REPUBLIC OF TURKEY
YUZUNCU YIL UNIVERSITY
INSTITUTE OF HEALTH SCIENCE

THE APOPTOTIC, CYTOTOXIC AND GENOTOXIC EFFECTS OF NOVEL FLUORINE BORON HYBRID COMPLEX (L_4BF_2) ON COLORECTAL CANCER

Hawro Dlawar Ismael MASSEH
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ABBREVIATIONS

Apaf-1	: Apoptosis protease-activating factor-1
APO-1	: Apoptosis Antigene-1
BH1-4	: Bcl-2 homology domains
CAD	: Caspase-activated DNase
CAM	: Cell Adhesion Molecule
CARD	: Caspase Recruitment Domain
Caspase	: Cysteinyl aspartate-specific protease
CD95	: Cluster of Differentiation 95
c-FLIP	: FLICE-inhibitory protein
CRC	: Colorectal Cancer
DAD	: Death Affecter Domain
DD	: Death Domain
DFI	: DNA Fragmentation Index
DIABLO	: Direct Inhibitor of apoptosis binding protein with low IP
DISC	: Death-inducing signaling complex
DMSO	: Dimethyl Sulfoxide
DR	: Death Receptor
FADD	: Fas-associated death domain
FAP	: Familial Adenomatous Polyposis
FasL	: Fas Ligand
FBS	: Fetal Bovine Serum
HNPCC	: Hereditary Non-Polyposis Colonic Cancer
HtrA2	: High-temperature-requirement protein

IAP	: Inhibitor of apoptosis protein
LMA	: Low Melting Agarose
MAP	: MYH-associated polyposis
MCAP	: Multiple Colorectal Adenomatous Polyps
NMA	: Normal Melting Agarose
N-terminus	: Amino terminus
PBS	: Phosphate-buffered saline
Puma	: p53-up-regulated modulator of apoptosis
ROS	: Reactive Oxygen Species
SLS	: Sodium Lauryl Sarcosinate
SMAC	: Second mitochondria-derived activator of caspases
TNF	: Tumor necrosis factor
TNFR	: Tumor necrosis factor receptor
TRADD	: Tumor necrosis factor receptor-associated death domain
TRAIL	: TNF-related apoptosis-inducing ligand

1. INTRODUCTION

Cancer considered one of the leading causes of death in the modern life, it has been estimated the second leading disease that cause death worldwide after cardiovascular diseases (heart diseases), with approximately 14 million new cases and 8.2 million deaths in 2012 according to the World Health Organization (Stewart and Wild, 2014). Most of the world cancer cases are stated in developing countries, with a 63% mortality rate (Ferlay et al., 2010).

Colorectal cancer (CRC) considered the second most frequently identified cancer in women and the third in men globally. In 2012, an estimated 1.36 million new cases (women: 614000; men: 746000) of CRC were diagnosed, which accounted for 9.7% of entire cancer cases. Eliminating nonmelanoma skin cancer, the rates differ more than 10 times across the world. High-income countries have nearly 2.5 times higher rates than do low-income countries, moreover, CRC is the third leading reason of cancer death in women and the fourth in men globally, with the combined number of total deaths reaching 694000 (8.5% of total cancer deaths) (American Cancer Society, 2015). The rate of cancer survivors has also increased significantly over the past several decades.

CRC is pronounced the fifth most common cancer in developing countries for both sexes. CRC frequency is about 7 per 100,000 persons, with approximately 5,000 new cases and 3,200 deaths yearly in Turkey (Tatar and Tatar, 2010). Usually, few CRC cases occur in persons younger than 40 years old. This rate grows up rapidly with age more obviously for colon than for rectal cancer (Parkin et al., 2002). The percentage of CRC is, therefore, expected to rise in the future as a result of increased life expectancy and population aging.

Despite progress in screening and treatment strategies, colorectal cancer remains a significant public health problem in the developed countries. While a proportion of patients are cured by surgical resection alone, a significant percentage go thru tumor relapse and ultimately die (Cassidy et al., 2007).

Etiological studies have shown that the occurrence of CRC varies between countries and between different cultural groups within the same country. The finding that immigrants show cancer frequency of their implemented country within a generation supports the important role of environmental and cultural factors in the etiology of CRC. The most powerful involved environmental and cultural cause of CRC is the diet which contains high fat, high protein and low fiber (Willett, 1989). In addition to environmental and diet factors, diseases such as inflammatory bowel also affect CRC. Ulcerative colitis increases the risk of developing CRC with tumors starting to appear 5–8 years after the beginning of the disease (Shi Yu Yang et al., 2009; Chen and Goeddel, 2002). Genetically, there are many hereditary disorders that might increase the risk of getting CRC. The main hereditary CRC includes hereditary non-polyposis colonic cancer (HNPCC or Lynch syndrome), familial adenomatous polyposis (FAP); and MYH-associated polyposis (MAP). HNPCC was first described in the early 20th century; later, it was more explained by Professor Dr. Henry T. Lynch and developed in the 1990s (Vasen et al., 1991;Vasen et al., 1999). This syndrome shows a preference for the right colon and accounts for 3–4% of all CRC cases. FAP is a disease permit to the development of several colonic cancers. It is inherited in an autosomal dominant mode with high penetrance but in variable expression, and account for about 1% of CRC (Rustgi, 2007). While MAP is an autosomal recessive inheritance disorder that has a connection with the appearance of multiple colorectal adenomatous polyps (MCAP) (Sieber et al., 2003).

Usually, chemotherapy alone or side by side with radiotherapy is used as adjuvant treatment for cancer. Unfortunately, adjuvant treatments are often ineffective in order to the development of resistance by cancer cells (Johnstone et al., 2002). By the meaning of chemoresistance, we point to the inhibition or prevention of apoptosis .Despite new methods and drugs in treating colorectal cancer and the good results that occur from them, we still didn't scratch the surface of therapeutic cancer medications and there is a wide range of chemicals and natural products have not yet studied and tested as anticancer agents. The novel fluorine boron hybrid complex (L_4BF_2) considered a new boron derivative with great anti-cancer potentials.

2. GENERAL INFORMATION

2.1. Cancer

According to National Cancer Institute (NCI), cancer is a group of diseases in which abnormal cells divide and proliferate uncontrolled and able to invade nearby tissues. A neoplasm or tumor is a group of cells that have undergone unregulated growth and undifferentiated to form a mass or bump and may spread diffusely (American Cancer Society, 2013).

Cancer considered the second lethal disease in nowadays worldwide after cardiovascular diseases, according to the WHO, about 14 million new cases are diagnosed with 8.2 million death incidents and that is just in 2012 (Stewart and Wild, 2014). Among them, CRC is the second most commonly diagnosed cancer in women and the third in men worldwide. In 2012, an estimated of 1.36 million new cases (women: 614 000; men: 746 000) of CRC were detected, which accounted for 9.7% of total cancers (American Cancer Society, 2015).

Colorectal cancer chances are increased due to lifestyle and aging factors with a minor number of cases attributed to genetic disorders, other factors like smoking, obesity, and absence of physical activity also dietary factors that elevate the risk of getting CRC include red and processed meat as well as alcohol (Stewart and Wild, 2014). Another risk factor is inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease (Lansdrop-Vogrlaar et al., 2012). Colorectal cancer cases can be also related to some of the inherited genetic disorders include familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC); however, these genetic diseases represent less than 5% of cases (Imperiale et al., 2014) it starts as a benign tumors, regularly in the form of a polyp, which by the time it becomes malignant. As for treatment, if the tumor was found at an early stage, it may remove by surgical operation, chemotherapy is used in addition to surgery depending on the stage of the disease (Cunningham et al., 2010).

2.2. Apoptosis

Apoptosis or programmed cell death is a word derived from Ancient Greek word means "falling off" which meant to describe the falling off the leaves from trees, it is a process of programmed cell death (suicide) that occurs in a multicellular organism (Clark, 2002). This process leads to series of biochemical and morphological events which eventually lead to cell death. These events include chromatin condensation, chromosomal DNA fragmentation, nuclear fragmentation, cell shrinkage and blebbing (Goldsby et al., 2002) (Figure 1).

In 1842 the principle of apoptosis was described by the German scientist Karl Vogt, and the term (Apoptosis) was firstly suggested by Kerr Wyllie and Currie paper in 1972 (Kerr et al., 1972). Apoptosis has an essential role in maintaining the cellular reliability of tissues and the functional capability of organs and it is a fundamental principle for embryogenesis, tissue homeostasis, and inhibition of oncogenesis (Preedy, 2010).

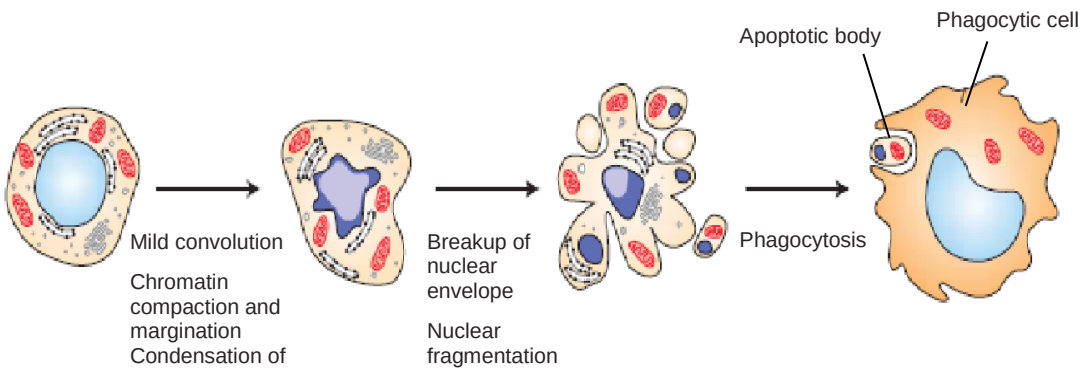


Figure 1. Steps of apoptosis (Goldsby et al., 2002)

Apoptosis is a specific morphological and biochemical changes mediated by a family of inactive zymogens called cysteine caspases (caspases), which are proteolytically processed to an active state. There are two main factors that cause the initiation of apoptosis in the cell which is either physiological or pathological. Physiological causes of apoptosis involve in the programmed elimination of cells during

embryogenesis and in proliferating cell populations to keep a constant cell number. While pathological causes include DNA damage (by free radicals, hypoxia, radiation, or cytotoxic drugs), accumulation of misfolded proteins (caused by inherited defects or free radical damage) and viral infections (Richard et al, 2012).

The main character of apoptosis is the absence of inflammatory reaction result from the escape of cytoplasmic contents to intracellular matrix due to loss of cellular membrane integrity and cause an immune response which can be seen in necrosis (Alberts et al., 2008).

