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M.Sc. - DEPARTMENT OF BIOLOGY

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UNIVERSITY OF GAZIANTEP

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NATURAL & APPLIED SCIENCES

INVESTIGATION OF THE BIOLOGICAL EFFECTS OF *XANTHIUM STRUMARIUM*

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DHIFAF FATLAWI

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Investigation of The Biological Effects of

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M.Sc. Thesis

in

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Prof. Dr. Mehmet OZASLAN
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This is to certify that we have read this thesis and that in our consensus/majority opinion, it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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Dhifaf FATLAWI

ABSTRACT

INVESTIGATION OF THE BIOLOGICAL EFFECTS OF *XANTHIUM STRUMARIUM*

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M.Sc. In Department of Biology

Supervisor: Assist. Prof. Dr. Isik Didem KARAGOZ

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50 pages

Xanthium strumarium, is known as a medicinal plant, a summer annual herb, a member of the Asteraceae family. It was originate in North America and has been extensively naturalized elsewhere. The burs of the plant samples were collected from the province of Kilis-Söğütlüdere locations, in November, 2013 and dried according to herbarium techniques. The acetone extract was done, then the silica gel column and thin layer chromatography used to isolate the xanthatin and xanthinosin. We used the MTT assay to determine the antiproliferative and cytotoxic activity of xanthatin and xanthinosin on A549 and H1299 cell lines. In addition, DNA protection activity was studied by pBR322 plasmid DNA assay. The results showed that, xanthatin in low concentrations (6.25, 12.5 μg/ml) had a good result on A549 whereas xanthinosin on A549 only in the high concentrations (50, 100 μg/ml) were worked. Both of xanthatin and xanthinosin at the same concentration (0.1 mg/ml) showed protection property against to plasmid DNA damaging in present of UV and H_2O_2. In conclusion, it was determined that *X. strumarium* has significante antitumor and DNA protection activities.

Key Words: Cancer, Xanthatin, Xanthinosin, MTT, DNA Protection.

ÖZET

XANTHIUM STRUMARIUM' un BİYOLOJİK AKTİVİTELERİNİN ARAŞTIRILMASI

FATLAWI, Dhifaf

Yüksek Lisans Tezi, Biyoloji Bölümü

Tez Yöneticisi: Yrd. Doç. Dr. Işık Didem KARAGÖZ

Ocak 2015

50 Sayfa

Xanthium strumarium tıbbi bir bitki olup, Asteraceae familyasının bir üyesidir ve yazın çiçek açan tek yıllık bitkilerdendir. Öncelikle Kuzey Amerika' dan orijinlenmiş olup, sonrasında başka habitatlarda da yayılış göstermiştir. Bitki diken örnekleri, Kasım, 2013'te, Kilis İli Söğütlüdere Mevkii'nden toplanmıştır ve herbaryum tekniklerine uygun olarak preslenip kurutulmuştur. Bitki aseton özütü çıkarılmıştır sonra bitkisinden ksantatin ve ksantinosin maddelerinin izole edilme için silika jel kolon ve ince tabaka kromatografisine tabi tutulmuştur. Özütünden elde edilen ksantatin ve ksantinosin A549 ve H1299 hücre hatları üzerindeki antiproliferatif ve sitotoksik aktivitesini tespit etmek için MTT testi kullanılmıştır. Ayrıca DNA koruyucu aktiviteleri için pBR322 plazmid DNA testi kullanıldı. Sonuçlara göre ksantatin düşük konsantrasyonda (6.25, 12.5 µg/ml) A549 üzerinde iyi bir sonuç gösterirken ksantinosin A549 üzerinde sadece yüksek konsantrasyonda (50, 100 µg/ml) çalışmıştı. Ksantatin ve ksantinosin (0.1 mg/ml) konsantrasyonda UV ve H_2O_2 ile oluşturulan plazmid DNA hasarına karşı koruyucu özellik gösterdi. *X. strumaruim* bitkisinin antitümör ve DNA koruyucu özellikleri sahip olduğu ortaya konulmuştur.

Anahtar Kelimeler: Kanser, Ksantatin, Ksantinosin, MTT, DNA Koruyucu.

To My Parents

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LIST OF SYMBOLS

AD	Anno Domini (After Christ)
ATCC	American Type Culture Collection
BC	Before Christ
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
CAT	Carboxyatractyloside
COPD	Chronic Obstructive Pulmonary Disease
CSIR	Council for Scientific and Industrial Research
CT	Computed Tomography
DAPI	4'-6-diamidino-2-phenylindole
DMSO	-Dimethyl sulfoxide
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium Bromide
FDP	Fructose diphosphate
GRP	Gastrin Releasing Peptide
IARC	International Agency for Research on Cancer
IC_{50}	The half maximal inhibitory concentration
linDNA	Liner form of DNA
MTT	(3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide)

NCI	National Cancer Institute
NICE	National Institiue for Health and Clinical Excellence
NSCLC	Non-Small Cell Lung Cancer
ocDNA	Open circular DNA
PBS	Phosphate Buffered Saline
PG	Propyl Gallate
ROS	reactive oxygen species
scDNA	Supercoiled Circular DNA
SCLC	Small Cell Lung Cancer
SDS	Sodium Dodecyl Sulfate
SRB	Sulforhodamine B assay
STL	Sesquiterpene lactones
TBHQ	Tertiary Butyl Hydroquinone
TCM	Traditional Chinese Medicine
TNM	Tumor, Lymph nodes, Metastasis
TP53	Tumor Protein p53
VIS	Visible Light
WHO	World Health Organization
ZFX	Zinc Finger Transcription factor

CHAPTER ONE

INTRODUCTION

1.1. CANCER

The body is made up of trillions of living cells. Normal cells grow, divide into new cells, and die in an orderly way. During the early years of human's life, normal cells divide faster to let the human grow. Subsequently the human becomes an adult, most cells divide for replacing worn-out or dying cells or to repair damages (American Cancer Society, 2013).

Cancer is medically known as malignant tumors, are a wide range of diseases involving unregulated cell growth. In cancer, the division and growth of cells uncontrollably, forming malignant tumors, which may cause invasion to adjacent regions of the body (National Cancer Institute, 2014).

Cancer may also spread to the further parts of the body by the bloodstream or lymphatic system. Some tumors are cancerous; and the others are benign tumors do not invade nearby tissues or spreads all over the body. There are more than 200 different types of cancer known which affect the humans (Cancer Research UK, 2013).

There is no single definition that describes all cancer cases, simply, we can describe cancer from the positions of different organizational levels:

1- Molecular, cancer is basically a genetic disease (Vogelstein and Kinzler, 2004).

2- Cellular, cancer is a disorder of unregulated proliferation of abnormal cells (Hanahan and Weinberg, 2000).

3- Personally, smoking causes by approximately 30% of all cancer deaths occur in the United States, and overweight and obesity caused by 15-20% (Peto, 2001; Calle and Kaaks, 2004).

4- Familial, inherited genetic actions contribute greatly to 5-10 % of breast cancer and 5-13% of cases of colon cancer (Emory et al., 2001; Balmain et al., 2003).

5- Environmental level: in industrialized countries, nearly 7% of cancer deaths can be attributed to viral infection; 4% of occupational risks; 2% of sunlight; 2% to the pollution of air, water and soil; and less than 1% of nutrient additives and industrial products (Peto et al., 1996).

1.1.1. History of cancers

There is some truth in the old adage that the cancer is as old as the human race, but the results indicate that paleopathologies tumors were found in animals in prehistoric times, long before the appearance of the men on the ground. In medicine, was found in the earliest written description of diseases and cancer, and breast cancer, in the Edwin Smith Papyrus which was written about 3000 BC. The author concluded that the swelling of the tumor from the breast was a serious disease and there is no cure for it (Breasted, 1930). In the Ebers Papyrus, dated around the year 1500 BC, contains the first reference to the presence of a tumor of soft tissue, fatty tumor, and with an indication of the possibility of the existence of cancer of the skin, uterus, stomach and rectum (Ebbell, 1937). The Egyptians tried to treat tumors and cancerous with ironing, knives, and salts, and made of arsenic paste, which remained in use as " Egyptian ointment" until the 19th century (Hajdu, 2004).

Sumerians, Chinese, Indians, Persians, and the Jews of the same era were used herbal remedies such as tea, fruit juices, figs, cabbage boiled, in the simple cases, but in advanced cases, they did not feel free to turn to solutions and pastes of iron, copper, sulfur, and mercury. A lot of these remained fabrications in the external and internal use, in different concentrations, for over 3000 years (Wolff, 1907- 1928).

The first picture of person that had cancer was taken to Clara Jacobi, a Dutch woman who had a tumor removed from her neck in 1689, as shown in Figure (1.1).

Figure 1.1: A Dutch woman who had a tumor removed in 1689.

1.1.2. Origin of the word cancer

The cancer word is returned to the Greek physician Hippocrates (460-370 BC), who is considered the "medicine's father". The terms of carcinos and carcinoma used by Hippocrates to describe non-ulcer forming and ulcer-forming tumors. In Greek, these words refer to a crab, most likely apply to the disease because the finger-like spreading prospects of cancer come to the mind the form of a crab (Littre, 1845; Castiglioni, 1931). The Roman physician, Celsus (28-50 BC), later translated the Greek term into cancer, the Latin word for crab (Eebbell, 1937). Galen (130-200 AD), another Roman physician, used the word oncos (Greek for swelling) to describe tumors. The crab analogy of Hippocrates and Celsus is still used to describe malignant tumors, whereas Galen's term is now used as a part of the name for cancer experts — oncologists (American Cancer Society, 2012).

1.1.3. Facts on cancers

According to GLOBOCAN, 2012, an estimated 14.1 million new cancer cases and 8.2 million deaths from cancer occurred in 2012, in comparison with 12.7 million and 7.6 million, in 2008. Prevalence estimates for 2012 show that there were 32.6 million people (over the age of 15 years) alive who had had a cancer diagnosed in the previous five years.

The most commonly diagnosed cancers worldwide were those of the lungs (1.8 million, 13.0% of the total), breast (1.7 million, 11.9%), and colorectum (1.4 million, 9.7%). The most common causes of cancer death were cancers of the lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%) (IARC, 2013).

