An-Najah National University

Faculty of Graduate Studies

Effect of New Copper Complexes on Cancer Cells in Inducing Caspase-Dependent Apoptosis and Binding to DNA

By

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Dedication

To all whom helped me to finalized this thesis, they earned my thankful and pray, Thank you all.

I am deeply gratified to my family for the support, patience and the inspiration they surrounded me with.

I am deeply grateful and thankful to my wife and children who inspired, supported and encouraged me to explore the best in me. I thank them for dedication and patience.

To myself, that sometimes/a lot of times give me hesitant and twice thinking, that it carries itself to finalize this work.

Thanks you all, ☺

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I have no words to express my sincere acknowledgement to Almighty, compassionate, and supreme Allah, who empowered me to complete this work. I also appeal peace for the last prophet of Allah, Muhammad (SAAW), who is forever a torch of guidance for humanity as a whole.

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Finally, I take great honor to prompt my sincere thanks to all the people who have been involved directly or indirectly with the completion of this work.

الإقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان

Effect of New Copper Complexes on Cancer Cells in Inducing Caspase-Dependent Apoptosis and Binding to DNA

أقر بأن ما شملت عليه الرسالة هو نتاج جهدي الخاص, باستثناء ما تمت الإشارة إليه حيثما ورد, وأن هذه الرسالة ككل أو أي جزء منها لم يقدم من قبل لنيل أي درجة أو لقب علمي أو بحثي لدى أي مؤسسة علمية أو بحثية

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degrees or qualifications.

Student's Name: **اسم الطالب:**

Signature: **التوقيع:**

Date: **التاريخ:**

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List of Abbreviations

µl	Microliter
Abs	Absorption
CC	Copper complex
CCs	Copper complexes
C_n	Complex number n
Colo205	Colon cancer cell line
CT-DNA	Circulating tumor DNA
Cu	Copper
Cu (I)	Reduced form of copper
Cu (II)	Oxidized form of copper
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
G	Gravitational force
Hs755	Liver cancer cell line
MC7	Breast cancer cell line
mg	Milligram
ml	Milliliter
MOH	Ministry of health
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenltetrazolium bromide
nm	Nanometer
PBS	Phosphate-buffered saline
pNA	p-nitroanilide
RNA	Ribonucleic acid
ROS	Reactive oxygen species
UV	Ultra Violet
UV-vis	Ultra Violet visibility
WHO	World health organization

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Abstract

Cancer spread worldwide as one of main reasons for mortality. There are more than 100 types of cancer. There were 14.1 million new cancer cases in 2012. By year 2030 new cases will reach to 21.7 million due to modernized lifestyles (such as smoking, poor diet, physical inactivity, and fewer pregnancies … etc.) that are known to increase cancer risk. Cancer treatment options may include chemotherapy, radiation, and/or surgery or a combination of all.

Chemotherapy is one of the major treatment options that are available to face this problem. One of the most noteworthy discoveries in cancer chemotherapy is cisplatin, which is very active against different types of cancer. Cisplatin has a cure rate exceeded 90% in some cancer types. However, the treatment option by using cisplatin is limited by its side effects, also the inherited and acquired resistance limits its usage too. These obstacles have led to the extensive search for other active antitumor metal-based complexes with improved pharmacological properties.

This research was aiming to study the anticancer activity of copper complexes that depended on activating apoptosis through caspases pathway. Nine copper-based complexes were prepared and screened in-vitro for

cytotoxic activities against three cancer cell lines (Colon cancer: colo205 cell line, Osteosarcoma cell line: Hs 755 and Breast cancer cell line: MC7 cell line) using caspase-3 colorimetric assay and cell viability test (MTT assay). Results showed that complexes 1,3,4,5,6 and complex 8 induced cytotoxic effect on colo205, while complex 2 and 7 have no cytotoxic effect on colon cancer cells. All the complexes that tested didn't show any sign of activating caspase 3 for Colon cell line or Breast cancer cell line MC7.

These complexes exhibited promising anti-proliferative activity against tested cell lines, which indicates that these complexes have anticancer effects.

Chapter One

Introduction

Introduction

1.1Cancer Preview:

Cancer spread worldwide as one of main reason for mortality. There are more than 100 types of cancer. Cancer has become a major cause of death around the world among all other non-communicable diseases, especially in low-income and middle-income countries (Torre, et al., 2015).

The low-income and middle-income nations will be the most one to be affected by this growth. These alterations is predicted to be the most important factor for the increase in global load of 21.7 million new cancer cases by 2030 compared with 14.1 million cases in 2012, Estimates for total cancer deaths in 2012 were 8.2 million (about 22,000 cancer deaths a day), in 2030, there will be around 13 million cancer deaths simply due to the growth and aging of the population (Ferlay, et al., 2010). With these increasing numbers massive efforts are being made with the objective to predict, prevent, and treat cancer through various methods (Torre, et al., 2015).

Those striking numbers of cancer cases are mainly results of modernized lifestyles (such as smoking, poor diet, physical inactivity, and fewer pregnancies … etc.) or environment, that are known to increase cancer risk (Torre, et al., 2015).

Cancer is a group of malicious diseases that could affect various parts of the human body. Cancer characterized by unspecific, uncontrolled cell growth and cell division that arise from uncontrolled cell cycle during cell division,

repair and growth leading to masses of tissue called tumors. Such masses may have the capability to invade the adjacent organs or even proliferate through the body, which called metastasis (Hardin, et al., 2012; Hanahan & Weinberg, 2000).

Tumors could be classified into benign and malignant tumors. Benign tumors are usually characterized by localization and lack the invasion capabilities which result in inability to invade nearby tissue and organs, also it could be removed while usually it does not need to be removed. Malignant tumors also known as cancer, characterized by rapid growth and invade nearby tissue and organs of the body. When tumor cells reach the blood stream or lymphatic system it spreads all over the body, this process called metastasis, lead to the inevitable death (Hardin, et al., 2012; Wujcik, 2011).

Cancer treatment may include surgery, radiation, chemotherapy, hormone therapy and biological therapy (Miller, et al., 2012). The most usable method nowadays to treat cancer use surgery in combination with radiation or chemotherapy (Miller, et al., 2012). However, the choices of treatment depend mainly on the type, location and the stage of the disease.

Type of cancer doesn't spread equally between population, lung cancer is the leading cause of death in men, while breast cancer is the leading cause of death in women (Torre, et al., 2015**a**; Torre, et al., 2015**b**).

According to studies the most common cancers types in men are prostate, lung and liver, while in women either breast or cervical cancer was the most common diagnosed neoplasm (Torre, et al., 2015).

1.2Cancer in Palestine (west bank):

According to annual reports of Palestinian MOH of 2015 released in 2016, cancer is the second cause of death after cardiovascular disease. There was 2400 new cancer registry in 2015, with 4.6% increase over 2014 that register 2294 new case, 6.8% of those new cases in patient less than 15 years old, 33.4% of those cases in people over 65 years old even those only represent 3.2% of the total population see Fig. 1.

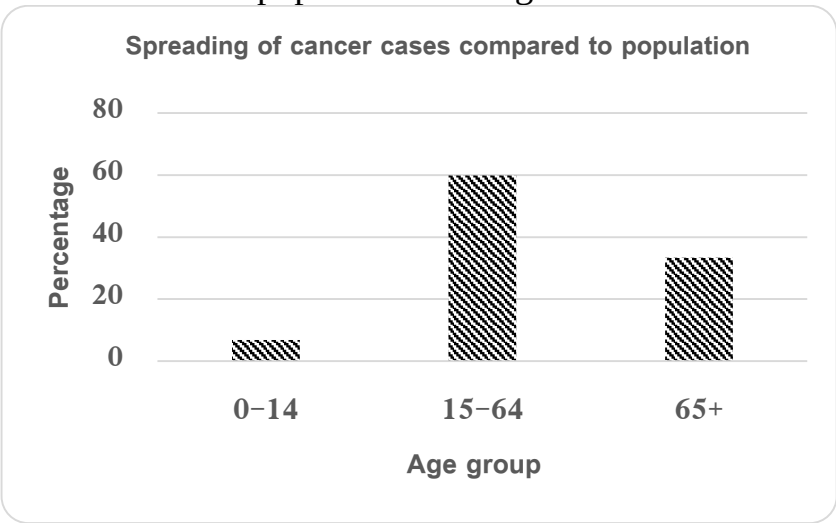

Figure 1. 1: Shows cancer percentage compared to population percentage in west bank, according to annual report published MOH 2016.

Ministry of health report also show that there were 1140 cases in male and 1260 in female, see Fig. 2.

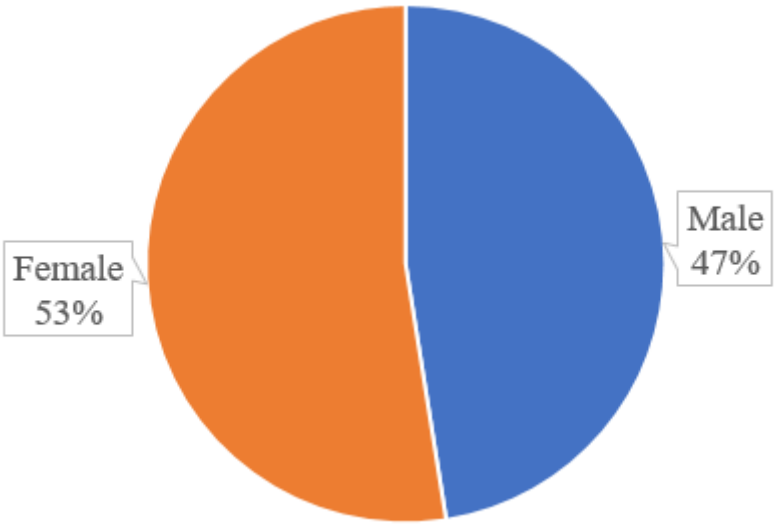

Figure 1. 2: Shows percentage of cancer cases in male compared to percentage of cancer cases in female percentage in west bank, according to annual report published MOH 2016.

Cancer in west bank spread across many types, breast cancer is #1 in cancer types as it took 17.8% of all cancer cases in 2015, then colon cancer by 9.4, see Fig. 3.

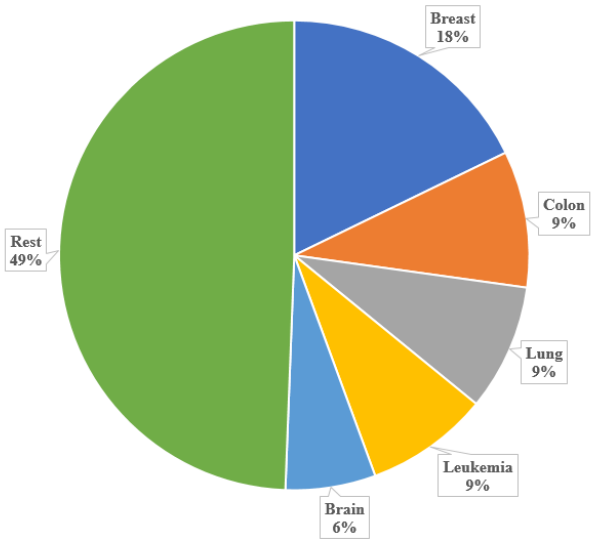

Figure 1. 3: Shows percentage of most common cancers in west bank, according to annual report published MOH 2016

MOH report shows that deaths due to cancer are spread among many type of cancers. Four type of cancer are responsible for half of deaths, lung, colon, breast and brain. This give us an idea that we should care more about some cancer types in west bank as lung and colon cancer. Those two types together form only 18% of cancer cases but responsible for 30% of deaths, while breast cancer grabbed 18% of cancer cases, but it responsible for 11% of cancer death.

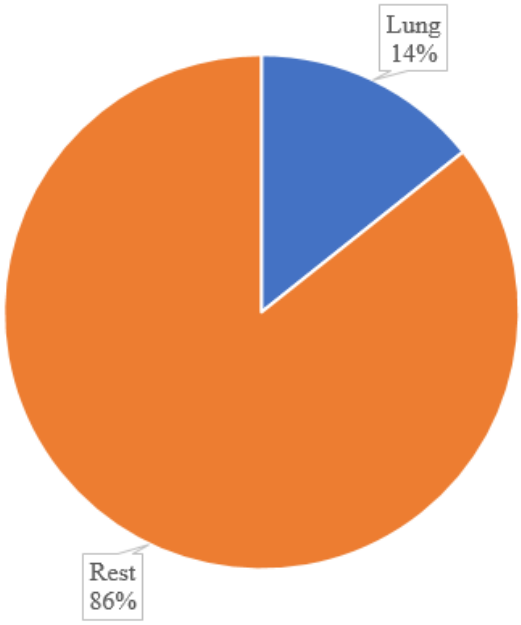

Figure 1. 4: Shows percentage of cancer's deaths in west bank, according to annual report published MOH 2016.

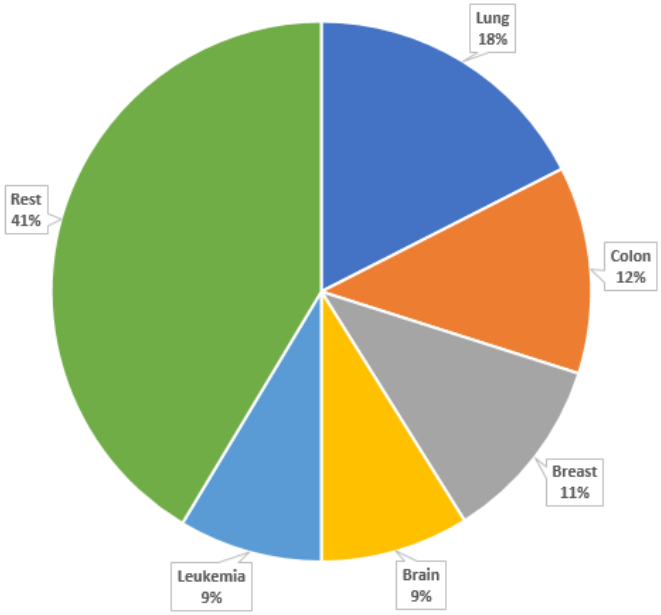

Figure 1. 5: Lung cancer is the most common cancer in males in west bank, this Fig shows that lung cancer taking 14% of all cancers cases, according to annual report published MOH 2016

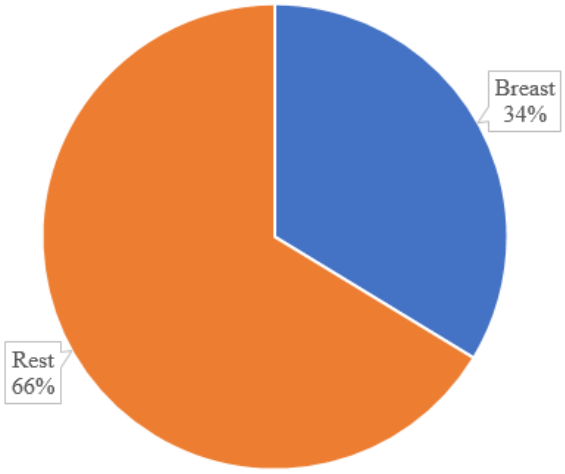

Figure 1. 6: Breast cancer is the most common cancer in women in west bank, this Fig shows that breast cancer taking 34% of all cancers cases in females according to annual report published MOH 2016.

Lung cancer is number one between men with 14%, see Fig. 6. While breast cancer is number one between women with 34%, see Fig. 5.

1.3 Cancer therapies and treatment types:

Cancer treatment may include chemotherapy, radiation, surgery, hormone therapy, immune therapy and targeted therapy (Torre, et al., 2015). Chemotherapy used through drugs that kill dividing cells.

Those drugs can be divided to 4 sub-categories: Anti-metabolites, that inhibit metabolic pathways needed for DNA synthesis. Antibiotics, that inhibit DNA function, leading to un-functional DNA that cannot do its genetic functions. Plant-derived drugs, that work on disrupting microtubules or inhibition of topoisomerases. And, Alkylating agents, those drugs chemically crosslinking the DNA double helix that lead to inhibition of the DNA function, cisplatin is an example of one of those drugs (Hardin, et al., 2012).

Every type of treatment may have its own problems and complications, this applied to chemotherapy also. Chemotherapy works on both normal and abnormal cells. Most side effects result from the inability to distinguish between active and inactive cells causing death of normal active cells (Hardin, et al., 2012). Those side effects also include vomiting, nausea, tiredness and hair loss (Coates, et al., 1983; Griffin, et al., 1996).