As far as we know there are two main pathways for apoptosis to take place, one is starting from inside the cell (intrinsic pathway) and the other signaled from outside (extrinsic pathway) (Green, 2000), each one of them is triggered by different inducer. (Figure 2).

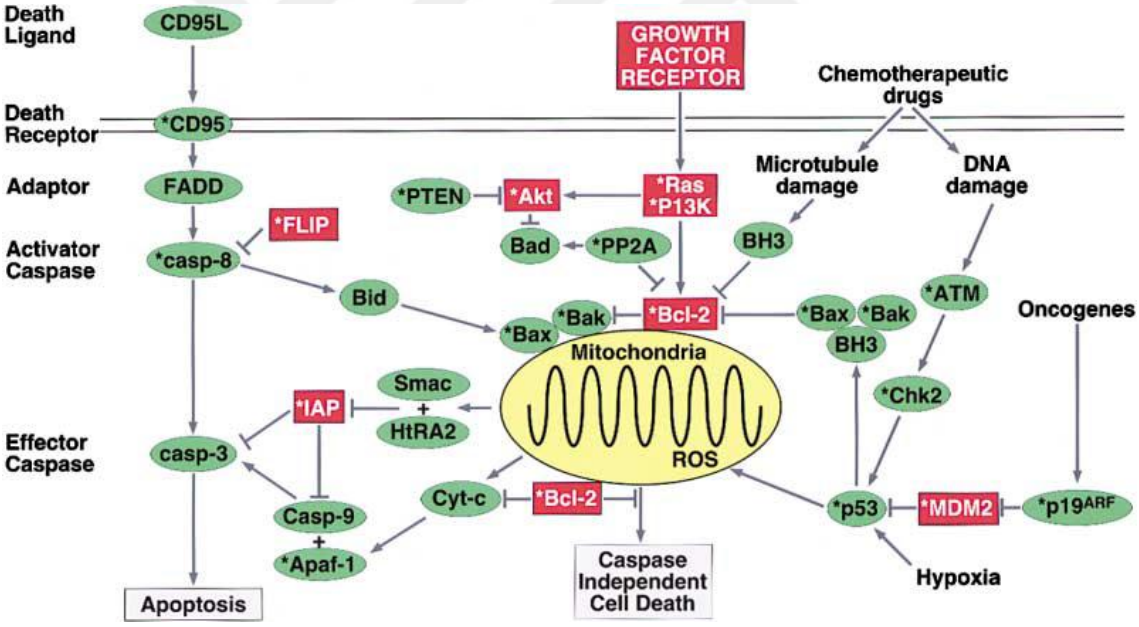


Figure 2. The integrated apoptotic pathways (Johnstone et al., 2002)

2.2.1. Intrinsic pathway

As the name refers, this pathway is initiated from the inside of the cell, at the beginning, the mitochondrial membrane permeability is decreased, and pro-apoptotic molecules such as Smac/DIABLO, HtRA2, and cytochrome c are released into the cytoplasm but no death receptors are involved. Cytochrome c binds with Apaf-1 to activate caspase-9 and initiate an apoptotic caspase cascade, while HtrA2 and Smac/DIABLO bind to antagonize inhibitor of apoptosis proteins (IAPs) (Suzuki et al., 2001).

Mitochondrial membrane permeability is controlled by the opposite actions of proapoptotic and antiapoptotic Bcl-2 family members. Multidomain proapoptotic Bcl-2 proteins such as Bak and Bax can be stimulated by BH3-only Bcl-2 protein Bid. In another way, the binding of other BH3-only proteins (e.g., Noxa, Puma, Bad, and Bim) to antiapoptotic Bcl-2 proteins (e.g., Bcl-2 and Bcl-XL) can cause the activation of Bax and Bak (Huang and Strasser, 2000). In the main time Bcl-2 proteins regulate mitochondrial membrane integrity by controlling the opening and closing of the permeability transition channel and/or directly making pores in the outer membrane, it is still the subject of much controversy (Martinou and Green, 2001).

One of the main factors that lead to loss of mitochondrial membrane integrity is endogenous reactive oxygen species (ROS), a byproduct of natural respiration process inside the mitochondria. There are also other inducing factors that cause alteration of the mitochondrial membrane and generate a stress on it such as heat shock, growth factor withdrawal, and DNA damage.

2.2.2. Extrinsic pathway

Extrinsic pathway, also called death receptor pathway, is activated by binding of death receptors in the transmembrane such as (TNF receptor, CD95 and TRAIL receptor) to stimulate caspase-8 and -10, which considered membrane-proximal caspases, that in turn split and activate effector caspases such as caspase-3 and -7. This pathway is controlled by c-FLIP, which inhibits upstream activator caspases, and

(IAPs), which affect both activator and effector caspases (Johnstone et al., 2002) (Figure 2).

The death receptor families members are known to be cysteine-rich repeats in their extracellular domain, those domains are known as death domain (DD) (Ashkenazi and Dixit, 1998). Death receptors belong to tumor necrosis factor receptors (TNFR) gene superfamily which contains TNFR-1, TRAIL receptors DR-4 and DR-5 and Fas/CD95 (Ashkenazi, 2002).

Tumor Necrosis Factor pathway: In this pathway, the main inducer is activated macrophages which produce a cytokine called TNF-alpha and considered the main extrinsic mediator of apoptosis. The TNF-alpha binds to either TNFR1 or TNFR2 those are the main receptors located on the human cell membrane, the linkage of TNF-alpha to TNFR1 has been observed to activate the pathway that leads to caspase initiation via the intermediate membrane proteins Fas-associated death domain protein (FADD) and TNF receptor-associated death domain (TRADD) (Chen and Goeddel, 2002).

Fas pathway: The other path of death domain is the first apoptosis signal (Fas) (also known as Apo-1 or CD95) which binds to Fas ligand (FasL), a transmembrane protein part of the TNF family, produce a complex of Fas/FasL lead to the formation of the death-inducing signaling complex (DISC), which compose of Fas-Associated Death Domain (FADD), caspase-8 and caspase-10. The Fas/Disc complex starts to activate caspase family by amplification the death signal which leads to releases the mitochondrial proapoptotic factors causing the activation of caspase 8 (Wajant, 2002).

2.3. Caspases

They are a family of protease enzymes have major roles in apoptosis as well as in necrosis and inflammation, the word (caspase) is derivated from cysteine-aspartic proteases and because of cysteine protease nucleophilic behavior, it attacks and cuts a target protein at the C-terminal of an aspartic acid amino acid. Usually caspases are present as pro-caspases a form of inactive zymogens and stimulated by intrinsic or extrinsic inducer, the activation process involves the dimerization of the pro-caspase and dissociation into a small and large subunit, and these units are associated together to

form an active heterodimer caspase, then each heterodimer combine to form a heterotetramer which is its biologically active form (Shi, 2004).

So far fourteen members of caspases have been discovered (Caspase-1 to Caspase-14) which are divided into two main groups according to their structure, caspases with long pro-domain have structural motifs such as caspase activation and recruitment domain (CARD) or death affecter domains (DADs) which give them the ability to interact with other proteins. Those three domains share the same 3D protein structure called death domain-fold, composed of six antiparallel α-helices organized in Greek key confirmation (Fan et al., 2005). While the second group of caspases have short pro-domains and activated by photolytic cleavages from other caspases. Caspases can be grouped according to their function too as an apoptotic or inflammatory (Table 1) (Figure 3).

Table 1. Caspase subfamilies divided according to pro domain structure and function

Members	Function	Pro-domain Structure	Subfamily
Caspase-1	Inflammatory	Long	I
Caspase-2	Effector		
Caspase-4	Inflammatory		
Caspase-5	Inflammatory		
Caspase-8	Effector		
Caspase-9	Effector		
Caspase-10	Effector		
Caspase-11	Inflammatory		
Caspase-12	Inflammatory		
Caspase-13	Inflammatory		
Caspase-14	Initiator		
Caspase-3	Initiator	Short	II
Caspase-6	Initiator		
Caspase-7	Initiator		

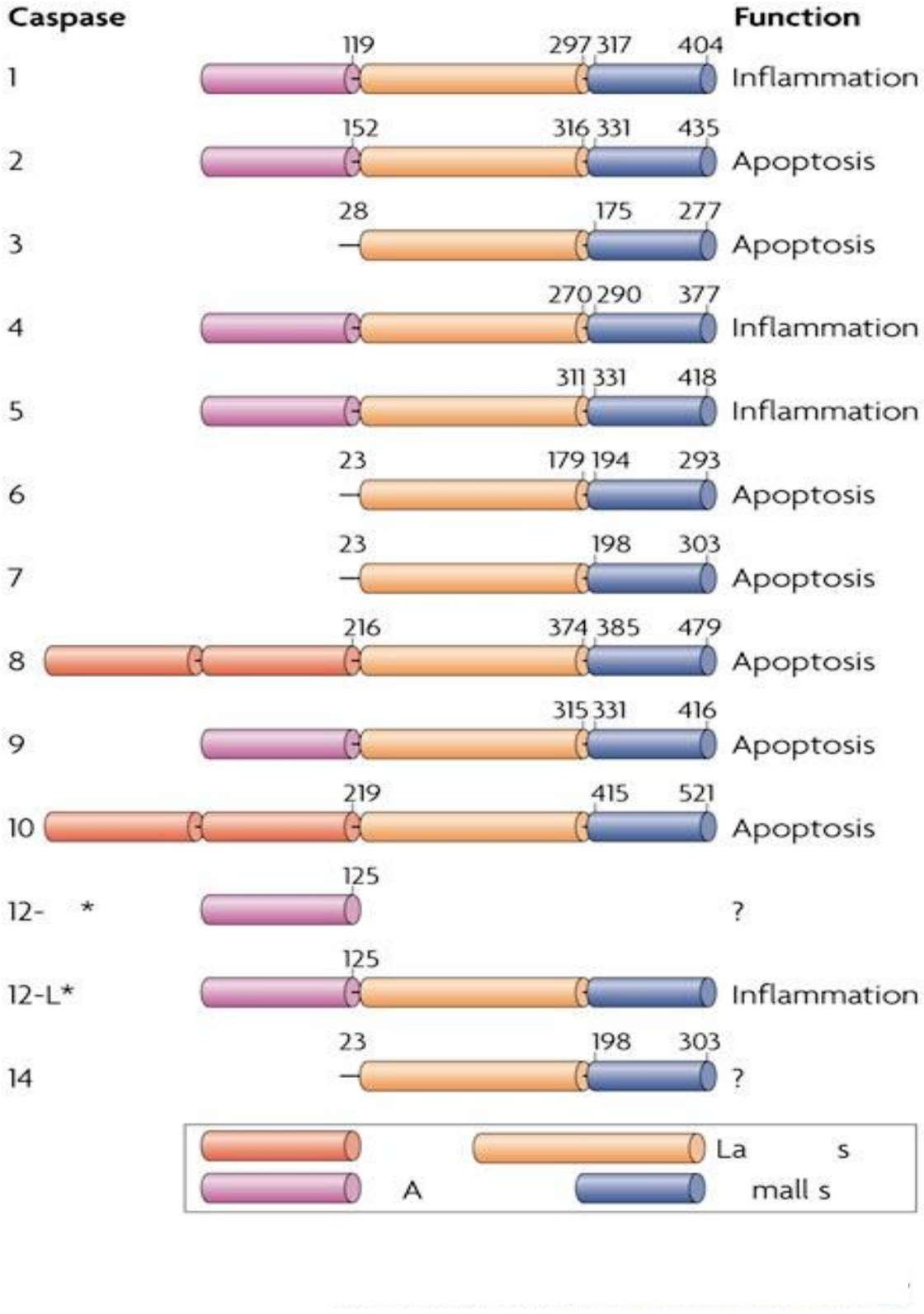


Figure 3. Caspase family members (Taylor et al., 2008)