1.1.4. Lung cancer

Lung cancer (also known as lung carcinoma) is a solid tumor that is characterized by uncontrolled cell growth in the lung tissue. Most types of cancer that start in the lung, known as primary lung cancers, which are derived from epithelial cells (Horn et al., 2012). Lung cancer begins in the cells lining the bronchi and parts of the lung like the bronchioles or alveoli.

Lung cancers are believed to begin as areas of pre-cancerous changes in the lung. The first changes in the genes (DNA) inside the lung cells may cause the cells to grow faster. These cells may look a little abnormal if seen under a microscope, but at this stage they do not form a mass or tumor. They cannot appear on an x-ray and they do not cause symptoms (American Cancer Society, 2013).

The most common symptoms are coughing, coughing up blood, wheezing or shortness of breath, weight loss, fever, clubbing of the fingernails, or fatigue, chest pain, bone pain, superior vena cava obstruction, difficulty swallowing coughing (Horn et al., 2012). However, they occur late in the cancerous process.

Lung cancer can spread by breaking away from a lung cell tumor. They can travel through blood vessels or lymph vessels to reach other parts of the body. Cancer cells may attach to other tissues after spreading, and grow to form new tumors that may damage those tissues (National cancer institute, 2012). Lung cancer is often a life-

threatening disease because it tends to spread in this way even before it can be detected on an imaging test such as a chest x-ray (American Cancer Society, 2013). When lung cancer spreads from its original location to another part of the body, the new tumor will have the same kind of abnormal cells and the same name as the primary (original) tumor. For example, if lung cancer spreads to the bones, the cancer cells in the bones are in fact lung cancer cells. The disease is metastatic lung cancer, not bone cancer. Therefore, it is treated as lung cancer, not bone cancer (National cancer institute, 2012).

The most common reason of lung cancer is long-term exposure to tobacco smoke, causes 80–90% of lung cancer (Merck Manual, 2013).

Tobacco smoke consists of over 7,000 chemicals, including over 70 material known to cause cancer (carcinogens). Some of these substances cause heart and lung diseases and all of them can be deadly. It may be surprising to know some of the chemicals found in tobacco smoke include: Cyanide, Benzene, Formaldehyde, Methanol (wood alcohol), Acetylene (the fuel used in welding torches) and Ammonia (American Cancer Society, 2014). According to the WHO, smoking is a risk factor for six of the eight leading causes of death in the world, including lung cancer, chronic obstructive pulmonary disease (COPD), ischemic heart disease, cerebrovascular disease, lower respiratory infections, and tuberculosis (WHO, 2008). Nonsmokers account for 10–15% of lung cancer cases (Thun et al., 2008), and these cases are often attributed to a combination of genetic factors, the other causes of lung cancer are exposure to; radon gas, air pollution (Alberg and Samet, 2010) and asbestos (O'Reilly et al., 2007), including second-hand smoke (Carmona, 2006; WHO, IARC, 2002). The diagnosis is made by performing chest radiographs the first investigative steps, computed tomography (CT) scans, then the diagnosis is confirmed by bronchoscopy sputum cytology (Lu et al., 2010). Treatment and long-term outcomes depend on the type of cancer, the stage (degree of spread) and the person's general health, measured according to situation of performance.

Common treatments include surgery, chemotherapy, radiotherapy and targeted therapy. (Chapman et al., 2009).

Generally, 15% of people in the United States diagnosed with lung cancer, they have five years after the diagnosis (Collins et al., 2007). In the developing world outcomes are

worse. All over the world, lung cancer is the most common cause of cancer-related death in men and women, and is responsible for 1.38 million from annual deaths, in 2008 (Ferlay et al., 2010).

1.1.5. Types of lung cancer

The most common types are named for how the lung cancer cells look under a microscope:

1- Small cell: The cells of small cell lung cancer look small under a microscope. About 1 from each 8 people with lung cancer has small cell lung cancer. About 10% to 15% of all lung cancers are small cell lung cancer (SCLC), other names for SCLC are oat cell cancer, oat cell carcinoma, and small cell undifferentiated carcinoma (American Cancer Society, 2013; National cancer institute, 2012).

SCLC is considered to be an aggressive form of lung cancer that grows quickly and has often spread (metastasis) beyond the lung at the time of diagnosis. SCLC responds often to treatment with chemotherapy, but in the most patients, whilst length of life can be extended, the cancer is not curable (NICE guideline, 2011). More common among men than women, small cell carcinoma is almost always caused by smoking and is rare among those who have never smoked (Horn et al., 2012).

The stage of small cell lung cancer:

- Limited stage: Cancer is found only on one side of the chest.
- Extensive stage: Cancer is found in the lung and also in tissues on the other side of the chest. Or, lung cancer is found in distant organs, such as the brain, or fluid between the two layers of pleura.

2- Non-small cell: The cells of non-small cell lung cancer is larger than the cells of small cell lung cancer. Most (about 7 of every 8) people diagnosed with lung cancer have non-small cell lung cancer. It doesn't grow and spread as fast as small cell lung cancer, and it's treated differently (National cancer institute, 2012).

About 85% to 90% of lung cancers are non-small cell lung cancer (NSCLC) (American Cancer Society, 2013).

The disease arises from epithelial cells of the lung, from the central bronchi to terminal alveoli. The histological type correlates with site of origin, reflecting the variation in respiratory tract epithelium by location. The most common types of NSCLC are:

- Adenocarcinoma: These cancers start in early versions of the cells that would normally secrete substances such as mucus (American Cancer Society, 2013). The adenocarcinoma has an increased incidence in smokers, and is the common type of lung cancer seen in non-smokers and women (Mitchell et al., 2007). People with a type of adenocarcinoma called adenocarcinoma in situ (previously called bronchioloalveolar carcinoma) tend to have a better outlook (prognosis) than those with other types of lung cancer (Raz et al., 2006).

- Squamous cell carcinoma: These cancers start in early versions of squamous cells, which are flat cells that line the inside of the airways in the lungs. They are often linked to a history of smoking and tend to be found in the middle of the lungs, near a bronchus (American Cancer Society, 2013).

- Large cell carcinoma: It can appear in any part of the lung. It tends to grow and spread quickly, which can make it harder to treat. A subtype of large cell carcinoma, known as large cell neuroendocrine carcinoma, is a fast-growing cancer that is very similar to small cell lung cancer (American Cancer Society, 2013).

These types of lung cancer and how many are non-smokers versus smokers was shown in Figure (1.2).

Several other types occur less frequently, all can occur in unusual histological variants (Jemal et al., 2010). The cells in these subtypes differ in size, shape, and chemical make-up when looked at under a microscope. But they are grouped together because the approach to treatment and prognosis (outlook) are often very similar (American Cancer Society, 2013).

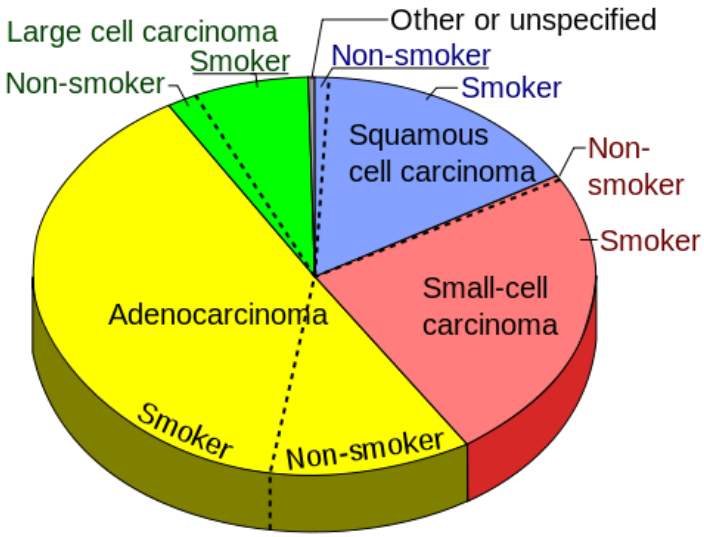

Figure 1.2: Incidence of lung cancer types, sorted by histological and smoking situation.

The initial evaluation of non-small-cell lung cancer (NSCLC) staging uses the TNM classification. This depends mainly on the size of the lung tumor, how deeply the tumor has invaded nearby tissue, such as the chest wall, whether lung cancer cells have spread to lymph nodes or other parts of the body. Ranging from occult cancer, through stages 0, IA (one-A), IB, IIA, IIB, IIIA, IIIB and IV (four). This stage group assists with the choice of treatment and estimation of prognosis (Rami-porta et al., 2009).

1.1.6. Cell lines

1.1.6.1. H1299

H1299, also known as NCI-H1299 or CRL-5803 (Phelps et al. 1996, ATCC 2009), is a human non-small cell lung carcinoma. This line was derived by A.F. Gazdar, H.K. Oie, J.D. Minna and associates from a lymph node metastasis of the lung from a patient who had received prior radiation therapy Caucasian male, 43 years old, which is widely used in research (Giaccone et al., 1992). These cells stain positive for keratin and vimentin but are negative for neurofilament triplet protein. As in other immortalized cell lines,

H1299 cells can divide indefinitely. These cells have a homozygous partial deletion of the TP53 gene and as a result of this, do not express the tumor suppressor p53 protein, which in part accounts for their proliferative propensity (Lin and Chang, 1996). These cells, as reported to secrete the peptide hormone neuromedin B (NMB), and not gastrin releasing peptide (GRP) (Giaccone et al., 1992). Figure (1.3) shown the H1299 cell line.

