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The Mono and Combined Anti-Proliferative Activity of D- Mannoheptulose and Newcastle Disease Virus Against Breast Cancer Cell Lines Targeting Glycolysis Inhibition

A Thesis

Submitted to the Council of College of Medicine, Al-Nahrain University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (PhD) in Medical Chemistry

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((قالُوا سُبحانَكَ لا عِلْمَ لَنَا إلاّ مَا علمتنا إنك أنْتَ العَلِيمُ الَحكِيم))

SUPERVISORS CERTIFICATION

We hereby certify that this thesis which is entitled **"The mono and combined anti-proliferative activity of D- Mannoheptulose and Newcastle disease virus against breast cancer cell line targeting glycolysis inhibition"** was carried out by the postgraduate student **"Ahmed Ghdhban Theiban AL-Ziaydi "** under our supervision, at College of Medicine, Al-Nahrain University. We do hereby recommend that it be accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy (PhD) in **Medical Chemistry.**

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DEDICATION

I dedicate this humble work,….To

My dear Father….

My beloved Mother….

My dear and beloved wife ……

My dear son and daughters…

My dear brothers and sisters….

My dear teachers…

To my lovely friends…

Ahmed Ghdhban Al-Ziaydi

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((Thanks to God for His entire blessing during
the pursuit of my academic and career goals))

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ABSTRACT

Recently, there have been several new approaches aimed at treating cancer, breast cancer in particular, by reducing the side effects of conventional therapies, other novel approaches such as targeted therapies, virotherapy and combinations of several of these new treatments. Synergism therapy reduce the side effects and toxicity, and overcome resistance of the cancer cells to the treatment.

Most cancer cells exhibit increased glycolysis, using this metabolic pathway to generate Adenosine tri phosphate for cell growth and proliferation. The Warburg effect illustrates that inhibition of glycolysis might have a therapeutic value in anti-tumor treatment. Newcastle disease virus (Iraqi strain) and enzyme inhibitor can modulate differentiation and induce apoptosis in breast cancer cell lines. D-Mannoheptulose functions biologically by inhibiting glycolysis, thus, inhibiting tumor growth.

Aim: To assess the synergistic effects of Newcastle disease virus as viral therapy and D-Mannoheptulose as a Hexokinase inhibitor, on the inhibition of the glycolysis pathway and eventually metabolism, to prevent proliferation and induce apoptosis in breast cancer cell lines, in comparison with normal Rat embryo fibroblast cell lines.

Method: Newcastle disease virus is propagated in embryonated chicken eggs and quantified using Hemagglutination assay and Median tissue culture infectious dose on the Vero cell line. The human breast cancer Ahmed Mortada jabria 2013 and Michigan cancer foundation-7, and normal Rat embryo fibroblast cell line were used. The cytotoxicity ratio of virus and inhibitor was measured against Ahmed Mortadha Jabria 2013, Michigan cancer foundation-7, and Rat embryo fibroblast cell lines for 72 hrs using the Methyl thiazolyl tetrazolium assay, to determine half maximal inhibitory concentration at different Multiplicity of infections (0.1-12.8 MOI) and diluted concentrations (13.125-1680 μg/ml) for virus and inhibitor, respectively, and

the Half maximal inhibitory concentration values were used for combination study. Using the CompuSyn software, the Combination index was measured to identify the effective doses of virus and inhibitor. Crystal violate/morphological changes, Acridine orange/Propidium iodide/apoptosis, Hexokinase activity, pyruvate, Adenosine tri phosphate concentration, and pH (acidity) were measured in treated and untreated breast cancer and normal cell lines, to investigate the effect on the glycolysis pathway.

Results: This study revealed that virus and inhibitor individually inhibited proliferation and growth, reduced cell viability, and induced morphological changes and apoptosis in breast cancer cell lines. Through glycolysis inhibition and apoptosis induction, there were no noticeable percentages of cytotoxicity for D-Mannoheptulose against normal Rat embryo fibroblast cells as the cytotoxicity ratio ranged from (1.67%, to 24.72%) at higher concentrations. While there was higher cytotoxicity against breast cancer cells ranged from (27.29% to 58.64%) for Ahmed Mortada jabria 2013 cell line; and (26.26% to 60.49%) for Michigan cancer foundation-7 cell line after inhibitor treatment. Virus did not induce cytotoxic effect against normal cell line as the cytotoxicity ratio ranged from (4.31% to 28.75%) for embryo fibroblast cells. Breast cancer cells were more sensitive to virus as the cytotoxicity ratio ranged from (24.69% to 64.26%) for Ahmed Mortada jabria 2013; and (23.95% to 62.02%) for Michigan cancer foundation-7 cell line after virus treatment. Glycolysis inhibition was observed by decreased Hexokinase activity from (3.2 U/ml as control to 2.0 U/ml with virus, 1.4 U/ml with inhibitor and 1.1 U/ml with combination in Ahmed Mortada jabria 2013 and from 3.0 U/ml as control to 1.9 U/ml with virus, 1.4 U/ml with inhibitor and 1.2 U/ml with combination in Michigan cancer foundation-7), pyruvate concentration from (0.48 μmol/ml as control to 0.28 μmol/ml with virus, 0.25 μmol/ml with inhibitor and 0.14 μmol/ml with combination in Ahmed Mortada jabria 2013 and from 0.5 μmol/ml as control to 0.23 μmol/ml with virus, 0.23 μmol/ml with inhibitor and 0.15 μmol/ml with

combination in Michigan cancer foundation-7) and Adenosine tri phosphate concentration from (1.2 mmol/L as control to 0.58 mmol/L with virus, 0.61 mmol/L with inhibitor and 0.39 mmol/L with combination in Ahmed Mortada jabria 2013 and from 1.3 mmol/L as control to 0.65 mmol/L with virus, 0.69 mmol/L with inhibitor and 0.35 mmol/L with combination in Michigan cancer foundation-7). Also, the decreased acidity was noticed by increasing pH from (6.3 as control to 7.3 with virus, 7.3 with inhibitor and 8.2 with combination in Ahmed Mortada jabria 2013 and from 6.3 as control to 7.4 with virus, 7.4 with inhibitor and 8.2 with combination in Michigan cancer foundation-7).

Combination therapy results revealed that the synergism between virus and inhibitor produced a significant anti-tumor effect than those of monotherapy with either. Their combination resulted in higher inhibition of cell viability, increase cytopathic morphological changes, and higher apoptosis induction in the treated cancer cell lines, via glycolysis pathway inhibition, as shown by decreased Hexokinase activity, pyruvate and Adenosine tri phosphate concentrations, and decreased environmental acidity which due to reduced lactate concentration, compared with normal cell lines. Moreover, this therapy was relatively safe in normal cells.

Conclusion: Synergism between Newcastle disease virus and D-Mannoheptulose increase the cytotoxicity and induce significant growth inhibition by glycolysis inhibition and promote apoptosis in breast cancer cells but not in normal cells in vitro, also it is better than individual treatment. A significant reduction was identified in the Hexokinase activity, Pyruvate, Adenosine tri phosphate concentrations, and acidity in all the treatment modalities in the breast cancer cells but not in the normal cells. Synergism of virus and inhibitor can be considered as a future treatment of cancer cells and a new strategic way to be used as an effective treatment.

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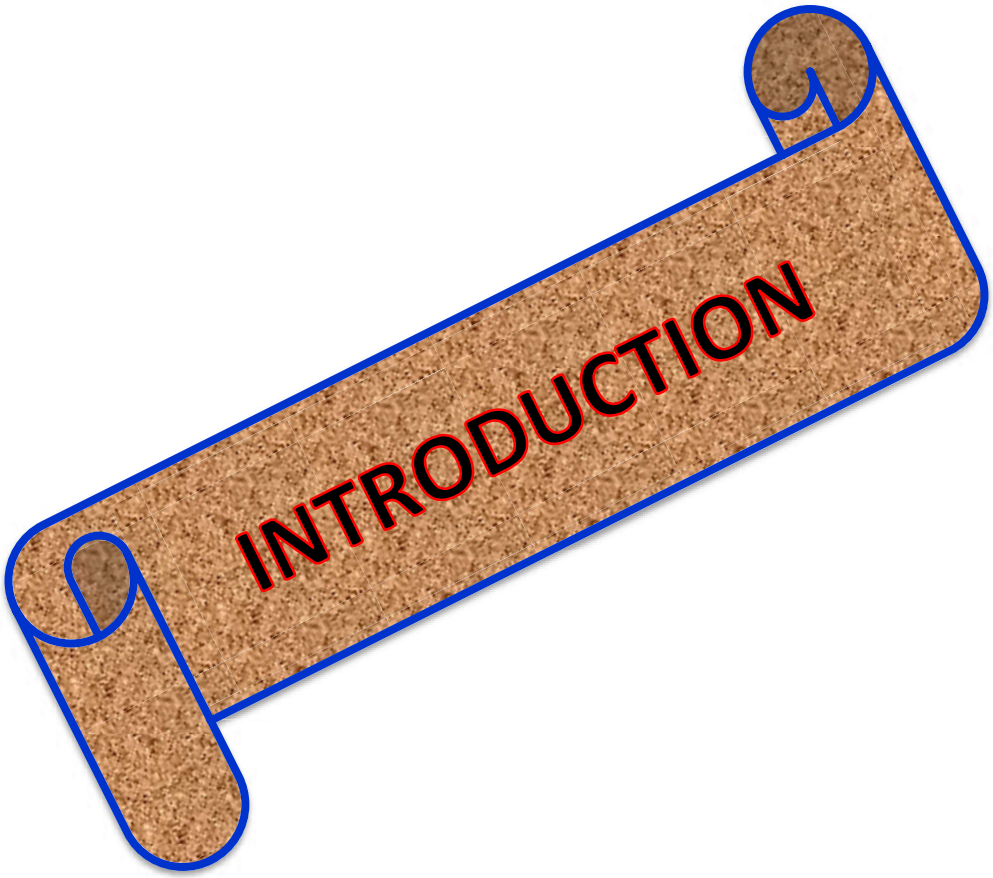
ABBREVIATIONS

