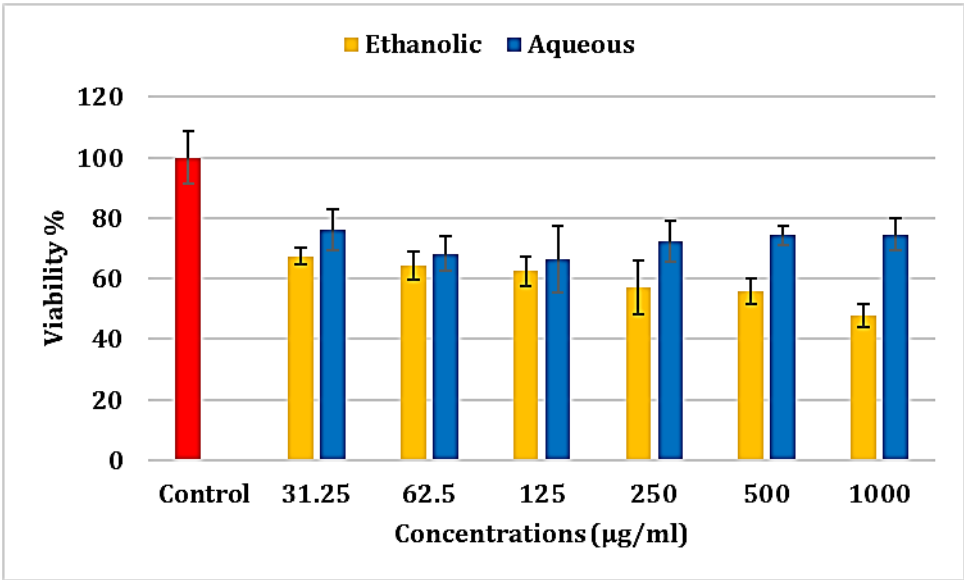


Figure 3.1: Effects of aqueous and ethanolic extracts of propolis on the viability of LNCaP cell line

The IC50 values of propolis ethanolic and aqueous extract on LNCaP were 717.36 µg/ml and 1416.43 µg/ml, respectively (figure 3.2).

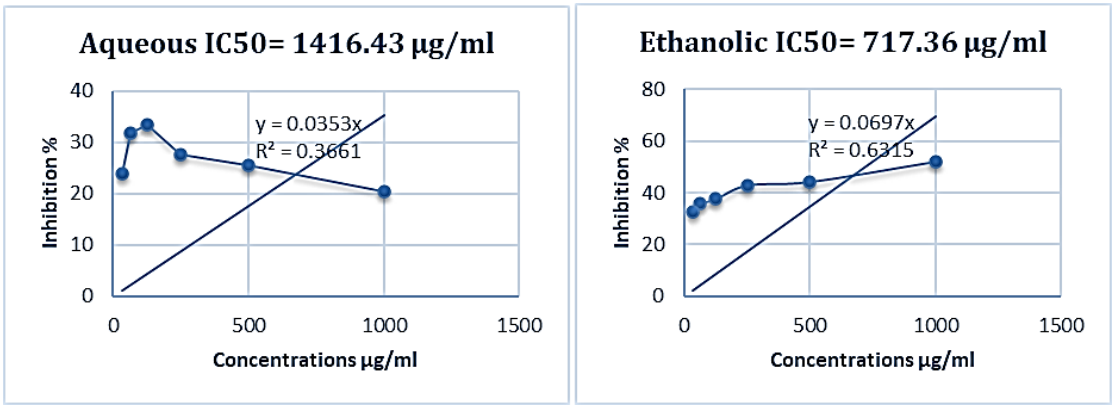


Figure 3.2: IC50 Values of aqueous and ethanolic extracts of propolis on LNCaP cell line

3.2 Effects of propolis extracts on the viability of Vero cell line

At concentrations of 1000 and 500 µg/ml, the aqueous extract of propolis reduced the viability of the Vero cell line significantly ($P \leq 0.050$). Ethanolic extract of propolis caused a significant ($P \leq 0.001$) decrease in the viability of Vero cell line at all concentrations used as compared to the control group (figure 3.3).

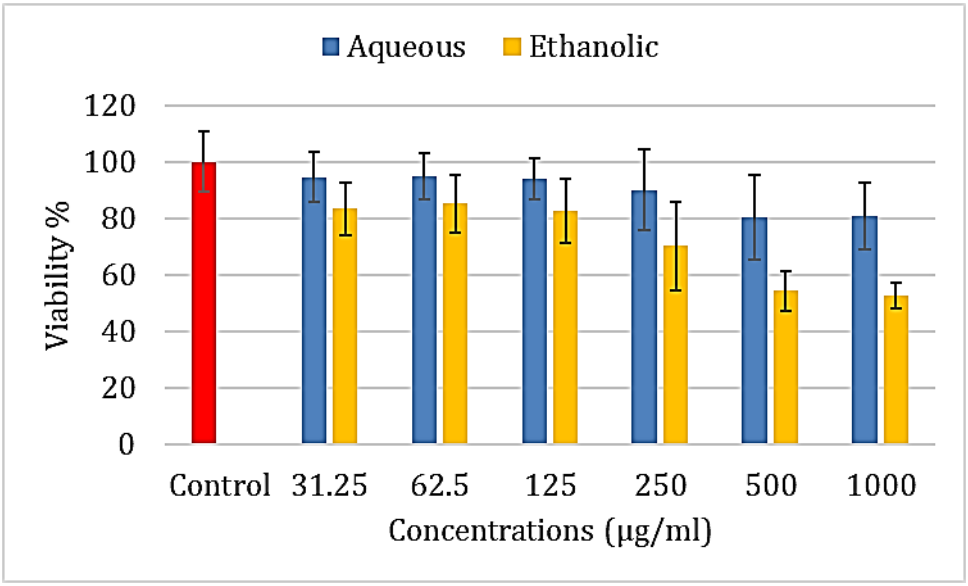


Figure 3.3: Effects of aqueous and ethanolic extracts of propolis on the viability of Vero cell line

The IC50 value of propolis aqueous extract was 2304 µg/ml while for ethanolic extract it was 821 µg/ml on Vero cell line (figure 3.4).

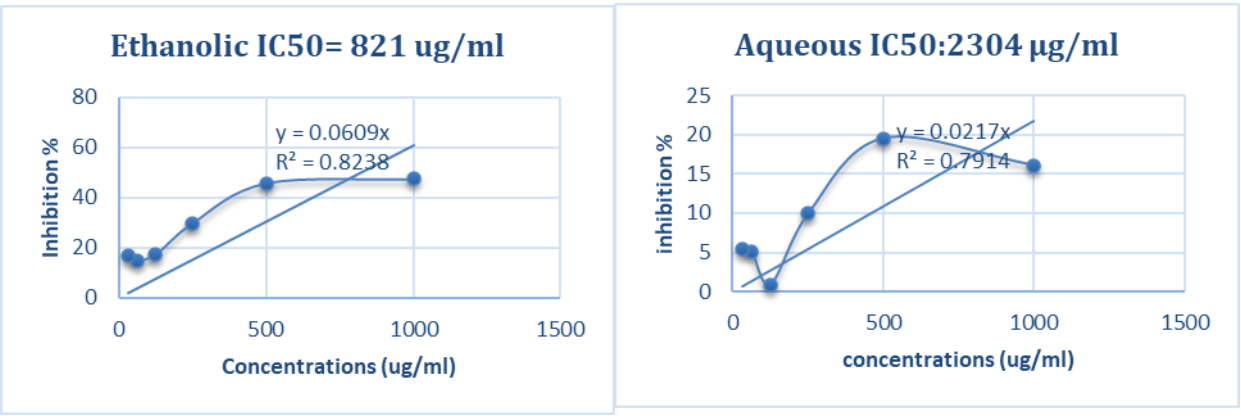


Figure 3.4: IC50 Value for aqueous and ethanolic extract of propolis on Vero cell line

3.3 Effects of propolis extracts on the viability of Raji cell line

Treatment with the aqueous extract of propolis results in a significant (P≤ 0.001) decrease in the viability of Raji cell line at concentrations of 1000, 500, and 250 µg/ml. While there was a significant (P≤ 0.001) decrease in the viability of Raji cell line at a concentration of 1000 µg/ml of the ethanolic extract of propolis when compared with the control group (figure 3.5).

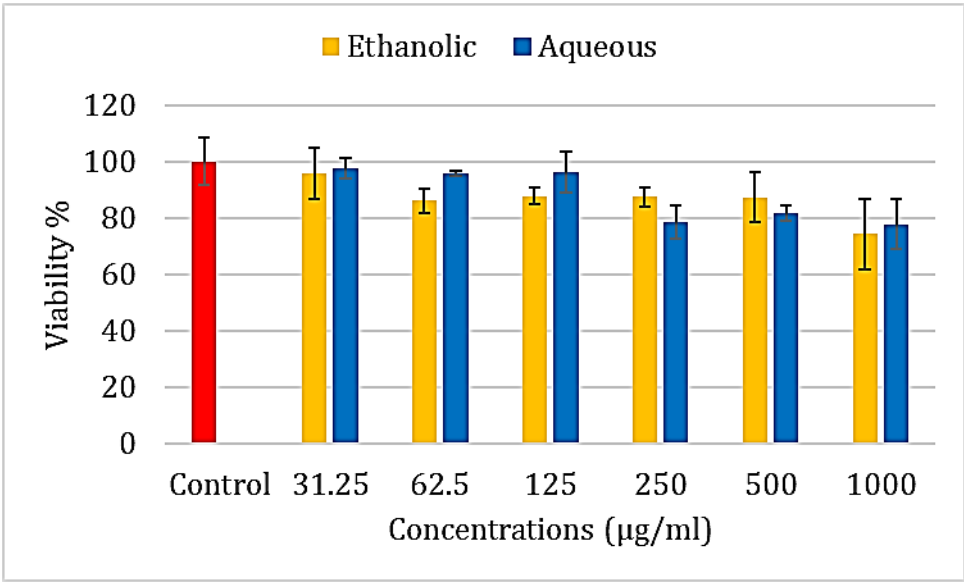


Figure 3.5: Effects of aqueous and ethanolic extracts of propolis on the viability of Raji cell line

The IC50 value of propolis aqueous extract on Raji cell line was 1773 µg/ml, and for ethanolic extract was 1845 µg/ml (figure 3.6).

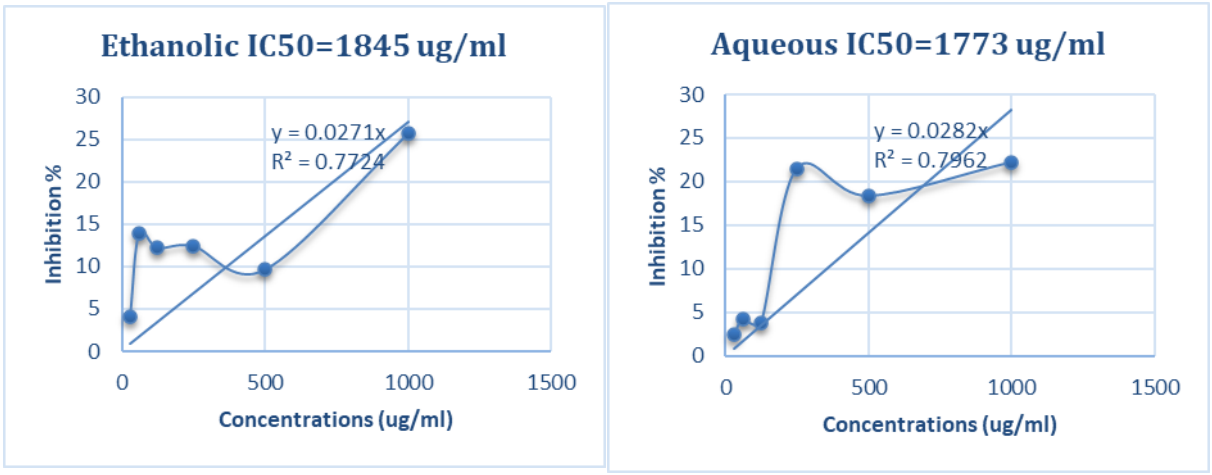


Figure 3.6: IC50 Values of aqueous and ethanolic extracts of propolis on Raji cell line

3.4 Isolation of peripheral blood lymphocytes

Results of the isolation technique used in this study listed in the table (3.1) show high lymphocyte yield (88%).

