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Department of Pharmacology

Study The Cytotoxic and Cytokines production modulation induced by Iraqi *Lantana Camara* alcoholic leaves extract. An in vitro study

A thesis

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By

Aula Ali Mohammed Kadhim

B. Pharmacy (2014-2015)

Supervised by

Prof. Dr.
Nisreen J. Mohammed
Ph.D. Pharmacology

Prof. Dr.
Kaiser N. Madlum
Ph.D. Biology

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2022 A.D

وَمِنَ النَّاسِ وَالدَّوَابِّ وَالْأَنْعَامِ مُخْتَلِفٌ أَلْوَانُهُ كَذَٰلِكَ ۗ إِنَّمَا يَخْشَى اللَّهَ مِنْ عِبَادِهِ الْعُلَمَاءُ ۗ إِنَّ اللَّهَ عَزِيزٌ غَفُورٌ ﴿٢٨﴾

صدق الله العلي العظيم

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Certification

We certify that this thesis entitled **"Study the Cytotoxic and Cytokines production modulation induced by *Lantana Camara* leaves extract. An in vitro"** was prepared by (Aula Ali Mohammed) under our supervision at the department of Pharmacology. College of Medicine, University of Babylon (Iraq) in partial fulfillment of the requirements for the master degree of sciences in pharmacology and toxicology.

Prof. Dr.
Nisreen J. Mohammed
Ph.D. Pharmacology

Prof. Dr.
Kaiser N. Madlum
Ph.D. Biology

The Recommendation of the Head of Department

In the view of the available recommendation, I forward this thesis for debate by the examining committee.

Assist. Prof. Dr.
Selman M. Selman
The Head of Department of Pharmacology
College of Medicine
University of Babylon, Iraq

Dedication

This study is lovingly dedicated to my respective and beloved family, friends and supervisors who have been my constant source of inspiration. They have given me the drive and discipline to tackle a task with enthusiast and determination. without their support and love this study would not have been made.

Aula Ali Mohammed

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Summary

The practical work of this study was performed at the Postgraduate Research laboratory /Department of Pharmacology/ College of Medicine/ University of Babylon during the period from October 2021- April 2022.

Plant chemical constituents play an important role in the treatment of different diseases, due its effects on immune system and cell proliferation. Thus, the aims of this study were to identify the cytotoxicity of *Lantana Camara* alcoholic leaf extracts on the cell lines including Vero cell line, LNCaP prostate cancer cell line and SW480 colon cancer cell line, Then, Study the immunological effect of alcoholic extract of *Lantana Camara* leaves on LNCaP cell line and SW480 cell line.

The cytotoxic part of the study was conducted to measure the cytotoxicity of plant extract on the viability in order to decide the useful range of concentrations used when performing immunomodulation assay using ELISA technique.

Vero, LNCaP and SW480 cell lines were seeded in tissue culture 96 well plate and treated with alcoholic leaf extracts in different concentrations (1000, 500, 250, 125, 62.5, 31.25) µg/mL (four replicates were used for each concentration of *Lantana Camara* for each type of cells) along with four replicates as a control group for each cell types and incubated for 24 hours. Then, the MTT (3-(4,5-Dimethyl thiazole- 2-yl)- 2,5- diphenyl-2H- tetrazolium bromide) assay was performed.

The results for Vero and SW480 cell lines showed that cells viability significantly reduced ($P \leq 0.001$) at concentrations (1000, 500, 250) µg /ml in comparison to the control group, while For LNCaP cell line, there was a significant ($P \leq 0.001$) decrement in the viability of those cells at (1000 ,500) µg /ml and significant increase in the viability of those cells at (250, 125, 62) µg /ml of plant extract after incubation 24 hour.

The cytokine's assay part of this study involves (LNCaP) and colon cancer (SW480). Cell lines were seeded in 96 tissue culture plates; all cells were treated with different concentrations of *Lantana Camara* at serial dilutions ranging from 1000 to 31 µg \ ml (four replicates were used for each concentration of *Lantana Camara* for each type of cells) along with four replicates as a control group for each cell types. Then the plate was covered with a self-plastic lid and incubated for 24 hours, at the end of the exposure period, the cell lines were taken for immunoassay by ELISA method.

For TNFα level in LNCAP cell line, it was significantly ($P \leq 0.001$) reduced at all concentrations of leaf extract of *Lantana Camara* when comparison with the control group after incubation period of 24 hours, while for SW480 cell line the level was significantly ($P \leq 0.001$) decrease at (1000) µg /ml only after incubation period of 24 hours. For IL-10, there was no change in its level at all concentrations used, While for SW480 cell line, its level significantly reduced at all concentrations of leaf extract of *Lantana Camara* ($p \leq 0.001$).

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List of abbreviations

Abbreviation	Meaning
µg	**Microgram**
5-FU	**5 fluorouracil**
Abs	**Antibodies**
AP-1	**Activator protein**
APCS	**Antigen presenting cells**
AR	**Androgen receptor**
BCL-2	**B-Cell lymphoma 2**
CAMS	**Cell-adhesion molecules**

CD4+	**Glycoprotein located on the surface of immune cells**
Cox-2	**Cyclooxygenase -2**
EGCE	**Epigallocatechin gallate**
ERK	**Extracellular -signal -regulated kinase**
FTIR	**Fourier transform infrared spectrometer**
HDACs	**Histone deacetylase**
HPLC	**High performance liquid chromatography**
HPTLC	**High performance thin layer chromatography**
iNOS	**Inducible nitric oxide synthase**
Lox-2	**Lipoxygenase -2**
LV	**Leucovorin**
MAPK	**Mitogen _activation protein kinase**
MMP-2	**Matrix metalloproteinase -2**
MMP-9	**Matrix metalloproteinase -9**
MTOR	**Mammalian target of rapamycin**
Nf-kb	**Nuclear factor kappa light chain enhancer of activated B cell**
Nrf2_ARE	**Nuclear factor -Antioxidant response element pathway**
PAMPS	**Pathogen associated molecular patterns**
PARP	**Poly ADP ribose polymerase**
PBS	**Phosphate buffer saline**
PDEF	**Prostate derived Ets factor**
PRS	**Pattern recognition receptors**
RAG	**Recombination activating gene**
ROS	**Reactive oxygen species**
RRS	**Response rates**
TLRS	**Toll like receptors**
TNF	**Tumor necrosis factor**

Chapter One

Introduction

&

Literature Review

1.introduction and literature review

1.1 Introduction:

Plants have always had a great position in any culture. Humans use them for their basic needs: feeding, clothing, sheltering, hunting and nursing. the interest in folk medicine has highly increased. It is a fact that 25% of all medical prescriptions are based on substances derived from plants or plant-derived synthetic analogues (Vitalini *et al*., 2009).

Herbal materials are used as a source of drugs for the treatment of different disorders on beliefs of its less side effect and economic values. Plant contains enormous number of phytoconstituent which is responsible for the different pharmacological activities. In the traditional medicine, we can easily find out various preparations for different disorders such as wounds, inflammation, skin infections, leprosy, diarrhea, etc. (Patel et al., 2016).

Traditional and conventional medicines are mostly based on numerous natural resources, including plants which are being used as a primary source in folkloric medicine. Before the discovery of chemical and synthetic compounds, these plants played a significant role as a traditional medicine for therapeutic various diseases throughout the world. However, with the emergence of modern techniques and equipment, synthesis of new classes of synthetic compounds has attracted attention among researchers during recent years. Some of these compounds were first isolated from the natural sources but further modified to improve their efficacy .(Abdul et al., 2018).

Many cancers arise from sites of infection, chronic irritation and inflammation. It is now becoming clear that the tumor microenvironment is largely composed by inflammatory cells. In addition, tumor cells have co-opted some of the

signaling molecules of the innate immune system, such as selectins, chemokines and their receptors for invasion, migration and metastasis. These insights are fostering new anti-inflammatory therapeutic approaches to cancer development (Coussens *et al* ., 2002).

1.2 Aim of study:

1- Study the cytotoxic effect of *Lantana Camara* leaf extract on normal and cancer cell lines (Vero, LNCAP prostate & SW480 colon cancers).

2- Study the effects of alcoholic extract of *Lantana Camara* leaf on the levels of IL10, TNF α levels.

1.3 Literature review

1.3.1 Description of *Lantana Camara plant*

The Verbenaceae family's native *Lantana Camara*, also known as wild or red sage, is a deciduous shrub that is native to tropical America. It is both a weed and a well-known exotic fruit plant. *Lantana Camara* is a robust shrub with a triangular shape, low, erect, or sub scandent stem, and bristly hairs while it is green. It also has weapons or is covered in small spines. The weed can grow anywhere between 1-3 meters tall and up to 2.5 meters wide. A rich smell emanates from leaves that are acute or subacute, crenate, ovate, scabrid serrate, and rugose above the leaves and stems are covered in coarse trichomes. The weed's petite, colorful, stalked, flat-topped clusters of flowers have a narrow tube and four short, spreading lobes on the corolla (Ntalo *et al* ., 2021).

The four spreading lobes measure 3-6 cm tall, 3-8 cm extended, and green. It features clusters of small blooms. The color is often orange and ranges from white to red. As people age, its color typically changes. Almost all of the time, flowers have yellow throats in the axillary head. The calyx is a 6-7 mm broad corolla tube that is divided into uneven lobes. There are pairs of inflorescences in the axils of the opposing leaves (Mukwevho et al., 2018).

Figure 1.1 of *Lantana Camara* from Al _ Ataba Al_Hussainiya plant nursery

Folk healers in Asia and South America have used lantana species including *Lantana Camara* for centuries to treat various human ailments such as dermatological diseases such as itches, cuts, ulcers, swellings, bilious fever, eczema and gastrointestinal diseases, tetanus, malaria and tumors (Pour *et al* ., 2011).

The *Lantana camera* is a significant medicinal plant with a number of folk and traditional therapeutic applications. It is clear that *Lantana Camara* includes several phytoconstituents, indicating that it may be used for a variety of therapeutic purposes. The plant or some portions of it can be used to treat a variety of human conditions, including ulcerative colitis, analgesic, anti-inflammatory, antimicrobial, anthelmintic, cancer, antifungal, and wound healing (Bairagi *et a*l., 2017).

Lantana oil is occasionally used to relieve skin itchiness, as an antiseptic for wounds, and topically for scabies and leprosy. Future research on this plant has

a ton of potential. The medicinal characteristics of *Lantana Camara have* been the subject of ethnomedical and scientific studies, identifying it as a useful plant and potential drug development candidate. Additional research on *Lantana Camara* plants' active chemicals can be done using a variety of investigative techniques, including HPLC, HPTLC, FTIR, NMR, and ultrasound Spectro photo -meter research (Ved *et al* ., 2018).

Lantana Camara is a plant from the family- Verbenaceae. The plant has various traditional uses. The plant containing many more phytoconstituents like alkaloids, glycosides, saponins, steroids, terpenoids, carbohydrates, flavonoids, coumarins, etc. It has various pharmacological activities like antioxidant, antimicrobial, antibacterial, antifungal, antiulcerogenic, anthelmintic, antihyperglycemic, anti-inflammatory, analgesic, anticancer, antitubercular, etc (Battase *et al* ., 2021).

1.3.2 Chemical constituents

The plant *L. Camara* was found to contain a variety of compounds, including alkaloids, glycosides, steroids, saponins, flavonoids, coumarins, tannins, carbohydrates, hydroxy anthraquinones, anthraquinone glycosides, proteins, phytosteroids, fixed oils, fats, and triterpenoids, according to a phytochemical analysis of the plant's leaves (Ganatra *et al.*, 2016).

An idea of similar carbohydrate and lipid contents was obtained from chemical analysis of the leaf and flower extracts. While the lipids were higher in the leaf extracts, the flowers had more carbs (Ganjewala *et al*., 2009).

Quantitative phytochemical screening of *Lantana Camara* by Asadu et al for example, flavonoids (11.08 0.05 mg/g), tannins (9.0 0.0 3 mg/g), alkaloids (9.76 0.0 2 mg/g), saponin (6.07 0.0 6 mg/g), reducing sugar (4.86 0.05 mg/g), carbohydrate (5.08 0.0 3 mg/g), vitamin A (0.50 mg/100 g), vitamins C (6.5

mg/100 g), and vitamin E (1.6 mg/100 g) are all present in (2.36 gallic acid equivalent) *(Asadu et al*., 2015). When *Lantana Camara* essential oil from Algeria was analyzed, it contained significant levels of sesquiterpene hydrocarbons, primarily -caryophyllene (Zoubiri *et al* ., 2012).

The examination of the hexane extract of *Lantana Camara* leaves from the Thanjavur area using gas chromatography-mass spectrometry revealed that the leaves contained eight chemicals, including isoterpinolene, santolina triene, elemene, germacrene, cadinene, bicycle, and katonic acid 2 (Mariajancyrani *et al* ., 2014).

In addition, From the aerial portions of *Lantana Camara* , pentacyclic triterpenoids (camaryolic acid, methyl*Camara* late, and camangeloyl acid), -sitosterol 3-O-beta-D-glucopyranoside, octadecanoic acid, docosanoic acid, palmitic acid, oleanolic acid, lantanolic acid, camaric acid, lantadene A (Qamar *et al*., 2005).

1.3.3 Biological effects of known phytochemicals in *Lantana Camara:*

The reported Lantana compounds all have a wide range of biological, therapeutic, and pharmacological properties. Here are a few of these biological uses and effects:

A- Katonic acid:

It is a cytotoxic triterpene that fights cancer in plants (Imran *et al* ., 2020).

B- Betulinic acid:

It demonstrates therapeutic effects on both healthy fibroblasts and breast cancer cells. (Hussein-Al-Ali *et al*., 2014) . It also acts as an antimelanoma agent (Yu *et al*., 2015).

C- Betulonic acid:

The biological activity and water solubility of Betulinic acid's ionic derivatives are both better. It has antiviral properties and combats type-2 herpes simplex virus (Visalli *et al*., 2015).