Symbol	Description
A	Absorbance
AMJ13	Ahmed Mortadha Jabria 2013 Cell line
AO	Acridine Orange
AO/PI	Acridine Orange- Propidium Iodide
Apoptosis	Programmed Cell Death
ATP	Adenosine Tri Phosphate
BRCA	Breast Cancer
BSH	Biosafety Hood
Caspases	Cysteine aspartyl-specific proteases
CEF	Chicken Embryo Fibroblast
CI	Combination Index
CM	Complete Media (Media contain PSS, TDW and Serum)
Conc.	Concentration
CRBC	Chicken Red Blood Cells
CT%	Cytotoxicity Ratio
CT_{MH}	Cytotoxicity MH
CV	Coefficient of variation
D.W	Distilled water
DCIS	Ductal Carcinomas In Situ
DXL	Docetaxel
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECE	Embryonated Chicken Eggs
ELISA	Enzyme-Linked Immunosorbent Analysis
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
GLUT	Glucose Transporter
HA	Hemagglutination Test
HAU	Hemagglutination Unit
HEPES	Hydroxyethyl Piperazine ethane sulfonic acid

Symbol	Description
HK	Hexokinase
HKI	Hexokinase Inhibitor
IC50	The half maximal Inhibitory Concentration
ICCMGR	Iraqi Center of Cancer and Medical Genetics Research
IDC	Invasive Ductal Carcinomas
ILC	Invasive Lobular Carcinoma
LCIS	Lobular Carcinoma In Situ
LDH	Lactate Dehydrogenase
LFH	Laminar Flow Hood
MCF-7	Michigan Cancer Foundation-7 Cell line
MEM	Minimum Essential Medium
MH	D - Mannoheptulose
MOI	Multiplicity of Infection
MTT	Methyl Thiazolyl Tetrazolium
NDV	Newcastle Disease Virus
OD	Optical Density
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate Buffer Saline
Pen-Strep	Penicillin-Streptomycin Solution
PSS	Penicillin - Streptomycin
R	Reagent
RBC	Red Blood Cell
REF	Rat Embryo Fibroblast
RIF	Rat Embryonic Fibroblast
RNA	Ribonucleic acid
RPMI	Rosswell Park Memorial Institute
SD	Standard Deviation
SEM	Standard Error Mean
SFM	Serum - Free Media
TCA	Tricarboxylic Acid Cycle
TCF	Tissue Culture Flask (Falcon).
TCID50	50% Tissue Culture Infective Dose
TCM	Tissue Culture Media (CM &cells)
TCP	Tissue Culture Plate (96 wells).
TDW	Trebled Distilled Water
VAF	Virus Allantoic Fluids
96WMP	96 wall Micro Plate

CHAPTER-ONE



1- Introduction

Cancer is a major public health problem worldwide and it is the second common cause of death. Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells, If the spread is not controlled, it can result in death (Siegel *et al.*, 2019). Cancer results from the accumulation of genetic and epigenetic changes in tumor suppressor genes (Wang *et al*., 2018). Cancer therapy aims at killing cancer cells while minimizing harm on normal cells (Fournier, 2013). Cancer treatment includes localized and/or systemic therapies or supportive therapies reduce side effects (Miller *et al*., 2019).

Europe accounts for 23.4% of the global cancer cases and 20.3% of the cancer deaths. America has 13.3% of the global population and accounts for 21.0% of the incidence and 14.4% of the mortality worldwide. The proportions of cancer deaths in Asia and Africa (57.3% and 7.3%, respectively). Female breast cancer (6.6%). Breast cancer is the most commonly diagnosed cancer in women (24.2%). In Iraq, breast cancer is the most common tumor in females, number of new cases in 2018, females, all ages (rank 1, 36.7%) of all malignant diseases, (rank 2, death 11.9%) and one-third of registered cancers in females (Bray *et al*., 2018). The rate of breast cancer is 30%, and the death rate is 15% of female for 2019 in USA according to World Health Organization (WHO).

This increment in the incidence rate of breast cancer may be due to exposure to environmental factors and many other factors (Al-Shammari, 2016). The methanolic extracts of Avocado fruit and leaves are highly promising for use in cancer therapy (Paul *et al.*, 2011). D-Mannoheptulose (MH) accumulates in avocado leaves and fruit. Phytochemicals extracted from avocado induces apoptosis in human oral epithelial cancer cell lines, but not normal cells (Ding *et al*., 2009). MH is a hexokinase inhibitor that blocks the enzyme hexokinase, which prevents glucose phosphorylation and breakdown (Tesfay *et al*. ,2012). Newcastle

disease virus (NDV) is a member of the genus avulavirus, in the family of Paramyxovirus (Jin *et al.*, 2017). NDV proved to have promising anti-tumor activity and great safety in lab animals (Costa-Hurtado *et al*., 2015). It is endemic in poultry, resulting in huge economic losses (Abdisa and Tagesu, 2017). Increased glycolysis is the main source of energy in cancer cells that use this metabolic pathway for ATP generation (Gill *et al*., 2016). The breakdown of glucose via glycolysis yields 2 molecules of pyruvate and 2 net molecules of ATP (Chaudhry & Varacallo, 2019). In the presence of oxygen, normal cells generate energy from glycolysis coupled with oxidative phosphorylation (Dhup *et al.*, 2012). Cancer cells mainly produce energy by increasing the rate of glycolysis compared with normal cells, even if oxygen is plentiful (Alfarouk *et al*. 2014). Relative to normal cells, cancer cells are highly proliferative and thus require increased ATP to meet their metabolic demand (Cairns *et al*., 2011). The Warburg effect promotes cancer cell proliferation. Cancer cells need high energy for continuous growth, and therefore rely on the Warburg effect (Koppenol *et al*., 2011).

Inhibiting the activity of glycolytic key enzymes, results in ATP depletion and decreased lactate generation (Guo *et al*., 2016). Cancer cells are disrupted by decreased lactate production (Lu *et al*., 2011). Starvation leads to a decrease in glucose uptake and lactate production (Gonin-Giraud *et al.*, 2002). Glucose enters the cell and generates pyruvate after a series of enzymatic reactions (Lu *et al*., 2015). In cancer cells, pyruvate is preferentially converted into lactate even in the presence of oxygen. Depletion of ATP by glycolytic inhibition potently induces apoptosis. Starvation induces apoptosis of breast cancer cells (Fu *et al*., 2007). Current strategies for cancer therapy are focused on apoptosis. Oncolytic virotherapy alone is not effective for tumor eradication (Chu *et al*., 2004). Combination therapies attack tumor cells and preventing treatment resistance development. The combination of chemotherapy and virotherapy is promising in cancer treatment. (Al-Shammari *et al*, 2016).

Aim of the study

1- To determine the anti-proliferative activity of NDV, D-Mannoheptulose (MH) and their combination.

2- Determination of $TCID_{50}$ of NDV and IC_{50} values of NDV and MH.

3- Determination of synergism, or antagonism between NDV and MH.

4- Study the morphological changes of cancer and normal cells after treatment with either NDV or MH, and their combination.

5- Investigate the apoptosis induction by mono- and combination therapies in cancer and normal cells.

6- To compare the effect of mono- and combination therapy on cancer cell glycolysis, by studying the effect on levels of glycolysis products such as hexokinase, [pyruvate], [ATP], and acidity (that represent lactic acid).

CHAPTER-TWO

2. LITERATURES REVIEW

2.1 Tumor (Neoplasia)

A tumor is a pathologic disturbance of cell growth, characterized by excessive and abnormal cell proliferation (Sinha, 2018). Tumor are classified as benign or malignant. Benign tumors are noninvasive, remain localized, and grow away from the surface because they cannot invade (Vinik *et al.*, 2018). Malignant tumors are invasive, capable of spreading directly or by metastasis, and have relatively rapid growth rates (Strayer *et al*., 2015; Davies, 2014). Most human tumors do not display pure logarithmic growth patterns, as not all cells within the tumor are actively dividing. (Grimes *et al*., 2016) (Figure (2-1)).

Benign neoplasms typically have smooth borders, are sharply demarcated from the normal tissue at the tumor site, and are encapsulated by a fibrous capsule that forms a barrier between the neoplastic cells and the host tissue (Coleman, 2018). Benign neoplasms typically grow slowly such as lipomas, whereas malignant neoplasms often demonstrate rapid growth. (Studdiford & Trayes, 2018).