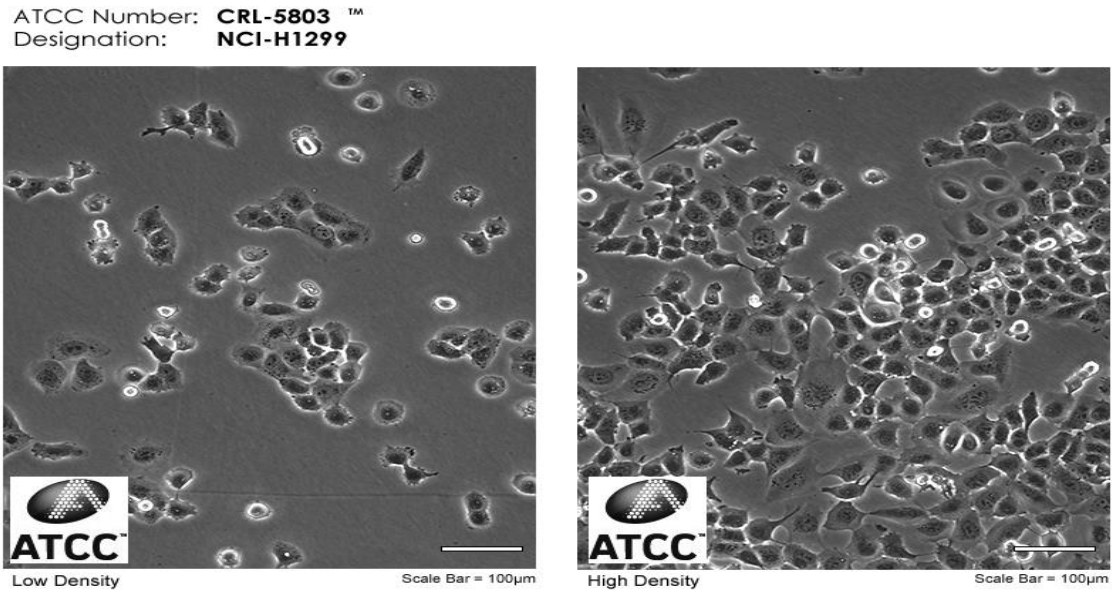

Figure 1.3: H1299 cell line.

http//:www.atcc.org

1.1.6.2. A549

Cells are adenocarcinoma human alveolar basal epithelial cells. The A549 cell line was first developed in 1972 by D. J. Giard, et al. through the removal and culturing of cancerous lung tissue in the explanted tumor a of a 58-year-old Caucasian male (Lin and Chang, 1996; A549 cell line, 2012). In nature, these cells are squamous as in Figure (1.4) and responsible for the diffusion of some substances, such as water and electrolytes, across the alveoli of the lungs. If A549 cells are cultured in vitro, they grow as monolayer cells, adherent or attaching to the culture flask (Lin and Chang, 1996). The

human alveolar epithelial cell line A549 may be anchored or suspended in a solution in vitro.

Another characteristic of these cells is that they are able to synthesize lecithin and contain high level of desaturated fatty acids, which are important to maintain the membrane phospholipids in cells (Giard et al., 1973). A549 cell line is widely used as an in vitro model for a type II pulmonary epithelial cell model for drug metabolism and as a transfection host (ATCC, 2012).

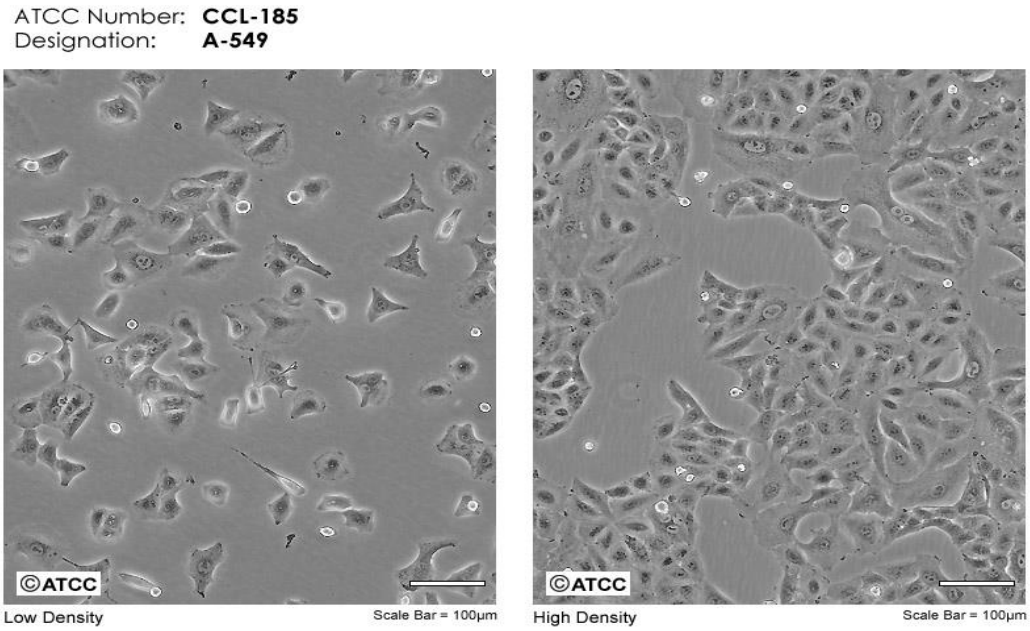

Figure 1.4: A549 cell line.

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1.2. MEDICINAL PLANTS

Medicinal plants are the richest bioresource of drugs for traditional systems of medicine, modern medicines, nutraceutical, food supplements, pharmaceutical intermediates, folk medicines and chemical entities for synthetic drugs. The development of systematic pharmacopoeias dates back to 3000 BC, when the Chinese were indeed using over 350 herbal remedies. Traditional systems of medicine continue to be practiced widely on many accounts. Population rise, Insufficient supply of drugs, high cost of treatments,

side effects of several drugs and development of resistance to drugs that are currently used for infectious diseases have led to increased focus on the use of plant materials as a source of medicines for a wide variety of human ailments (Handa et al., 2008).

In many of the developing countries the use of plant drugs is increasing because modern life saving drugs are beyond the reach of three quarters of the third world's population, although many such countries spend 40-50% of their total wealth on drugs and health care. As a part of the strategy to reduce the financial burden on developing countries, it is clear that an increased use of plant drugs will be trending in the future (Joy et al., 1998).

1.2.1. *Xanthium strumarium*

The other names of the plant are rough cocklebur, clotbur, common cocklebur, large cocklebur, woolgarie bur and in turkish pıtrak. Table (1.1) illustrates the scientific classification of *X. strumarium.*

Table 1.1: Scientific classification (Everitt et al., 2007).

Kingdom	Plantae
Order	Asterales
Family	Asteraceae
Subfamily	Asterodieae
Genus	*Xanthium*
Species	*Xanthium strumarium*

X. strumarium (Compositae), is a summer annual herb, it probably originates in North America and has been extensively naturalized elsewhere (Wunderlin and Hansen, 2008; Calflora 2014). The word "xanthium" is derived from an ancient Greek word "xanthos" meaning yellow and "strumarium" means "cushion like swelling", with reference to the

seed pods which turn from green to yellow as they ripen (later they become deep yellow to brown) (Dharmananda, 2003).

With 4.5ft tall with green leaves and stems without spines. The stems are thick, branch, often slightly fleshy, reddish or black-spotted or tinged dull red. The leaves have a distinctive scent, are broadly triangular, 1 to 6 inches long and wide, often weakly three-lobed with three main veins from the base. Upper and lower surfaces are green and rough to the tough (Ditomaso et al., 2013).

In addition, the flowers develop into hard woody burrs (fruits), 1.2 cm to 2 cm long, with numerous hooked spines Figure (1.5) shown the plant. *X. strumarium* is extremely competitive with other crops (Bozsa and Oliver, 1993). Cocklebur is a widespread weed in temperate to subtropical regions nearly worldwide. Ingestion of seedling and seeds at 1% of body weight or more can be fatally toxic to livestock, especially pigs and calves. Seed and sprout contain a high concentration of diterpene glycoside that cause acute metabolic disorder characterized by a sudden drop in blood glucose and an increase in certain liver enzymes. In humans, dealing with cocklebur can cause contact dermatitis in sensitive individuals (Ditomaso et al., 2013).

Figure 1.5: The whole plant of *X. strumarium.*

http//: www it.wikipedia.org

The spines and seeds of this fruit are rich in a chemical called carboxyatractyloside (CAT), formerly referred to as xanthostrumarin, which is the chemical that responsible for most of the harmful effects from the use of *X. strumarium* CAT has been shown to be a growth inhibitor in *Xanthium* and other plants, had two functions, delaying seeding germination and inhibiting the growth of other plants. Most of the chemical is concentrated in the spines. When the bur is prepared as an herbal remedy, the spines are usually removed, reducing the CAT content of the finished product (Cutler and Cole, 1983).

X. strumarium and other species of the genus *Xanthium* are known for the production of sesquiterpene lactones (STL) termed xanthanolides, which are responsible for most of the biological activities of *Xanthium* species.

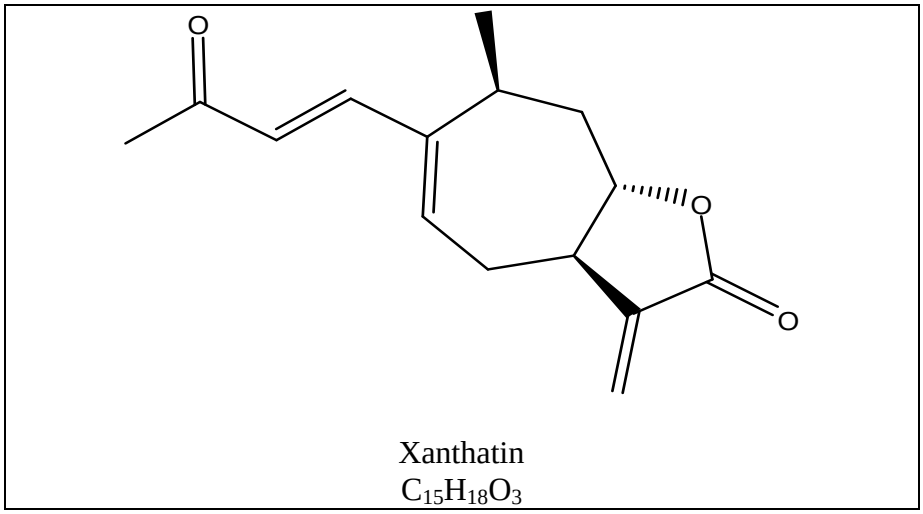
STLs are an exceptionally diverse class of plant secondary metabolites, with close to 5000 structures elucidated to date (Harborne et al., 1999; Schmidt, 1999). Laboratory assays have indicated that STL exhibit activity against bacteria, fungi, mollusks, insects, and mammals (Rodriguez et al., 1976; Picman, 1986; Schmidt, 2006).