2.4. ROS and Oxidative Stress

Free radicals and other reactive oxygen species (ROS) are continually produced in vivo by the meaning of mitochondrial respiration as a secondary product. In living cells, when the formation of intracellular reactive oxygen species (ROS) exceeds, the oxidative stress rise, causing damage to cellular macromolecules such as lipids, proteins, and DNA (Halliwell, 1991; Halliwell, 1994; Sailaja Rao et al., 2011). DNA is a sensitive cellular target molecule because of the possibility of creating mutations that can disturb cellular homeostasis (Hazra et al., 2007). The damage of DNA has also been realized as an important inducer of cancer, as well as being associated with the aging process (Bjelland and Seeberg, 2003). In another hand sometimes cells have particular enzymatic and non-enzymatic defense mechanisms, known as antioxidants to neutralize ROS, these mechanisms are not sufficient to prevent oxidative alterations in lipids, proteins and DNA (Giorgio et al., 2007).

2.5. Novel Fluorine Boron Hybrid Complex (L_4BF_2)

The progress of metal-based chemicals had a great role in developing new cancer chemotherapy drugs, one of a well-known example of metal-based anticancer agents is Schiff based metal complexes (Hajrezaie et al., 2015). The Schiff base ligands' fluorine boron hybrid complex has been newly synthesized and well characterized by both analytical and spectroscopic methods (Ozbahceci, 2015) The Spectroscopic results for novel fluorine boron hybrid complex (L_4BF_2) indicate that the colour is white with the purification percentage of 89%, and a melting point of 115-117 ° C. The elemental analysis showed the following formula ($C_{43}H_{68}B_2F_4N_2O_6$) with a molecular weight of (806.63 g / mol) (Figure 4).

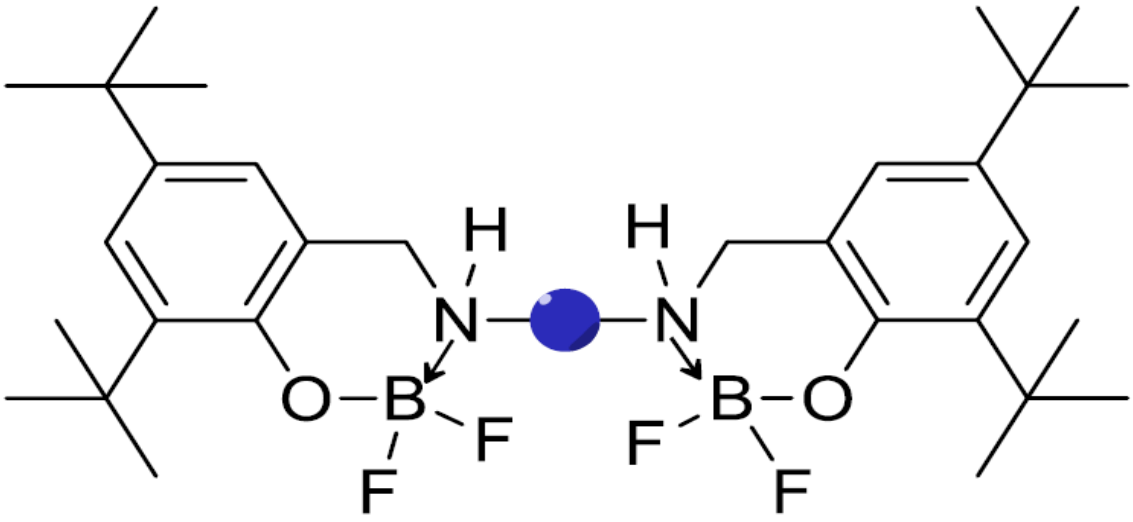


Figure 4. Molecular Structure of L_4BF_2 (Ozbahceci, 2015)

In our study two cell lines and two chemicals have been used, colorectal adenocarcinoma cell line (HT-29) 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_4BF_2) as a new agent and cisplatin as a control. The aim of this study is to estimate the apoptotic, cytotoxic and genotoxic effects of L_4BF_2 by determining the MTT, ELISA, DNA laddering, COMET assay and acridine orange results on the colorectal cancer cell line HT-29. Also by measuring the total oxidant status (TOS), total antioxidant status (TAS) and ferric reducing ability of plasma (FRAP) we estimated the oxidative status index (OSI).

3. MATERIALS AND METHODS

3.1. Cell Culture

Human colorectal adenocarcinoma cell lines (HT-29) were cultured in RPMI-1640 containing filter-sterilized fetal bovine serum (10%) FBS and penicillin/streptomycin (1% P/S 50 U/ml / 50 microgram/ml) plus additives L-glutamine in a humidified atmosphere with 5% CO_2 in 37^oC. Since L-glutamine is an unstable amino acid, the medium was supplemented with L-glutamine just prior to use to a final concentration of 2 mM (0,292 g/L).

When cells were grown to confluence, they were detached with sterilized trypsin/EDTA solution. As the cells were detached in 37^oC 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 plated onto new dishes or flasks in appropriate densities.

3.2. Cell Viability Assay (MTT Assay) and IC_{50} Measurement

The cytotoxicity and IC_{50} of L_4BF_2 were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay which is based on the cleavage of tetrazolium salts by mitochondrial succinate-tetrazolium reductase in viable cells to form formazan dye (Mosmann, 1983). HT-29 colorectal cancer cells were plated in 96-well plates at a density of 5 x 10^3 cells/well and allowed to attach. After incubation at 37^oC 5% CO_2 for 24 hours with exposure to the L_4BF_2 at different concentrations (2-200 µM), the medium was then removed and the cells were slightly washed with PBS. MTT (5 mg/mL) was added, and after 3 h of incubation at 37 °C, the resulting formazan crystals were dissolved in DMSO. Cell viability was measured in terms of absorbance at 540 nm in a microplate reader (Varioskan Flash Multimode Reader, Thermo, Waltham, MA) and, the reduction of cell viability was expressed as

the percentage compared with 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 is two kinds of lysis solution; alkaline lysis solution to detect double and single strand breaks or 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 cisplatin 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 ^{o}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 human colorectal cell line HT-29 as target cells,

Dilute exponentially growing HT-29 cells with culture medium to obtain a cell concentration of 1x10^{5} cells/ml, we transferred into Eppendorf tubes (500 µl/tube 5 × 10 ^{4}cells/tube), we add 500 µl culture medium with different concentrations of CAM (0 µg CAM/ml to 4 µg CAM/ml, closing tubes loosely to allow further exchange of gas and incubate at 37°C in a CO_2 -incubator for 4 h. Then centrifuge the cells in an Eppendorf centrifuge at 200× g for 5 min and discard the supernatant and resuspend the

cells pellet in 1 ml culture medium. Centrifuge 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°C (= lysis), then centrifuge the lysate at 20,000× g for 10 min and remove 400 µl of the supernatant (= cytoplasmic fraction) carefully. Then we predilute the resulting supernatant 1:10 with incubation buffer (= 1 × 10^4 cell equivalents/ml) and detect the nucleosomes in the sample by immunoassay.

To read the sample by ELISA we start to pipette 100 µl coating solution into each well of the MP-modules, then cover MP-modules tightly with the adhesive cover foil included after incubate for 1 h at 15–25°C we remove coating solution thoroughly by tapping or suction then pipette 200 µl incubation buffer into each well of the MP-modules. Cover MP-modules tightly and incubate for 30 min at 15 – 25°C then we remove 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 cover tightly and incubate 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)

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 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) (Erel, 2004; Dikilitas et al., 2011). OSI value was calculated according to the following formula:

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

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. Effect of L_4BF_2 on Cell Proliferation

In cytotoxicity experiments, the anti-proliferative activities of L_4BF_2 were examined in 7 different cell lines, cervix adenocarcinoma HELA, prostate cancer DU-145, prostate cancer PC3, Dukes' type C, colorectal DLD-1, endometrial adenocarcinoma ECC, normal prostate epithelium cell line PNT1-A, and colorectal adenocarcinoma HT-29. According to the results it has been observed the half maximal inhibitory concentration IC_{50} value for HT-29 cells treated with L_4BF_2 was the least compares to the other cell lines and a tiny difference in compare to HELA cell line (Table 2). IC_{50} is a measure used to estimate the efficiency of a material in inhibiting a specific biochemical or biological function; it is usually used for measuring antagonist drug effectiveness in pharmacological research. According to the FDA, IC_{50} represents the concentration of a drug that is required for 50% inhibition in vitro. The result reveals the high sensitivity rate of an HT-29 cell line to our chemical (Figure 5). Depend on this results we decided to use L_4BF_2 in HT-29 cell line for further experiments.