D- Camaric acid:

It is a nematicidal agent that was isolated from a methanolic extract of lantana (Qamar *et al*., 2005).

E- Lantadene A:

Rehmannic acid is yet another name for it. It is the *Lantana Camara* L. active principle, which performs certain oxygen functions (Imran *et al* ., 2020).

F- Lantoic acid:

It is a pentacyclic triterpenoid derived from Lantana aerial parts and has nematicidal action (Begum *et al*., 2008).

G- Luteolin:

It thwarts the development and dissemination of human lung cancer cells. It has antioxidant, anti-inflammatory, anti-tumor, anti-cancer(Zhao *et al*., 2011). It is a kind of flavonoid that prompts peripheral blood mononuclear cells to respond immunomodulatory (Sternberg *et al.*, 2009). It is a kind of polyphenolic flavone that inhibits cell metastasis and programmed cell death to promote the anticancer impact of cisplatin in drug-resistant ovarian cancer (Wang *et al*., 2018) . On human gastric cancer cell lines, it exerts chemo-stimulating and antiproliferative effects (Wu *et al.*, 2008). By causing specific signaling in adipose tissue macrophages, it also lowers obesity-associated insulin resistance in mice and is important in insulin resistance (Zhang *et al.*, 2016).

H- Martynoside:

These plant phenylpropanoid glycosides have cytotoxic, antimetastatic, and anticancer properties. As a natural selective estrogen receptor modulator, it is also a possibility (Papoutsi *et al.*, 2006) . In accordance with their antioxidant properties, it also assists in delaying skeletal muscle exhaustion (Liao *et al.*, 1999).

I- Oleanolic acid:

It has a variety of pharmacological properties including a naturally occurring pentacyclic triterpene that prevents prostate cancer cells from proliferating and surviving (Li *et al*., 2016) . Additionally, it prevents lung cancer cells from proliferating, demonstrating its antitumor efficacy. (Zhao *et al.*, 2015) . Its mixture with metals like iron or zinc exhibits strong immunomodulatory, anti-inflammatory, and antiasthma tic properties (Jehangir *et al*., 2019).

J- p-Coumaric acid:

It is a particular variety of cinnamic acid with antioxidant properties. Additionally, it can stop bone loss and encourage osteoblastogenesis. *(Yamaguchi et al.*, 2013). Additionally, both in vitro and in vivo, it has an antiproliferative effect on (Sharma *et al*., 2018).

K- p-Hydroxybenzoic acid:

It is a fungicide and has potential biotechnological uses in food, medicine, cosmetics, and other industries (S. Wang *et al.*, 2018).

L- Pomolic acid:

Human platelet aggregation was caused by a competing antagonist of norepinephrine and adenosine triphosphate (Alvarado-Castillo *et al.*, 2012).

M- Salicylic acid:

It is a crucial plant hormone that facilitates the immune response to microbial diseases (D. Kumar *et al* ., 2014).

N- Theviridoside:

It is discovered in the *Lantana Camara* roots, and it is also cytotoxic to human cancer cell lines (Gorantla *et al*., 2014).

O- Ursolic acid:

It is a pentacyclic triterpenoid carboxyl acid derived from plants that protects mice's livers from oxidative stress and vascular endothelial damage (Li *et al.*, 2016). Additionally, it works well as a neuroprotective medication against inflammatory reactions in the cerebral region of the brain. It has the capacity to cause inflammation (Wang *et al*., 2016).

1.3.4 Pharmacological activity of *Lantana Camara*:

1.3.4.1 Anti-inflammatory activity:

Methanolic extracts of the leaves and bark of lantana were screened for anti-inflammatory activity by carrageenan and histamine-induced paw edema models. Methanolic leaf and bark extracts possessed significant anti-inflammatory effects (Nema *et al* ., 2017).

The Rats with carrageenan-induced paw oedema were used to assess the anti-inflammatory effects of petroleum ether, ethanol, acetone, methanol, hydroalcoholic, and aqueous extracts of *Lantana Camara* leaves. The findings showed that treatment with *Lantana Camara* 's aqueous extract (300 mg/kg) caused a slight reduction in paw volume, whereas 500 mg/kg significantly reduced inflammation (BK *et al* ., 2009).

1.3.4.2 Antioxidant activity:

One essential characteristic of all food ingredients is their antioxidant capacity. Estimated in terms of its capacity to scavenge free radicals (Mahdi-Pour et *al.,* 2012*).*

Lantana Camara leaf extracts contain high levels of flavonoids, it is thought that these flavones trigger cellular antioxidant defense mechanisms that aid in scavenging reactive oxygen molecules. According to reports, phenolic components including Caffeic acid and Rosmarinus acid are abundant in the methanolic extract of lantana, which adds to the plant's antioxidant properties. In addition to free radical scavenging activity, Lantana leaf extract showed lipid peroxidation in a test tube. As a result, it shields against conditions that affect humans, including cancer, rheumatoid arthritis, and cardiac infection (Kumar *et al.*, 2014) .

The primary phenolic chemical found in Lantana species, especially Lantana montevidensis, is Verbascoside, which prevents the oxidative damage-related disease Konzo (Kotz *et al.*, 2017).

At a dosage of 0.2-0.8 mg/ml, *Lantana Camara* leaf extract exhibited strong hydroxyl radical scavenging capabilities (45-73%). The capacity to reduce was concentration-dependent in leaf extracts. At 0.8 mg/ml, it caused the greatest reducing power (Naz *et a*l ., 2013).

1.3.4.3 Antimicrobial activity:

Flavones, a compound found in the leaves of some lantana species, have antifungal and antibacterial activities (Bokhari *et al* ., 2009). Additionally, it has been demonstrated that these plants' extracts can treat gastrointestinal diseases. Lantana has also been shown to have antifungal properties against specific fungi.

The primary elements of Lantana species' essential oils include E-nerolidol, phytol, and E-caryophyllene, which offer resistance against a variety of fungi infections (Passos *et al*., 2012).

1.3.4.4 Anticancer:

Terpenoids, primarily triterpenes and sesquiterpenes, found in Lantana leaf extract have been shown to have anticancer potential in animal models and are poisonous to tumor cells (Silva *et al.*, 2005). Lantana is rumored to contain anthraquinones. As an anticancer agent, anthraquinones are widely employed in the pharmaceutical industry. This shows a possible use for lantana as an anti-cancerous agent. It has been suggested that this plant's phenolic chemicals aid in the prevention of cancer. It has also been demonstrated to prevent cancer cells from proliferating (Bisi -Johnson *et al.*, 2011). Low quantities of *Lantana Camara* have cytotoxic effects on cancer cell lines, demonstrating the plant's potential for application in the treatment of cancer (Passos *et al.*, 2012) .

1.3.4.5 Antidiabetic activity:

It has been demonstrated that the saponins in lantana extract can lower cholesterol and blood sugar levels (Sen *et al* ., 2010).

1.3.4.6 Antipyretic activity:

Lantana plant ethanolic extract has been found to have antipyretic properties. It decreases body temperature by preventing the production of prostaglandins (Ved *et al* ., 2018).

1.3.4.7. Antispasmodic activity:

Lantana contains oleanolic acid, which has been extracted and utilized as an antispasmodic by Asian traditional medicine practitioners (Rahmatullah *et al*., 2011).

1.3.4.8 Antidiarrheal activity:

Diarrhea has traditionally been treated with the stem of the *Lantana Camara* plant. Scientific research demonstrated that taking *Lantana Camara* reduces feces weight and frequency by a significant amount. This effect may attribute to the antibacterial activity against the causative bacteria. On the other hand, Additionally, the flammable and laxative qualities of lantana have been demonstrated (Tadesse *et al*., 2017).

1.4 Cancer:

Cancers resemble group of diseases that occur as a result of uncontrolled growth and the spread of abnormal cells. The spread of cancer cells to other parts of the body is called metastasis, which may lead eventually to death. Many factors can cause cancer including external factors such as tobacco smoking, radiation chemicals, and infections), while internal factors include inherited mutations, hormones, immune conditions, and random mutations (Mathur et al., 2015).

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

1.4.1 Cancer epidemiology:

According to the International Agency for Research on Cancer, an estimated 19.3 million new cancer cases (18.1 million excluding nonmelanoma skin cancer)

and almost 10.0 million cancer deaths (9.9 million excluding nonmelanoma skin cancer) occurred worldwide in 2020. Female breast cancer has surpassed lung cancer as the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%), followed by lung (11.4%), colorectal (10.0 %), prostate (7.3%), and stomach (5.6%) cancers. Lung cancer remained the leading cause of cancer death, with an estimated 1.8 million deaths (18%), followed by colorectal (9.4%), liver (8.3%), stomach (7.7%), and female breast (6.9%) cancers (Sung *et al.*, 2021).

1.4.2 Cancer pathogenesis:

Cancer is a genetic disorder, any alterations that result in dysregulating gene structure and function can cause cancer. The damage to the cellular genetic material alteration in the expression of genes is a common feature for virtually all neoplasms. When there is an inherited error in DNA replication, the cells of multicellular organisms face the same certainty of developing neoplasms if they survive long enough. Many mutations may be inconsequential, but cancer can develop when non-lethal mutations occur in a small subset of the coding and noncoding regions of the genome (Cullen *et al* ., 2016).

The ordinary natural physiological tissue repair mechanisms may become pathophysiological when microdamage occurs due to exogenous factors including chemical, physical and biological carcinogens. as well as the presence of imbalances in the autonomic nervous system stimulation. sympathetic/hyper sympathetic dominance may cause tissue ischemia and consequently hypoxia. As a result, tissue micro damage can occur resulting in chronic inflammation with the possible formation of cancer cells that could be permanent (Bukhtoyarov *et al* ., 2015).

Major advances in our understanding of cancer pathogenesis and therapy have come from efforts to catalog genomic alterations in cancer. A growing number of large-scale genomic studies have uncovered mutations that drive cancer by perturbing transcriptional and post-transcriptional regulation of gene expression. These include alterations that affect each phase of RNA processing, including splicing, transport, editing, and decay of messenger RNA. The discovery of these events illuminates a number of novel therapeutic vulnerabilities generated by aberrant RNA processing in cancer, several of which have progressed to clinical development (Obeng *et al.*, 2019)

1.4.3 Prostate cancer

In prostate cancer, transformed changes in the prostate follow several pathways, beginning with prostatic intraepithelial neoplasia with minimal prostate cancer and then prostatic adenocarcinoma with localized invasion and metastatic prostate cancer (Tika *et al*., 2019).

Prostate cancer (PCa) is the second most frequent malignancy in men worldwide, in 2018 it is estimated that 1,276,106 cases of newly diagnosed prostate cancer (7.1% of whole cancer in men) and result in 358,989 deaths (3.8% of all cancer death in men) (Bray *et al.*, 2018).

The incidence rate of prostate cancer differs from region to region in each population, so there is a different age-standardized rate in many countries. Globally, the incidence and mortality of prostate cancer increase with the increase in age. Only one case of PCa in 350 men below the age of 50 years has been diagnosed, but the incidence value increases up to one case of PCa for every 52 men with ages 50to 59 years. The incidence rate is about 60% in men older than 65 years (Seer *et al.*, 2015).

1.4.3.1 Pathogenesis of prostate cancer:

The prostate gland requires androgen to function optimally, this is why hormonal therapy (testosterone deprivation) is so effective except for castrate-resistant tumors which can generate intracellular androgens (Alukal *et al.*, 2016).

PCa is mostly present in the peripheral zone which is primarily that portion of the prostate that can be palpated via digital rectal examination (DRE), PCa is an adenocarcinoma as it develops mainly from the glandular part of the organ and shows typical glandular patterns on microscopic examination, such a tumor may grow outside the prostate (extracapsular extension) or may remain localized within the prostate for decades, PCa commonly metastasizes to the bones and lymph nodes. Metastases to bone are thought to be at least partially a result of the prostatic venous plexus draining into the vertebral veins (Alonso *et al* ., 2016).

1.4.3.2 Treatment of prostate cancer:

Some generally safe cases would now be able to be followed with active surveillance. In active surveillance, patients are typically required to have a standard, intermittent PSA testing, and in any event one extra biopsy 12 to 18 months after the first determination (Filson *et al* ., 2018).

A. Surgery

A surgeon may carry out a radical prostatectomy to remove the tumor in addition to removing the prostate ,the procedure may also involve the removal of the surrounding tissue seminal vesicles ,and nearby lymph nodes , a doctor can perform this procedure using either open ,laparoscopic or robot -assisted laparoscopic surgery (Boni *et al.*, 2020).

B. Hormonal Therapy:

Approximately 80–90% of PCa are dependent on androgen at the initial diagnosis, and endocrine therapy of PCa is directed toward the reduction of serum androgens and blockage of androgen receptor (AR) (Heinlein *et al* ., 2004). Hormonal therapy (testosterone deprivation) is so effective except that castrate-resistant tumors can generate intracellular androgens resulting in decreased effectiveness (Alukal *et al* ., 2016).

Beginning treatment with leuprolide, goserelini and comparable luteinizing hormone-releasing hormone (LHRH) agonists have to be proceeded by anti-androgen therapy like bicalutamide in cases where PSAi level is greater than 10 ng/ml to avoid any clinical response to initial testosterone surge which occurs at initial hormonal therapy. LHRH antagonist such as Degarelix does not result in testosterone surge (Crawford *et al*., 2019).

Abiraterone acetate is an enzyme inhibitor, irreversibly bind to CYP17 (17α-hydroxylase and 17,20- lyase), an essential enzymes for androgen biosynthesis, it results in blockage of androgen production in the testis and adrenal gland, then prevents the growth of PCa (Scott *et al*., 2017).

Prednisone was used for handling the possible side effects which result from increased mineralocorticoid levels after the inhibition of CYP17A1, The Food and Drug Administration (FDA) first authorized abiraterone acetate for late-stage Castration-Resistant Prostate Cancer (CRPC) patients who received docetaxel in 2011 (Alex *et al.*, 2016) and before docetaxel therapy in 2012 (Ryan *et al.*, 2013).