Table (3.1) peripheral blood cell count analyzed by CBC

Peripheral Blood cells fractions	Average ± SD
lymphocyte count (Million / ml)	1.34 ± 0.52
Lymphocyte (%)	0.88 ± 0.04
WBC count (Million / ml)	1.48 ± 0.6
Platelets (Million /ml)	34.7 ± 16.8
RBC (%)	0

3.5 Effect of propolis extracts on the viability of peripheral blood mononuclear cells

Compared to the control group, results showed a statistically significant ($p \leq 0.001$) increase in cells viability at concentrations of 1000 and 500 µg/ml and a significant ($p \leq 0.05$) decrease in cells viability at concentrations of 125, 62.5, and 31.25 µg/ml of the aqueous extract, while results showed statistically significant ($p \leq 0.001$) increase in cells viability at 1000, 500, 250, and 125 µg/ml for the ethanolic extract figure (3.7).

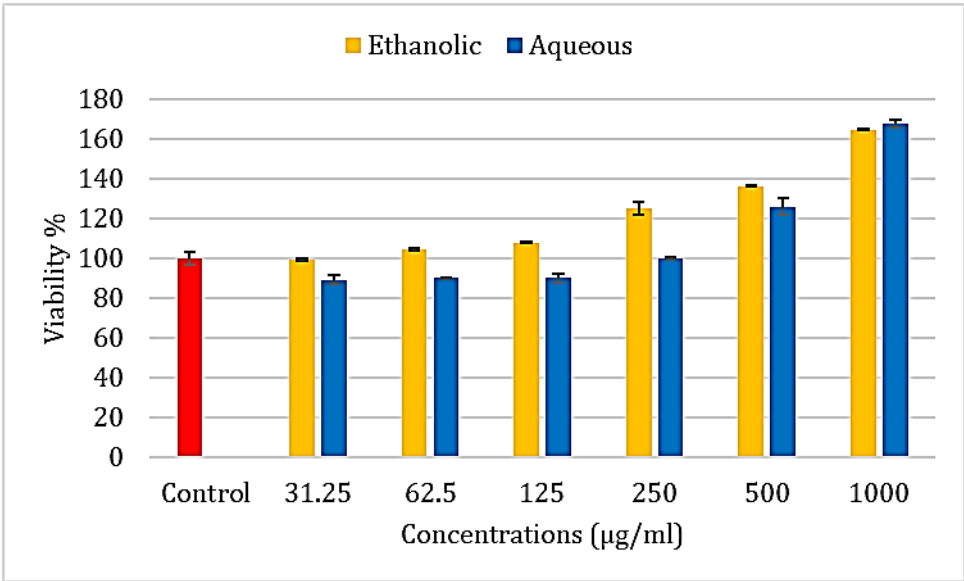


Figure 3.7: Effects of aqueous and ethanolic extracts of propolis on the viability of the peripheral blood mononuclear cells

3.6 Effect of doxorubicin on the viability of prostate cancer cell line (LNCaP)

There was a significant ($P \leq 0.001$) decrease in the viability of LNCaP cell line at all concentrations used of doxorubicin (figure 3.8).

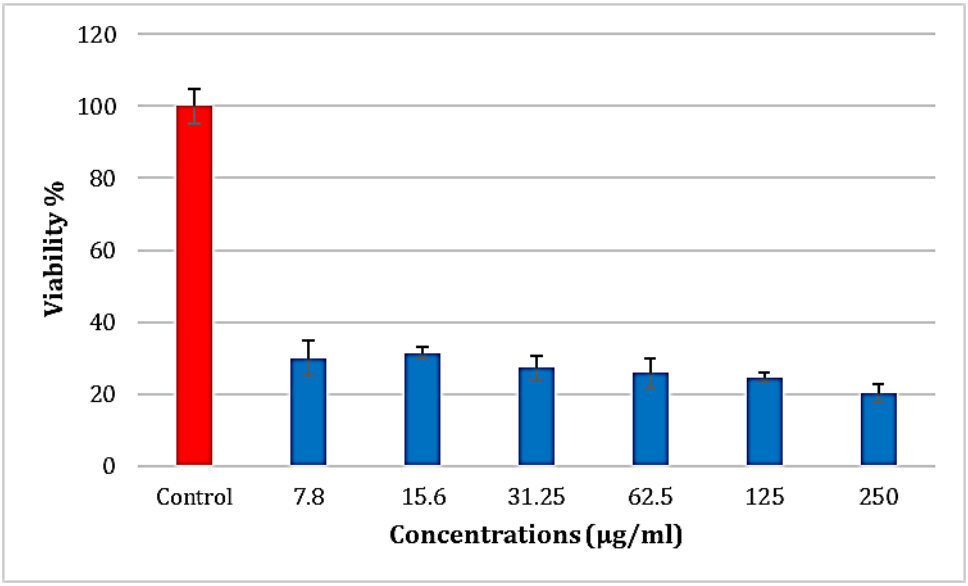


Figure 3.8: Effect of doxorubicin on the viability of LNCaP cell line

3.7 Effect of doxorubicin - propolis combination on the viability of prostate cancer cell line (LNCaP)

There was a significant (P≤0.001) decrease in the viability of LNCaP cell line when treated with the combination of doxorubicin and different concentration of ethanolic extract of propolis as shown in figure 3.9.

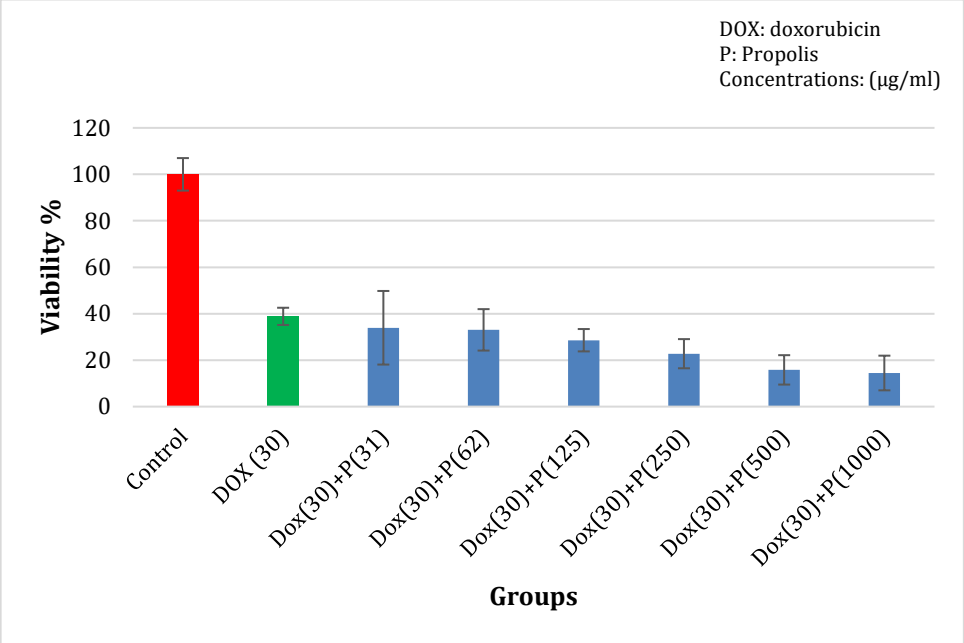


Figure 3.9: Effect of doxorubicin and ethanolic extract of propolis combination on the viability of LNCaP cell line

3.8 Immunomodulatory effect of propolis extracts on lymphoma cell line (Raji) and peripheral blood mononuclear cells

3.8.1 Level of interleukin 4 in the propolis extracts treated Raji cell line

The results showed a significant (P≤ 0.001) decrease in IL-4 levels in the aqueous extract at concentrations of 1000, 250, and 50 µg/ml and ethanolic extract at the concentration of 1000 µg/ml when compared to the control group (figure 3.10).

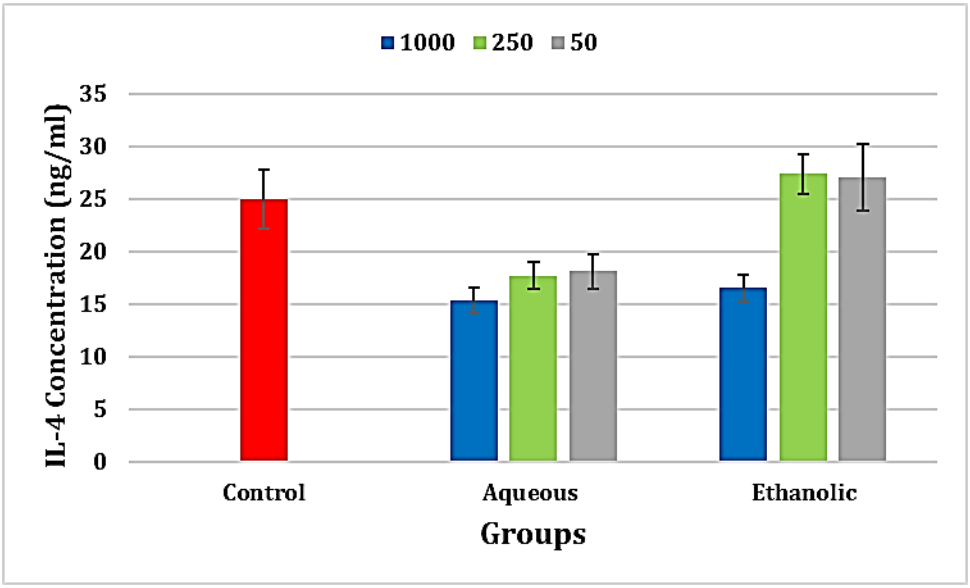


Figure 3.10: Level of interleukin 4 in the propolis extracts treated Raji cell line

3.8.2 Level of interleukin 4 in the propolis extracts treated peripheral blood mononuclear cells

When compared to the control group, the results showed a significant (P≤ 0.001) increase in IL-4 levels in both aqueous and ethanolic extracts of propolis at concentrations of 50 and 250 µg/ml, and a significant (P≤ 0.001) decrease in IL-4 levels in ethanolic extract of propolis at concentration 1000 µg/ml (figure 3.11).

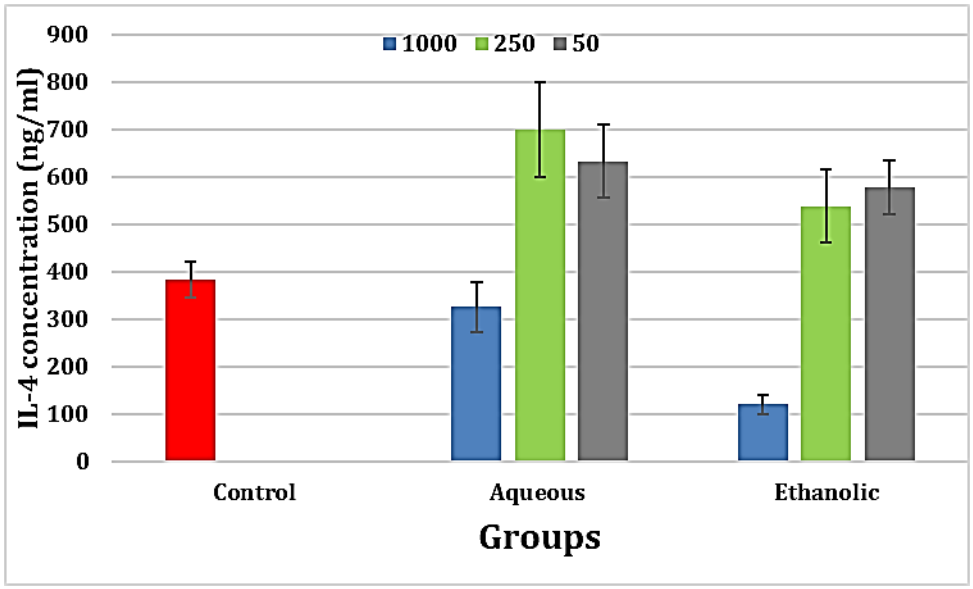


Figure 3.11: Level of interleukin 4 in the propolis extracts treated peripheral blood mononuclear cells

3.8.3 Level of interleukin 10 in the propolis extracts-treated Raji cell line

When compared to the control group, the results demonstrated a significant (P≤ 0.001) increase in IL-10 levels in the ethanolic extract at a concentration of 50 µg/ml and a significant (P≤ 0.001) decrease in IL-10 levels in the ethanolic extract at concentration 1000 µg/ml (figure 3.12).