The interest in these compounds is high because of the historical discovery of artemisinin, an endoperoxide sesquiterpene lactone, from *Artemisia annua* for the treatment of malaria (Malik et al., 1993). In addition to STL, *X. strumarium* has been reported to contain carboxyatractyloside (Cole et al., 1980), caffeoylquinic acid (Agata et al., 1993) and thiazinediones (Han et al., 2006).

1.2.1. Xanthatin

Xanthatin is a bicyclic STL biosynthesized mainly by *Xanthium* plants (Asteraceae). It was first isolated from *X. strumarium* at 1960s (Geissman et al., 1954). The chemical structure of xanthatin was given in Figure (1.6).

Recent studies demonstrated that xanthatin had significant antitumor activity in a variety of cell culture systems implicated in colon, breast, lung, cervix and skin cancers (Kovacs et al., 2009).



Xanthatin
$C_{15}H_{18}O_3$

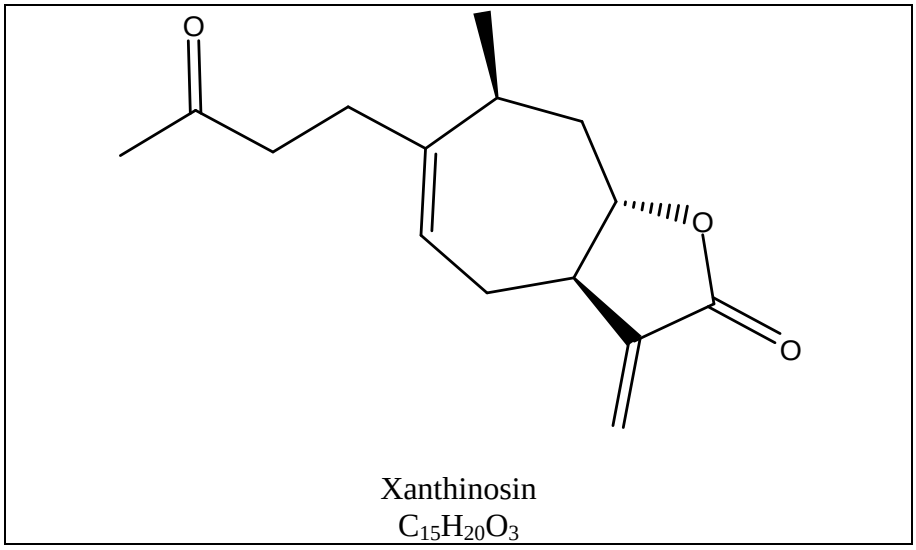
Figure 1.6: The chemical structure of xanthatin.

http://www.chemical-reagent.com.

1.2.2. Xanthinosin

Xanthinosin - phytotoxin- a known sesquiterpene lactone. Xanthinosin has been previously isolated from *X. italicum*, *X. macrocarpum* and *X. strumarium*, xanthinosin reported to possess plant inhibitory activity (Geissman et al., 1954; Khan, 1963; Shao et al., 2012) and it was confirmed to possess antileishmanial and antitumor activity (Marco et al., 1993).

Antitumor activity of xanthinosin was demonstrated high in vitro cytotoxic activity in the human cancer cell lines WiDr ATCC (colon), MDA-MB-231 ATCC (breast), and NCI-417 (lung) (Irving et al., 2007).



Xanthinosin

$C_{15}H_{20}O_3$

Figure 1.7: Chemical structure of xanthinosin.

(Kamboj, A. And Saluja, A.K., 2010).

1.2.BIOACTIVATION

1.3.1. MTT assay

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium salt that is reduced to yield a purple-colored water-insoluble formazan product (Altman, 1976). Whereas MTT is cleaved only by active mitochondria in living cells and not from dead cells or erythrocytes, MTT reduction is the most common used method for measuring cell proliferation and viability (Mosmann, 1983; Hansen et al., 1989). The formazan salt is produced in proportion to the active cell number, and accumulates within the cell because it does not membrane permeable. However, when dimethyl sulfoxide (DMSO), isopropanol or other suitable solvent is added, formazan salt can be quantified colorimetrically. The MTT assay is simple and suitable for a wide variety of cell lines (Barile, 1994). However, the MTT assay requires monitoring as duration of MTT treatment, the concentration of MTT used, and the number of test cells. These defined experimental conditions must be taken into consideration when comparing inter-laboratory results (Berridge et al., 2005).

1.3.2. DNA Protective Activity

UV rays that reach the earth from destruction of the stratospheric layer are known to produce adverse effects on living creatures. UV rays, causes skin cancer, skin aging and other serious diseases. Antioxidants provide protection against the damaging effects of UV rays. Enzymatic and non-enzymatic antioxidants are an effective approach to topically (on the skin) implementation to protect the skin against the damaging effects of UV rays (Tepe et al., 2011).

In fact, human skin has a number of mechanisms to reduce the damaging effects of VIS (visible light) and UV rays. However, the exposure to high levels of UV rays can lead to reduce the amount of cellular antioxidants, and this will cause oxidation, which resulting reactive oxygen species (ROS) and DNA damage. In addition, UV rays can cause the free radicals that lead to DNA damaging. DNA damage can caused by transforming a free radical species hydrogen peroxide, guanine and 8-hydroxy guanine (Gutteridge, 1984). To investigate novel compounds derived from medicinal plants extracts a detailed study are performed to take under control oxidative DNA damage which cause cancer (Feig et al., 1994).

The safety and toxicity of synthetic antioxidants have been important concerns; therefore, attention has been focused on the use of natural antioxidants for inhibition or protection from oxidative damage. Synthetic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tertiary butyl hydroquinone (TBHQ), often used in foods to prevent oxidative degradation, are known to have toxic and carcinogenic effects on human health (Chan, 1987; Barlow, 1990). Therefore, substitution of synthetic antioxidants by natural and the screening of plant species for identifying new antioxidants have become critically important in recent years (Elzaawely et al., 2005). For that reason, many researchers have focused on natural antioxidants and in the plant kingdom numerous crude extracts and pure natural compounds were formally reported to have antioxidant properties (Rajeshwar et al., 2005).

CHAPTER TWO

LITERATURE REVIEW

In the previous study, they isolated xanthatin from the fruits of *X. strumarium*, as MRSA inhibitors. MIC of xanthatin against twenty strains of MRSA and seven strains of MSSA (methicillin-susceptible *S. aureus*) was between 7.8-15.6 μg/ml they described xanthatin as an active compound with IC_{50} in 3.0, 2.2 and 1.5 μg/ml against MCF-7, A431 and HepG2 cell lines (Sato et al., 1997).

In another study the antifungal activity of xanthatin derivatives was also investigated. Tomentosin, xanthumin and 8-epi-xanthatin, isolated from the aerial parts of *X. strumarium*, showed antimalarial activity against chloroquine-resistant Plasmodium falciparum strain (Joshi et al., 1997).

An analysis of the available scientific research on botanically derived compounds that have potential efficacy in the treatment of lung cancer. The mechanisms of activity reviewed include alkylating agents, topoisomerase poisons, DNA synthesis inhibitors, protein synthesis inhibitors, immunoceuticals, and lipoxygenase inhibitors. Selection criteria include: (1) products whose activity have at least minimal scientific confirmation - preclinical (in vitro, in vivo) or clinical; (2) products with a well-defined chemical composition; or (3) products with a well-known or scientifically plausible mechanism of activity (Ancuceanu and Istudor, 2004).

The synthesis of new xanthanolide derivatives is reported starting from xanthatin, a STL isolated from *Xanthium macrocarpum* (Asteraceae). In vitro evaluation of their antifungal activity has been investigated. The antifungal activity of xanthatin (1) and its reduced derivatives (2, 3 and 8) was evaluated in vitro against three strains of fungal

species, *Candida glabrata*, *C. albicans* and *Aspergillus fumigatus*. (1 and 8) revealed an antifungal activity similar to the positive control (amphotericin B) (Pinel et al., 2005).

Their study suggests that the inhibitions of iNOS, COX-2 expression, and TNF- ∝ release by the methanol extract of the semen of *X. strumarium* L. are achieved by blocking NF- k k B activation, and that this is also responsible for its anti-inflammatory effects (Kim et al., 2005).

Collaborative research programme between the Council for Scientific and Industrial Research (CSIR) in South Africa and the National Cancer Institute (NCI) in the USA aimed at the screening of plant extracts and identification of potentially new anticancer drug leads was initiated in 1999 Plant extracts that exhibited anticancer activity against a panel of three human cell lines (breast MCF7, renal TK10 and melanoma UACC62) at the CSIR were screened by the NCI against sixty human cancer cell lines organized into sub panels representing leukaemia, melanoma and cancer of the lung, colon, kidney, ovary and central nervous system. *X. strumarium* one of these plants were dichloromethane and methanol extracts of its stems were tested at a single concentration (100 ppm) found as moderate activity (6,25 ppm< TGI <15 ppm) (Fouche et al., 2006).

In this study many medicinal plants have been tested for anticancer activity such as antofine, acutiaporberine, etc. The compounds which have been isolated from these medicinal herbs include a variety of alkaloids, triterpenoids, flavonoids, terpenoids, and polysaccharides. Some of the formulas used in clinical tests have been combined with chemotherapy. Traditional Chinese Medicine (TCM) formulae such as shenyi capsule and Aidi injection contain numerous ingredients so that they may be considered to have anti-lung cancer activity as well as serving as immunomodulators. They have been demonstrated to ameliorate or prevent adverse effects as a result of the use of single drugs. Research on Chinese herbal medicine for the treatment of lung cancer has not only been shown to affect lung cancer, but also important to provide methods for the study of lung cancer therapy. This report reviews some of the findings resulting from the use of Chinese herbal medicine in the treatment of lung cancer (Zhou et al., 2008).

