REPUBLIC OF TURKEY
YUZUNCU YIL UNIVERSITY
INSTITUTE OF HEALTH SCIENCE

THE APOPTOTIC, CYTOTOXIC AND GENOTOXIC EFFECTS OF NOVEL FLUORINE BORON HYBRID COMPLEX (L_5BF_2) ON ENDOMETRIAL CANCER

Pawan Tareq Ahmed LAK
DEPARTMENT OF MEDICAL BIOLOGY
MASTER THESIS

SUPERVISORS
1. Assoc. Prof. Dr. Yasin TÜLÜCE
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Assoc. Prof. Dr. Yasin TÜLÜCE

Head of Jury

Asst. Prof. Dr. Gökhan GÖRGİŞEN

Member

Assoc. Prof. Dr. Zafer ÇETİN

Member

THESIS ADMISSION DATE

/ / 2017

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CONTENTS

CONTENTS IV
ACKNOWLEDGMENTS III
FIGURES VI
TABLES VII
ABBREVIATIONS VIII
1. INTRODUCTION 1
2. GENERAL INFORMATION 3
2.1. Epidemiology 3
2.2. Risk Factors 3
2.3.1. Radiotherapy 3
2.3.2. Hormonal therapy 4
2.3.3. Chemotherapy 4
2.4. Apoptosis 4
2.5. Apoptotic Pathways 6
2.5.1. Intrinsic pathway (mitochondrial pathway) 6
2.5.2. Extrinsic pathway 7
2.6. Novel Fluorine Boron Hybrid Complex (L_5BF_2) 10
3. MATERIALS AND METHODS 12
3.1. Cell Culture 12
3.2. Cell Viability Assay (MTT Assay) 12
3.3. Single Cell Gel Electrophoresis Assay (Comet Assay) 13
3.4. Apoptotic Analysis by ELISA 14
3.5. Apoptotic DNA fragmentation (DNA Laddering) 15
3.6. Acridine Orange and Ethidium Bromide Apoptotic Cell Analysis 16
3.7. Determination of intracellular TAS 17
3.8. Determination of intracellular TOS 18
3.9. Ferric Reducing Ability of Plasma (FRAP) Assay 18
3.10. Statistical Analysis 19
4. RESULTS 20
4.1. Measuring Cell Proliferation Rate by the Meaning of MTT Assay 20

4.2. Determination of DNA Damage in ECC-1 cells by the Meaning of Single Cell Gel Electrophoresis assay 24
4. 3. Determination of Apoptosis by Using Acridine Orange 26
4.4. Determination of DNA Damage Using DNA Fragmentation Technique 28
4.5. Measuring Apoptosis of ECC-1 by ELISA 29
4.6. Determination of intracellular TAS , TOS, and OSI 31
4.7. Ferric Reducing Ability of Plasma (FRAP) Assay 35
5. DISCUSSION AND CONCLUSION 37
SUMMARY 40
ÖZET 41
REFERENCES 42
CURRICULUM VITAE 46
SUPPLEMENTS 1: ETHICS REPORT 47
SUPPLEMENTS 2: PLAGIARISM REPORT 48

FIGURES

Figure 1. Apoptosis vs. Necrosis 5

Figure 2. Apoptotic pathways 8

Figure 3. Domain organization of human caspases 10

Figure 4. Novel fluorine boron hybrid complex (L_5BF_2) 11

Figure 5. IC50 value of different cell lines treated with L_5BF_2 21

Figure 6. Effect of L_5BF_2 and cisplatin on ECC-1 cells viability 23

Figure 7. Effect of L_5BF_2 and cisplatin on PNT1-A cells viability 23

Figure 8. DNA damage rate by the mean of comet formation in ECC-1 cells treated with L_5BF_2 and cisplatin 25

Figure 9. A compare between the ratio of necrotic, living and apoptotic ECC-1 cells, treated with L_5BF_2 27

Figure 10. DNA laddering of ECC-1 cells treated with L_5BF_2 and cisplatin 28

Figure 11. The effect of different concentrations of L_5BF_2 on ECC-1 DNA damage measured by ELISA 30

Figure 12. TAS value according to the elevation of L_5BF_2 concentration 32

Figure 13. TOS value according to the elevation of L_5BF_2 concentration 33

Figure 14. OSI values of ECC-1 treated with L_5BF_2 34

Figure 15. The effect of various concentrations of L_5BF_2 and cisplatin on ECC-1 36

TABLES

Table 1. Subfamily members of caspase family 9

Table 2. IC_{50} value of different cell lines treated with L_5BF_2 20

Table 3. ECC-1 Cell line treated with various concentrations of L_5BF_2 22

Table 4. PNT1-A Cell line treated with various concentrations of L_5BF_2 22

Table 5. ECC-1 Cell line treated with various concentrations of cisplatin 22

Table 6. PNT1-A Cell line treated with various concentrations of cisplatin 22

Table 7. Results of comet assay test of ECC-1 cells treated with L_5BF_2 25

Table 8. Growth rate of necrotic, vital and apoptotic ECC-1 cells treated with L_5BF_2 . 27

Table 9. ELISA measurements of ECC-1 cell apoptosis treated with L_5BF_2 30

Table 10. TAS value of ECC-1 treated with L_5BF_2 31

Table 11. TOS value of ECC-1 treated with L_5BF_2 33

Table 12. FRAP values of ECC-1 treated with L_5BF_2 and cisplatin 35

ABBREVIATIONS

Apaf-1	: Apoptosis protease-activating factor-1
APO-1	: Apoptosis Antigene-1
CAM	: Cell Adhesion Molecule
CARD	: Caspase Recruitment Domain
Caspase	: Cysteinyl aspartate-specific protease
CD95	: Cluster of Differentiation 95
DAD	: Death Affecter Domain
DD	: Death Domain
DISC	: Death-inducing signaling complex
DMSO	: Dimethyl Sulfoxide
DR	: Death Receptor
EC	: Endometrial Cancer
FADD	: Fas-associated death domain
Fas	: First Apoptotic Signal
FasL	: Fas Ligand
FBS	: Fetal Bovine Serum
HNPCC	: Hereditary Non-Polyposis Colonic Cancer
IAP	: Inhibitor of apoptosis protein
LMA	: Low Melting Agarose
MAC	: Mitochonderial Apoptosis-Induced Channal
NMA	: Normal Melting Agarose
PBS	: Phosphate-buffered saline
PCOS	: PolyCystic Overy Syndrum
RIP1	: Receptor Interacting Protein 1

ROS	: Reactive Oxygen Species
SMAC	: Second mitochondria-derived activator of caspases
TNF	: Tumor necrosis factor
TNFR	: Tumor necrosis factor receptor
TRADD	: Tumor necrosis factor receptor-associated death domain
TRAF2	: Tumour Receptor Associated Factor 2
TRAIL	: TNF-related apoptosis-inducing ligand

1. INTRODUCTION

Cancer is defined as an illness in which assemblies of abnormal cells proliferate uncontrollably by ignoring the normal rules of cell division. Normal cells are controlled by signals that command either the cell have to divide, differentiate or die. Cancer cells are kind of independent from these signals, resulting in uncontrolled growth and proliferation. If this proliferation is permitted to continue and extent, it can be lethal. In fact, almost 90% of cancer disease are due to tumor dispersal in the mean of metastasis (Hejmadi, 2010).

Cancer is the world's second biggest killer after cardiovascular disease, but one of the most preventable noncommunicable chronic diseases. Cancer caused the death of 7.6 million people in 2005, three-quarters of whom were in low- and middle- income countries. By 2012, that number has risen to 8.2 million deaths (Stewart and Wild, 2014). Up to 40% of all cancer deaths can be avoided by decreasing tobacco smoking use, healthy diets, and physical activity, lowering alcohol consumption, eliminating workplace carcinogens. Infection considered as a major cause of cancer globally and prevention in addition to treatment of infectious agents have already had a substantial effect on cancer avoidance (De Martel et al., 2012).

Endometrial cancer (EC) (also called uterine cancer) is the sixth most common type of cancer among women and fourteenth overall, thru approximately 60,000 new cases are diagnosed with endometrial cancer in 2016 and estimation of 10,000 deaths in U.S. alone (American Cancer Society, 2016). Endometrial cancer considered the most common gynecologic cancer of the female genital tract, with an incidence of approximately 30 per 100000 cases in western women (Quinn, 1996). About 40% of EC cases are associated with obesity, high estrogen exposure, diabetes, and high blood pressure (National Cancer Institue, 2014).

The main treatment for EC is surgery; 90% of women with this type of cancer are treated with some form of surgery (Vale et al., 2012), while chemotherapy considered as post-operative adjuvant treatment, and it has a limited room in the controlling of advanced EC, the commonly used drugs include cisplatin and

doxorubicin, but the side-effect may not be acceptable for quite a lot of patients (Humber et al., 2007).

Developing new anti-cancer agents with more efficiency and fewer side-effects are becoming one of the priorities of the researchers in nowadays, and novel fluorine boron hybrid complex (L_5BF_2) gives us that new hope in the field of treating EC due to its anticancer properties and open future doors for new chemotherapeutic molecules.

2. GENERAL INFORMATION

2.1. Epidemiology

Endometrial cancer (EC) is the widest malignancy of the female genital tract, it has been estimated the sixth common type among women after breast, colorectal, lung, cervix and stomach cancer, with an incident of 319,600 new cases worldwide (Torre et al., 2015). An average of 2-3% of women can be diagnosed with EC in their lifetime, early diagnosis can extend the life expectancy for affected women, and due to the new methods of diagnosis and modern medical instruments it is possible nowadays to reach that aim, however some other risk factors like obesity, inactive and unhealthy lifestyle etc, may be associated with increasing cancer incidence (Schouten et al., 2004).

2.2. Risk Factors

Epidemiological studies reported that physical activity, mass and weight change, alcohol consumption, smoking, the presence of overweight/obesity and diabetes mellitus are found to increase the risk for endometrial cancer (Terry et al., 1999; Weiderpass et al., 2000; Gates et al., 2006)**.** A family history of hereditary non-polyposis colorectal cancer (HNPCC) may also increase the cancer risk rate by 40-60%. However, less than 5% of endometrial cancer is related to HNPCC (Hampel et al., 2006). Hormonal irregularity might also rise the opportunity to develop EC in women such as in polycystic ovary syndrome (PCOS) (Pillay et al., 2006)**.**

2.3. Treatment

Surgery is still the best choice of treatment, but the post-operative management methods such as chemotherapy, radiotherapy, hormonal therapy, are not less important than the operation itself, to ensure the total elimination of all neoplasms as well as the tumor.

2.3.1. Radiotherapy

Primary radiation therapy alone is usually a poor choice for surgery patients. Pelvic external beam radiation alone or intracavitary brachytherapy is a typical method. Overall survival rate is less than 10-15% compare to surgery (Fishman et al., 1996).

2.3.2. Hormonal therapy

In some rare cases of excessive operative risk, a primary dose of progesterone is used, particularly in grade 1 cervical adenocarcinoma which shows great benefits in adjuvant progesterone treatment.Tamoxifen is used to increase the progesterone receptors in EC hormonal therapy (Özdemgr, 2015).

2.3.3. Chemotherapy

Chemotherapy alone or in combination with radiotherapy is usually used as adjuvant treatment for advanced disease. Unfortunately, adjuvant treatments are often ineffective due to the development of resistance. A major contributor to chemoresistance is the inhibition or avoidance of apoptosis.

2.4. Apoptosis

Apoptosis is also called programmed cell death, have a great role in cell limitation and decency by regulating the number and function of cells and as a defensive mechanism against cancer cell formation by eliminating needless or surplus cells to maintain a healthy balance between cell existence and cell death (Cotter, 2009). In 1842 the principle of apoptosis was described by the German scientist Karl Vogt, and the term (Apoptosis) was originally proposed by Kerr Wyllie and Currie paper in 1972 (Kerr et al., 1972).

Lately, there was a great deal of focusing on the relationship between apoptosis and cancer, apoptosis provides many appreciable suggestions about a therapy's efficiency, and many anticancer effects of numerous chemotherapeutic agents are by means of apoptosis (Hassan et al., 2014). Therefore, the stimulation of apoptosis has become a major emphasis when developing anticancer therapies.

Unlike necrosis, apoptosis produces cell wastes called apoptotic blebs that phagocytic cells are able to engulf and quickly remove before the contents of the cell can leak out onto the intracellular matrix and cause inflammation (Alberts et al., 2008) (Figure 1).