Constantly scientists are promoted and motivated to develop new drugs or drug combinations that have fewer problems or side effects.

There are a lot of metal complexes that could be used in chemotherapy that categorized into two groups: Inorganic and complex compounds, and organometallic compounds.

Inorganic and complex compounds, two classes: main group metal compounds and transition metal compounds, which include titanium, zirconium, chromium, iron, cobalt, copper, zinc and a lot of other metals (Haiduc, 1990).

1.3.1 Chemotherapy as a methodical cancer treatment

Increase in cancer incidence and mortality rate had lead toward the most radical treatment choices.

Chemotherapy is a systemic treatment using chemical compounds meant to terminate and constrain cancer cells growth and dispersal in the human body, leading to extend the patient life or to decrease the symptoms (Denmeade & Isaacs, 2002).

Chemotherapy is a powerful tool in the war against cancer, but it has a major problem when to be consider as a cancer treatment, because such highly effective compounds can be toxic to the patient.

Many studies reported that less than 1% of the administrate compound will be transported to the target cells, while the rest room free in our body, damaging the other healthy not affected cells and tissue especially bone marrow, epithelial tissue, reticuloendothelial system and gonads (Hardin, et al., 2012; Coates, et al., 1983; Griffin, et al., 1996).

Off target effect is due to the lack of chemotherapy drug agent's selectivity and the inability to distinguish between affected cancer cell and the normal not affected cancer cells in mammalian cells (Daniel, et al., 2004).

Drug resistance is one of the main side effects of using chemotherapy, which could occur after long time of treatment by chemotherapeutic agent (Luqmani, 2005). Resistance to the therapeutic drug the obligate scientific community and the researchers to continue the search for new anti-cancer agents, with an improved selectivity toward malignant cells and counter act the mechanisms of resistance formed by malignant cells (Torre, et al., 2015; Denmeade & Isaacs, 2002; Godwin, et al., 1992; Zhang & Lippard, 2003).

Cisplatin $Pt(Cl_2)(NH_3)_2$ is one of the most substantial discoveries in the war against cancer in the 20th century (Ho, et al., 2003). Cisplatin is so effective against many types of cancer cells especially testicular cancer, which has a cure success rate of over 90% (Tisato, et al., 2010). Though, the treatment is limited by numerous side effects including emetogenesis, nephrotoxicity, neurotoxicity and hepatotoxicity (Zhang & Lippard, 2003).

One of the drawback of using cisplatin is its oral bioavailability. Cisplatin is not orally bioavailable, taking this in consideration with the inherited and acquired resistance, lead to minimize its usage (Farrell, 2004).

1.4 Anticancer activity of metal ions:

Medicinal inorganic chemistry is a branch of chemistry. Provide the possibility to design and manufacture wide range of novel metal-based therapeutic agents, that are difficult to be available as organic compounds.

Medicinal inorganic chemistry can utilize the unique properties of metal ions to design several derivatives of new drugs (Bruijnincx & Sadler, 2008).

It has been 5000 years since the introduction of metals to the medical application (Orvig & Abrams, 1999).

The discovery of cisplatin in 1965 had promoted and lead the current effort in the search for metal based treatments. Cisplatin has led to a new journey to explore the world of metal based therapeutic agents with possible diverse kinetics and mechanism of action from those in conventional organic drugs (Muhammad & Guo, 2014).

Metal ions have crucial roles in osmotic balance, signal transduction, and functions of various lipids, glycans, nucleic acids and proteins. About third of all proteins present in mammalians contain metal ions and metallo-enzymes catalyze some of the most enigmatic reactions found in nature for example methane oxidation, nitrogen fixation and water splitting (Chang & He, 2013).

One of the most significant feature of metal ions is the ability to lose electron from its elemental state leading to the formation of positively charged ions, which improve its solubility in biological fluids. The conversion of the metal into a cationic form result in metal become more active in biological reactions.

This lead to electron deficient positively charged state, whereas most of biomolecules such as DNA and proteins are electron rich negatively charged, the attraction among these opposing charges leads to a general inclination for metal ions to bind to and interact with biomolecules (Warad, et al., 2013).

The possibility of adding ligands to metal complexes increase its tendency to enhance ligand substitution reactions with bio-molecular targets. One of the possibility is to bind to selenium, nitrogen or sulfur atoms of selenocysteine, histidine or cysteine residues in protein leading to enormous wanted therapeutic action (Che & Siu, 2010).

Metal ions such as cisplatin had been used as anticancer agents in chemotherapy, the mode of action is believed to be through targeting the DNA directly. Cisplatin is covalently bond to DNA which form adducts that lead to different crosslinks between the drug and the DNA leading to significant distortion of helical structure of DNA, causing inhibition and distortion of DNA replication and transcription processes. Which finally trigger cell death through activating a cell suicide mechanism (Pruefer, et al., 2008; Jamieson & Lippard, 1999).

Advancements occur continuously in the development of other metal based drugs with different mode of actions such as better DNA interactions (non-covalent binding to DNA), targeting proteins that lead to broad results such as histone deacetylase modification, telomerase inhibition, topoisomerase and protein kinases inhibition and inactivation.

A wide range of metal complexes initiate cytotoxic activity through enzyme inhibition, superoxide dismutase mimic and producing oxygen radicals and others using metal as support rather than as reactive center (Zhang & Lippard, 2003; Bruijnincx & Sadler, 2008; Che & Siu, 2010).

To discover and prepare new metal complexes with fewer side effects and comparable or improved cytotoxicity a wide variety of metal complexes

based on gold, manganese, gallium, titanium, ruthenium, iron, germanium, cobalt, palladium and copper are being in intensively investigated as platinum substitutes (Zhang & Lippard, 2003; Ott & Gust, 2007).

Copper based complexes appear to be very promising candidate for anticancer treatment and cure (Zhang & Lippard, 2003; Warad, et al., 2013).

1.5 Copper complexes as anticancer agents

Copper is an essential trace element exists in all living organisms. It is important in redox chemistry, development and growth (Harris & Gitlin, 1996).

Copper has several important rules in enzyme function and proteins required for DNA synthesis, cytochrome oxidase, energy metabolism, respiration, ascorbate oxidase, superoxide dismutase and tyrosinase (Halliwell & Gutteridge, 1990).

Copper as a metal found in cell in both oxidation states, oxidized Cu (II) and reduced Cu (I). Copper complexes has a long history of medical application, coordination compounds of copper (I, II) have been used as enzyme inhibitors, anti-inflammatory, antitumor agents, antiviral, antimicrobial or chemical nucleases. Which made them attractive to medicinal chemistry to explore the anticancer effects of those potent complexes (Weder, et al., 2002).

Copper complexes were investigated as potential anti-proliferative agents only in the last few decades, particularly after the discovery of cisplatin the most widely used anticancer metallo-drug (Marzano, et al., 2009).

Daily intake of copper in healthy adults is between 1.5 and 3.0 mg/day (Milne, 1998).

Abnormal accumulation of copper by cancer cells proliferation might be one of the characteristics of mutated vs. healthy cells. Elevated levels of copper have been found in many types of human cancers, including breast, prostate, colon, brain and lung cancer (Daniel, et al., 2004).

A precise local amount of copper is also crucial for angiogenesis to occur, these facts made targeting cancer cells by copper complexes very attractive towards finding replacement to the cisplatin (Goodman, et al., 2004).

Copper complexes at molecular level interact directly with proteins and DNA, leading to cleavage and dysfunction of macromolecular structure, or indirectly producing reactive oxygen species that attack and degrade biomolecules such as DNA and protein (Iakovidis, et al., 2011). Some copper-complexes cause DNA fragmentation leading to apoptosis, but in other CCs DNA fragmentation and apoptosis were not observed (Marzano, et al., 2009). On the other hand, a non-apoptotic form of programmed cell death has been evidenced recently in human cancer cells treated with both copper (I) and copper (II) complexes (Tardito, et al., 2006; Tardito S, 2007).

In metal-based drugs, the metal can organize ligands in a specific three-dimensional configuration, allowing the structure of the molecule to recognize and interact with a specific molecular target and adjust the properties of the molecule (Marzano, et al., 2009).

Some of those metals are essential metals and one of those is copper. Even though if essential metal escapes its very specific metabolic pathway; could

be very toxic to the surrounding cells. Complexes of those metal may serve as powerful cytotoxic drugs (Halliwell & Gutteridge, 1990; McQuitty, 2014).

Properties of copper-coordinated compounds are largely determined by the nature of ligands and donor atoms bound to the metal ion. Copper-biological compound reacts with molecular oxygen to produce free radicals. Copper plays an important role in cell as a catalytic cofactor in the redox chemistry of mitochondrial respiration, iron absorption, free radical scavenging, and elastin cross-linking (Halliwell & Gutteridge, 1990; Arredondo & Núñez, 2005; Miller, et al., 1965).

Copper toxicity comes from its ability to produce reactive oxygen species (ROS), displace other metal ions, peroxidize lipids and directly cleave DNA and RNA (Halliwell & Gutteridge, 1990).

Copper complexes bio-distribution, bioavailability and toxicity differ from those of drugs and might be effective against human cancers that are poor chemo-sensitive or have become resistant to conventional chemotherapy drugs like platinum drugs (Gaetke & Chow, 2003). Copper is an essential metal so it could be less toxic than non-essential metals such as platinum. Copper complexes may provide a broader spectrum of antitumor activity.

1.5.1 Copper interaction with DNA:

Copper bind to DNA with high affinity than any other divalent cation, thus promoting DNA oxidation (Burkitt, 1994).

Many hypotheses are trying to explain copper compounds mechanism of action on DNA, those hypotheses include: phosphate hydrolysis, reactive oxygen species and/or base-pair intercalation (Rodrigo G, 2015).

Copper is capable of inducing DNA strand breaks and oxidation of bases by producing ROS. Due to this, intracellular free copper is limited to less than one free copper ion per cell. Some copper-complexes cause DNA fragmentation leading to apoptosis, but in other CCs DNA fragmentation and apoptosis were not observed (Marzano, et al., 2009).

1.5.2 Chromatic effect and DNA binding:

DNA is very stable structure. This stability depends on the integrity of the DNA double helix and how much the double strand is stable (Watson, 2004). DNA has a very stable light absorbance, when DNA double strand separate due to external force (ex, thermal, chemical) this results in increase in optical density (hyperchromicity). When DNA strands re-associate reduction in optical density occur (hypochromic shift) (Thompson, 1981).

The interaction between copper complexes and DNA can be detected and evaluated through measuring the optical density.

1.5.3 Copper and cancer development and angiogenesis

Increased ceruloplasmin and copper levels in various tissues have been linked to cancer progression (Gupte & Mumper, 2009).

Copper role in cancer development isn’t fully understood. But copper play an important role in angiogenesis through stimulation, proliferation and migration of human endothelial cells (Wang & Guo, 2006).

Copper involves in ROS production, so one of proposed method for cancer therapy is to deplete copper from our systems. This could be a successful anticancer strategy, but clinical studies have not been especially encouraging, even though some studies reach phase II in preclinical animals (Wang & Guo, 2006)

Two proposed strategies to try against cancer, copper depletion and copper-complexes.

1.5.4 Copper complexes:

In the field of non-platinum compounds that are showing an antitumor potential, copper-based complexes have been investigated on the assumption that endogenous metals may be less toxic. Coordination chemistry of copper is dominated by Cu (II) derivatives with little, but important examples of Cu (I) compounds (Marzano, et al., 2009).

The properties of copper-coordinated compounds are mostly determined by the ligands and donor atoms linked to the metal ion (Marzano, et al., 2009).

Copper complexes could inhibit proteasome and induce apoptosis in human cancer cells (Daniel, et al., 2004).

Cu complexes can be used as anti-viral, anti-cancer, anti-microbial, chemical nucleases and enzyme inhibitor (Iakovidis, et al., 2011).

1.6 Apoptosis and Cancer:

Apoptosis, a crucial biological process of multicellular organisms is facilitated by the activation of proteases called caspases (Hardin, et al., 2012). Apoptosis through caspase activation may take place via two major

signaling pathways: First, the extrinsic or death receptor pathway, which is triggered via specific cell membrane receptors. Second, the intrinsic which also called mitochondrial pathway induced upon disruption of mitochondria and release of cytochrome c through the mitochondria gates (Hardin, et al., 2012; Elmore, 2007).

Apoptosis is regulated either by loss of pro-apoptotic signals or by gains of anti-apoptotic signals and can lead to initiation, promotion and progression of cancer. Successful eradication of cancer cells from the human body achieved and depends on activation of cell death by apoptosis either the intrinsic or extrinsic pathways (Mohammad, et al., 2015; Hassan, et al., 2014; Jordan, 2015; Miguel, 2016).

Caspase-3 was used in several studies. That determine apoptosis induction following treatments by new anticancer drugs. Detection of caspase 3 levels is an indication of caspase-dependent apoptosis initiation (Hardin, et al., 2012; Alberts, et al., 2004).

1.7 The aim of the study:

This research study was aimed to screen the anticancer effects of the newly prepared copper complexes. In addition to that; the study was to evaluate the death mechanism of the newly prepared complexes on cancer cells, to determine if the death of cancer cells was due to caspase-3 dependent apoptosis or something else.

Chapter Tow

Material and Methods

Material and Methods

2.1 Copper complexes:

Copper complexes were prepared in An Najah National University, department of chemistry under the supervision of Prof. Ismail Warad and his team and available as a crystalized green and blue crystals in 9 containers numbered from 1 to 9 and ready to prepare stock solution. (AL-Noaimi, et al., 2014)

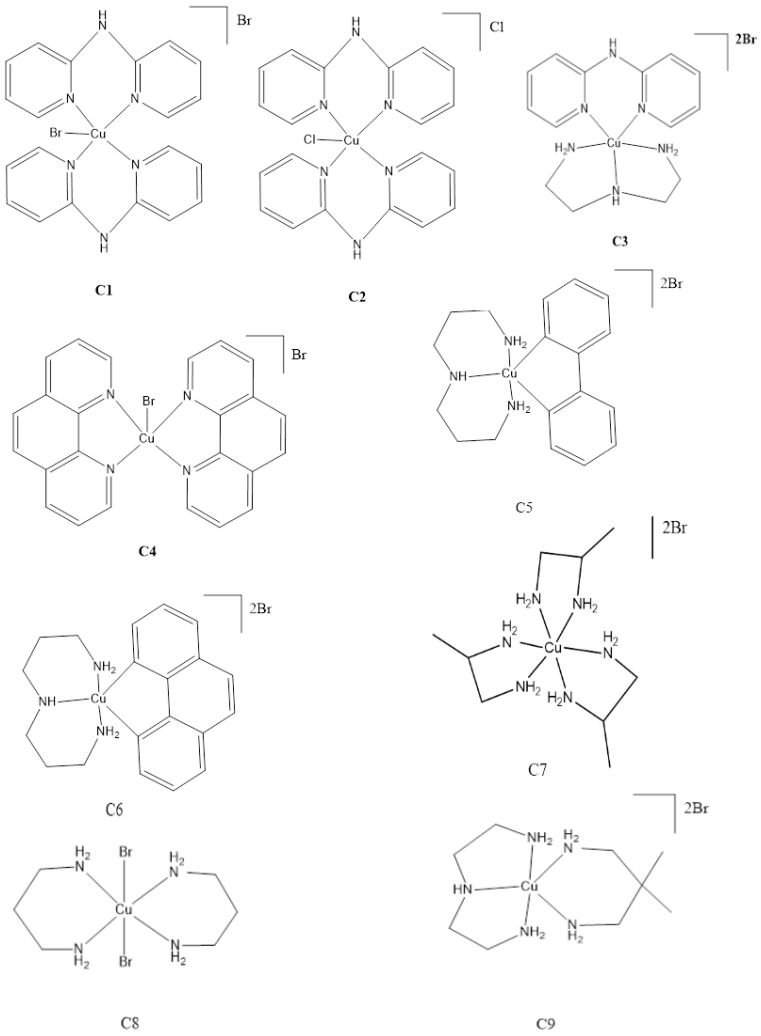

Figure 2. 1: Copper complexes used in this research, that are based on Cu(II).

2.2 Preparation of solutions:

Various weight of each complexes where dissolve in proper size of solvent (PBS) to get stock concentration for each complex, and those solutions will be used as stock solutions as shown in table 1 and 2, to prepare the working solutions.