Enzalutamide is an oral second-generation, it is competitive AR antagonist, approved by the FDA in 2012 and 2014 for the treatment of metastatic CRPC (mCRPC) after docetaxel therapy, respectively. (Poon *et al*., 2018) Enzalutamide strongly improves metastasis-free survival (MFS) in mCRPC cases, fatigue is one

of the most side effects occurring during treatment with Enzalutamide due to its ability to pass through the blood-brain barrier (BBB) (Moreira *et al.*, 2017).

Darolutamide is another oral competitive AR antagonist of the second generation with a unique structure, it does not cross BBB (Fizazi *et al.*, 2015). It strongly binds to Ligand Binding Domain of AR (AR LBD), causing a potent anticancer effectiveness in the preclinical trial and distinct action against AR mutations shown in patients with resistant PCa (Borgmann *et al*., 2018).

C. Chemotherapy

Androgen deprivation therapy (ADT) has been a conventional treatment of newly diagnosed metastatic PCa for more than 70 years, however, all patients eventually become castration-resistant, the hypothesis that a significant level of resistance to ADT already exists in newly diagnosed metastatic PCa and ADT exhibits synergistic antitumor activity with toxins (Iczkowski *et al.*, 2021).

Every three weeks, Docetaxel and Cabazitaxel are intravenously delivered to prevent tubulin depolymerization and subsequent mitotic cell division, which results in cell death. (Martin *et a*l ., 2015). Docetaxel and cabazitaxel, respectively, were approved in 2004 and 2010 for the treatment of mCRPC (Fernández *et al*., 2014), (Francini *et al* ., 2016).

Mitoxantrone is an inhibitor of the topoisomerase enzyme, in comparison to docetaxel, it has limited survival benefit in PCa but with some symptomatic relief (Summers *et al.*, 2017) Mitoxantrone in combination with cabazitaxel, it offers a durable efficacy in mCRPC patients, but side effects limit its use *(Nevedomskaya et al*., 2018).

1.4.4 Colorectal cancer:

Colorectal cancer is the fourth most commonly diagnosed cancer. Colorectal cancer consists of four stages depending on the location of the tumor. In stage 0, the cancer is found only in the inner lining of the colon or rectum. In stage I, the tumor has grown into the inner wall of the colon or rectum. In stage II, the tumor extends more deeply through the wall of the colon or rectum. It may have invaded nearby tissue but does not extend to the lymph nodes. In stage III, cancer has spread to nearby lymph nodes, but not to other parts of the body. In stage IV, cancer has spread to other parts of the body, such as the liver (Li *et al.*, 2016).

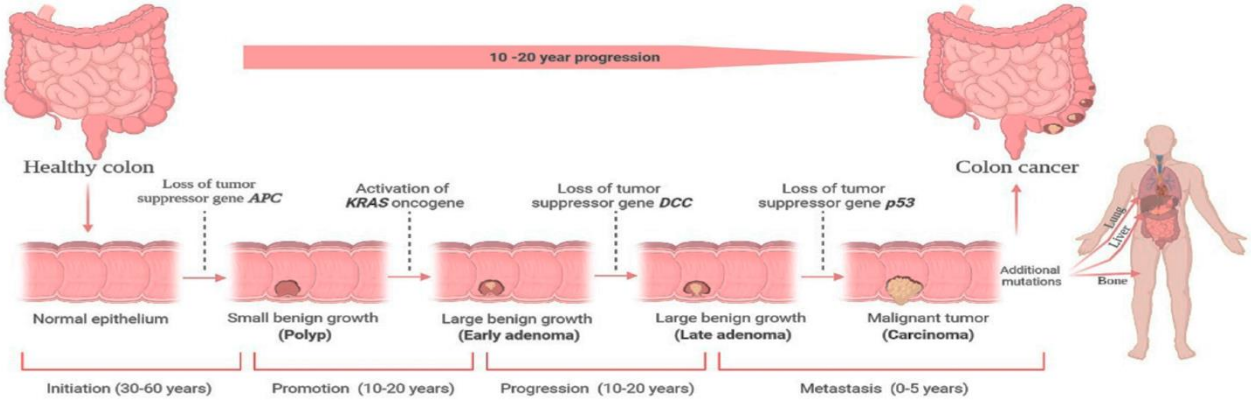

Figure 1.2 Colorectal cancer (CRC) stages and development (Hossain et al., 2022)

1.4.4.1 Epidemiology of Colon cancer

According to GLOBOCAN data, in 2020, there were an estimated 19.3 million new cases and 10 million cancer deaths worldwide, of which CRC contributed about 1.93 million (10 %) further incidences and 0.94 million (9.4%) deaths. Between nations and global regions, there are significant differences in the incidence and mortality of CRC. They are connected to the nation's socioeconomic position as well. The World Bank reports that new cases and

deaths are more notable in higher-income areas and less noticeable in lower-income places. Despite being much higher in high-income nations, the incidence and mortality of CRC are rising in developing countries for both men and women. The highest occurrences (45.94%) and deaths (49.37%) were found in upper-middle-income nations. Compared to upper middle-income nations, higher-income countries recorded fewer instances (42.43%), but mortality were much lower (36.40%), possibly as a result of greater medical facilities. More than 88 and 85% of incidence and mortality, respectively, were covered by the high-income and upper-middle income countries (Hossain *et al*., 2022).

1.4.4.2 Treatment of colon cancer:

A. Chemotherapy:

The backbone of first-line palliative chemotherapy alone is composed of intravenous fluoropyrimidines (FP) and 5-fluorouracil (5-FU) or capecitabine an oral FP in various combinations and schedules (Vodenkova *et al*., 2020**).**
Intravenous 5-FU/leucovorin regimens are less toxic than bolus regimens and have to be preferably used. The oral FP capecitabine is alternative to I.V. 5-FU/LV (Affleck *et al* ., 2022).

Combined chemotherapy of f-FU/LV/oxaliplatin (FOLFOX) or 5-FU/LV/irinotecan (FOLFIRI) provides higher response rates (RRs), longer progression-free interval (PFS) and better survival than 5-FU/LV alone (Ullah *et al*., 2022).

A Triplet combination of chemotherapeutic treatment with 5-FU, oxaliplatin and irinotecan (FOLFOXIRI**)** is interesting, where an Italian randomized phase III study showed a better outcome for patients treated with FOLFOXIRI compared to FOLFIRI (Folprecht *et al.*, 2022).

The 5-FU is converted intracellularly to several active metabolites including Fluorodeoxyuridine monophosphate (FdUMP), Fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP), these active metabolites disrupt RNA synthesis and the action of thymidylate synthetase (TS). The rate-limiting enzyme in 5-FU catabolism is dihydropyridine dehydrogenase (DPD), which converts 5-FU to Dihydrofluorouracil (DHFU). More than 80% of administered 5-FU is normally catabolized primarily in the liver, where DPD is abundantly expressed (Vodenkova *et al*., 2020).

B. Biologic therapy:

Monoclonal antibodies (bevacizumab) or proteins (aflibercept) against vascular endothelial growth factor (VEGF) and cetuximab against the epidermal growth factor receptor (EGFR), in combination with chemotherapy, should be considered in patients with metastatic CRC (mCRC), since they improve the outcome of mCRC. Only trials with a combination of chemotherapeutic agents and a biological targeted treatment consistently reported a median survival exceeding 24 months. The antibody Bevacizumab binds to the circulating VEGF-A, increases the activity of any chemotherapeutic regimen. Bevacizumab has been shown to increase the survival, PFS and RR in first-line treatment in combination with i5-FU/LV/ irinotecan and in combination with 5-FU/LV or capecitabine alone (Tebbutt *et al.*, 2010).

Aflibercept, a recombinant fusion protein, that blocks the activity of VEGF-A, VEGF-Bi and placenta growth factor, improves survival, PFS and RR when combined with first-line therapy (FOLFIRI) (Eric Van Cutsem et al., 2012).Cetuximab is anti-EGFR monoclonal antibody and is available for the treatment of patients with mCRC, Where EGFR is expressed in approximately 85% of patients with mCRC, the clinical efficacy of treatment with anti-EGFR

antibodies is limited to a subset of patients due to the resistance to anti-EGFR monoclonal antibodies (Normanno *et al*., 2009).

1.5 Immune system:

Innate immunity and adaptive immunity are the two layers of defense provided by the immune system, in addition to the chemical and structural barriers that shield us from harm and keep out invaders and pathogens. The first immunological defense mechanism against an invading disease, innate immunity is non-specific (antigen-independent). It is a quick immunological reaction with no immunologic memory that takes place minutes or hours after aggression. Contrarily, adaptive immunity is antigen-dependent and antigen-specific; it has the ability to form memories, allowing the host to generate a more effective immune response when exposed to the antigen again. The innate immune system and the adaptive immune system work in close harmony, and deficiencies in either system can result in illness or disease, such as immune system problems, autoimmune illnesses, and hypersensitivity reactions (Marshall *et al*., 2018).

1.5.1 Types of immunity:

The immune system consists of two components including both innate and adaptive arms of the immune responses which can be activated, suppressed or modulated by substance, biological or synthetic, this is defined as immunomodulation (Kumar *et al*., 2018).

1.5.1.1 Innate immunity:

The innate immune system is an evolutionarily conserved host defense system with key features being shared between plants, invertebrates, and mammals, which has been developed and evolved to protect the host from the surrounding environments. The innate or nonspecific immunity, which consists of the activation and participation of preexistent mechanisms including the

natural barriers (skin and mucosa) and secretions; when a particular pathogen is newly introduced to the host, it is initially recognized by the host innate immune system and then the adaptive immune response is activated. Innate immunity is the host's first line defense that intended to prevent infection and attack the invading pathogens. This type of immunological response typically has a broad scope and acts quickly (minutes to hours) while the adaptive response takes longer ranging from days to weeks (Gasteiger *et al*., 2017).

The innate immunity response to pathogens depends on specific receptors called pattern recognition receptors (PRRs) which allow a limited range of immune cells to detect and respond rapidly to a wide range of pathogens with the same and common structures, known as pathogen associated molecular patterns (PAMPs) (Marshall *et al*., 2018).

This system consists of a variety of components including the physical barriers such as skin, mucosal and epithelial membranes, mucus, 19 anatomical barrier, lysozymes, phagocytes and its subsets (neutrophils, monocytes and macrophage), inflammatory proteins such as C-reactive protein, complement, anti-microbial granule peptides such as defensins and cell receptors that senses micro-organisms and signal the defense mechanism such as Toll like receptors (TLRs) and cells that secrete cytokines and inflammatory mediators (macrophage, mast cells, natural killer cells). When invader micro-organism enters the body, the immune identifies the invader and initiate a signaling cascade which enhance the immune response and activate specific mechanisms, this natural immune response is designed to fight and prevent infections, eliminate the invader micro-organisms and triggers the immune response, This form of immunity includes myeloid and lymphoid cell types; unlike adaptive immunity, these cells are generated by the hematopoietic system but lack antigen receptors and immunological memory (Gasteiger *et al*., 2017).

1.5.1.2 The adaptive Immunity:

Vertebrates have developed an additional protective mechanism including lymphocytes that respond to specific antigens using receptors generated from rearranged gene segments. These cells provide an immunologic memory, allowing to robust immunity when the same pathogen re-encounter the immune system and provide an enormous repertoire of antigen specific immune responses, these are collectively known as adaptive or specific immune response (LaRosa *et al* ., 2008).

Adaptive immune recognition is mediated by T-cell receptors and B-cell receptors, two different categories of antigen receptor. The genes encoding antigen receptors are assembled from variable and constant fragments through recombination-activating gene (RAG)-protein-mediated somatic recombination, a process that yields a diverse repertoire of receptors (Medzhitov *et al* ., 2007).

Antibodies, B lymphocytes, T lymphocytes and antigen- presenting cells (APCs) are the primary effectors of the adaptive immune system. T and B lymphocytes express surface antigen receptors that are specific through consequence of receptor -gene rearrangements. Expansion and wide spread clones of lymphocytes that are specific for particular antigen is stimulated by antigen that encounter and resultant activation and proliferation, this collectively constitute the basis of immunological memory (Nikbakht *et al*., 2019).

1.5.2 Cytokines

Cytokines are regulatory proteins, that are secreted by various cells, which control immune response, hematopoiesis, inflammation, wound repair and tissue morphogenic. Cytokines may be secreted, or membrane-bound. The secreted cytokines may act at the same location in which they are produced as autocrine

or paracrine, or over some distance as would a hormone. The cytokines that bound to the cell membrane act by cell-to-cell contact, allowing the transfer of information from one side to another and often bidirectionally(Morris *et al.*, 2022).

Generally, cytokines involve several types according to the cells that secrete them and their function such as lymphokines which are secreted by lymphocytes, monokines which are secreted by monocytes, and chemokines which possess chemotactic ability and interleukins in which they are produced by one leukocyte and act on other leukocytes. Generally autocrine refers to the cytokines that act on the cell that secreted them, paracrine is referred to the cytokines acting on the nearby cells, while endocrine refers to cytokines that act on distant cells. There are both proinflammatory cytokines and anti-inflammatory cytokines (Karki *et al*., 2021).

Cytokines considered to be the corner stone in the regulation of inflammation by participating in both acute and chronic inflammation by sophisticated network of interactions and communications and can be simply classified according to the nature of the immune response they elicit depending on their cell type and location. The major proinflammatory cytokines include interleukin-1, interleukin-6 and tumor necrosis factor (TNFα) in which they signals through type-1 cytokine receptor which is different from other cytokine receptors, on the other hand the critical pro-inflammatory chemokines such as interleukin-8 signals through G protein couple receptors (Turner *et al*., 2014).

Measurements of cytokines are considered complex and problematic and this can be done by different techniques such as reverse transcription PCR technique, in situ hybridization, enzyme-linked immunosorbent assays which are mostly used for measuring cytokines in body fluids, flow cytometry and enzyme-linked immunes (Kouwenhoven *et al*., 2001).