Table 2. IC_{50} value of different cell lines treated with L_4BF_2

No.	Cell Line	IC_{50} (µM)
1	HELA	27.65
2	DU-145	39.02
3	PC3	37.19
4	DLD-1	86.264
5	ECC	40.383
6	PNT1-A	34.22
7	HT-29	26.49

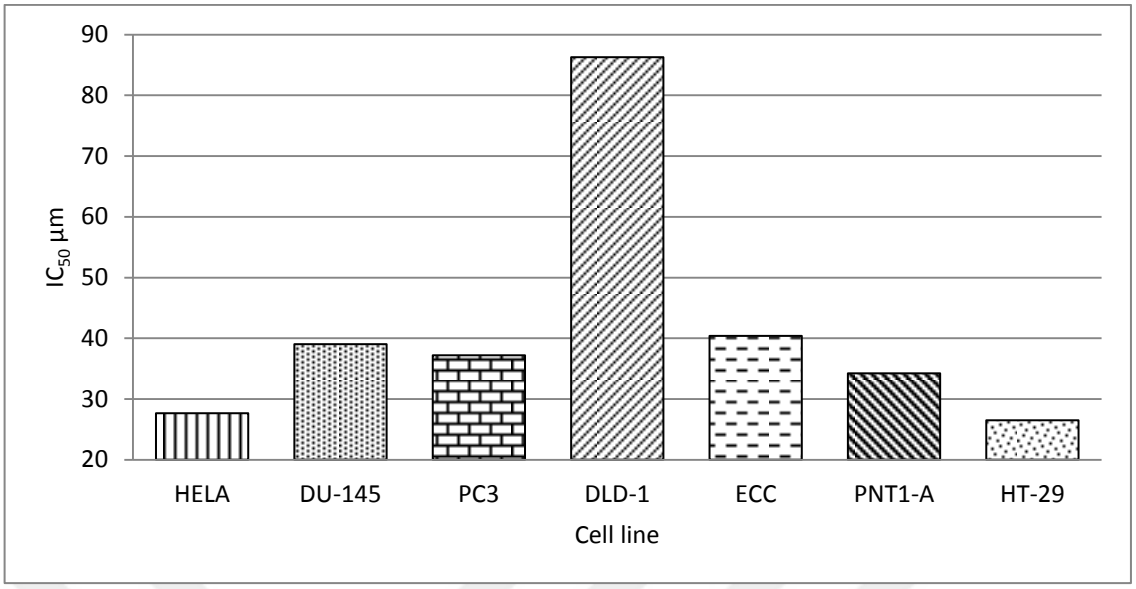


Figure 5. IC_{50} value of different cell lines treated with L_4BF_2

Multiple concentrations of L_4BF_2 were applied (0, 2, 5, 10, 25, 50, 100 and 200 µM) to HT-29 and PNT1-A, the apoptotic analysis showed that L_4BF_2 caused high apoptotic rate for both HT-29 and PNT1-A (Table 3 and 4). For positive control same concentrations of cisplatin applied to both cell lines (Table 5 and 6), in negative control the applied cells did not show any notable change, similar to negative control, however, in 2 µM concentration there was a slight difference between HT-29 and PNT1-A compare to cisplatin of the same dose. In HT-29, no distinguishable variation seen while in PNT1-A a small alteration observed (Figure 6). The same pattern repeated in the 10 µM reciprocally, as there was no significant change in PNT1-A compares to HT-29 cells, but distinguishable changes were seen in the value of HT-29 cells, even in 25 and 50 µM the effect of L_4BF_2 was more efficient than cisplatin (Figure 7). In general, the cell viability percentage decreased in counter to the increasing of the concentrations of L_4BF_2 in both cell lines as a consequence of the rise in apoptotic cell rate.

Table 3. MTT results of HT-29 treated with L_4BF_2

Dose (μM)	1	2	3	Mean ± STD
0	1.0121	1.0392	1.0684	1.04 ± 0.0282
2	1.0538	1.0146	1.0403	1.04 ± 0.020
5	0.9902	0.7586	1.021	0.92 ± 0.143
10	0.7869	0.6338	0.8178	0.75 ± 0.099
25	0.5042	0.4126	0.3947	0.44 ± 0.059
50	0.3252	0.2649	0.3325	0.31 ± 0.037
100	0.2711	0.2858	0.2773	0.28 ± 0.007
200	0.17	0.174	0.1738	0.17 ± 0.002

Table 4. MTT results of PNT1-A treated with L_4BF_2

Dose (μM)	1	2	3	Mean ± STD
0	1.1558	1.1697	1.1925	1.17267 ± 0.019
2	1.092	1.0517	1.0778	1.07383 ± 0.020
5	0.9377	0.8666	0.90864	0.90431 ± 0.036
10	0.9009	0.8733	0.9353	0.90317 ± 0.031
25	0.7635	0.7996	0.7216	0.76157 ± 0.039
50	0.5655	0.4757	0.5734	0.53820 ± 0.054
100	0.3858	0.2922	0.2808	0.31960 ± 0.058
200	0.1913	0.1814	0.1953	0.18933 ± 0.007

Table 5. MTT results of HT-29 treated with cisplatin

Dose (μM)	1	2	3	Mean ± STD
0	1.0038	1.0521	0.9891	1.015 ± 0.0330
2	0.955	1.024	0.925	0.968 ± 0.0508
5	0.786	0.902	0.760	0.816 ± 0.0755
10	0.661	0.701	0.646	0.669 ± 0.0284
25	0.510	0.572	0.509	0.530 ± 0.0363
50	0.347	0.403	0.325	0.358 ± 0.0401
100	0.154	0.167	0.144	0.155 ± 0.0112
200	0.015	0.015	0.015	0.015 ± 0.0004

Table 6. MTT results of PNT1-A treated with cisplatin

Dose (μM)	1	2	3	Mean ± STD
0	1.0235	1.0599	0.9934	1.0256 ± 0.0333
2	0.874	0.8646	0.88644	0.875 ± 0.0109
5	0.5045	0.4712	0.4556	0.4771 ± 0.0250
10	0.3284	0.3443	0.31455	0.3291 ± 0.0149
25	0.1979	0.2231	0.23278	0.2179 ± 0.0180
50	0.1079	0.1042	0.10322	0.1051 ± 0.0025
100	0.0334	0.035	0.03153	0.0333 ± 0.0017
200	0.0126	0.0134	0.01241	0.0128 ± 0.0005

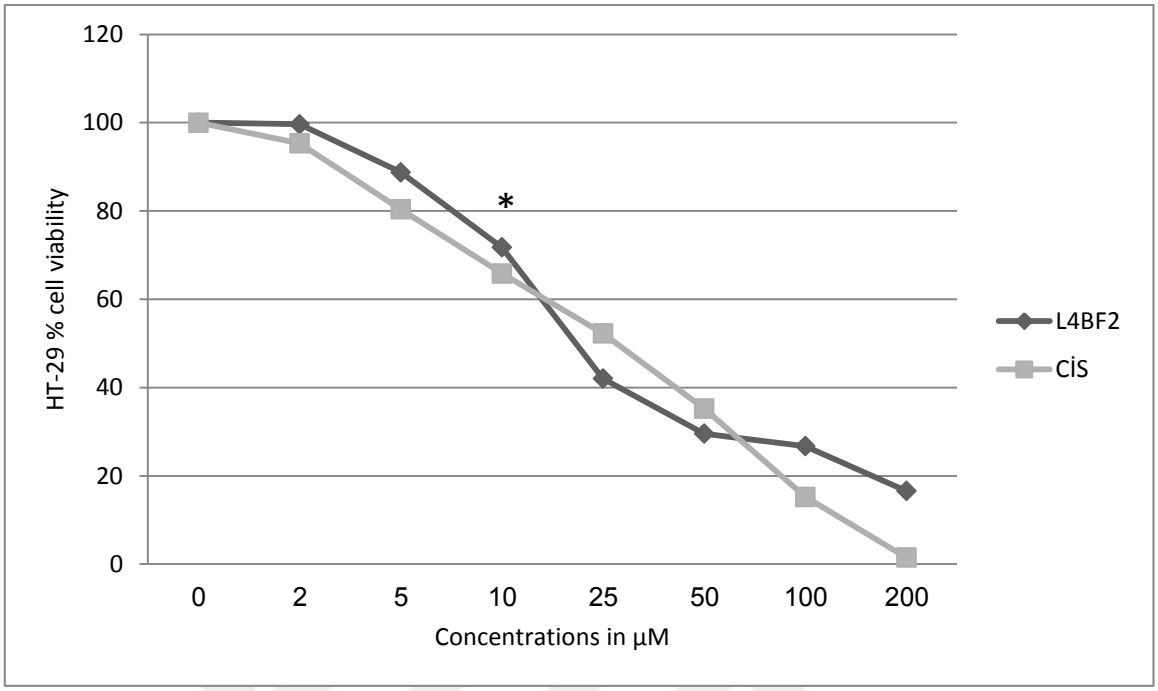


Figure 6. L_4BF_2 and cisplatin on HT-29 viability for 24h (*= effective dose)

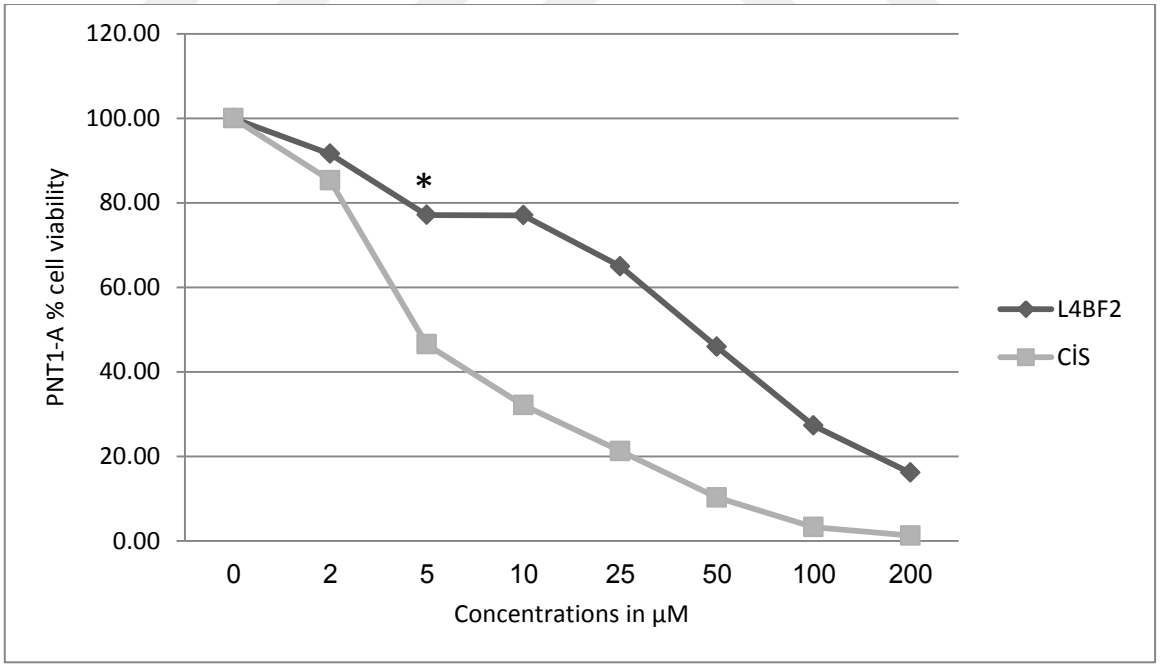


Figure 7. L_4BF_2 and cisplatin on PNT1-A viability for 24h (*= effective dose)

4.2. Effect of L_4BF_2 in DNA Fragmentation of Single HT-29 Cells

Apoptosis can be indicated by many various methods and comet assay is one of these methods, the fragmentation of DNA is determined by the comet assay because it is a sensitive method to examine DNA fragmentation in the level of the individual cell. The technique is based on microgel electrophoresis of DNA cell. Essentially cells were set in a low melting agarose, lysed and electroporation was applied. While damaged DNA moves in the electric field and forms a tail like a comet, the undamaged DNA stays its place like the head of the comet.