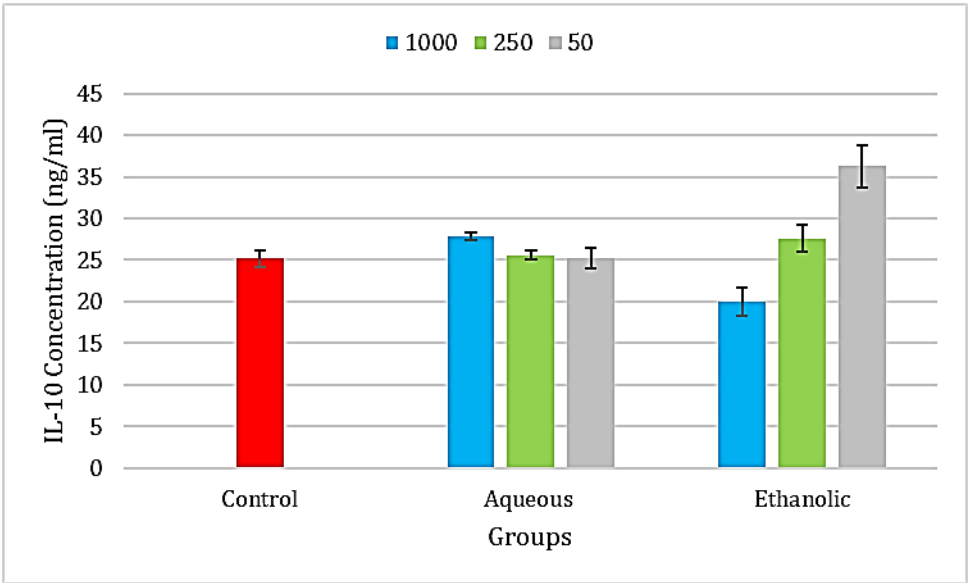


Figure 3.12: Level of interleukin 10 in the propolis extracts-treated Raji cell line

3.8.4 Level of interleukin 10 in the propolis extracts treated peripheral blood mononuclear cells

The results showed a significant (P≤ 0.001) increase in IL-10 levels in both aqueous and ethanolic extract at a concentration of 250 µg/ml when compared to the control group (figure 3.13).

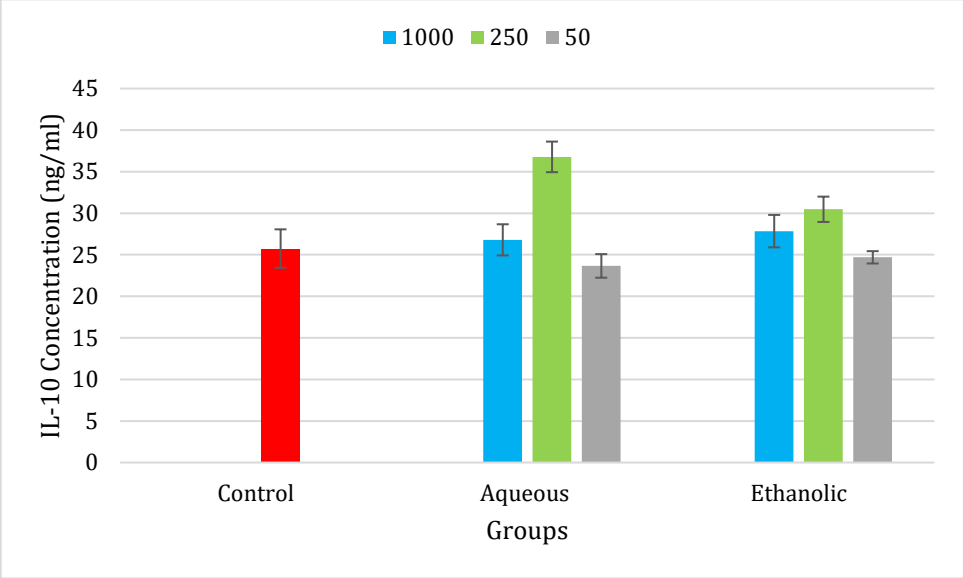


Figure 3.13: Level of interleukin 10 in the propolis extracts-treated peripheral blood mononuclear cells

3.8.5 Level of interleukin 17 in the propolis extracts treated Raji cell line

The results demonstrated a significant (P≤ 0.001) decrease in IL-17 levels in both aqueous and ethanolic extracts of propolis at all concentrations when compared to the control group (figure 3.14).

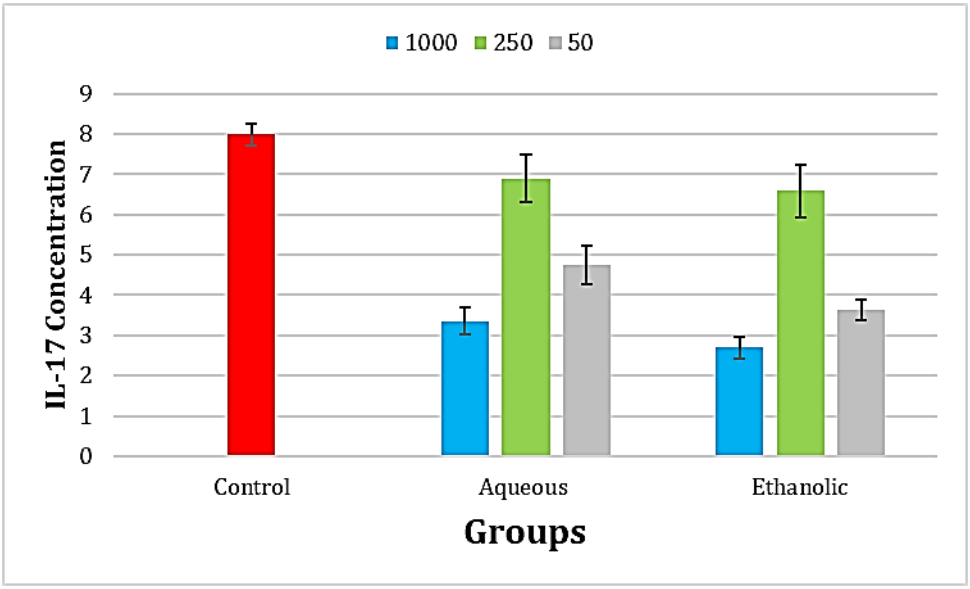


Figure 3.14: Level of interleukin 17 in the propolis treated Raji cell line

3.8.6 Level of interleukin 17 in the propolis extracts treated peripheral blood mononuclear cells

The results demonstrated a significant (P≤ 0.001) increase in IL-17 levels in both aqueous and ethanolic extracts at concentrations of 50 and 250 µg/ml, while there was a significant (P≤ 0.05) decrease in IL-17 levels in the ethanolic extract at concentration 1000 µg/ml (figure 3.15).

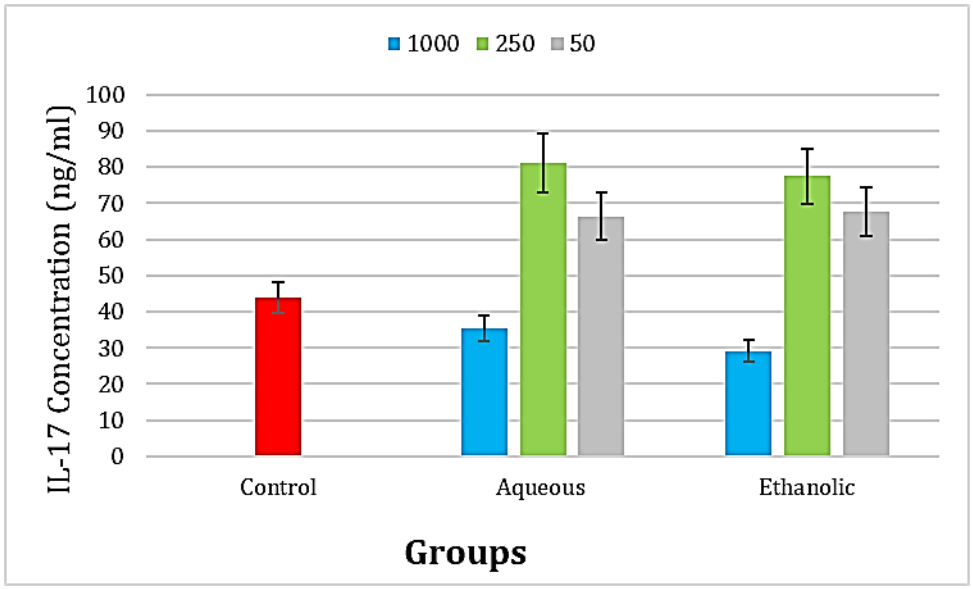


Figure 3.15: Level of interleukin 17 in the propolis extracts treated peripheral blood mononuclear cells

3.8.7 Level of interferon-gamma in the propolis treated Raji cell line

The results showed a significant (P≤ 0.001) decrease in INF-γ levels in concentrations 50 and 1000 µg/ml of aqueous extract and 1000 µg/ml of the ethanolic extract, while there was a significant (p≤ 0.05) decrease in INF-γ levels in concentrations 250 µg/ml of aqueous extract and 250, 50 µg/ml of the ethanolic extract of propolis when compared to the control group (figure 3.16).

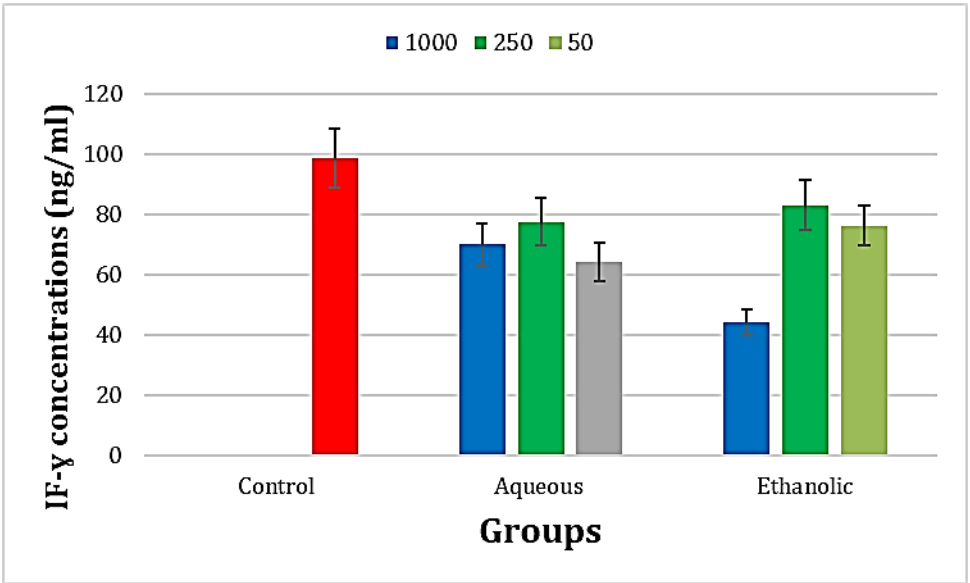


Figure 3.16: Level of interferon-gamma in the propolis treated Raji cell line

3.8.8 Level of interferon-gamma in the propolis treated peripheral blood mononuclear cells

The results demonstrated a significant (P≤ 0.001) increase in INF-γ levels in the aqueous extract of propolis at concentrations of 50 and 250 µg/ml and the ethanolic extract at the concentration of 1000 µg/ml when compared to the control group (figure 3.17).