1.5.2.1 Interleukins family:

Interleukins, a family of numerous proteins that functions as an immune regulator in the body, and also involved in autoimmune diseases and are implicated in other conditions as cancer, myocardial infarction, and depression; and have value in the diagnosis and prognosis of many diseases (O'Neill *et al*., 2001). Interleukins are integral part of our immune system. Leukocytes produce interleukins in order to clear infections and foreign bodies to initiate healing process (Salajegheh *et al*., 2016).

1.5.2.1.1 Interleukin 4 (IL-4):

Interleukin-4 is a type of cytokine that functions as a strong regulator of the immune system and is secreted majorly by mast cells, Th2 cells, eosinophils, and basophils (Gadani *et al*., 2012).

1.5.2.1.2 Interleukin 10 (IL-10):

Interleukin-10 is an anti-inflammatory interleukin, in which its production was first determined in Th2 cells and was initially thought to be only produced by immune cells, but later studies demonstrated that IL10 is also produced by non-immune cells. Monocytes, T cells (mostly type 1 T regulatory cells), B cells (primarily B regulatory cells), a tiny portion of NK cells, macrophages, and dendritic cells are among the immune cells involved in secretion (Akdis *et al.*, 2016).

While Non-immune cells that produce IL-10 include keratinocytes, epithelial cells, and tumor cells. IL-10 primarily exerts its anti-inflammatory effect by inhibiting pro-inflammatory cytokines such as IL1, IL-6, IL-12, and TNF as well as chemokines. IL-10 also inhibits antigen presentation by blocking MHC class II expression (Chatterjee *et al.*, 2014).

This cytokine can attenuate the production of inflammatory cytokines. IL-10 is a prominent participant in human inflammatory diseases (Holdsworth *et al* ., 2015).

In allergy and asthma, IL-10 is found to have a great role in controlling the disease severity by inhibiting many effector cells and disease processes, hence, the levels of IL-10 are inversely correlated with the disease incidence, progression and severity. It inhibits the production of Th1 and Th2 cell activation and pro-inflammatory cytokine production, justifying the consequent effects of IL-10 on antigen-presenting cells and its direct action on T cells (Shahbazi *et al*., 2020).

Mast cells can also produce IL-10, which limits the leukocyte infiltration rate, inflammation, and skin disorders, such as contact dermatitis. IL-10 also has a direct effect on antigen-presenting cells function by downregulation and limits the expression of molecular histocompatibility complex class II on the surface of macrophages and monocytes. IL-10 decrease the expression of proinflammatory cytokines, chemokines, and chemokine receptors. The activation of T cells is highly affected by IL-10 through suppression of CD28, CD2. In contrast to the inhibitory effects of IL-10 on T cells, it is also promoting the survival, proliferation, and differentiation of human B cells and increases IgG4 production (Akdis *et al*., 2016).

1.5.2.1.3 Interleukin 17 (IL-17):

Interleukin-17 is the founding member of this structurally distinct cytokine family, IL-17A binds with IL-17F to its receptor, IL-17RA (Akdis *et al.*, 2016).

1.5.2.1.4 tumor necrosis factor:

TNF-alpha was initially identified by its anticancer effect, but it is now understood to be one of the most pleiotropic cytokines, serving as a host defense component in inflammatory and immunologic responses. Among its many distinct functions, TNF has effects on the vascular endothelium that may contribute to tissue harm in illnesses such cerebral malaria or disseminated intravascular coagulation. It may also play a role in hemorrhagic tumor necrosis in animal models. TNF causes endothelial cells to release a number of substances, such as GM-CSF, PAI-1, IL-1, and IL-6 (Ulich *et al*., 2022).

This substance was named "Tumor Necrosis Factor" (TNF-alpha) in 1975. It was discovered that well-known immune stimulants such bacillus Calmette-Guerin (BCG), zymosan, and corynebacterial could also induce this endotoxin-induced factor. In vitro, the separated factor could directly kill tumor cells, but it had no negative effects on non-cancerous murine embryonic cells that were actively multiplying. Cloning of the cDNA and revelation of the molecule were made possible by the molecular analysis was made up of 233 amino acids, with the first 76 amino acids making up the leader sequence. It was interesting to see that the identical sequence belonged to Cachectic, another element linked to cancer. The effects of cachectic on the central nervous system (CNS) and peripheral tissues were first shown to mediate weight loss and change normal metabolic priorities (Josephs *et al*., 2018).

TNF (tumor necrosis factor alpha) is also referred to as differentiation-inducing factor, cachectic, and endotoxin-induced factor in serum. TNF are the most significant cytokines involved in physiological processes, systemic inflammation, tumor lysis, apoptosis. They are members of the tumor necrosis factor (TNF) family. TNFα is mainly produced by macrophages, other cells can also express TNFα at low levels. The human TNFα genes are located on

chromosome 6, TNFα comes in two different forms: membrane-bound (m TNF) and soluble (s TNF). TNFα is produced during synthesis and translocate to the cell membrane, where it is converted into s TNF by the TNF converting enzyme (TACE). TNFβ, in contrast to TNFα, only comes in soluble form (s TNF). All species have the same TNF gene. For instance(Chu *et al*, 2013).

The physician William B. Coley first recognized the idea of an immune system-mediated antitumoral response in vivo over 100 years ago. A soluble cytokine called tumor necrosis factor (TNF), which is produced when the immune system is activated and has been shown to trigger tumor necrosis in some animal model systems, was first discovered about 30 years ago. TNF's structural and functional resemblance to lymphotoxin (LT) was discovered in 1984, and a few years later, two membrane receptors that can bind both cytokines were discovered. The TNF ligand family of big cytokines, of which TNF is the prototypical member, was later discovered TNF is primarily synthesized as a type II transmembrane protein organized in stable homotrimers. The metalloprotease TNF alpha converting enzyme releases the soluble homotrimer cytokine (s TNF) from its membrane-integrated form through proteolytic cleavage (TACE). At doses below the nanomolar range, the soluble 51 k Da trimeric s TNF has a tendency to dissociate and lose its bioactivity. The 17 k Da TNF protomers have a "jelly roll" structure that is distinctive of the TNF ligand family and is also present in viral capsid proteins. It is made up of two antiparallel pleated sheets with antiparallel strands (Wajant *et al*., 2003).

1.6 Cell culture:

Cell culture is the process by which human, animal, or insect cells are grown in a favorable artificial environment. Because many animal cells may be made to

grow outside of their organs or tissue of origin under specific conditions when supplemented with a nutrient-containing and growing media, animal cell culture is currently one of the primary techniques utilized in the biological sciences in areas of inquiry. The culture settings must replicate in vivo parameters with regard to temperature, pH, carbon dioxide (CO2), oxygen (O2), osmolality, and nutrition for cells to grow in vitro (Capes-Davis *et al*., 2010).

1.6.1 Primary Cultures:

Primary cell culture is the term used to describe the cells that are collected directly from the patient's tissues and organs through mechanical, chemical, or enzymatic digestion. Then, using a complicated medium, these cells are stimulated to grow in appropriate glass or plastic containers. The preparation of primary cultures is intensive labor and they can be maintained in vitro only for a limited period. During their relatively limited lifespan, primary cells usually retain many of the differentiated characteristics of the cell in vivo. Primary cultures by definition have not been passaged, as soon as they are passaged, they become a cell line and are no longer primary. The primary cell culture, when sub-cultured, Depending on how long it remains in culture, it develops into a cell line or cell strain that can either be continuous or finite(Walker *et al .,* 2009).

1.6.2 Cell line:

Cell lines are in vitro model frameworks that are generally utilized in various fields of clinical research, particularly fundamental malignant growth research and medication disclosure. Their convenience is basically connected to their capacity to give an inconclusive wellspring of natural material for exploratory purposes. Under the correct conditions and with suitable controls, verified malignancy cell lines hold the greater part of the hereditary properties of the disease of inception *(Mirabelli et al.,* 2019).

Normally, regular cells are divided only for a small number of times before they lose their capacity to proliferate, which is a genetically defined phenomenon known as senescence; these cell lines are considered finite. However, some cell lines become immortal through a mechanism called transformation which may occur naturally or can be caused either chemically or virally. When a finite line or strain of cells goes through a transformation (J. Singh1 *et al*., 2011).

1.6.2.1Vero cell:

Derived from the kidney of an African green monkey (Cercopithecus aethiops) in the 1960s, Vero cells are a common mammalian continuous cell line used in research. Vero cells have been licensed in the United States for production of both live (rotavirus, smallpox) and inactivated (poliovirus) viral vaccines, and throughout the world Vero cells have been used for the production of a number of other viruses, including Rabies virus, Reo virus, and Japanese encephalitis virus. For long-term storage, Vero cells are kept either in liquid nitrogen or at −80◦C. This protocol describes how to start growing vero cells obtained from frozen stock. After recovery from frozen stock, Vero cells usually take 2 to 3 passages to reach their regular growth rate, and this should be taken into account if planning to use the cells for experiments, infections, etc(Ammerman *et al*., 2008).

1.6.2.2 LNCaP prostate cancer cell line:

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

LNCaP cell line requires androgens for its growth, unless these cells have been transformed to androgen-independent clones. Androgen-dependent and androgen-independent LNCaP cell lines have previously been used. (Vaarala et al., 2000).

1.6.2.3 SW480 cell line:

One of the most fatal and prevalent cancerous world-wide is the colorectal cancer, despite the great advance in the medical field and chemotherapeutic agents, the drug resistance remains the unresolved problem in cancer. Therefore, establishing an effective compound with lowest side effects to fight cancer is of central priority. Herbal products have been traditionally used to prevent and treat a variety of diseases (Shanehbandi *et al.*, 2021).

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

1.6.3 Applications of cell culture:

The production of animal cells has been used in numerous areas, from basic to advanced science. It provides a model system for a variety of research efforts including (Hudu *et al*., 2016):

1. Study of basic cell biology, cell cycle mechanisms, and specialized celli function.
2. Testing for toxicity to understand new pharmacological effects.
3. Gene therapy replaces non-functional genes with cells that carry functional genes.
4. Cancer research to characterize cancer cells and investigate how different chemicals, medications, viruses, and radiation affect cancer cells.
5. Manufacturing of hormones, interferons, clotting factors, monoclonal antibodies, and vaccinations.

1.6.4 Advantage of cell culture:

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

1. Control of the environment by altering pH, temperature, O_2/iCO_2 ratio, and osmotic pressure of the culture media to study their effects on the cell culture.
2. provides a useful tool for investigating cell physiology and biochemistry for studying cell metabolism.
3. Investigate the cytotoxic effect of various compounds or drugs on specific types of cells such as liver cells.
4. Enable the study of biology and origin of the cells obtained from homogen cell culture
5. Specific proteins can be synthesizedi in large quantities from genetically modified cells in large-scale cultures.
6. Consistency and reproducibility of the results can be obtainedi by the use of a single clonal population.
7. Specific cell types can be detected by the presence of markers such as molecules or by karyotyping.

1.6.5 Disadvantage of cell culture:

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

1. Cost and knowledge: Cell culture is a specialist process that calls for sterile conditions, skilled labor, and pricey equipment.

2. Dedifferentiation: After a time of continuous cell growth in cultures, cell features are altered, resulting in differentiated qualities compared to the original cell strain.
3. Small quantity of product: The production of monoclonal antibodies and recombinant proteins, along with downstream processing to isolate pure products, significantly raises costs.
4. Contamination: Mycoplasma and viral infections are extremely infectious and difficult to detect.
5. Instability: In continuous cell lines, aneuploid chromosomal makeup causes instability.

Chapter Two

Materials

&

Methods

2. Materials and methods

2.1 Introduction

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

2.2 Materials

2.2.1 Chemicals

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

Table (2.1): Chemicals used in the study

Chemical	Company	Country
Alcohol liquid 99.9% Ethanol solution	France Alcohols	France
Alcohol spray (ethanol 70%)	Aljoud	Iraq
Dimethyl sulfoxide	Sigma Aldrich	Germany
Fetal bovine serum (FBS)	Capricorn	Germany
MTT (3-(4,5- Dimethyl thiazole-2-yl)- 2,5- diphenyl-2H-tetrazolium bromide) dye powder	Roth	Germany
Penicillin- streptomycin solution	Capricorn	Germany
Phosphate buffer saline packets	Bio PLUS 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

2.2.2 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
Cell culture flask (25ml)	SPL	Korea
Cell culture plate (96-wells)	SPL	Korea
Centrifuge	Rotana	Germany
Distiller	ROWA	Germany
Double distillation	GFL	Germany
water stills		
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 air flow	Labtech	Korea
Cabinet		
Magnetic stirrer	Scotech	Germany
Micropipettes (different sizes)	Dragon-Med	India
Millipore filter (0.45,0.22µm)	Biofil	Australia
Neubauer Hemocytometer	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

2.2.3 Assay kit:

The assay kits used in this study include:

Table 2.3 list of ELISA assay kits used in the present study

ELISA kit tumor necrosis factor α	**Elabscience**	**USA**
ELISA kit Interleukin 10	**Elabscience**	**USA**

Kit contents include the following:

Table 2.4 List of contents of the ELISA assay kit

Biotinylated Detection Ab Diluent	**1 vial, 14 mL**
Certificate of Analysis	**1 copy**
Concentrated Biotinylated Detection Ab (100×)	**96T: 1 vial, 120 mL 48T: 1 vial, 60 mL**
Concentrated HRP Conjugate (100×)	**96T: 1 vial, 120 mL 48T: 1 vial, 60 mL**
Concentrated Wash Buffer (25×)	**1 vial, 30 mL**
HRP Conjugate Diluent	**1 vial, 14 mL**
Manual	**1 copy**
Micro ELISA Plate (Dismountable)	**96T: 8 wells ×12 strips 48T: 8 wells ×6 strips**
Plate Sealer	**5 pieces**
Reference Standard	**96T: 2 vials 48T: 1 vial**
Reference Standard & Sample Diluent	**1 vial, 20 mL**
Stop Solution	**1 vial, 10 mL**
Substrate Reagent	**1 vial, 10 mL**