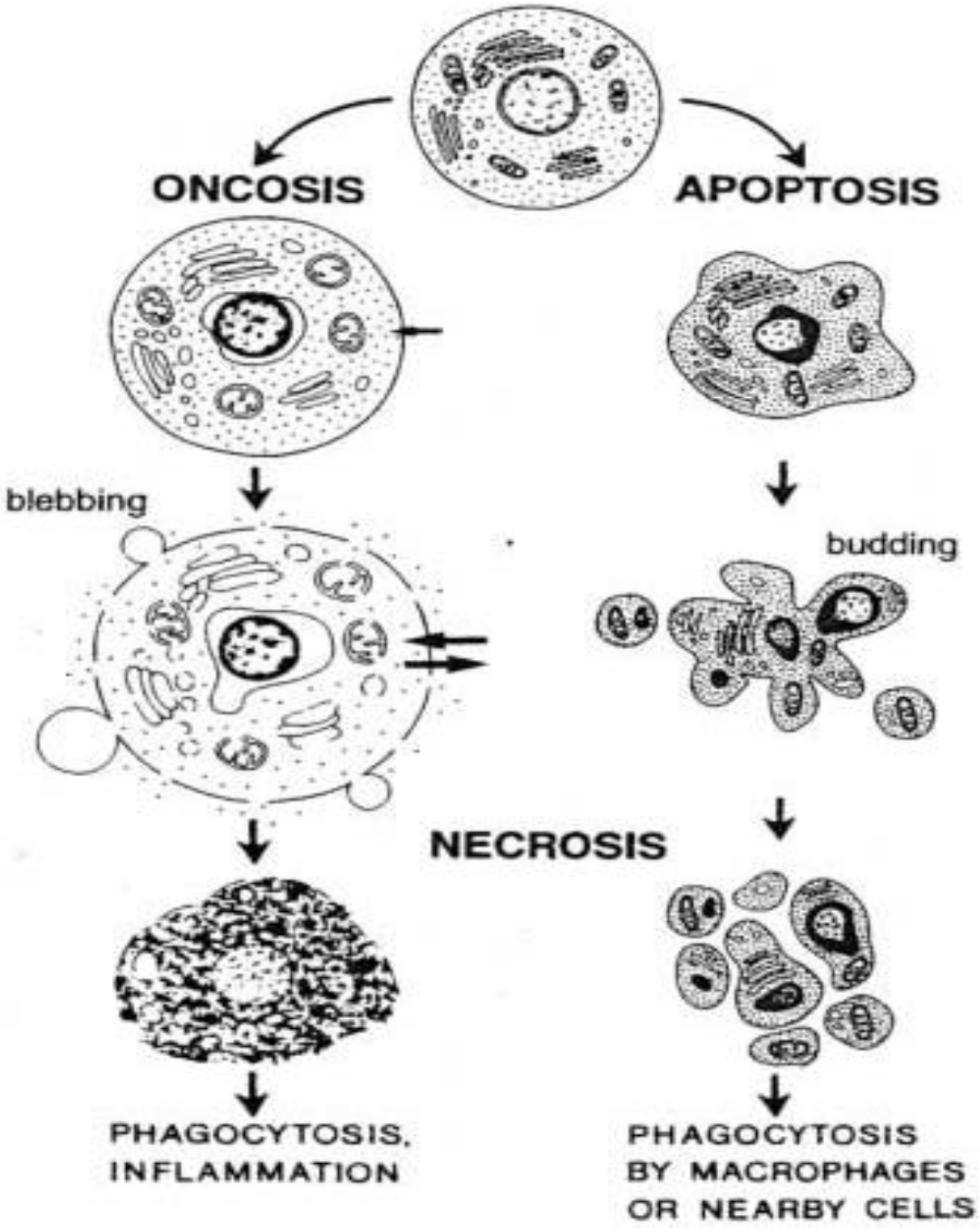


Figure 1. Apoptosis vs. Necrosis (Majno and Joris, 1995)

2.5. Apoptotic Pathways

The activation of apoptosis is highly restricted by initiation mechanisms, because once apoptosis has begun, it irreversibly leads to the death of the cell, the two best-known activation mechanisms are the intrinsic pathway and the extrinsic pathway (Alberts et al., 2008). The intrinsic pathway (also called mitochondrial pathway) is triggered by intracellular signals produced when cells are stressed and release proteins from the intermembrane space of mitochondria (Abd El-Ghany et al., 2009; Bishayee et al., 2013), while the extrinsic pathway is initiated by an extracellular ligands binding to death receptors on the surface of cell membrane which leads to the formation of the death-inducing signaling complex (DISC) (Friesen et al., 1996; Hyer et al., 2008) (Figure 2).

2.5.1. Intrinsic pathway (mitochondrial pathway)

The mitochondria are essential to eukaryotic cell life, due to their important role in cell respiration and energy production. Apoptotic proteins that affect mitochondria cause swelling of mitochondria by forming membrane pores and may increase the membrane permeability of the mitochondria and cause the leakage of apoptotic factors (González et al., 2010). Mitochondrial proteins known as second mitochondria-derived activator of caspases (SMACs), are released into the cell's cytoplasm due to the increasing of mitochondrial membrane permeability then bind to other apoptotic proteins such as Inhibitors of apoptosis proteins (IAPs) preventing them from arresting the process and as a result allowing to activate a group of cysteine proteases called caspases and proceed apoptosis (Callus and Vaux, 2007). Another apoptotic inducer is the cytochrome c which also released from mitochondria due to loss of membrane impermeability, through the mitochondrial apoptosis-induced channel (MAC) in the outer membrane of mitochondria (Dejean et al., 2006). Once cytochrome c is released to cytosol it binds with apoptotic protease activating factor – 1 (Apaf-1) and ATP, which then bind to pro-caspase-9 to create a protein complex known as an apoptosome (Cotran et al., 2002). Intracellular signals that initiate intrinsic pathway are playing a positive or negative roles, for example, negative signals include the reduction of some growth factors, cytokines and hormones can suppress the cell aging and apoptosis, while positive stimuli includes factors that induce cell apoptosis such as

toxins, radiation, hypoxia, hyperthermia, viral infections, and free radicals (Elmore, 2012). free radicals are one of the effective characters that induce intrinsic apoptosis, but also it is created in mitochondria in the mean of reactive oxygen species (ROS) as byproduct of cellular respiration, and in the absence or reduced amount of anti-oxidants, an excess ROS may accumulate and causing irreversible damage to DNA and mitochondrial membrane initiating cell death cascade (Hazra et al., 2007).

2.5.2. Extrinsic pathway

As the name refers this pathway starts from the outer cell, according to (Wajant, 2002) there is two hypothesis to initiate direct apoptotic mechanisms from outside of the cells:

Tumor Necrosis Factor (TNF) Path: The tumor necrosis factor alpha (TNF-alpha) is a strong cytokine produced monocytes, macrophages, keratinocytes, lymphocytes, and fibroblasts, as a response to injury, infection, inflammation. TNF-alpha activates a cascade of cellular responses participate in cell proliferation, differentiation, and apoptosis (Baud and Karin, 2001). TNF-alpha binds to two distinct receptors in cell membrane, tumor necrosis factor receptor 1 (TNFR1) and tumor necrosis factor receptor 2 (TNFR2), by binding of TNF-alpha to TNFR1 it induces receptor trimerization and activates TNFR1-associated death domain protein (TRADD), which serves as a intermediate to recruit three additional mediators: TNF-receptor-associated factor 2 (TRAF2), receptor-interacting protein 1 (RIP1), and Fas-associated death domain protein (FADD), activated receptor TNFR1 will lead to some signaling caspase cascade translated to apoptosis (Baud and Karin, 2001).

First apoptotic signal (Fas) path: the other extrinsic pathway, also known as apoptosis antigen 1 (Apo-1) or cluster of differentiation 95 (CD95) is a death domain–consists of a group of the tumor necrosis factor receptor (TNFR) superfamily. It has a major role in the physiological regulation of apoptosis (Peter and Krammer, 1998). The interaction between Fas and Fas ligand (FasL) forms the death-inducing signaling complex (DISC), which contains the caspase-8, caspase-10, and FADD. In some types of cells, activated caspase-8 directly affect other caspase family members, and lead to apoptosis of the cell. In other cells, the Fas-DISC starts an increasing release of

proapoptotic factors from mitochondria and the amplified activation of caspase-8 (Wajant, 2002) (Figure 2).

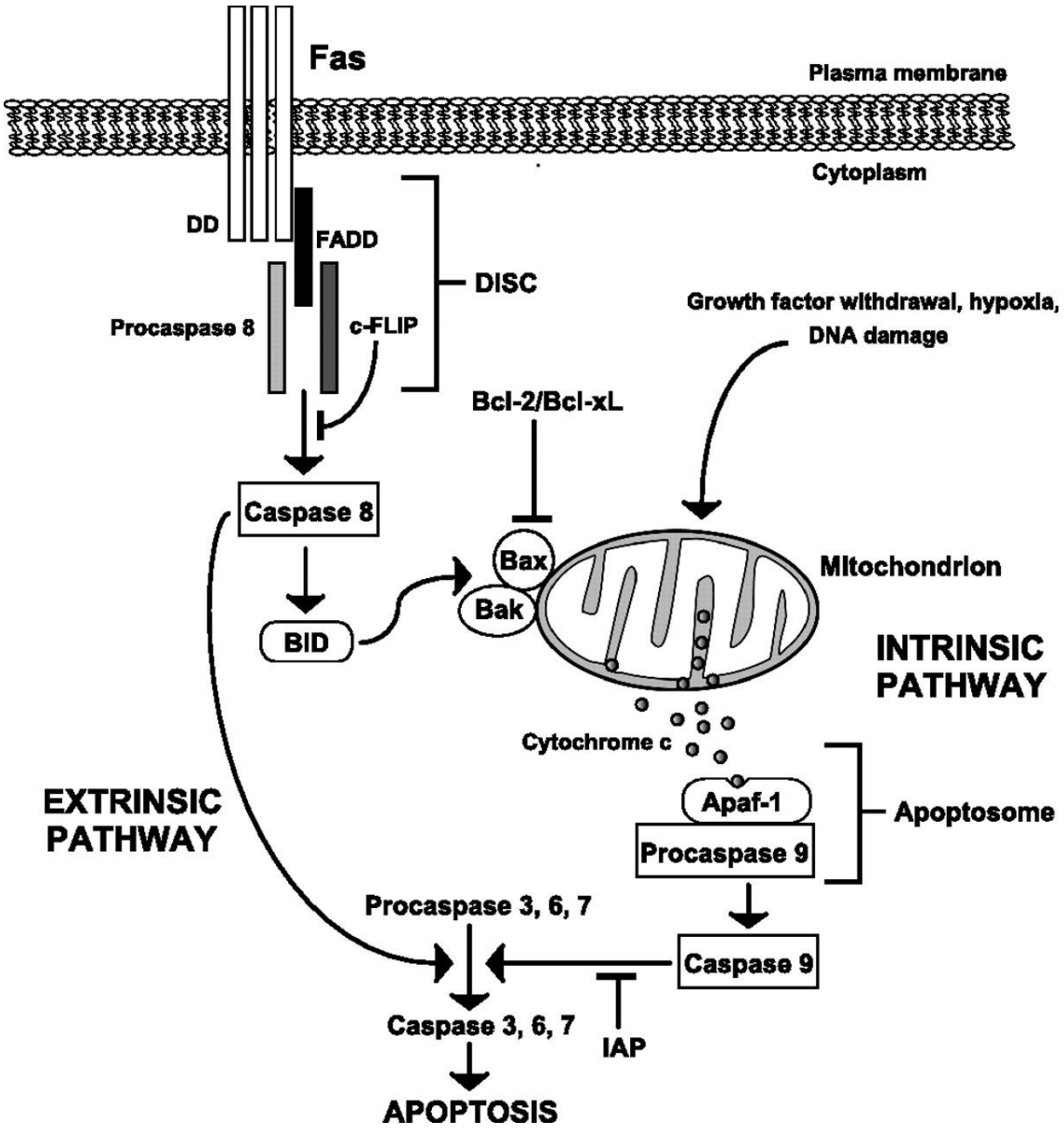


Figure 2. Apoptotic pathways (Choi and Woo, 2010)

2.6. Caspases

Caspases are a family of endoproteases that have great roles in controlling cell death and inflammation, and the name (caspase) is derived from cysteine-aspartic proteases. Activation of apoptotic caspases leads to creating a cascade of signals allowing the destruction of cellular components. Inflammatory caspases activations produce an active pro-inflammatory cytokines and promote native immune responses to many inner and exterior insults (McIlwain et al., 2015).

Until now there have been 14 types of caspases discovered (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14). Caspases are divided into two subfamilies according to their structure. Caspases with long pro-domain have structural designs such as caspase activation and recruitment domain (CARD) or death affecte domains (DADs) which give them the ability to interact with other proteins, the second group of caspases have short pro-domains and activated by photolytic cleavages from other caspases. The caspases also divided into three subfamilies according to their function (Figure 3). The first subfamily is called apoptotic initiator contains caspase (2, 8, 9, and 10). The second is known as apoptosis executioner of caspases (3, 6, and 7), and finally the third and the largest group is inflammatory mediator contain caspase (1, 4, 5, 11, 12, 13, and 14) (T. J. Fan et al., 2005; Callus and Vaux, 2007; Galluzzi et al., 2016) (Table 1).