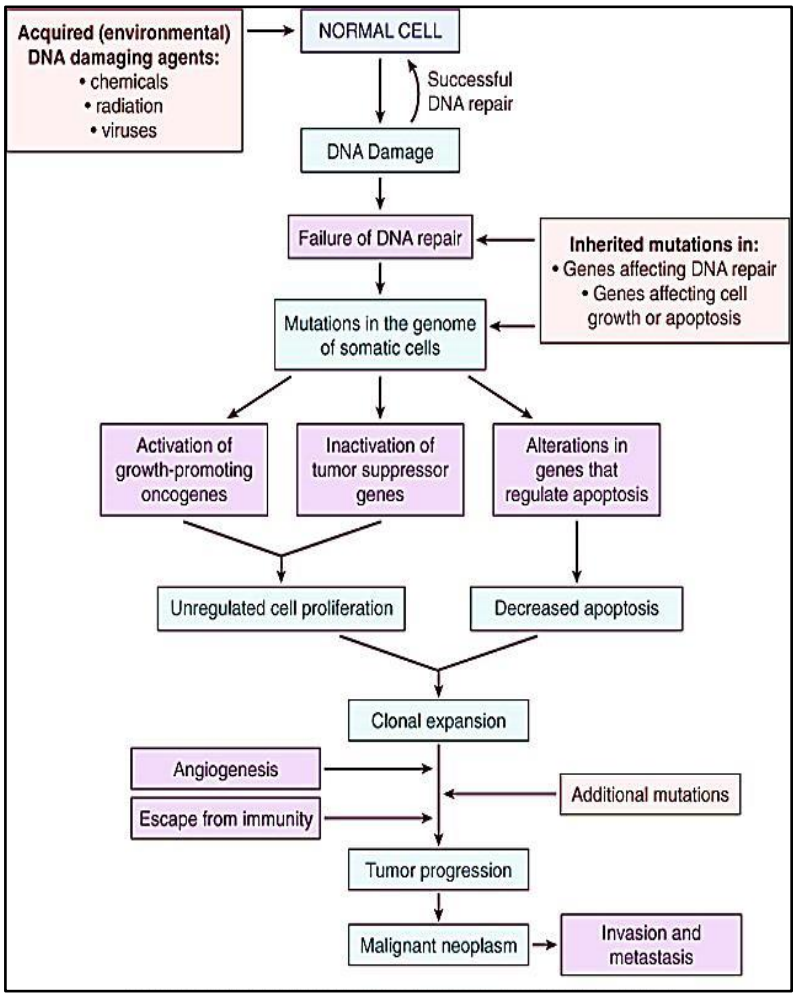


Figure (2-1) Scheme of molecular basis of cancer (*Stricker and Kumar, 2007*)

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells, which if not controlled can result in death. Cancer is caused by external factors and internal factors. Cancer cells are generally hungrier for nutrients than normal cells, in order to sustain their high proliferative rates. This is shown by their higher consumption of glucose, due to the lower efficiency in energy production by anaerobic glycolysis, and increased extracellular acidosis, (Tarver, 2012). Many factors contribute to the cell changes that lead to cancer. (Fernandes *et al*., 2015). The interplay between the intrinsic and extrinsic factors is the major determinant of an individual's cancer risk (Wang and Karin, 2015). Risk factors vary widely worldwide based on differences in lifestyle and in social, economic, and political development (Wu et al., 2016). The most important modifiable risk factors are infection with cancer-causing viruses or bacteria (Pacheco *et al*., 2016).

As normal cells divide, mature, and die, abnormal cells that do not follow this progression lead to cancer development (*Kim et al., 2015*). Cancer begins when damage is caused to 1 or more genes in a single cell. This harm will cause the cell to divide incorrectly, producing abnormal cells (Marzouk and Schofield, 2011). Cancer cells divide more rapidly than normal cells (El Mesallamy *et al*., 2011). During early stages of cancer, a person may have just 1 small cancerous tumor. More advanced stages may involve a larger tumor (Hong *et al*., 2014). Cancer is caused by alterations in oncogenes, tumor-suppressor genes, and microRNA genes. (Croce, 2008).

Nutrition and physical activity directly affect cancer risk. Physical activity reduces the risk of breast. Eating a diet high in fruits and vegetables is associated with lower risk of cancers (Smith *et al.*, 2018). The goal of cancer therapy is to push the death of cancer cells while minimizing the harm on normal cells. (Fournier and Schirrmacher, 2013). When cancer spreads from its original location to another part of the body, the new tumor has the same kind of abnormal cells and name as the primary tumor. (Santa-Maria, 2015).

2.2 Carcinogenesis

Carcinogenesis or neoplastic pathogenesis is a process caused by a series of mutations in the genetic material of normal cells (Dermadi Bebek, 2014). Neoplastic pathogenesis can be described in 3 stages: initiation, promotion, and progression (Trosko, 2001). During initiation and promotion, apoptosis and cell proliferation can occur at various rates; this balance is modified during progression so that malignancy can arise (Hanahan and Weinberg, 2011).

2.3 Cancer and cell death

Abnormalities in cell death regulation can be a significant component of diseases such as cancer. regulated cell death can occur in the absence of any exogenous environmental perturbation (Galluzzi *et al*., 2016). Classify cell death into three different forms: type I cell death or apoptosis, exhibiting cytoplasmic shrinkage, chromatin condensation, nuclear fragmentation , and plasma membrane blebbing with the formation of apoptotic bodies that are efficiently taken up by neighboring cells with phagocytic activity, type II cell death or autophagy, manifesting with extensive cytoplasmic vacuolization with phagocytic uptake and consequent lysosomal degradation; and type III cell death or necrosis, displaying no distinctive features of type I or II cell death and terminating with the disposal of cell corpses in the absence of obvious phagocytic (Lorenzo *et al*., 2017).

2.4 Breast cancer

Breast cancer is the most important and frequently diagnosed cancer in women worldwide (24.2%, i.e. about 1 in 4 of all new cancer cases diagnosed in women worldwide are breast cancer), and it is the leading cause of cancer-related deaths in developing countries and women (15.0%), (Bray *et al.*, 2018). In Iraq, breast cancer is the most common tumor in female, number of new cases in 2018, females, all ages (rank 1, 36.7%) of all malignant diseases, (rank 2, death 11.9%) and one-third of registered cancers in females. Number of new cases in 2018, both sexes, all ages

(rank 1, 20.3%) (Bray *et al*., 2018). This increment in the incidence rate of breast cancer may be due to exposure to environmental and other factors such as life style, chemicals, and radioactive pollution (*Hassan et al., 20*14). The poor survival is a consequence of the late stage at presentation reflecting the limited access to screening, diagnostic, and treatment facilities together with the lack of awareness on the significance of early detection of breast cancer among the population (Sankar *et al*., 2013) (Figure (2-2)).

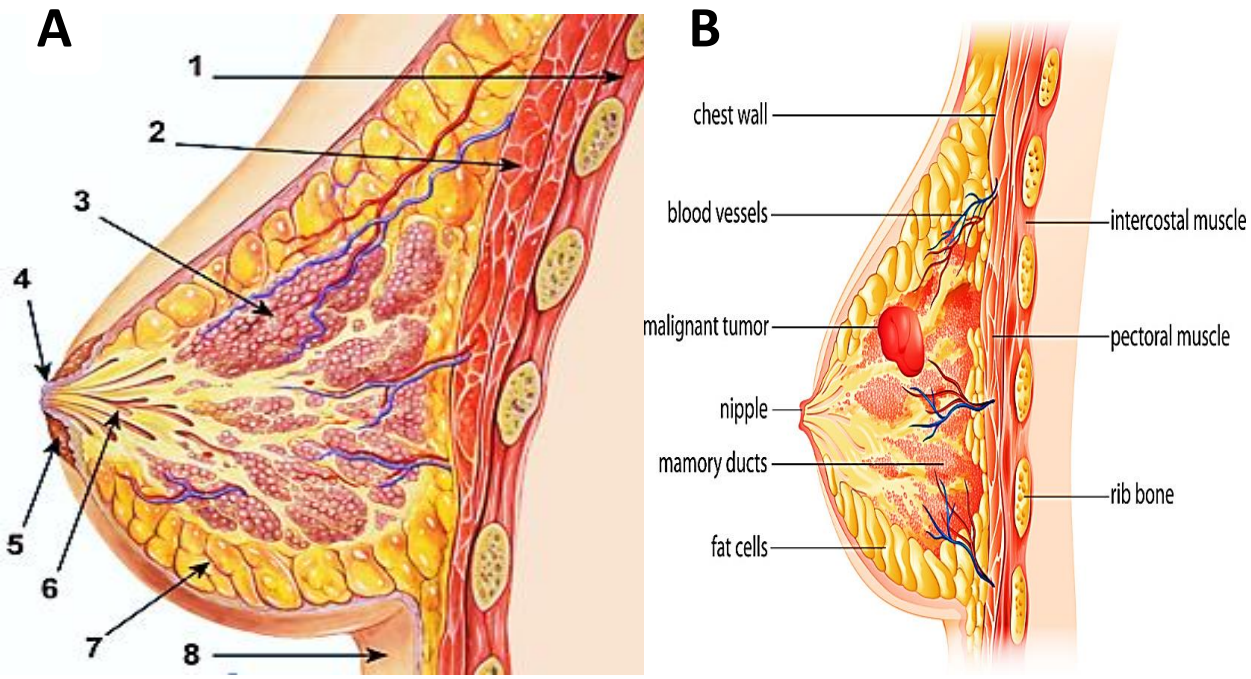


Figure (2-2): A- Structure of the normal female breast, cross-section scheme of the mammary gland. Chest wall, 2- Pectoralis muscles, 3- Lobules, 4- Nipple, 5- Areola, 6- Milk duct, 7- Fatty tissue and 8- Skin (Love, 2015). B- Breast cancer

2.4.1 Etiology of breast cancer

In Iraq, breast cancer is the most common cancer in women, accounting for 36.7% of all malignant diseases (Bray *et al*., 2018). Breast cancer can be diagnosed in women of all ages, but the risk of developing breast cancer increases with age, and

most cases (80%) occur in women over the age of 50 years. Several factors that increase a woman's risk of developing breast cancer (Parkin *et al*., 2011). Breast cancer is a complex, heterogeneous, and multifactorial disease. Although some genetic factors have a strong and well-defined impact, such as mutations in the breast cancer 1 (BRCA1) and BRCA2 genes, only 5–10% of all breast cancers are considered to be due to mutations in inherited high penetrance genes (Di Sibio *et al*., 2016). The risk of breast cancer has been consistently associated with age, a family or personal history of breast cancer, reproductive and hormonal factors, hormone replacement therapy, obesity, alcohol consumption, physical inactivity, exposure to ionizing radiation, and genetic predisposition (Lauby-Secretan *et al*., 2015).