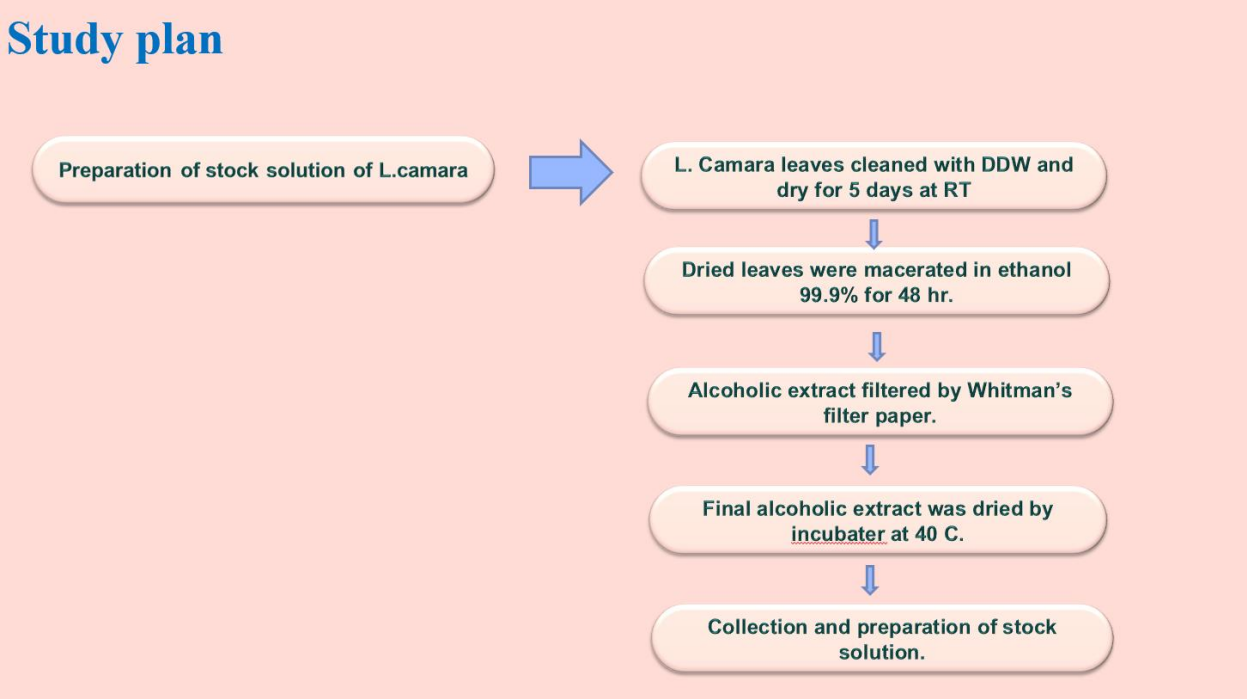

Figure 2.1 Preparation of Stock Solution of *Lantana Camara*

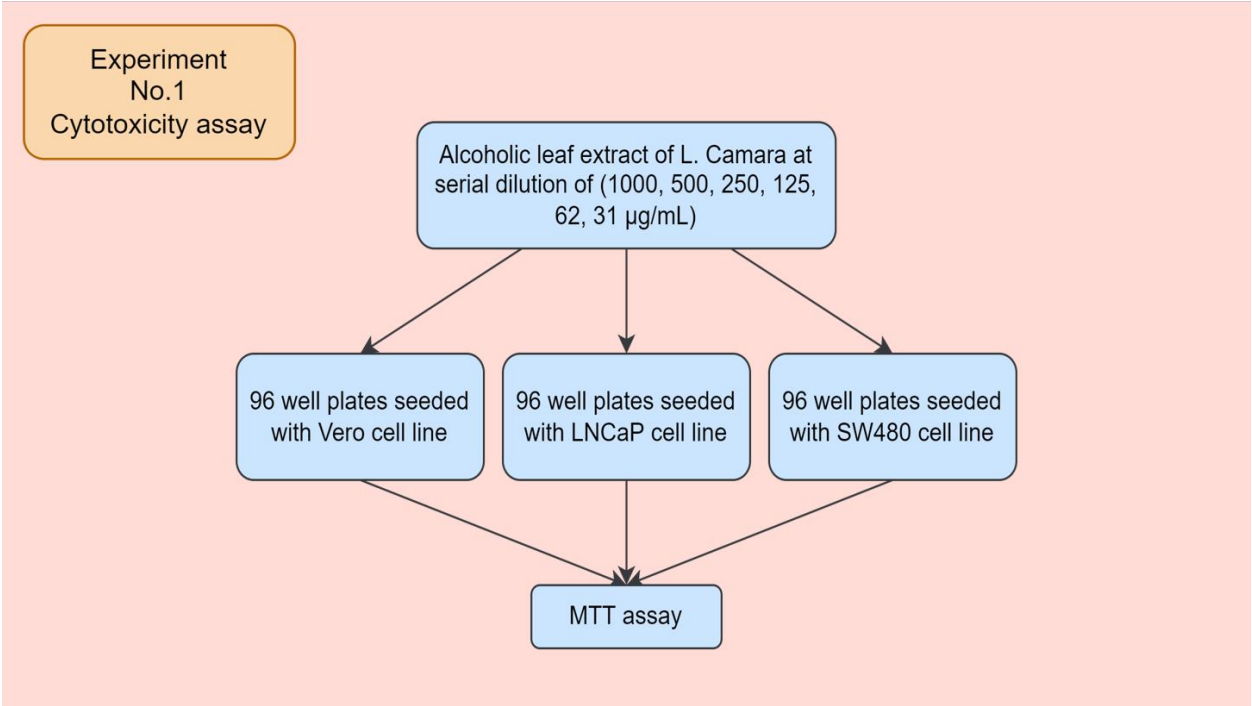

Figure 2.2 Cytotoxicity Assay of *Lantana Camara*

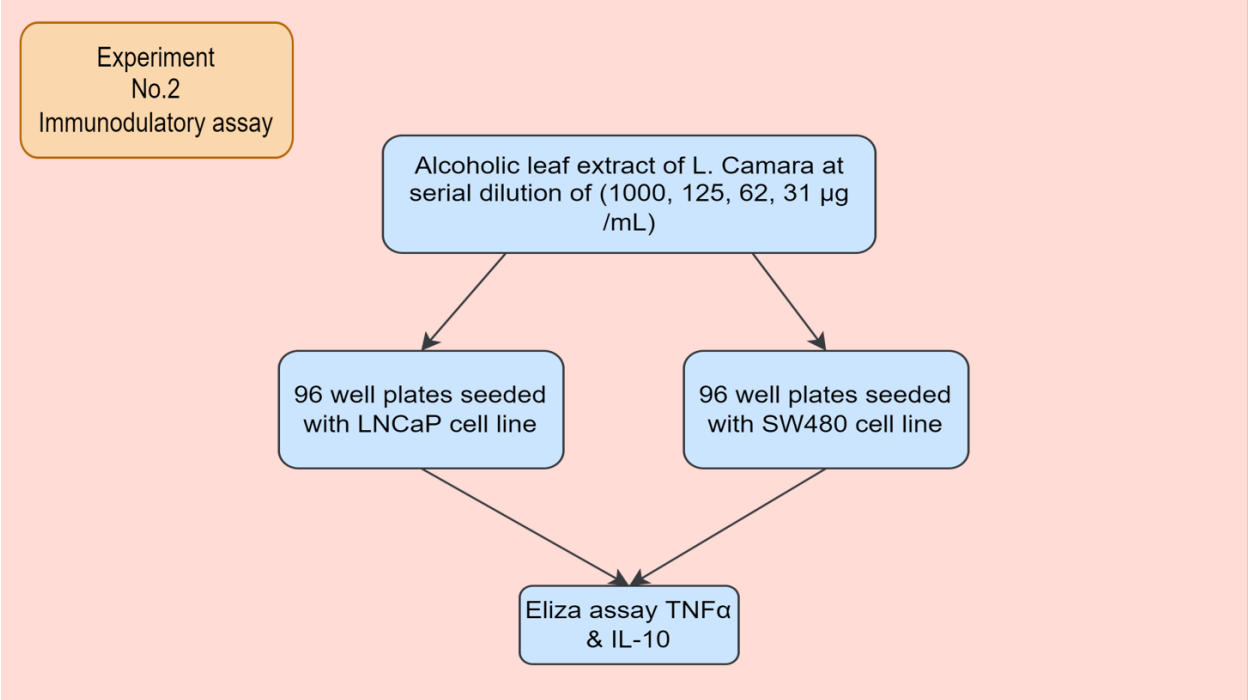

Figure 2.3 immunomodulatory Assay of *Lantana Camara*

2.2.4 Cell lines:

Frozen vials of human prostate cancer LNCaP, SW480 and Vero cell lines were gathered from the College of Medicine/University of Babylon's Tissue Culture lab.

2.3 Methods:

2.3.1 Preparation of Reagents and Solutions

2.3.1.1 Phosphate Buffer Saline (PBS):

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

2.3.1.2 Trypsin-(EDTA) Solution:

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

2.3.1.3 Preparing MTT assay solution:

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

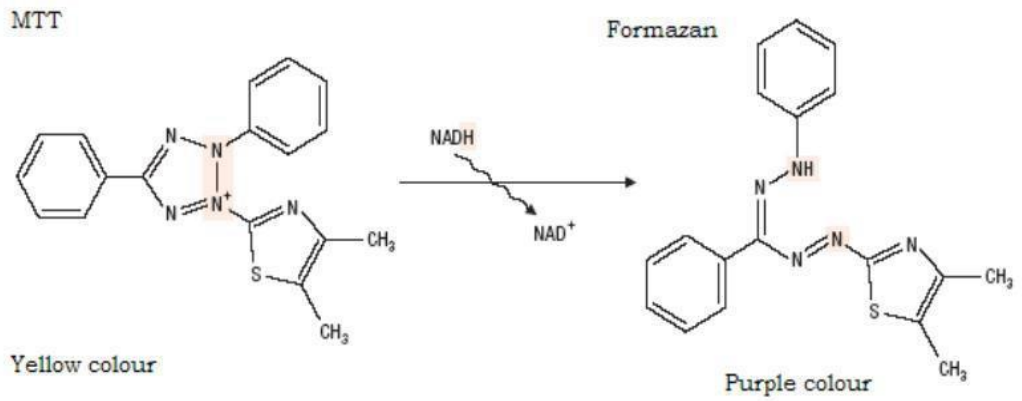

Figure 2.1: Principle of MTT Assay (Sukhramani *et al.*, 2011).

Procedure (Kasper *et al.*, 2011):

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

2- To get a final concentration of 0.5 mg/ml, 1.2 ml of MTT solution (5 mg/ml) was added to 10.8 ml of medium. The resultant solution was then poured 200 µl into each well.

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

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

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

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

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

$$\% \text{ viability} = (AT - AB) / (AC - AB) \times 100\%$$

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

AB = Absorbance of blank (only medium).

AC = Absorbance of control (untreated).

% Inhibition = 100 – % viability.

2.3.2 Preparation of Tissue Culture Medium:

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

Weighing RPMI-1640 powder to obtain 16.353 grams and dissolved in 900ml of ddH2O without heating, 2 g of sodium bicarbonate can beadded if required with gentle stirring to adjust pH additional water was added to obtain 1 liter then filtered using 0.22-micron membrane. Penicillin-Streptomycin at 1% was added and 10% fetal bovine serum also added, then filter-sterilized using 0.22-micron membrane filter. The mixture contains heat labile compounds that can be damaged with autoclaving.

The prepared media should be kept at 4°C and used within a short period of time.

2.3.3 colletion and Preparation of *Lantana Camara* leaf extract:

The plant leaves were carefully cleaned with deionized water, allowed to dry for 5 days at room temperature in a dark spot, then ground into a fine powder and stored in a dry, airtight container for later use.

The plant leaves then soaked and macerated in alcoholic (ethanol 99.9%) in concentration of 100 gm of leaf powder1 liter of Ethanol and left for extraction at room temperature for 48 hours.

Alcoholic extract is separated into two layers, draw the upper layer and filtered using Whitman filter paper NO.1 and then the final alcoholicliquid extract is then placed in oven at 40 c until drying then collectedand stored (Saraf *et al*., 2011) .

Preparation of stock solution of ethanolic extracts of *Lantana Camara by* weighing 10 mg of the dried extract, which is then diluted in 0.5 ml of 70% ethanol and 4.5 ml of serum-free RPMI to produce a final concentration of 2000 µg/ml. The mixture is then filtered through a Millipore filter syringe to remove any contaminants (Aritonang *et al*., 2019) .

2.3.4 Preparation of cell line:

2.3.4.1 Thawing of Vero, LNCaP, and SW480 cell lines

The frozen Cell line vial was carefully taken out of the liquid nitrogen bottle and put into a beaker with pre-warmed (37°C) sterile DDW. Before the ice floccule entirely disintegrated, the vial was taken out of the solution and cleaned with 70% ethanol. The vial's cell suspension was immediately pipetted into a 15 ml sterile plastic centrifuge tube that had 10 ml of pre-warmed serum-free medium in a laminar flow cabinet. The supernatant was aspirated and decanted after 5 minutes of centrifugation at 1000 rpm. In 5ml of warm water, the cell pellet was re-suspended. (37C) serum-medium was prepared, transferred into a 25 ml cell culture flask, and the serum medium was changed the following day. (Phelan *et al*., 2017).

2.3.4.2 Sub-culturing of cell culture:

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

- The growth medium is removed from the flask using a pipette and washthe monolayer with a sufficient volume of PBS to ensure the removal ofall media from the flask.
- an appropriate volume of the trypsin/EDTA solution is added to the flask and incubate at 37 c° to allow the cells to detach from the inside surface of the flask (within 2-10 min).
- The cells were examined using an inverted microscope to ensure that all the cells are detached and in suspension. Gently tap the flask with thepalm of the hand a couple of times to release any remaining detachedcells.
- the trypsin is inactivated by adding an equal volume of serum-containing media to the flask.
- then the cell suspension is divided into two flasks and each new flask are label with cell line name, passage number, and date.
- The Cell line then incubated at 37C° for 24 hr (Dixit *et al.*, 2018).

