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M.Sc. THESIS

IN VITRO ASSESSMENT OF CYTOTOXIC AND APOPTOTIC EFFECTS OF URTICA DIOICA AGGLUTININ ON DIFFERENT CELL LINES

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FOREWORD

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May 2016 **Tutku ÖZARPACI**

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF SYMBOLS AND ABBREVIATIONS

Symbol **Abrevation**

DNA :Deoxyribonucleic acid

EC :European Commission

FDA :United States Food and Drug Administration

FBS :Fetal Bovine Serum

PBS-T :Phosphate Buffered Saline Containing Tween 20

RPMI :Roswell Park Memorial Institute

ROS :Reactive Oxygen Species

UDA :Urtica Dioica Aglutunin

ÖZET

YÜKSEK LİSANS TEZİ

URTİCA DİOİCA AGLUTİNİNİN FARKLI HÜCRE HATLARI ÜZERİNE SİTOTOKSİK VE APOPTOTİK ETKİLERİNİN BELİRLENMESİ

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Günümüzde tamamlayıcı tıpta bitkisel tedavilerin önemi gittikçe artmaktadır. Isırgan otu olağanüstü bir lektin içermektedir.*Urtica dioica* bitkiler aleminde Urticaceae ailesinden izole edilen ilk lektindir ve moleküler yapısı diğer lektinlerden kesinlikle saygı duyulacak kadar farklılık gösterir. UDA bilinen en ufak (8.5kDa) lektin olmasıyla kalmayıp, ayrıca bitkilerde bulunan ilk tek zincirli lektindir. Lektinler multivalent karbohidrat bağlanma proteinleridir.Hücre yüzeyindeki glikanlara bağlanarak çeşitli biyolojik aktivitelere neden olurlar. Bu çalışmada, *Urtica dioica*' yı en güzel temsil eden içeriklerinden biri olan UDA'nın sitotoksik ve apoptotik etkilerini; farklı konsantrasyonlarda (5, 10, 20 µg/ml) ve farklı kanser hücre hatları olan, servikal kanser (HeLa), meme kanser (MCF-7) ve sağlıklı hücre olan fare fibroblast (L929) hatları üzerinde sırası ile MTT (kolorimetrik hücre yaşayabilirliği testi), xCelligence (gerçek zamanlı hücre sayımı), TUNEL (apoptotik test) testlerini sırasıyla uygulayarak araştırdık. Sonucunda tüm bu süreçler toplu olarak; UDA'nın en yüksek konsantrasyondaki (20 µg/ml) uygulanışının hücre hatları üzerinde 24 saatlik süreç sonrasında apoptotik ve hücre büyümesini durdurucu etkileri olduğunu gösterdi. Bu yüzden; bizim UDA hakkındaki güncel bilgimiz, UDA'nın gelecekte kanser tedavi stratejileri arasında kullanılabileceğini doğruluyor.

Mayıs 2016, 48 Sayfa

Anahtar kelimeler: Isirgan Otu, Lektin, L929, HeLa, MCF-7, sitotoksisite, apoptoz.

SUMMARY

M. Sc. THESIS

IN VITRO ASSSESSMENT OF CYTOTOXIC AND APOPTOTIC EFFECTS OF URTICA DIOICA AGGLUTININ ON DIFFERENT CELL LINES

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In the world, there is incomparably increasing interest in medical botanicals as part of complementary medicine. Rhizomes of stinging nettle contain an extraordinary lectin. In Urticaceae family, *Urtica dioica* agglutinin is the first isolated lectin and because of the molecular structure it is decidedly different from all other acknowledged plant lectins. UDA is not only the smallest (8.5kDa) lectin known at present, but also the first single chain lectin to be found in plants. Lectins are well known group of multivalent carbohydrate binding proteins that bind glycans of cell surface and mediate a variety of biological functions. In this study, we investigated the cytotoxic and apoptotic effects of one of a good representative ingredients of *U. dioica*, UDA, at different concentrations (5, 10, 20 μg/ml) on discrepant types of cancer cell lines which are human cervical cancer (HeLa), human breast cancer (MCF-7) and also non-cancer mouse fibroblast (L929) cell lines by carrying out MTT (colorimetric assay for assessing cell viability), xCelligence (real-time cell counter), TUNEL (apoptosis) assays, respectively. Very recently all together, these processes suggested that UDA in highest concentration, 20 μM, has antiproliferative and apoptotic effects on MCF-7, Hela cell lines after 24 hours. Therefore, our current information regarding UDA confirms that it may be used in cancer therapeutic strategies as a primary cure in the future.

May 2016, 48 pages.

Keywords: *Urtica Dioica*, *Urtica dioica agglutinin*, stinging nettle, L929, HeLa, MCF-7, cytotox

1. INTRODUCTION

Urtica dioica, is known by various vernacular names that are; stinging nettle, common nettle, big string nettle, urtiga, isirgan, ortiga and much more. *U. dioica* is an herbaceous perennial flowering plant that is belonging to Urticacea plant family. It has opposite, cardate, deeply serrate, pointed leaves which are downy underneath. Stinging nettle thrives in damp and nutrient rich soil. This plant widely grown through the temperate region. Also, it is adapted to a wide range of climatic conditions. *U. dioica* is a native plant of Europe and Asia (Borchers et al., 2000). Also, it is overspread in North and South Africa, India, China, New Zealand, Australia, North and South America (Otles and Yalcin, 2012). Additionally, stinging nettle grown in Turkey and it has reach chemical composition in Black sea region.

There is significantly increasing interest in medical botanicals as part of complementary medicine in the world. It has become a source of folk medicine for the treatment of many diseases. Stinging nettle has antioxidant, antimicrobial, antiulcer, and analgesic properties (Gulcin et al., 2004). In addition, the extract of stinging nettle shows *in vitro* inhibition of several key inflammatory events that cause the symptoms of seasonal allergies Also it has been used improve kidney function, helps prevent hemorrhoids, cleans the digestive tract, helps stomach problems, aids with diarrhea and dysentery. Stinging nettle contain A, C and D vitamins and minerals that are; iron, manganese, potassium, and calcium. Therefore, this herb is used for anemic children (Clapham et al., 2011).

The leaves, roots and seeds of *Urtica dioica* contain different chemical compounds. Well known constituents of *U. dioica* are; rutinosides, quercetin, caffeic acid, caffeoylmalic acid, chlorogenic acid, flavonoids, glucosides, kaempferol, isorhamnetin, neochlorogenic acid, scopoletin, polysaccharides, fatty acids, glycoproteins, histamine, lectins, UDA, tannins, essential oils, vitamin A, B1, B2, C, K1, acetic acid (Borchers et al., 2000; Mahmoud et al., 2005).

A rhizome conventionally rootstalk are underground horizontal stems. They are vegetative storage organ of perennials that amasses significant amount of both carbohydrate and proteins

when the upper parts of the plants undergo senescence (Pryme et al., 2006). Rhizomes stock their reserves at the beginning of the next growing season.

U. dioica roots are one of the most strategic options that respect on life promising cancer treatments (Ganzera et al., 2005). Only a few rhizome specific lectins have been isolated to date which are; *U. dioica* from stinging nettle and *Aegopodium podpraria* from ground elder (Pryme et al., 2006). UDA from stinging nettle rhizomes, is a small 8.5kDa monomeric protein. UDA is not one of the smallest phytohemagglutinin known at present, but also the first single chain lectin to be found in plants (Peumans et al., 1984). These protein structures induce a cascade of events at the end leading to cell activation processes, including cell proliferation, production of lymphokines and cell differentiation (Le Moal and Truffa-Bachi, 1988). It is a well-known fact that plant lectins are one of the most studied ones. Against this background, there is only one study has found about UDA on cancer. Based on this idea, the scope of this thesis is examined the cytotoxic and apoptotic effects of UDA at different concentration on two cancers and one standard cell line in vitro.

1.1 SCIENTIFIC CLASSIFICATION OF URTICA DIOICA

Urtica dioica stands for Urticales, fall into the class Magnoliopsida due to is dicotyledons. *U. dioica* is belongs to the family of Urticaceaea because of the members of the nettle family. According to researchers, they list 48 genera with 1050 species for Urticaceae (Coile, 1999). (See Table 1.1) *U. dioica* commonly termed stinging nettle is the best-known member of the nettle genus Urtica.

Table 1.1: Scientific classification of *Urtica dioica*

KINGDOM	Plantae
PHYLUM	Magnoliophyta
CLASS	Rosopsida
ORDER	Rosales
FAMILY	Urticaceae
GENUS	Urtica
SPECIES	*Urtica dioica*

1.2 DESCRIPTION OF URTICA DIOICA

In Latin, Urtica means “burn” and dioica means “two houses” that refers monoecious. Namely, they have both male and female genders in reference to their flowers. The flowers of stinging nettle look like inconspicuous drooping clusters that grow from leaf axils (Figure 1.a) *U.dioica* is a dioecious herbaceous perennial flowering plant with amspacious sympodial system of rhizomes and stolons, rooting at the nodes and that rise in leap to aerial shoots up to 1,5 - 2m or 3m (Kavalali et al., 2003).