Evidence indicates that breastfeeding reduces the risk of developing breast carcinoma (Zhou *et al*., 2015). Epidemiological evidence has suggested that the use of contraceptives is associated with an increased risk of breast cancer among carriers of BRCA1 or BRCA2 mutations (Friebel *et al*., 2014). It was found that a 5 kg/m^2 increase in BMI increases the risk of postmenopausal breast cancer by 12% (Arnold, M., *et al*., 2015), and that physical inactivity increase the risk of breast cancer by 33% (Pena *et al*., 2014).

2.4.2 Signs and symptoms of breast cancer

The first sign of breast cancer is a new lump or mass in the breast. Breast cancers show up as red or thickened skin rather than a lump. The most common physical sign is a painless lump. Sometimes it can spread to underarm lymph nodes and cause a lump or swelling. It is important to note that pain does not indicate the presence or the absence of breast cancer. Less common signs and symptoms include breast pain or heaviness; persistent changes to the breast, such as swelling, thickening, or redness of the breast's skin; and nipple abnormalities such as spontaneous discharge, erosion, inversion, or tenderness, irritation and nipple discharge other than breast milk, including blood, or dimpling of breast skin (Berry Khatri, 2019).

2.4.3 Genetics and breast cancer

Cancer is fundamentally a disease of tissue growth regulation. In order for a normal cell to transform into a cancer cell, the genes that regulate cell growth and differentiation must be altered (Croce, 2008). Oncogenes are genes that promote cell growth and reproduction. The first major gene associated with hereditary breast cancer is BRCA1, or BRCA2 confers an increased risk of breast cancer. Female carriers of mutations in BRCA1 or BRCA2 have a lifetime risk (50–85%) of breast cancer. Male carriers of BRCA1 have an increased risk of breast cancer (Shiovitz and Korde, 2015).

Genetic changes can occur at different levels and by different mechanisms. (Colotta *et al*., 2009). Disruption of a single gene may also result from integration of genomic material from a DNA virus or retrovirus, leading to the expression of viral oncogenes in the affected cell and its descendants (Borrello *et al*., 2008).

2.4.4 Breast cancer Spread and metastasis

Breast cancer can spread through the lymph system, which includes the lymph nodes, lymph vessels, and lymph fluid found throughout the body. Lymph nodes are small, bean-shaped collections of immune system cells that are connected by lymph vessels. Lymph vessels are like small veins, except that they carry a clear fluid called lymph away from the breast. Lymph contains tissue fluid and waste products, as well as immune system cells. Breast cancer cells can enter lymph vessels and grow in lymph nodes. Most of the lymph vessels of the breast drain into lymph nodes under the arm, around the collar bone, and inside the chest near the breast bone (Smith *et al*., 2011).

2.4.5 Types of breast cancer

Breast cancer is often classified into 2 types: non-invasive and invasive (infiltrating).

2.4.5.1 Non-invasive breast cancer or in situ (in the same place) carcinoma: In situ carcinoma is defined by the clonal proliferation of neoplastic epithelial cells within the ducts. It is located in the ducts and lobules of the breast. They do not grow into or invade normal tissues within or beyond the breast. Tumor cells of in situ carcinomas are confined to the breast epithelial cells where they started (Phuc Van Pham, 2017).

A. Ductal Carcinomas in Situ (DCIS): It is the cells that line the milk ducts of the breast have become cancer, but they have not spread into surrounding breast tissue. It is the most common type of non-invasive breast cancer and attributed about 15-20% of new breast cancer cases (Figure (2-3)) (Phuc Van Pham, 2017).

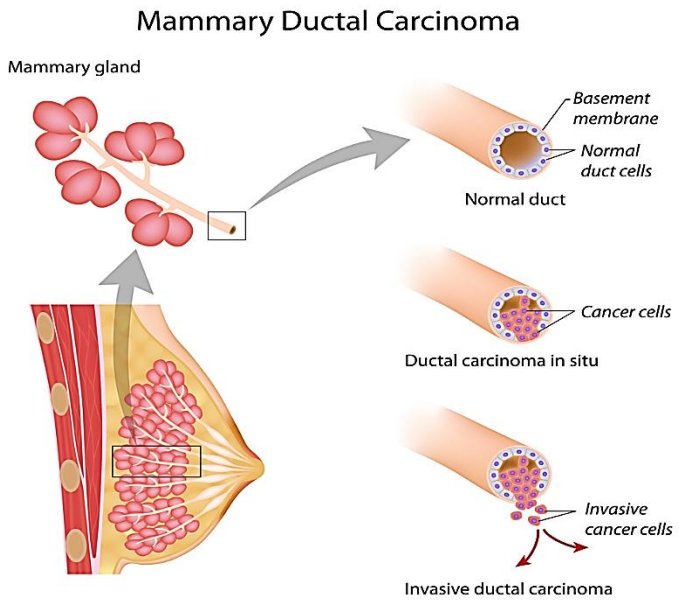


Figure (2-3): Ductal carcinoma in situ (DCIS). (Phuc Van Pham, 2017).

B. Lobular Carcinoma in Situ (LCIS):

It is an uncommon condition in which abnormal cells. It arises inside the milk-producing glands of the breast and constitutes about 12% of female in situ breast cancers. Therefore, Patients diagnosed with LCIS have an increased risk of developing invasive cancer at a later point in time. It is an area of abnormal cell growth that increases a person's risk of developing invasive breast (Figure (2-4)), (Phuc Van Pham, 2017).

2.4.5.2 Invasive Carcinoma

The breast cancer epithelial cells spread outside the basal membrane that covers a duct or alveoli and invade the surrounding breast cancer tissues (Sharma *et al*., 2010). Most breast cancers are invasive and are of different types. The 2 most common are invasive ductal carcinoma and invasive lobular carcinoma (Visser *et al*., 2019).

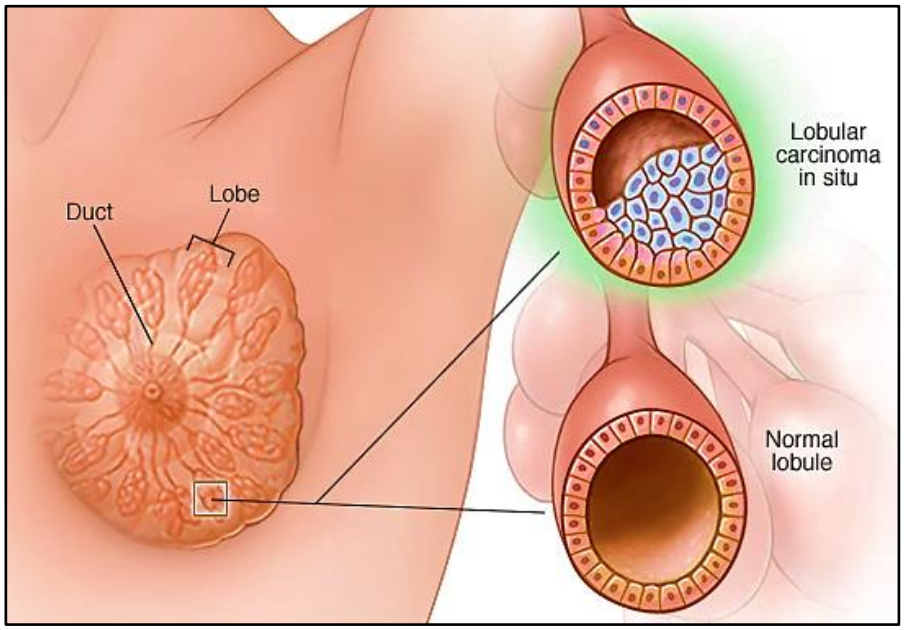


Figure (2-4): Lobular carcinoma in situ (LCIS). (Phuc Van Pham, 2017).

A. Invasive Ductal Carcinomas (IDC)

This is the most common type of breast cancer. IDC arises in the epithelial cells within the ducts and starts in the cells lining the milk duct in the breast. It may be able to spread to other organs such as liver or lungs through the lymph system and blood stream (Dillon *et al*., 2014). IDC often presents alone or with a co-existing ductal carcinoma in situ component (IDC + DCIS) (Kole *et al*., 2019). Treatment of invasive breast cancer depends on how advanced the cancer and the other factors. (Overmoyer and Pierce, 2015).

B. Invasive Lobular Carcinoma (ILC)

The invasive tumor cells of this cancer type are originated in lobules and inevitably metastasize to other body parts. It is the second most frequent invasive carcinoma of breast cancer, contributing to about 10-15% of invasive cases (Watheq, 2016).

C. Less Common Types of Invasive Breast cancer

Special types of breast cancer that are sub-types of invasive carcinoma, each typically makes up less than 5% of all breast cancers. They include tubular, papillary, adenocystic, mucinous, and medullary carcinomas. Some sub-types, which include metaplastic, micropapillary, and mixed carcinomas have the same prognosis as IDC (Jagsi *et al*., 2019).

2.5 Glycolysis Pathway

Glycolysis (Embden-Meyerhoff Pathway (EMP)) is a metabolic pathway and an anaerobic source of energy. In glycolysis, 1 glucose molecule utilizes 2ATP molecules to produce 4ATP, 2NADH, and 2 pyruvates. The pyruvate can be used in the citric acid cycle, or serve as a precursor for other reactions (Chaudhry and Varacallo, 2019). Glycolysis is an inefficient means of energy production, producing only 2 net molecules of ATP per molecule of glucose (Melkonian and Schury, 2019). Glycolysis is an oxygen independent metabolic pathway, that occurs in the cellular cytosol of most organisms (Keller, *et al*., 2014). If a cell has mitochondria and oxygen, glycolysis is aerobic. If either mitochondria or oxygen is lacking, glycolysis may occur anaerobically although some of the available energy is lost (Sam Turco, *et al*., 2016).