Table 1. Subfamily members of caspase family

Subfamily	Role	Members
I	Apoptotic initiator	Caspase-2
		Caspase-8
		Caspase-9
		Caspase-10
II	Apoptotic executioner	Caspase-3
		Caspase-6
		Caspase-7
III	Inflammatory mediator	Caspase-1
		Caspase-4
		Caspase-5
		Caspase-11
		Caspase-12
		Caspase-13
		Caspase-14

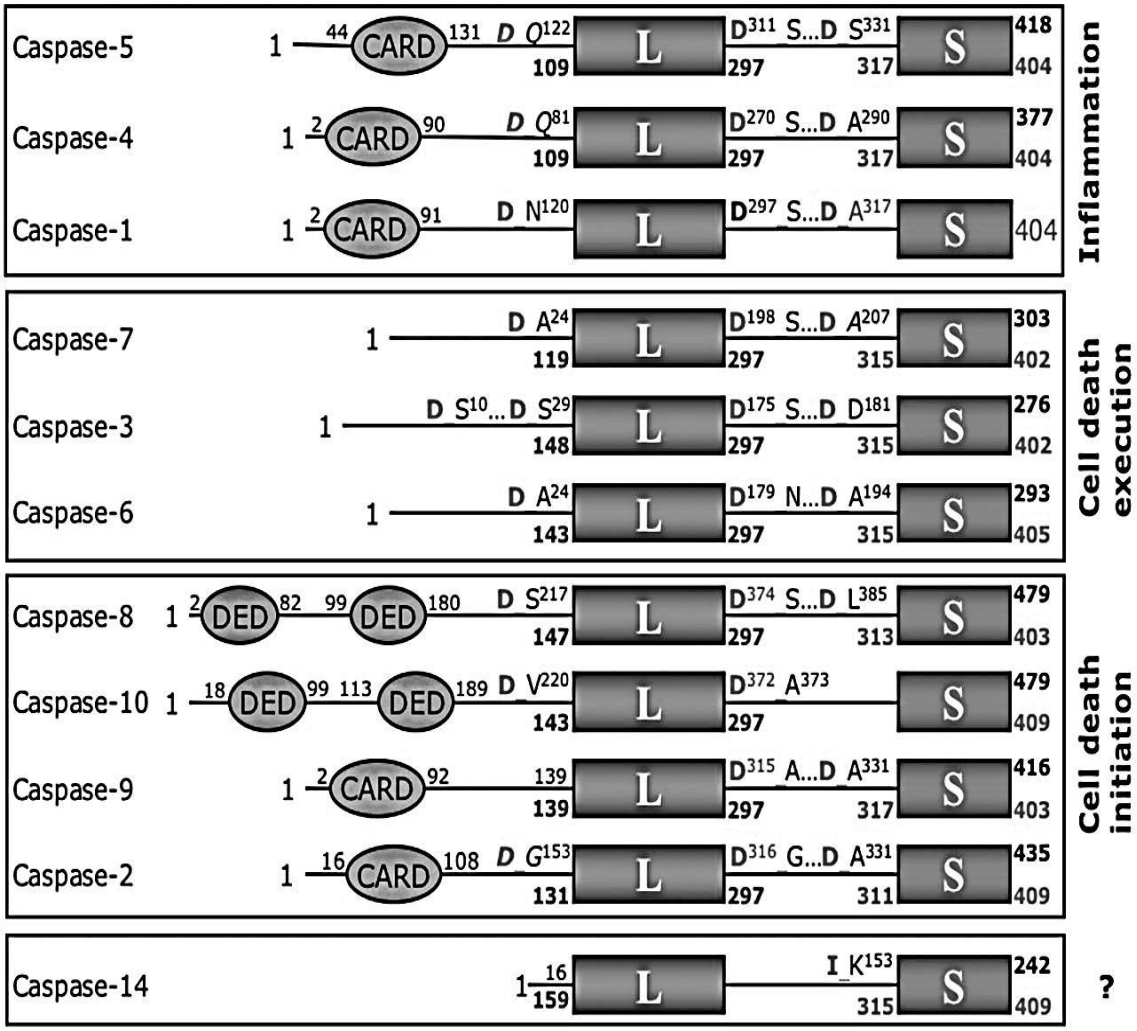


Figure 3. Domain organization of human caspases (Fuentes-Prior and Salvesen, 2004)

2.6. Novel Fluorine Boron Hybrid Complex (L_5BF_2)

Boron-based drugs represent a new class of molecules that have several biomedical applications including as therapeutic agents with anticancer, antiviral, antibacterial, antifungal and other medical purposes (Das et al., 2013). The novel fluorine boron hybrid complex (L_5BF_2) has been used in this study. The Schiff base ligands' corresponding fluorine boron hybrid complex has been newly synthesized and well characterized by both analytical and spectroscopic methods (Ozbahceci, 2015) (Figure 4).

In this study two cell lines and two chemicals have been used, adenocarcinoma of human endometrial cell line ECC-1 as the target cell and normal prostate epithelium

cell line PNT1-A as control, while the chemicals which used as anticancer agents were novel fluorine boron hybrid complex (L_5BF_2) as a new agent and cisplatin as a positive control. The aim of this study is to estimate the apoptotic, cytotoxic and genotoxic effect of L_5BF_2 by determining the MTT, ELISA, DNA laddering, COMET assay, acridine orange , total antioxidant status (TAS), total oxidant status (TOS) and Ferric Reducing Ability of Plasma (FRAP) results on the endometrial cell line ECC-1.

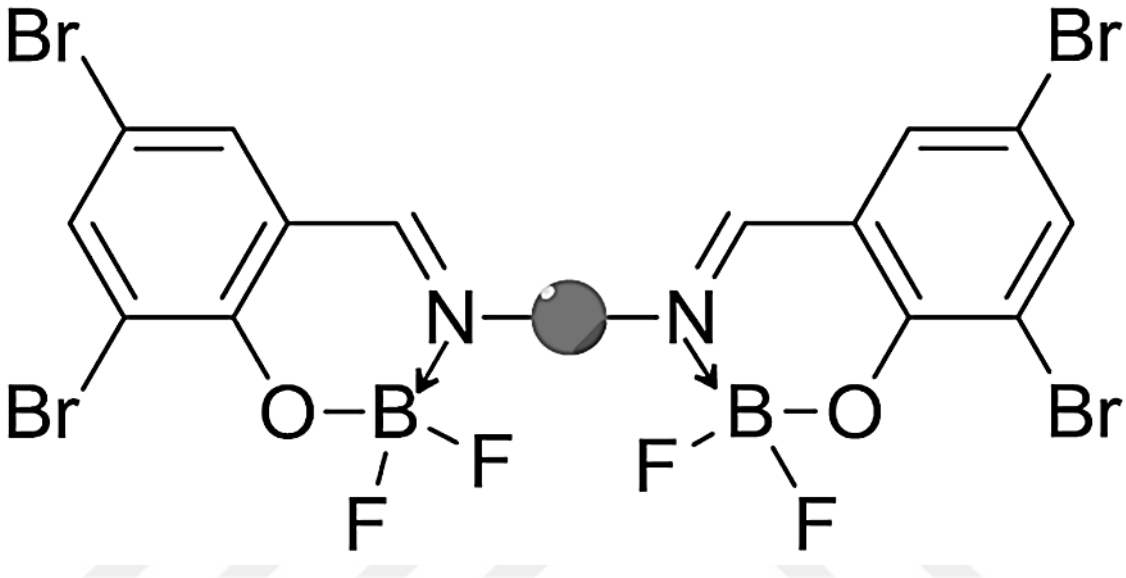


Figure 4. Novel fluorine boron hybrid complex (L_5BF_2) (Ozbahceci, 2015)

3. MATERIALS AND METHODS

3.1. Cell Culture

Normal human prostate epithelial cells (PNT1-A cells) and human endometrial adenocarcinoma cells (ECC-1 cells) were obtained and maintained in RPMI 1640 supplemented with heat in activated FBS (10%), streptomycin (100 mg/mL), and penicillin G (100 U/mL). The cells were cultured at 37^{o}C in a humidified atmosphere of 95% air/5% CO_2.

When cells were grown to convergence, they were detached with sterilized trypsin/EDTA solution. As the cells were detached in 37^{o}C incubator, trypsin activity was inhibited by the addition of complete medium. Cells were counted with a hematocytometer by the following formula:

Cells/ml= average count per square X dilution factor X 10

Equal volumes of cell suspension were plotted onto new dishes or flasks in appropriate densities.

3.2. Cell Viability Assay (MTT Assay)

The cell viability was analyzed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide] assay. ECC-1 endometrial cancer cells were cultured in 96-well plates at a population of 5 x 10^3 cells/well and allowed to attach. After incubation at 37^{o}C 5% CO_2 for 24 hours with exposure to the L_5BF_2 at different concentrations (2-200 µM), then stained with MTT solution (10 mL; 5 mg/mL in phosphate-buffered saline) for 3 h to dissolve the dark formazan crystals. The absorbance was then measured at 540 nm using a microplate reader (Varioskan Flash Multimode Reader, Thermo, Waltham, MA). The IC_{50} value was evaluated as the concentration of the complex required to reduce the absorbance of the treated cells to 50% of that of the dimethyl sulfoxide DMSO-treated control cells. Control group designated as 100%. Three individual wells were measured per treatment point.

3.3. Single Cell Gel Electrophoresis Assay (Comet Assay)

The comet assay is a sensitive assay to examine DNA fragmentation at the level of the individual cells. In this assay, there are two types of lysis solutions; alkaline lysis solution to detect double and single strand breaks and neutral lysis solution to detect double strand breaks (Nature Protocol). In this experiment, alkaline lysis solution was applied to examine any effect of L_4BF_2 that causes DNA damage.

Samples were prepared as followed: $5x10^5$ cells in 1980 µL was cultured in a 6-well plate (Corning® Costar®) and incubated at 37°C 5% CO_2 for 24 hours. Next day 3 concentrations of the L_4BF_2 were prepared by dissolving with sterile DMSO and filtered. Then 20 µL of each concentration of L_4BF_2 were applied to wells and incubated at 37°C 5% CO_2 for 24 hours. 20 µL 50 µM of H_2O_2 as a positive control was applied for last 2 hours of incubation (Benhusein et al., 2010). After incubation mediums of each well were collected to falcons, cells were trypsinized and collected in the same falcon. Then falcons were centrifuged at 1800 rpm for 10 minutes then pellet were washed with 5 mL PBS and centrifuged again. Pellets were dissolved in 500 µL of PBS and each sample was counted as explained above.

After sample preparation, slides were prepared as following: 1% normal melting agarose (NMA) was prepared in 50 mL of PBS, microwaved until near boiling and dissolve agarose. 1% low melting agarose (LMA) was prepared in 10 mL of PBS, microwaved until near boiling and dissolve agarose. LMA was aliquoted in 5 mL samples and refrigerated until needed. When needed, melt in microwave and keep in a water bath and stabilize temperature as 40°C. Slides were dipped in methanol and burned with a flame to remove dust. Then slides were dipped in hot NMA, the underside of slides was wiped to remove agarose and laid on a flat surface to dry (Slides can be prepared before the day of experiment). 75 µL of LMA was mixed with about 10.000 in 5-10 µL of cells, pipetted on NMA covered slides and covered with a coverslip. Slides were placed on ice pack until second agarose layer hardened (5-7 minutes). Then coverslips were removed gently and samples were placed in freshly prepared cold lysis solution (1,2 M NaCl, 100 mM Na2EDTA, %0,1 Sodium Lauryl Sarcosinate (SLS), 0,26 M NaOH, pH adjusted 13 or higher) and incubated at +4 °C for overnight. After incubation slides were removed, rinsed with fresh made cold

alkaline/electrophoresis solution dropwise onto slides kept for 20 minutes and repeated 2 more times (0,03 M NaOH, 2 mM Na2EDTA, pH adjusted to 12,3). Then slides were placed in fresh electrophoresis solution in electrophoresis tank run for 20 minutes at 0,6 V/cm and the current was 40 mA. After electrophoresis slides were gently removed from electrophoresis tank, neutralized with neutralization buffer by dropwise coat slides for 5 minutes 3 times and let them dry (0,4 M Tris-Cl, ph adjusted to 7,4). 100 µL of 10 µg/mL PI solution pipetted onto slides, incubated for 20 minutes and rinsed with dH2O. Comets were analyzed with a microscope under UV light.

3.4. Apoptotic Analysis by ELISA

The assay is based on the quantitative sandwich enzyme- Two immunoassay-principle using mouse monoclonal anti- bodies directed against DNA and histones, respectively. Specificity anti-histone antibody reacts with the histones H1, H2A ,H2B, H3 and H4 of various species, e.g., man, mouse, rat, hamster, cow, opossum, Xenopus. Anti-DNA POD antibody binds to single- and double- stranded DNA. Therefore, the ELISA allows the detection of mono and oligonucleosome from various species and may be applied to measure apoptotic cell death in many different cell systems.

To prepare the sample we introduce dilute cells with culture medium to obtain a suitable cell concentration, the cell number per test has to be determined and optimized. As a model system for cell death, camptothecin (CAM) was used as the apoptosis-inducing drug. The tests were performed with the ECC-1 cell line as target cells,

Dilute exponentially growing ECC-1 cells with culture medium to obtain a cell concentration of $1x10^5$ cells/ml, we transferred into Eppendorf tubes (500 µl/tube 5×10^4cells/tube), we added 500 µl culture medium with different concentrations of CAM (0 µg CAM/ml to 4 µg CAM/ml, closed tubes loosely to allow further exchange of gas and incubated at 37°C in a CO_2 -incubator for 4 h. then centrifuged the cells in an Eppendorf centrifuge at 200× g for 5 min and discarded the supernatant and resuspend the cell pellet in 1 ml culture medium. We centrifuged the cells again in an Eppendorf centrifuge at 1500× g for 5 min; resuspend the cell pellet in 500 µl Incubation buffer per tube (1×10^5 cell/ml and we mix thoroughly. Incubate the sample for approx. 30 min at 15 to 25^{o}C (= lysis), then centrifuged the lysate at 20,000× g for 10 min and remove 400

µl of the supernatant (= cytoplasmic fraction) carefully. Then we prediluted the resulting supernatant 1:10 with incubation buffer (= 1×10^4 cell equivalents/ml) and detected the nucleosomes in the sample by immunoassay.

To read the sample by ELISA we started to pipette 100 µl coating solution into each well of the MP-modules, then covered MP-modules tightly with the adhesive cover foil included after incubate for 1 h at 15–25°C we removed coating solution thoroughly by tapping or suction then pipette 200 µl incubation buffer into each well of the MP-modules. Covering MP-modules tightly and incubate for 30 min at 15 – 25°C then we removed the solution. Rinse wells three times with 250 – 300 µl washing solution per well and remove washing solution carefully. Pipette 100 µl of sample solution into each well of the MP-modules, then covered tightly and incubated for 90 min at 15 – 25°C, remove solution and rinse wells three times with 250–300 µl washing solution per well, pipette 100 µl of conjugate solution into each well of the MP-modules, except the blank position. For determination of the background of the immunoassay, pipette 100 µl per well of Incubation buffer into two wells. Cover MP-modules and incubate for 90 min at 15 – 25°C. 12 then remove the solution, rinse wells three times with 250 – 300 µl washing solution per well and remove washing solution carefully. pipette 100 µl of substrate solution into each well of the MP-modules and incubate on a plate shaker at 250 rpm until the color development is sufficient for a photometric analysis (approx. after 10 – 20 min). 15 Homogenize well contents by careful tapping the side of the MP, before measuring at 405 nm with respect to a substrate solution blank. Optionally, use 490 nm as the reference wavelength.