The soft green leaves are 3 to 15 cm long (Figure1.b) and born oppositely (Figure 1.c). Leaves and stems covered with stinging hairs, which is called trichomes (Figure 1.d) that cause pain because of the histamine based. Also include several chemicals that are; acetylcholine, serotonin, moroidin, lekotrieves and formic acid. *U. dioica* is distributed itself to variety of regions which are rich in light, nutrients, moisture (Borchers et al., 2000). Stinging nettle burgeon from the ground are often reddish early to mid-spring and become green as they mature.

a)  **b)** 

Figure1.1: Male and female genders (a), and leaf of U. dioica (b)

c)  d) 

Figure 1.2: Opposite positions leaves (c) and Trichomes, stinging hairs of U. Dioica

1.3 DISTRIBUTION OF U. DIOICA

U. dioica are exhibited distribution of the world. They found in countryside. Also stinging nettle is found meadows as understory plant in wet environments. They prefer nutrient riches, plentiful sunlight and flourishes in temperate climates such as North and South Africa, India, China, Australia, New Zealand, North and South America, but not found in the tropics (Taylor 2009) (Figure 1.3). In Turkey, *U.dioica* is especially inhabits in Black Sea Region (Kultur, 2007)**.**

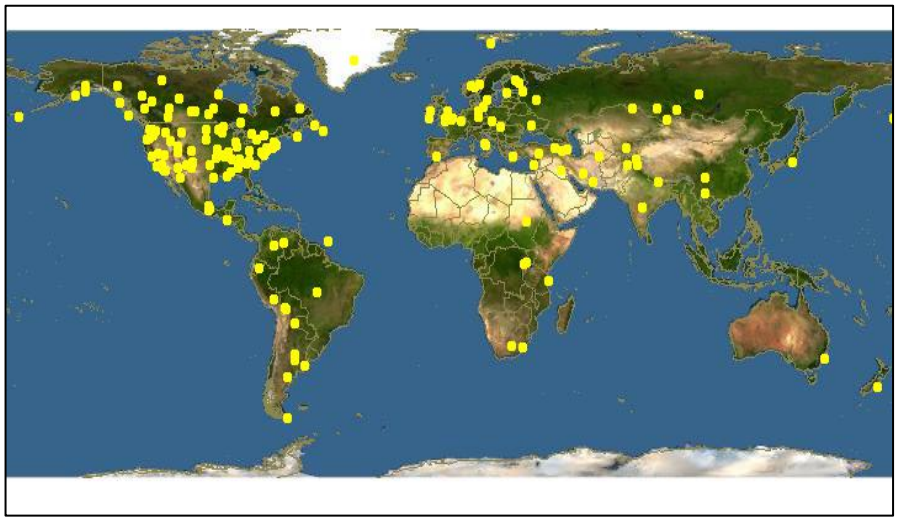

Figure 1.3: Distribution of U. dioica throughout the world

1.4 USAGE AREAS OF U. DIOICA

As predicted, there is a significantly increasing interest in medical botanicals as part of the complementary medicine in the world. *U. dioica* play important role to herbal cure and nutrition's addition to the diet which is easily digested. *U. dioica* is used as an alternative cure in gout, hair loss, mild bleeding, eczema, anemia, muscle and joint paints, rheumatism has been confirmed for many years. In today, *U. dioica* is contained into a number of herbal medicinal preparations (Ozen and Korkmaz, 2003).

Also, powerful antioxidant property of water extracts of nettle reveals free radical inhibition or scavenger activity as well as a primary antioxidant that reacts with free radicals, which may prominently restrict free radical damage occurring in the human body. In cardiovascular disease, platelet aggregation is shown to be markedly inhibited in animal studies (Gulcin et al., 2004).

Epidemiological studies indicate that the consumption of plant-based diet is strongly associated with a reduced risk of developing certain types of cancer (Roberto A. Ferriz-Martinezz et al., 2010). The plant generally has been used in soup, a tea made from leaves. Additionally, it is used such as a drug, food, fibrous, dye and cosmetic. Pharmacologic researches about nettle are increased day by day. It is noteworthy that *U. dioica* extract is used most of the cancer drug.

The best-researched pointer to the use of nettle roots for men with symptomatic benign prostatic hyperplasia. Thus, the root has been shown to have a beneficial effect upon enlarged prostate glands. The extract from roots of *U.dioica* has been succeed to use in clinical for the treatment of prostatic hyperplasia in Europe (Kavalali et al., 2003; Akbay et al., 2003; Guil-Guerrero et al., 2003).

1.5 THE CONTENTS OF *U. DIOICA*

U. dioica contains different chemical compounds. Well known constituents of *U. dioica* are, rutinosides, quercetin, caffeic acid, caffeoylmalic acid, chlorogenic acid, Flavonoids, glucosides, kaempferol, isorhamnetin, neochlorogenic acid, scopoletin, polysaccharides, fatty acids, glycoproteins, histamine, lectins, UDA, tannins, essential oils, vitamin A, B1, B2, C, K1,

acetic acid. The roots of *U.dioica* includes steroids, terpenoids, phenylpropanoids, lignans, coumorins, polysaccharides and lectins (Urtica dioica agglutinin) (Borchers et al., 2000; Mahmoud et al., 2005).

1.6 CANCER

Cancer is a term used for the general name for a group of more than 100 diseases in which abnormal cells divide without control and are able to invade other tissues or spread to other parts of the body through the blood and lymph systems. This spreading is called metastasis. Types of cancers always named based on the place where it started.

In 2012, there were 14.1 million new cancer cases, 8,2 million cancer deaths and 32,6 million people living with cancer in worldwide (Globacan, 2012). Incidence data quality of Turkey is C (high quality regional, coverage lower than 10%) in Global cancer atlas (Figure 1.4). In 2012, there were 58.715 cancer deaths in males and 33.111 deaths in females in Turkey.

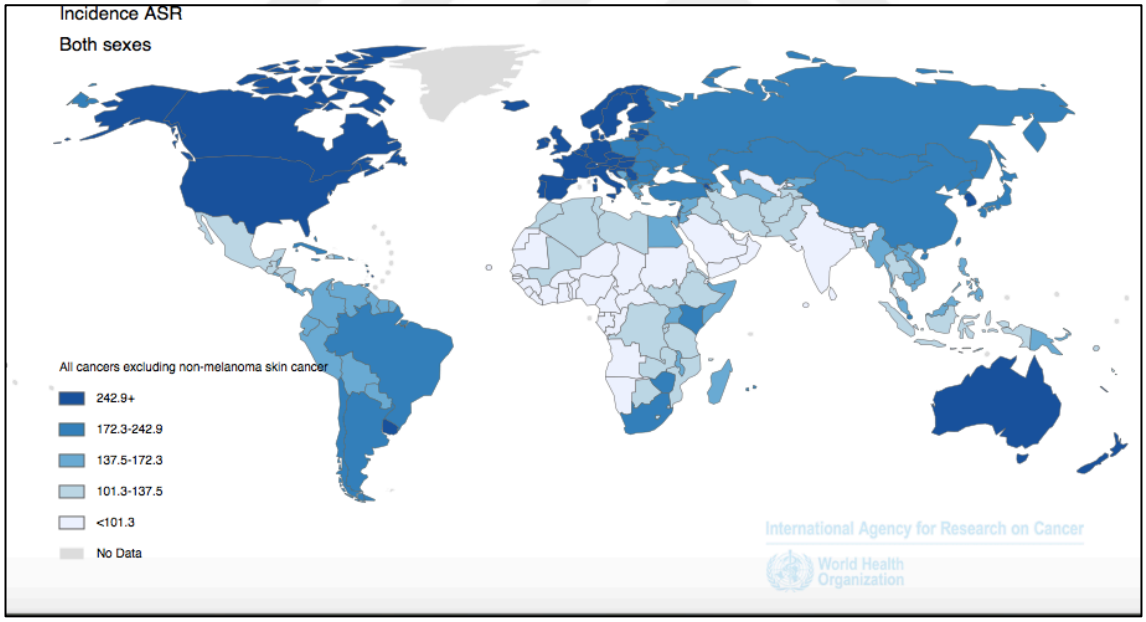

Figure 1.4: Cancer rates of World

1.6.1 Cancer Rates of Estimated New Cases and Deaths

In worldwide, breast cancer is the most frequently diagnosed cancer and the leading cause of cancer deaths in females. 23% of the total new cancer cases and 14% of the total cancer death in 2008. Lung cancer is the leading cancer site in males worldwide, comprising 17% of the total new cancer cases and 23% of the total cancer deaths (Figure 1.5) (Jemal et al., 2011). According to WHO report (2014) cancer incidence will increase in next two decades and 25 million new cancer case will appear (WHO, 2014).