The aim of this work was to evaluate the antimicrobial activity of *X. strumarium L*. leaf extracts against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Clostridium perfringens*, as well as to investigate the presence of the toxic compound carboxyatractyloside in different plant parts. *S. aureus* and *C. perfringens* were more sensitive to non-polar than to polar fractions, and there was no difference between extracts for the remaining bacteria. All extracts had strong antimicrobial activity against the evaluated microorganisms. Carboxyatractyloside was found in cotyledons and seeds, but not in adult leaves and burrs. Thus, only *X. strumarium* leaves in adult stage can be used for medicinal purposes (Scherer et al., 2009).

The isolating of four xanthanolides from $CHCl_3$ extract of the leaves of *Xanthium italicum* : xanthatin , 4-epixanthanol , 4-epi- isoxanthanol , and 2-hydroxyxanthinosin were evaluated for their antiproliferative activities, and were demonstrated to exert significant cell growth inhibitory activity against human cervix adenocarcinoma (HeLa), skin carcinoma (A431), and breast adenocarcinoma (MCF7) cell (Kovácsa et al., 2009).

The objective of the present work was to evaluate the biological activities of the major bioactive compound, xanthatin, and other compounds from *X. strumarium* (Asteraceae) leaves. Inhibition of bloodstream forms of *Trypanosoma brucei brucei* and leukaemia HL-60 cell proliferation was assessed using resazurin as a vital stain. Xanthatin was found to be the major and most active compound against *T. b. brucei* with an IC_{50} value of 2,63 mg/ml and a selectivity index of 20. The possible mode of action of xanthatin was further evaluated. Xanthatin showed antiinflammatory activity by inhibiting both PGE2 synthesis (24% inhibition) and 5-lipoxygenase activity (92% inhibition) at concentrations of 100 mg/ml and 97mg/ml, respectively. Xanthatin exhibited weak irreversible inhibition of parasite specific trypanothione reductase. Unlike xanthatin, diminazene aceturate and ethidium bromide showed strong DNA intercalation with IC_{50} values of 26,04 mg/ml and 44,70 mg/ml, respectively. Substantial induction of caspase 3/7 activity in MIA PaCa-2 cells was observed after 6h of treatment with 100 mg/ml of xanthatin. All these data taken together suggest that xanthatin exerts its biological activity by inducing apoptosis and inhibiting both PGE2 synthesis and 5-lipoxygenase

activity thereby avoiding unwanted inflammation commonly observed in diseases such as trypanosomiasis (Nibret et al., 2011).

Index of ethanolic leaves extract of *X. strumarium* (ELEXS). In vitro antiplasmodial activity of ELEXS has been evaluated through Schizont inhibition assay. The cytotoxicity of plant extracts has been determined through standard colorimetric methyl thiazole tetrazolium (MTT) assay by using Hela cancer cell line. ELEXS has been found to exert significant in vitro antiplasmodial activity with an IC_{50} 4 µg/ml and cytotoxicity with an IC_{50} 40 µg/ml. The selectivity index was found to be 10, which offers potential safer therapy. The significant in vitro antiplasmodial activity and high selectivity index stresses the need to isolated active antiplasmodial components and characterize their exact mechanism of action (Chandel and Bagai, 2012).

The ethyl acetate fraction (8,03 g) and methanol extract (12,5 g) on repeated columns chromatography over Si-gel and sephadex LH20 afforded Caffeic acid, Xanthiazone, and Xanthiazone-(2-O-caffeoyl)-b-D-glucopyranoside, The identification of these compounds was made by concerted use of 1D- and 2D-NMR, Mass, UV and IR spectroscopy and chemical methods (Pandey and Rather, 2012).

They were found that the whole plants as well as leaves of *X. strumarium* are used by hakims as a blood purifier and in scabies. The plant is rich in potassium and containing flavonoids, catechol, alkaloids, cardenoloids and many free amino acids (Bhogaonkar and Ahmad, 2012).

The antimicrobial potential of alcoholic extract and various fractions of *X. strumarium* (XS) and *Duchesnea indica* (DI) against different strains of bacteria and fungi was investigated. The chloroform fraction from *X. strumarium* was found to be the most active among the fractions, showing good activity against *Escherichia coli*, *Shigella flexneri*, *Bacillus subtilus* and *Staphylococcus aureus*. Interestingly, most of the activity detected was against gram-positive (*S. aureus*) bacteria. The chloroform fraction exhibited significant antibacterial activity (19 mm zone of inhibition) against gram negative (*S. flexneri*), bacteria. Promising level of antifungal activity was observed in all fractions against *Aspergillus flavus*, *Fusarium solani* and *Microsporum canis*. Looking

at these results it may be concluded that XS and DI can be a potential source for activity guided isolation of lead compounds with antimicrobial properties (Khuda et al., 2012).

In this work the researchers, isolated a phytotoxin—Xanthinosin—from fruits of X. italicum, which exhibited phytotoxic activity on four test plants (*Amaranthus mangostanus* L., *Lectuca sativa* L., *Triticum aestivum* Linn, *Lolium multiforum*) and the possibility of utilizing xanthinosin as an ecofriendly herbicide was discussed (Shao et al., 2012).

Previously, they reported that (-)-xanthatin, a naturally occurring xanthanolide present in the cocklebur plant, exhibits potent anti-proliferative effects on human breast cancer cells, accompanied by an induction of the growth arrest and DNA damage-inducible gene 45(GADD45), recognized recently as a novel tumor suppressor gene. However, the mechanisms mediating this activation were unknown (Takeda et al., 2013).

In addition to the anti-inflammatory response, sesquiterpene lactones like xantholides have been found to sensitize tumor cells to conventional drug treatments. This review explores the varied ecological roles of sesquiterpenes in the plant producer, depending upon the plant and the compound. Some sesquiterpenoid lactones are antimicrobial, disrupting the cell wall of fungi and invasive bacteria, whereas others protect the plant from environmental stresses that would otherwise cause oxidative damage. The implications of sesquiterpenoid lactone qualities for future crop production are discussed (Chadwick et al., 2013).

X. stramarium and *Psoralea corylifolia* extracts in combination with UVA1 irradiation suppressed cell proliferation and induced apoptosis of keloid fibroblasts. Furthermore, the XAS and PSC in combination with UVA1 irradiation inhibited TGF-β1 expression and collagen synthesis in keloid fibroblasts (Park et al., 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1. MATERIALS

Table 3.1: The Materials That Used In The Work

MATERIAL	COMPANY	ORIGIN
L-glutamine	Thermo scientific-Hydon	Germany
Penicillin/ streptomycin	Biological Industries	Turkey
Fetal Bovine Serum	Sigma-AppliChema	Germany
MTT	Sigma-Aldrich	USA
DMEM	Sigma-Aldrich	USA
Tris base	AMRESCA	USA
Boric acid	Scharlu	Spain
SDS	Sigma-Aldrich	USA
EtBr	Sigma-AppliChema	Germany
Agarose	Sigma-Aldrich	USA

pBR322 DNA	Vivantis	USA
Triton X100	Sigma-AppliChema	Germany
DMSO	Sigma	USA
Ethanol	Sigma- Aldrich	USA
Tripsin EDTA	Sigma-Life Science	USA
Trypan blue solution	Roche	Germany
HCl	Biolab	Turkey
PBS	Gibco	UK
H_2O_2	Biolab	Turkey

3.2. EQUIPMENTS AND APPARATUS

Table 3.2: The Equipments and Apparatus

EQUİPMENTS AND APPARATUS	COMPANY	ORIGIN
Micropipettes (1-10, 10-100, 100-1000 µl)	Thermo Scientific	Germany
Micropipettes tips (10, 100, 1000 µl)	Isolab	Germany
Multichannal pipette	Eppendorf	USA

96-Well Plate	Plasma Indicator	Turkey
Refrigerator (-20 °C)	Vestel	Turkey
Refrigerator (+4 °C)	Vestel	Turkey
Vortex	Lab Companion	Korea
UV Apparatus	BLX-254	France
Distillator	Microline	Turkey
Incubator	Memmert	Germany
Sensitive Balance	Denver Instrument	Germany
Autoclave	Hirayama	Japan
Microwave Oven	Archelik	Turkey
Spectrophotometer	Thermo Scientific	Germany
Invert Microscope	Nikon	Japan
Electrophoresis	Cleaver Scientific	Germany
Gel Doc™ EZ Imaging System	Bio-rad	Germany
Magnetic Stir Plate	Biolab	Turkey
Magnetic Stir Bar	Biolab	Turkey
Filter 25/0,45 µMm	Agilent Technologies Econofilter	Germany
Hood MSC- Advantage	Thermo scientific	Germany
Cooling Centrifuge	Universal 320R-Hettich Zentril	Germany

3.3. METHODS

3.3.1. Materials Preparations

3.3.1.1. SDS 20% (w/v) (Sodium Dodecyl Sulfate)

We took 25 g from SDS then dissolved in 250 ml dH_2O and 207.5 µl from HCl 37% was added then mixed with magnetic stir plate in existence of magnetic stir bar. Stored at room temperature (Hames and Rickwood, 1990).

3.3.1.2. Triton X-100 10%

From Triton 100 µl was dissolved in 10 ml dH_2O and mix very well, if it need, we can use the bath water (37 ºC) to dissolve and kept the solution in +4ºC (Goldstein and Blecher, 1975).

3.3.1.3. MTT (5mg/ml) (Thiazolyl Blue Tetrazolium Bromide)

We were taking 0.25 mg from the MTT powder and dissolved in 5ml from PBS, then mixed very well then filtrate by 25/0.45µMm filter. Stored in dark at +4°C (Mosmann, 1983).

3.3.1.4. Ethidium Bromide (0.5 µg/ml)

The working solution (0.5 µg/ml) was prepared by taking 10 µl from EtBr stock solution (10 mg/ml) in 200 ml dH_2O. Both the stock and working solutions were kept in dark at room temperature (Severini and Morgan, 1991).

3.3.1.5. TBE 10X

We mixed the following components Tris base 108 g, boric acid 55 g and EDTA (pH 8,0) 40 ml to make 1 liter 10X TBE then autoclaved for 20 min and stored at room temperature (Rolland-Valognes, 2004).