3.5. Apoptotic DNA fragmentation (DNA Laddering)

Fragmentation of DNA and chromatin is an integral process during apoptosis and has been reported to occur in more than one distinct stage (Suman et al., 2012).The apoptotic DNA ladder kit (Roche®) is designed for the purification of nucleic acids cultured cells to detect the typical DNA ladder, which is the hallmark of apoptotic cells. DNA can be applied to the agarose gel directly after its elution from the column. Thus the purification procedure is less time consuming compared to alternative methods which require extraction with organic solutions or DNA precipitation.

We prepared working solutions for DNA gel electrophoresis EDTA solution (0.5 m) by dissolving 18.6 g EDTA in 80 ml distilled water (D.W.) and stir. pH was adjusted to 8.0 ± 0.1 with 1 M NaOH, because EDTA solubilizes at alkaline pH only. After solubilization, we fill up to 100 ml with D.W. to prepare TBE-buffer we dissolve 5.4 g Tris, 2.8 g boric acid in 800 ml D.W. and added 2 ml of 0.5 M EDTA solution. Stir until dissolved, final pH 8.0 ± 0.1. Fill up to 1 liter with D.W. to prepare ethidium bromide stock solution we dissolve 50 mg ethidium bromide in 5 ml D.W. and to prepare loading buffer we dissolved 0.1 g sodium dodecyl sulfate, 25 mg bromophenol blue in 7 ml D.W. and added 3 ml glycerol.

To prepare the 1% agarose DNA gel we fill 1 g agarose in an Erlenmeyer-flask and add 100 ml TBE- buffer. We place the flask into a microwave oven and heat at maximum energy level. We shake the solution when boiling occurs. Agarose microwaved until it is completely dissolved then cooled to +60°C and add 5 l ethidium bromide stock solution. We poured the solution into the prepared electrophoresis chamber and place the gel comb. After the gel has hardened, we removed the tape from the open ends of the gel platform and withdraw the gel comb, taking care not to tear the sample wells.

To prepare the sample we mixed 1–3 g of purified DNA and 15 l of positive control DNA with loading buffer to fill the loading pocket not completely. Then we add sufficient TBE buffer to cover the gel to a depth of about 1 mm and plugged the electrophoresis tank. Loading samples carefully into the sample pockets using a micropipette. Turn on power supply and set the voltage to 75 V (typically 5 V/ cm) for 1.5 hours, then turn off power supply when bromophenol blue dye from the loading buffer has migrated to the end of the platform. Visualizing the DNA by placing the gel onto a UV light source and photograph.

3.6. Acridine Orange and Ethidium Bromide Apoptotic Cell Analysis

Acridine Orange (AO) is a nucleic acid selective metachromatic stain useful for cell cycle determination. AO interacts with DNA and RNA by intercalation or electrostatic attraction respectively. DNA intercalated AO fluoresces green (525nm); RNA electrostatically bound AO fluoresces red (>630nm). It may distinguish between

quiescent and activated, proliferating cells, and may also allow differential detection of multiple G1 compartments (Darzynkiewicz, 1990). AO may also be useful as a method for measuring apoptosis, and for detecting intracellular pH gradients and the measurement of proton-pump activity (Darzynkiewicz et al., 1992).

Acridine orange/ propidium iodide double staining. To detect the early and late apoptotic properties of the treated ECC-1 cells, a propidium iodide (PI) and acridine orange (AO) double staining assay was performed using a fluorescent microscope according to the standard procedure. Briefly, the cells were incubated with 3 mg/mL L_5BF_2 complex for 24 hrs. The harvested cells were then stained with the AO/PI fluorescent dyes and observed under a UV-fluorescent microscope (Olympus BX51) within 30 min.

Ethidium bromide staining with methods to detect apoptosis morphologically whether treated with the lamellar material is washed 2 times with PBS for 2 minutes. Were fixed in 70% ethanol for 5 minutes, the cells were washed 5 times with distilled water. They are then allowed to stand at room temperature for 15 minutes two times in PBS acridine orange/ethidium bromide stained with study solution. Paint kept upright by the fins on the blotter paper was removed and washed 4 times with PBS. Covered over the PBS installed a drop fluorescence microscope slide to be examined in lamellar.

3.7. Determination of intracellular TAS

Intracellular cell total antioxidant status TAS level was measured using a novel automated colorimetric measurement method developed by (Erel, 2004). In this method, the hydroxyl radical, the most potent biological radical, is produced by the Fenton reaction and reacts with the colorless substrate O-dianisidine to produce the dianisyl radical, which is bright yellowish-brown in color. Upon the addition of a sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction mix are suppressed by the antioxidant components of the lysate, preventing the color change and thereby providing an effective measure of the TAS of the lysate. The assay results are expressed as mmol Trolox equiv/mg protein.

3.8. Determination of intracellular TOS

Intracellular total oxidant status TOS (Sakihama et al., 2000) level was measured using an automated colorimetric measurement method developed by (Erel, 2005). In this method, oxidants present in the sample oxidize the ferrous ion-O-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules (e.g. lipids, proteins) present in the sample. The assay is calibrated with HP, and the results are expressed in terms of micromolar hydrogen peroxide per mg protein (lmol HP/mg protein). The percent ratio of TOS to TAS level has been accepted as the oxidative stress index (OSI) (Dikilitas et al., 2011; Erel, 2004). OSI value was calculated according to the following formula:

$$OSI = \frac{\text{TOS, } \mu \text{ mol HP equiv / g}}{\text{TAS, } \mu \text{ mol Trolox equiv / g}} \text{ X } 10$$

3.9. Ferric Reducing Ability of Plasma (FRAP) Assay

A simple, automated test measuring the ferric reducing ability of plasma, the FRAP assay, is presented as a novel method for assessing ‘‘antioxidant power.’’ Ferric to ferrous ion reduction at low pH causes a colored ferrous-tripyridyltriazine complex to form (Benzie and Strain, 1996).

The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method employing an easily reduced oxidant, Fe(III). Reduction of a ferric tripyridyltriazine complex to ferrous-(2,4,6-tripyridyl-s-triazine)2 ie: Ferric (Ill) colorless to Ferrous (II) blue can be monitored by measuring absorbance at 593 nm. The absorption readings are related to the reducing power of the electron-donating

antioxidants present in the test compound. Hence the FRAP assay can rank the reducing power and the antioxidant potential of a wide range of test Compounds.

To get the readings, 100 µL of our sample is added to 900 µL of DW, then 2 mL of FRAP reagent is added as well all into the cuvette. After mixing leave for 30 min in dark. Then the mixture is transferred to cuvettes and read at 593 nm in a spectrophotometer. The calculation is done by the following equation:

$$\text{FRAP value } \mu M = \frac{\text{Abs.(sample)} \times \text{FRAP value of Std } (\mu M)}{\text{Abs. of Std}}$$

3.10. Statistical Analysis

All outcomes including the measured concentrations of TAS and TOS were evaluated statistically and the final results were expressed as mean± SD. The results are presented as three replicates. Data in the experiments were analyzed using analyses of variance (one-way ANOVA). The $p < 0.05$ value was considered as statistically significant. All statistical analyses were performed using MINITAB 14® package program for Windows.

4. RESULTS

4.1. Measuring Cell Proliferation Rate by the Meaning of MTT Assay

L_5BF_2 was examined in 8 different cell lines, cervix adenocarcinoma HELA, prostate cancer DU-145, prostate cancer cell line PC3, Dukes' type C, colorectal DLD-1, endometrial adenocarcinoma ECC, breast cancer MCF-7, normal prostate epithelium cell line PNT1-A, and colorectal adenocarcinoma HT-29, the results from the MTT assays on the ECC-1 and PNT1-A cell lines demonstrated that the L_5BF_2 complex exhibit cytotoxic effect on both normal cells and ECC-1 cells. As shown in (Table 2), the L_5BF_2 complex provoked the half maximal inhibitory (IC_{50}) value of 38.36 µM against ECC-1 cells, indicating significant inhibitory effect, and it was the minimum among other treated cells rendering to IC_{50} value (Figure 5). According to the previous outcomes we preferred to continuing further tests on ECC-1 treating it with various concentrations of L_5BF_2.

Table 2. IC_{50} values of different cell lines treated with L_5BF_2

No.	Cell Line	IC_{50} (µM)
1	Hela	123.8041
2	DU-145	43.85
3	PC3	61.31
4	DLD-1	52.751
5	ECC	38.36
6	PNT1-A	41.42
7	HT-29	164.857
8	MCF-7	74.81

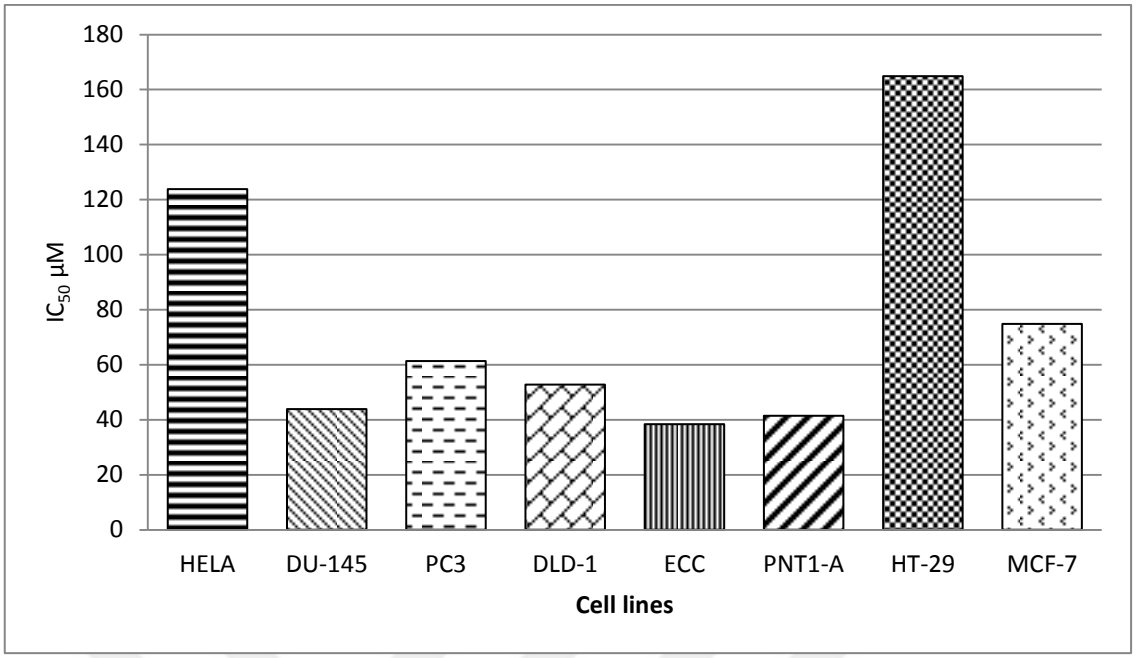


Figure 5. IC_{50} value of different cell lines treated with L_5BF_2

Both cell lines of ECC-1 (as a test subject) and PNT1-A (as normal control) were treated with different dosages (0, 2, 5, 10, 25, 50, 100 and 200 µM) of L_5BF_2 (Table 3 and 5) and cisplatin (Table 4 and 6). In the start, the two cell lines were treated with L_5BF_2 complex show no change in 0 µM concentration, a stable state of limited but not significant descending pattern was seen in both cell lines in 2, 5 and 10 µM doses, though in 25 µM dosage there was a significant drop in ECC-1 cell as same as PNT1-A cells, determining the effective dose of $p < 0.001$, this continued in last three doses to reach the least MTT value in 200 µM for both types of cells attaining cisplatin value of the same dose with lesser side effects compare to previous doses of cisplatin (Figure 6 and 7).