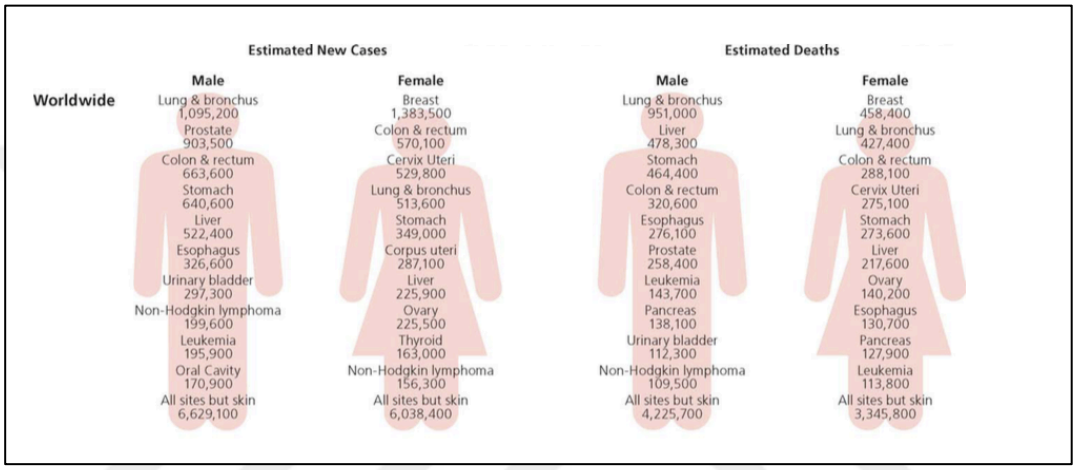

Figure 1.5: Total new cancer cases and deaths in males and females

1.7 LECTINS

Lectins are very important group of bioactive proteins that found in almost all organisms with the inclusion of plants, vertebrates, invertebrates, bacteria and viruses. Lectins are well known group of multivalent carbohydrate binding proteins that bind glycans of cell surface and mediate a variety of biological functions. Most of lectins contain two or more sugar binding sites and can agglutinate cells and/or precipitate complex carbohydrate conjugates (De Mejia and Prisecaru, 2005).

This binding gave birth to the word “lectin” which is come from “legere” which is Latin verb for “to select”. Lectins are the most widely studied molecules in glycobiology. All plant lectins are artificially classified together solely on the basis of their tendency and ability to bind specific sugars. Lectins have been isolated from various sources, such as bacteria, plants and

animals, and are grouped into five classes on the basis of their preferential binding to type of D-pyranose sugars (Ghazarian et al., 2011). Classification of plant lectins is based on different criteria. On the basis of the overall structure of the mature lectins they can be divided in four groups but analysis of the avaliable sequences distinguishes seven families of evolutionary related proteins (Table 1.2) (Roberto A. Ferriz-Martinezz et al., 2010).

Table 1.2: Plant lectins classification based on mature lectin structure

Lectin type	Definition
Merolectins	Single carbohydrate-binding domain, they are monovalent and hence cannot precipitate in glycoconjugates or agglutinate cells.
Hololectins	Contain at least two carbohydrate-binding domains that are either identical or very homologous and bind either the same or structurally similar sugars. They are di- or multivalent and hence agglutinate cells and/or precipitate glyco-conjugates.
Chimerolectins	They are fusion proteins consisting of one or more carbohydrate-binding domains and a well-defined enzymatic domain or another biological activity that act independently from the carbohydrate-binding domain. Depending on the number of carbohydrate-binding sites, chimerolectins behave as merolectins or as hololectins.
Superlectins	Consist of at least two carbohydrate-binding domains that recognize structurally unrelated sugars. They can also be considered a special group of chimerolectins.

One lectin molecule specific for external saccharides can also block intra cytoskeleton reactions, which are manifested as shape changes (Anderson et al., 2002). The interaction between lectins and their ligands are mainly achieved through a network of hydrogen bounds with added contributions from van der waals and hydrophobic interactions. Even small changes in the structure of the binding sites may result in significant changes in lectin specifityn and consequently in its biological activity (De Mejia and Prisecaru, 2005). Lectins are used as tools in the field of biochemistry, cell biology, and immunology as well as for diagnostic and therapeutic purposes in cancer research. Lectins are currently being employed as therapeutic agents in cancer treatment studies (De Mejia and Prisecaru, 2005).

The authors of more recent studies have proposed that; lectins have anticancer properties *in vitro, in vivo*, preferentially binding to cancer cell membranes or their receptors, causing

cytotoxicity, apoptosis, and inhibition of tumor growth. Lectins from different sources as cytotoxic or anti-tumoral agents inhibit cancer cell growth depending on lectin source, concentration and cancer type. First studies had focused on cytotoxic properties of lectins like Ricin (RCA) and Abrin (APA) for human cancer treatment. Con A from *Concanavalin ensiformis* showed tumor growth inhibition in hamsters. Mistletoe lectin (MC) is one of the most studied lectin in clinical trials and has demonstrated beneficial effects against cancer development. In studies on VAA (*Viscum album agglutinin)* which is used a complementary cancer therapy more than 80 years in Europe, showed cytotoxic activity. Also Chinese mistletoe exhibited significant effect on human T cells cytotoxicity, apoptosis and cytokine production (Roberto A. Ferriz-Martinezz et al., 2010). On one hand, WGA (wheat germ agglutinin) has shown on inhibitory effect on the rat pancreatic tumor cell line AR429.

Additionally, WGA significantly influenced the cell growth of several human breast cancer cell lines *in vitro* (MCF-7, T47D, HBL100, BT20). WGA proved highly toxic to human pancreatic carcinoma cells *in vitro* with high membrane binding. It's clear that many of lectins may cause different degrees of toxicity in cancer cell. In spite of not whole lectins are toxic (Valentiner et al., 2003). Also, authors of more recent studies showed that some lectins are highly allergenic. Because of the properties of some lectins as RIP, some studies have focused on using them production of immunotoxins against cancer cells, where the lectin is attached to monoclonal antibody, which has specific receptor site for tumor cells (Roberto A. Ferriz-Martinezz et al., 2010).

On the other hand, there are some lectins (PNA (*Phaseolus vulgaris*), GSA (*Griffonia simplicifolia*), Con-A (Concavalina A), WGA (*Triticum vulgare*), PNA (*Arachis hypogea*)) which are studied their effects on ; three colon cancer cell lines (Lovo, HCT-15, SW837) and proved the growth of cancer cells affected in different ways depending on the concentration and type of lectin tested (Lorea et al., 1997).

1.8 UDA

As has been reported previously that rhizomes of stinging nettle contain a striking amount of a lectin. UDA present oneself in rhizomes and in outer exodermises cell layer of roots in spite of

it is absent from leaves. UDA is the first lectin to be isolated from a member of Urticaceae family and differs definitely from all other known plant lectins with respect to its molecular structure (Peumans et al., 1984). UDA is not only the smallest (8.5kDa) lectin known at present, but also the first single chain lectin to be found in plants. Native form of UDA composed of a single polypeptide chain of less than 100 aa residues. UDA possess carbohydrate binding specifity for N-acetlyglucosamine (NAG) oligomers and chitin (Peumans et al., 1984). UDA has been suggested to have a role in plant defence and has shown to possess both antifungal and insecticidal activities. Additionally, *in vitro* studies has been showed that UDA have growth inhibiting activities against several plant pathogenic fungi which contain chitin in their cell walls (Broekaert et al., 1989). The authors of more recent studies have proposed unexpected peculiarity of UDA that it's complex isolectin composition. The nettle lectin contains of least six molecular forms which exhibit identical agglutination properties and carbohydrate binding specifity and in addition have the same molecular structure and virtually identical biochemical properties. UDA which sugar binding experiments have suggested, has two sugar binding sites that have intrinsically different affinities on the molecular surface (Anderson et al., 2002; Broekaert et al., 1989). UDA agglutinates erythrocytes nonspecifically, induces the production of γ-interferon in human lymphocytes and acts as a super antigen in T cell activation (Le Moal and Truffa-Bachi, 1988). UDA inhibited the binding of EGF/bFGF (basic fibroblast growth factor) to Hela cells, binding of EGF to membranes of A431 cells and EGF receptor tyrokinase activity (Wagner et al., 1995). Also, only one time to be researched on AGS cell line and this study showed that UDA at different concentrations has anti-proliferative and apoptotic effects on AGS cell line (Çagıl et al., 2015). In the light of this study, we investigated cytotoxic and apoptotic effects of UDA on two cancer cell lines that are; MCF-7, Hela and one standard control cell line L929.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General Chemical Reagents

General laboratory chemical reagents are given in the list below (Table 2.1)

Table 2.1: List of general chemical reagents and their brands

Chemical Reagents	
PBS	Biochrome AG
FBS	Biochrome AG
DMEM	Biochrome AG
Trypsin	Biochrome AG
DMSO (Dimethyl sulphoxide)	Merck
Urtica dioica agglutinin	Biological Industries
Penicillin	Biological Industries
Prymocin	Biological Industries
Mitomycin C	Sigma
Paraphormaldehyde 4%	(4 g paraformaldehyde-100 ml PBS)
Triton X 0.1%	Roche

2.1.2 Equipments

General laboratory equipments are given in the list below (see Table 2.2).