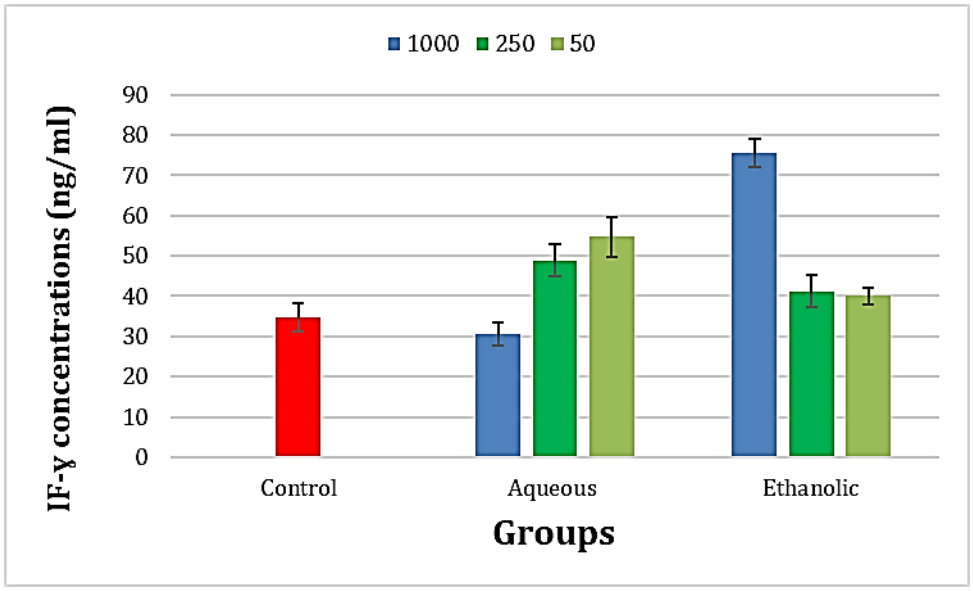


Figure 3.17: Level of interferon-gamma in the propolis treated peripheral blood mononuclear cells

Chapter Four

Discussion

4. Discussion

4.1 Effect of propolis extracts on the viability of prostate cancer cell line (LNCaP)

The result of the current study revealed that all concentrations of both aqueous and ethanolic extracts caused a significant decrease in the viability of the LNCaP cell line (figure 3.1), although ethanolic extract was more effective as it had lower IC50 (figure 3.2) in comparison to aqueous extract (717.36 µg/ml versus 1416.43 µg/ml).

Depending on a previous Iraqi study the anti-proliferative effects of aqueous and ethanolic extracts of propolis could be related to an overall effect of the phenolic compounds present in these extracts. The growth inhibition induced by propolis or its compounds can result in cancer cell death. The investigated propolis extracts significantly had less toxicity on normal cells, which could be explained by selective toxicity of the chemical constituents of propolis, especially phenolic acids and flavonoids against cells depending on the type of cells. Furthermore, as propolis was less cytotoxic to normal cells, it is also possible to consider propolis a potential anti-tumor agent (Ghassan, 2010).

Apoptotic pathways that are disturbed play a key role in the beginning and progression of prostate cancer. It's been proposed that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) plays a crucial role in immunological homeostasis, tumor surveillance, and cancer cell defense (Szliszka *et al.,* 2012). TRAIL is a naturally occurring anticancer agent that induces apoptosis in

cancer cells with no toxic effect on normal cells (Watanabe *et al.*, 2011).

Artepillin C is one of the major phenolic compounds found in green propolis (which also have been used in our study), it has a wide range of pharmacological effects such as antioxidant, antimicrobial, anti-inflammatory, immunomodulatory, and anti-cancer effects (Shahinozzaman *et al.*, 2020). In vitro arte C can cause suppression of tumor growth, and in vivo, it can cause an increase in the ratio of CD4/CD8 T cells, therefore arte C can activate the immune system (Ghassan, 2010).

It has been documented that LNCaP cell line is resistant to apoptosis mediated by TRAIL. Inactivation of the TRAIL pathway and escape from the TRAIL-mediated immunosurveillance may play an important role in the onset and progression of the tumor. In the present study, the ability of propolis to inhibit the growth of LNCaP cell line may be attributed to the synergistic effect between TRAIL and propolis extracts or phenolic compounds like arte C. Artepillin C exhibits mainly indirect antitumor action by stimulating the TRAIL-mediated apoptotic pathway (Szliszka *et al.*, 2012).

Caffeic acid phenethyl ester (CAPE) presented in the propolis can block the proliferation of LNCaP cells and reduces the tumor growth of LNCaP xenografts in nude mice (Zabaiou *et al.*, 2017). CAPE can inhibit the growth of mutated cells without harming normal cells (Desai *et al.*, 1993). Both CAPE - based and arte C -based propolis extracts selectively block oncogenic serine/threonine-protein kinase (PAK1) signaling pathways, more

than 70% of human cancers, such as breast and prostate cancer, require PAK1, an enzyme encoded by the PAK1 gene, for their growth (Farooqui and A. Farooqui, 2010).

Another component to which the cytotoxic effect of propolis on LNCaP cell line may attribute is pinocembrin which is one of the major flavonoids of propolis it exhibits many pharmacological effects, including anti-inflammatory, antioxidant, antimicrobial, anti-allergic, anti-viral, and anti-asthmatic effects (Yang *et al.*, 2013). Pinocembrin exerts a cytotoxic effect and attenuates cell viability of LNCaP cell lines, inducing apoptosis and arresting the cell cycle at both S and G2/M phases (Zabaiou *et al.*, 2017). Also, the result of the current study goes with that found by Szliszka *et al*. (2011) study who pointed out that 50 mg/ml of ethanolic extract of propolis from southern Poland showed 25% cytotoxicity in prostate cancer cells. In addition, Seda Vatansever *et al.* (2010) study showed that 125 µg/ml of ethanolic extract of propolis is toxic to MCF-7 breast cancer cell lines. Also, they documented differentiation in the cytotoxicity on MCF-7 cells after the use of seven various samples of ethanolic extract of propolis that were collected from different locations.

In the present study, the high significant decrease in the viability of LNCaP cell line after the treatment with different concentrations of ethanolic extract of propolis (figure 3.1) is in agreement with Salim *et al.* (2015) study which demonstrated that the treatment of PC3 prostate cancer with ethanolic extract of propolis resulted in cellular morphological changes and toxic effects in the prostate cancer cell lines. It also found that the percentage of surviving cells was reduced in a dose-dependent

manner. These findings show that the ethanolic extract of propolis is effective against the PC3 prostate cancer cell line.

According to the results achieved in many cell cultures, it is known that propolis from different origins have different types of compounds, enable control of cell growth, and distinguish the normal cell from the cancer cell (Najafi *et al.,* 2007). The inhibitory effects of propolis on the growth of cells were reported for different cell lines such as K-562 by Aliyazicioglu *et al.* (2005), HL-60 cells by Akao *et al.* (2003), and Mishima *et al.* (2005) or some other leukemia cells (Hamblin, 2006). Artepillin C has been shown to have a cytotoxic effect on human gastric carcinoma cells, human lung cancer cells, and mouse colon carcinoma cells (Kimoto *et al.,* 2001).

Estrogens play a role in a variety of physiological processes, including reproduction, growth, and development. Estrogen signaling disruption is linked to several adverse health outcomes, including carcinogenesis. Estrogen activities are primarily mediated by two nuclear estrogen receptors, α(ESR1) and β(ESR2). ESR1 is thought to have pro-proliferative effects, particularly on cells of the reproductive tissues, increasing pro-carcinogenic activity, whereas ESR2's actions appear to be more complex, as ESR2 has anti-proliferative and pro-apoptotic activities in breast and prostate tumor cells. ESR2 appears to be the major ESR expressed in peripheral blood leukocytes, tonsils, and spleen. ESR-binding properties have been observed in natural dietary xenoestrogens such as isoflavones, with genistein being one of the most active so-called phytoestrogens. Genistein binds to both ESR1 and ESR2 and activates them, but it has a preference for ESR2. Ingestion of

genistein and other isoflavones has been linked to a lower risk of estrogen-dependent malignancies including breast and prostate cancer as indicated by many studies. In the instance of prostate carcinogenesis, the suppressive effects of genistein have been linked to the activation of ESR2 and the inhibition of tumor cell proliferation (Yakimchuk *et al.,* 2018).

4.2 Effect of propolis extracts on the viability of Vero cell line

In the present study, ethanolic extract at all concentrations caused a significant decrement in the viability of Vero cell line, this result is in agreement with that reported by Calhelha *et al.* (2014) which demonstrated that the ethanolic extract of propolis had high toxicity for non-tumor (normal) liver primary culture (PLP2). Lopez *et al.* (2015) also observed a cytotoxic effect of ethanolic extract of propolis against human keratinocytes (HaCaT cells) and murine fibroblasts (BALB/c 3T3 cells).

In the present study, the cytotoxic effect of both the aqueous and ethanolic extracts of propolis was observed, especially with the ethanolic extract which had lower IC50 (figure 3.4) in comparison to the aqueous extract (821 µg/ml versus 2304 µg/ml). Frozza *et al.* (2013) obtained an IC50 > 150 µg/ml of ethanolic extract of propolis with human normal epithelial embryonic kidney (HEK-293) cells.

The inhibitory impact of propolis ethanolic extract was dosage and time-dependent on human laryngeal epidermoid carcinoma HEp-2 cell growth. The IC50 value of propolis towards HEp-2 cells at 24 hours was 218.5 µg/ml (da Silva *et al.*, 2017).

A study performed by Rufatto *et al.* (2018) pointed out that the hydroalcoholic extract of propolis had a cytotoxic effect against the non-tumor Vero cell line.

In the current study, only high concentrations of aqueous extract (1000 and 500 µg/ml) caused a significant decrement in the viability of these cells (figure 3.3).

A study by Pontin *et al.* (2008) indicated that hydroalcoholic extract of propolis had no toxicity to these cells. Also, a study by Friõn-Herrera *et al.* (2015) pointed out that propolis hydroalcoholic extract at concentrations of 6.25, 12.5, 25, 50, and 100 µg/ml displayed anti-proliferative and cytotoxic effects on A549 cells in a dose- and time-dependent manner, but it did not suppress the growth of normal Vero cells.

4.3 Effect of propolis extracts on the viability of Raji cell line

Natural products have provided a rich source of substances that have been used in cancer chemotherapy in a variety of ways. Chemicals derived from natural sources account for more than 70% of anticancer compounds. Propolis and its separated components have been demonstrated to have cytotoxic effects on several tumor cells in vitro. Propolis and its isolated constituents have anti-tumor effects in vivo, owing to an immunomodulatory effect, primarily due to the enhancement of non-specific antitumor immunity via macrophage activation, which could then produce soluble factors and interfere directly with tumor cells or other immune cells (Watanabe *et al.*, 2011).

In the present study, the IC50 of propolis aqueous extract on Raji cell line was less than ethanolic extract (1773 versus 1845 µg/ml) respectively (figure 3.6), this result indicates that propolis aqueous extract is more potent than the ethanolic extract as it caused a highly significant decrease in Raji cell line viability at the concentrations (1000, 500 and 250 µg/ml) of propolis' aqueous extract, this result is inconsistent with the result found by Spiridonov *et al.* (1992) study in which propolis aqueous extract cause complete suppressing to the growth of Raji cell line after treatment with the concentrations ranging from 50-500 µg/ml.