2.3.4.3 Maintenance of cell culture:

Cells were routinely checked under an inverted microscope for any contamination and the cells were given new medium (RPMI) every 2 to 3 days based on color changes. The cells were maintained in medium supplemented with 10 % serum and kept at 37 ºC in an incubator, After the cells have achieved more than 80 % confluence, they were subculture (Freshney *et al* ., 2002).

2.3.4.4 Harvesting of cultured cells:

Harvesting is a technique that uses proteolytic enzymes to detach adherent cells from the surface of a cell culture flask. First, the growth

medium in the vessel was aspirated and discarded. PBS was used to wash the cells twice. Afterward, the enzymatic harvesting solution was added tothe 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 Ethylenediaminetetraacetic acid (EDTA) (Viazzi *et al.*, 2015) .

2.3.5 Study the cytotoxic effect of alcoholic extracts of *Lantana Camara* leaves on (Vero, LNCaP, and SW480) cell lines.

In 96 tissue culture plates, cell lines such as Vero, prostate cancer (LNCaP), and (SW480) cell lines were sown and marked. Four repetitions of each concentration of ethanolic extract were employed for each kind of cell, and four replicates served as the control group for each cell type. The plant extracts were applied to all of the cells at serial dilutions ranging from 1000 to 31.25 µg/ml. Following a 24-hour incubation period, the plate was covered with a self-plastic lid, and the proliferation of the cell lines was evaluated using an MTT assay to determine cytotoxicity.

2.3.6 Study the immunological effect of alcoholic extract of *Lantana Camara leaves* on LNCAP and SW480 cell line

Cell lines including prostate cancer (LNCaP), and (SW480) cell lines were seeded and labeled in 96 tissue culture plates. all cells were treated with different concentration of *Lantana Camara* at serial dilutions ranged from 1000 to 31.25 µg\ml (four replicates were used for *Lantana Camara* for each type of cells) along with four replicates as a control group for each cell type. Then the plate was covered with a self-plastic lid and after a 24-hour

incubation period following the exposure period, the cell lines were taken for immunoassay by ELISA method using TNF α, IL10 according to the protocol mentioned below:

Test principle:

This ELISA kit uses the sandwich-ELISA principle in which the plateis precoated with an antibody specific to the human cytokine of interest. Samples or standards are added to the plate and combined with the antibody. The biotinylated detection antibody and avidin-HRP conjugate are added tothe plate changing the color to blue. The enzyme-substrate reaction is terminated by addition of stop solution and the color turns yellow.

The optical density is measured at wavelength of 450 nm. The concentration of cytokine of interest is measured by comparing the OD of sample to the standard curve, Assay technique Before use, all samples and reagents are brought to room temperature. After the material has thawed before the test, centrifuge it once more. Before pipetting, all the chemicals should be properly mixed by gently swirling; foaming should be avoided. It is suggested that all samples and standards be analyzed twice (Alhajj *et al.*, 2021).

1. **Addition of Sample**: Each well receives 100 µl of Standard, Blank, or Sample. The Reference Standard and Sample diluent is added to the blank well. The bottom of the micro-ELISA plate is filled with solutions; care is taken to prevent inside wall contacting and foaming. Stir it slowly. Apply the sealant that provided to the plate. At 37 °C, incubate for 90 minutes.

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: As was done in the third step, the wash procedure is repeated five times.

6. Substrate: To each well, 90 µL of the substrate solution are added. Add a fresh Plate sealer on top. At 37°C, incubate for approximately 15 minutes. shield the plate from the sun. Depending on the actual color shift, the reaction time may be cut or prolonged, but not beyond 30 minutes. The user should stop the reaction when an apparent gradient appears in conventional wells.

7. Stop: To each well, 50µL of Stop Solution are put. The color then abruptly changes to yellow. The substrate solution and stop solution should be added in the same order.

8. OD Measurement: A microplate reader tuned to 450 nm is used to determine the optical density (OD value) of each well simultaneously. The user should

preheat the instrument, open the microplate reader in advance, and configure the testing parameters.

9. After experiment, until they expire, all of the unused reagents are returned to the refrigerator at the appropriate storage temperature (Alhajj *et al.*, 2021).

2.3.7 Statistical Analysis:

Sigma plot version 12.5 and Microsoft Office Excel 2016 were used to collect and analyze all the data. To evaluate whether there were any significant variations in the data means, a one-way ANOVA test was utilized. statistical significance was assigned to the p-values ($p \leq 0.001$), ($p \leq 0.05$.

CHAPTER

THREE

RESULTS

3. Results

3.1 Cytotoxicity evaluation of *Lantana Camara* alcoholic leaf extracts on normal Vero cell line:

There was significant (P≤ 0.001) decrement in the viability of Vero cell line when the cells treated with alcoholic extract of *Lantana Camara* leaf with concentrations 1000,500 and 250 µg/ml in comparison with the control group after incubation period of 24 hours (Fig 3.1).

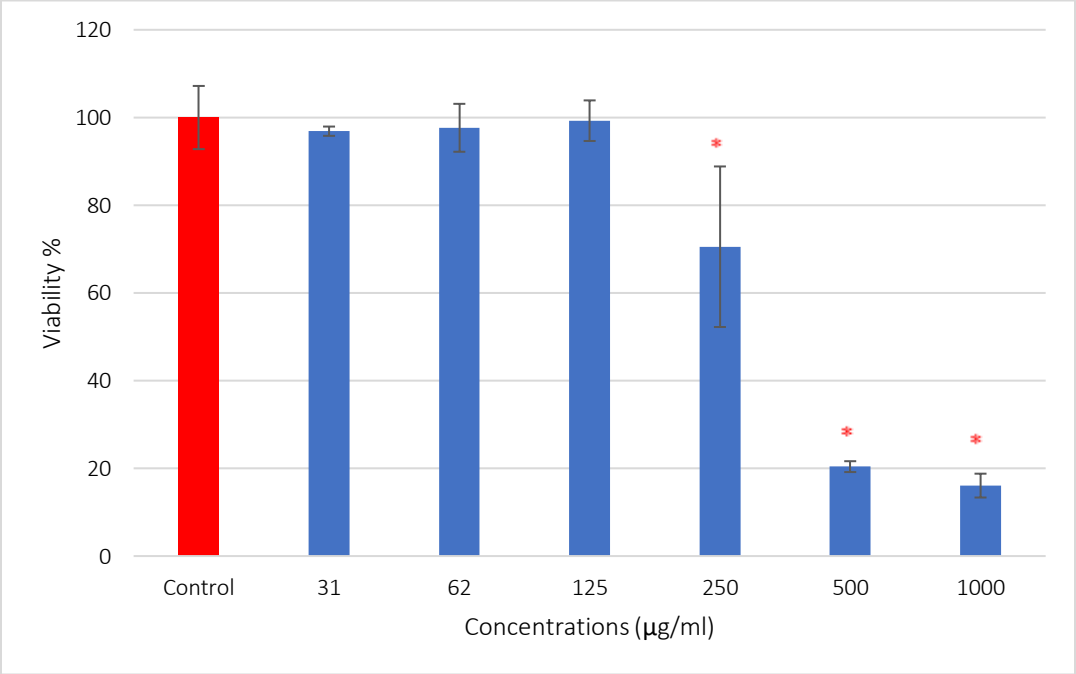

Figure 3.1 Effects of different concentrations of alcoholic leaf extracts of *Lantana Camara on* the viability of Vero cell line by MTT assay.

* **mean significant difference (P≤0.001).**

3.2 Evaluation of *Lantana Camara* alcoholic leaf extracts cytotoxicity on the viability of LNCap cell line:

Regarding the effects of alcoholic extract *of Lantana Camara* leaf on the viability of LNCap cell line, there was a significant (P≤ 0.001) decrement in the viability of those cells at (1000 and 500) µg/ml and significant increase (p≤0.001) at concentration (250 ,125, 62) µg /ml in comparison to the control group, after incubation period of 24 hours.

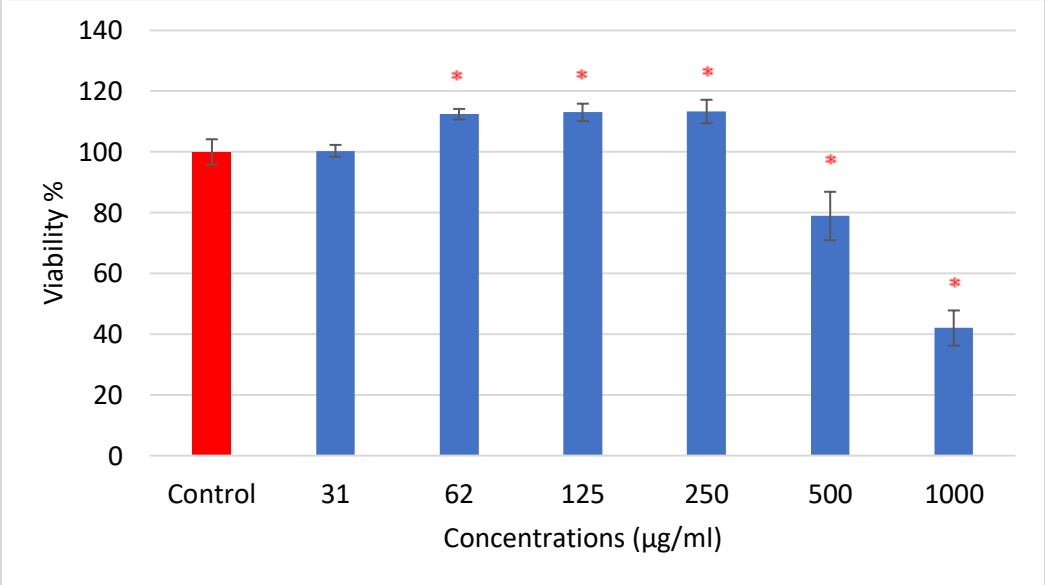

Figure 3.2: Effects of different concentrations of alcoholic extracts of *Lantana Camara* leaf on the viability of LNCap cell line by MTT assay.

* **mean significant difference (P≤0.001) .**

3.3 Evaluation of *Lantana Camara alcoholic* leaf extracts cytotoxicity on the viability SW480 cell line:

There was significant (P≤ 0.001) decrement in the viability of SW480 cell line when alcoholic extract of *Lantana Camara* leaf with concentrations 1000,500 and 250 µg/ml was added, in comparison with the control group after incubation period of 24 hours. (fig3.3).

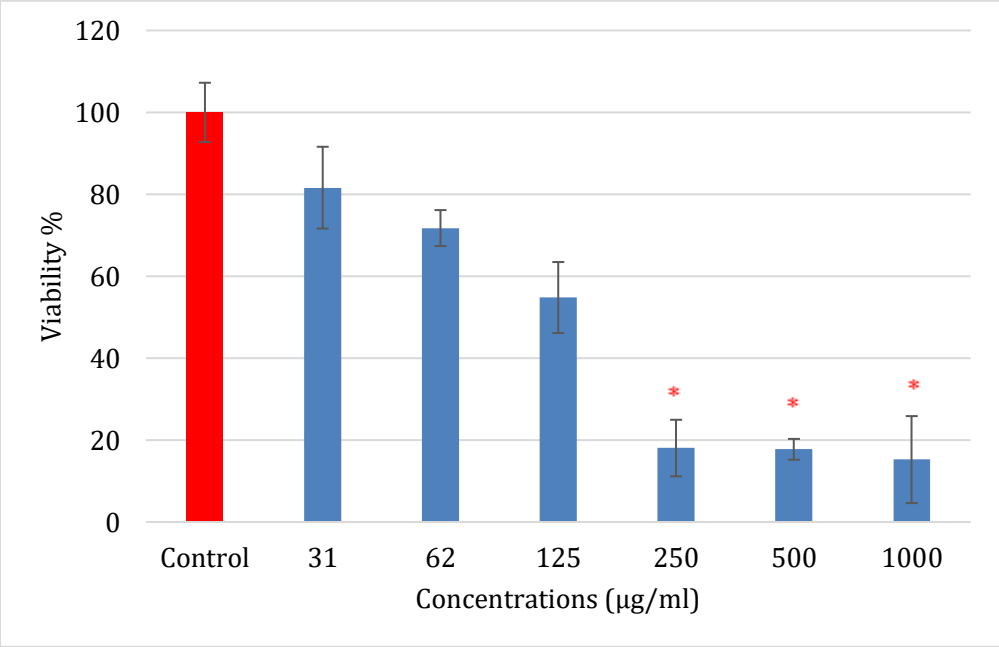

Figure 3.3 Effects of alcoholic leaf extracts of *Lantana Camara* on the viability of SW480 cell line by MTT assay.

* **mean significant difference (P≤0.001).**

3.4 Evaluation of *Lantana Camara alcoholic* leaf extracts effect on the levels of TNFα in LNCAP cell line:

After a 24-hour incubation period, there was a significantly (P≤ 0.001) lower level of TNFα than in the control group at all used concentrations. (fig3.4).