Under certain conditions, especially under oxygen deprivation, OXPHOS cannot take place, so pyruvate is instead converted to lactate by LDH. Lactate is then ejected out of the cell by monocarboxylate transporter 4 (MCT4), in order to maintain the intracellular pH within acceptable levels (Knobloch *et al*., 2019). Disposal of lactate from the cell is one of the main causes of extracellular acidosis. Anaerobic glycolytic pathway is much less efficient than OXPHOS in producing energy, as only 2 molecules of ATP are produced per glucose molecule, versus the ~36 ATP units usually produced in OXPHOS. Glycolysis generates ATP more rapidly than OXPHOS, and this offers a selective advantage to rapidly growing tumor cells.

Glycolytic inhibitors are being designed that target the enzymes involved in the glycolysis pathway (Fadaka *et al*., 2017). D-Mannoheptulose, a heptose (monosaccharide), is a hexokinase inhibitor found in avocados. By blocking the enzyme hexokinase, it prevents glucose phosphorylation, inhibiting the breakdown of glucose. It behaves as a competitive inhibitor of hexokinase, an enzyme that phosphorylates hexoses such as glucose. The inhibition of hexokinase leads to the prevention of glycolysis (Liu *et al*, 2002).

2.5.1 Glycolysis in cancer cells

Cancer cells mainly produce energy by an increased rate of glycolysis (200 times more as compared to normal tissues of origin) followed by fermentation of lactate in the cytosol of the cell, even if oxygen is plentiful (Alfarouk *et al*., 2014). Increased glycolysis is the main source of energy supply in cancer cells that use this metabolic pathway for ATP generation. The immune system can prevent tumor growth by eliminating cancer cells (Kheshwant *et al.*, 2016).

Lactate dehydrogenase (LDH) is a tetrameric enzyme with 2 subtypes: LDH-A and LDH-B. In hypoxic cells, the conversion of pyruvate into lactate is catalyzed by LDH-A, whilst LDH-B catalyzes the conversion of lactate into pyruvate. Lactate accumulation substantially reduces intracellular pH which is detrimental to the cell. Production of NAD+ from the oxidation of cofactor NADH is necessary at the reduction step of pyruvate into lactate, to continue glycolysis. Evidence suggests that LDH-A, which is upregulated in invasive glycolytic cancers, plays a critical role in cell proliferation. This allows the tumors to survive even in low oxygen levels (Jin *et al*, 2013).

2.5.2 Metabolic pathways in cancer

Cancer metabolism refers to the alterations in cellular metabolism pathways. The metabolism of cancer cells is glycolysis dependent. Cancer cells continuously rely on glycolysis rather than oxidative phosphorylation even under aerobic conditions,

which require high quantities of glucose to produce energy and support the metabolic function (i.e., the Warburg effect) (Warburg, 1956). This effect shows that glucose uptake of cancer cells is higher than that of normal tissues. Thus, the glucose metabolism could be targeted as a site for chemotherapeutic intervention by agents (Al-Shammari *et al*., 2019).

Cancer is a disease condition arising from uncontrolled division of cells in the body to form lumps of tissues called tumors. There are many different types of cell in the body, and many different types of cancer which arise from different types of cell. Some are more easily treated than others, particularly if diagnosed at an early stage (Fadaka *et al*., 2017).

2.5.3. Glycolysis and differences between cancer cells and normal cells

Aerobic glycolysis occurs when oxygen is plentiful, and the final product is pyruvate along with the production of 8 ATP molecules. Anaerobic glycolysis occurs when oxygen is scarce, and the final product is lactate along with the production of 2 ATP molecules (Chaudhry and Varacallo, 2019). In normal cells, the last step in the breakdown of glucose is called oxidative phosphorylation (OXPHOS). It takes place in the mitochondria, produces a large amount of ATP, and requires oxygen for completion (Urry *et al*., 2017).

Aerobic breakdown of glucose consists of four stages: glycolysis, pyruvate oxidation, Krebs cycle and Electron transport chain (ETC). Increased glycolysis is the main source of energy supply in cancer cells that use this metabolic pathway for ATP generation (Kheshwant *et al*., 2016). Cancer cells only partially break down sugar molecules unlike healthy cells that burn the entire molecule of sugar to capture a large amount of energy as ATP. They overuse the first step of respiration, glycolysis, and frequently do not complete the second step, oxidative phosphorylation. As a result, cancer cells need to use a lot more sugar molecules to grow (Elf and Chen, 2014).

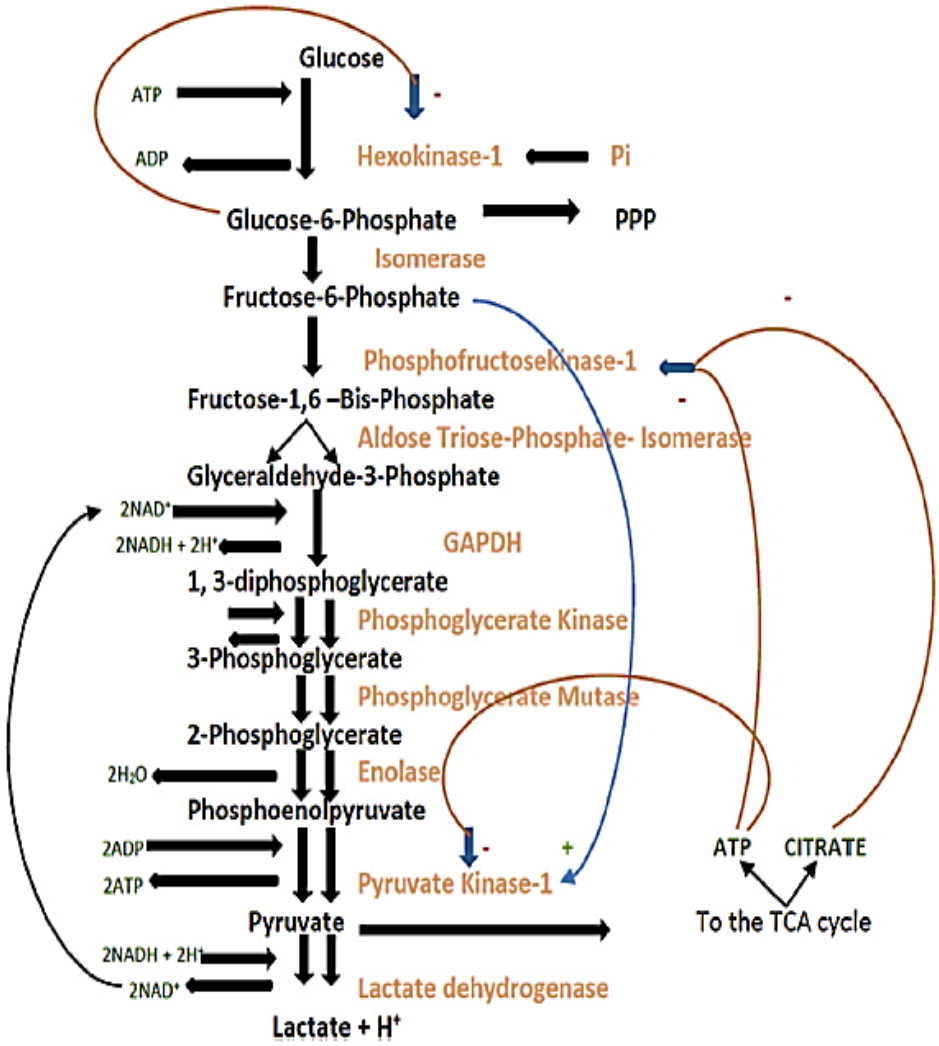


Figure (2-5): Glycolysis in tumor cell (Fadaka et al., 2017).

This produces far less energy than oxidative phosphorylation and as cancer cells require a lot of energy to grow, this seems paradoxical. The Warburg effect may be beneficial to cancer cells because it provides precursors for many biosynthetic pathways. These precursors include amino acid precursors and NADPH and ribose sugars for DNA and RNA synthesis. The Warburg effect may be caused by impaired oxygen sensing in cancer cells. Glycolytic enzymes such as Glucose transporter 1 (GLUT1), lactate dehydrogenase, pyruvate kinase and the lactate exporter are unregulated in cancer cells, whilst pyruvate dehydrogenase is inhibited leading to increased glycolytic flux and impaired ability of pyruvate to enter oxidative phosphorylation (Fadaka et al., 2017) (Figure (2-5)).

2.5.4 Warburg effect and the glycolytic inhibitors

Warburg effect phenomenon, is considered one of the most fundamental metabolic alterations during malignant transformation. One hallmark of cancer is the shift from aerobic to anaerobic metabolism seen within tumor cells, referred to as the Warburg Effect (Melkonian and Schury, 2019). The increased dependence of cancer cells on glycolytic pathway for ATP generation provides a biochemical basis for the design of therapeutic strategies to kill cancer cells by pharmacological inhibition of glycolysis (Kheshwant, *et al*., 2016). In the presence of oxygen, nonproliferation (differentiated) tissues first metabolize glucose to pyruvate via glycolysis and then completely oxidize most of that pyruvate in the mitochondria to CO_2 during the process of oxidative phosphorylation, (Figure (2-6)).