Table 3. ECC-1 Cell line treated with various concentrations of L_5BF_2

Dose (μM)	1	2	3	Mean ± STD
0	0.7865	0.7356	0.743	0.75503 ± 0.028
2	0.7866	0.73	0.7155	0.74403 ± 0.038
5	0.6672	0.6847	0.7352	0.69570 ± 0.035
10	0.6675	0.7316	0.6333	0.67747 ± 0.050
25	0.5616	0.5409	0.5295	0.54400 ± 0.016
50	0.3639	0.3246	0.3129	0.33380 ± 0.027
100	0.1691	0.1738	0.1903	0.17773 ± 0.011
200	0.0769	0.0754	0.0747	0.07567 ± 0.001

Table 4. PNT1-A Cell line treated with various concentrations of L_5BF_2

Dose (μM)	1	2	3	Mean ± STD
0	0.5847	0.5968	0.6325	0.60467 ± 0.025
2	0.5597	0.5445	0.6387	0.58097 ± 0.051
5	0.53018	0.5437	0.5556	0.54316 ± 0.013
10	0.5377	0.5127	0.5208	0.52373 ± 0.013
25	0.4339	0.4092	0.4413	0.42813 ± 0.017
50	0.2436	0.2323	0.2449	0.24027 ± 0.007
100	0.1864	0.1674	0.1938	0.18253 ± 0.014
200	0.1184	0.11856	0.0889	0.10862 ± 0.017

Table 5. ECC-1 Cell line treated with various concentrations of cisplatin

Dose (μM)	1	2	3	Mean ± STD
0	0.7446	0.7219	0.7985	0.7550 ± 0.039
2	0.7178	0.776	0.6865	0.7267 ± 0.045
5	0.6385	0.6125	0.6165	0.6225 ± 0.014
10	0.5212	0.4952	0.5809	0.5324 ± 0.044
25	0.4238	0.4608	0.4348	0.4398 ± 0.019
50	0.3186	0.3012	0.2919	0.3039 ± 0.014
100	0.1587	0.1637	0.1837	0.1687 ± 0.013
200	0.0199	0.0189	0.0191	0.0193 ± 0.001

Table 6. PNT1-A Cell line treated with various concentrations of cisplatin

Dose (μM)	1	2	3	Mean ± STD
0	0.5710	0.5904	0.6436	0.6017 ± 0.0376
2	0.4852	0.5126	0.4689	0.4889 ± 0.0221
5	0.2688	0.2479	0.2279	0.2482 ± 0.0205
10	0.1659	0.1885	0.1912	0.1818 ± 0.0139
25	0.1411	0.1608	0.1341	0.1453 ± 0.0138
50	0.0579	0.0554	0.0561	0.0565 ± 0.0013
100	0.0247	0.0268	0.0253	0.0256 ± 0.0011
200	0.0120	0.0116	0.0124	0.0120 ± 0.0004

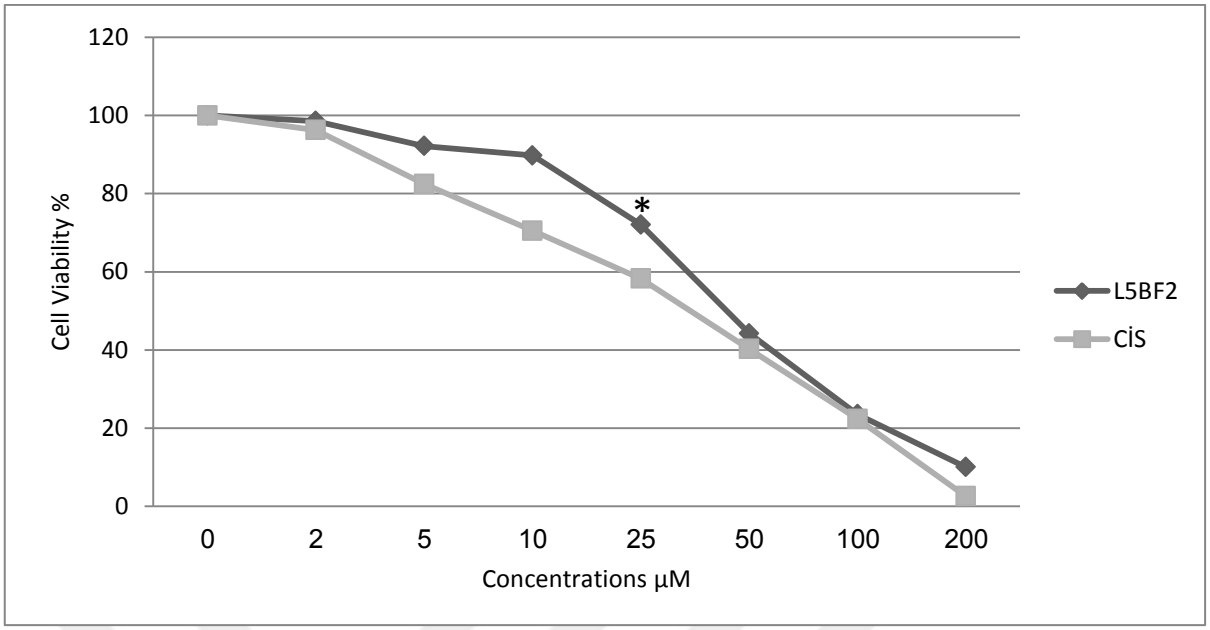


Figure 6. Effect of L_5BF_2 and cisplatin on ECC-1 cells viability (* = effective dose)

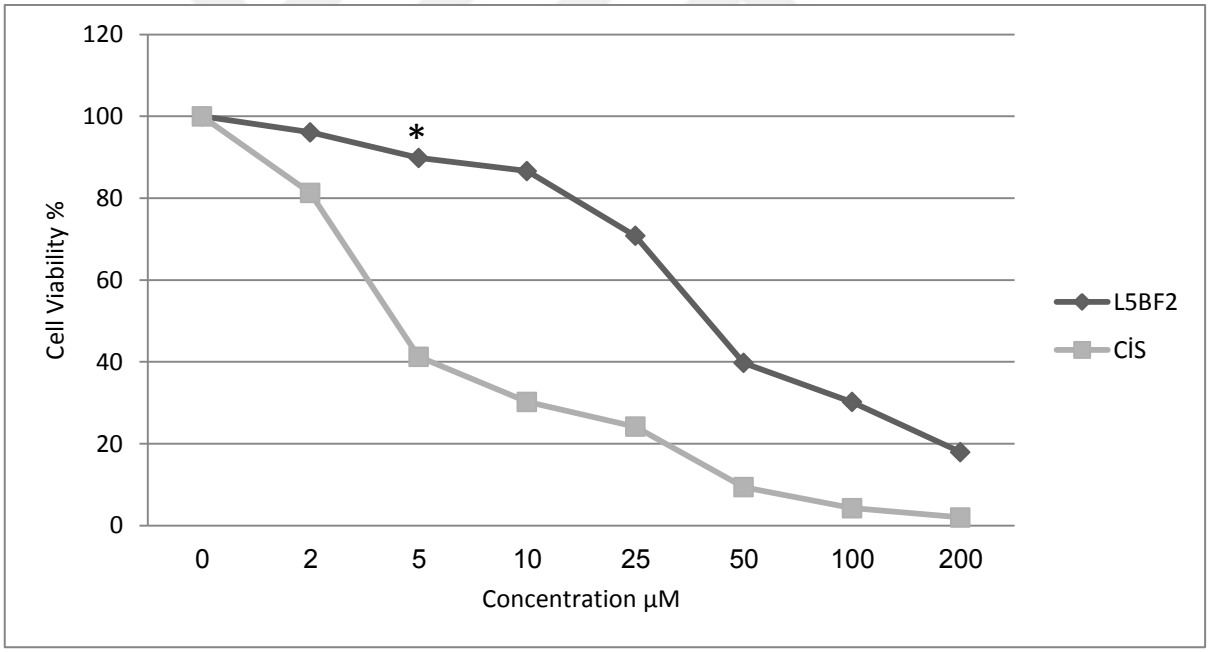


Figure 7. Effect of L_5BF_2 and cisplatin on PNT1-A cells viability (* = effective dose)

4.2. Determination of DNA Damage in ECC-1 cells by the Meaning of Single Cell Gel Electrophoresis assay

The comet is a rapid and sensitive method for the detection of DNA damage in individual cells, induced by a variety of genotoxic agents. It was first introduced by (Ostling and Johanson, 1984), and later modified independently by (Singh et al., 1988) and (Olive, 1989). Image analysis provides three important parameters for each comet: tail length, tail fluorescence intensity and tail moment (Collins et al., 1997).

The test was initialized with six different concentrations of L_5BF_2 on ECC-1 cells (0, 5, 10, 25, 50, and 100 μM) (Table 7). In the binging, no changes observed, yet in the first increasing doses there was a notable alteration in apoptotic value depending on comet extension tails, to reach the top value of 52.3 arbitrary units in the last dose of 100 μM recording a high significant rate precede the typical control drug cisplatin with a p-value of $p<0.0001$ (Figure 8).

Table 7. Results of comet assay test of ECC-1 cells treated with L_5BF_2

Doses µM	Arbitrary Unit			Mean ± STD
0	3.0	4.0	6.0	4.3 ± 1.53
5	12.0	9.0	8.0	9.7 ± 2.08
10	15.0	18.0	14.0	15.7 ± 2.08
25	20.0	24.0	29.0	24.3 ± 4.51
50	30.0	32.0	36.0	32.7 ± 3.06
100	52.0	46.0	59.0	52.3 ± 6.51
Cis	32.0	46.0	50.0	42.7 ± 9.45

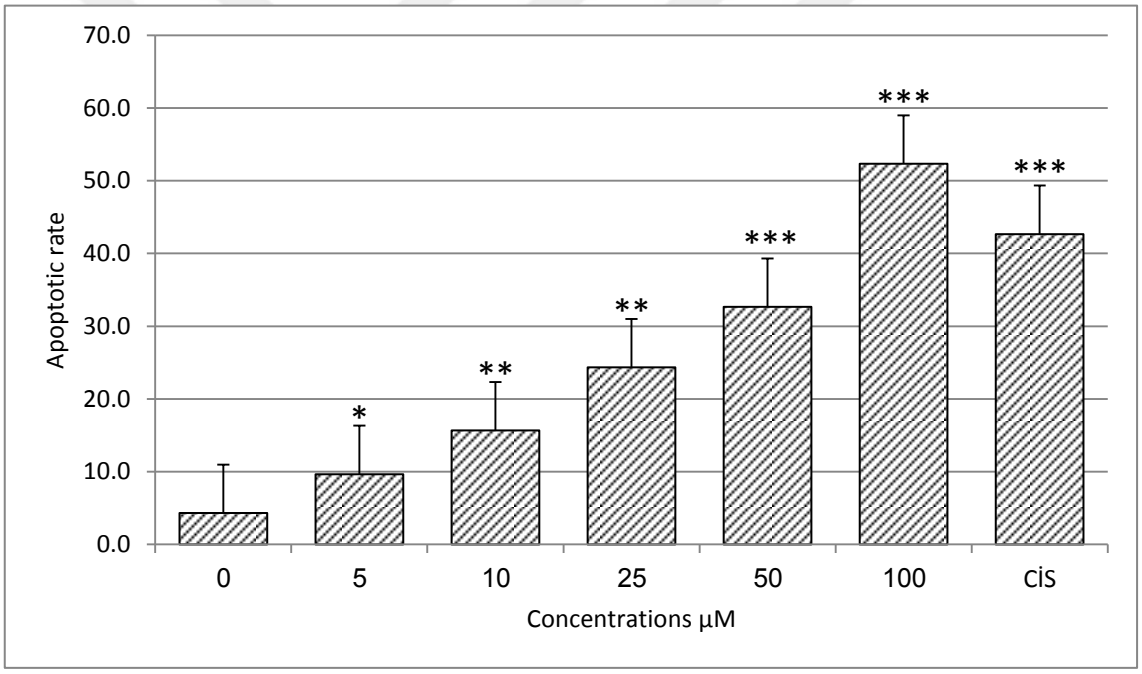


Figure 8. DNA damage rate by the mean of comet formation in ECC-1 cells treated with L_5BF_2 and cisplatin (*=P<0.05, **=P<0.005, ***=P<0.0005)

4. 3. Determination of Apoptosis by Using Acridine Orange

Acridine Orange (AO) is an organic compound used as a nucleic acid-selective fluorescent cationic dye useful for cell cycle determination and because it is cell-permeable, it reacts with DNA and RNA by interjection or electrostatic attractions respectively. AO has two various dyeing features, in fixed tissues, it is used as a metachromatic stain, by differentiating single-stranded nucleic acids with orange and double-stranded nucleic acids with green. However, in living cells it serves as a pH indicator, becoming stuck in acidic compartments such as lysosomes and phagosomes, which then fluoresce a brilliant orange-red (Mirrett, 1982).

To determine the relation between vital, necrotic and apoptotic cells of ECC-1 treated with L_5BF_2, we performed an AO test using various concentrations of L_5BF_2 (Table 8). According to the results, a descending pattern is observed in vital cells except in the concentration of 50 µM in which a fluctuation was recorded as same as apoptotic cells in which have an adverse ascending pattern, not forget to mention the slight differences between the 10 and 25 µM concentration in total cell ratio showing no changes in cell percentage values (Figure 9). Also, we got to declare that even in the highest dose of 100 µM, the apoptotic percentage didn't reach the typical cisplatin dosage for the same cells. Either for necrotic cell percentage, there were no dramatical changes in cell percentage in any of different concentrations of L_5BF_2.

Table 8. Growth rate of necrotic, vital and apoptotic ECC-1 cells treated with L_5BF_2

Dosage µM	Necrotic %	Vital %	Apoptotic %
0	1.655	94.563	3.783
10	4.167	68.750	27.083
25	3.673	68.163	28.163
50	4.484	70.852	24.664
100	4.000	60.000	36.000
Cis	3.960	54.455	41.584

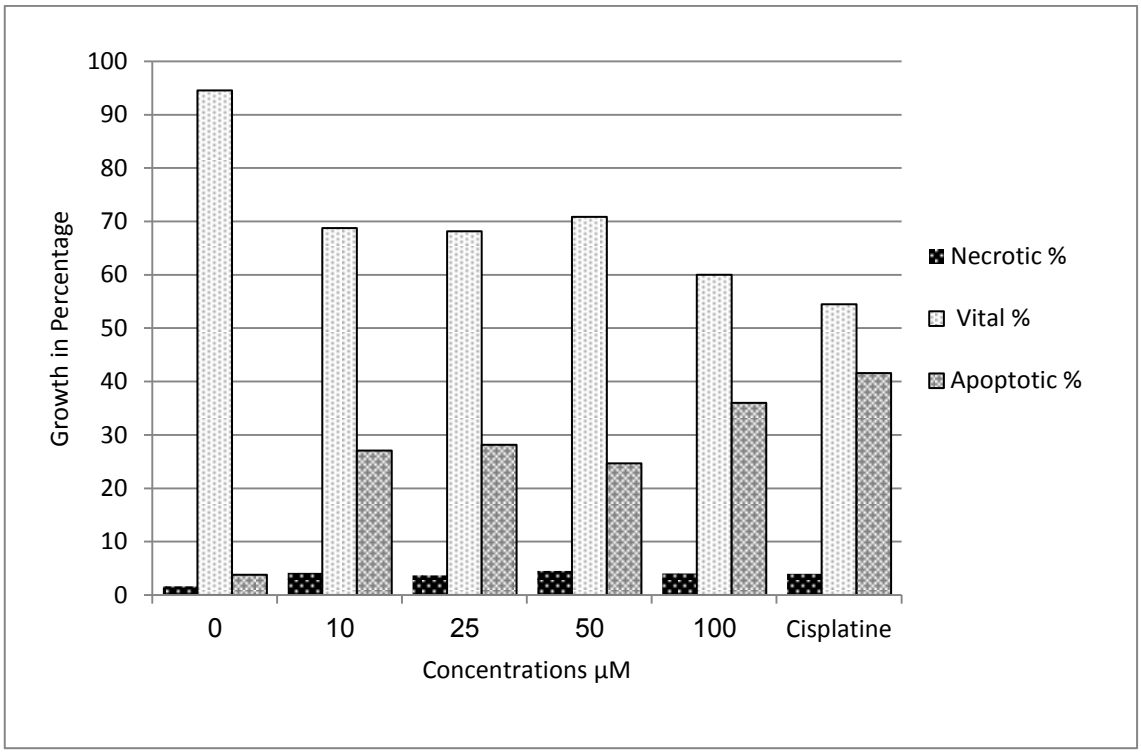


Figure 9. A diagram compare between the ratios of necrotic, living and apoptotic ECC-1 cells, treated with L_5BF_2

4.4. Determination of DNA Damage Using DNA Fragmentation Technique

The biochemical hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die. Separation of the fragments by low melting agarose gel electrophoresis and subsequent visualization, for example by ethidium bromide staining, results in a distinctive "ladder" pattern, Its main units of measurement is the DNA Fragmentation Index (DFI) (Speyer et al., 2010).