Various concentrations of L_4BF_2 were used in HT-29 cell line (0, 5, 10, 25, 50 and 100 μM) (Table 7). From the first ascending dose, the efficiency of L_4BF_2 has been observed in the mean of comet formation. The number and shape of comets increased compatible with the doses to reach the pick in 100 μM, nevertheless, the last concentration didn't extend to the best DNA damaging rate compare to cisplatin (Figure 8).

Table 7. Comet assay result of HT-29 treated by L_4BF_2 and cisplatin

Doses (µM)	Group 1	Group 2	Group 3	Mean ± STD
0	3.0	2.0	2.0	2.3 ± 0.577
5	8.0	5.0	9.0	7.3 ± 2.082
10	12.0	10.0	16.0	12.7 ± 3.055
25	18.0	14.0	22.0	18.0 ± 4.000
50	24.0	25.0	33.0	27.3 ± 4.933
100	32.0	40.0	30.0	34.0 ± 5.292
Cis	36.0	42.0	38.0	38.7 ± 3.055

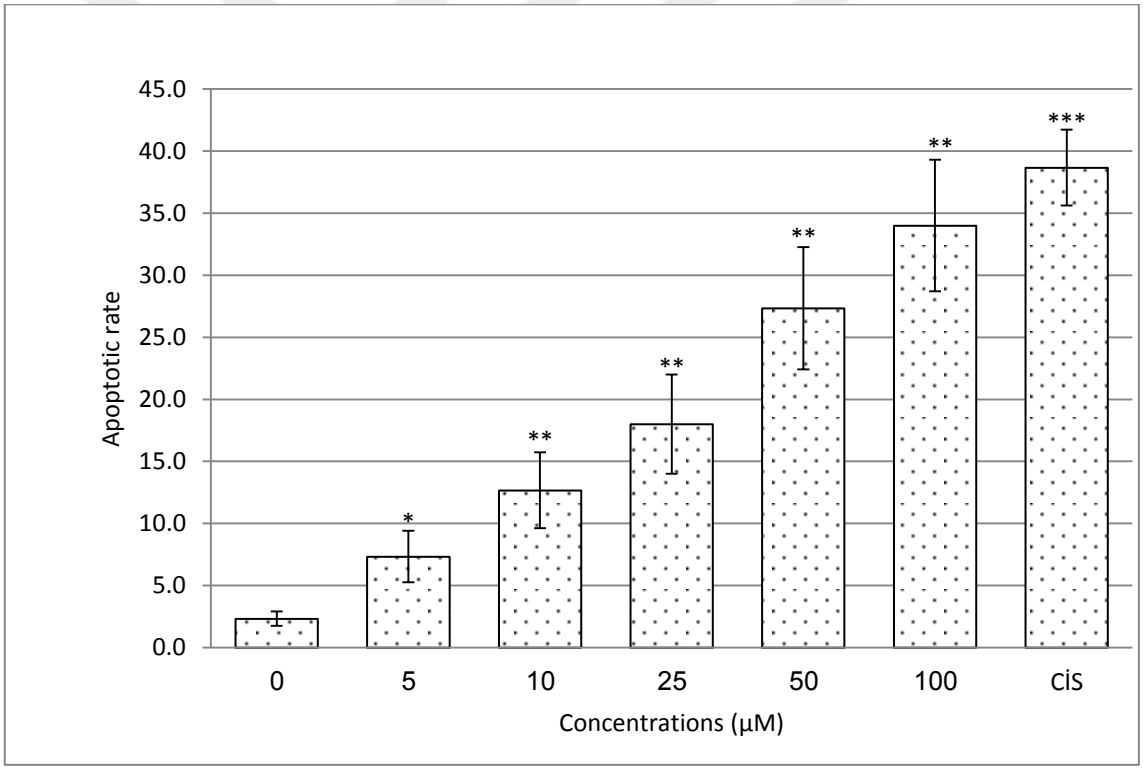


Figure 8. DNA damage rate by the mean of comet formation in HT-29 cells treated with L_4BF_2 and cisplatin (*=P<0.01, **=P<0.001, ***=P<0.0001)

4.3. Determining Apoptosis Levels in HT-29 Cells by using ELISA

The role of L_4BF_2 induced cell apoptosis was determined by ELISA apoptotic assay. Apoptosis is characterized by blebbing of the cell membrane, cytoplasm condensation and the activation of an endogenous nuclease enzyme, generating mono- and oligonucleosome. In contrast the DNA of the nucleosomes is firmly bind with the core histones H2A, H2B, H3, and H4, therefore, protected from cleavage by the endonuclease enzyme (Burgoyne et al., 1974) Anti-histone antibody reacts with the histones H1, H2A, H2B, H3 and H4 of human, anti-DNA-POD antibody binds to single- and double- stranded DNA, therefore, the ELISA allows the detection of mono and oligonucleosome and used to measure apoptotic cells.

A group of different concentrations (0, 5, 10, 25, 50 and 100 μM) of L_4BF_2 was applied in HT-29 cells (Table 8), from the first ascending dose there was a dramatical rise in DNA damage and according to the relativity, it gives a significant p-value ($p=0.0001$) and continue to increase in both doses (10 and 25 μM) recording higher apoptotic rate. However, the last two doses (50 and 100 μM) show a little notable increase rate in apoptosis (Figure 9). Despite the vibration in the p-value of the different concentrations, the increasing of doses affected DNA damage progressively, and show almost the same value of cisplatin in (100 μM).

Table 8. Effect of various doses of L_4BF_2 on HT-29 cell line measured by ELISA

Doses (µM)	1 Abs	2 Abs	3 Abs	Mean	Relative
0	0.975	0.825	0.812	0.871	1.0
5	1.7865	1.658	1.625	1.690	1.9
10	1.8675	1.895	1.902	1.888	2.2
25	2.6335	2.544	2.68	2.619	3.0
50	2.7785	2.78	2.52	2.693	3.1
100	2.8215	2.989	3.12	2.977	3.4
Cis	2.99	2.86	3.41	3.087	3.5

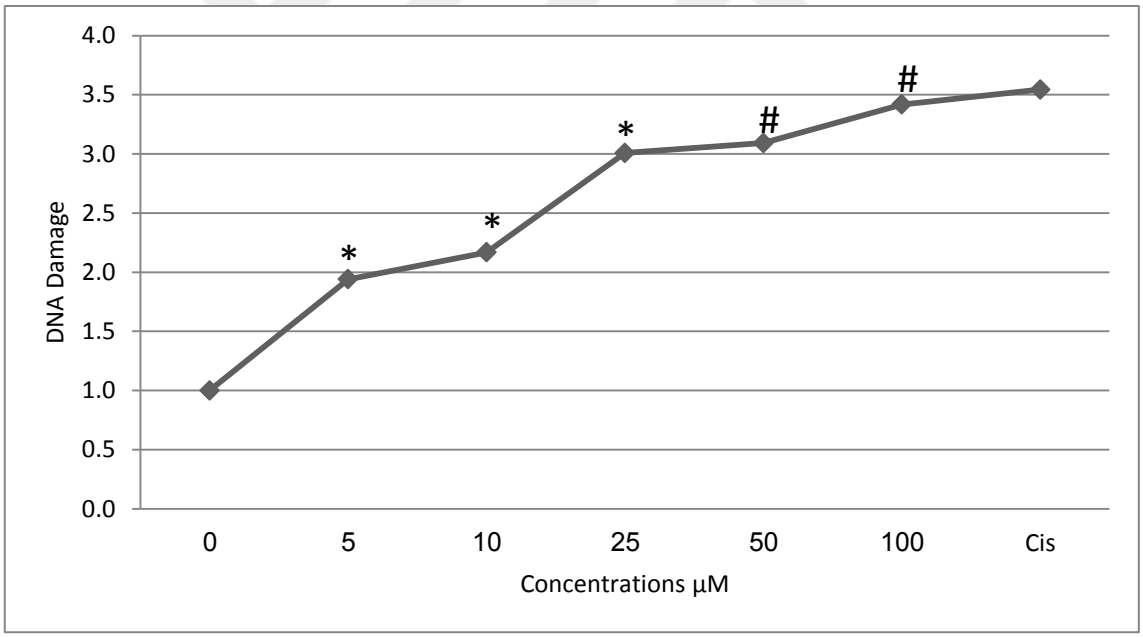


Figure 9. The effect of different concentrations of L_4BF_2 on HT-29 DNA damage measured by ELISA (* = $p<0.05$ compare to the previous concentration, # = $p>0.05$ compare to the previous concentration)

4.4. Measuring DNA Fragmentation (DNA laddering) in HT-29 Cells

DNA laddering is a characteristic feature of DNA degradation by caspase-activated DNase (CAD) and forms fragments of smaller base pairs (bp), which is an important incident during apoptosis. CAD cleaves genomic DNA at internucleosomal linker regions, resulting in DNA fragments that are multiples of 180–185 base-pairs in length. 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 show such DNA damage various concentrations of L_4BF_2 was examined (0, 10, 25, 50, and 100 µM) on HT-29 cells, the first concentration of 0 µM show no extension as in control, while in the first ascending of 10 µM dose there were ladder formation which was indicator of DNA fragmentation and begin to increase to reach longest extension in 100 µM, however, it doesn't appear clearly in the readings but we assume the fragmentation of 50 and 100 µM was beyond the reader depending on the reading result of 25 µM DFI in its best performance (Figure 10)