3.3.1.6. Agarose gel

To prepare 1.25% agarose gel, we added 1.25 g agarose to 100 ml of 1X TBE. The gel solution placed in the microwave oven then boil and swirl the solution until all of the small translucent agarose particles are dissolved.When the molten agarose cooled to 60 °C we added 0.25µl from ethidium bromide to the solution before pouring an agarose gel on the tray then let the gel to be cool at room temperature (Pharmacia LKB Biotechnology, 1991).

3.3.2. Plant Extraction

The burs of *X. strumarium* plant was collected from Kilis-Sogutludere province, in November, 2014 and dried according to the herbarium techniques. The plant was identified by Assic. Prof. Dr. Mustafa PEHLIVAN faculty member at Nurdagı MYO Gaziantep University. The plant extraction and isolation of the two materials have be done in guidance by Prof. Dr. Ahmet CAKIR faculty member of Kilis 7 Aralık University. The healthy burs from fruit have been washed with tap water before used and dried in a cool and shade environment then pulverized into powder. Dried and powdered burs (1600 g) were placed on shaking water bath for 5 days and the acetone extraction was done. We placed the resulting extract in the rotary evaporator at 40º C to remove aseton by evaporation method in the end of this work we found 129,03 g liquid and the extract was stored at +4 ° C (Handa et al., 2008).

Xanthatin and xanthinosin were isolated from the crude acetone extract of *X. strumarium*. The two materials were isolated by taking 5 g from the extract which subjected to silica gel column chromatography, and the chromatography outcome was about 1 g. Its chemical structure was illuminated with UV, IR, ^{1}H-NMR, ^{13}C-NMR and ^{2} D-NMR spectroscopic methods. For isolation greater amount of this illuminated structure thin layer chromatography (TLC) was used. The materials spots in TLC were visualized by UV_{254} and UV_{366} nm. For this purpose, the column (2.5 x 70 cm) was filled with 250 grams of silica gel (70-230 mesh) that suspended with chloroform-ethyl acetate (8:2) solvent. The crude acetone extract dissolved in a sufficient hot volume of chloroform-ethyl acetate (8:2) then was applied as drop by drop to the column.

Chloroform-ethyl acetate (8:2) were continued with the mobile phase to elution and the fractions were collected in 50 ml volume. Purity of collecting fractions was checked and determined by TLC and chloroform-ethyl acetate (8:2) solvent system. These fractions were diveded according to their sizes. Then to separate the materials we used chloroform- ethyl acetate (9,5: 0,5) then chloroform- ethyl acetate (9:1). The result of this mothod were 61,8 mg from xanthatin and 2,02 g from xanthinosin (Wagner and Bladt, 1996).

3.3.3. Cell lines and culture

Two cancer cell lines, adenocarcinoma human alveolar basal epithelial cells A549 and non-small cell lung carcinoma cells H1299, were kindly provided by Prof. Dr. Engin ULUKAYA at the University of Uludag, Faculty of Medicine, Department of Medical Biochemistry. These cell lines were cultured in DMEM containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L- glutamine and kept in an incubator at 37°C and 5% CO_2. When the cells grew to reach about 90% confluence, they were detached from the culture dish with 0.5 ml trypsin-EDTA, to ensure the removal of any residual media, tap the flask, then incubated at 37º C, for approximately 4 minutes, until all of the cells detached from the inside surface of the flask. The trypsin was deactivated by adding an equal volume of complete media to the flask (3-4 ml). The cell suspension was removed from the flask and placed in a sterile universal container and centrifuged at 8000 r.p.m. for 5 minutes. The supernatant was then discarded from the universal container and the pellet was suspended in complete medium. A cell count was performed manually by trypan blue and chamber slide and then an aliquot of cells was used to reseed a flask at the required density (Langdon, 2004).

3.3.4. Cytotoxicity assay (MTT)

We weighed 0.1 g from xanthatin and xanthinosin then dissolved it in 1 ml from DMSO, after complete dissolving, 10 ml DMEM was added. 100 µL of the cell suspension was seeded on a flat bottom 96-well plate with a concentration of 5×10^3 cell/well from A549 and H1299 cell lines, respectively, and incubated for 24 hr. at 37°C and 5% CO_2. After incubation, serial dilutions of xanthatin and xanthinosin were done for each well, except

the control wells (MI-maximum inhibition, MO-maximum growth, Blank). The plates were incubated for 48 hr. Then 25µl from MTT was added to the all the wells. The plates were incubated for 4 hr. before addition of 100 µL solubilization solution (10% SDS) and mix thoroughly. After 18 hr. incubation, the absorbance at 570 nm was measured for each well by spectrophotometer. The absorption intensities were averaged and normalized to the untreated cells (negative controls) to generate the cell viability (Carmichael et al., 1987).

Data Analysis

The relative viability of the treated cells as compared to the control cells was expressed as the % viability, using the following formula (Mosmann, 1983):

Cell viability (%) = Sample absorbance /Control absorbance *100

3.3.5. DNA Protection Activity

pBR322 plasmid DNA (Vivantis) was used for detection the activity of xanthatin and xanthinosin for DNA protection from UV-induced damage and oxidation. Plasmid DNA has been subjected to damage in the presence of the materials by applying H_2O_2 and UV. We used UV Transsidelight (DNR-IS) equipment that generating wavelength 302 nm and light intensity 8,000 µw/cm and the room temperature was used as light source. After electrophoresis administration in gel documentation system of 1,5% agarose gel and 100 Volt 100 minutes, images were obtained by displaying (DN-IS, minibis Pro). In this test the UV and H_2O_2 (2.5 mmol/ L) were used as the control before application pBR322 plasmid DNA. According to the method determined by Russo et al. (2000), the imaging was performed in 1,5% agarose gel.

For preparation of 0,1% concentration stock, respectively by, weighing 0,1 mg from xanthatin and xanthinosin then 100 µL DMSO was added over them. Dilution process was realized after ensuring complete dissolution of the material. Dilution rate 1/5, 1/2,5 were made from this stock 0,1% xanthatin, xanthinosin solutions. In this work we used these dilutions for 1/5 dilution 40µl of dH_2O was added on 10µl from the stock. For

1/2,5 dilution 30µl from dH_2O was added on 20 µl from the stock. For this assay, experimental groups as shown below:

C1. Control: Plasmid DNA (3µl) + dH_2O (6µl)

C2. Control: Plasmid DNA (3µl) + dH_2O (6µl) + UV + H_2O_2 (1µl)

C3. Control: Plasmid DNA (3µl) + dH_2O (6µl) + H_2O_2 (1µl)

C4. Control: Plasmid DNA (3µl) + dH_2O (6µl) + UV

C5: Plasmid DNA (3µl) + DMSO (6 µl)

X1: Plasmid DNA (3µl) + 1/5 rate (0.02 mg/ml) xanthatin (5µl) + UV + H_2O_2 (1µl)

X2: Plasmid DNA (3µl) + 1/2.5 rate (0.04 mg/ml) xanthatin (5µl) + UV + H_2O_2 (1µl)

X3: Plasmid DNA (3µl) + 1/10 rate (0.1 mg/ml) xanthatin (5µl) + UV + H_2O_2 (1µl)

XS1: Plasmid DNA (3µl) + 1/5 rate (0.02 mg/ml) xanthinosin (5µl) + UV+ H_2O_2 (1µl)

XS2: Plasmid DNA (3µl) + 1/2.5 rate (0.04 mg/ml) xanthinosin (5µl) + UV+ H_2O_2 (1µl)

XS3: Plasmid DNA (3µl) + 1/10 rate (0.1 mg/ml) xanthinosin (5µl) + UV+ H_2O_2 (1µl)

(C: control groups; X: xanthatin groups; XS: xanthinosin groups)

We put 5µl from xanthatin dilutions 1/5, 1/2.5 of the X1 and X2 tubes, but we put 5µl from xanthatin stock (without dilution) to X3 tube. In the same way we put 5µl from xanthinosin dilutions 1/5, 1/2.5 to XS1 and XS2 tubes then we put 5µl from the xanthinosin original (without dilution) stock to XS3 tube. To the all tubes we added 3µl pBR322 DNA plasmid (172 mg. µl) and 1µl from 30% H_2O_2. Whereas we put just 3µl pBR322 DNA plasmid and DMSO in C5 tube. The tubes that containing xanthatin and xanthinosin, in addition to the control tubes C2, C4 were placed in the UV device for 5 minutes. After exposure to UV add 2 µl load dye to all tubes then we loaded all tubes contents on the 1.25% agarose gel for 100 minutes.

CHAPTER FOUR

THE RESULTS

4.1. Cytotoxicity Results

4.1.1. Effects of Xanthatin on H1299:

When applying the MTT assay were used the following concentrations from xanthatin on the H1299 cell line respectively (6.25, 12.5, 25, 25,50 and 100 µg/ml). As shown in Figure (4.1).