To investigate DNA damage by the meaning of DNA laddering, we used various concentrations of L_5BF_2 in this test (0, 10, 25, 50, and 100 µM) in EEC-1 cells, the first concentrations of 0 µM show no significant extension in DNA matter as in control, though there was a distinguishable DNA matter extension in 10, in 25 µM concentration it showed the best results among all other doses of L_5BF_2 and lead to ladder formation which indicates DNA fragmentation. However, it was observed that the fragmentation of 50 and 100 µM to be less than previous doses, and the best DNA laddering result belong to cisplatin with most extended area of DNA compare to earlier results (Figure 11)

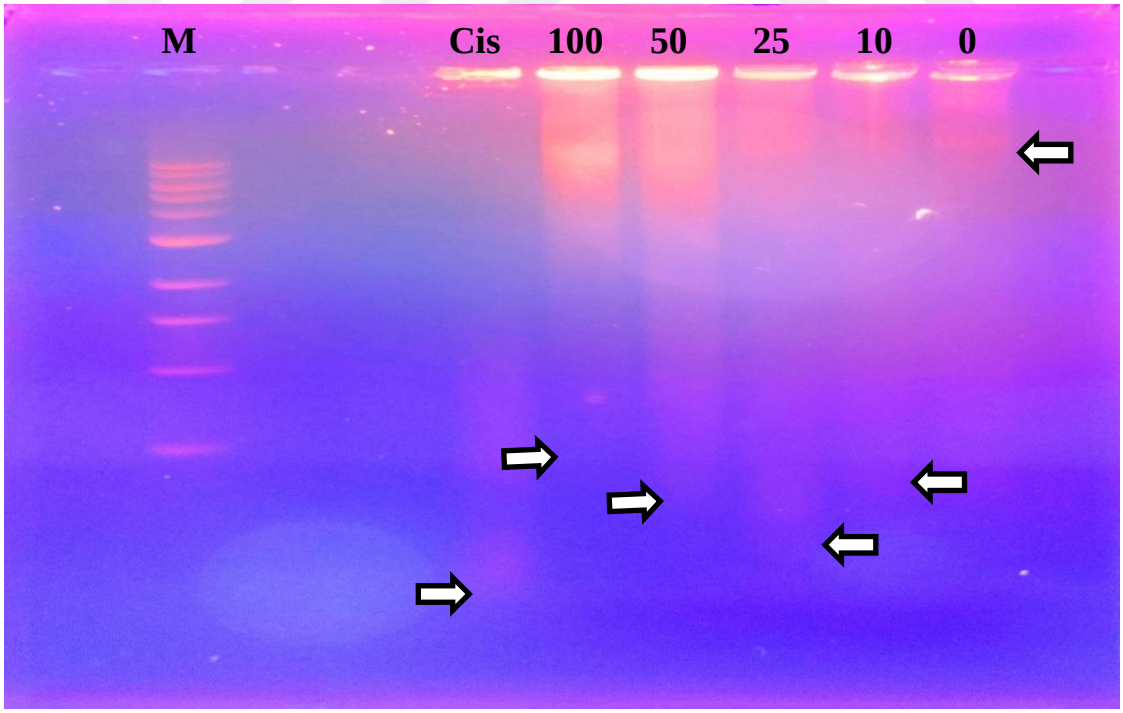


Figure 10. DNA laddering of ECC-1 cells treated with L_5BF_2 and cisplatin (the index show the last point of DNA extension)

4.5. Measuring Apoptosis of ECC-1 by ELISA

The role of L_5BF_2 induced cell apoptosis was determined by ELISA apoptotic assay. In apoptosis there are some specific events that determined by the mean of cytoplasmic condensation cell membrane blebbing and the activation of the endogenous endonuclease, causing mono- and oligonucleosome to form. In contrast, the DNA of the nucleosomes is strongly compacted with the core histones H2A, H2B, H3, and H4, therefore, protected from disassembling by the endonuclease (Burgoyne et al., 1974).

We applied different doses of L_5BF_2 to ECC-1 cells (Table 9), 0 μM considered the control dose and after first ascending dose of 5 μM concentration there was a dramatical increase in DNA damage showing the first effective dose, followed by 10 μM recording the second effective dose according to the p-value of p=0.0001 and p=0.006 respectively. Continuing the elevation pattern in the last three concentrations with no significant chang until it reached the pick in the 100 μM concentration, almost equal to same in cisplatin with a slight difference of p=0.939 indicating the highest effect of L_5BF_2 on ECC-1 cells.

Table 9. ELISA measurements of ECC-1 cell apoptosis treated with L_5BF_2

Dose (μM)	1 Abs	2 Abs	3 Abs	Mean	Relative
0	1.00795	0.985	1.15	1.0477	1.0
5	2.3025	2.215	2.342	2.2865	2.2
10	2.615	2.78	2.596	2.6637	2.5
25	2.8255	2.985	2.789	2.8665	2.7
50	2.9665	2.89	3.05	2.9688	2.8
100	2.9265	3.215	3.185	3.1088	3.0
Cis	3.02	3.19	3.142	3.1173	3.0

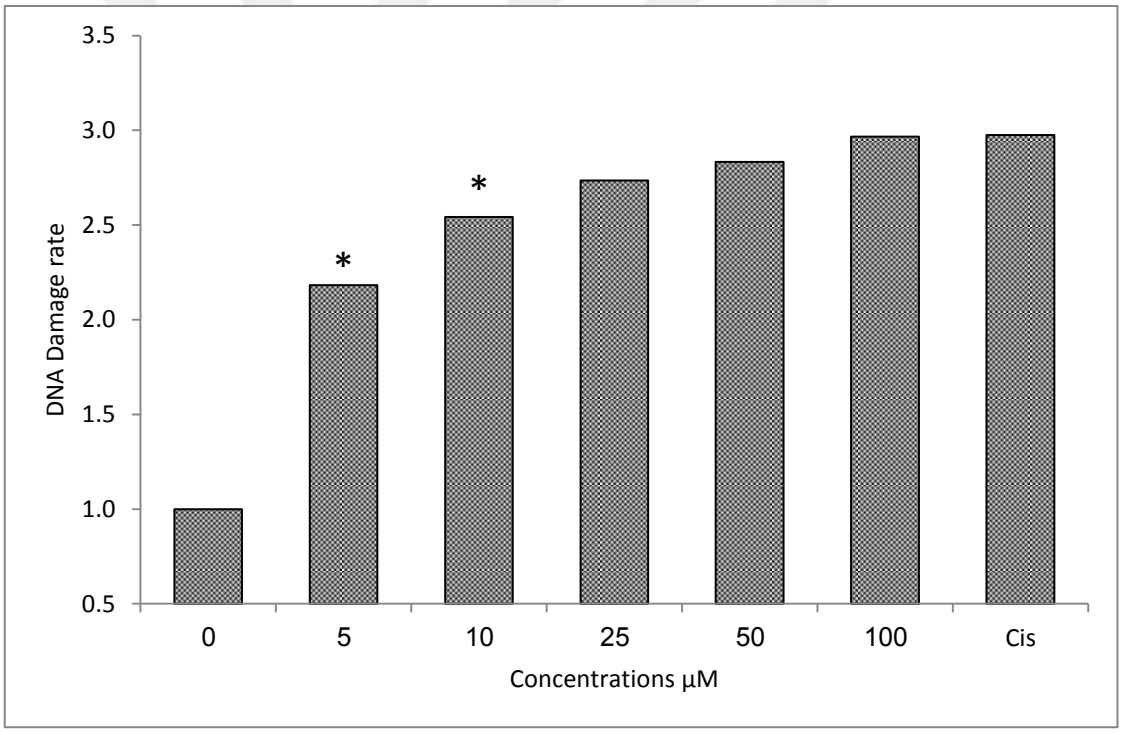


Figure 11. The effect of different concentrations of L_5BF_2 on ECC-1 DNA damage measured by ELISA (* = indicates the effective dose depending on $p < 0.01$compared to the previous dosage)

4.6. Determination of intracellular TAS, TOS, and OSI

Total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) are oxidative profiles determining the intracellular oxidative posture and reveals their role in apoptosis.

To evaluate intracellular TAS value, we measured the effect of different concentrations of L_5BF_2 used in ECC-1 cells, each concentration groups was in triple (Table 10). From 10 µM dose, there was a proper reduction in the value of TAS with a significant p value of <0.019. Continuing in 25 µM with p<0.036. the dropping in TAS value carries on till the last L_5BF_2 concentration of 100 recording the least value of TAS mean = 0.783 superior compared to cisplatin of mean = 0.812 (Figure 12). Overall the TAS values showed a significant p-value of $p < 0.004$ and give us a pre-expectation of TOS value.

Table 10. TAS values of ECC-1 treated with L_5BF_2

Dose µM	1	2	3	Mean ± STD
0	1.100	1.090	1.220	1.137 ± 0.072342
10	0.986	0.980	0.960	0.975 ± 0.013614
25	0.940	0.850	0.860	0.883 ± 0.049329
50	0.934	0.890	0.780	0.868 ± 0.079322
100	0.850	0.800	0.700	0.783 ± 0.076376
Cis	0.876	0.800	0.760	0.812 ± 0.058924

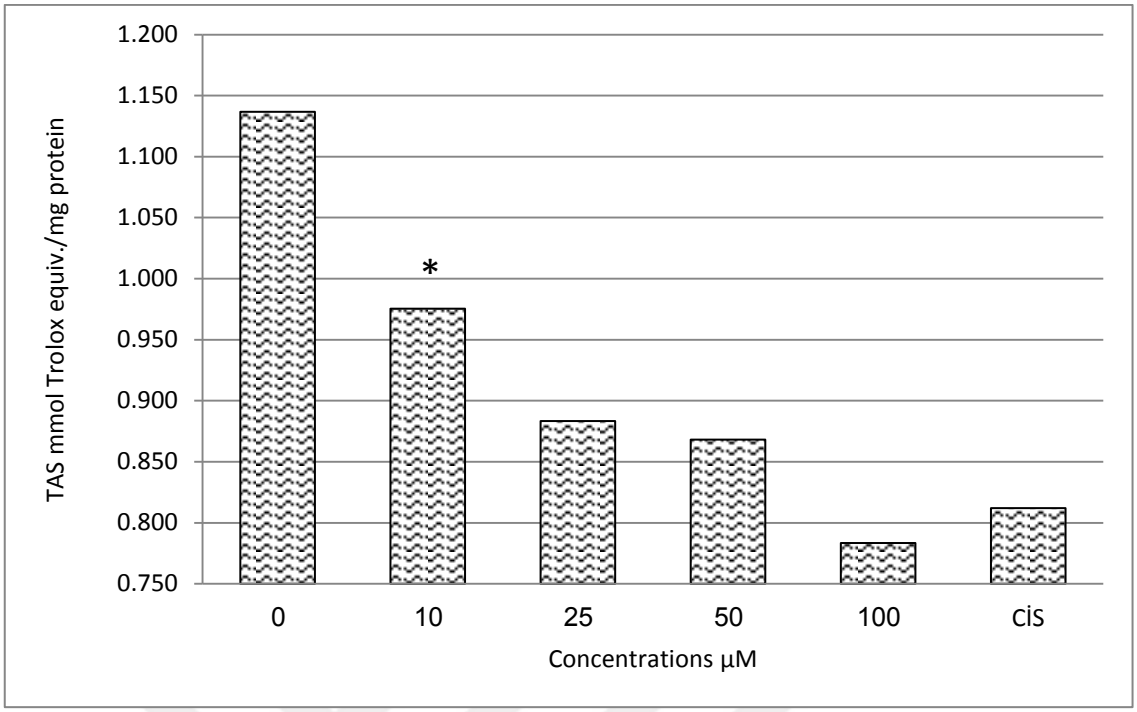


Figure 12. TAS values according to the elevation of L_5BF_2 concentration (*= effective dose)

Intracellular total oxidant status (TOS) of the ECC-1 cell line was measured as same as in TAS concentrations (Table 11). The TOS values, unlike TAS, did boost with the increasing of L_5BF_2 concentration, from first ascending concentration of 10 µM there was a significant elevation in TOS value with a $p<0.041$ keeping in this manner with lesser significance in each followed doses compare to its previous dose (Figure 13). In fact, the total $p<0.005$ showed the high efficiency of the last dose of 100 µM but didn't reach the control dose of cisplatin with $p<0.0001$.