Table 2.2: List of the general equipments and their brands

Equipments	Brand and Model
Centrifuge	Hettich , Mikro 22
Vortex	Vortex IKALABOTECHNIK
Water Bath	Water Bath Nuve
Inverted Light Microscope	Inverted Light Microscope Zeiss
CO^2 Incubator	CO^2 Incubator Thermo ,Sanko
Microfilter (1.0/0.45 µm) GF/PET	Microfilter (1.0/0.45 µm) GF/PET
Pipettor	Reddot Hirschmann Laborgerate
Centrifuge Tubes Falcon	Micropipettes Nichiryo
Trypan blue	Sigma
Water purification system	Millipore
Laminar Flow Hood	Esco , Kotterman
Equipments	Brand and Model
Serological Sterile, Plastic pipets	(2 mL, 5 mL, 10 mL)
Autoclave	CERTO CLAW A-4050 Traun, Austria
Water Purification System	Millipore
Flask	(25, 75, 150 cm^2)
96-well plates	(25, 75, 150 cm^2)
ELISA reader	Roche

2.2 METHODS

2.2.1 Cell Culture

Human cervical cancer (HeLa), human breast cancer (MCF-7) and also non-cancer mouse fibroblast (L929) cell lines were chosen to observe the effects of UDA *in vitro*.

2.2.2 Thawing of Cell Lines

Previously 10 ml of warmed up Dulbecco's Modified Eagle's Medium (DMEM-LG, Gibco) at 37ºC was transferred to the 15 ml falcon tubes. Then, cryovial tubes were taken from the liquid nitrogen tank (-196ºC). They were transferred into the 37ºC water bath and shaken slowly until melting. The contents in the cryovial were transferred to the 15 ml falcon tube bearing DMEM as quickly as possible and centrifuged at 2000 rpm for 3 min. The supernatant was discarded to get rid of DMSO (Dimethyl Sulfoxide, Applichem) and other unwanted cellular products from the medium. The remaining part is pellet was resuspended by finger mixing. Then living cells were counted. After that cells were seeded into a 25 cm^2 tissue culture flask in 10 ml DMEM including 20% Fetal Bovine Serum (FBS, Biochrom) and 1% Penicillin/Streptomycin (Biochrom). Cells were incubated in a humidified atmosphere (37°C, 5% CO_2) and the screw cap of the flask was kept flexible to allow circulation of CO_2 into flask. The day after medium was refreshed to allow eliminate the dead cells.

2.2.3 Subculture of Cells Lines

After seeding of cells, the cells in the flask, the cells were attached and confluenced on the surface of the flask. When the cells confluence %80-90, subculture will have to be done. Before DMEM, FBS, Phosphate Buffered Saline (PBS, Biochrom) and trypsin were heated up to 37ºC at water bath. Medium was removed by a sterile pipette and cells were washed with 5 ml of PBS in the flask. Then PBS was removed, cells were trypsinized with pre-warmed 4 ml of 0.2 % Trypsin/EDTA solution (Gibco) and cells were incubated in humidified atmosphere (37°C, 5% CO_2) for 5-10 min. after that the cells were observed under the inverted microscope. When the cells detached on the surface of flask, FBS (1.5 ml) was added in the flask to neutralize effect of Trypsin/EDTA solution. The neutralized cells in the flask with trypsin and FBS were transferred into a 15 ml centrifuge tube and then centrifuged at 2000 rpm for 3 min at room

temperature. After centrifugation, supernatant was discarded until 0.5 ml of the cell suspension at the bottom of the tube. Pellet was finger mixed and the tube was completed 10 ml with DMEM medium in order to washing cells. Centrifuge was repeated. Then cells were counted with hemocytometer. After counting, cells were seeded at a density of 1×10^4 cells/cm^2 with DMEM containing 10% FBS for expansion. Subculture of cells was repeated at about 3-4 day intervals according to the cell types and doubling times.

2.2.4 Freezing of Cell Lines

For the freezing process cells were re-suspended in FBS at density of 1-2x10^6 cells/ml. Cryovial tubes were placed on ice and 950µl cell suspension was transferred into each tube. 50 µl DMSO (Dimethyl Sulfoxide, Applichem) was added into each tube drop by drop and cell suspension was mixed by pipetting. Tubes were left at -20^{0}C for 1 h and then kept at -80^{0}C overnight. Then cryovial tubes were transferred to the liquid nitrogen tank (-196 ^{0}C) to protection for a long time.

2.2.5 Adding Urtica dioica agglutinin

Urtica dioica agglutinin were prepared at different concentration with serial dilutions to add the cell lines (Table 2.3 and Table 2.4). This proportion was estimated with xCelligence system and proliferation tests.

Table 2.3: Used concentration of *Urtica dioica* agglutinin (UDA)

Groups	Concentrations
UDA 1	20 µg/ml
UDA 2	10 µg/ml
UDA 3	5 µg/ml

2.2.6 MTT Assay

Human cervical cancer (HeLa), human breast cancer (MCF-7) and also non-cancer mouse fibroblast (L929) cell lines were seeded in triplicate at 1x10^4 cells/well to the 96 well plates in 100 µl medium and then pre-incubated for 24 h before the treatment. Following exposure to the

determined concentrations of UDA, cytotoxicity for cell lines at 24th and 48th hours were analyzed with MTT. The assay conducted immediately by mixing the media with the assay reagent which was prepared by mixing two separate solutions. This was incubated for 30 minutes, protected from light, and the absorbance was then read at 490 nm, with a reference reading at a wavelength above 600 nm.

2.2.7 XCelligence System for Analyzing the Cell Proliferation

Human cervical cancer (HeLa), human breast cancer (MCF-7) and also non-cancer mouse fibroblast (L929) cell lines were grown and expanded in tissue-culture flasks. After reaching 75% confluence, the cell lines were washed with PBS, afterwards detached from the flasks by a short time treatment with trypsin/EDTA. Subsequently, 100 µL of cell culture media at room temperature was added into each well of E-plate 96. After this the E-plate 96 was connected to the system and checked in the cell culture incubator for proper electrical-contacts and the background impedance was measured during 24h The cell lines were resuspended in cell culture medium and adjusted to 10.000cells/mL at the same time. 100 µL of each cell suspension was added to the 50 µL medium containing wells on E-plate 96, in order to determine the optimum cell concentration. After 30 minutes of incubation at room temperature, E-plate 96 was placed into the cell culture incubator. Finally, adhesion, growth and proliferation of the cells was monitored every 15min for a period of up to 24h via the incorporated sensor electrode arrays of the E-Plate 96. The electrical impedance was measured by the RTCA-integrated software of the xCelligence system as a dimension less parameter termed CI. The proliferation, attachment and spreading of the cells was monitored for each 15 minutes by the xCelligence system. Approximately 24h after seeding the medium in each well was discarded in order to get rid of the dead cells. When the cells were in the log growth phase, the cells were exposed to 100 µL refresh DMEM containing the following substance: UDA (5, 10, 20 µg/ml). DMEM and 10% FBS were used as control group. All experiments were run for 48h. Cell index (CI) is derived to represent cell status based on the measured relative change in electrical impedance that occurs in the presence and absence of cells in the wells Impedance is measured at 3 different frequencies (10, 25 or 50kHz) and a specific time.

2.2.8 TUNEL Assay

DNA fragmentation is an indicator for detecting the late apoptosis. TUNEL (Roche) is an assay that is composed of an enzyme solution and labeling solution which detects the nicks (single strand breaks) in DNA and binds to free 3'-OH ends. Adding of dUTPs to 3'-OH ends by terminal deoxynucleotidyl transferase cause labeling of DNA. Fluorescently labeled ends were detected by using confocal microscopy (Leica). Cell lines were used in this technique. The cell lines were grown for TUNEL assay. cell lines were seeded at $1x10^4$ cells/well to the 96 well plates in final volume of 100 µL/well culture medium in a humidified atmosphere (37°C, 5%CO_2) for 24h before the treatment. Then the next day the highest concentration of UDA, 20 µM, was chosen for all of the cell lines. The cells were treated with 20 µg/ml UDA for 48h. Mitomycin C was used for the positive control. After the incubation period, the cells were washed twice with phosphate buffer solution (PBS), then fixed with 5% paraformaldehyde for 60 minutes and washed again twice with PBST (PBS+0,1% Triton-X-100) on the shaker. 0,1% Triton X were treated with 200 µL with cells on the ice for 10 minutes. After that the cells were washed twice with PBST on shaker. TUNEL reaction mixture was added 50 µl and incubated at 37°C in a humidified atmosphere with 0.5% CO_2 for an hour. After that the cells washed with PBST. And then DAPI was applied on the cells for 15 minutes then washed once with PBST and once with distilled H_2O. The results were analyzed by fluorescence microscopy.