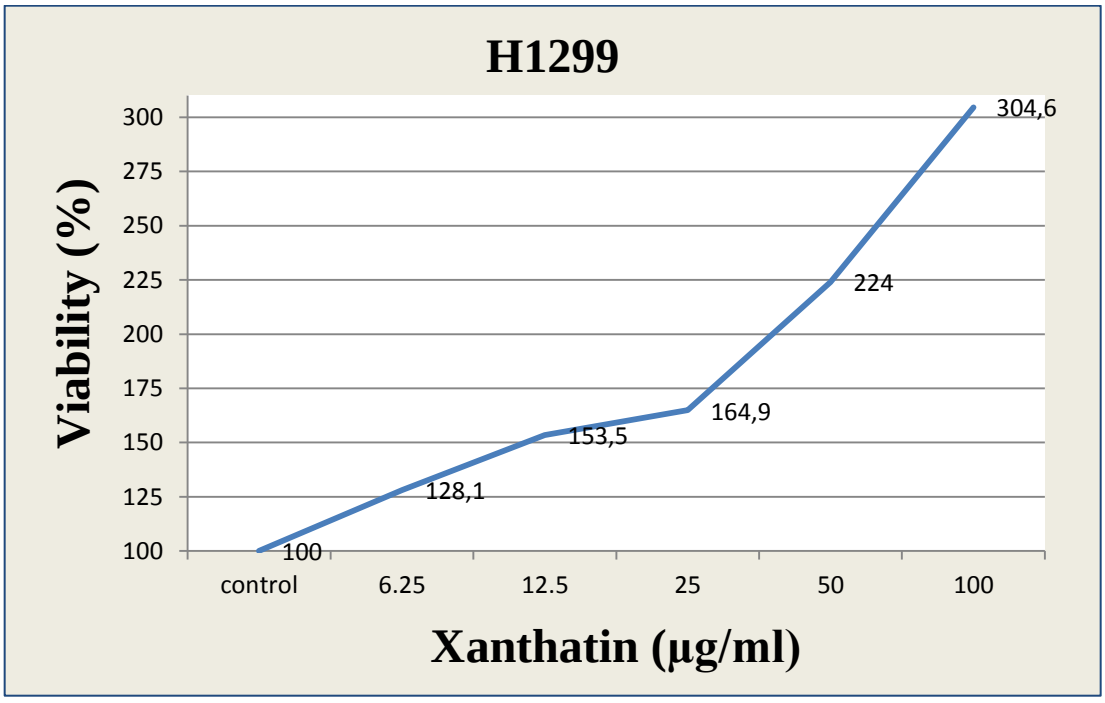


Figure 4.1: Viability of H1299 According to Different Concentrations of Xanthatin

4.1.2. Effects of Xanthatin on A549:

In application of MTT on the A549 cell line that treated with (6.25, 12.5, 25, 25,50 and 100 µg/ml) concentrations of xanthatin for 48 hr. viability was decreased slightly in low concentrations (6.25 and 12.5). As shown in Figure (4.2):

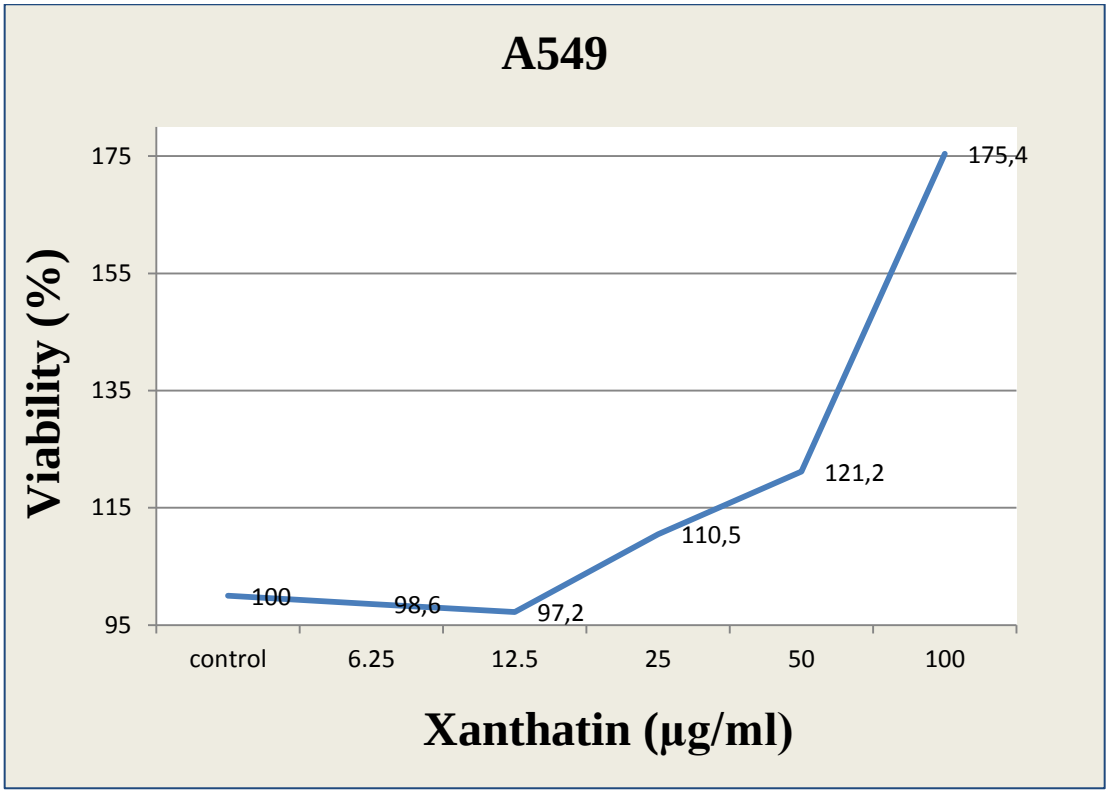


Figure 4.2: Viability of A549 According to Different Concentrations of Xanthatin

4.1.3. Effect of xanthinosin on H1299 Cell Line:

When applying the MTT assay on the same concentrations were used. Viability ratios were found in minor decreasing in all concentrations. As shown in Figure (4.3):

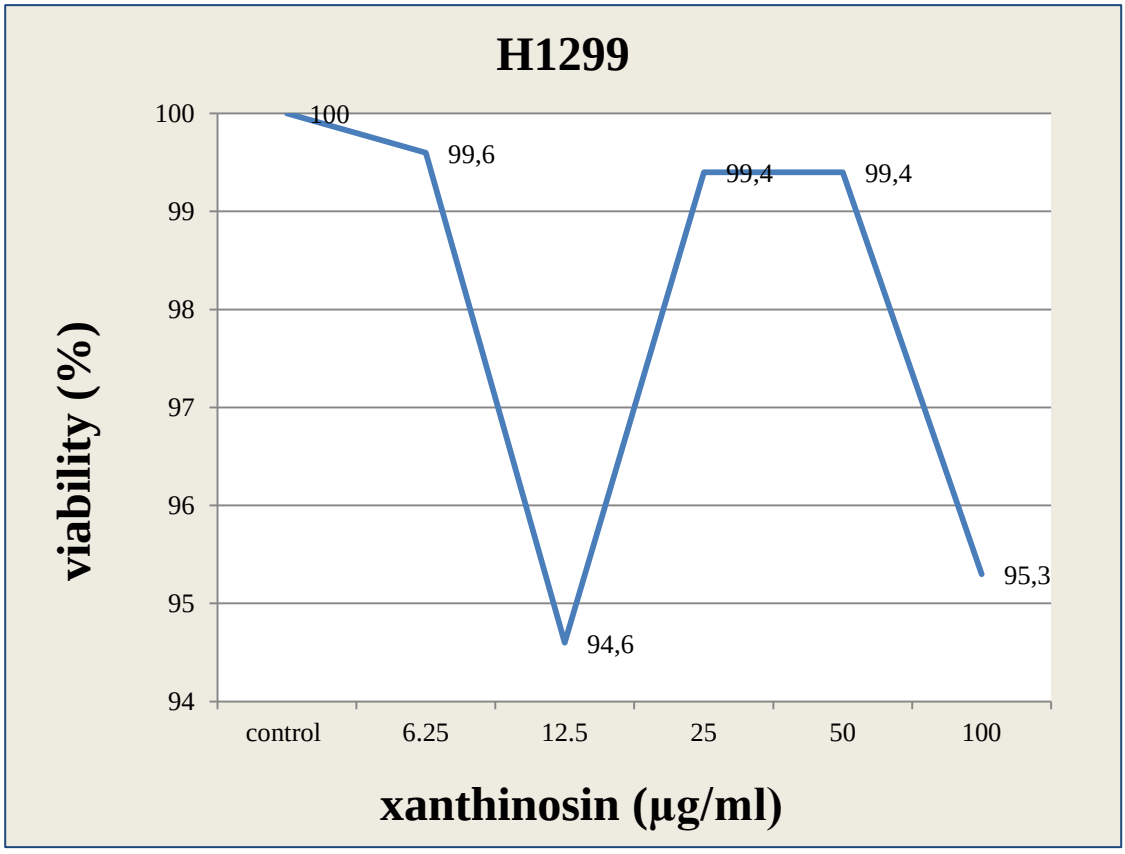


Figure 4.3: Viability of H1299 According to Different Concentrations of xanthinosin

4.1.4. Effect of xanthinosin on A549 Cell Line:

xanthinosin applied in same concentrations was found to be effective at high doses (50-100 µg/ml). It inhibited the cell viability at a 16.2 % rate. As shown in the Figures (4.4):