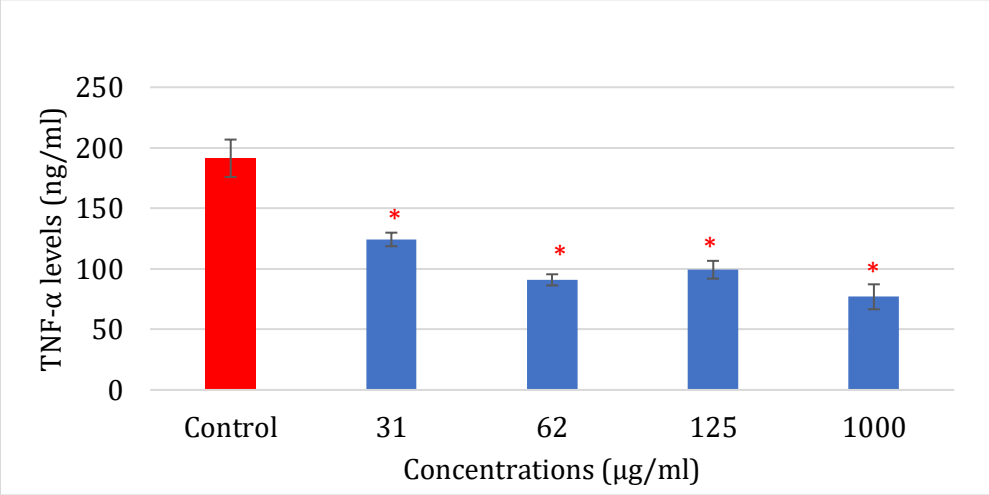

Figure 3.4 Effects of alcoholic leaf extract of *Lantana Camara* on TNFα in LN cell line.

*** mean significant difference (P≤0.001).**

3.5 Evaluation of *Lantana Camara alcoholic* leaf extracts effect on the levels of TNFα in SW480 cell line:

After a 24-hour incubation period, the high concentration of alcoholic leaf extracts (1000 g/ml) caused a considerably (P 0.001) lower level of TNF in SW480 cell line compared to other concentrations and the control group (fig3.5).

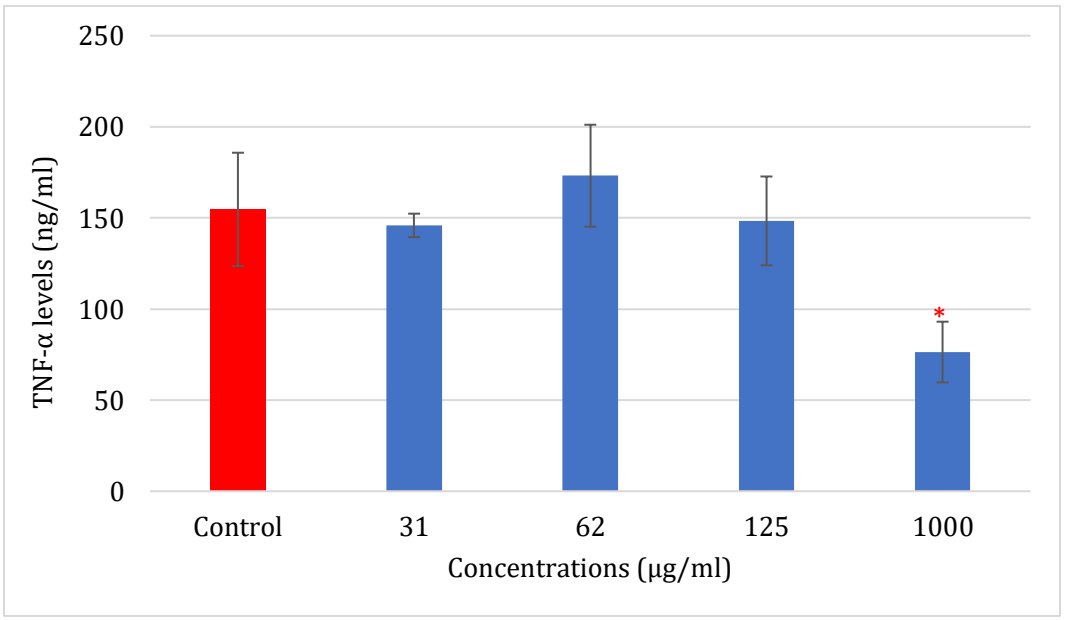

Figure 3.5 Effects of alcoholic leaf extract of *Lantana Camara* on TNFα in SW480 cell line.

*** mean significant difference (P≤0.001).**

3.6 Evaluation of *Lantana Camara* alcoholic leaf extracts effect on the levels IL-10 in LNCAP cell line.

The different in the levels of IL-10 in all concentration of alcoholic leaf extracts in LN cap is not statistically significant, as shown in (fig3.6).

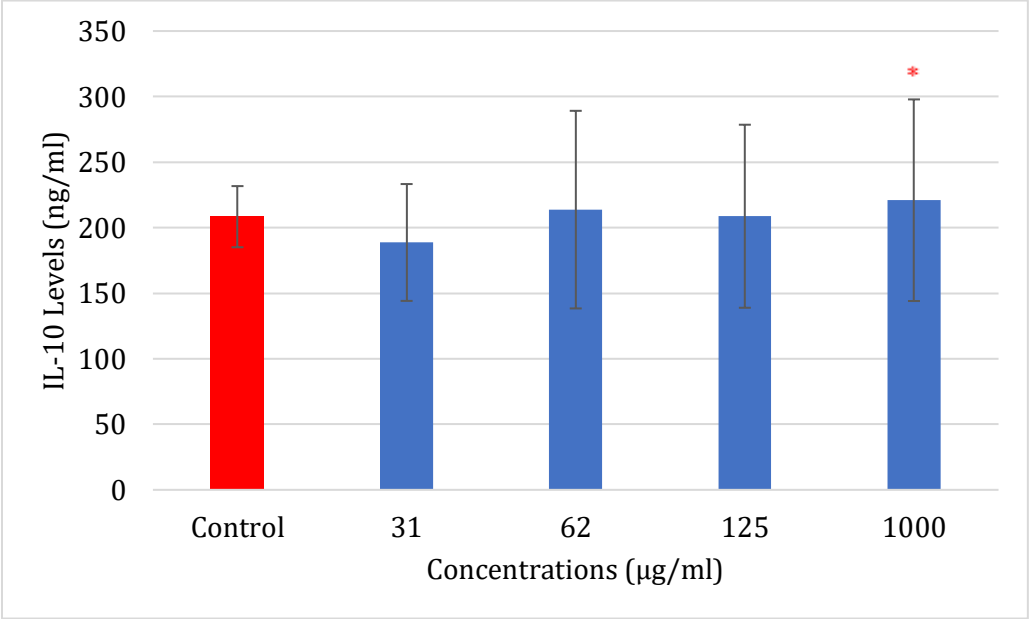

Figure 3.6 Effects of alcoholic leaf extract of *Lantana Camara* on IL_10 in LN cell line.

*** mean significant difference (P≤0.001).**

3.7 Evaluation of *Lantana Camara* alcoholic leaf extracts effect on the levels IL-10 in SW cell line:

After a 24 hours incubation period, there was a significantly (P≤ 0.001) lower level of IL 10 in all concentrations of the *Lantana Camara* alcoholic leaf extracts used compared to the control group (fig3.7)

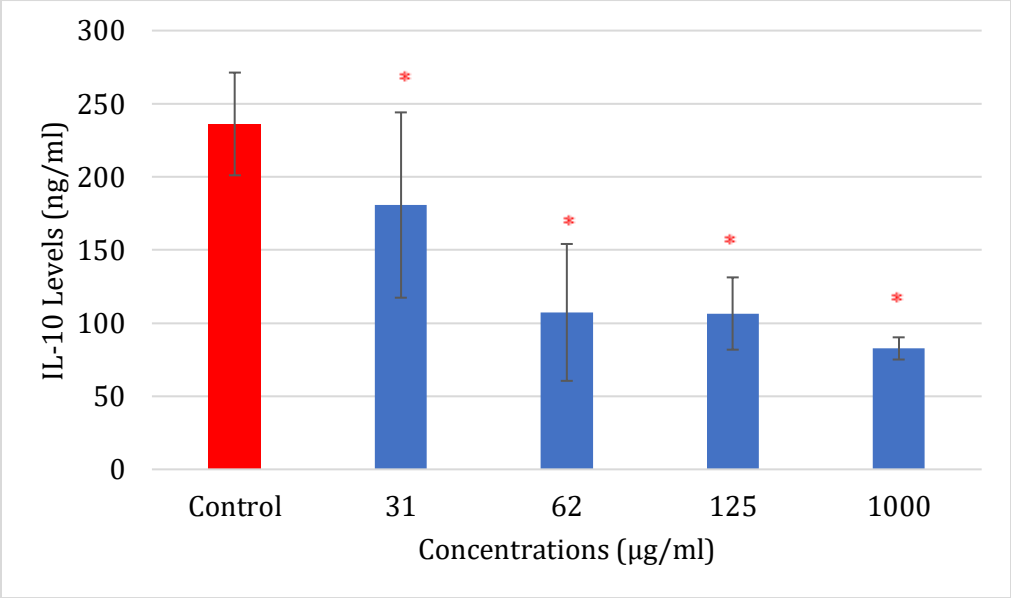

Figure 3.7 Effects of alcoholic leaf extract of *Lantana Camara* on IL_10 in SW480 cell line.

* **mean significant difference (P≤0.001).**

Chapter

Four

Discussion

4.Discussion:

4.1 Cytotoxicity evaluation of *Lantana Camara alcoholic* leaf extracts on the viability Vero cell line:

extract at concentrations above 250 µg/ml decreased vero cells viability, as previously illustrated in Fig 3.1.

One of the main pentacyclic triterpenoids found in *Lantana Camara* leaves is lantadene A (LA). It has been shown to have anticancer properties. LA effect has been evaluated against LNCap, SW480, and Vero cell lines. It was found that as the LA concentration increased, the cells' viability drops in a dose-dependent manner. For Vero cells, the effect was more pronounced.(Dawood *et al.*, 2021).

Studies suggest possible mechanism of anticancer effect is due to A pentacyclic triterpenoid called lantadene A was discovered in the weed *Lantana Camara*. Potential was seen when female Swiss albino mice (LACCA) were given Lantadene A twice a week for 20 weeks at a dose of 50 mg/kg body weight chemopreventive activity. This chemopreventive activity could be linked to the expression of transcriptional factors and a significant decrease in the mRNA expression of AP-1 and c-fos), NF-kB (p-65) and p53 was observed in Lantadene A treated mice skin tumors (Fernando *et al* ., 2013) .

The alcoholic extract results showed significant cytotoxic effects ($P \leq 0.001$) causing decreasing in cell line viability up to 80% reduction in the viability at conc. (1000, 500) µg/ml._The presence of phytochemicals in the leaves of *Lantana Camara* could be the main reason leading to the

cytotoxicity of alcoholic extract, especially due to the presence of toxic lantanoids and alkaloids from this plant (Hariharapura *et al*., 2004) .

Alkaloids are among the most essential active ingredients in botanical herbs that have a biological effect. Some of these substances have also come into the therapeutic arena as a cancer treatment medication, such as vinblastine and vincristine (isolated from Vinca rosea) that interacts with tubulin, camptothecin (extracted from Camptotheca acuminate), a potent topoisomerase I inhibitor and berberine from Rhizoma coptidis that has a chemo-preventive effect against colon cancer as well as an antiangiogenic effect (Al-Hakeim *et al.*, 2021) .

Phytochemicals induce Endoplasmic reticulum stress and apoptotic cell death. However, some phytochemicals modulate mitochondrial biogenesis and ensure apoptosis-autophagic cell death. Phytochemicals regulate the cell cycle and microRNA as well as cause apoptosis-autophagic cell death in cancer cells.(Rahman *et al*., 2021) .

Studies demonstrated that the *Lantana Camara* induced apoptotic pathway is mediated by the Bcl-2 family, caspases, and PARP. These studies also found that treatment with the *Lantana Camara* extract induced the expression of the proapoptotic protein Bax and suppressed the expression of the anti-apoptotic protein Bcl-2 (Han *et al.*, 2015) .

4.2 Evaluation of *Lantana Camara alcoholic* leaf extracts cytotoxicity on LNCap cell line:

Regarding the effects of alcoholic extract *of Lantana Camara* leaf on the viability of LNCap cell line, there was a significant ($P \leq 0.001$) decrement in the viability of those cells at (1000 and 500) µg / ml and significant increase

($p \leq 0.001$) at concentration (250 ,125, 62) µg /ml in comparison to the control group, after incubation period of 24 hours .

This result is in consistence with study of (Dawood et al 2021), which can be explained by that Lantadene A specifically inhibited the growth of LNCaP cells. The intrinsic pathway of a mitochondria-dependent mechanism was used by Lantadene A to induce apoptosis. This was confirmed by the substantial, dose-dependent activation of caspases -3/7 and 9. Clearly, cell cycle arrest occurred during the G0/G1 phase (Dawood *et al.,* 2021) .

Flavonoids have the potential of modulating many biological events in cancer such as apoptosis, vascularization, cell differentiation, cell proliferation, etc. A strong correlation persists between flavonoid-induced modulation of kinases with apoptosis, cell proliferation and tumor cell invasive behavior in vitro (Batra *et al.,* 2013).

In 2022 , study was performed by tong and his co-worker to extract Oleanonic acid from *Lantana Camara* and evaluated its anticancer properties ,they found that Oleanolic acid and its derivatives primarily suppress tumor cell migration and invasion by decreasing tumor cell proliferation, causing tumor cell death, inducing tumor cell autophagy, controlling cell cycle regulatory proteins, and inhibiting vascular endothelial growth (Tang *et a*l., 2022) .

Another study by (Li *et al*.,2016) in prostate cancer PC-3, DU145, and LNCaP cells, they discovered that oleanolic acid promoted the G0/G1 phase cell cycle arrest by the dose-dependent regulation of the expression levels of cell cycle-related proteins. Oleanolic acid also reduced the activity of the PI3K/Akt pathway.

In the process of carcinogenesis, flavonoids interfere with multiple signal transduction pathways and thus limit proliferation, angiogenesis, and metastasis or increase apoptosis (Abotaleb *et al*., 2019) .

About 30 flavones have been isolated from Lantana species. Additionally, it is thought that these flavones stimulate certain antioxidant response mechanisms in the cell, aiding in the removal of reactive oxygen molecules from the cell. Antitumor, anti-inflammatory, anti-cancer, and antioxidant properties are all present in luteolin when it is extracted from lantana species (Imran *et al* ., 2020) .