Table 11. TOS values of ECC-1 treated with L_5BF_2

Dosage µM	1	2	3	Mean ± STD
0	7.25	7.5	6.34	7.03 ± 0.610492
10	8.25	8.43	7.87	8.18 ± 0.28589
25	8.95	8.5	8.8	8.75 ± 0.229129
50	9.35	9.45	9.54	9.45 ± 0.095044
100	10.52	10.2	9.24	9.99 ± 0.666133
Cis	11.35	11.59	11.76	11.57 ± 0.205994

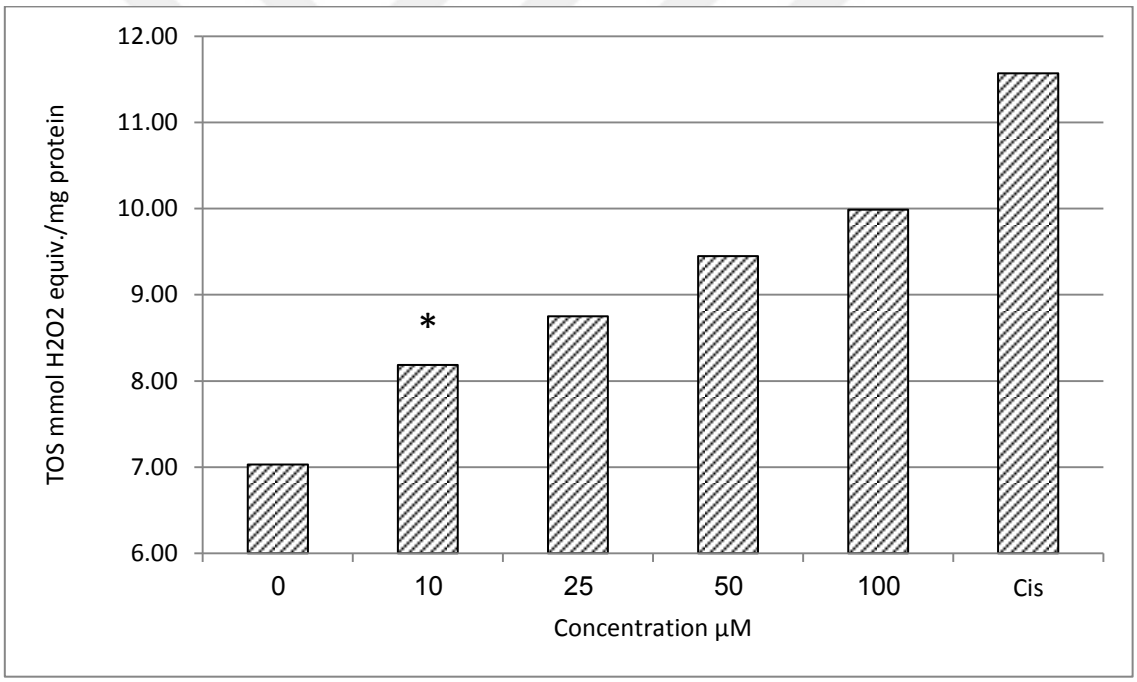


Figure 13. TOS values according to the elevation of L_5BF_2 concentration (*= effective dose)

Depending on the TAS and TOS results we achieved to calculate oxidative stress index (OSI) value and since OSI is an index of oxidative stress obtained by dividing TOS to TAS (lmol HP equivalent/lmol Trolox equiv), there is no definite unit to express the OSI results, thus these results are being reported as arbitrary unit (AU). As the result shows in (Figure 14) and according to the calculation result, there was an ascending pattern explains the increasing in oxidative stress and reduction in antioxidant which eventually induce the process of apoptosis.

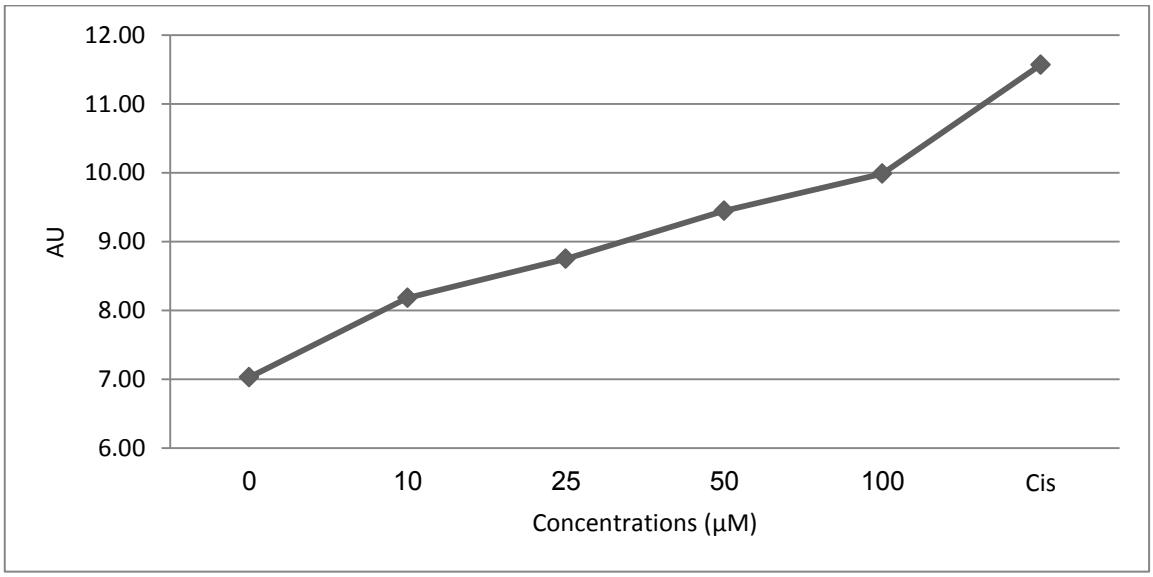


Figure 14. OSI values of ECC-1 treated with L_5BF_2

4.7. Ferric Reducing Ability of Plasma (FRAP) Assay

As we mentioned previously in the introduction, the free radicals are a secondary product of mitochondrial respiration activity. These free radicals are usually removed or inactivated in vivo by antioxidants (Frei et al., 1989; Gutteridge, 1994). The total amount of those antioxidants is called (antioxidant power).This can be measured by the meaning of FRAP assay.

Five different concentrations of L_5BF_2 were applied to ECC-1 cells in addition to cisplatin as a control (Table 12), by measuring the FRAP we observed a descending pattern started from 10 µM with great decreasing supported by p value of <0.05 the same fashion seen in the 100 µM recording the lowest oxidative power value of all including the cisplatin with $p<0.001$, proving that L_5BF_2 is more effective than control (Figure 15).

Table 12. FRAP values of ECC-1 treated with L_5BF_2 and cisplatin

Dose µM	1	2	3	Mean ± STD
0	0.167	0.165	0.158	0.163 ± 0.004726
10	0.151	0.152	0.148	0.150 ± 0.002082
25	0.144	0.145	0.139	0.143 ± 0.003215
50	0.133	0.140	0.152	0.142 ± 0.009609
100	0.121	0.119	0.122	0.121 ± 0.001528
Cis	0.118	0.120	0.126	0.121 ± 0.003884

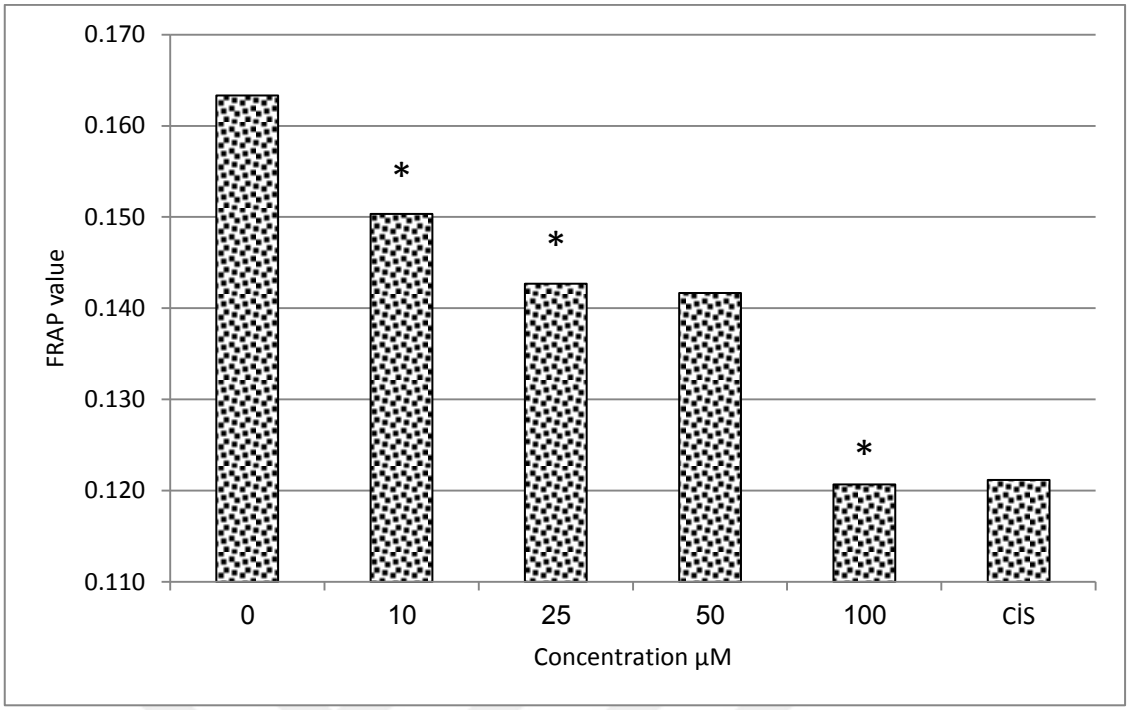


Figure 15. Shows the effect of various concentrations of L_5BF_2 and cisplatin on ECC-1 (*= $p<0.05$ according to the previous value)

5. DISCUSSION AND CONCLUSION

Regardless of the developed and advances in understanding the molecular features of cancer, traditional therapies have not changed dramatically within the past 25 years (Shaker and Melake, 2012). The toxicities and negative side effects of these therapies and the frequent appearance of resistance in persistent tumors have led to a substantial increase in the clinical testing of new drugs.

A wide range of reactions in biology is ruled by the metal ions. Latest developments in the biochemistry opened an interest out of the scientists of an inorganic field to judge in a new area known as "Bioinorganic Chemistry", which is mainly based on the synthesis of metal complexes able to mimic the functional features of natural metalloproteins (Chatterjee and Bhattacharyya, 2015), examples of Schiff bases or porphyrins are used as models in biological sciences (Sadeek et al., 2013). .Most of the Schiff base compounds and their metal complexes presented decent anticancer activities (Murtaza et al., 2014).

The boron compounds revealed anticancer properties to many cancer cell lines and there are researches in which boron compounds work as a proteasome inhibitor in cancer cells on diverse cancer cell lines including lung, leukemia and pancreatic cells (Bone, Shenvi, Kettner, and Agard, 1987). In this study, we focused on the apoptotic, cytotoxic and genotoxic effect of Schiff base ligands' corresponding fluorine boron hybrid complex L_5BF_2 on endometrial cancer cell line.

Experimentally, ECC-1 cells showed high sensitivity to L_5BF_2 compares to other cell lines with an IC_{50} value of 38.36 µM, which is slightly different from PNT1-A cells IC_{50} value used as a normal control model for this research. From first observation of the ECC-1 cells exposed to 2, 5 and 10 µM of L_5BF_2, the growth patterns did not appear to be different from the control cells, whereas 25 µM L_5BF_2 caused an obvious decrease in cell proliferation in both cell lines, in some points it has the same effectiveness of cisplatin which used as a drug control model, we also can note that the effect of our compound is less than cisplatin on PNT1-A which lean the balance to our advantage (Figure 6). This encouraged us to go along with a further test to determine its effect on ECC-1 cell proliferation.

In order to determine the mechanism behind the notable decrease in cell proliferation, we looked for DNA laddering 24 h after exposure of the cells to different concentrations of L_5BF_2, and then we succeeded to display DNA fragmentation with all applied dosages in various degrees, proving L_5BF_2 is inducing cell apoptosis. For more confirmation we also carry out comet assay to see the DNA fragmentation in single cell levels, we used the same concentrations applied in the previous test started from 5 µM, also showed evidence of DNA disintegration, all the previous has shown an important impact of the boron compound on DNA causing disintegration and fragmentation in the genetic material.

ELISA readings removes all doubts by showing the high rate of DNA damage in ECC-1 cells from first ascending dose to the last dose that equalized the cisplatin (Figure 11), proposes that DNA damage was past repair stage, and L_5BF_2 induced cell apoptosis by activating pro-apoptotic proteins and reducing anti-apoptotic ones, in a way had no effect on necrotic cell percentage as seen in AO test, moreover it display a significant decrease in vital cell percentage as well.

Reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radicals, and superoxide anion, are highly reactive molecules with unpaired electrons in outer orbit, they produced in normal physiological processes such as aerobic metabolism or inflammation, in the case of exceeding ROS production and lake of cellular antioxidant defenses, result in cell damage (Renschler, 2004). Now it is obvious that the generation of ROS can be translated therapeutically in the treatment of cancer and that the ability of a cell to defend itself against ROS is associated with resistance to chemotherapy. To determine the oxidative stress we estimated oxidative stress index (OSI) relying on TAS and TOS results.