Table 2. 1: 1st patch of cooper complexes stock solutions.FC: fully crystalized, SC: semi-crystalized, NC: non-crystalized

Cc	Color crystal	Wight (mg) **±0.5 mg**	Solvent vol. (ml) **±0.1 ml**	Conc. (mg/ml)	Notes
C1	Brown	6	3	2	FC
C2	Olive	10	5	2	FC
C3	Blue	18	5	3.6	NC
C4	Green	6	3	2	FC
C5	Blue	5	2.5	2	FC
C6	Blue	8	4	2	SC
C7	Green	6	3	2	FC
C8	Blue	9	4.5	2	FC
C9	Blue	10	5	2	FC

Table2. 2: 2nd patch of cooper complexes stock solutions for the complexes we need more from it.

Cc	Color crystal	Wight mg **±0.5 mg**	Solvent vol. ml **±0.1 ml**	Conc. Mg/ml	Notes
C1	Brown	11	5.5	2	FC
C2	Olive				FC
C3	Blue				NC
C4	Green	13	6.5	2	FC
C5	Blue	33	8	4	FC
C6	Blue	21	7	3	SC
C7	Green	16	8	2	FC
C8	Blue				FC
C9	Blue				FC

FC: fully crystalized, SC: semi-crystalized, NC: non-crystalized

Table2. 3: The concentration of copper complexes.

Copper complex	Dilutions mg/ml			
C1	1.0	0.5	0.25	0.125
C2	1.0	0.5	0.25	0.125
C3	1.0	0.5	0.25	0.125
C4	1.0	0.5	0.25	0.125
C5	1.0	0.5	0.25	0.125
C6	1.0	0.5	0.25	0.125
C7	1.0	0.5	0.25	0.125

After preparation stock and working solutions was kept at room temp.

2.3 Cell culture

Colon cancer: colo205 cell line, Osteosarcoma cell line: Hs 755 and Breast cancer cell line: MC7 cell line, were grown in Dulbecco's modified Eagle's medium (DMEM) enriched with 10% fetal calf serum, 1% non-essential amino acid, 1% l-glutamine, 1% penicillin streptomycin and 1% amphotericin B. All cell lines were grown in incubator with a humidified atmosphere of 95% air, 5% CO_2 at 37ºC, the culture medium was changed every 3-4 days as needed. Materials used in all experiment were purchased from Biological Industries except for the amphotericin B and MTT reagent from SIGMA Company, Caspase-3 colorimetric assay kit from MBL, and cell lines were provided by Dr. Ashraf Sawafta.

2.4 Determination of cell viability

2.4.1 MTT Assay

Viable cells can produce formazan, through decrease of the yellow colored water soluble 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl-tetrazoliumbromide (MTT) to a water insoluble purple colored that we know it as formazan crystals by functional mitochondria through succinate dehydrogenase that provide a quantitative measurement of a live cells (Sieuwerts, et al., 1995).

Cells in culture with more than 70% confluence were detached from culture flask by removing the culture medium, then adding 0.05% trypsin- EDTA. A suspension of 100 µl (2.5×10^4 cells/well) of viable cells were seeded in a 96-well plate, then treated with several concentrations as shown in table 3 for concentrations and results, then incubated for another 24h. MTT Solution (5mg/ml) was added in amount equal to 10% of culture volume, which is 20 µl, then the 96-well plate was incubated for 4 hours. After the 4 hours, the culture fluid was removed, and MTT solvent was added to the 96-well plate. Gentle stirring was applied by gyratory shaker.

The absorbance of the MTT formazan was measured by an enzyme linked immunosorbent assay (ELISA) reader at 600nm wavelength in less than an hour of adding MTT solvent.

2.5 Caspase activity:

Caspase-3 induction following the treatment of cancer cell lines by different concentrations of the newly prepared complexes was evaluated using caspase-3 colorimetric assay kit from MBL company code No. 4800.

2.5.1 Caspase-3 Activity Assay

Intracellular caspase-3 activity was quantified by colorimetric assay kit using synthetic substrate that contain 4 labeled amino acids with pNA (p-nitroanilide) at the C-terminal side, induction of caspase-3 and the synthetic substrate will release the 4 labeled residues that quantify its amount in ELISA reader at 400-405 nm wavelength.

Cells (1-5*10^{6} cells/well) that were treated with copper complexes was seeded in 16-well plates (Corning) for 3 hours for colo205 and 24 hours for MC7.

Plates were treated to harvest the cells using trypsin to detach the cell from the wells. Cells was harvested by centrifugation at 400X g for 5 minutes. Harvested cells were suspended in ice-cold Cell Lysis Buffer (50-500 µL), and incubated on ice for 10 minute. Cell lysates was centrifuged at 10,000X g for 5 minute with temp inside the centrifuge is 4C degree to obtain the supernatant of those cells. Supernatant was transfer to a new tube and put on ice while we work on it.

96 wells plate was used to measure the absorbance on ELISA reader. 50 µL of 2X Reaction Buffer containing 10mM DTT prepared just before the experiment was added to each well. 50 µL of cell lysates was added to each well. 50µL was added to the blank wells instead of cell lysate. 5 µL of Caspase-3 Substrate was added to each well. MC7 cell line was incubated for overnight, and colon cancer cell line was incubated for 3 hours.

100 µL of pNA standards were add to empty wells just before reading on ELISA reader at 405nm.

2.6 DNA binding

Experimental absorption titration spectral was carried out in pH 7.2 buffer solution of a Tris–HCl [5 mM Tris–HCl, 50 mM NaCl] and with a Cu(II) complex concentration of 1.0 x 10^{-4} M. CT-DNA concentrations were varied between 0 to 1.0 ×10^{-3} M by keeping the total volume of mixture constant to 10.0 mL. The mixed solution of C7 and CT-DNA was allowed to equilibrate for 10 min at room temperature for each trial before being subjected to absorption measurements.

Chapter Three

Results and Discussion

Results and Discussion

Cancer is one of the main health issues worldwide and chemotherapy is extensively used to treat cancer even with it's all side effects.

9 novel copper (II) complexes were used to test their anti-tumor effect on different cancer cell lines, human colon cancer cells, human breast cancer and osteosarcoma cell line.

Caspase -3 colorimetric assay kit was used to evaluate the apoptosis induction through programed cell death mechanism. MTT assay was used to evaluate the cytotoxic and cytostatic effects of copper complexes on cancer cell lines. DNA binding and cleavage assay were also used to study the interaction of the newly prepared copper complexes with DNA.

Copper complexes activity on cancer cells were observed under inverted microscope. Following treatment by different concentration of each complex it was clear that most of the complexes caused cell death and cell damage or loss of its cellular membrane after 24 hours of incubation with the complexes, with different degree of activity depend on copper complexes concentration and structure.

3.1 Cytotoxic effect of copper on colon cancer cell line

Cancer cells were seeded on 96 well plate, 20,000 cell/well with 40-50% confluence, was incubated overnight. Cancer cells were treated with 100 µl stock solution serially diluted to reach concentrations of 1.0, 0.5, 0.25, and 0.125 mg/ml for all complexes then was incubated overnight.

Most of the tested complexes show a strong anti-proliferation activity against the tested cancer cell lines. Results indicate that colon cancer cells were not able to survive the treatment by copper complexes even at the lowest concentration. Complexes 1,2,3,4,5,6,7 and 8 end up with nearly total death of the tested cell line -as it shows only 0-10% confluence- compared to full growth and activity of the untreated cancer cells, while complex 9 ended up with 20-40% confluence.

Pictures of this treatment available in pics in appendix A.

3.2 Cytotoxic effect of copper on osteosarcoma cancer cell line:

Osteosarcoma cancer cells were seeded on 96 well plate, 20,000 cell/well with 40-50% confluence, and left for 24 hours. Cancer cells were treated with 100 µl stock solution serially diluted to reach concentrations of 1.0, 0.5, 0.25, and 0.125 mg/ml for all complexes then incubated for 24 hours.

Most of the tested complexes show a strong anti-proliferation activity against the tested cancer cell lines. Results indicate that osteosarcoma cancer cells were not able to survive the treatment by most of copper complexes even at the lowest concentration. Complexes 2,4,5, and 8 ended up with 0-10% confluence. Complexes 1,3,6 and 7 ended up with 10-30% confluence. Complex 9 ended up with above 75% confluence.

Pictures of this treatment available in appendix A

3.3 Detection of cytotoxic effect of the complexes on colon cancer cells using MTT assay:

Copper complexes 1,3,5,7,8 and 9 killed the colon cancer cell line in MTT assay completely, that we find that no viable cells in those complexes wells, but C2 and C7 gave same results as the control sample, as show in Fig 7.

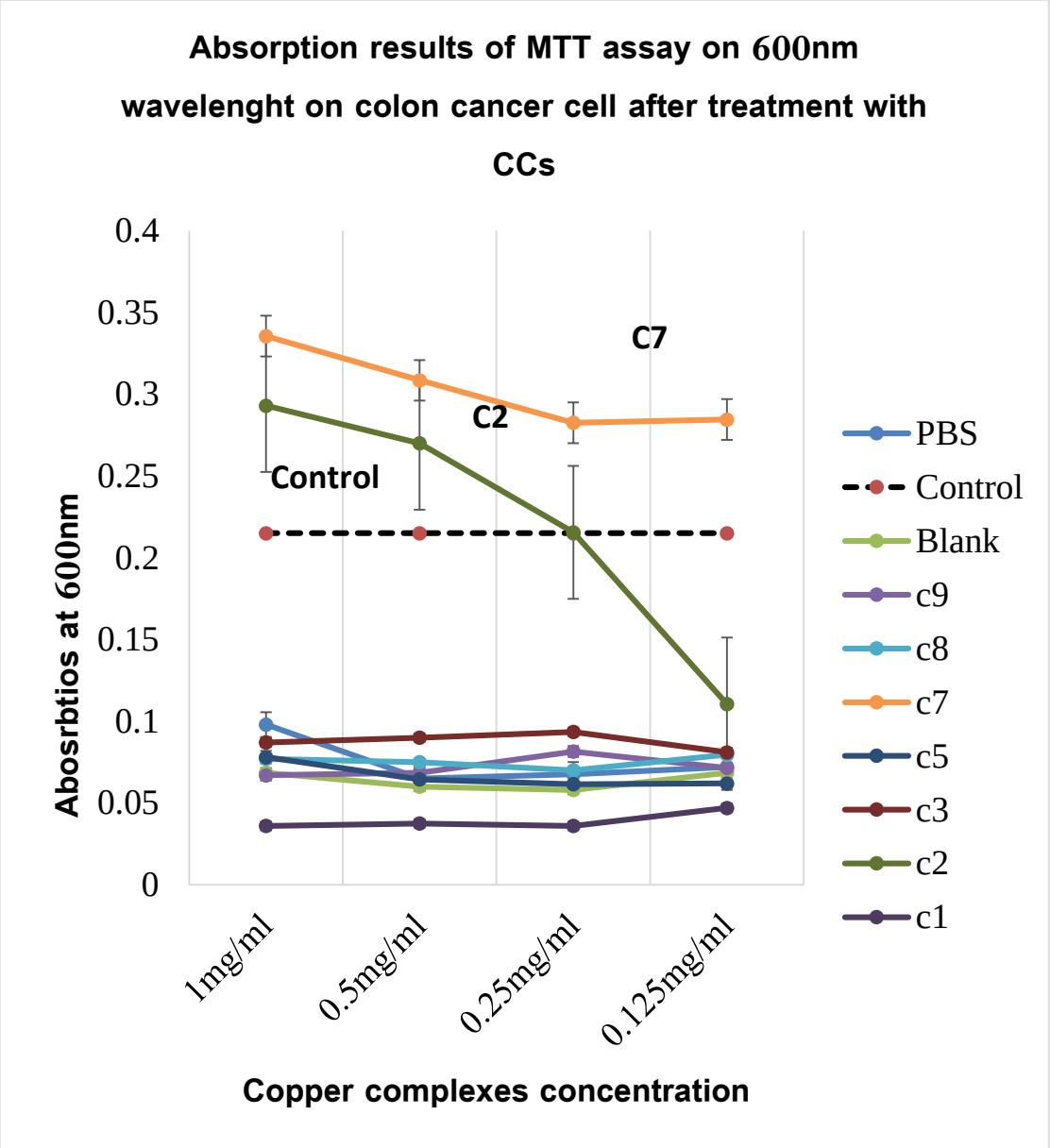

Figure 3. 1: Absorption results of MTT assay for Colon cancer cell line after treating with copper complexes at 4 concentrations.

MTT assay results indicate that concentrations of copper complexes should be much lower. IC50 could not be calculated. C2 and C7 results should be retested, as it contradicts with microscopic inspection. 570nm and 690nm, wasn't available to be used, it's recommended that the experiment re-done at 570nm and 690nm.

3.4Caspase activity detection:

As shown in the tables 4 & 5, all the results of absorbance or relative absorbance "Ar" are around zero, detection of caspase activity in those cells after the treatment with copper complex for both MC7 cell line (table 5) and colon cancer cell line (table 6) cell lines couldn't be quantified.

Control = cells with treatment. Blank = cell lysis buffer. Ar = sample absorbance – control absorbance

Table 3. 1: Absorbance measurements for Caspase-3 activity assay kit, after treating MC7 cell line with 2 different concentrations of different copper complexes.

MC7 cancer cell line caspase-3 activity

CCs / Concentrations	C1	C2	C3	C4	C5	C6	C7	C8
1mg	0.35	0.316	0.317	0.328	0.316	0.311	0.309	0.325
0.5 mg	0.303	0.305	0.299	0.292	0.292	0.274	0.318	0.324

Control	Blank
0.3155	0.301

pNA conc µM	500	250	125	62.5	31.25	16.625		
pNA reading	1.707	1.011	0.784	0.464	0.5	0.579		

Ar for 1 mg	0.0345	0.0005	0.0015	0.0125	0.0005	-0.005	-0.007	0.0095
Ar for 0.5 mg	-0.013	-0.011	-0.017	-0.024	-0.024	-0.042	0.0025	0.0085
Ar for pNA standard	1.406	0.71	0.483	0.163	0.199	0.278		

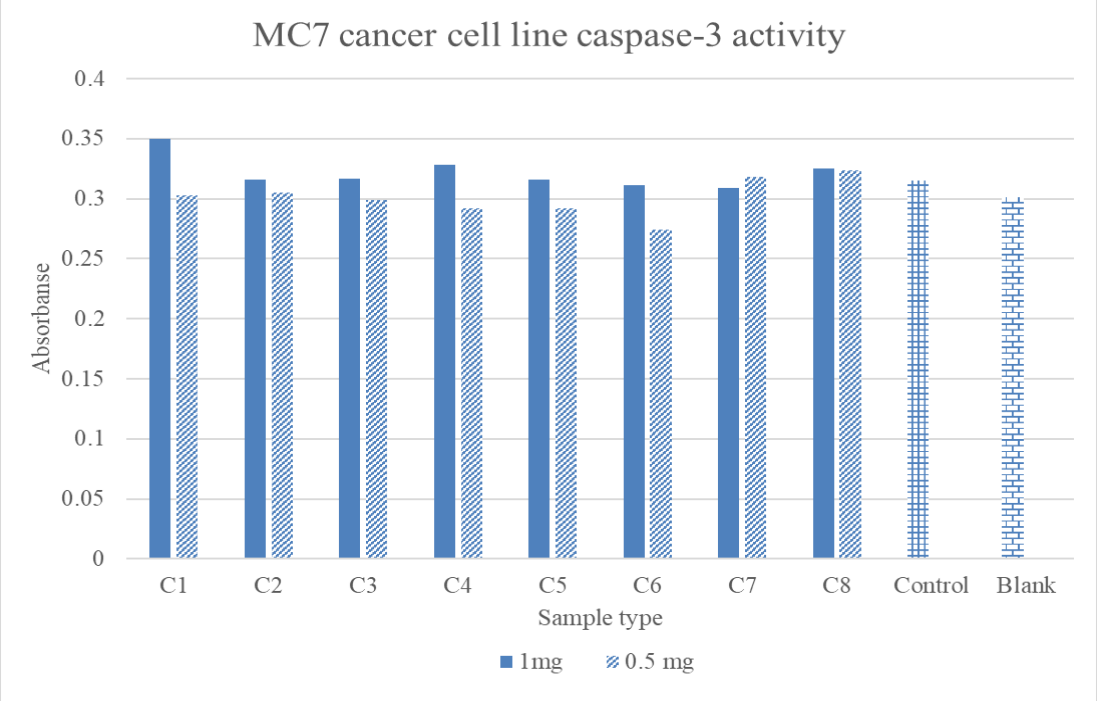

Figure 3. 2: Absorbance measurements after treating MC7 cell line with 2 different concentrations of copper complexes

Table 3. 2: Absorbance measurements after treating Colon cell line with 2 different concentrations of copper complexes.