In the present study, only the concentration of 1000 µg/ml of the ethanolic extract caused a highly significant decrease in the viability of Raji cell line (figure 3.5) and this effect may be attributed to arte C, found in the ethanolic extract of propolis, which has antitumor activity in lymphocytic leukemia (both T-cell and B-cell lines), myeloid leukemia, and induces apoptotic cell death (marked by the appearance of apoptotic bodies and DNA fragmentation) in all tested cells; and it has been suggested that this effect may be partly associated with up-regulation of Fas expression and loss of mitochondrial membrane potential. In addition, the ability of propolis in decreasing the viability of Raji cell line may be related to the presence of genistein an isoflavone that had been present in aqueous and ethanolic extracts of propolis (Abubakar *et al.*, 2014). Also, the result of the current study is in agreement with the study of Campos *et al.* (2014, 2015) which reported that ethanolic extract of propolis was capable of reducing 50% of the K562 (leukemia) tumor cells growth.

Flavonoids have been demonstrated to stop cancer and leukemia cells from proliferating. Raynal *et al.* (2008) showed that

genistein induced a time and dose-dependent effect on both myeloid and lymphoid leukemic cell lines. Plasma concentrations of geinsten that can be obtained from a soy-enriched diet led to a loss of clonogenicity by leukemic cells. In the in vivo model, the leukemic mice fed with a 0.5% genistein-enriched diet showed a significant increase in survival time as compared to control mice on a normal diet (Oršolić, 2010).

The antitumor properties of aqueous extract of Iraqi propolis on acute myeloid leukemia (HL-60) and human colon carcinoma (HCT-116) cell lines have been evaluated. Propolis induced an antitumor effect against HL-60 cells and HCT-116 cells. Moreover, the apoptosis in HL-60 cells was associated with down-regulation of Bcl-2 and the activation of Bax, whereas in HCT-116 cells necrotic features were observed. The size of cells was also dramatically increased by swelling of cytoplasm and loss of membrane integrity, cell rupture, and release of cellular contents; similarly, the propolis induced cell cycle perturbations in both cell lines. The exposure of HL-60 cells to propolis caused an increase in the levels of γ-H2AX (it is Phosphorylated H2AX which is an early cellular response to the induction of DNA double-strand breaks) in a dose-dependent manner and was associated with the induction of apoptosis, indicating that Iraqi propolis could be a promising antitumor agent. Diverse research had reported that propolis from distinct countries (Brazil, Mexico, and Thailand) exhibit antitumor activity in different tumor cell lines (human ovarian cancer cells, human colon cancer cells, HCT-116; human promyelocytic leukemia cells, HL-60; human glioblastoma cells, human cervical cancer cells, HeLa, and human adenocarcinoma alveolar basal epithelial cells) (Rivera-Yañez *et al.*, 2021).

Another study found that the ethanolic extracts of both Chinese and Brazilian propolis inhibited the growth of the human colon carcinoma cell line. The anti-proliferative activity of propolis is consistent with those reported to exert biological activities in other types of carcinoma cells. For instance, the Netherlands and Brazilian propolis exhibited antiproliferative activities in murine colon carcinoma, murine melanoma, human HT1080 fibrosarcoma, and human A549 lung adenocarcinoma cell lines. Also, the Chilean propolis inhibited the growth of human KB mouth epidermoid carcinoma and DU145 prostate carcinoma cell line (Aso *et al.*, 2004).

4.4 Effect of propolis extracts on the viability of the isolated peripheral blood mononuclear cells

Fatty, aliphatic, and aromatic acids, flavonoids, alcohols, sugars, and esters are the primary components of propolis. Several studies have confirmed the differences in percentages of individual components of propolis, depending on the origin of the plants from which the resin is collected and the species of bees (Sawicka *et al.*, 2012).

It has been reported that flavonoids, phenolics, and aromatics compounds are the most important pharmacological active constituents of propolis, and it is well known that these products are powerful anti-oxidants. For this reason, propolis is considered as being a natural source of anti-oxidant compounds that may play an important role in the anti-proliferative activity of propolis (Ghassan *et al.*, 2012).

In the present study the ethanolic extract of propolis at concentrations of 1000, 500, 250, and 125 µg/ml caused a significant increase in the proliferation of PBMNs (figure 3.7), this result agrees with the result of the study by Arslan *et al.* (2021) which pointed that the ethanolic extract of propolis enhanced human PBMNs viability at concentrations 500, 250, 125, and 62.5 µg/ml and protect the cells against mitomycin cytotoxicity.

Propolis promotes PBMNs proliferation, this effect is correlated to the flavonoids content. Propolis´s flavonoids are difficultly dissolved in water (Tao *et al.*, 2014), thus the low influence of aqueous extract at low concentrations is reported by the present study. At higher concentrations (1000 and 500 µg/ml) a significant increase in the viability of PBMNs agrees with the result of the study performed by Mojarab *et al.* (2020) which indicated that using the aqueous extract of propolis as an adjuvant in HIV vaccine and using this formulation in mice model led to increasing the proliferation of PBMNs.

4.5 Effect of doxorubicin on the viability of prostate cancer cell line (LNCaP)

As had been reported by the current study all concentrations of DOX cause a significant decrease in the viability of LNCaP cell line (figure 3.8), this effect may be related to the ability of anthracycline DOX to sensitize several types of cancer cells to TRAIL-mediated apoptosis, while it does not sensitize normal human prostatic stromal cells to TRAIL-mediated cytotoxicity. The initiative caspase-8 which plays a crucial role in TRAIL-mediated

apoptosis was significantly activated after the treatment of LNCaP cell line with DOX (Wu *et al.*, 2002).

Kang *et al.* (2005) pointed out that treatment of LNCaP cells with 0.86 mmol/L DOX led to a decrease in the viability of these cells and this decrement would be enhanced by adding 100 ng/ml human recombinant TRAIL. Also, a study by Tsakalozou *et al.* (2012) indicated that DOX causes dose-dependent cytotoxicity in different prostate cancer cell lines such as PC3 and DU145.

Treatment of PC3 cells with chemotherapy, such as DOX, revealed that the vitality of the cells reduced as the DOX concentration increased (Salim *et al.*, 2015).

4.6 Effect of doxorubicin-propolis extract combination on the viability of prostate cancer cell line (LNCaP)

The current study found that the treatment of LNCaP cell line with a combination of both DOX and ethanolic extract of propolis results in a dose-dependent decrement in the viability of these cells (figure 3.9), this result is in agreement with that reported by Salim *et al.* (2015) which revealed that the percentage of surviving cells was reduced with the elevation in the doses of the combination of DOX and ethanolic extract of propolis.

Shu *et al.* (2018) indicated that DOX therapy for prostate cancer is frequently leading to acquired DOX resistance which is a major problem. Doxorubicin which interacts with DNA by intercalation and inhibition of macromolecular biosynthesis is commonly used to treat some leukemia and Hodgkin's lymphoma, as well as other cancers, e.g., prostate, bladder, breast, stomach, lung, and ovaries. However, its use is associated with numerous side effects such as

induction of testicular toxicity and infertility. In a rat model, propolis co-administered with DOX protect against testicular toxicity (Zabaiou *et al.,* 2017). Thus, the combination of low doses of DOX with other agents that have an anti-proliferating effect against cancer cells may help in decreasing DOX resistance and adverse effects associated with higher doses of DOX.

Regarding the synergistic effect of the DOX-propolis combination reported by the present study, it is consistent with a study performed by Shu *et al.* (2018) which revealed that quercetin can reverse DOX resistance in DOX-resistant PC3 cell line (PC3/R) by increasing the sensitivity of PC3/R cells to DOX-induced apoptosis.

Quercetin is a natural predominant flavonoid found in propolis that is greatly contributing to its antioxidant activity (Zheng *et al.,* 2017). Quercetin is involved in the induction of the apoptosis pathway in cancers. Therefore, the combination of quercetin with several anti-tumor agents such as TRAIL has been proved to be an effective synergistic treatment for prostate cancer.

Consistent with this respect, the synergistic effect of the DOX-propolis ethanolic extract combination goes with that reported by Alsherbiny *et al.* (2021) study which found that the Australian propolis extracts significantly enhanced the inhibitory effect of DOX against MCF7 breast adenocarcinoma cell line proliferation in a dose-dependent manner. It enhanced the apoptotic effect of DOX after 24 hours of treatment with significant up-regulation of DR5 and DR4 (death receptors), TRAIL-mediated apoptosis, contributing to the anti-proliferative activity of Australian propolis extracts. Significant up-regulation of pro-apoptotic p27, PON2, and catalase with down-regulated anti-apoptotic XIAP, HSP60, and

HIF-1, and increased antioxidant proteins (catalase and PON2) may be responsible for the enhancement of the apoptosis of MCF7 cell line after the treatment with a combination of propolis and DOX in comparison to the mono-therapy of each one, moreover, it was found that the Australian propolis extract potentiated the anticancer activity of DOX by promoting apoptosis versus DOX-mediated necrosis, which may be advantageous to declining DOX-related side effects. The observed necrotic to an apoptotic shift of DOX by the synergistic combination may be attributed to the antioxidant profile of Australian propolis extracts and the resultant antioxidant-related apoptotic pathways in the MCF7 cells.

4.7 Immunomodulatory effects of propolis extracts on Raji cell line and peripheral blood mononuclear cells

The immunomodulatory effects of aqueous and ethanolic extracts of propolis on Raji Cell line and PBMNs were evaluated by measuring the level of the following:

4.7.1 Interleukin-4

Mast cells, Th2 cells, eosinophils, and basophils are the main producers of IL-4 which act as a strong immune system regulator (Gadani *et al.*, 2012).

Macrophages are a major source of many cytokines involved in immune response, hematopoiesis, and inflammation. Upon stimulation by microorganisms, microbial products (for example lipopolysaccharide (LPS), or endogenous factors (including cytokines), macrophages synthesize and release a large variety of cytokines such as IL-4 (Szliszka *et al.*, 2013).

Propolis contains anti-inflammatory substances such as CAPE and has been reported to restore the balance between pro-inflammatory and anti-inflammatory mediators. In addition, propolis has a direct regulatory effect on the basic functional properties of immune cells, suppressing the release of pro-inflammatory cytokines and stimulating the production of anti-inflammatory cytokines like IL-4 (Elswefy *et al.,* 2020). Caffeic acid phenethyl ester increases the production of anti-inflammatory cytokines mainly IL-4 (Pahlavani *et al.,* 2020).

In the current study, the significant decrease in the level of IL-4 after the treatment of Raji cell line with aqueous (50, 250, and 1000 µg/ml) and ethanolic (1000 µg/ml) extracts of propolis (figure 3.10) goes with that found by Szliszka *et al.* (2013) study which pointed that the level of IL-4 in RAW264.7 cells [monocyte/macrophage-like cells, originating from Abelson leukemia virus-transformed cell lines derived from mice (Taciak *et al.,* 2018)] was reduced by arte C. Also it goes with Szliszka *et al.* (2013) study which observed that the level of IL-4 in J774A.1 cells (Reticulum cell sarcoma) was also reduced when treated with ethanolic extract of propolis.

The increment in the level of IL-4 after the treatment of isolated PBMNs with both aqueous and ethanolic extracts of propolis at concentrations (50 and 250 µg/ml) (figure 3.11) that noticed in the current study agrees with Magnavacca *et al.* (2021) study which pointed that subcutaneous injection of liposomal preparations of Chinese propolis flavonoids with ovalbumin to mice efficiently activated the cellular and humoral immune response, boosting the level of IL4 in the serum. Also, it agrees with Sforcin (2007) in vivo study which revealed that different doses of CAPE

administered to mice increased T lymphocyte proliferation and secretion of IL-4 by splenocytes. Moreover, the result of the current study is inconsistent with Soudi *et al.* (2020) in vitro study which pointed out that treatment of human myelomonocytic cell line U937 cells with ethanolic extract of propolis at a concentration of 40 µg/mL causes a significant increment in the level of IL-4.