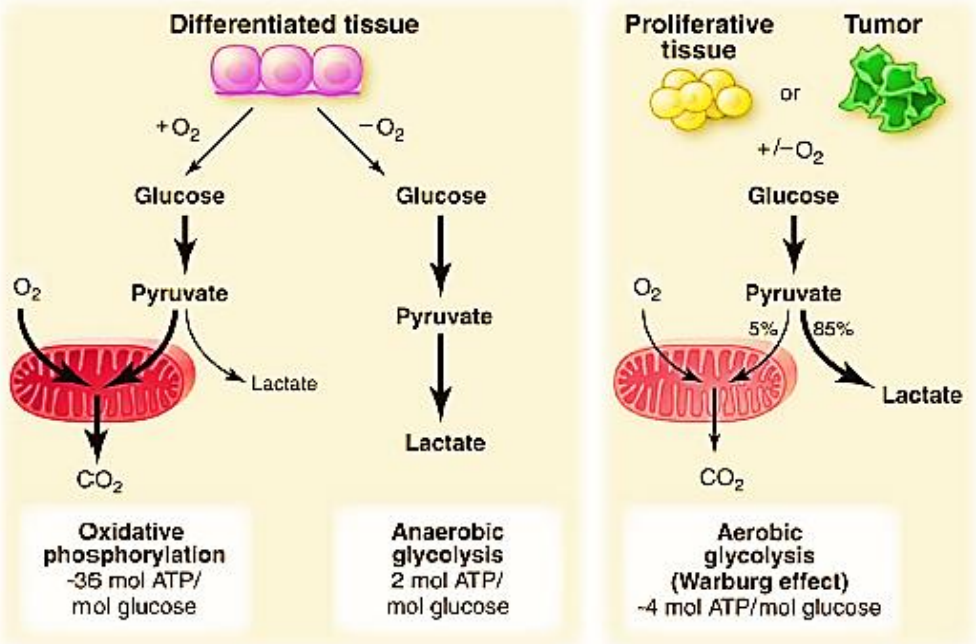


Figure (2-6): Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect) (Vander Heiden *et al*, 2009).

Oxygen is essential for this process as it is the final electron acceptor to completely oxidize the glucose. When oxygen is limited, cells can redirect the pyruvate generated by glycolysis away from mitochondrial oxidative phosphorylation by generating lactate (anaerobic glycolysis). This generation of

lactate during anaerobic glycolysis allows glycolysis to continue (by cycling NADH back to NAD+), but results in minimal ATP production when compared with oxidative phosphorylation. Warburg originally proposed that the aerobic glycolysis in cancer cells was due to a permanent impairment of mitochondrial oxidative phosphorylation (Kroemer and Pouyssegur, 2008). Malignant cells exhibit significantly elevated glycolytic activity even in the presence of sufficient oxygen, and this phenomenon is considered as the most fundamental metabolic alteration in malignant transformation, or 'the origin of cancer cells' (Hammoudi et al., 2011).

Warburg observed that cancer cells tend to convert most glucose to lactate regardless of whether oxygen is present (aerobic glycolysis). This property is shared by normal proliferative tissues. The mitochondria remain functional and some oxidative phosphorylation continues in both cancer cells and normal proliferating cells. Nevertheless, aerobic glycolysis is less efficient than oxidative phosphorylation for generating ATP. Under anaerobic conditions, pyruvate has a different fate i.e., instead of entering the mitochondria, the cytosolic enzyme lactate dehydrogenase converts it to lactate. Although lactate itself is not utilized by the cell as a direct energy source, this reaction allows the regeneration of NAD+, an oxidizing cofactor necessary to maintain the flow of glucose through glycolysis, from NADH. Glycolysis produces 2 ATP molecules per glucose molecule, providing a direct means of producing energy in the absence of oxygen. This process of breaking down glucose in the absence of oxygen is aptly named anaerobic glycolysis (Chen *et al*., 2017).

In proliferating cells, ~10% of the glucose is diverted into biosynthetic pathways upstream of pyruvate production. Cancer cells produce large amounts of lactate regardless of the availability of oxygen; hence their metabolism is often referred to as aerobic glycolysis. Warburg originally hypothesized that cancer cells develop a defect in the mitochondria that leads to impaired aerobic respiration and a subsequent reliance on glycolytic metabolism. There is an advantage to oxidative

metabolism during nutrient limitation and non-oxidative metabolism during cell proliferation (Vander Heiden *et al*., 2009). The glycolytic inhibitors are particularly effective against cancer cells with mitochondrial defects. Because increased aerobic glycolysis is commonly seen in human cancers and hypoxia is present in most tumor microenvironment, development of novel glycolytic inhibitors as a new class of anticancer agents is likely to have broad therapeutic applications. The biological differences between normal and cancer cells are essential for the design and development of anticancer drugs with selective anticancer activity. Each reaction in the glycolytic pathway is catalyzed by a specific enzyme or enzyme complex. (Pelicano *et al*., 2006).

This effect means higher glucose uptake by cancer cells compared to normal tissues, which can be used as target for selective therapy by targeting glucose metabolism as a site for chemotherapeutic intervention. Targeting cellular metabolism can improve cancer therapeutics (Zhao *et al*., 2013). Warburg hypothesis explains that cancer cells mainly produce energy by an increased rate of glycolysis followed by fermentation of lactate in the cytosol of the cell, even if oxygen is plentiful (Alfarouk *et al*., 2014).

Hexokinase is a tissue-specific isoenzyme that catalyzes the phosphorylation of glucose to glucose-6-phosphate (G6P), after glucose enters the cell via glucose transporters (GLUT). This ATP-dependent reaction is the first step in glycolysis, and it is also a rate limiting step as hexokinase possesses the ability to transfer an inorganic phosphate group from ATP to a substrate. (Kheshwant *et al*., 2016). ATP is a key indicator of cellular activity and has been utilized as a measure of cell viability and cytotoxicity in research and drug discovery (Schwarzer *et al,* 2008). The electron transport chain requires oxygen as the final electron acceptor, inadequate tissue oxygenation inhibits the process of oxidative phosphorylation (Melkonian and Schury, 2019). Anaerobic glycolysis serves as a means of energy production in cells that cannot produce adequate energy through oxidative

phosphorylation (Chen et al., 2017). As anaerobic glycolysis allows more rapid production of ATP, glycolysis is approximately 100 times faster than oxidative phosphorylation (Melkonian and Schury, 2019).

2.6 Programmed cell Death (Apoptosis)

Cell death was once believed to be the result of one of two distinct processes, apoptosis (programmed cell death) or necrosis (uncontrolled cell death); in recent years, several other forms of cell death have been discovered highlighting that a cell can die via a number of differing pathways. Apoptosis is characterized by a number of characteristic morphological changes in the structure of the cell, together with a number of enzymes-dependent biochemical processes. Failure of apoptosis and the accumulation of damaged cells in the body can result in various forms of cancer. An understanding of the pathways is therefore important in developing efficient therapeutics (D'Arcy, 2019). The increased understanding of how environmental exposures cause diseases through apoptosis disruptions could ultimately lead to the discovery of therapeutic strategies (Rager, 2015).

Programmed cell death is a natural process of eliminating cells that have been produced redundantly, developed improperly or have genetic damages. Apoptosis can be initiated by 3 alternative pathways: the extrinsic pathway, which is mediated by death receptors on cell surface; the intrinsic pathway, which is mediated by permeabilization of the mitochondria and the stress signaling pathway of endoplasmic reticulum (ER) and mitochondria (Kuwana *et al*., 2016). Apoptosis, the cell's natural mechanism of death, is a promising target for anticancer therapy. Both the intrinsic and extrinsic pathways use caspases to carry out apoptosis through the cleavage of hundreds of proteins. In cancer, the apoptotic pathway is typically inhibited through a wide variety of means. Many of these changes cause intrinsic resistance to the most common anticancer therapy (Pfeffer and Singh, 2018). Initiation of apoptosis occurs principally by signals from 2 distinct but convergent

pathways; the extrinsic (receptor-initiated) and intrinsic (mitochondrial) pathway (Pistritto *et al*., 2016). The last phase of apoptosis (Execution Phase) is mediated by a proteolytic cascade of proteases that belongs to the caspase (cysteine-aspartic proteases) family (Salvesen *et al.*, 2016). At early stages of apoptosis, dying cells secrete soluble factors that induct phagocytes and facilitate clearance of apoptotic cells before they undergo secondary necrosis and release their cellular contents that can result in inflammation. Also, apoptosis may be essential for the prevention of tumor formation (Poon *et al*., 2014).

The apoptotic bodies are gulped by macrophages and discarded from the tissue unless they give rise to an inflammatory response. Apoptotic cells can be recognized by the following stereotypical morphological changes. The cell shrinks, shows deformation, and loses contact with its neighboring cells, its chromatin condenses and marginates at the nuclear membrane, the plasma membrane is budding, and finally the cell is fragmented into compact membrane-enclosed structures called 'apoptotic bodies' which contain cytosol, the condensed chromatin, and organelles (Salvesen *et al*., 2016) (Figure (2-7)).