Colon cancer cell line caspase-3 activity

CCs / Concentraons	C1	C2	C3	C5	C6	C7	C8	C9
0.5mg	0.299	0.292	0.295	0.304	0.294	0.302	0.293	0.292
0.125 mg	0.29	0.295	0.326	0.282	0.29	0.299	0.291	0.295

Control	Blank
0.3065	0.2917

pNA conc	500	250	125	62.5	31.25	16.625
pNA Elisa reading	1.597	0.896	0.593	0.431	0.403	0.37

Ar for 0.5 mg	-0.008	-0.015	-0.012	-0.003	-0.013	-0.005	-0.014	-0.015
Ar for 0.125 mg	-0.017	-0.012	0.0195	-0.025	-0.017	-0.008	-0.016	-0.012
Ar for pNA	1.3053	0.6043	0.3013	0.1393	0.1113	0.0783		

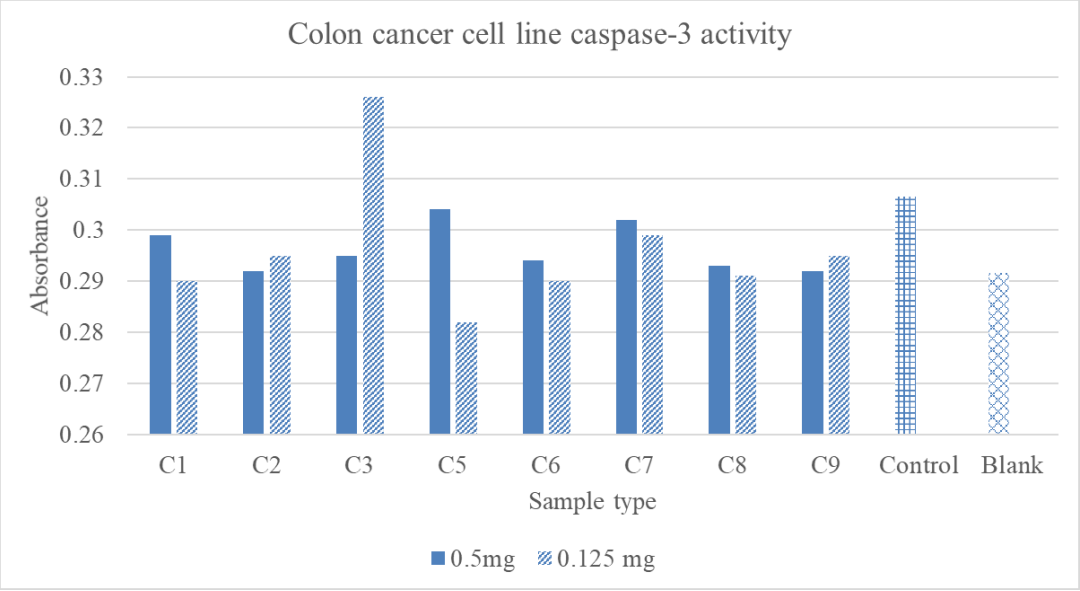

Figure 3. 3: Caspase-3 activity experiment with Colon cells using different types of copper complexes was done in a humidified atmosphere of 95% air, 5% CO_2 at 37ºC.

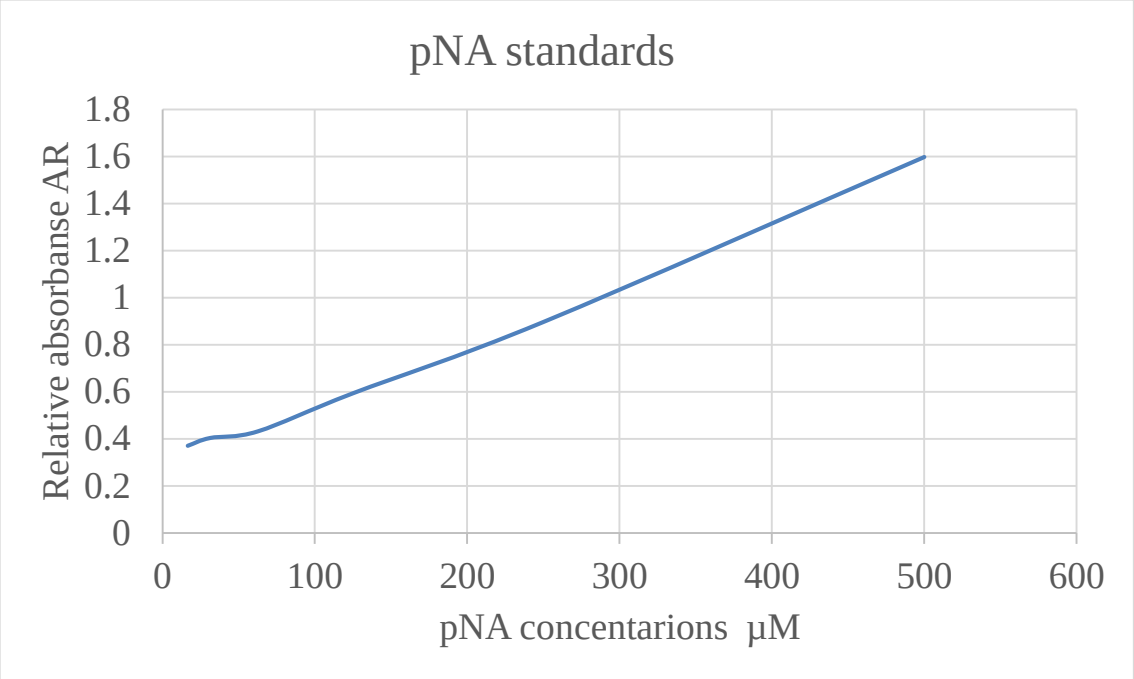

Figure 3. 4: Results of pNA standards, pNA standards prepared prior to caspase-3 activity test and kept at 6C, and was ready to be used when needed.

Results of both MC7 and colo205 cell after it was treated shows no difference between treated cells, non-treated cells and blank. This mean that there wasn't a measured caspase activity in tested sample. Lack of measured caspase activity could be for many reason. First: copper complexes didn't provoke caspase-dependent apoptosis. Second: copper complexes concentrations may be too much high that it didn't give the cells the opportunity to go under apoptosis process. Third: this cell line lack functional caspase pathways.

3.5 Absorption titration CT-DNA binding of complex C7

UV-vis. titration spectroscopy considers to be is one of the famous and fast method to evaluated DNA-binding ability. The affinity of C7 toward CT-DNA was monitored by UV-visible titrations using Tris–HCl buffer solution. Typically, changes are expected in UV spectra of the desired

compound by drug-DNA binding (Abdel-Rahman, et al., 2016). Fig. 12 showing the UV-Visible spectra titration of complex C7 upon CT-DNA addition.

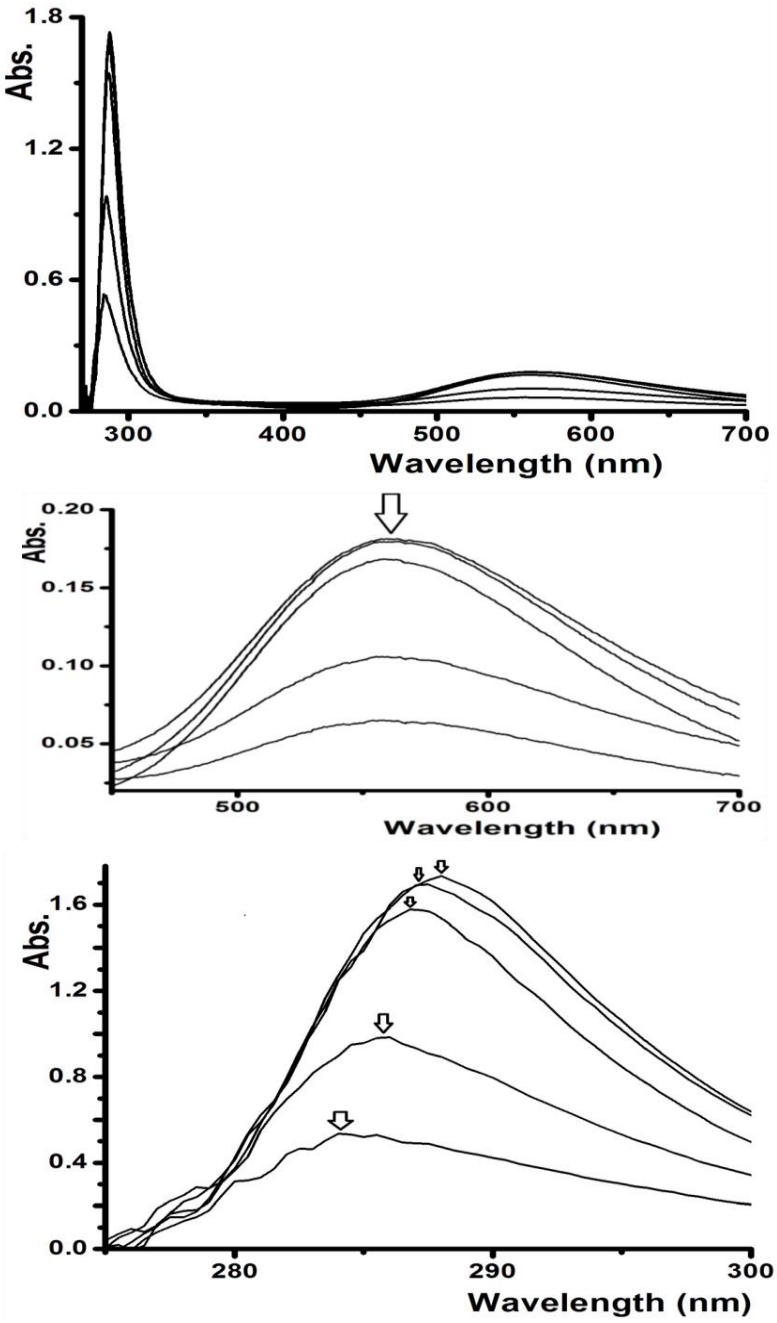

Figure 3. 5: 1.0 ×10-4 M of complex x UV-Vis. spectra interacted with 0, 1.0 × 10-5, 2.0 × 10-5, 1.0 × 10-4 and 1.0 × 10-3 M [DNA] at RT.

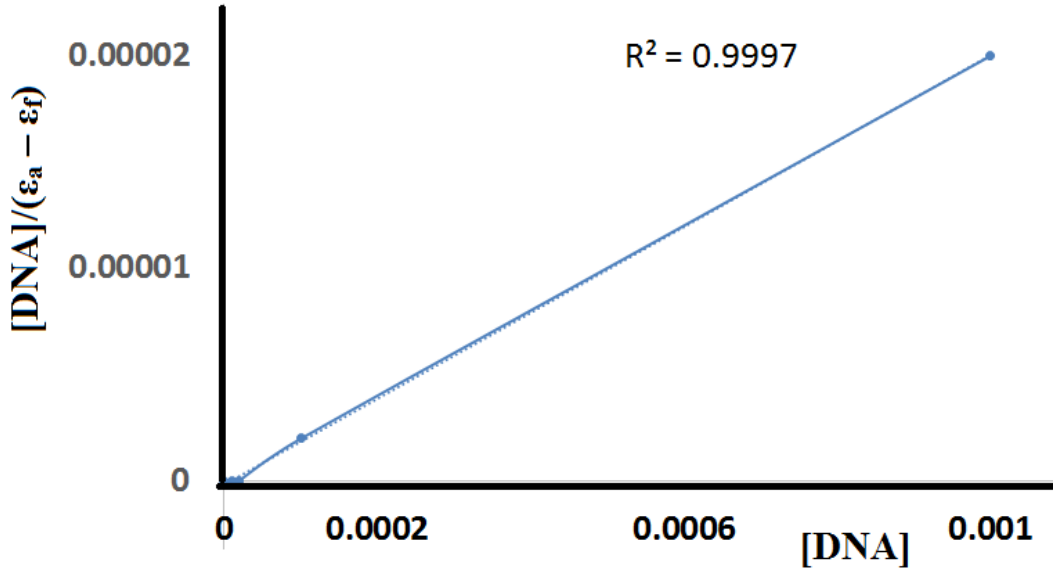

Figure 3. 6: Plot of [DNA]/(εa- εf) vs.[DNA] at λmax = 565 nm to establish the Kb value

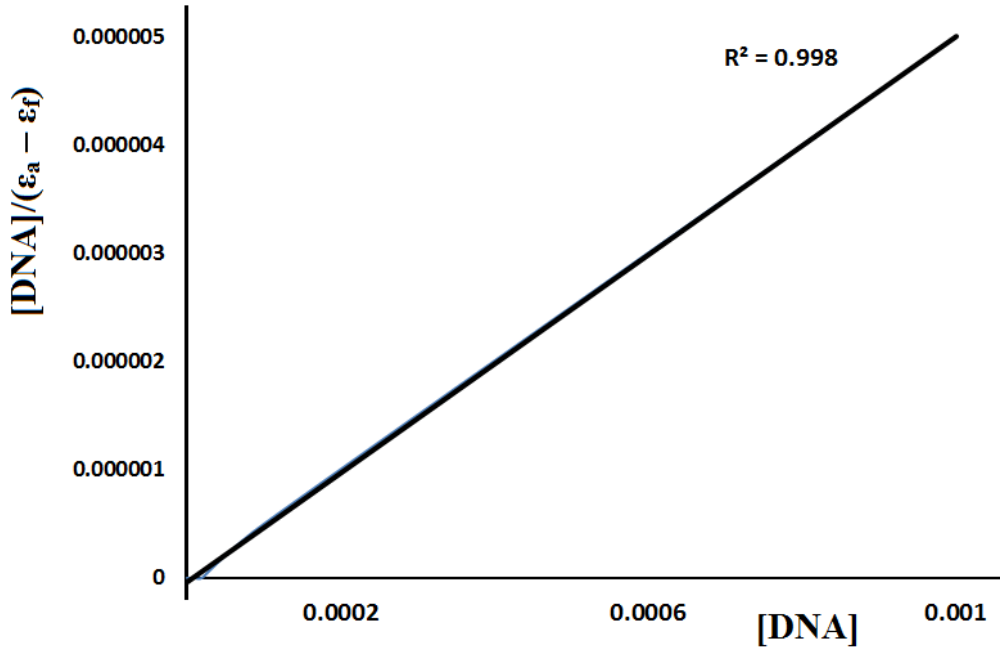

Figure 3. 7: Plot of [DNA]/(εa – εf) vs. [DNA] at λmax = 288 nm to determine the intrinsic binding constant Kb.

$1x10^{-4}$ M of the complex was treated with several DNA concentrations ranging from 0 to 1 x 10^{-3} M in order to monitor the decrease in absorption at λ_{max} = 288 nm, as seen in Fig. 14. To evaluate the binding ability of investigated complexes, K_b (intrinsic binding constant) for both complexes were evaluated by observing the changes in Abs. *vis.* CT-DNA concentrations by using the following equation] (Abdel-Rahman, et al., 2016):

$$[DNA] / (\varepsilon_a - \varepsilon_f) = [DNA] / (\varepsilon_b - \varepsilon_f) + 1 / K_b (\varepsilon_b - \varepsilon_f)$$

[DNA] is the concentrations of DNA in base pairs, ε_f, ε_a, and ε_b are the free, apparent-, and metal-bound-complex extinction coefficients, respectively. K_b is the equilibrium binding constant (in M^{-1}) of complex binding to DNA. When plotting [DNA] / (ε_a–ε_f) *vs* [DNA], K_b was obtained from the ratio of the slope to intercept. K_b for complex **C7** = 1.4×10^4 M^{-1} (as seen in Fig. 12). These results are like those obtained by other researchers for Cu(II) complexes] (Abdel-Rahman, et al., 2016; Inamdar, et al., 2016; Linert, et al., 1993).