Regarding the dropping in the level of IL-4 after the treatment of PBMNs with a high concentration (1000 µg/ml) of ethanolic extract of propolis reported by the current study, it is consistent with the result of Chan *et al.* (2012) study which indicated that propolis suppresses cytokines production from Th2 such as IL-4, and this can be explained by the presence of chrysin and kaempferol which are flavonoids of propolis reported inhibiting the release of cytokines from mast cells. A study by Nakamura *et al.* (2010) pointed out that giving these flavonoids to mice caused suppression of Th2 cytokines, such as IL-4. Also, this result goes with Kusnul *et al.* (2017) study which pointed out that propolis extracts or the active compounds (Quercetin and hesperidin) were able to reduce cytokine production, such as IL-4 in PBMCs from a healthy donor.

Also, this result could be attributed to the presence of arte C which modulates the immune system by normalizing IL-4. Arte C affects various immune cells, such as immunosuppression of T lymphocytes and activation of macrophage function. The exact underlying mechanisms of these actions remain unclear till now (Beserra *et al.*, 2020).

4.7.2 Interleukin-10

Interleukin-10 plays very important immune-regulatory roles in host defense and immune homeostasis. IL-10 is a major immunosuppressive cytokine. Almost all cells of the innate and adaptive arms of the immune system can produce IL-10, including macrophages, mast cells, NK cells, eosinophils, neutrophils, and B cells. The major role of IL-10 is to limit the extent of the activation of both the innate and the adaptive immune cells to maintain a homeostatic state. This role of IL-10 is very important in protecting the host from infection-associated immunopathology, autoimmunity, and allergy, such as arthritis, inflammatory bowel disease, and so on. In addition to these activities, IL-10 regulates the growth and/or differentiation of B cells (Ma *et al.*, 2015).

Regarding the effect of propolis extracts on Raji cell line, the decrease in the level of IL-10 after the treatment of these cells with 1000 µg/ml of ethanolic extract of propolis is in agreement with which have been reported by Szliszka *et al.* (2013) study which indicated that treatment of J774A.1 macrophage with ethanolic extract of propolis would cause slightly down-regulates in IL-10 production in culture supernatants derived from these cells. This may be attributed to the inhibitory effect of arte C on the production of IL-10 in vitro and in vivo models (Shahinozzaman *et al.*, 2020).

Regarding the increment in the level of IL-10 after treatment of Raji cell line with ethanolic (50 µg/ml) of propolis reported by the current study, it is in agreement with what has been found by Machado *et al.* (2012) in vivo study in which the mice with cotton pellet granuloma given propolis orally for six days increase IL-10 production. Also, it agrees with Missima *et al.* (2010) in vivo study

which pointed out that treatment of melanoma-bearing mice with propolis for 14 days would induce IL-10 production.

Most of the research about propolis's effect was using experimental animals, mainly mice or rats, and little is known concerning propolis's effect on human cells (Conti *et al.*, 2013).

The significant increment in the level of IL-10 after the treatment of PBMNs with both aqueous and ethanolic extracts of propolis at a concentration of 250 µg/ml (figure 3.13) found by the current study is in agreement with that reported by Conti *et al.* (2015) who observed that Brazilian green propolis stimulated IL-10 production by human monocytes. Another in vitro study done by Conti *et al.* (2015) supports the result of the present study as it shows that the monocytes treated either with Brazilian propolis (2, 10, and 20 µg/ml) or Mexican propolis (1, 2, 10 and 20 µg/ml exhibited a stimulatory effect in IL-10 secretion in a dose-dependent manner.

This result may be related to the effect of CAPE as having been found by Conte *et al.* (2021) in vivo study in which the CAPE was administered to LPS-treated rats resulting in a decrease in the production of pro-inflammatory cytokines and an increase in the production of the anti-inflammatory cytokines such as IL-10. Another in vivo study performed by Liberio *et al.* (2011) supported the result of the current study as it showed that propolis increases the production of IL-10 in a mice model suggesting anti-inflammatory activity.

Propolis' ability to increase IL-10 development demonstrates its ability to control inflammation. After an inflammatory stimulus, many immune cells produce IL-10, which is essential for

maintaining homeostasis by controlling both innate and adaptive immune responses (Ma *et al.,* 2015).

The immunomodulatory action of propolis depends on its chemical composition and the synergistic or antagonistic effects between its compounds (Conti et al., 2015). The details of the mechanisms of actions of propolis and its components on immune cells are still unknown (Ansorge *et al.,* 2003).

The result of the current study regarding the effect of 250 and 1000 µg/ml of ethanolic extract of propolis disagrees with the result of the study done by Conti *et al.* (2013) which indicated that treatment of PBMNs with ethanolic extract of propolis in which the concentration of cinnamic acid was 25, 50, and 100 μg/ml would inhibit the production of IL-10.

4.7.3 Interleukin-17

Interleukin-17 (IL-17, also known as IL-17A) is a key cytokine that links T cell activation to neutrophil mobilization and activation. IL-17 can mediate protective innate immunity to pathogens and contribute to the pathogenesis of inflammatory diseases, such as psoriasis and rheumatoid arthritis (Zenobia and Hajishengallis, 2016).

Interleukin-17 production and/or release is strongly influenced by propolis extracts and substances within this bee product (Ansorge *et al.,* 2003).

Regarding the significant decrease in the level of IL-17 after the treatment of Raji cell line with both aqueous and ethanolic extracts of propolis at all concentrations found by the current study,

it is in agreement with that reported by Szliszka *et al.* (2013) and her colleagues which pointed that the level of IL-17 in activated RAW264.7 cells was reduced by arte C. Also the result of the current study is in agreement with that of Khosravi *et al.* (2014) in vivo study in which the mice with invasive ductal carcinoma given propolis orally for 10 days result in a decrease in IL-17 production. While the result of the study made by Szliszka, *et al.* (2013) on J774A.1 macrophages is disagree with the result of the current study as it pointed out that the ethanolic extract of propolis did not influence the level of IL- 17.

In the present study, the significant decrease in the level of IL-17 after the treatment of PBMNs with 1000 µg/ml of ethanolic extract of propolis is in agreement with the result of Cheung *et al.* (2011) who found that both arte C and ethanolic extract of propolis can significantly inhibit T cell proliferation, activation, and suppress the expression of IL-17. Moreover, a recent clinical study demonstrated that the level of IL-17 released from PBMNs in HIV patients taking propolis at a dose of 500 mg/day for 3 months was significantly reduced (Conte *et al.*, 2021).

The significant decrease in the level of IL-17 may be attributed to the inhibitory effect of high concentration (1000 µg/ml) of ethanolic extract of propolis on Th2 cells as had been found by recent a study in which the ethanolic extract of Iranian propolis inhibits the release of IL-17 prompted by Aspergillus fumigatus conidia in mice lung epithelial cells (Magnavacca *et al.*, 2021).

Another in vivo previous study confirm the result of the current study as it examined the effect of ethanolic extract of propolis on the production of IL-17 from splenocytes of normal mice and it

observed a concentration-dependent decline in the production of IL-17 (Tanaka *et al.*, 2012).

Regarding the significant increase in the level of IL-17 after the treatment of PBMNs with low concentrations (250 and 50 µg/ml) of both aqueous and ethanolic extracts of propolis found by the current study it is consistent with a recent in vivo research performed by Mohammed *et al.* (2021) which indicated that the oral administration of propolis (30, 40, and 50 mg/kg) to mice with wound infected with *Acinetobacter baumannii* would cause an increment in the level of IL-17. The result of this study showed that the low concentrations (30, 40, 50) mg/kg of propolis caused a higher effect than the high concentrations of (100, 150, 200) mg/kg in stimulating the production of IL-7.

4.7.4 Interferon-gamma

Inflammation is a natural response of the innate immune system to protect the organisms by cleaning the cellular and extracellular debris (Zhao *et al.*, 2016).

Interferon-gamma is a cytokine that is primarily produced by cells of the immune system, including natural killer (NK) cells and T helper 1 (TH1) cells, and CD8+ cytotoxic T lymphocytes (CTLs). It signals through the IFN-γ receptor (IFN-γR; comprising the IFNγR1 and IFNγR2), which can be expressed in most cell types (Ivashkiv, 2018).

In the current study, the treatment of Raji cell line with 50, 250 and 1000 µg/ml from the aqueous and ethanolic extracts caused a significant decrease in the level of INF-γ, this result explains the anti-inflammatory and antitumor effects of propolis as it causes a

decrease in the level of IFN-γ which acts as a proinflammatory cytokine in immune responses (Lee *et al.*, 2017).

In the current study, the decrease in the level of IFN-γ after the treatment of Raji cell line with propolis extracts may be attributed to arte C as have been reported by a recent study which revealed that arte C can inhibit IFN-γ and affects various immune cells, such as the suppression of T lymphocytes. The exact mechanisms of these actions remain unclear till now (Beserra *et al.*, 2020).

Results of the above-mentioned studies suggest that the propolis extracts can maintain immunological homeostasis so that it causes different effects when administered under different conditions (Kusnul *et al.*, 2017).

The present study's findings on Raji cell line contradict those of a previous study made by Szliszka *et al.* (2013) which pointed out that treatment of stimulated RAW264.7 cells with arte C led to an increase in IFN-γ level.

In the current study, there was a significant increase in the level of IFN-γ after treatment of PBMNs with aqueous (250 and 50 µg/ml) and ethanolic (1000 µg/ml) extracts of propolis (figure 3.15). This result is consistent with a study done by Magnavacca *et al.* (2021) which demonstrated that the phagocytic function of peritoneal macrophages harvested from mice was greatly enhanced by liposomal preparations of Chinese propolis flavonoids, which also stimulated IFN-γ production. Subcutaneous injection of a liposomal mixture of Chinese propolis flavonoids with ovalbumin to mice activated the cellular and humoral immune responses and increased blood IFN-γ levels. Another in vivo study done by Sá-

nunes *et al.* (2003) demonstrated that the production of IFN-γ in the supernatant of splenocytes culture taken from propolis-treated mice (mice were treated daily with 2.5, 5, and 10 mg/kg of propolis hydroalcoholic solution for 3 days intraperitoneally) was increased.

Conclusions and Recommendations

Conclusions

1. Both aqueous and ethanolic extracts of propolis have anti-proliferative effects against Raji and LNCaP cancer cell lines.
2. On Raji cell line aqueous extract has more anti-proliferative effect than ethanolic extract of propolis, while on LNCaP cell line ethanolic extract has more anti-proliferative effect than aqueous extract.
3. On Vero cell line (normal cells) ethanolic extract has more cytotoxicity than aqueous extract of propolis. On PBMNs, both aqueous and ethanolic extracts of propolis have proliferative effects at high concentrations, although aqueous extract has a less potent effect than ethanolic extract. At low concentrations, the aqueous extract has an anti-proliferative effect against PBMNs.
4. Aqueous and ethanolic extracts of propolis have immunomodulatory effect in both Raji cell line and PBMNs:
    - In Raji cell line both propolis extracts exert less anti-inflammatory effect especially aqueous extract. On PBMNs both aqueous and ethanolic extracts of propolis had anti-inflammatory properties, but a high concentration of ethanolic extract exert less anti-inflammatory effect.
    - In Raji cell line the lowest concentration of ethanolic extract had anti-inflammatory property, while the highest concentration exerts a less anti-inflammatory effect. In PBMNs both aqueous and ethanolic extracts of propolis exert an anti-inflammatory effect.
    - In Raji cell line both aqueous and ethanolic extracts had anti-inflammatory properties. In PBMNs, the highest concentration of ethanolic extract had anti-inflammatory property, while other concentrations of both extracts exert a pro-inflammatory effect.

- In Raji cell line both aqueous and ethanolic extracts had anti-inflammatory properties. In PBMNs, aqueous extract exerts a pro-inflammatory effect more potently than ethanolic extract.