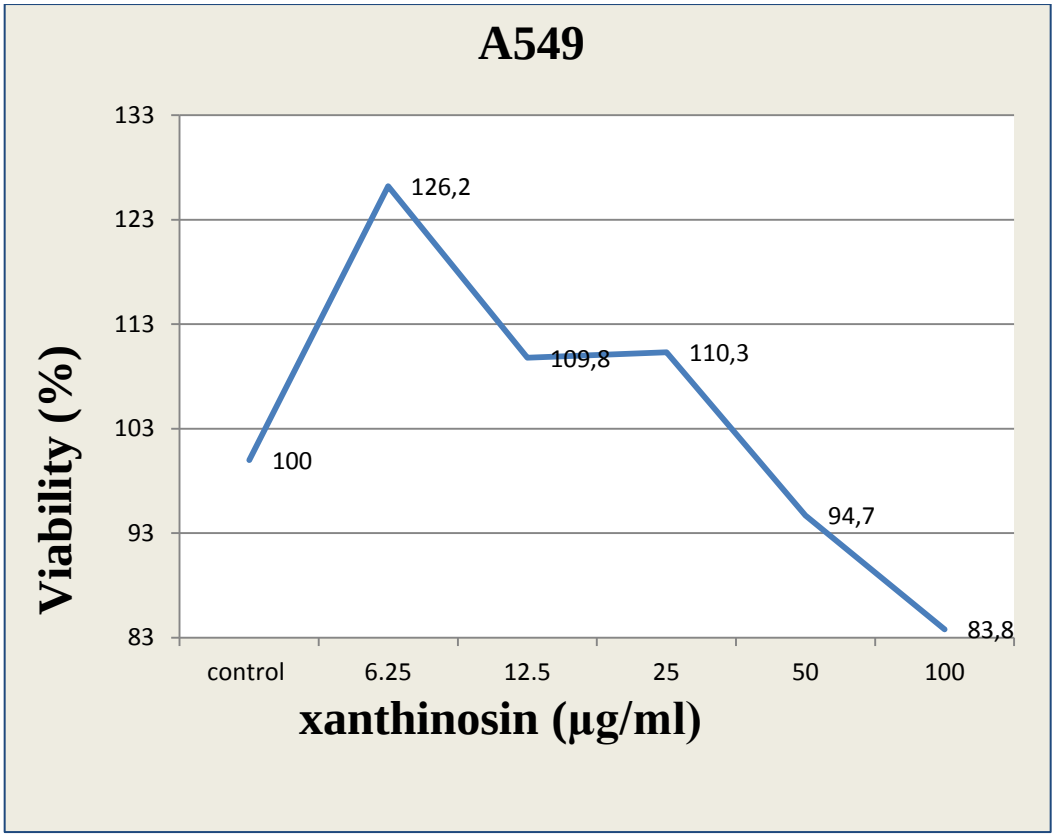


Figure 4.4: The Viability of A549 Cell Line Treated with Xanthinosin

4.2. DNA Protection Assay Results:

The DNA protective activity of xanthatin and xanthinosin obtained from the leaves of *X. strumarium* were tested by using pBR322 DNA plasmid. According to this method, the ability of xanthatin and xanthinosin which is a sesquiterpene from lactones that not cause DNA damage was evaluated in the presence of the UV rays and H_2O_2. Xanthatin and xanthinosin was determined to be dose-dependent protective against the damaging effects on DNA (Figure 4.5). An 0.1 µg/ml doses from xanthatin and xanthinosin which was determined to be a DNA protective activity, while the other doses 0.02 mg/ml and 0.04 mg/ml were found to not be protective of DNA.

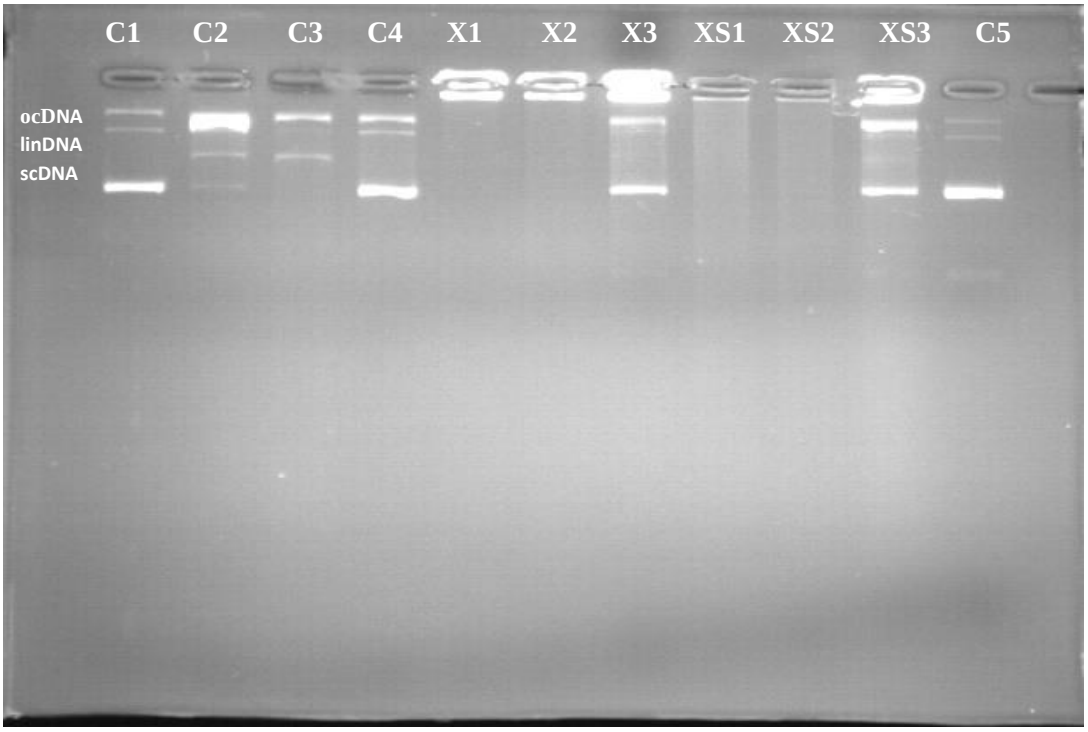


Figure 4.5: UV Protection Activity of Xanthatin and Xanthinosin

(C: control; X: xanthatin; XS: xanthinosin).

CHAPTER FIVE

DISCUSSION AND CONCLUSION

The search for medicines, which undoubtedly began in prehistorically times, has led to compounds such as morphine, atropine, tubocurarine, digoxin, quinine and artemisinin like compounds (Rosenthal, 2003; Chandel and Bangui, 2010). According to the World Health Organization (WHO), 25 percent of modren medicines are made from plants that were previously used in traditional medicine (WHO, 2008).

Xanthanolides, a bicyclic subtype of STL, a lot of xanthanolide lactones have been isolated from *Xanthium* plants, and many of them have been proven to have a variety of biological activities including antimicrobial, anti-inflammatory, anti-ulcerogenic, antitumor and plant growth regulatory activity (Irving et al., 2007; Yoon et al., 2008).

Two human lung cancer cell lines, H1299 and A549, were used in this study to investigate the cytotoxicity of xanthatin and xanthinosin against cancer cells over time. We can say that the increase in absorbance lead to an increase in viability. The data were obtained from three independent biological replicas, presented as mean values relative to the untreated, negative control cells. Viability of H1299 cell lines that treated with xanthatin was increasing by the high concentration of xanthatin but all of it was higher from control viability. The outcome of viability of A549 cell line that treated with xanthatin was less than the control viability (98.6% and 97.2%) in the concentrations (6.25, 12.5 μg/ml). The viability of all H1299 cell lines treated by xanthinosin was less than the control viability, but the best outcome was on (12.5 and 100 μg/ml) doses, it was respectively (94.6% and 95.3%). The viability of A549 cell line treated by xanthinosin for all concentrations was decreased with increases in xanthinosin concentrations where the low viability was (94.7% and 83.8%) in the doses (50 and 100 μg/ml). That lead us to the conclusion the best results of cytotoxicity (16.2 %) obtained from xanthinosin in (100 μg/ml) concentration. These data suggest xanthatin and

xanthinosin were limited the cell viability and showed cytotoxicity in low degree, but it's not at the required level, that can be attributed to material concentrations that used in the methods.

In two previous studies, researchers reported parallel results to our results. Kovacs and colleagues (2009) showed that xanthatin had the highest activity with IC_{50} values of 8.00, 3.44 and 5.19 μM on the HeLa, A431 and MCF7 cell lines, respectively; its effect was close to that of the positive control, cisplatin. The results of another work showed that xanthatin led to apparent morphological changes in a dose-dependent manner. These changes became increasingly visible with the dose increased, but were absent in the control cells. They subsequently used the MTS assay to determine xanthatin effects on A549 cell growth at different intervals. The data showed that xanthatin had inhibitory effects on A549 cell growth both dose- time-dependently. After 12 h treatment, xanthatin at 5 μM inhibited cell growth significantly compared with the control ($p < 0.05$). The IC_{50} values of xanthatin inhibition of A549 cell growth at 12, 24 and 48 h were 36.2, 21.1 and 8.3 μM, respectively. Also the study demonstrated that xanthatin selectively induced G2/M arrest in A549 cells, leading to decreased cell viability and growth in a dose- and time-dependent manner. That suggested the antitumor properties of xanthatin were consistent with the criterion for developing selective agents for cancer treatment (Zhang et al., 2012).

Moreover, xanthatin showed strong gastric protective activity (Penissi et al., 2009) and exhibited little or no toxicity to animals, with an LD_{50} value of 800 mg/kg so they may be a highly effective inhibitor of tumor cell proliferation (Roussakis et al., 1994).

To confirm our findings, xanthatin and xanthinosin will be tested on other cancer cell lines also further activity evaluation will be done by in vivo studying.

There were no previous literatures about DNA protection activity of the extracts of *X. strumarium* or from xanthatin and xanthinosin. So our study considered as a first in this purpose.

UV photolysis and H_2O_2, produce OH radical which causes DNA cleavage. The antioxidants are neutralized or prevented the harmful effects of the free radical

molecules in the cell. Therefore plants containing antioxidant compounds may provide protection against DNA damage. The active substance must have a high antioxidant capacity for protecting DNA against the oxidative damage induced by hydrogen peroxide (H_2O_2) (Emen et al., 2009).

DNA derived from pBR322 plasmid showed two bands on agarose gel electrophoresis (C1), the faster moving band corresponded to the native form of supercoiled circular DNA (scDNA) and the slower moving band was the open circular form (ocDNA). The UV irradiation of DNA in the presence of H_2O_2 (C2) resulted in the cleavage of scDNA to linear form (linDNA), indicating that OH radical generated from UV photolysis of H_2O_2 produced DNA strand scission.

In our study, the addition of xanthatin (X1, X2, X3) and xanthinosin (XS1, XS2, XS3) in 0.1 mg/ml dose to the reaction mixture suppressed the formation of linDNA and induced a partial recovery of scDNA.

Our results revealed the DNA protective efficacy of xanthatin and xanthinosin, this property could be capitalized to develop another researches to get a clear results on having natural antioxidants for the prevention/control against to free radical that induced cancer. In addition, recent related studies based on the negative effects of substances contained in the sun protection cream continue to work (Pelle et al., 2002) and as a result, we believe that the need to alternative substances to use in cosmetics is an urgent need and these materials can be used for this purpose.

In the other works about DNA protection activity Kalita and others (2012), their observations confirm that aqueous extract *Lantana camara* L. (Verbenaceae) leaves have high antioxidant and DNA damage. Sharma and others (2011), they found on DNA protective effect of different fractions from the crude methanolic extract of *B. Variegate*. According to Ul-Haq and others (2012) promising results were obtained from DNA protection assay demonstrated that the crude methanolic root extract of *Euphorbia wallichii* has concentration dependent DNA protection properties.

Thus, the identification of natural products able to provide protection against UV radiation-induced inflammatory responses and the generation of oxidative stress may

have important human health implications. The DNA protection activity results demonstrated the strong antioxidant properties of xanthatin and xanthinosin which derived from *X. strumarium*.

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