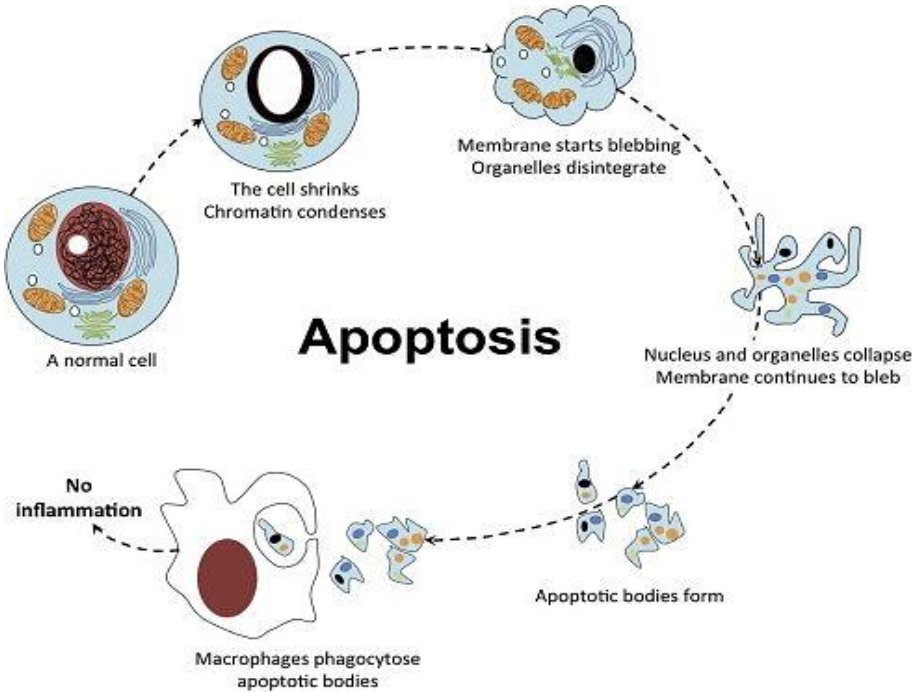


Figure (2-7) Programmed cell Death (Apoptosis) (Srivastava, 2007).

2.7 Cancer Therapy

2.7.1 Conventional cancer therapeutics

There are widely accepted therapies and used by most healthcare professionals (NCI., 2016). The spectrum of the conventional cancer treatment includes 3 basic methods: operations, chemotherapy, and radiotherapy (Damyanov *et al*., 2018).

2.7.1.1 Surgical Therapy

Surgical therapy is considered the oldest type of cancer treatment. It had been an effective treatment to cure patients from cancer (Tai, 2013). Randomized trials have shown that the addition of preoperative chemotherapy to surgery significantly improves survival (Ajani *et al*., 2019). The primary goals of breast cancer surgery are to remove the cancer from the breast and determine the stage of the disease. Surgical treatment for breast cancer involves breast-conserving surgery, a surgery in which only the part of the breast containing the cancer is removed, or mastectomy. The presence of cancer cells in the lymph nodes will help in determination the need for subsequent therapy (Miller *et al*., 2019). Surgical therapy is very unlikely to cure breast cancer that has spread to other parts of the body (Harris *et al*.,2014).

2.7.1.2 Radiotherapy

Radiotherapy utilizes ionizing radiation to control or kill malignant cells. Ionizing radiation works by damaging the DNA of cancerous tissue leading to cellular death (Borrego-Soto *et al*., 2015). Despite the fact that radiation damages both cancer and normal cells. The goal of radiation therapy is to damage as many cancer cells as possible. (McIntyre and Harris, 2015). It is mainly applied to primarily treat solid tumors when combined with the other conventional methods. It is used as a palliative treatment to reduce the disease symptoms. (Christo *et al*., 2018).

2.7.1.3 Chemotherapy

Chemotherapy agents can be divided into many types: Alkylating agents such as cyclophosphamide and antibiotics (Chock *et al*., 2010), and platinum compounds

such as cisplatin and antimetabolites that have an effect on nucleic acids (Torrence, 2012).The benefit of chemotherapy depends on multiple factors, including the size of the cancer, the number of lymph nodes involved, and the presence of estrogen or progesterone receptors (Cortazar *et al.*, 2014). Drug combinations are more effective than a single drug for breast cancer treatment, and many combinations are being used (Moura *et al*., 2015).

One problem with chemotherapy is that they are toxic to normal dividing cells as their high growth fraction is as that of cancer cells (Mitchison, 2012). Chemotherapy can be given before surgery (neoadjuvant) or after surgery (adjuvant). In most cases, chemotherapy is most effective when drug combinations are used. Drugs for breast cancer that has spread include taxanes, such as paclitaxel, docetaxel and albumin-bound paclitaxel, anthracyclines, platinum agents (cisplatin and carboplatin), vinorelbine and capecitabine. Although drug combinations are often used to treat early breast cancer, advanced breast cancer more often treated with single chemo drugs. (Smith *et al*., 2018).

2.7.1.4 Hormone therapy

Hormone therapy is significantly less applicable additional method to treat cancer (Damyanov *et al*., 2018). Hormone therapy is often used after surgery to help reduce the risk of cancer recurrence. Sometimes it is started before surgery (Miller et al., 2019). Women whose breast cancers test positive for estrogen or progesterone receptors can be given hormone therapy to lower estrogen levels or to block the effects of estrogen on the growth of breast cancer cells. Tamoxifen and toremifene are drugs that prevent the binding of estrogen to breast cancer cells and are effective in both postmenopausal and premenopausal women (Nicholas, 2019).

2.7.1.5 Immunotherapy

Immunotherapy is the treatment of disease by activating or suppressing the immune system. Immune system plays a major role in rejecting transformed cells

(Smyth et al., 2006). The disadvantages of surgery such as recurrence of tumor or toxicity of radiotherapy or chemotherapy can be substantially reduced by immunotherapy when used in combination with these treatment modalities (Mohan et al., 2019). Immunotherapy is a significantly less applicable additional method. This application is more in the field of the experimental research (Damyanov *et al*., 2018). Immunotherapy involves manufacturing antitumor effects primarily through the action of natural defense processes (Smith and Andreansky, 2014). The work with IL-2 in treating cancer substantiated the important role of adaptive immunity in cancer therapy (Guo, 2018).

Normal antibodies bind to external pathogens, but the modified immunotherapy antibodies bind to the tumor antigens marking and identifying the cancer cells for the immune system to inhibit or kill (Nielsen and Hawkes, 2019). Immunotherapies can be categorized as active, passive, or hybrid. Active immunotherapy directs the immune system to attack tumor cells by targeting tumor antigens. Passive immunotherapies enhance existing anti-tumor responses (Korneev *et al*., 2017). Passive antibody therapies involve targeting of cell surface receptors (Espinoza-Sánchez and Götte, 2019).

2.7.2 Novel therapeutics

Alternative or complementary therapies, which are not as widely used (NCI., 2016)

2.7.2.1 Anti-Angiogenesis Therapy

Angiogenesis permits growth of cancer mass. Once the mutated cell growth starts, the body recognizes this by cellular communication and starts transmitting new micro-blood vessels to the region. Anti-angiogenesis will inhibit the expansion of these new blood vessels and inhibit the growth of the cancer (Negro, 2015).

2.7.2.2 Biological Therapy

Biological therapy has a very important modality for cancer treatment that produces antitumor effects primarily through the action of natural host defense

mechanisms (Devita *et al*., 2015). A type of treatment that uses substances made from living organisms to treat disease. Some biological therapies stimulate or suppress the immune system to help the body fight cancer, infection, and other diseases. They may also lessen certain side effects caused by some cancer treatments (NCI, 2016). Biological therapy is an effective treatment for a range of immune-mediated inflammatory diseases (Baumgart et al., 2019).

2.7.2.3 Gene Therapy

Gene therapy can be broadly defined as the transfer of genetic material to cure a disease or at least to improve the clinical status of a patient. One of the basic concepts of gene therapy is to transform viruses into genetic shuttles, which will deliver the gene of interest into the target cells (Verma and Mishra, 2016). Gene therapy is a replacement of a deficient gene product or a correction of an abnormal gene. It can be done either in vitro, or in vivo, where the cells cannot be cultured, or are replaced in the affected individual (Mueller and Young, 2012).

2.7.2.4 Virotherapy

The use of viruses for cancer treatment began before the 1950s (Kelly and Russell, 2007). It has been identified for over 70 years that some viruses like Newcastle disease virus (NDV) have the ability to destroy cancer cells. These viruses are units either used with non-genetic manipulation or biotechnology for increasing properties in animal models and human clinical trials (Delwar *et al*., 2016; Kaufman *et al*., 2016).

NDV has been classified beside alternative viruses like reovirus and parvovirus as viruses with inherent oncolytic effects; these viruses were manipulated in to how they are attenuated in normal cells while not losing their ability to lyse tumor cells. (Zimmermann *et al.,* 2014). Numerous viruses are tested in various animals and human models for their anti-cancer efficiency and tumor specificity (Addisu *et al.,* 2016). These viruses are used for cancer treatment either with or without genetic

manipulation (Wong, *et al.,* 2010). Oncolytic virotherapy divided in two main groups, oncolytic wild natural occurring viruses with preferential replication in human cancer cells and gene-modified viruses engineered to achieve selective oncolysis (Garcia et al, 2008).

2.7.2.4.1 Newcastle Disease Virus (NDV)

NDV, a member of the Avulavirus genus of the Paramyxoviridae family (Meng *et al*., 2019), infects a number of avian species. An oncolytic virus selectively kills tumor cells, and it is not a natural human pathogen (Bai *et al*., 2015). NDV causes highly infectious and economically significant Newcastle disease in birds of various species worldwide (Costa-Hurtado *et al*., 2015).

Pathogenic NDV causes an economically necessary animal disease in various bird species worldwide (Miller and Koch, 2013). Based on the virulence in infected birds, NDV has been categorized into 3 pathotypes: Lentogenic which causes moderate respiratory disease, Mesogenic which causes output respiratory and nervous signs and the velogenic which causes severe gastrointestinal lesions and neurological disease (Pantin-Jackwood *et al*., 2015). NDV selectively replicates in human cancerous cells stinting normal cells and it activates apoptosis programmer in cancerous cells (Biswas *et al*., 2012). The anti-cancer effect of NDV has been tested on various human cancers (Farkona *et al*., 2016).