2.2.9 Cellular Binding: Fitch Conjugated Lectin

Human cervical cancer (HeLa), human breast cancer (MCF-7) and also non-cancer mouse fibroblast (L929) Passaged cells were seeded in 96 well-plate for each plate to be 10.000 cells. 96 well-plate was incubated for 24 h to let the cells grow and attach themselves inside the plates. 24 h later diluted fluorescent labeled lectin was applied on cell culture at 20µg/ml concentration. After 24 h of incubation, cells were washed with buffer solution three times. Cells were examined with and without fixation under fluorescent microscope. Appropriate filter was used and FITCH absorption was 492 nm and emission rate was 517 nm. FITCH conjugated and pure UDA from stinging nettle obtained from Ey Laboratories, North Amphlett Blvd. San Matea, CA**.**

2.2.10 Statistical Analysis

The statistical analysis of WST-1 and MTT values Mann– Whitney U-test was used. Also for TUNEL assay values Chi-square (X^2) test was used. Statistical differences between time and dosage were analyzed. A value of P less than 0.05 was accepted as statistically significant. Results were expressed as mean ± SE. For these procedures, SPSS 11.5 version for Windows (SPSS Inc, Chicago, Illinois, USA) was used.

3. RESULTS

3.1 xCelligence Result

To define optimum cell number for cytotoxic experiment of xCelligence assay, L929, MCF-7 and HeLa were seeded at numbers ranging from 60.000, 40.000, 20.000, 10.000 and 5.000 in each well of an E-plate and automatically monitored over 24 hours. Subsequently, the CI value were calculated, and the highest value found for each of them. According to these results, the highest CI values are; 20.000 for L929, 40.000 for MCF-7 and 40.000 for HeLa (Figure 3.1).

RTCA results marked that there was a significant change on MCF-7 cells for all concentrations throughout 24^{th} hours. It can be seen from the graphic that the number of cell viability has decreased in 20µg/ml when compare with control group. xCelligence pointed out that as the concentration rises up cell number goes down during 24^{th} hour. For HeLa cell, xCelligence data showed that UDA was also effective on HeLa cells. Especially, 20µg/ml dosage was more effective than other dosages. Also, there is clear difference between control and other concentrations. As can be seen from xCelligence results for L929, except 20µg/ml concentration treated with UDA had more cell numbers than control group. According to these results, concentration under the 20µg/ml were increased the cell proliferation. But 20µg/ml have slight antiproliferative effect on L929 (Figure 3.2).

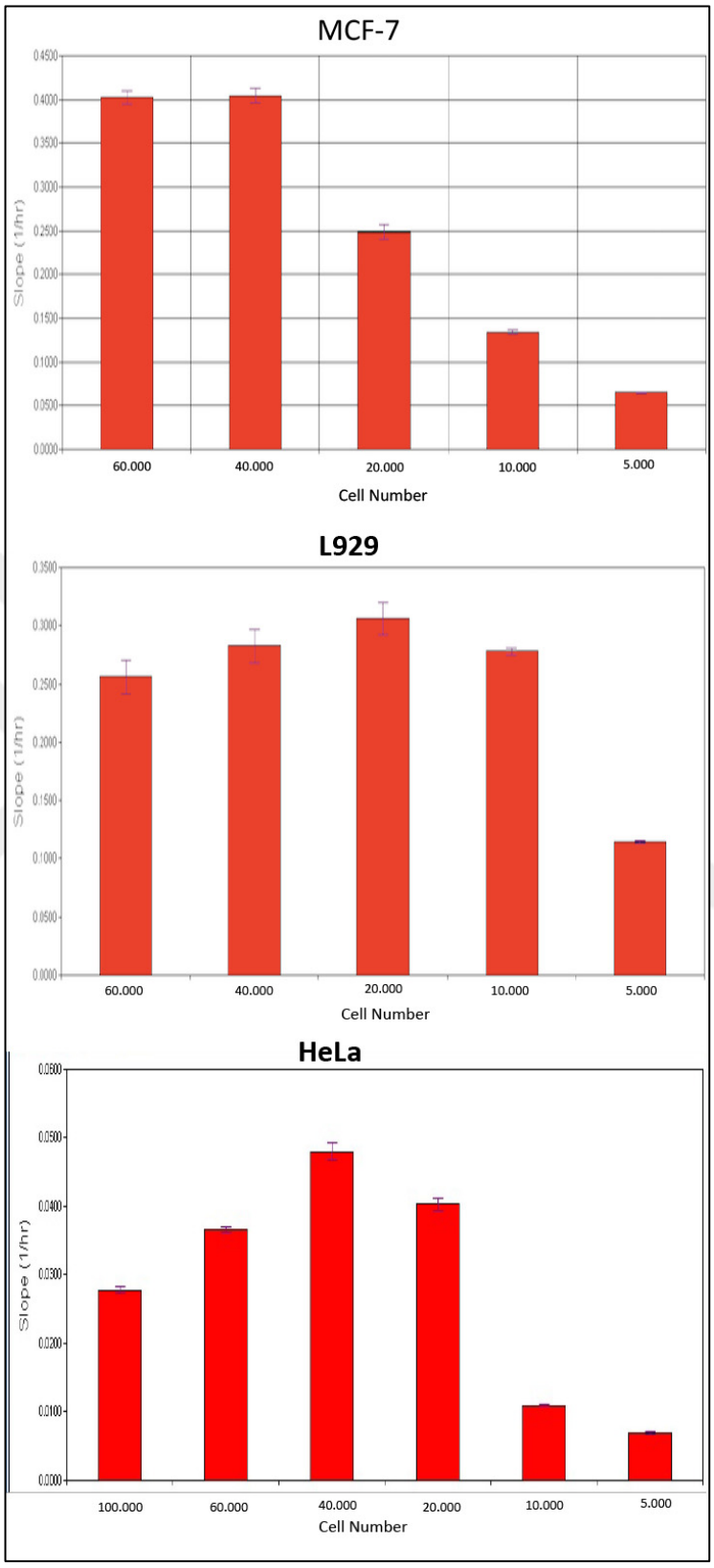

Figure 3.1: RTCA Opimization Results

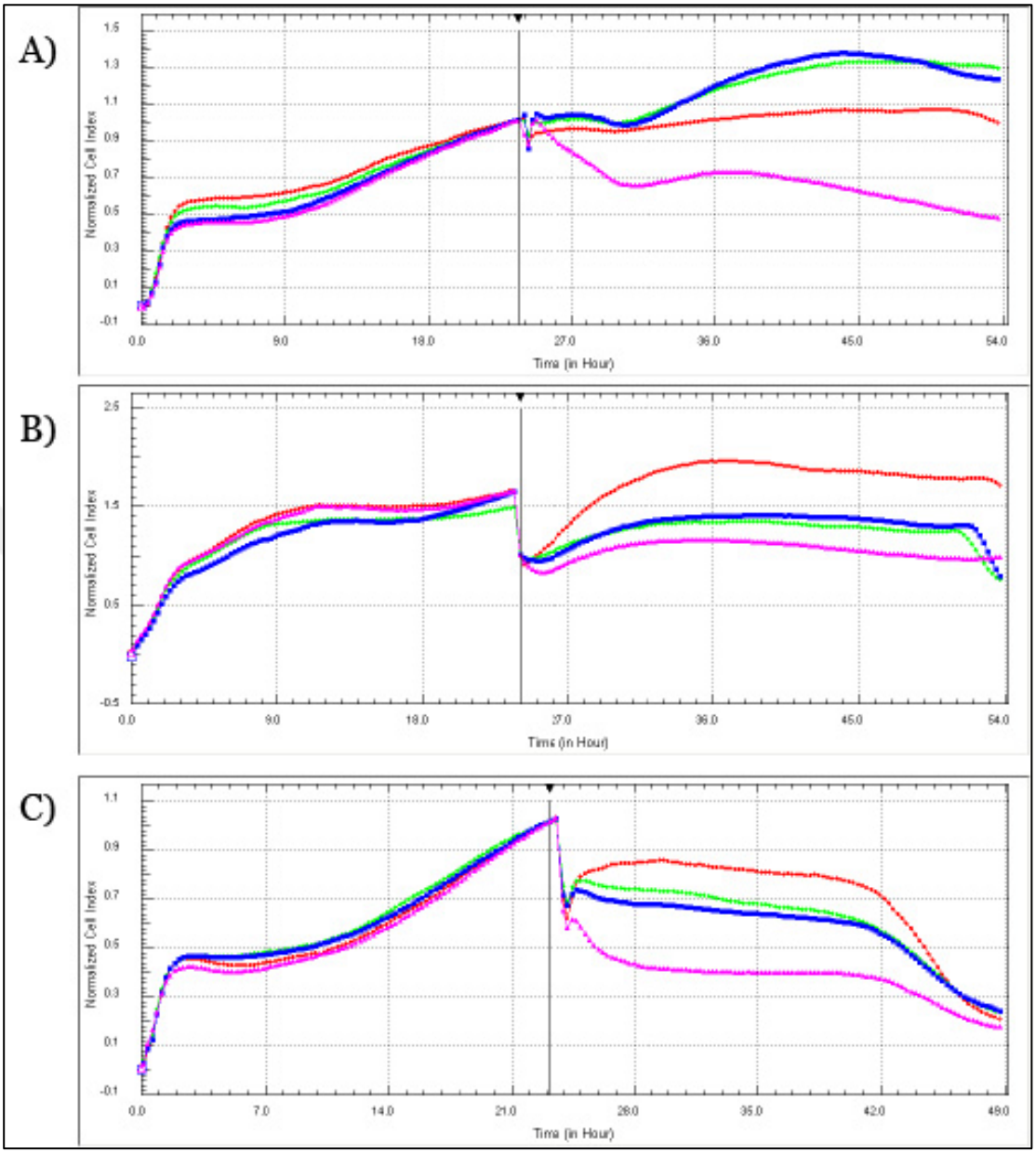

Figure 3.2: RTCA results on cell lines. A) L929, B) MCF-7, C) HeLa. Red lines show control group. Green lines show 5µg/ml. Blue lines show 10 µg/ml. Pink lines show 20µg/ml.