TAS results showed a decent decrease in the value rate from the first dose giving a significant $p < 0.019$ continuing to precede cisplatin in 100 µM boosting our expectations of TOS to be raised at the same time, giving a rising model of OSI as seen in (Figure 14) proving that our chemical drive the oxidative stress to rise and antioxidant defenses to be reduced. Moreover, the results of FRAP test supported our theory by estimating the antioxidant power in ECC-1 cells compare to cisplatin. As it was shown in (Figure 15) the effect of L_5BF_2 was dramatically dropped the value of

antioxidant power advancing cisplatin not forgetting to mention there was no adequate change between 25 and 50 μM concentrations.

Overall, our results display a significant effect of L_5BF_2 to increase intracellular oxidative stress and cause unrepairable DNA damage leading to induction of cell apoptosis and for that, we see high potentials of our chemical to replace the traditional chemotherapeutic agents used in endometrial cancer treatments after further tests to determine its clinical properties.

Altogether the data shows novel fluorine boron hybrid complex (L_5BF_2) has possible therapeutic properties as an alternative treatment for endometrial cancer. However, more studies need to be done to determine clinical characteristics of this novel compound and its mechanism of action in vivo models, since this was an in vitro study. As a suggestion, future work should determine the effect of methylation of L_5BF_2 on epigenetic changes and its effect on histones and microRNA expression. DNA relaxation assays will be used to evaluate its effect on topoisomerase activity in different cancer and non-cancer cell lines.

SUMMARY

The apoptotic, cytotoxic and genotoxic effect of novel fluorine boron hybrid complex (L_5BF_2) on endometrial cancer. Y. Y. U, Institute of Health Science Department of Medical biology MSc Thesis, Van, 2016. Endometrial cancer (EC) is one of the most common types of gynecologic cancer of the female genital tract; it considered to be the fourth leading death among other cancers. Based on the potential of Schiff based complexes for the induction of apoptosis, Schiff base compounds, and their metal complexes displayed good anticancer properties. This study meant to examine the cytotoxic, genotoxic and apoptotic activity of a novel florin boron hybrid complex (L_5BF_2) on ECC-1 cells. The complex exerted a potent suppressive effect on ECC-1 cells. In this study L_5BF_2 was examined in seven different cell lines, HELA, DU-145, PC3, DLD-1, ECC, PNT1-A and HT-29, later investigated its apoptotic, cytotoxic, and genotoxic properties on human endometrial adenocarcinoma cell line ECC-1 in different concentrations, by using standard apoptotic and cytotoxic tests such as single cell gel electrophoresis assay (comet assay), DNA fragmentation laddering, acridine orange test for DNA damage, and ELISA for apoptotic measuring. We also measured the oxidative status by evaluating total antioxidant status (TAS) and total oxidant status (TOS). Oxidative stress index (OSI) was calculated too. In conclusion, we found a marvelous effect of L_5BF_2 on ECC-1 cells especially in damaging their DNA and cause a series of reactions lead to apoptosis. The results suggest that the L_5BF_2 complex is able to induce the apoptotic pathway of endometrial cancer cells and is a possible candidate for future cancer treatment studies.

Key Words: Apoptosis, Cytotoxicity, Endometrial cancer, DNA damage, Chemotherapy

ÖZET

Yeni fluor boron hibrit kompleksinin (L_5BF_2) endometrial kanser üzerindeki apoptotik, sitotoksik ve genotoksik etkisi. Y. Y. U, Sağlık Bilimleri Enstitüsü Tıbbi Biyoloji Bölümü Yüksek Lisans Tezi, Van, 2016. Endometrial kanser (EC), kadın genital sisteminin jinekolojik kanserinin en sık görülen tiplerinden biridir. Diğer kanserler arasında önde gelen dördüncü ölüm olarak değerlendirilmiştir. Schiff bazlı komplekslerin apoptoz indüksiyonu potansiyeline dayanılarak, Schiff bazlı bileşikler ve bunların metal kompleksleri, iyi anti kanser özellikleri sergilemektedir. Bu çalışma, yeni bir florin bor hibrit kompleksinin (L_5BF_2), ECC-1 hücreleri üzerindeki sitotoksik, genotoksik ve apoptotik aktivitesini incelemek amacıyla yapılmıştır. Kompleks, ECC-1 hücreleri üzerinde güçlü bir baskılayıcı etki yapmıştır. Bu çalışmada L_5BF_2, HELA, DU-145, PC3, DLD-1, ECC, PNT1-A ve HT-29 olmak üzere yedi farklı hücre dizisinde incelenmiş ve daha sonra insan endometriyal adenokarsinoma hücre dizisi üzerindeki apoptotik, sitotoksik ve genotoksik özellikleri araştırılmıştır. ECC-1 hücre hattı üzerinde Tek hücreli jel elektroforez testi (comet testi), DNA parçalanma laddering, DNA hasarı için akridin orange testi ve apoptotik ölçüm için ELISA gibi standart apoptotik ve sitotoksik testleri L_5BF_2'in farklı konsantrasyonları için kullanılmıştır. Ayrıca toplam antioksidan durumu (TAS) ve toplam oksidan durumu (TOS) değerlendirerek oksidatif durumu ölçülmüştür. Oksidatif stres indeksi (OSI) de ayrıca hesaplanmıştır. Sonuç olarak, L_5BF_2'nin ECC-1 hücrelerine, özellikle de DNA'larının zarar görmesinde olağanüstü bir etki bulunmuştur ve bir dizi reaksiyonun apoptozise yol açtığı görülmüştür. Sonuçlar, L_5BF_2 kompleksinin endometrial kanser hücrelerinin apoptotik yolağını indükleyebildiğini ve gelecekteki kanser tedavisi çalışmalarında olası bir aday olduğunu ortaya koymaktadır.

Anahtar Kelimeler: Apoptoz, Sitotoksisite, Endometrial kanser, DNA hasarı, Kemoterapi

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CURRICULUM VITAE

Pawan Tareq Ahmed LAK, I am from Erbil city in North Iraq. I was born on April 3, 1981, in Erbil. I finished Primary, Secondary and High Schools in my home city of Erbil. After that I started undergraduate admission process at the University of Salahaddin - College of Science, Biology department during the years (2000-2005), and I got my Bachelor's degree in Science of Biology. While I got my Diploma I worked as an employer (Biologist assistant) at the Republic Hospital Internal Laboratory- Erbil, and I have rotated on every department from Microbiology to parasitology to hematology to virology …. etc, then I was sent to Kasnazan Health Center in Kasnazan town in the city of Erbil to finish up my trainee till 2011, then I have been transferred to Tarin Health Center in Kasnazan to work as a (Biologist) till now I work there. Finally, I came to the Republic of Turkey to begin the postgraduate admission process at the Yuzuncu Yil University-Institute of Health Science, Faculty of Medicine-Medical Biology Department during the years (2014-2016). I am married and I have two children.

SUPPLEMENTS 1. ETHICS REPORT

T.C.
YÜZÜNCÜ YIL ÜNİVERSİTESİ
TIP FAKÜLTESİ
GİRİŞİMSEL OLMAYAN
KLİNİK ARAŞTIRMALAR ETİK KURULU
KARAR FORMU

KARAR BİLGİLERİ	Karar No: 14	Tarih: 03.06.2016
	Doç.Dr. Yasin TÜLÜCE sorumluluğunda yapılması tasarlanan ve yukarıda başvuru bilgileri verilen **"Schiff bazı – Bor kompleksinin Endometriyal Kanser Hücre Hattında Apoptotik, Sitotoksik ve DNA Hasarı Üzerine Etkisi "** isimli bilimsel araştırma başvuru dosyası ve ilgili belgeler araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiştir. Araştırmacıların Yüzüncü Yıl Üniversitesi Girişimsel Olmayan Klinik Araştırmalar Etik Kurulunun Çalışma Esasları Hakkında Yönergesinde belirtilen hususları yerine getirdikleri belirlenmiş olup, çalışmaları ile ilgili tüm sorumluluk araştırmacılara ait olmak üzere, söz konusu çalışmanın gerçekleştirilmesinde sakınca bulunmadığına, toplantıya katılan Etik Kurul üye tam sayısının salt çoğunluğu/oy birliği ile karar verilmiştir.	

GİRİŞİMSEL OLMAYAN KLİNİK ARAŞTIRMALAR ETİK KURULU	
ETİK KURULUN ÇALIŞMA ESASI	Klinik Araştırmalar Hakkında Yönetmelik, İyi Klinik Uygulamaları Kılavuzu
BAŞKANIN UNVANI / ADI / SOYADI:	Prof.Dr. Oğuz TUNCER

Unvanı/Adı/Soyadı	Uzmanlık Alanı	Kurumu	Cinsiyet		Araştırma ile ilişki		Katılım *		İmza
Prof.Dr. Oğuz TUNCER	Çocuk Sağlığı ve Hastalıkları	Yüzüncü Yıl Üniversitesi Tıp Fakültesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Yrd.Doç.Dr. Şükran SEVİMLİ	Tıp Tarihi ve Etik	Yüzüncü Yıl Üniversitesi Tıp Fakültesi	E ☐	K ☒	E ☐	H ☒	E ☐	H ☒	
Prof.Dr. Sıddık KESKİN	İstatistik Uzmanı	Yüzüncü Yıl Üniversitesi Tıp Fakültesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Doç.Dr. Hasan Ali GÜMRÜKÇÜOĞLU	Kardiyoloji	Özel Van Lokman Hekim Hastanesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Doç.Dr. Murat DOĞAN	Çocuk Sağlığı ve Hastalıkları	Yüzüncü Yıl Üniversitesi Tıp Fakültesi	E ☒	K ☐	E ☐	H ☒	E ☐	H ☒	
Doç.Dr.M. Fatih GARÇA	KBB	Yüzüncü Yıl Üniversitesi Tıp Fakültesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Doç.Dr. Hüseyin BEĞENİK	Dahiliye	Yüzüncü Yıl Üniversitesi Tıp Fakültesi	E ☒	K ☐	E ☐	H ☒	E ☐	H ☒	
Yrd.Doç.Dr. M.Bilal ÇEĞİN	Anesteziyoloji ve Reanimasyon	Yüzüncü Yıl Üniversitesi Tıp Fakültesi	E ☒	K ☐	E ☐	H ☒	E ☐	H ☐	
Yrd.Doç.Dr. Numan ÇİM	Kadın Hastalıkları ve Doğum	Yüzüncü Yıl Üniversitesi Tıp Fakültesi	E ☒	K ☐	E ☒	H ☐	E ☐	H ☒	
Yrd.Doç.Dr. Ramazan ÜSTÜN	Fizyoloji	Yüzüncü Yıl Üniversitesi Tıp Fakültesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Yrd.Doç.Dr. Ersoy ÖKSÜZ	Farmakoloji Uzmanı	Yüzüncü Yıl Üniversitesi Tıp Fakültesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Fatma PEKER	Hukuk	Van Güvenlik Meslek Yüksekokulu	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Çiğdem ÖNER	Üniversite Mezunu	-	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	

Adres : Yüzüncü Yıl Üniversitesi Tıp Fakültesi Dekanlığı Merkez Kampüsü Van
Tel : 432- 2150470
Faks : 432-2168352
e-posta: etikkurull@gmail.com

SUPPLEMENTS 2. PLAGIARISM REPORT

YÜZÜNCÜ YIL ÜNİVERSİTESİ
SAĞLIK BİLİMLERİ ENSTİTÜSÜ
LİSANSÜSTÜ TEZ ORİJİNALLİK RAPORU

Tarih: 15/12/2016

Tez Başlığı / Konusu: THE APOPTOTIC, CYTOTOXIC AND GENOTOXIC EFFECTS OF NOVEL FLUORINE BORON HYBRID COMPLEX (L5BF2) ON ENDOMETRIAL CANCER

Yukarıda başlığı/konusu belirlenen tez çalışmamın Kapak sayfası, Giriş, Ana bölümler ve Sonuç bölümlerinden oluşan toplam 33 sayfalık kısmına ilişkin, 15/12/2016 tarihinde şahsım/tez danışmanım tarafından Turnitin intihal tespit programından aşağıda belirtilen filtreleme uygulanarak alınmış olan orijinallik raporuna göre, tezimin benzerlik oranı % 11 (on bir) dir.
Uygulanan filtreler aşağıda verilmiştir:
- Kabul ve onay sayfası hariç,
- Teşekkür hariç,
- İçindekiler hariç,
- Simge ve kısaltmalar hariç,
- Gereç ve yöntemler hariç,
- Kaynakça hariç,
- Alıntılar hariç,
-Tezden çıkan yayınlar hariç,
- 7 kelimeden daha az örtüşme içeren metin kısımları hariç (Limit match size to 7 words)

Yüzüncü Yıl Üniversitesi Lisansüstü Tez Orijinallik Raporu Alınması ve Kullanılmasına İlişkin Yönergeyi inceledim ve bu yönergede belirtilen azami benzerlik oranlarına göre tez çalışmamın herhangi bir intihal içermediğini; aksinin tespit edileceği muhtemel durumda doğabilecek her türlü hukuki sorumluluğu kabul ettiğimi ve yukarıda vermiş olduğum bilgilerin doğru olduğunu beyan ederim.

Gereğini bilgilerinize arz ederim.

Tarih ve İmza 15.12.2016

Adı Soyadı: Pawan Tareq Ahmed LAK
Öğrenci No: 159302007
Anabilim Dalı: Tıbbi Biyoloji Anabilim Dalı
Programı: Tıbbi Biyoloji
Statüsü: Y.Lisans☒ Doktora☐

DANIŞMAN ONAYI
UYGUNDUR

Doç. Dr. Yasin TÜLÜCE
(Unvan, Ad Soyad, İmza)

ENSTİTÜ ONAYI
UYGUNDUR

(Unvan, Ad Soyad, İmza)