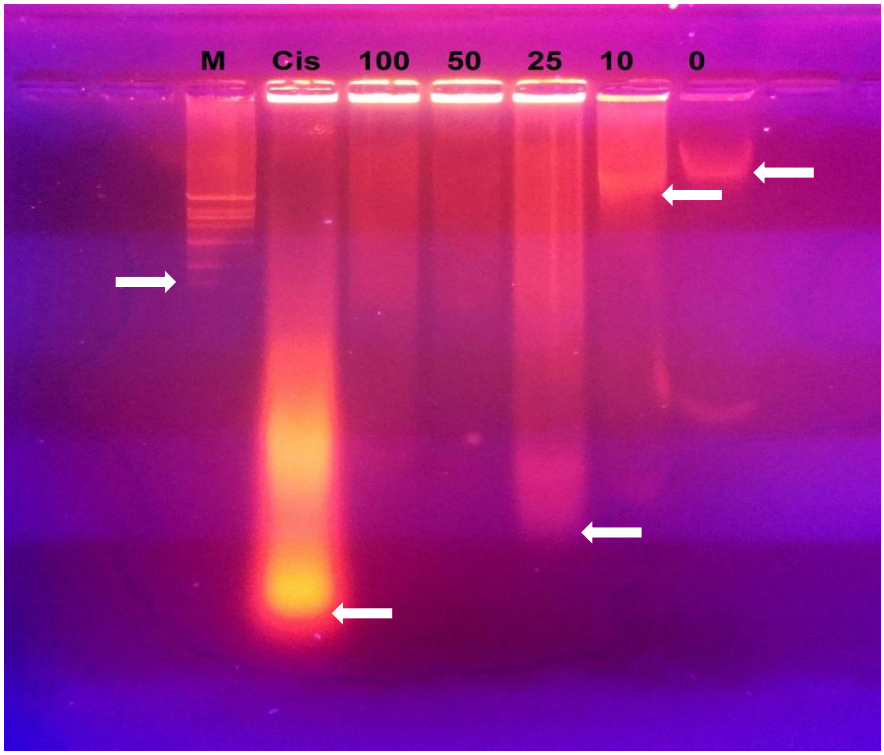


Figure 10. DNA laddering of HT-29 cells treated with L_4BF_2 and cisplatin (the index show the last point of DNA extension)

4.5. Measuring Apoptotic effect of L_4BF_2 by using Acridine Orange (AO) and Ethidium Bromide (EB)

AO is an organic compound used as a nucleic acid-selective fluorescent cationic stain used to determine cell cycle and because it is cell-permeable, it interacts with DNA and RNA by intercalation or electrostatic attractions respectively. When bound to DNA, it is very similar spectrally to fluorescein, with an excitation maximum at 502 nm and an emission maximum at 525 nm (green). In acidic conditions, the dye will produce orange light when treated by blue light. Thus, acridine orange can be used to identify apoptotic cells (Kasibhatla, 2006)

In this test we have five groups of different concentrations of L_4BF_2 applied in HT-29 cells (0, 10, 25, 50 and 100 μM) (Table 9), according to the results the first two ascending doses (10 and 25 μM) showed a little effect on vital cell percentage, but gives a decent increase in apoptotic cells, especially 25 μM. But from that on there was a significant descending rate of living cells and an upsurge of apoptotic cells, precisely in 100 μM which considered the closest to the same cell line treated with cisplatin. However, there was a frequent change in the percentage of necrotic cells, but within the normal range compare to 0 μM (Figure 11).

Table 9. Percentile growth of necrotic, vital and apoptotic HT-29 cells treated with different concentrations of L_4BF_2

Doses (μM)	Necrotic %	Vital %	Apoptotic %
0	4.815	92.593	2.593
10	6.573	88.263	5.164
25	4.399	84.164	11.437
50	5.904	70.111	23.985
100	3.327	53.620	43.053
Cis	7.229	49.799	42.972

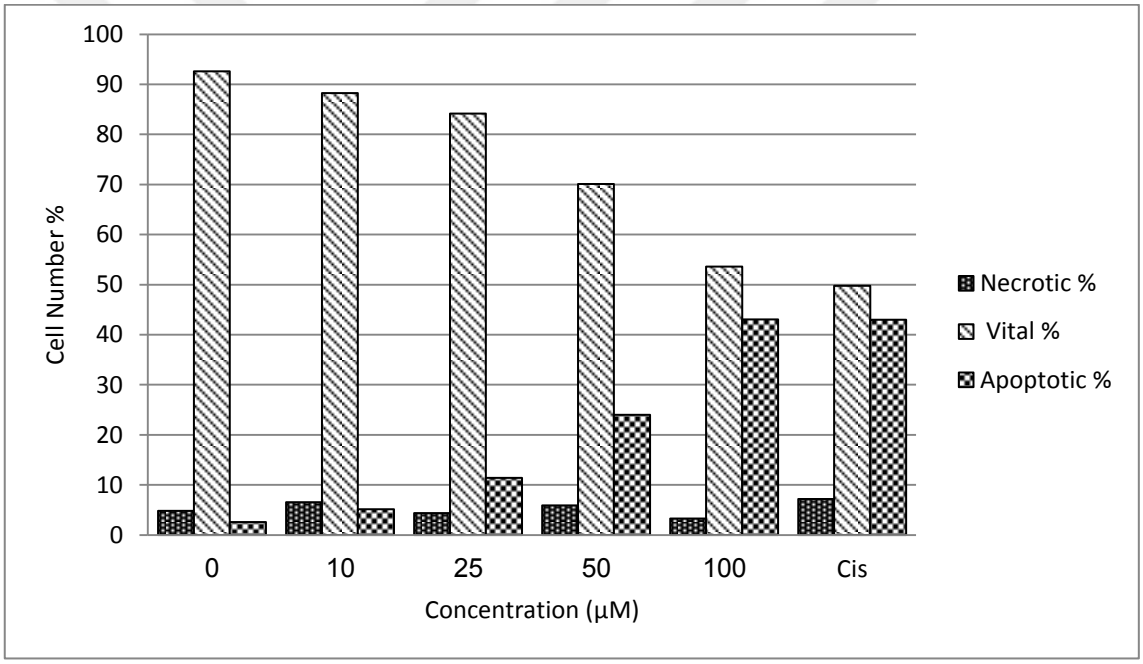


Figure 11. A comparative diagram between the ratio of necrotic, living and apoptotic HT-29 cells, treated with different doses of L_4BF_2

4.6. Determination of Intracellular TAS , TOS, and OSI

Intracellular total antioxidant status (TAS) level was measured for five different concentrations (0, 10, 25, 50 and 100 µM) of L_4BF_2 used on HT-29 cell line (Table 10), in 10 µM dose there was a decent decrease in the value of TAS, however, in 25 µM a fluctuation has been observed, but another drop seen in 50 µM and continued in 100 µM to reach the nearest point compare to cisplatin (Figure 12). Overall the TAS values showed a significant p-value of $p < 0.008$.

Table 10. TAS value of different concentrations of L_4BF_2 used in HT-29 cell line

Doses (µM)	1	2	3	Mean ± STD
0	1.042	1.03	0.99	1.02 ± 0.0272
10	1.031	1	0.85	0.96 ± 0.097
25	1.007	0.98	0.95	0.98 ± 0.029
50	0.946	0.85	0.89	0.90 ± 0.048
100	0.83	0.88	0.75	0.82 ± 0.066
Cis	0.82	0.8	0.81	0.81 ± 0.010

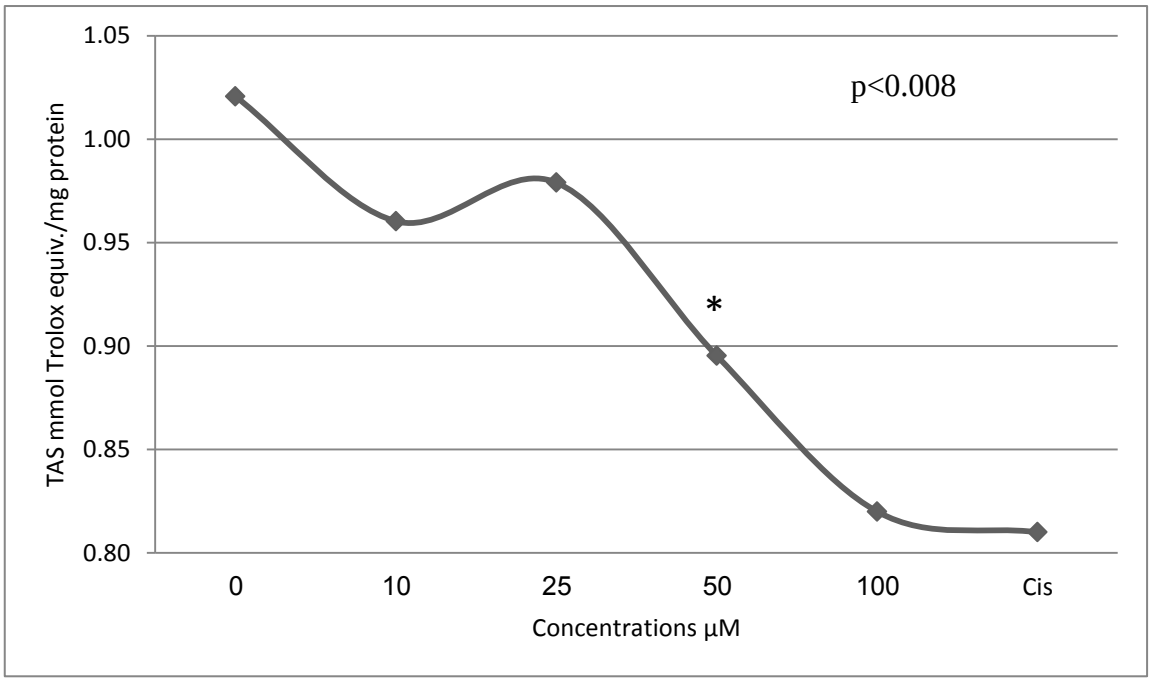


Figure 12. Shows the TAS value according to the elevation of L_4BF_2 concentrations (*= $p < 0.05$)

Intracellular total oxidant status (TOS) of the HT-29 cell line was measured as same as TAS concentrations (Table 11). The value of TOS was increased as expected, contrary to TAS there was no fluctuation of the indicator but doesn't reach cisplatin value even in 100 µM dose (Figure 13).