3.2 MTT Results of UDA on MCF-7, HeLa and L929

At 24th hour in MTT assay, UDA had considerable effect on MCF-7 cells. In other words, this MTT result showed that number of cells has decreased in all concentrations regularly along with the treatment. Also, this MTT result confirmed with xCelligence result. Considering the MTT data, we can summarize that; concentrations from 5µg/ml to 20µg/ml were decreased the

cell proliferation on HeLa cells. specially the concentration of 20µg/ml. The result of the MTT study demonstrate that, UDA increased cell proliferation for all concentrations except 20µg/ml on L929 cells. But 5µg/ml and 10µg/ml concentrations cell number were higher than cell number of control group. (Figure 3.3).

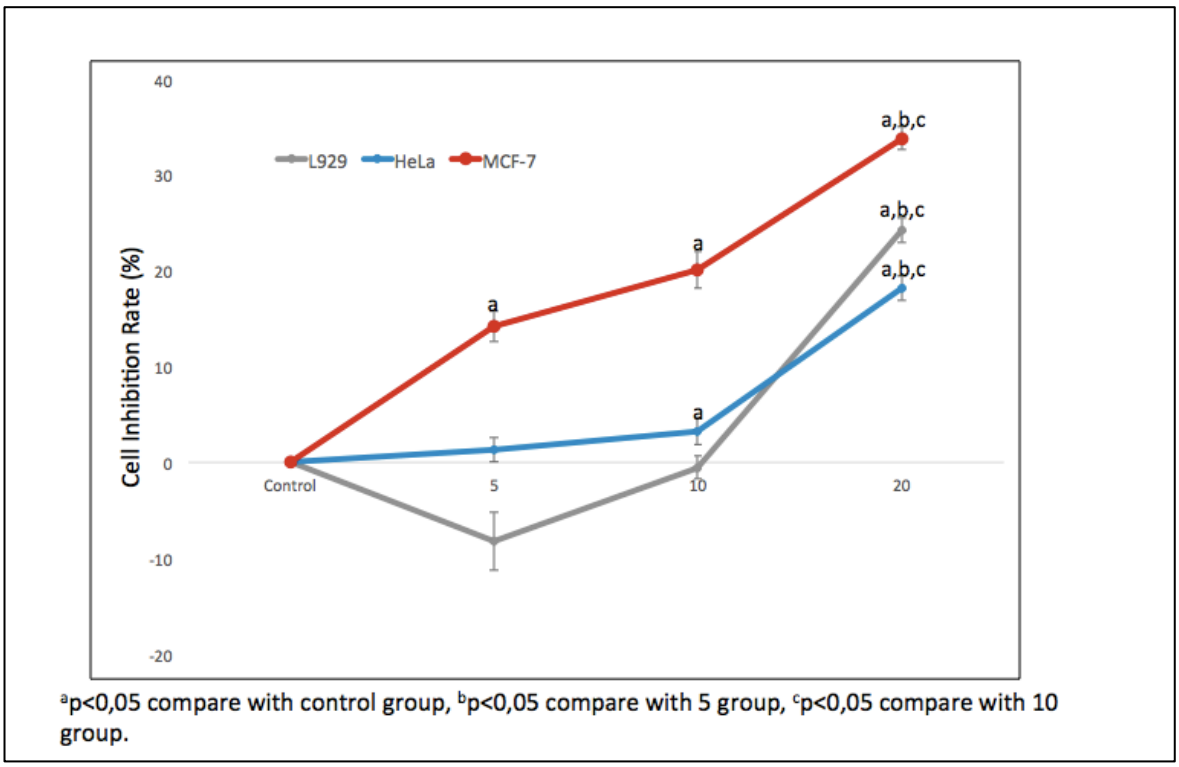

Figure 3.3: MTT results of MCF-7, HeLa and L929

3.3 TUNEL Results of MCF-7, HeLa and L929

To determine quantity of apoptotic cells we used TUNEL method. The cells exposure to highest concentrations of UDA (20µg/ml). Considering the TUNEL data, we can summarize that; UDA is lead to apoptosis on HeLa and MCF-7 cells (Figure 3.4). When we look at the Table 3.3 application of 5µg/ml concentration resulted apoptotic cells 36.27%, 10µg/ml concentration resulted apoptotic cells 46.80% and 20µg/ml concentration resulted apoptotic cells 78.26% in HeLa cells (Figure 3.4, 3.5). Application of UDA resulted in apoptotic cells 63.79% in 5 µg/ml, 68.69% in 10µg/ml and 80% in 20µg/ml on MCF-7 cells (Table 3.1, Figure 3.6).

When we look at the L929, application of 5µg/ml concentration resulted apoptotic cells 17.80%, 10 µg/ml concentration resulted apoptotic cells 23.28% and 20 µg/ml concentrations resulted. Apoptotic cells 20.54% (Figure 3.4, Figure 3.7). Figure.4 Bar graph shows percentage of values apoptotic cells in total cells which treated with different concentration of UDA at 24 hrs. [a]p≤0.05 compared with negative control group, [b]p≤0.05 compared with positive control, [c]P≤0.05 compared with 5 concentration group, [d]P≤0.05 compared with 10 concentration group by using one-way ANOVA test.

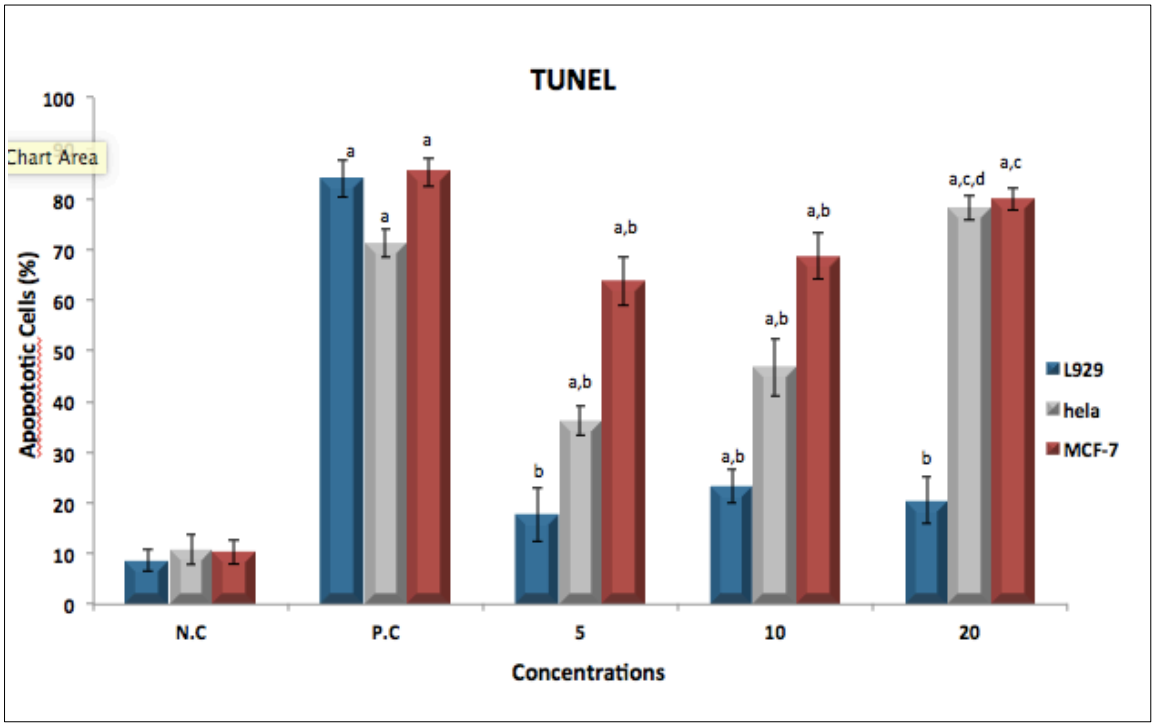

Figure 3.4: Bar graph shows percentage of values apoptotic cells in total cells which treated with different concentration of UDA at 24 hrs. [a]p≤0.05 compared with negative control group, [b]p≤0.05 compared with positive control, [c]P≤0.05 compared with 5 concentration group, [d]P≤0.05 compared with 10 concentration group by using one-way ANOVA test.