Chapter Four

Conclusions and Recommendations

Conclusions and Recommendations

Based on our experiments, copper complexes killed the cell lines that was used, but didn't prove that our newly prepared complexes induce apoptosis through activation of caspase-3 pathway. Copper complexes kill the cancer cell by inducing a binding of the complexes to DNA causing cell cycle arrest. Due to those results the researcher suggested that future studies use lower concentrations of copper complexes, different incubation times for cell lines with copper complexes and more cell lines.

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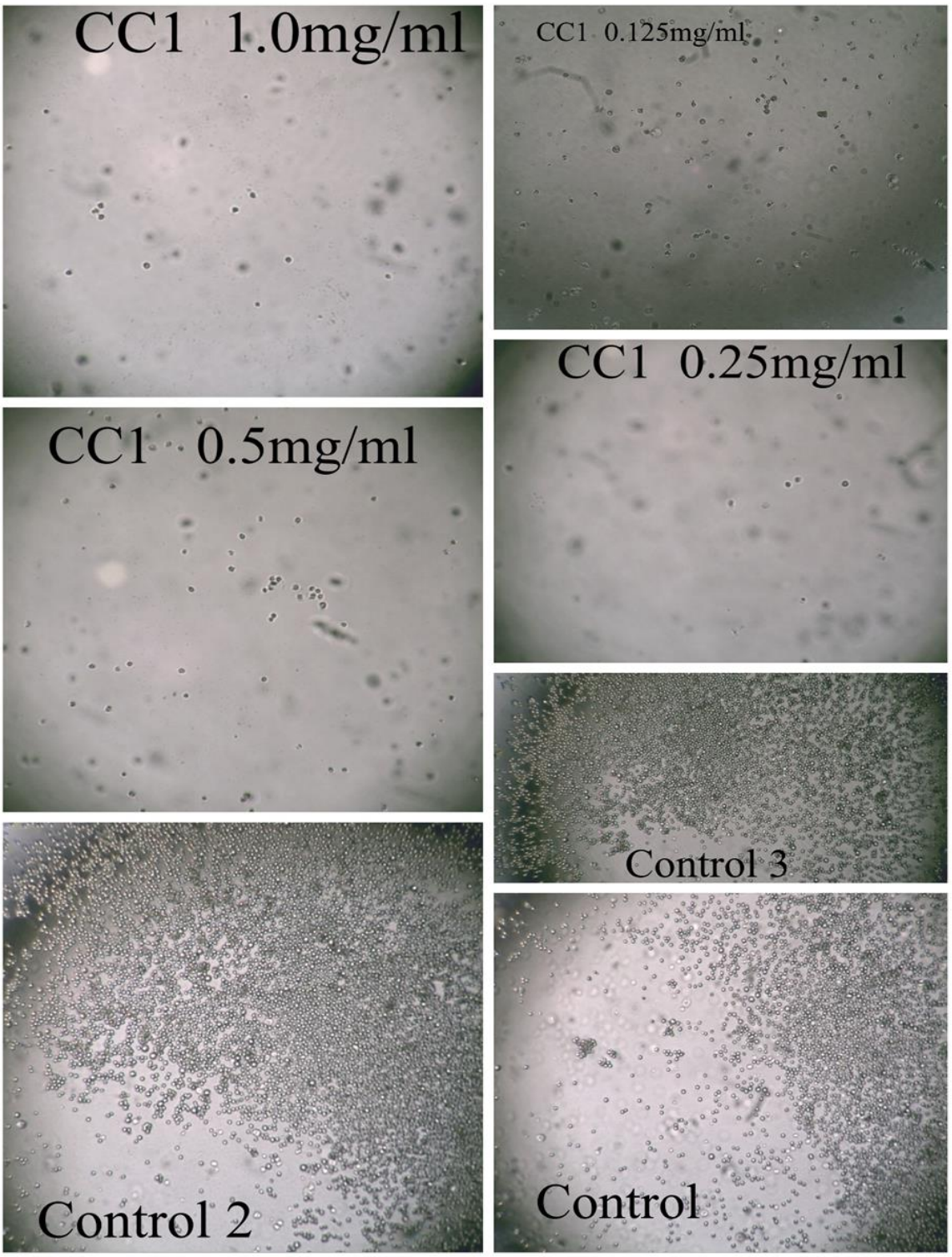
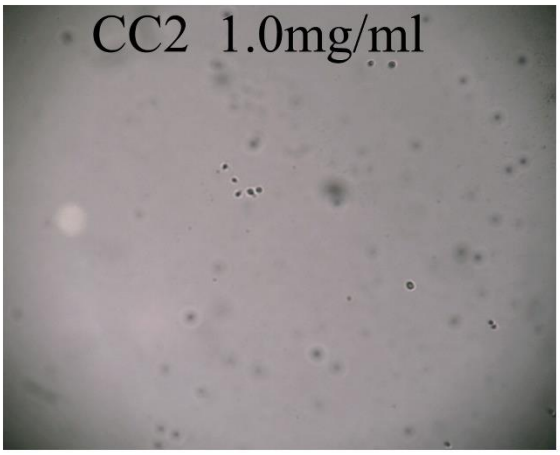
Appendix

Appendix A:

1: Pictures of Cytotoxic effect of copper on colon cancer cell line, each picture labeled by copper complex number and concentrations.