Table 11. Values of different concentrations of L_4BF_2 used in HT-29 cell line

Doses (µM)	1	2	3	Mean ± STD
0	6.5	6.25	5.95	6.23 ± 0.2754
10	7.2	6.85	7.35	7.13 ± 0.257
25	7.9	7.85	7.99	7.91 ± 0.071
50	8.8	8.56	9.025	8.80 ± 0.233
100	9.6	9.56	9.85	9.67 ± 0.157
Cis	11	10.85	10.95	10.93 ± 0.076

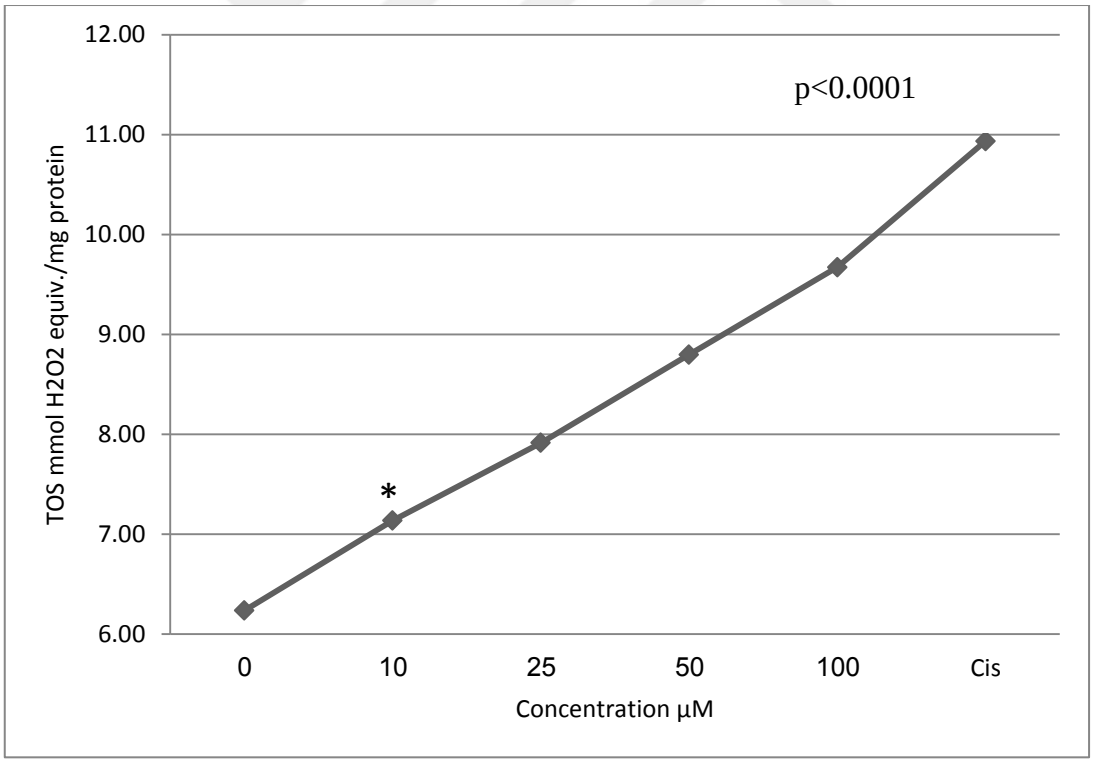


Figure 13. Shows the TOS value according to the elevation of L_4BF_2 concentrations (*= $p < 0.05$)

Depending on the results of TAS and TOS we managed to gain oxidative stress index (OSI) value. 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, so these results are being stated as arbitrary unit (AU). The results are presented in (Figure 14). As seen in the figure, the OSI value increased parallel to the concentration of L_4BF_2 with a substantial peak in 100 µM.

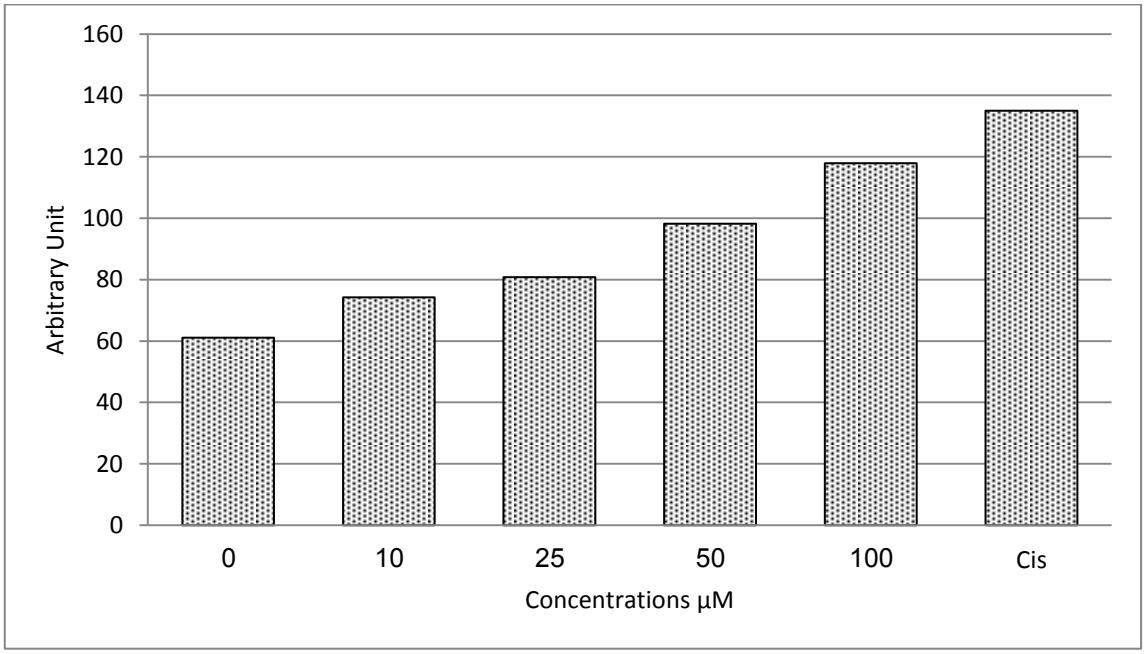


Figure 14. OSI values of HT-29 treated with L_4BF_2 in different concentrations compare to cisplatin as a positive control

4.7. Estimating Antioxidant Power by measuring Ferric Reducing Ability of Plasma

Free radicals are produced as a side effect of normal aerobic metabolism. Reactive oxygen species (ROS) are one of these free radicals that formed as a consequence of this process (Gutteridge, 1994; Halliwell, 1994). These free radicals are usually removed or inactivated in vivo by a team of antioxidants (Frei, England, and Ames, 1989; Gutteridge, 1994). FRAP assay provides the ability to measure the amount of those antioxidants in samples.

For this test, we applied various concentrations of L_4BF_2 in HT-29 cell line (0, 10, 25, 50 and 100 μM) (Table 12). At first we measured the FRAP value without adding any agents to determine the negative control, then we started with the concentration 10 μM, and observed a descending in the value of FARP but not significant, the rate continued in this order in 25 μM with a significant p-value of $p<0.048$, moreover, in the last two doses (50 and 100 μM) the FRAP value keep decreasing recording a high significant p-value of $p<0.013$ in 100 μM which considered the nearest to cisplatin FRAP value in the same cell line (Figure 15).

Table 12. FRAP values of HT-29 cells treated by L_4BF_2 in different concentrations

Doses (µM)	1	2	3	Mean ± STD
0	0.16	0.158	0.169	0.16 ± 0.0059
10	0.15	0.145	0.155	0.15 ± 0.005
25	0.155	0.145	0.14	0.15 ± 0.008
50	0.147	0.135	0.145	0.14 ± 0.006
100	0.148	0.135	0.138	0.14 ± 0.007
Cis	0.132	0.129	0.142	0.13 ± 0.007

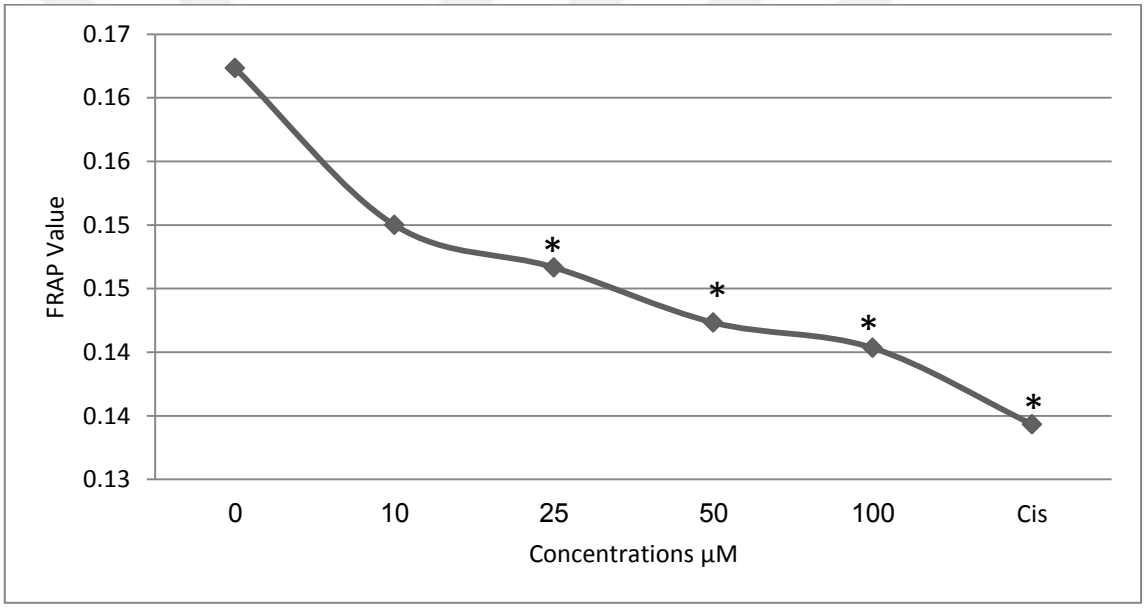


Figure 15. FRAP values in respect to L_4BF_2 concentrations used in HT-29 cell line (*= $p<0.05$) compare to negative control

5. DISCUSSION AND CONCLUSION

If we can define cancer in a short term as it is a result of a non-equilibrium homeostasis between cell proliferation and cell deaths. Apoptosis plays a controlling role of unwanted cells to each vital organelle within the cell, such as the mitochondria and chromosomes (Wallace, 2012; Jeon et al., 2013). With the increasing incidence rate of cancer in type and range, the need to find new anti-cancer drugs became a priority for researchers in the field. Apoptosis provides many appreciable hints about a therapy's efficiency, and many anticancer effects of numerous chemotherapeutic agents are by means of apoptosis (Hassan, Watari, Abualmaaty, Ohba, and Sakuragi, 2014). Therefore, the stimulation of apoptosis has become a major emphasis when developing anticancer therapies. Many studies have been performed on transitional metal chemistry and biological action of numerous synthesized Schiff based compounds to regulate their role in inducing apoptosis in cancer cells and to identify the key actions and molecules that are regulated by the pathway (Sergent et al., 2012; Ihnen et al., 2013).