Table 3.1: TUNEL apoptotic counting results of MCF-7, HeLa and L929

Cells	Values	Apoptotic	Normal	DNA Fragmentation Cell (%)
HELA	Neg. control	13	108	%10,74
	Positive control	134	54	%71,27
	5	37	65	%36,27
	10	44	50	%46,80
	20	90	25	%78,26
MCF-7	Negative control	12	104	%10,34
	Positive control	139	24	%85,27
	5	74	42	%63,79
	10	79	36	%68,69
	20	100	25	%80
L929	Negative control	10	105	%8,69
	Positive control	153	32	%84,06
	5	13	60	%17,80
	10	17	56	%23,28
	20	15	81	%20,54

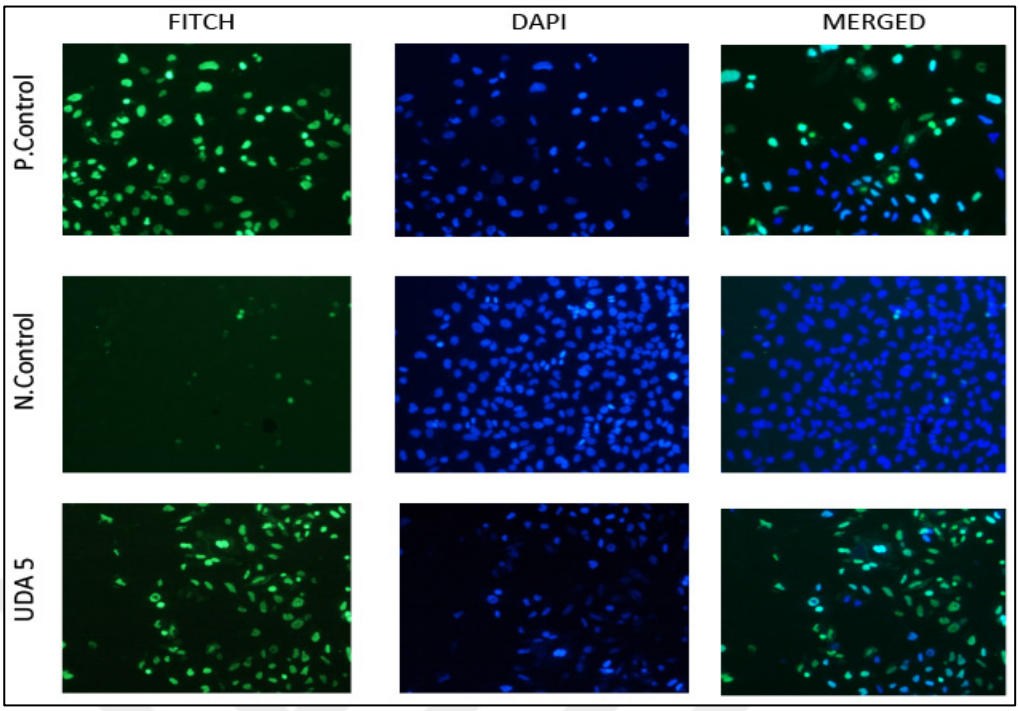

Figure 3.5: TUNEL images of HeLa cell incubated with 5 µg/ml UDA. Yellow dots show apoptosis. Blue dots show nucleus (10X).

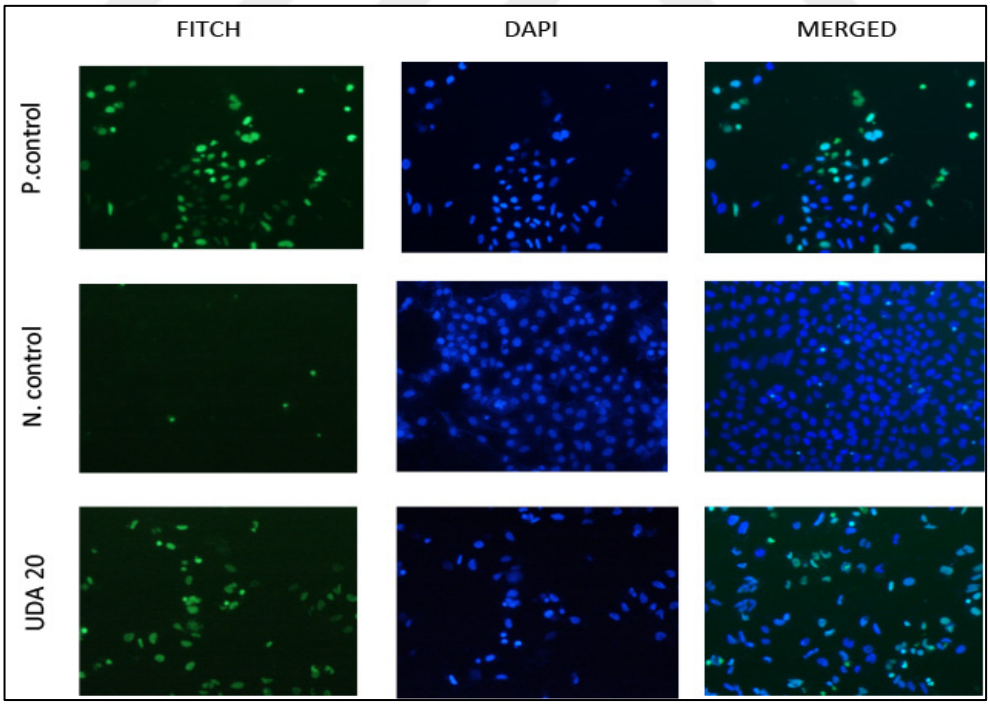

Figure 3.6: TUNEL photos of MCF-7 incubated with 5 µg/ml UDA. Yellow dots show apoptosis (10X).

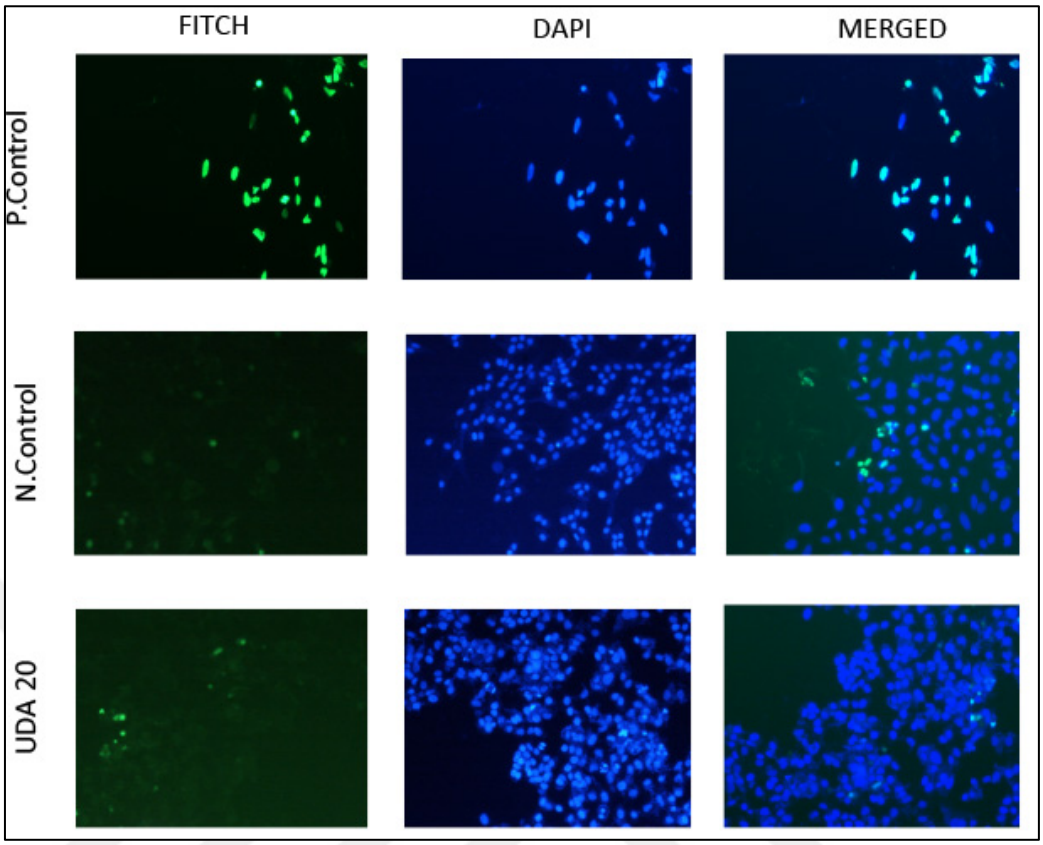

Figure 3.7: TUNEL photos of L929 incubated with 5 µg/ml UDA (10X).