Recommendations

1. Isolation, identification, and studying the biological activities and mechanism of action of the bioactive compounds in propolis.
2. Study the effect of propolis in combination with another type of chemotherapy like vincristine.
3. Study the immunomodulatory effect of aqueous and ethanolic extract of propolis on animal models.

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Appendix

Appendix

ELISA procedure

Test principle: This ELISA kit uses Sandwich-ELISA as the method. The micro-ELISA plate provided in the kit has been pre-coated with an antibody specific to the test of interest. Standards or samples are added to the appropriate micro-ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for test of interest and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain test or protein of interest, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of test of interest. You can calculate the concentration of test of interest in the samples by comparing the OD of the samples to the standard curve.

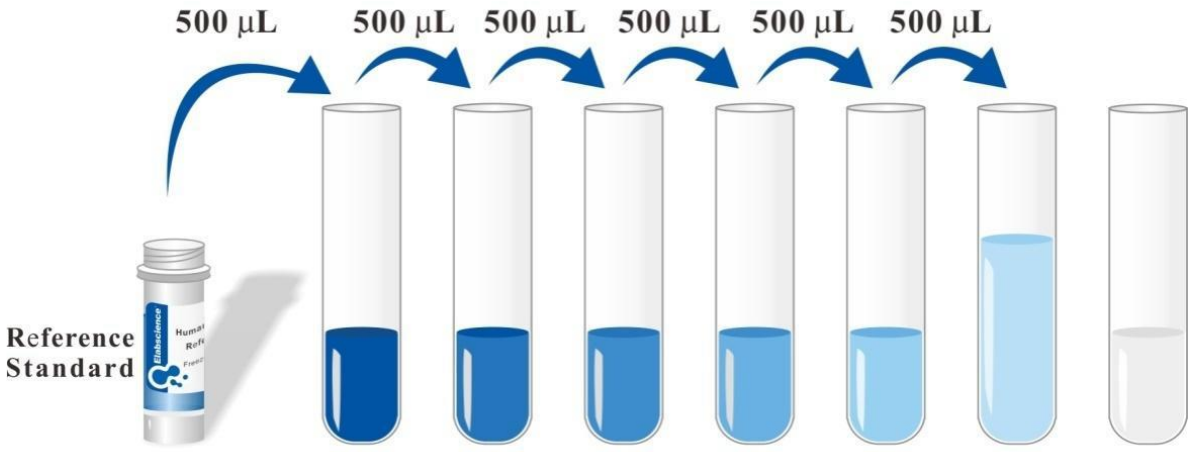
Reagent preparation Bring all reagents to room temperature (18-25℃) before use.

Wash Buffer - 30 mL of Concentrated Wash Buffer is diluted into 750 mL of Wash Buffer with deionized or distilled water. Then the unused solution is placed back at 4℃. If crystals have formed in the concentrate, you can warm it with 40℃ water bath (Heating temperature should not exceed 50℃) and mix it gently until the

crystals have completely dissolved. The solution should be cooled to room temperature before use.

Standard –standard is prepared within 15 minutes before use and centrifuged at 10,000×g for 1 minute, then reconstitute the Standard with 1.0 mL of Reference Standard & Sample Diluent. Tighten the lid, let it stand for 10 minutes and turn it upside down for several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a stock solution of 10ng/ml. Then make serial dilutions as needed (making serial dilution in the wells directly is not permitted). The recommended concentrations are as follows:10、5、2.5、1.25、0.625、0.313、0.156、0 ng/ml. If you want to make standard solution at the concentration of 5ng/mL, you should take0.5mL standard at 10ng/mL, add it to an EP tube with 0.5mL Reference Standard &Sample Diluent, and mix it. Procedures to prepare the remained concentrations are all the same. The undiluted standard serves as the highest standard (10ng/mL). The Reference Standard &Sample Diluent serves as the zero (0 ng/mL).

(Standards can also be diluted according to the actual amount such as 200μL/tube).



1	2	3	4	5	6	7	8	tube
10	5	2.5	1.25	0.625	0.313	0.156	0	ng/mL

serial dilution of reference reagent

Biotinylated Detection Ab – the required amount is calculated before experiment (100µL/well). Then the stock tube is centrifuged before use, and the concentrated Biotinylated Detection Ab is diluted to the working concentration using Biotinylated Detection Ab Diluent (1:100).

Concentrated HRP Conjugate –the required amount is calculated before experiment (100µL/well). the Concentrated HRP Conjugate is diluted to the working concentration using Concentrated HRP Conjugate Diluent (1:100).

Substrate Reagent: As it is sensitive to light and contaminants, so the vial should not be opened until there is a need. The needed dosage of the reagent can be aspirated with sterilized tips and the unused residual reagent should not be dumped back into the vial again.

Washing Procedure:

1. **Automated Washer**: 350μL wash buffer is added into each well, the interval between injection and suction should be set about 60s.

2. **Manual wash**: 350μL Wash Buffer is added into each well, then soaked for 1~2minutes. After the last wash, decant any remaining Wash Buffer by inverting the plate and blotting it dry by rapping it firmly against clean and toweling absorbent paper on a hard surface.

Assay procedure all reagents and samples are brought to room temperature before use. Centrifuge the sample again after thawing before the assay. All the reagents should be mixed thoroughly by gently swirling before pipetting, foaming should be avoided. It is recommended that all samples and standards be assayed in duplicate.

1. Addition of Sample: 100μL of Standard, Blank, or Sample is added per well. The blank well is added with Reference Standard & Sample diluent. Solutions are added to the bottom of micro-ELISA plate well, avoid inside wall touching and foaming as possible. Mix it gently. Cover the plate with sealer we provided. Incubate for 90 minutes at 37℃.

2. Biotinylated Detection Ab: then the liquid of each well is removed, without washing. Addition of 100μL of Biotinylated Detection Ab working solution to each well. Then the plate is covered with the Plate sealer. Gently tap the plate to ensure thorough mixing. Incubate for 1 hour at 37°C.

3. Wash: each well is aspirated and washed three times, the wash process is done by filling each well with Wash Buffer

(approximately 350μL) (a squirt bottle, multi-channel pipette, manifold dispenser or automated washer are needed). Complete removal of liquid at each step is essential. After the last wash, the remained wash Buffer is removed by aspirating or decanting. Then the plate is inverted and placed against thick clean absorbent paper.

4. HRP Conjugate: 100μL of HRP Conjugate working solution is added to each well, then incubate for 30 minutes at 37°C.

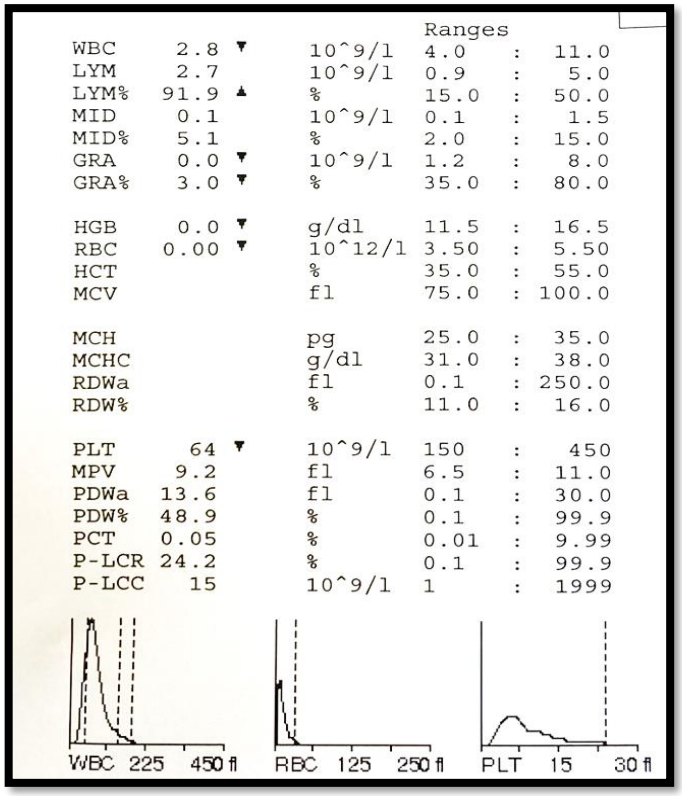
5. Wash: the wash process is repeated for five times as conducted in the third step.

6. Substrate: 90μL of Substrate Solution is added to each well. Cover with a new Plate sealer. Incubate for about 15 minutes at 37°C. Protect the plate from light. The reaction time can be shortened or extended according to the actual color change, but not more than 30minutes. When apparent gradient appeared in standard wells, user should terminate the reaction.

7. Stop: 50μLof Stop Solution is added to each well. Then, the color turns to yellow immediately. The order to add stop solution should be the same as the substrate solution.

8. OD Measurement: the optical density (OD value) of each well is determined at once, using a micro-plate reader set to 450 nm. User should open the micro-plate reader in advance, preheat the instrument, and set the testing parameters.

9. After experiment, all the unused reagents are placed back into the refrigerator according to the specified storage temperature respectively until their expiry.



				Ranges		
WBC	2.8	▼	10^9/l	4.0	:	11.0
LYM	2.7		10^9/l	0.9	:	5.0
LYM%	91.9	▲	%	15.0	:	50.0
MID	0.1		10^9/l	0.1	:	1.5
MID%	5.1		%	2.0	:	15.0
GRA	0.0	▼	10^9/l	1.2	:	8.0
GRA%	3.0	▼	%	35.0	:	80.0
HGB	0.0	▼	g/dl	11.5	:	16.5
RBC	0.00	▼	10^12/l	3.50	:	5.50
HCT			%	35.0	:	55.0
MCV			fl	75.0	:	100.0
MCH			pg	25.0	:	35.0
MCHC			g/dl	31.0	:	38.0
RDWa			fl	0.1	:	250.0
RDW%			%	11.0	:	16.0
PLT	64	▼	10^9/l	150	:	450
MPV	9.2		fl	6.5	:	11.0
PDWa	13.6		fl	0.1	:	30.0
PDW%	48.9		%	0.1	:	99.9
PCT	0.05		%	0.01	:	9.99
P-LCR	24.2		%	0.1	:	99.9
P-LCC	15		10^9/l	1	:	1999

				Ranges		
WBC	1.7	▼	10^9/l	4.0	:	11.0
LYM	1.6		10^9/l	0.9	:	5.0
LYM%	86.7	▲	%	15.0	:	50.0
MID	0.1		10^9/l	0.1	:	1.5
MID%	8.3		%	2.0	:	15.0
GRA	0.0	▼	10^9/l	1.2	:	8.0
GRA%	5.0	▼	%	35.0	:	80.0
HGB	0.0	▼	g/dl	11.5	:	16.5
RBC	0.00	▼	10^12/l	3.50	:	5.50
HCT			%	35.0	:	55.0
MCV			fl	75.0	:	100.0
MCH			pg	25.0	:	35.0
MCHC			g/dl	31.0	:	38.0
RDWa			fl	0.1	:	250.0
RDW%			%	11.0	:	16.0
PLT	30	▼	10^9/l	150	:	450
MPV			fl	6.5	:	11.0
PDWa			fl	0.1	:	30.0
PDW%			%	0.1	:	99.9
PCT			%	0.01	:	9.99
P-LCR			%	0.1	:	99.9
P-LCC			10^9/l	1	:	1999

Isolated PBMN cells count of two apparently healthy donors using Swelab® apparatus.

الخلاصة

السرطان: مرض يتميز بالنمو الغير منضبط للخلايا الغير طبيعية التي يمكن أن تنتشر إلى أنسجة أخرى من الجسم. سرطان البروستات يبدأ عندما تبدأ الخلايا الطبيعية للبروستات بالتغير والنمو بشكل لا يمكن السيطرة عليه. سرطان الغدد الليمفاوية بوركيت هو نوع من سرطان الغدد الليمفاوية اللاهودجكينية، وهو سرطان يصيب الأنسجة الليمفاوية، يحدث أثناء تكوين الجسم للخلايا اللمفاوية البائية غير الطبيعية.