Nowadays most promising strategies to invent new chemotherapy drugs is to find novel DNA-targeted anticancer drugs by the meaning of searching and designing new molecules enable of reacting with nucleic acids and induce apoptosis (Maheswari et al., 2008), . Recently, Reedjik and co-workers reported a novel Schiff base class of DNA binding and cleaving agents that could cleave DNA catalytically in the absence of reductants showing high to modest cytotoxicity against selected cancer cell lines (Roy et al., 2009).

The combination of boron in some Schiff base boron compounds showed antitumor properties to various cancer cell lines and there are experiments in which boron compounds work as a proteasome inhibitor in cancer cells on different cancer cell lines including lung, leukemia and pancreatic cells (Bone et al., 1987). Boron is known to be important for animal cell replication and growth, but the fundamental mechanism remains unclear (Eckhert, 1998). Studies on HL-60 and U-937 cell lines treated by some boron compounds such as using guanidine biboric acid, dihydroxy boron hydrochloride monohydrate and hydroxo salicyl hydroxomate boron derived from new boron compounds show boron compounds have an apoptotic effect on cancer cells and have a damaged apoptosis mechanism (Canturk et al., 2014). The purpose of this study was to

determine whether the L_4BF_2 complex has a cytotoxic and genotoxic effect and initiation of the apoptotic pathway in a colon cancer cell line.

The cell line model which used in this study was colorectal adenocarcinoma cell line HT-29, which was recently used as a model in different other studies (Perego et al., 2012; Paydar et al., 2014). For control, we used cisplatin as an anticancer agent due to its roles in cross-linking DNA strands through organization of nucleic acid bases, which can consequently induce apoptosis in cancer cell (Wong and Giandomenico, 1999),

The low molecular weight of L_4BF_2 had a great advantage for our compound because it had a spectacular effect in our study on cancer cells HT-29. Pharmacologically most drugs have small molecular weight and have a variety of biological functions, serving as cell signaling molecules, drugs in medicine, and in many other roles. In our case, the novel fluorine boron hybrid complex (L_4BF_2) acted directly on DNA damage activation route. DNA-damaging agents have a long time of use in the history of cancer chemotherapy but the full level of their cellular mechanisms, which is crucial to stabilizing efficacy and toxicity, is often not totally understandable (Cheung-Ong et al., 2013).

The MTT cytotoxic assay displays a dose depended on growth inhibition of HT-29 cells with IC_{50} value of 26.49 µM treated with L_4BF_2. we suggested that increasing the concentration of L_4BF_2 decreases cell viability in a dose-dependent manner in most cell lines as it demonstrated in (Figure 6). The L_4BF_2 treatment has more effect on HT-29 ,cell viability reduced by 83.40 %, showing that HT-29 is highly affected by the compound. To further confirm the accruing of apoptosis more tests display extra detailed apoptotic and cytotoxic effects of L_4BF_2, to be more precise the tests of single cell gel electrophoresis showed a pattern of DNA damages seen in some anticancer drugs such as cisplatin with a high rate efficiency to reach a signification value of $p<0.001$ in last concentrations..

Anticancer therapies such as radiotherapy and chemotherapy make use of ROS overproduction to prevent the proliferation of cancer cells (Renschler, 2004), Hence it has been observations in elevation of L_4BF_2. Downregulation of antioxidant defense systems is responsible for ROS elevation as seen in the reduced levels of TAS in cancer

cells and FRAP value which showed a distinct decrease in a parameter for antioxidant amount in HT-29 cells while there was a significant increase of TOS and OSI in cancer cells, as well as an induction of chronic oxidative damage on biomolecules such as DNA (Li et al., 2011; Tiloke et al., 2013).

The mitochondria have a great role in the organizing of cell death and existence (Bernardi et al., 2006). The interaction between ROS and the phospholipids in the mitochondrial membrane cause an opening of transmission pores in the first stages of apoptosis (Trushina and McMurray, 2007; Chen et al., 2001). As an important triggers in cell apoptotic pathways, the production of ROS along with other intracellular cascade reactions such as loss of mitochondrial transmembrane integrity, order of caspase-3, down-regulation of Bcl-2, and deficiency of p-53, would finally lead to cell apoptosis (Trejo-Solís et al., 2005; Carvallo-Chaigneau et al., 2008)

In another hand ELISA readings confirmed last results by showing the high rate of DNA damage in HT-29 cells; this suggests DNA damage was beyond repair, induced cell death through activation of pro-apoptotic proteins and reduction of anti-apoptotic proteins. To be more accurate we run a DNA laddering test on HT-29 treated with different concentrations of L_4BF_2, and according to the results they appear to be promising molecules and studies should proceed to determine the potential target or targets of the molecule.

We demonstrated the anticancer properties of novel fluorine boron hybrid complex L_4BF_2 and due to its ability to induce cell apoptosis in colorectal cancer cells through activation of oxidative stress and reduction in expression of anti-apoptotic proteins and antioxidant defense proteins. This resulted in DNA damage and subsequently increased expression of pro-apoptotic proteins and apoptosis executioner molecules. These data shows that L_4BF_2 possesses potential pharmaceutical properties as an alternative treatment for colorectal cancer. However, since this was an in vitro study, more studies need to be done to further characterize this novel compound and its mechanism of action in vivo models. Future work includes determining the effect of methylation of L_4BF_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_4BF_2) on colorectal cancer. Y. Y. U, Institute of Health Science Department of Medical biology MSc Thesis, Van, 2016. Colorectal cancer (CRC) is one of the most common types of cancer in both sexes; it considered to be the third leading death among other cancers. Based on the potential of Schiff based complexes for the initiation of apoptosis, this study meant to examine the cytotoxic, genotoxic and apoptotic activity of a novel florin boron hybrid complex (L_4BF_2) on HT-29 cells. The complex applied a potent suppressive effect on HT-29 cells. In this study L_4BF_2 was examined in seven different cell lines, HELA, DU-145, PS3, DLD-1, ECC, PNT1-A and HT-29, later investigated its apoptotic, cytotoxic, and genotoxic properties on human colorectal adenocarcinoma cell line HT-29 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 tremendous effect of L_4BF_2 on HT-29 cells especially in damaging their DNA and cause a series of reactions lead to apoptosis. The results suggest that the L_4BF_2 complex is able to induce the apoptosis of colorectal cancer cells and is a possible candidate for future cancer studies.

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

ÖZET

Yeni fluor boron hibrit kompleksinin (L_4BF_2) kolorektal kanser üzerindeki apoptotik, sitotoksik ve genotoksik etkisi. Y. Y. U, Sağlık Bilimleri Enstitüsü Tıbbi Biyoloji Bölümü Yüksek Lisans Tezi, Van, 2016. Kolorektal kanser (CRC), her iki cinsiyette en sık görülen kanser türlerinden biridir; Diğer kanserler arasında önde gelen üçüncü ölüm olarak değerlendirilmiştir. Schiff bazı komplekslerin apoptozu indükleme potansiyeline dayanan bu çalışma, HT-29 hücrelerinde yeni florin bor hibrid kompleksinin (L_4BF_2) sitotoksik, genotoksik ve apoptotik aktivitesini incelemeyi amaçlamıştır. Kompleks, HT-29 hücreleri üzerinde güçlü bir baskılayıcı etki yapmıştır. Bu çalışmada, HELA, DU-145, PS3, DLD-1, ECC, PNT1-A ve HT-29 olmak üzere yedi farklı hücre dizisinde L_4BF_2 incelendi ve daha sonra insan kolorektal adenokarsinoma hücre dizisi üzerindeki apoptotik, sitotoksik ve genotoksik özellikleri araştırıldı. Bunun için de Tek hücreli jel elektroforez testi (comet testi), DNA fragmentasyon laddering, DNA hasarı için akridin orange testi ve apoptotik ölçüm için ELISA gibi standart apoptotik ve sitotoksik testleri L_4BF_2 farklı konsantrasyonlarda HT-29'un üzerinde uygulanmıştır. Ayrıca toplam antioksidan durum (TAS) ve toplam oksidan durum (TOS) değerlendirerek oksidatif durumu ölçülmüştür. Oksidatif stres indeksi (OSI) de ayrıca hesaplanmıştır. Sonuç olarak, HT-29 hücrelerinde L4BF2'nin çok büyük bir etkisini özellikle DNA'larına zarar vermede buldu ve apoptozise yol açan bir dizi reaksiyona neden oldu. Sonuçlar, L_4BF_2 kompleksinin kolon kanseri hücrelerinin apoptozunu indükleyebildiğini ve gelecekteki kanser çalışmalarında potansiyel bir aday olduğunu ortaya koymaktadır.

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

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CURRICULUM VITAE

Hawro Dlawar Ismael MASSEH, I am from Erbil city in North Iraq. I was born on April 15, 1981, in Baghdad. 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 (2000-2004), 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 Medical Central Laboratory- Erbil, and I have rotated on every department from medical biology to food and water quality controlling, then I was sent to Sidakan town in the city of Soran to work in a health center and finish up my trainee till 2007, then I came back to Erbil to work as a (Biologist) in Nanakaly Hospital for Blood Diseases until 2008, then transferred to Medical Technical institute where I began my teaching carrier as a research assistant, 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.

SUPPLEMENT 1. ETHICS REPORT

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

KARAR BİLGİLERİ		
	Karar No: 12	**Tarih: 03.06.2016**
	Doç.Dr. Yasin TÜLÜCE sorumluluğunda yapılması tasarlanan ve yukarıda başvuru bilgileri verilen **"Bor İçeren İndirgenmiş Schiff Bazı Türevinin Kolorektal 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 ☐	

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SUPPLEMENT 2. PLAGIARISM REPORT

YÜZÜNCÜ YIL ÜNİVERSİTESİ
SAĞLIK BİLİMLERİ ENSTİTÜSÜ
LİSANSÜSTÜ TEZ ORİJİNALLİK RAPORU

Tarih: 09/12/2016

Tez Başlığı / Konusu: THE APOPTOTIC, CYTOTOXIC AND GENOTOXIC EFFECTS OF NOVEL FLUORINE BORON HYBRID COMPLEX (L4BF2) ON COLORECTAL CANCER

Yukarıda başlığı/konusu belirlenen tez çalışmamın Kapak sayfası, Giriş, Ana bölümler ve Sonuç bölümlerinden oluşan toplam 33 sayfalık kısmına ilişkin, 09/12/2016 tarihinde şahsım/tez danışmanım tarafından Turnitin intihal tespit programından aşağıda belirtilen filtreleme uygulanarak alınmış olan orijinallik raporuna göre, tezimin benzerlik oranı % 8 (sekiz) dır.
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 09.12.2016

Adı Soyadı: Hawro Dlawar Ismael MASSEH

Öğrenci No: 159302006

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)