3.4 Binding Results of UDA on MCF-7, HeLa and L929

The aim of the binding study is show interactions between lectin and ligands. Lectins are the carbohydrate binding protein. Our FITCH binding method is showing our UDA binding MCF-7, HeLa and L929 cells's membranes proteins which is specifically N-acetlyglucosamine on cell surface. Fitch conjugated lectin, display to increasing fluorescent intensity. So that, we can understand to UDA binding cells surface. The fluorescence image determines the effect of UDA lectin affinity on cell lines. Figure 3.8 show that FITCH labeled UDA bound MCF-7, HeLa and L929 cell surface.

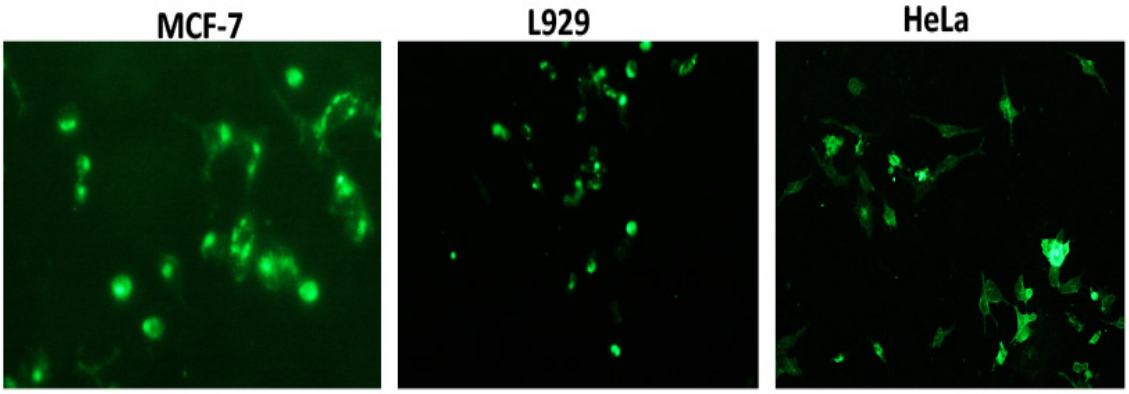

Figure 3.8: Binding results of UDA on MCF-7, HeLa and L929

4. DISCUSSION

In former studies it has been showed that lectins from plants bind to the sugar or glucose residues of the polysaccharides, glycoproteins or glycolipids. By this conjugation, they stimulate immune cells and result in cell count and activity increase. The lectins binding to their ligand was provided by hydrogen bonds. Moreover, Van der Waals and hydrophobic interactions also attenuate this subjected ligand formation. Although ligand between membrane proteins and plant lectins seems to weak, multiple binding points that formed by weak bounds is giving to them strong bonds properties (De Mejia and Prisecaru, 2005).

On the other hand, previous studies showed that binding capacity of lectins depends on cancer types. Each structural differences within the cell membrane effects the ligand formation. For instance in a research, binding effect of three different lectin such as UDA, WGA (wheat germ agglutinin) and Con A (concanavalin A) on I-125 labelled A431 were studied and UDA (5 ng/ml to 10 µg/ml) showed the strongest bond with its ligand (Wagner et al., 1995).

In our results, we found that UDA bound to the MCF-7 rather than the HeLa thus we may specify that cell vitality of MCF-7 decreased in each concentration comparing to the other cell type. After binding test by FITCH bound lectin, images supporting this outcome. Besides, TUNEL-counting results also supported to this finding. Moreover, less binding to the L929 because of its normal cell type property is another promising result. Although, not all the lectins are toxic, some of them may show the toxicity in different degrees and causing the death. Because of this property lectins are studied against cancer by immunotoxin production (Roberto A. Ferriz-Martinezz et al., 2010). For instance, Ricin which is the firstly found lectin in 1888, were found to be showing toxic effect by collapsing the erythrocytes Furthermore, in other study, two different lectin PNA and DBA tested on non-cancer (Anderson et al., 2002; Franz, 1986). CRL-1459/CCD-18Co and 8CCL-220/Colo320DM cancer cell line. As a result, non-cancer cells effected rather than cancerous cell type In most of the lectin studies only cancer cells were used. However normal cells generally ignored. This kind of experiments especially needed for the cancer drug production (Petrossian et al., 2007).

In this study we showed the binding properties of UDA to the normal cells and determine the toxic effects. Stinging nettle (*U. dioica*) is used as an extract in most of the studies but lectin containing *Urtica dioica agglutinin* never studied before. However only our group studied *U. agglutinin* before on AGS cells and found as effective (Çagıl et al., 2015). As a result we found the UDA is effective on MCF-7 cell line even in lowest dose 5μg/ml and lead to L929 cell count increase. We may admit the highest effective dose is 20μg/ ml which is the highest concentration at the same time on cancer cells. Nonetheless, we found it may affect the L929 negatively. Underlying mechanism of the UDA not clear yet. Nonetheless, there are information pertaining to effecting mechanism of Con A (Concanavalin A) and other some lectins. Related studies showed that plant lectins are leading cell death by inducing apoptotic and autophagic signaling pathways including the Bcl-2, caspase family, p53, PIJK/Akt, ERK, BNIP3, Ras-Raf and ATG protein families. Despite the fact that, lectins are known since long time, their roles in programmed cell death is not clear yet (Roy et al., 2014).

In a study with A375 and HepG2, Con A caused mitochondrial membrane disrupting leads to the caspase activation by cytochrome c release and subsequently result in mitochondria mediated apoptosis. Beside, this result may give us an understanding of lectin mechanism (Liu et al., 2010; Liu et al., 2009). Another example of lectin mechanism studied on U87 (Pratt et al., 2012). In the study, lectin bound to the outer membrane surface site of the SHPS-1 (multifunctional transmembrane protein) and caused to MMP-9 release herewith COX-2 upregulated and Akt downregulated as a result induced IKK/NF αB dependent apoptosis(Biswas et al., 2006). In another study, Bax/Bcl-2 inhibited and resulted to apoptosis by Foxola-Bim signaling pathway upregulation in SKOV3 (ovarian cancer) (Amin et al., 2007). In Jeurkat leukemic T cells, ML-I (ribosome inactivating protein) induce the apoptosis by activating caspase 8 (Pryme et al., 2006). ML-I degrading the MMP and activate caspase 3, provoke the apoptosis by APAF-1 pathway (apoptosis associated fator-1) in human p53 mutated adenocarcinoma cells but ML-I changed MMP levels and lead to the cytochrome c release and ROS increase in Hep3B cells. Finally, ML-II induced apoptosis by activating the SAPK/JUNK & p53 signal pathway (Figure 4.1) in U987 cells (Hostanska et al., 2003; Lyu et al., 2002; Pae et al., 2001).

We observed the cytotoxic, apoptotic and proliferative effects of UDA on HeLa, MCF-7 and L929 for 24 hours and our results showed UDA decreases the cell proliferation of MCF-7 and HeLa in each of three concentrations (5, 10, 20 µg/ml). MTT and xCelligence results are supportive to each other. According to xCelligence , all concentrations of MCF-7 and HeLa is under the control group and highest dose (20 µg/ml) is most effective in each of tests. If we look the ratios it can be seen that MCF-7 was affected rather than HeLa cells. In normal L929 cells, according to MTT outcomes highest concentration (20 µg/ml) resulted to cell death whilst 5, 10 µg/ml concentrations of UDA assisted to cell proliferation. xCelligence outcomes showed the UDA exposed cell vitality closed to control group vitality. According to TUNEL results, apoptosis positively correlated with the concentrations as supportive to the MTT and xCelligence of MCF-7 and HeLa. However, in L929 cell line each concentration resulted to apoptosis slightly. This result is not consisted with the MTT and xCelligence outcomes.

As a result, HeLa, MCF-7 and L929 weren't studied before and in this study, we showed the antiproliferative and apoptotic effects of UDA on these two-cell line and UDA increased the apoptosis by decreasing the cell proliferation in each of two cell lines. However, we found also UDA increases the cell proliferation at low concentrations and slightly causes the cell death at high concentrations in L929 cell line.

5. CONCLUSION AND RECOMMENDATIONS

By and large, in this thesis we investigated cytotoxic and apoptotic effects of UDA on L929, HeLa and MCF-7 cell lines. Consequently, UDA in 5µg/ml which is lowest concentrations display antiproliferative and apoptotic effects on MCF-7 and Hela cancer cell lines, the most effective concentration was 20µg/ml has found to be the most effective concentration. But, between to three concentrations 20µg/ml also showed antiproliferative effect on the standard control cell L929.This thesis only the second UDA study on cancer cell line. In the first study, UDA had antiproliferative and apoptotic effects on AGS cells.

Because of the smallness and biorecognition of specific carbohydrates UDA may also be used as diagnostic tools for the early recognition of different types of cancer cells. Also, UDA shows future at drug delivery.

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