لسنوات عديدة، لعبت الطبيعة دورًا واعدًا في اكتشاف المركبات الطبيعية المختلفة التي يمكن استخدامها كأدوية جديدة لعلاج العديد من الأمراض بما في ذلك السرطان الذي يحظى باهتمام خاص في الطب. العكبر، مادة راتنجية طبيعية معروفة منذ قرون، هو منتج معقد يحصل عليه نحل العسل من مواد تم جمعها من أجزاء من نباتات مختلفة في مناطق جغرافية مختلفة.

كان الهدف الأساسي من هذه الدراسة هو تقييم التأثير المضاد للتكاثر للمستخلصات المائية والإيثانولية للعكبر ضد خط خلايا الراجي وخط خلايا سرطان البروستات LNCaP وخط خلايا فيرو وخلايا الدم المحيطية. والهدف الثانوي هو تقييم التأثيرات المناعية لمستخلصات العكبر على خط خلايا الراجي وخلايا الدم المحيطية من خلال قياس مستويات السايتوكينات المحددة (IL-4 وIL-10 وIL-17 وIFN-γ) التي تنتجها هذه الخلايا.

تم عزل خلايا الدم المحيطية من متبرع شاب سليم باستخدام وسط متدرج الكثافة، ثم تمت تنقية الخلايا الليمفاوية المعزولة والتحقق من صلاحيتها. ثم زُرعت خلايا من كل من خلايا الراجي وLNCaP وفيرو وخلايا الدم المحيطية في مزرعة الأنسجة ٩٦ طبقًا جيدًا وعولجت بتراكيز مختلفة (١٠٠٠، ٥٠٠، ٢٥٠، ١٢٥، ٦٢,٥، ٣١,٢٥ ميكروغرام / مل) من كل من خلاصة العكبر المائي والإيثانولي. وحضنت لمدة ٢٤ ساعة. ثم تم استخدام مقايس السمية الخلوية MTT لتقييم تأثير المستخلصات على حيوية هذه الخلايا، وتم قياس التركيز المثبط نصف الأقصى (IC50) للمستخلصات المائية والإيثانولية من العكبر. وبالمثل، تم تقييم النشاط مضاد السرطان الدوكسوروبيسين بمفرده وبالاقتران مع مستخلص العكبر الإيثانولي ضد خط الخلايا LNCaP.

من أجل تقييم التأثير المناعي للمستخلصات المائية والإيثانولية للعكبر على خط خلايا الراجي وخلايا الدم المحيطية، تمت معالجة هذه الخلايا بـ ١٠٠٠ و٢٥٠ و٥٠ ميكروغرام / مل من كلا المستخلصين. تم إجراء اختبار ELISA للكشف عن مستويات IL-4و IL-10 وIL-17 وIFN-γ.

اظهرت النتائج أن المستخلص المائي من العكبر بتراكيز ١٠٠٠ و٥٠٠ و٢٥٠ ميكروغرام / مل تسبب في انخفاض معنوي عالي ($P \leq 0.001$) في حيوية خط خلايا الراجي. بينما تسبب التركيز العالي فقط للمستخلص الإيثانولي (١٠٠٠ ميكروجرام / مل) في انخفاض معنوي عالي ($P \leq 0.001$) في حيوية خط خلايا راجي.

كما أوضحت النتائج أن جميع تراكيز المستخلصين المائي والإيثانولي من العكبر تسببت في انخفاض معنوي عالي ($P \leq 0.001$) في حيوية خط الخلايا LNCaP. فيما يتعلق بالنشاط المضاد للسرطان للدوكسوروبيسين فقد تسبب في انخفاض معنوي كبير ($P \leq 0.001$) في حيوية خط الخلايا LNCaP في جميع التركيزات (٢٥٠، ١٢٥، ٦٢,٥، ٣١,٢٥، ١٥، ٧,٥ ميكروغرام / مل). أدى الجمع بين دوكسوروبيسين (٣٠ ميكروغرام / مل) والمستخلص الإيثانولي من العكبر (١٠٠٠، ٥٠٠، ٢٥٠، ١٢٥، ٦٢,٥، ٣١,٢٥ ميكروغرام / مل) إلى انخفاض معنوي كبير ($P \leq 0.001$) في حيوية خط الخلايا LNCaP في كل التراكيز المستخدمة.

فيما يتعلق بتأثير مستخلصات العكبر، أوضحت النتائج أن حيوية خط خلايا فيرو انخفضت معنويا ($p \leq 0.05$) بتراكيز ١٠٠٠ و٥٠٠ ميكروغرام / مل من مستخلص العكبر المائي، بينما كان هناك انخفاض معنوي عالي ($P \leq 0.001$) في حيوية هذه الخلايا بتراكيز ١٠٠٠ و٥٠٠ و٢٥٠ ميكروغرام / مل من المستخلص الإيثانولي وانخفاض معنوي ($p \leq 0.05$) في حيوية هذه الخلايا بتراكيز ١٢٥ و٦٢,٥ و٣١,٢٥ ميكروغرام / مل من نفس المستخلص.

كان التركيز المثبط نصف الأقصى لمستخلصات العكبر المائي والإيثانولي ١٧٧٣ ميكروغرام / مل مقابل ١٨٤٥ ميكروغرام / مل لخط خلايا الراجي، و ٧١٧,٣٦ ميكروغرام / مل مقابل ١٤١٦,٤٣ ميكروغرام / مل لخط خلايا LNCaP و ٢٣٠٤ ميكروغرام / مل مقابل ٨٢١ ميكروغرام / مل لخط خلايا الفيرو.

فيما يتعلق بنتائج خلايا الدم المحيطية أظهرت فصلًا فعالًا مع ارتفاع عدد الخلايا الليمفاوية، وانخفاض عدد الصفائح الدموية، ولم يتم العثور على خلايا دم حمراء مع نسبة حيوية أكثر من ٩٥ ٪. ادت التراكيز ١٠٠٠ و٥٠٠ ميكروغرام / مل من المستخلص المائي إلى زيادة معنوية عالية ($P\leq 0.001$) في حيوية خلايا الدم المحيطية، بينما ادت التراكيز ١٢٥ و٦٢,٥ و٣١,٢٥ ميكروغرام / مل الى انخفاض معنوي ($p\leq 0.05$) في حيوية هذه الخلايا. أنتج المستخلص الإيثانولي للعكبر بتراكيز ١٠٠٠ و٥٠٠ و٢٥٠ و١٢٥ ميكروغرام / مل زيادة معنوية عالية ($P\leq 0.001$) في حيوية خلايا الدم المحيطية.

بالنسبة للتأثير المناعي لمستخلصات العكبر على خلايا الدم المحيطية وخط خلايا الراجي، أظهرت نتائج خلايا الدم المحيطية زيادة معنوية عالية ($P\leq 0.001$) في مستوى IL-4 بعد معاملة الخلايا بالمستخلص المائي بتراكيز ٥٠ و٢٥٠ ميكروغرام / مل، وكانت هناك زيادة معنوية ($p\leq 0.05$) في مستوى IL-4 بعد معاملة الخلايا بـ ٥٠ و٢٥٠ ميكروغرام / مل من المستخلص الإيثانولي، بينما كان هناك انخفاض معنوي عالي ($P\leq 0.001$) في المستوى IL-4 بعد معاملة الخلايا بـ ١٠٠٠ ميكروغرام / مل من المستخلص الإيثانولي. فيما يتعلق بخط خلايا الراجي، أظهرت النتائج انخفاضًا معنويًا عاليًا ($P\leq 0.001$) في مستوى IL-4 بعد معاملة الخلايا بـ ٥٠ و٢٥٠ و١٠٠٠ ميكروغرام / مل من المستخلص المائي و١٠٠٠ ميكروغرام / مل من المستخلص الإيثانولي.

أظهرت النتائج بالنسبة لخلايا الدم المحيطية زيادة معنوية عالية ($P\leq 0.001$) في مستوى IL-10 بعد معاملة الخلايا بـ ٢٥٠ ميكروغرام / مل من المستخلص المائي وزيادة معنوية ($p\leq 0.05$) في مستوى IL-10 بعد معاملة الخلايا بـ ٢٥٠ ميكروغرام / مل من المستخلص الإيثانولي. فيما يتعلق بخط خلايا الراجي، أظهرت النتائج انخفاضًا معنويًا عاليًا ($P\leq 0.001$) في مستوى IL-10 بعد معاملة الخلايا بـ ١٠٠٠ ميكروغرام / مل من المستخلص الإيثانولي، بينما كانت هناك زيادة معنوية عالية ($P\leq 0.001$) في مستوى IL-10 بعد معاملة الخلايا بـ ٥٠ ميكروغرام / مل من المستخلص الإيثانولي.

أظهرت النتائج بالنسبة لخلايا الدم المحيطية زيادة معنوية عالية ($P\leq 0.001$) في مستوى IL-17 بعد معاملة الخلايا بـ ٥٠ و٢٥٠ ميكروغرام / مل من كلا المستخلصين، بينما كان هناك انخفاض معنوي ($p\leq 0.05$) في مستوى IL-17 بعد معاملة الخلايا بـ

١٠٠٠ ميكروغـرام / مـل مـن المسـتخلص الإيثـانولي. فيمـا يتعلـق بخـط خليـة الراجـي، أظهـرت النتـائج انخفاضًـا معنويًـا عاليًـا($P\leq0.001$) فـي مسـتوى IL-17 فـي جميـع التراكيز المستخدمة لكلا المستخلصين.

أظهـرت النتـائج بالنسـبة لخلايـا الـدم المحيطيـة زيـادة معنويـة عاليـة ($P\leq0.001$) فـي مستوى IFN-γ بعـد معاملـة الخلايـا بـ ٥٠ و٢٥٠ ميكروغـرام / مـل مـن المسـتخلص المـائي و١٠٠٠ ميكروغـرام / مـل مـن المسـتخلص الإيثـانولي. فيمـا يتعلـق بخـط خلايـا الراجـي، أظهـرت النتـائج انخفاضًـا معنويًـا عاليًـا ($P\leq0.001$) فـي مسـتوى IFN-γ بعـد معاملـة الخلايـا بـ ٥٠ و١٠٠٠ ميكروغـرام / مـل مـن المسـتخلص المـائي و١٠٠٠ ميكروغـرام / مـل مـن المسـتخلص الإيثـانولي. كـان هنـاك انخفـاض معنـوي ($p\leq0.05$) فـي مسـتوى IFN-γ بعـد معاملـة الخلايـا بـ ٢٥٠ ميكروغـرام / مـل مـن المسـتخلص المـائي و٥٠، ٢٥٠ ميكروغرام / مل من المستخلص الإيثانولي.

يسـتنتج مـن الدراسـة الحاليـة أن المسـتخلص المـائي والإيثـانولي لمـادة العكبـر لهمـا تـأثير مضـاد لتكـاثر الخلايـا السـرطانية فـي خـط خلايـا سـرطان البروسـتات(LNCaP) وخـط سـرطان الخلايـا اللمفاويـة راجـي (Raji) بالإضـافة الـى التـأثيرات المناعيـة المختلفـة علـى الخلايا اللمفاوية وخط خلايا راجي.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامـــعة بابـل
كليـة الطب

دراسة مختبرية لتقييم تأثير مستخلصات العكبر على الخلايا الطبيعية والسرطانية

رســـالـــــــة

مقدمـــــة إلـــى مجلس كليـــة الطـــب / جامعـــة بابـــل

كجزء من متطلبـــات نيـل درجـــة الماجستيـر في الادوية / الأدوية والسموم

من قبل

مها حسين عبد الكاظم حمد

(بكالوريوس صيدلة, ٢٠١٣-٢٠١٤)

إشراف

أ.د. إنتصار جواد حمد المختار

أ.د. قيصر نعمة مظلوم

١٤٤٣ هـ

٢٠٢٢م