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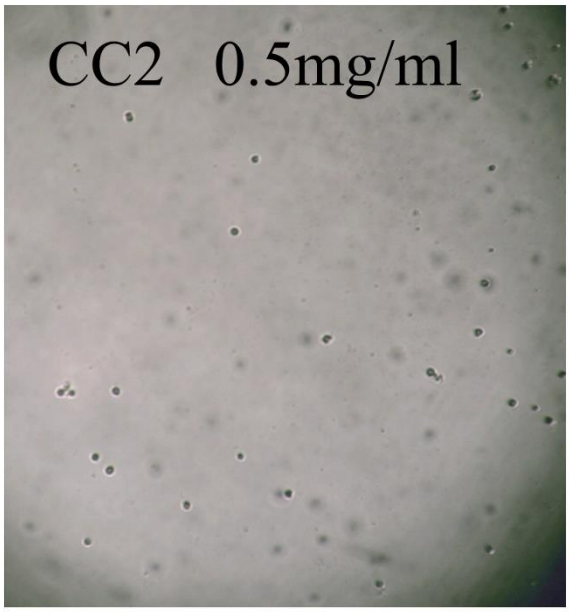
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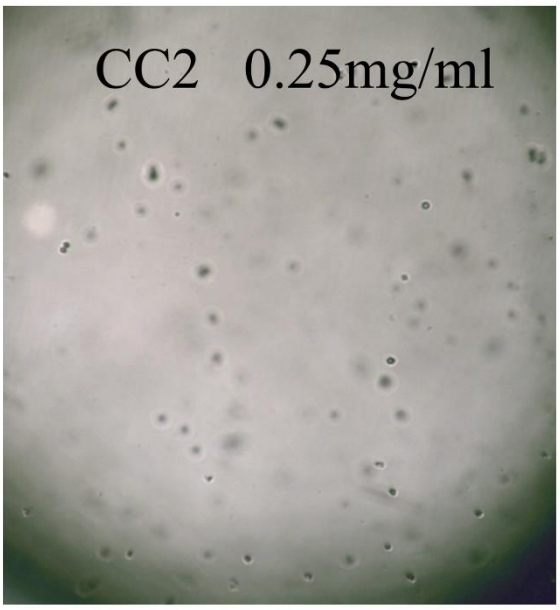
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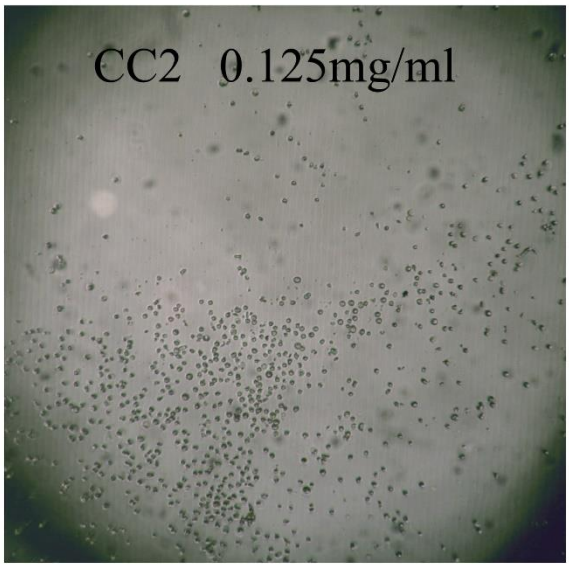
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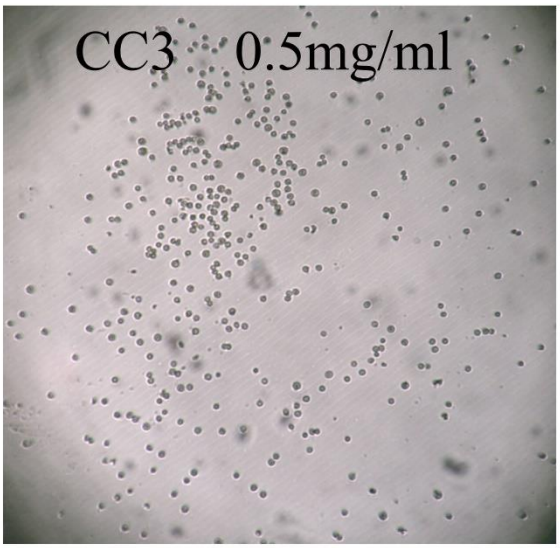
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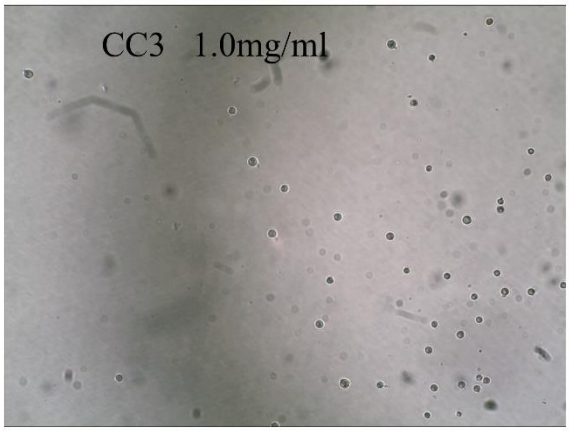
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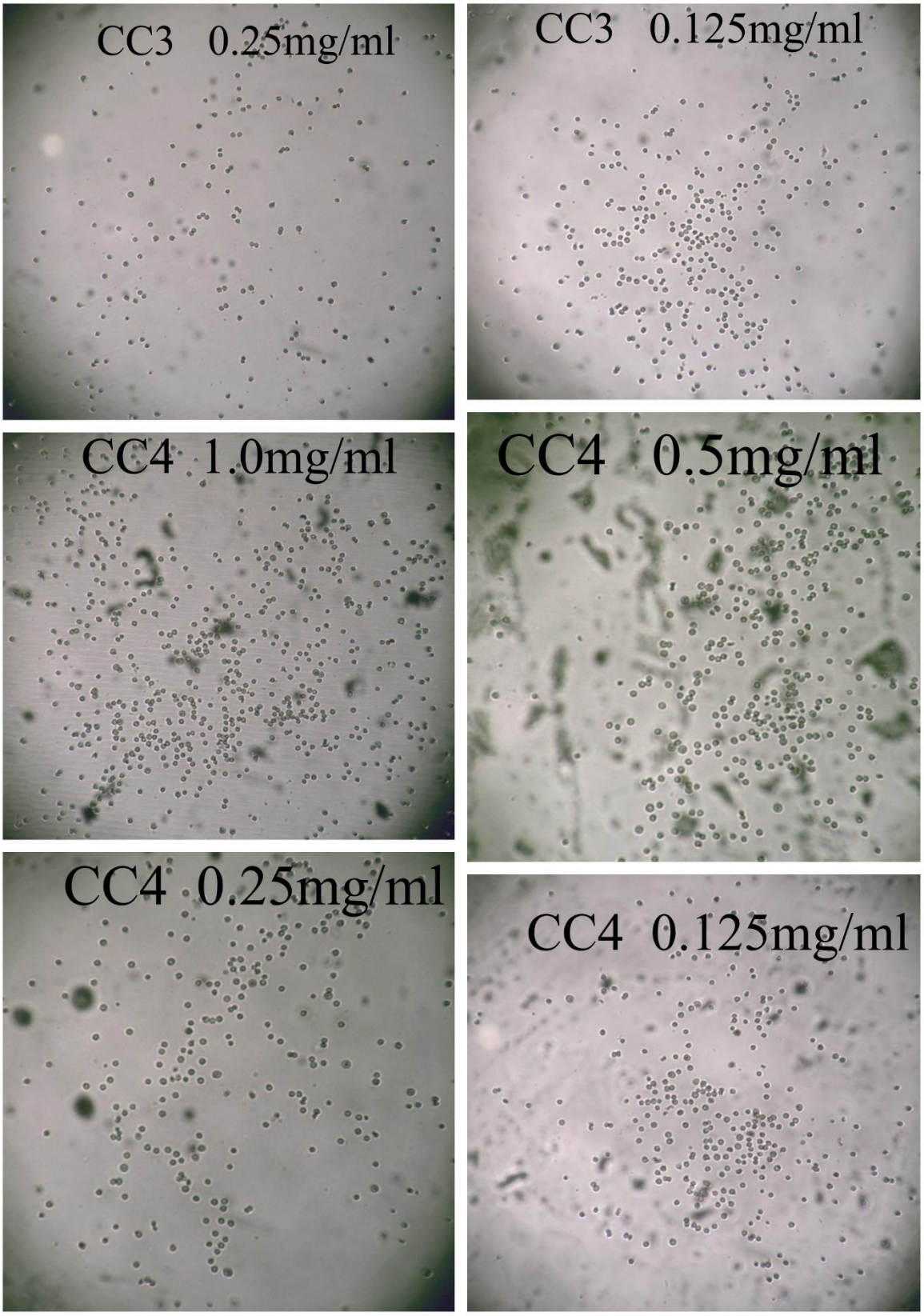
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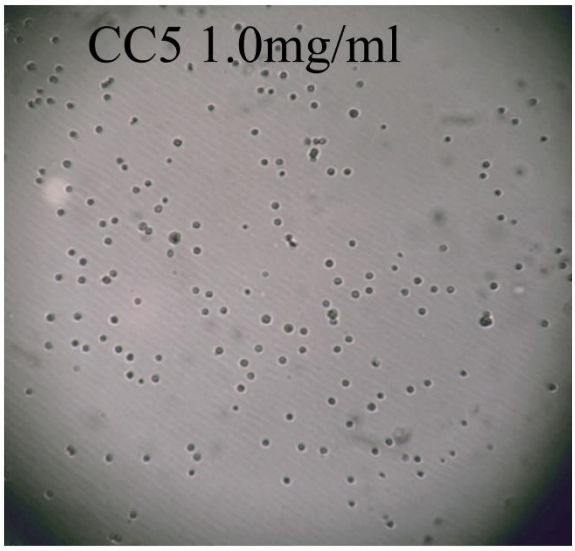
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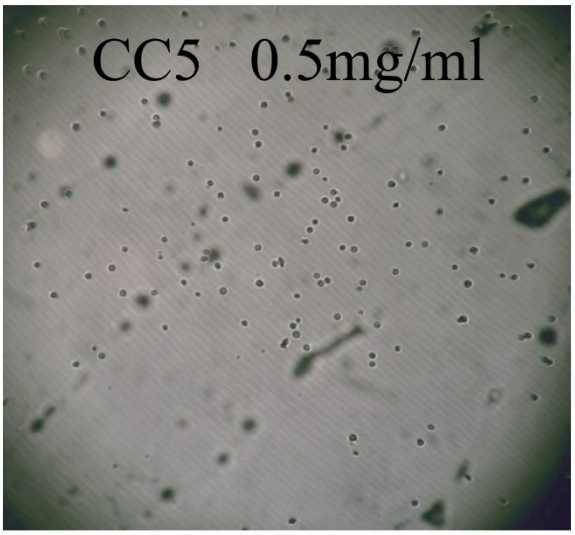
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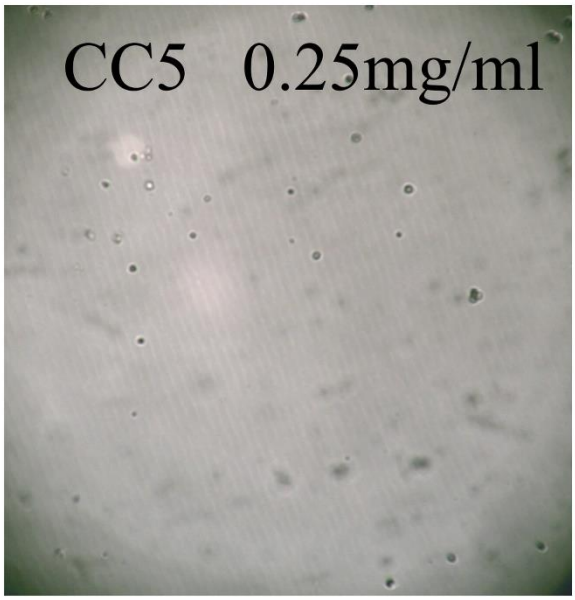
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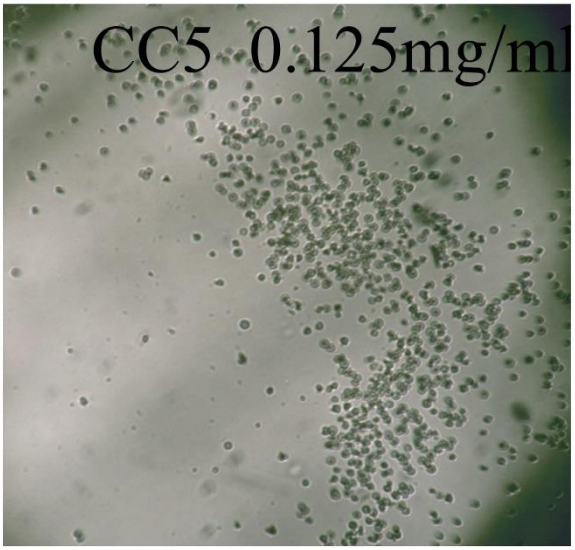
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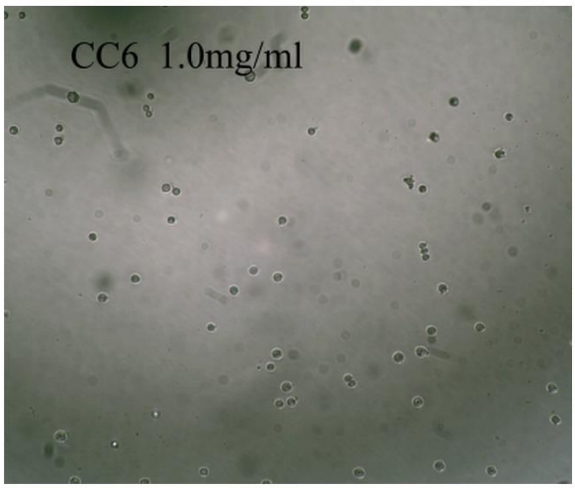
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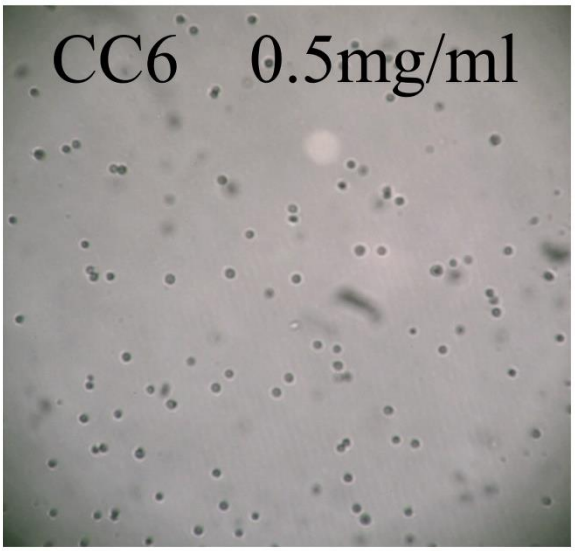
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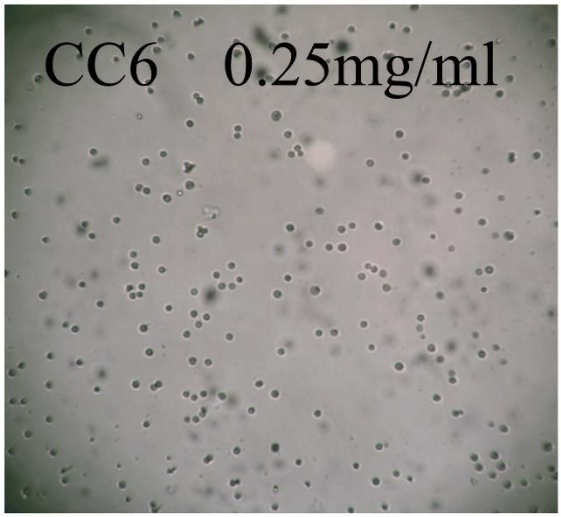
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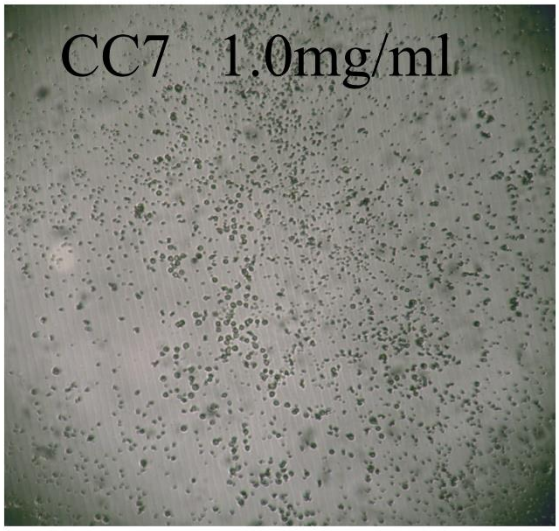
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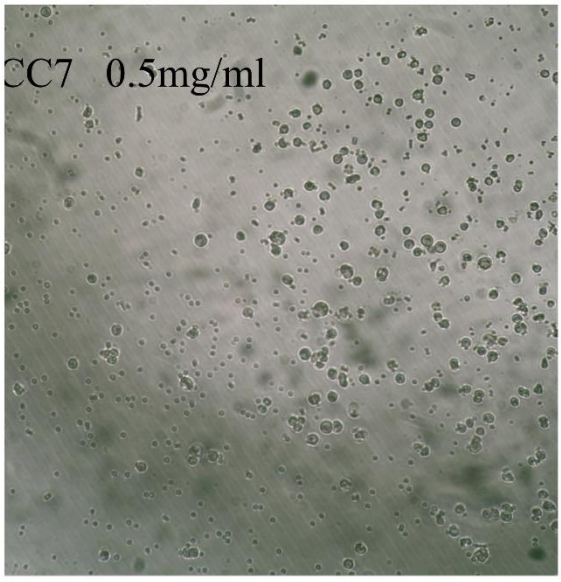
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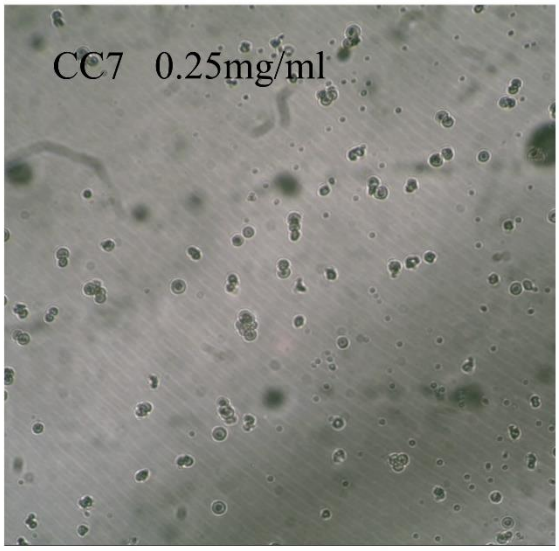
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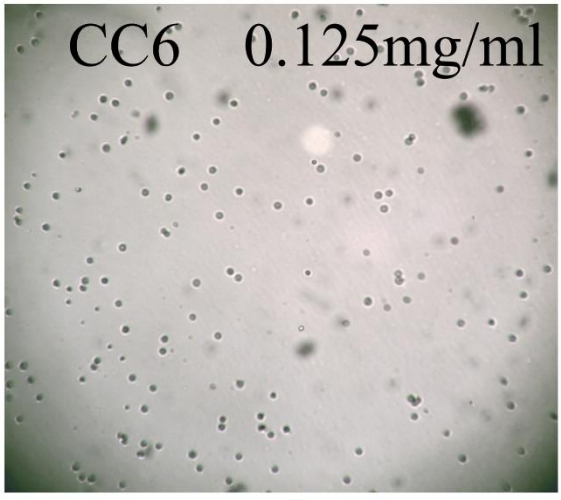
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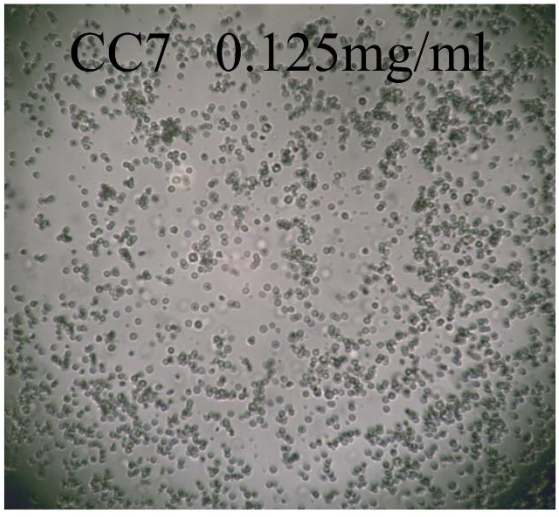
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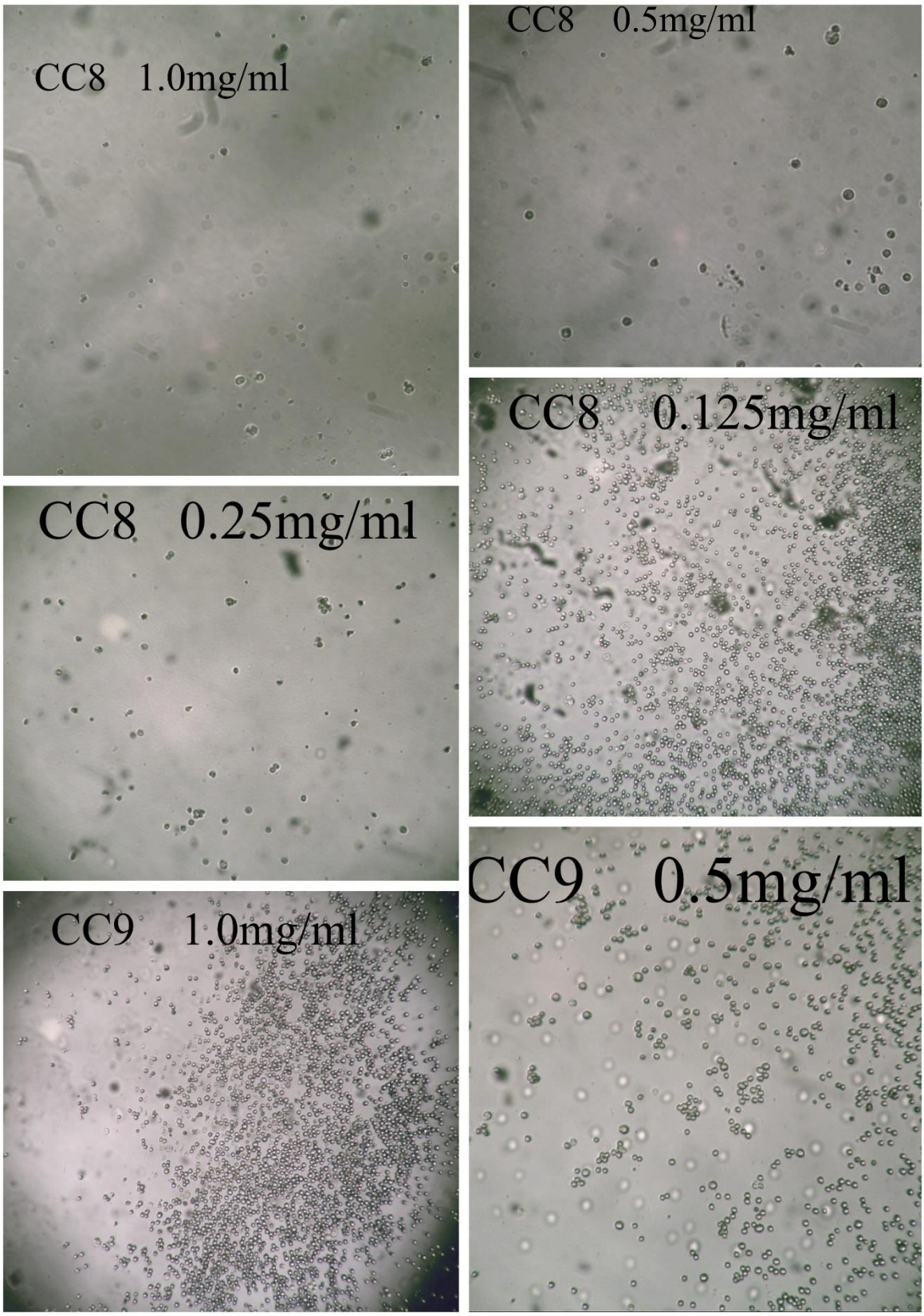
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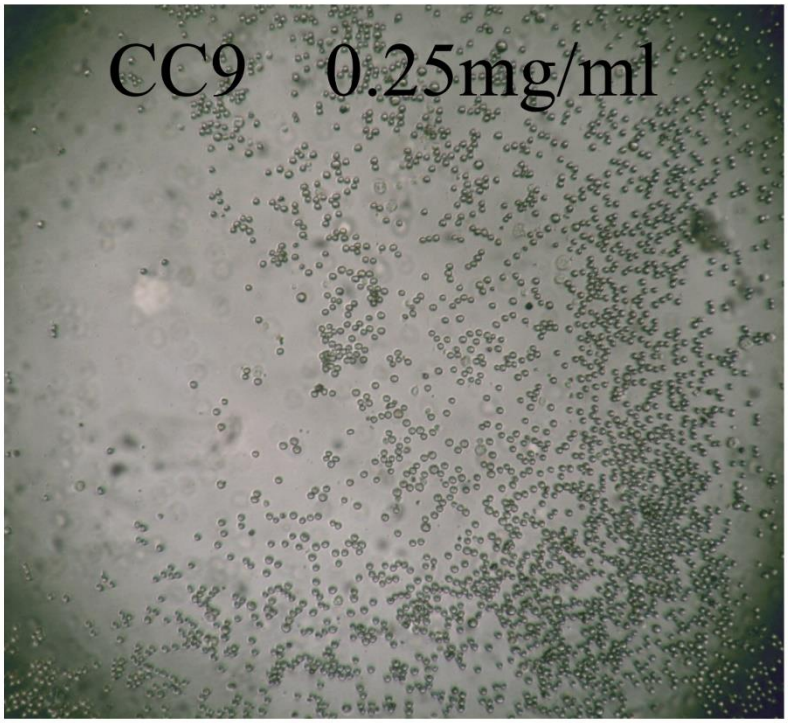
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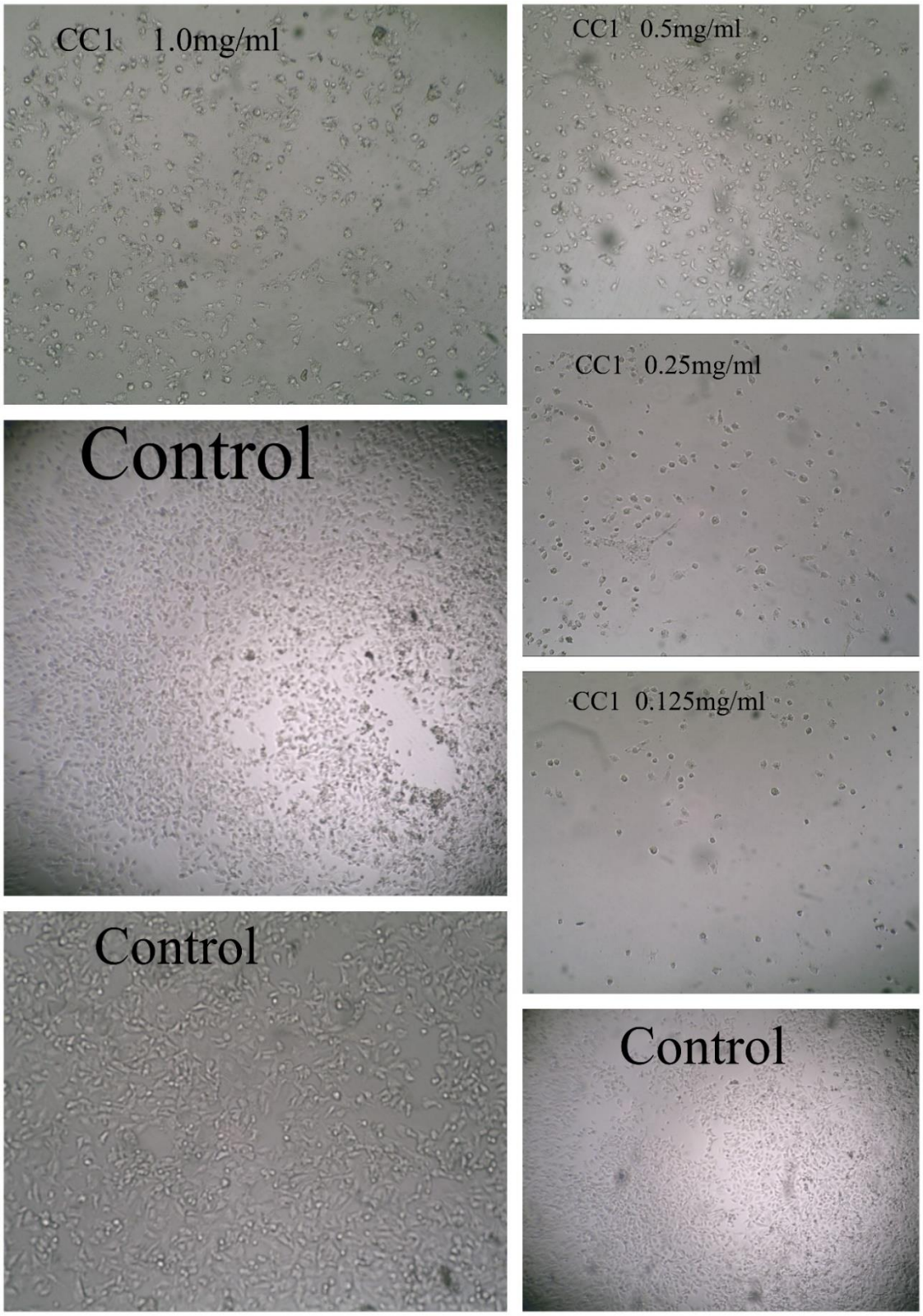
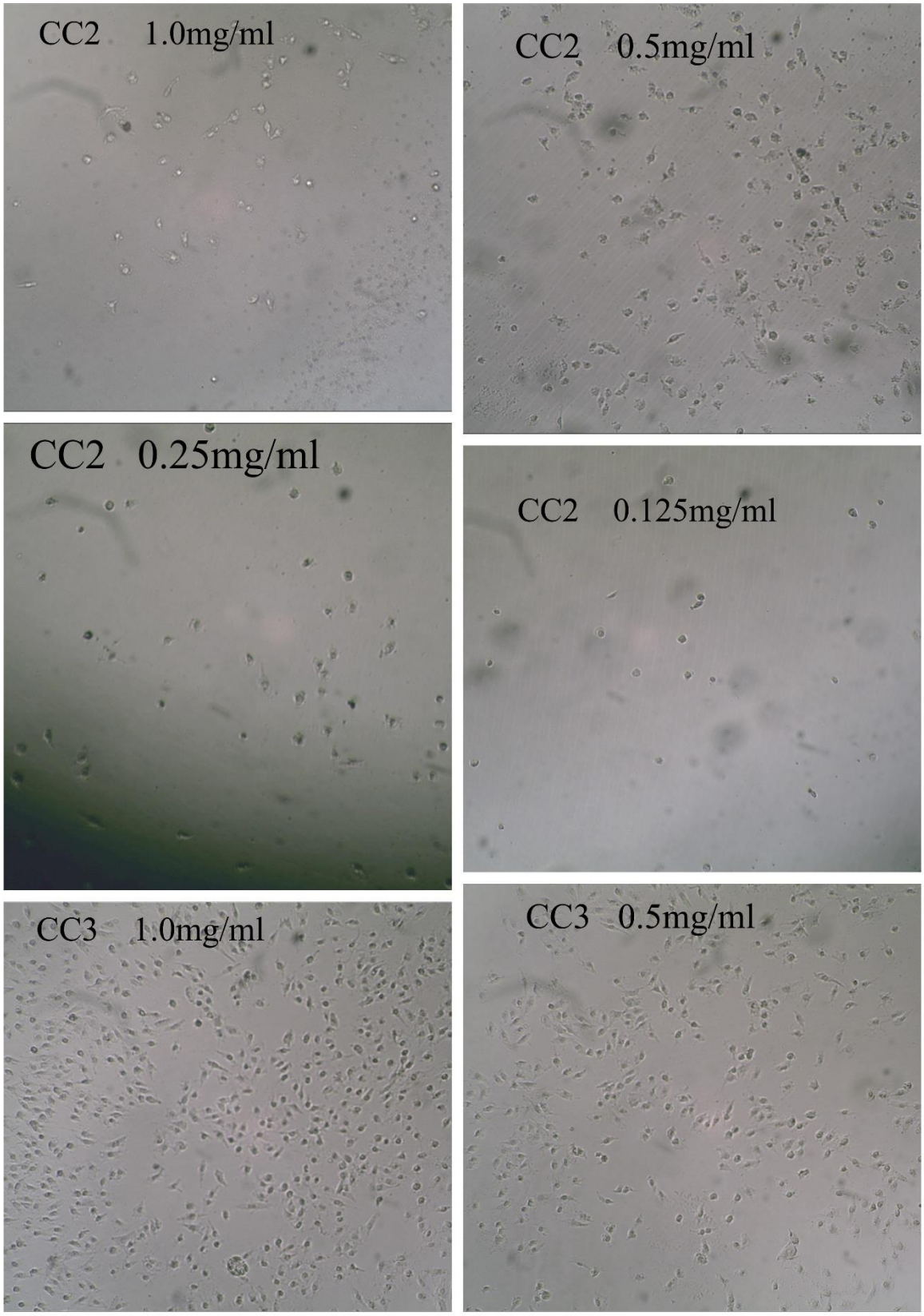
2: Cytotoxic effect of copper on osteosarcoma cancer cell line, each picture labeled by copper complex number and concentrations:

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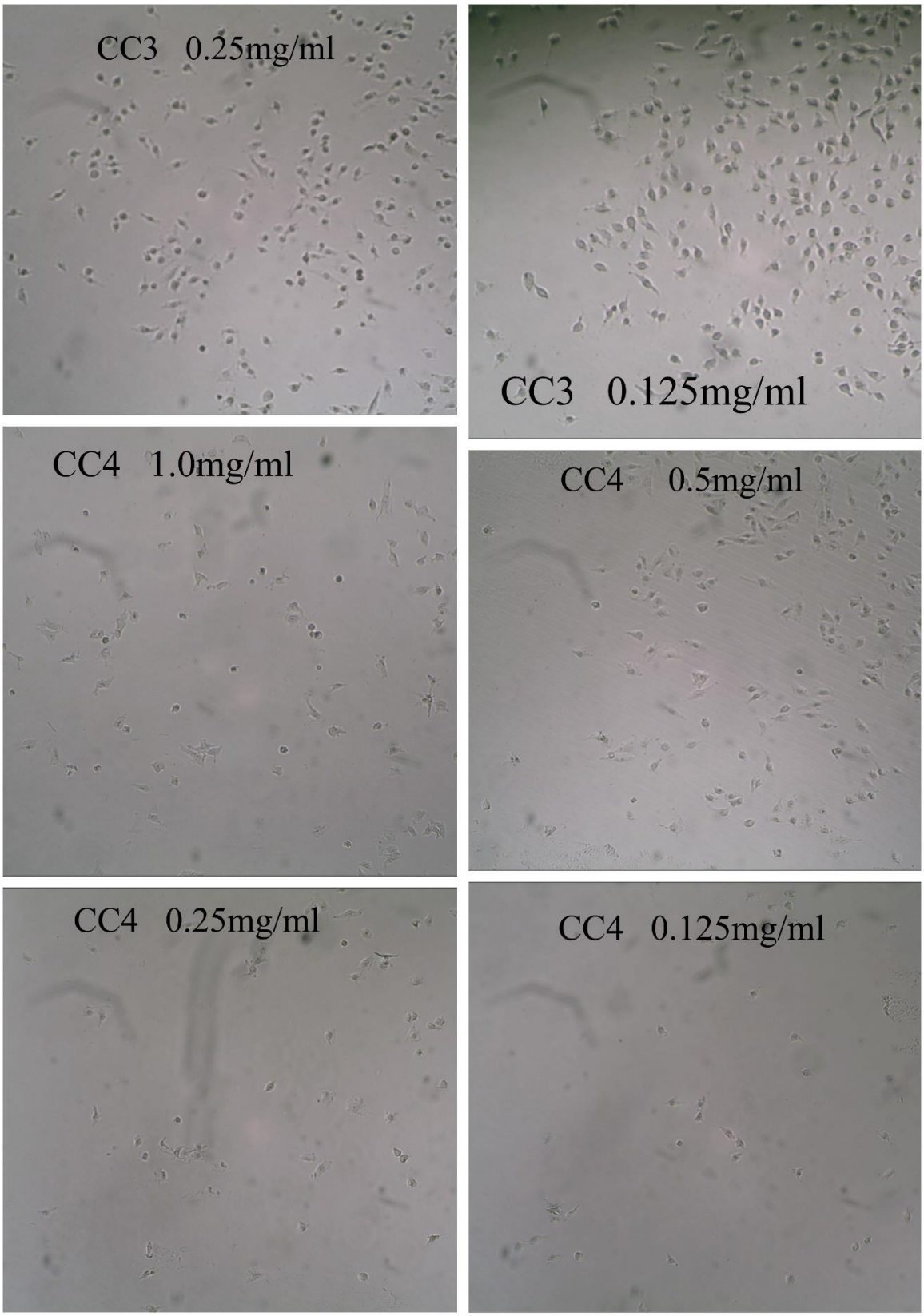
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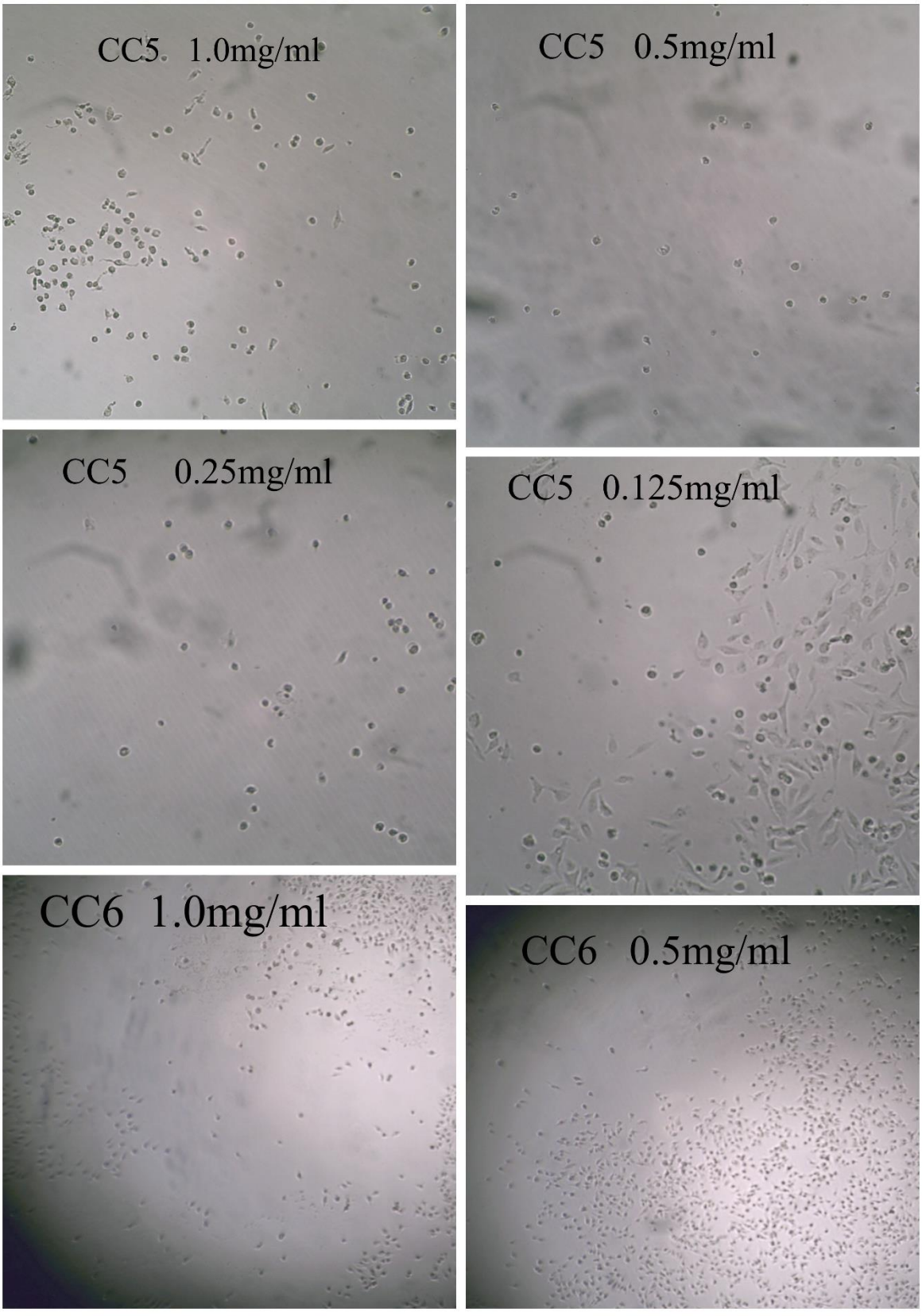
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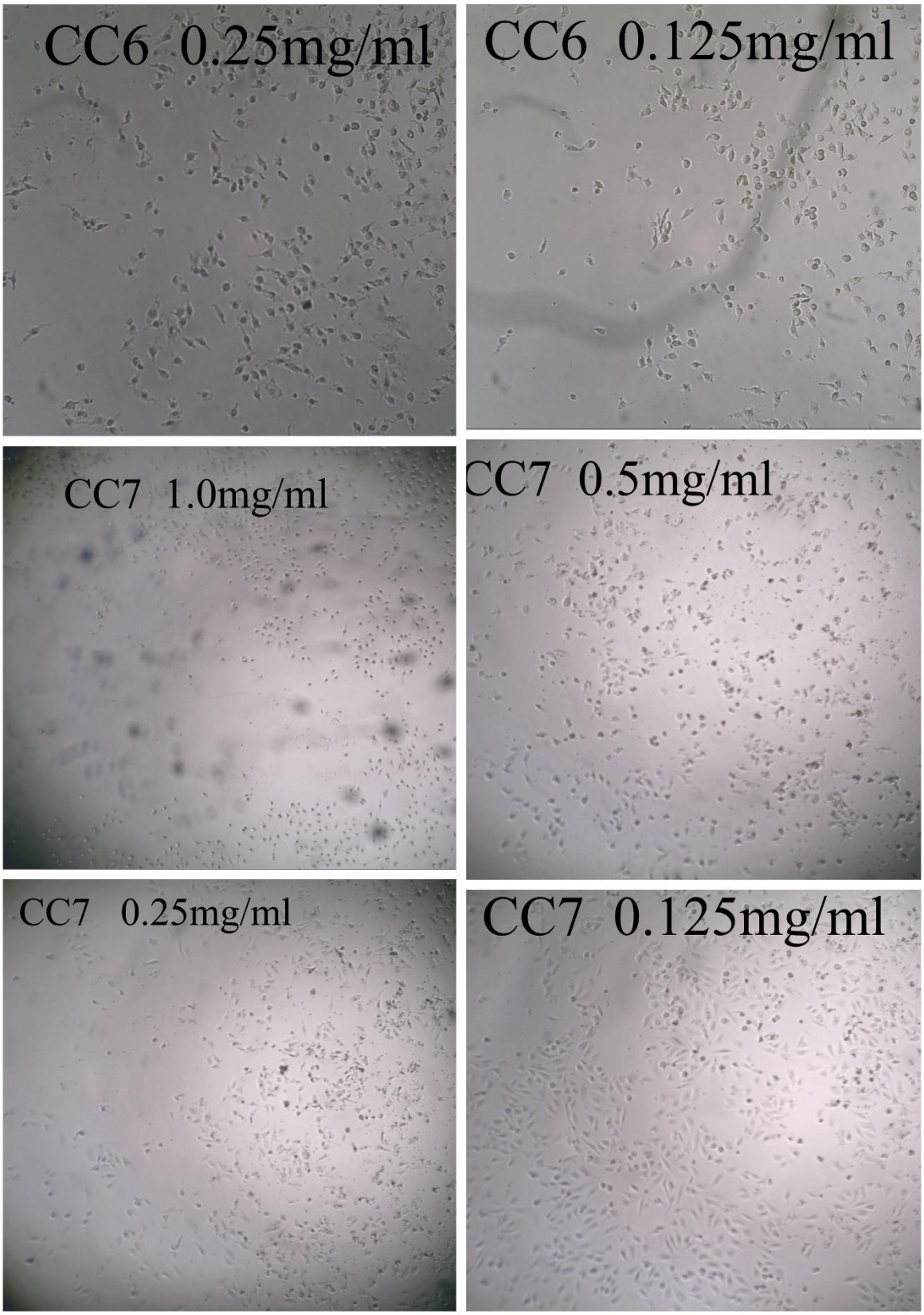
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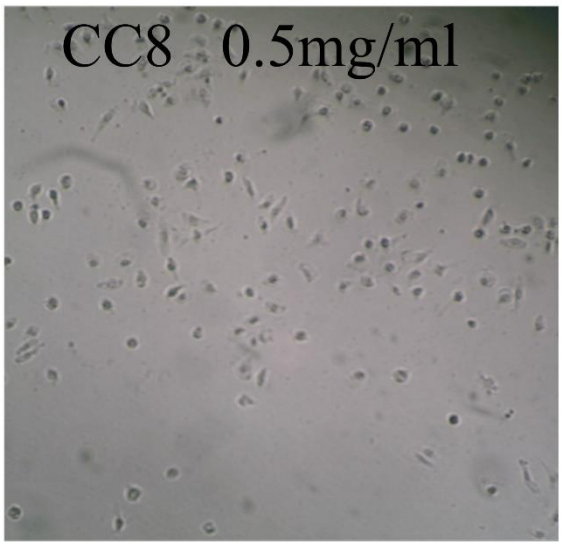
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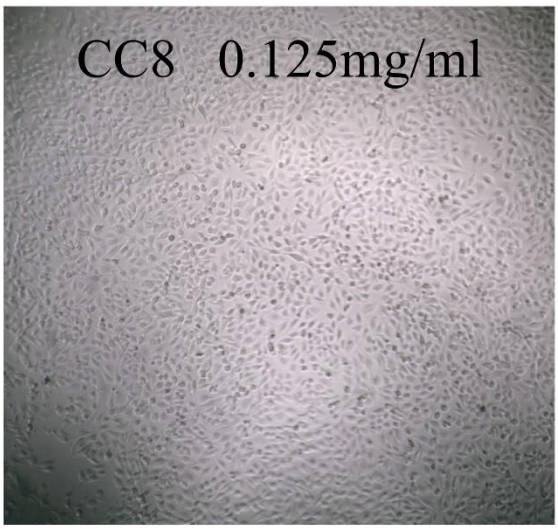
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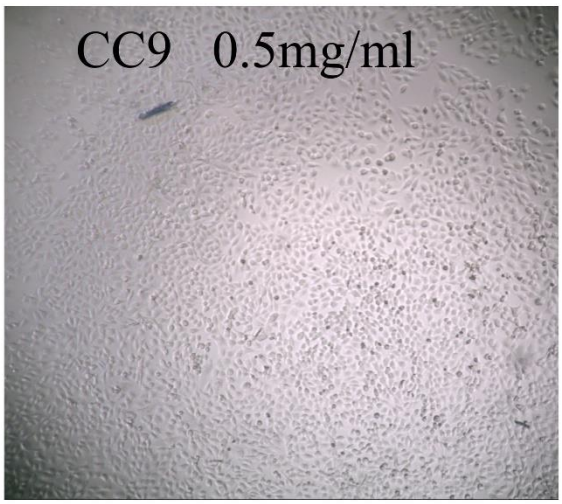
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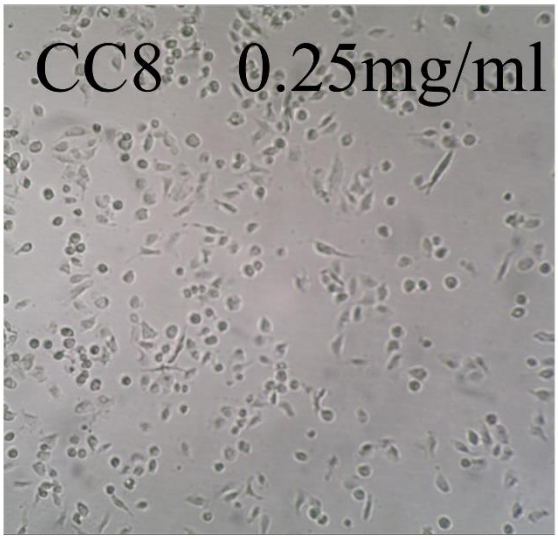
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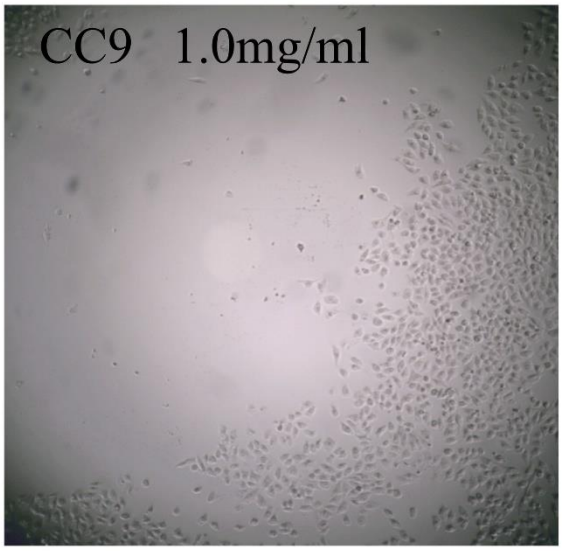
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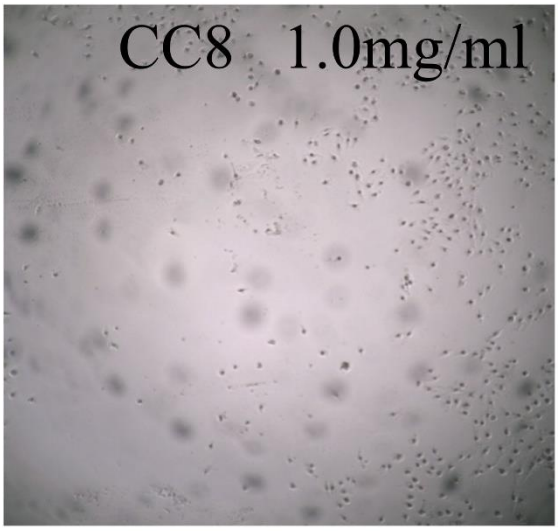
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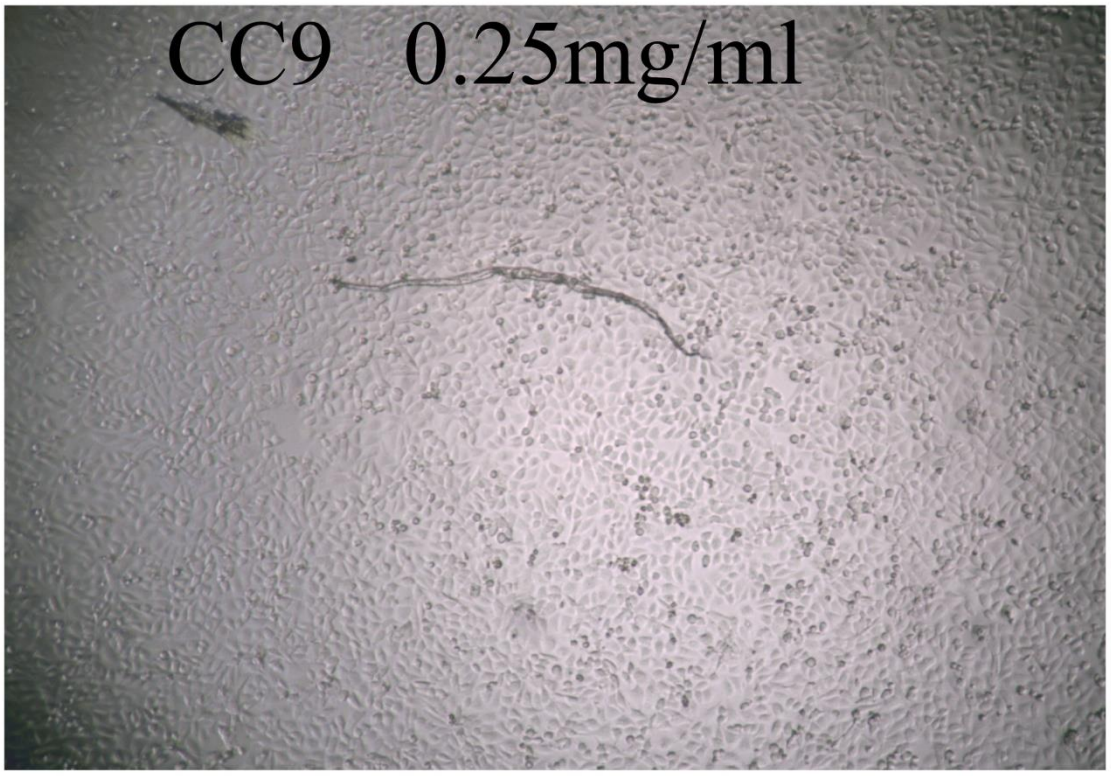
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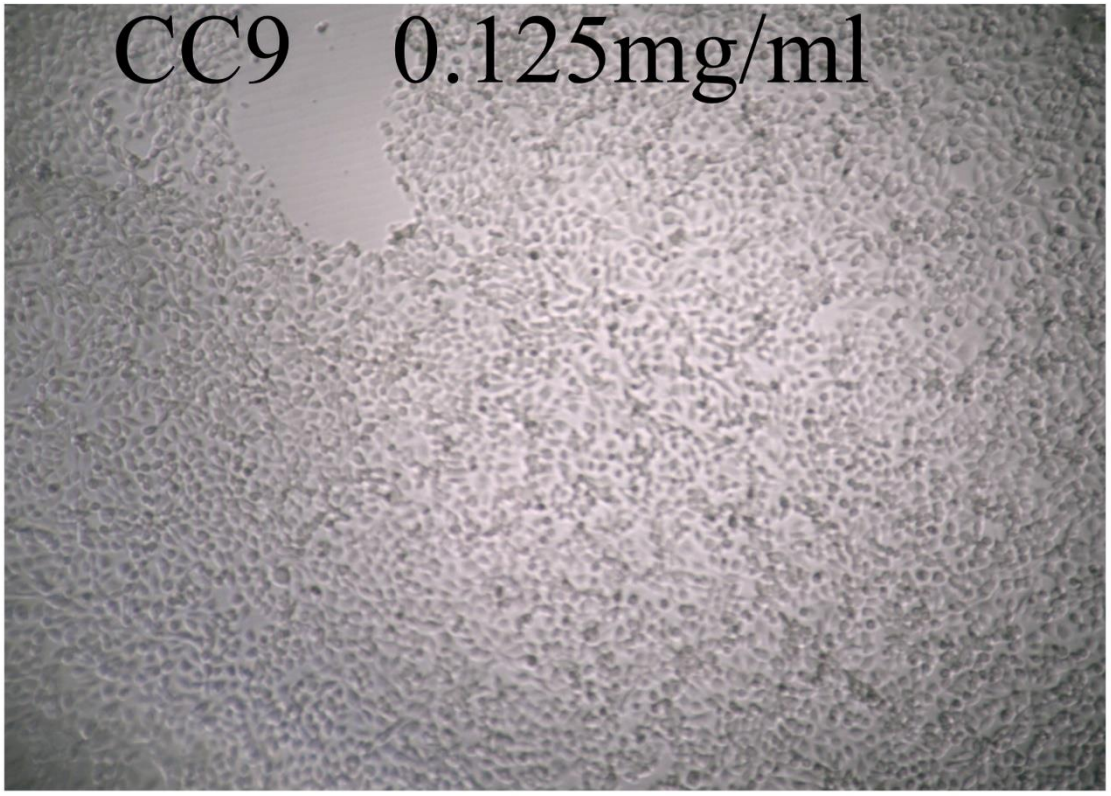
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جامعة النجاح الوطنية
كلية الدراسات العليا