It is widely known that the androgen receptor (AR) plays a significant role in the initiation and spread of prostate cancer. Luteolin exerted an antiproliferative effect and caused the activation of apoptosis in prostate cancer cells, including LNCaP, PC-3, and DU145 cell lines. Luteolin also downregulated the levels of AR's mRNA and protein in a dose- and time-dependent manner while suppressing PSA levels (both intracellular and secreted) (Ganai *et al.*, 2021) .

4.3 Evaluation of *Lantana Camara* alcoholic leaf extracts cytotoxicity on the viability SW480 cell line:

There was a significant ($P \leq 0.001$) decrement in the viability of SW480 cell line when alcoholic extract of *Lantana Camara* leaf with concentrations 1000,500 and 250 µg/ml was added, in comparison with the control group after incubation period of 24 hours, (fig3.3).

It has been established that lantana leaf extracts contain high levels of flavonoids, about 30 flavones have been isolated from Lantana species. Additionally, it is thought that these flavones stimulate certain antioxidant

response mechanisms in the cell, aiding in the removal of reactive oxygen molecules from the cell. As a result, it guards against several human diseases including cancer. Additionally, it includes p-coumaric acid, which both in vitro and in vivo has an antiproliferative effect on colon cancer. Phytochemicals Pharmacological properties and composition of Lantana species (Imran *et al* ., 2020).

A Review could suggest the existence of pro- and anti-toxic phytochemicals in the leaf extract that interfere with the cytoprotective or cytotoxic effects of each other in a concentration _ dependent manner (Pour *et al*., 2011).

Luteolin as with other types of flavonoids have shown to have anti-cancer activity against increasing evidence points to luteolin's ability to inhibit colon cancer carcinogenesis via activating the Nrf2/antioxidant response element (ARE) pathway. Lutein inhibited cellular transformation and proliferation in HCT116 and HT29 cells in a dose-dependent manner. Importantly, luteolin altered these cells' epigenetic regulators to provide its therapeutic effects. It is clear that luteolin therapy stimulates the Nrf2 pathway by reducing the activity of some conventional HDACs and DNMTs while downregulating their expression (Ganai *et a*l., 2021) .

According to studies, the main characteristic of phenolic phytochemicals is their antioxidant activity. This is a result of the hydroxyl groups in phenols' capacity to offer hydrogen atoms for scavenging ROS. Therefore, it is hypothesized that phenolic phytochemicals could scavenge ROS molecules, inhibit mitogen-activated protein kinase (MAPK) signaling, impede the activation of nuclear factor kappa B and activator protein 1, and therefore decrease the proliferation of cancer cells. It was

also discovered that the *Lantana Camara* extract of p-coumaric acid increased ROS formation in a time-dependent way. Through the activation of MAPK, excessive ROS production by phenolic phytochemicals causes apoptosis. Ras/MAPK kinase/MAPK pathway simultaneously boosted p53 activation, as seen in the apoptosis of EGCG and resveratrol (Jaganathan *et al*., 2013) .

4.4 Evaluation of *Lantana Camara* alcoholic leaf extracts on the levels of TNFα in LNCAP cell line:

There was a significantly ($P \leq 0.001$) decrease in the levels of TNFα at all concentrations used in comparison with the control group after incubation period of 24 hours, (fig3.4).

Studies showed that luteolin treatment significantly decreased the elevated levels of proinflammatory cytokines, IL-1β, IL-6, IL-8, and TNF-α in prostate cancer (Pratheeshkumar *et al*., 2012).

In a human study model, researchers found that luteolin at a concentration of 30 μg is effective against prostate carcinoma LNCaP cells by inducing cell apoptosis, up-regulating prostate-derived Ets factor (PDEF), and down-regulating androgen receptor (AR) gene expression It additionally, caused significant reduction in weight and volume of solid tumors, and suppressed the micro-vessel density. It also decreased cell viability, induced apoptosis, and down-regulated the ERK, AKT, P70S6K, mTOR, MMP-2, and MMP-9 expressions (Imran *et al*., 2019).

Also, according to this study, Alkaloids have been found to suppress tumorigenesis by targeting the NF-κB pathway, namely by suppressing its activation and regulating its gene expression.(Habli *et a*l., 2017).

4.5 Evaluation of *Lantana Camara* alcoholic leaf extracts on the levels of TNFα in SW480 cell line:

The high concentration of alcoholic leaf extracts 1000 µg/ml a significantly ($P \leq 0.001$) decrease in the levels of TNFα in SW cell line in comparison with other concentration and control group after incubation period of 24 hours (fig3.5).

Studies have shown that *Lantana Camara* L. Leaves extract can suppress the TNF-a-induced activation of NF-kB by inhibiting IKK activation and IkBa degradation. also inhibited the NF-kB regulated protein expression of COX-2, which regulates inflammation and cyclin D1, which in turn regulate the proliferation (Ashal *et al*., 2020).

There are many genes known to be regulated by NF-jB including TNFα itself and those that play roles in inflammation, such as cyclooxygenase-2 (COX-2), lipoxygenase-2 (LOX-2), cell-adhesion molecules (CAMs), inflammatory cytokines and inducible nitric oxide synthase (iNOS). Thus, the proinflammatory effects of TNF-a are mainly due of its ability to activate NF-Jb (Iqbal *et a*l., 2013) .

Ursolic acid is a plant _based pentacyclic triterpenoid carboxyl acid derived from plants called ursolic acid can be found in *Lantana Camara* and according to this study it can inhibit tumor cell proliferation, induce apoptosis, resist mutation, resist oxidation, and resist angiogenesis. The key targets of Ursolic acid in the treatment of colon cancer mainly included interleukin-6 (IL-6), mitogen-activated protein kinase 3 (MAPK3), vascular endothelial growth factor receptor (VEGFA), prostaglandin endoperoxide synthase 2 (PTGS2/COX2), caspase-3 (CASP3), mitogen-

activated protein kinase 8 (MAPK8), tumor necrosis factor (TNF), cyclin D1 (CCND1), JUN, and signal transducer and transcription activator 3 (STAT3) (J. Zhao *et al*., 2021).

4.6 Evaluation of *Lantana Camara* alcoholic leaf extracts on the levels IL-10 in LN cap cell line.

There is not a statistically significant difference ($P \leq 0.001$) in the levels of IL-10 in all concentration of alcoholic leaf extracts of *lantana camara* in LNCap cell lines.

IL10, which is elevated in PCa patients, may directly act on some PCa cells to increase PDL1 expression ,IL10 is best studied for its inhibitory action on immune cells such as macrophage ,but IL10 can also stimulate CD8+ T cell antitumor immunity and was tested in a clinical trial of multiple tumor types.IL10 treatment increased CD8+ activity and prolonged patient survival in some cancer types (Samiea et al., 2020).

M1 and M2 macrophages produce IL-12 (anti-tumor) and IL-10, respectively. M2 macrophages play a key role in tumor progression and growth. IL-10 concentration is elevated in a variety of solid tumors. The ratio of IL-12 and IL-10 concentration is an important predictor of tumor progression. Some phytochemicals can increase M1 and decrease M2 macrophages, resulting in a decrease in STAT3, IL-10, and arginase I gene expression and secretion, by reducing IL-10 levels, phytochemicals were also shown to inhibits Janus Kinase-STAT signaling and increases tumor apoptosis (hamid et al.,2019)

4.7 Evaluation of *Lantana Camara* alcoholic leaf extracts on the levels IL-10 in SW480 cell line.

The levels of IL10 at doses decreased considerably ($P \leq 0.001$) after the treatment with *Lantana Camara* alcoholic leaf extracts in comparison with the control group after incubation period of 24 hours.

IL-10 levels were significantly higher in patients with worse prognosis and circulating IL-10 levels can be prognostic in CRC patients, along with number of lymph nodes involved, penetration of tumor through bowel wall, bowel perforation, vascular invasion, and serum CEA levels, Interactions between neoplastic cells and other cells in tumor microenvironment contribute to cancer progression, and inflammatory infiltrates in the tumor microenvironment are essential for tumor metastasis. As the tumor progresses, more IL-10+ tumor-associated macrophages and monocytes and other immune cells accumulate in the tumor microenvironment. This fact is probably the reason of higher IL-10 levels in more progressed colorectal tumors (Shabnam et al ., 2017).

In cancer cells, STAT3 promotes the expression of factors that are both immunosuppressive and STAT3 activating, including vascular endothelial growth factor (VEGF) and IL-10. These tumor-derived factors, in turn, up-regulate STAT3 signaling in various immune-cell subsets in the tumor microenvironment, which produce more immunosuppressive factors, including IL-6, IL-10, transforming growth factor-β (TGF-β), and VEGF, thereby abrogating the function of various immune effector cells. Several studies demonstrated that phytochemicals were able to inhibit STAT3 activation. Thus, we can speculate that the decreased level of IL-10 may be attributed to the negative effect on STAT3 activation and consequently on the synthesis and secretion of IL-10 (hala et al., 2019)

Conclusions
&
Recommendations

Conclusions:

1- Alcoholic leaves extract of *Lantana Camara* showed significant cytotoxic effects on Vero, prostate cancer (LNCaP), and colon cancer (SW480) cell lines.

2- Alcoholic leaves extract caused a significant decrease in TNFα levels in the LNCaP cell line at all concentrations used. While for SW480 cells, only higher concentration (1000 µg/ml) caused significant decrease in TNFα levels.

3- Plant extract decreased the levels if IL-10 in SW 480 colon cancer cells at all concentration used. While for LNCaP cell line, there was no effect.

Recommendations:

1- Study the cytotoxic and anticancer activity of different plant parts on different cell lines.
2- Study the cytotoxicity and anticancer activity of this plant on animal models
3- Study the interaction of plant extract with known anticancer drugs on cell lines and animal models.
4- Study the immunomodulatory effect of the plant on cultured cells and on animal models using other markers and study the molecular mechanisms responsible for these immunomodulatory effects.

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الخلاصة

تم تنفيذ الجزء العملي للدراسة البحثية في مختبر بحوث طلاب الدراسات العليا /فرع الادوية/ كلية الطب / جامعة بابل خلال الفترة من أكتوبر 2021 –ابريل 2022.

تلعب المكونات الكيميائية النباتية دوراً مهماً في العلاج، وذلك لتأثيرها على الجهاز المناعي وتكاثر الخلايا، لذلك كانت أهداف هذه الدراسة كان تحديد السمية الخلوية لمستخلصات أوراق المينا الكحولية على خطوط الخلايا بما في ذلك خط خلايا Veroوخط خلايا سرطان البروستات LNCaPوخط خلايا سرطان القولون SW480.

كذلك هدفت الى دراسة التأثير المناعي للمستخلص الكحولي لأوراق المينا على خط خلايا سرطان البروستات LNCaPوخط خلايا سرطان القولون SW480.

اجري اختبار السمية للخلايا لقياس سمية المستخلص النباتي من أجل تحديد النطاق المفيد للتراكيز المستخدمة عند إجراء فحص المناعة باستخدام تقنية.ELISA

تم زرع سلالات خلايا Vero cell و LNCaPو SW480في اطباق الزرع الخلوي ذات 96 حفرة ومعالجتها بمستخلصات كحولية للاوراق بتركيزات مختلفة (1000، 500، 250، 125، 62.5، 31.25) ميكروغرام / مل وحضنت لمدة 24 ساعة. بعد ذلك، تم إجراء فحصMTT

اظهرت نتائج السمية الخلوية لخطوط خلايا Vero و 480 SWانخفاضاً كبيرا (P <0.001) في اعداد الخلايا بتراكيز (1000، 500، 250) ميكروغرام / مل مقارنةً بمجموعة السيطرة، بينما بالنسبة لـ LNCaP خط خلايا سرطان البروستات، كان هناك انخفاض كبير في قابلية هذه الخلايا للحياة في التراكيز(1000 و500) و زياده في قابليه الخلايا للحياه في التراكيز (250 و125و62) ميكروغرام / مل وحضنت لمدة 24 ساعة.

تم زرع الخلايا خطوط خلايا سرطان البروستات (LNCaP) وسرطان القولون (SW480) من اجل قياس السيتوكينات في اطباق الزرع الخلوي ذات 96 حفرة، وعولجت جميع الخلايا بتركيزات مختلفة من نبات المينا بتراكيز تتراوح من 1000 إلى 31.5 ميكروغرام / مل (استخدمت أربع مكررات لكل تركيز من نبات المينا لكل نوع من الخلايا) جنبًا إلى جنب مع أربع مكررات كمجموعة سيطرة لكل نوع من الخلايا. ثم تم تغطية الصفيحة بغطاء بلاستيكي ذاتي وحضنت لمدة 24 ساعة، في نهاية فترة التعرض، أخذت خطوط الخلايا للمقايسة المناعية بطريقة ELISA.

انخفض مستوى TNFα في خط خلية LNCAP بشكل ملحوظ ($P\leq 0.001$) في جميع تركيزات مستخلص أوراق نبات المينا عند مقارنته مع مجموعة التحكم بعد فترة حضانة لمدة 24 ساعة، بينما في خط خلية SW480 كان هناك انخفاض ملحوظ ($P). \leq 0.001$) عند المعاملة بتركيز (1000) ميكروغرام / مل فقط.

لم يقل مستوى IL-10 في خط خلية LNCAP في اي تركيز لمستخلصات الأوراق الكحولية من نبات المينا، بينما في خط الخلية SW480 انخفض مستواه بشكل كبير في كل تركيز من مستخلص النبات.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل

كلية الطب

دراسة تحوير إنتاج السيتوكينات والسموم للخلايا الناجم عن مستخلص أوراق لانتانا كامارا الكحولية العراقية. دراسة في المختبر

رســـالـــــة

مقدمـــة الى مجلس كلية الطب/جامعة بابل

كجزء من متطلبات نيل درجة الماجستير في الأدوية / الأدوية والسموم

من قبل

عـلا علــي محــمد كاظم

بكالوريوس صيدلة،

2015-2014

إشراف

أ.د. نسرين جلال محمد

دكتورا ادويه وسموم

أ.د قيصر نعمة مظلوم

دكتورا علم الخليه

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