تأثير معقدات نحاسية جديدة على الخلايا السرطانية في استحثاث الموت المبرمج للخلايا المعتمد على الكاسباز وارتباطها بال DNA

اعداد
جهاد شناعة

اشراف
د. أشرف صوافطة
أ.د. اسماعيل ورّاد

قدمت هذه الأطروحة لمتطلبات الحصول على درجة الماجستير في العلوم الحياتية بكلية الدراسات العليا في جامعة النجاح الوطنية، نابلس- فلسطين.
2017

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اعداد

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اشراف

د. أشرف صوافطة

أ.د. اسماعيل ورّاد

الملخص

السرطان ينتشر في العالم كاحد الاسباب الرئيسية للوفاة، على هيئة أكثر من مئة نوع. حالات السرطان في تزايد مستمر بحيث يتوقع ان يكون هناك 21.7 مليون حالة جديدة سنويا بحلول العام 2030 بعد ان كانت الحالات الجديدة في 2012 تبلغ 14.1 مليون حالة فقط، وذلك بسبب نمط الحياة الحديث (التدخين، سوء التغذية، الخمول، انخفاض عدد حالات الحمل .... الخ) المرتبط بازدياد احتمالية السرطان. خيارات معالجة السرطان يمكن ان تتنوع وتشمل العلاج الكيميائي، الاشعاعي، الجراحي، او خليط منها.

العلاج الكيمائي واحد من اهم طرق علاج السرطان. ومن أهم الإكتشافات في العلاج الكيميائي للسرطان هو ال سيسيبلاتين (cisplatin) النشط جدا ضد انواع مختلفة من السرطان، حيث يتمتع بنسب شفاء تتجاوز ال 90% في بعض انواع السرطان. رغم ذلك، فالخيارات العلاجية بواسطة ال سيسيبلاتين (cisplatin) تواجه بعض المحددات بسبب اعراضه الجانبية وكذلك المناعة المكتسبة او المورثة والتي تلعب دورا في زيادة المحددات على استعماله. هذه المعيقات أدت الى ابحاث مستفيضة للبحث عن بدائل أخرى، كـ معقدات كيميائية على اساس معدني ذات خصائص افضل.

هذا البحث هدف لدراسة نشاط معقدات النحاس المضاد للسرطان والمعتمد على تفعيل الموت المبرمج للخلايا المعتمد على الكاسباز. تسعة معقدات نحاسية تم تجهيزها وفحصها في المختبر مع ثلاثة سلالات من الخلايا السرطانية (خلايا سرطان القولون، سرطان الثدي وسرطان العظام) بواسطة فحص المقياس اللوني للكاسباز-3 و فحص مقياس الطيف لل MTT.

النتائج بينت ان المعقدات 1,3,4,5,6 و 8 أظهرت سمية قاتلة للخلايا السرطانية من القولون، بينما المعقدات 2 و 7 لم تظهر سمية قاتلة للخلايا السرطانية من القولون. كما لم تظهر المعقدات اي نشاط للكاسباز-3 لكل من الخلايا السرطانية من القولون او الثدي. هذه المعقدات أظهرت قدرة مشجعة على قتل وايقاف نشاط الخلايا التي تم اختباراها، أيضا اظهرت المعقد النحاسي رقم 7 قدرة على الارتباط ب ctDNA واتلافه، وهذا يحدد أن لهذه المركبات تأثير مضاد للسرطان.