2.7.2.4.2 Molecular Biology of NDV

NDV is spherically shaped and surrounded with a lipid bilayer underneath which is the matrix protein that forms the inner layer of virus (Battisti *et al*., 2012), (Figure (2-8)). The genomic RNA in NDV consists of 6 genes encoding at least 8 proteins (Jin *et al*., 2016). An interesting aspect of NDV is its ability to selectively replicate in tumor cells. site-specific alterations can be happened in the genomes of RNA-virus by mutagenesis of single nucleotides or insertion of foreign genes. (Bai et al., 2015).

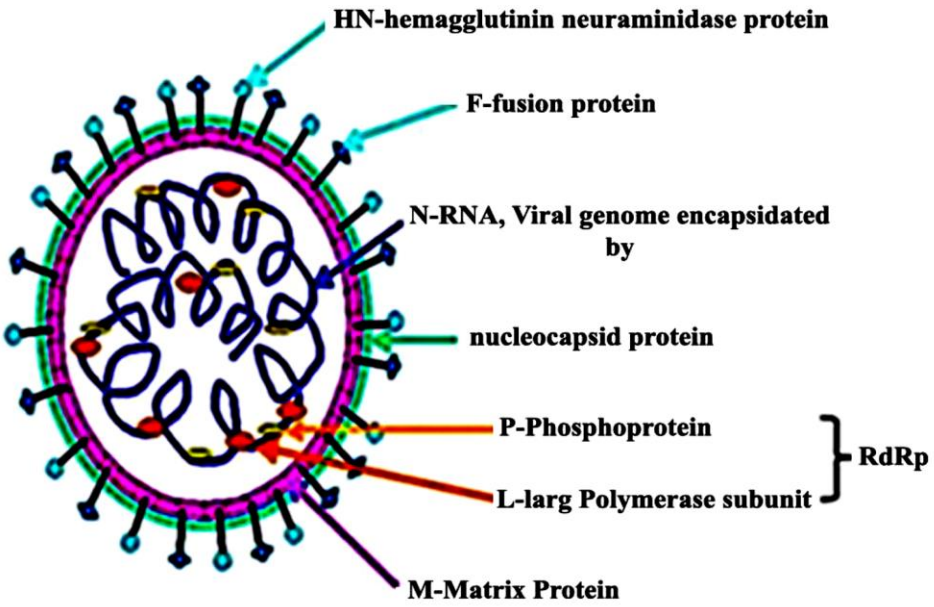


Figure (2-8): Structure of Newcastle Disease Virus (Battisti *et al*., 2012).

2.7.2.4.3 Mechanisms of NDV-induced Oncolysis

When infecting a cancer cell, it replicates speedily and infects neighboring tumor cells through the discharge of progeny virions which are noticeable 3 hrs post-inoculation, and plaques develop within 2 days post-inoculation (Elankumaran *et al*., 2010). Several mechanisms for its anti-tumor effect have been proposed in humans. Firstly, the lytic strains of the virus may artlessly kill tumor cells by a direct route and initiate effectiveness in preventing tumors. Secondly, the non-lytic strains injected into the tumor cell membrane after infection by the viral protein may develop an immune response. Finally, the virus itself may stimulate the host to produce effector cytokines such as interferons (IFN-γ) or tumor necrosis factors α (TNF-α), which activate natural killer (NK) cells, macrophages, and sensitized T-cells. (Sweiss *et al*., 2011). Apoptosis is susceptible to disruption by mutations as it is a gene-controlled process (Hassan *et al*., 2014). Critical linkage between apoptosis and cancer indicates that any curative planning aimed at specifically triggering apoptosis in cancer cells will have a potential therapeutic effect (Su *et al*., 2015). It was reported that NDV induces expression of varied cytokines which may activate the host cell death pathway inflicting oncolysis (Lam *et al*., 2014).

NDV might activate programmed cell death through the extrinsic and intrinsic death pathway. Additionally, there are reports that NDV augments the expression of evoked nitric oxide synthase in infected cells (Hrabak *et al.*, 2006). Viruses are utilized as possible anticancer therapeutics for cancer treatment (Assi, 2013), (Figure (2-9)).

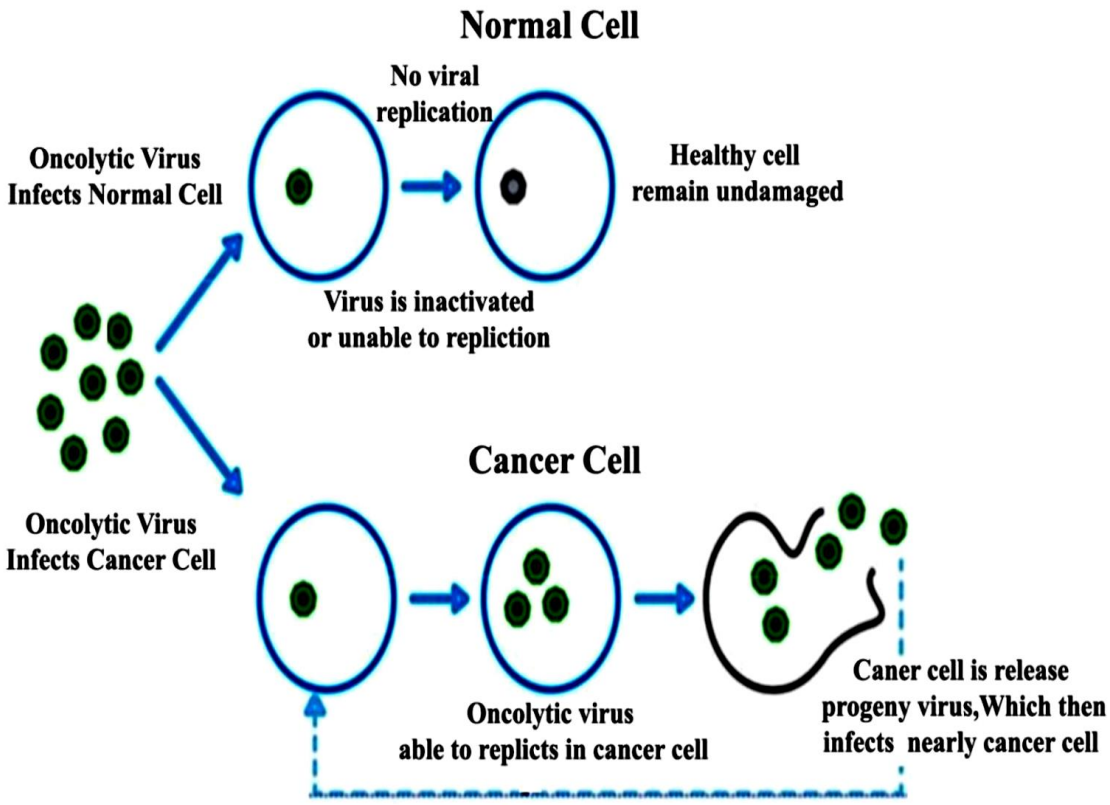


Figure (2-9): Oncolytic virus function (Assi *et al*., 2013)

2.7.2.4.4 Safety of NDV Administration as Anticancer Agent

Scientists are interested in the therapeutic effect of NDV because of its tumor selectivity (Lam *et al*., 2014). NDV strains can selectively replicate up to 10000 times better in tumor cells, but not in normal cells (Alamares *et al*., 2010). Numerous reports had shown the inability of the virus to replicate in non-transformed cells, such as fibroblast, resting T lymphocytes, and normal primary culture (Fábián et al., 2007). NDV is an immunostimulatory agent, as it can induce anti-tumor activities of a variety of effector cells, including NK cells, macrophages, and cytotoxic T lymphocyte (CTL) (Assayaghi *et al*., 2016).

Iraqi strain of Newcastle disease virus (NDV) is a novel anti-tumor therapy. Virulent strains of NDV are being used in gene therapy as possess a number of desirable oncolytic properties. NDV Iraqi strain can reduce tumor cell infiltration, invasion, and metastasis (Al-Shammari *et al*., 2013). Virotherapy is a promising agent against cancer because of its safety and selectivity. NDV is safe, it selectively targets tumor cells, and it could be used to augment other chemotherapeutic agents and reduce their toxicity by halving the administered dose and replacing the eliminated chemotherapeutic agents with it (Al-Shammari et al., 2016).

2.7.2.4.5 Replication of NDV

NDV selectively infects growing cells to produce more viral copies. The infection process starts when the virus interacts with a target receptor on the host cell, after which the initial steps of virus entry begin. The whole replication process of NDV occurs in the cytoplasm. The replication process can be divided into phases:1-initiation attachment, penetration 2- Replication genome synthesis, RNA production with protein synthesis and finally 3-Release assembly, maturation exit from the cell. NDV generates dsRNA during replication (Chu *et al*., 2019). Replication of Newcastle disease virus explain the NDV-infection and cellular responses. NDV attaches via the hemagglutinin-neuraminidase (HN) protein to sialic acid containing host cell surface receptors. The viral fusion protein (F) initiates fusion of viral and host cell membranes. The viral RNA polymerase starts replication in the cytoplasm of the target cell by transcription of viral negative single-strand RNA into positive single-strand RNA necessary as template for mRNA and protein synthesis. The viral proteins undergo processing in the rough endoplasmic reticulum and the Golgi apparatus. In tumor cells they are assembled at the host cell membrane where budding leads to newly produced virions. In nontumorigenic cells, endocytosis of viral components bound to Toll-like receptors (TLR) and the appearance of intermediate double strand RNA during the process of viral replication induces danger signals to the host cell, triggering the transcription of various

protective genes. DsRNA is recognized by RNA helicase (Fiola et al., 2006). (Figure (2-10)).

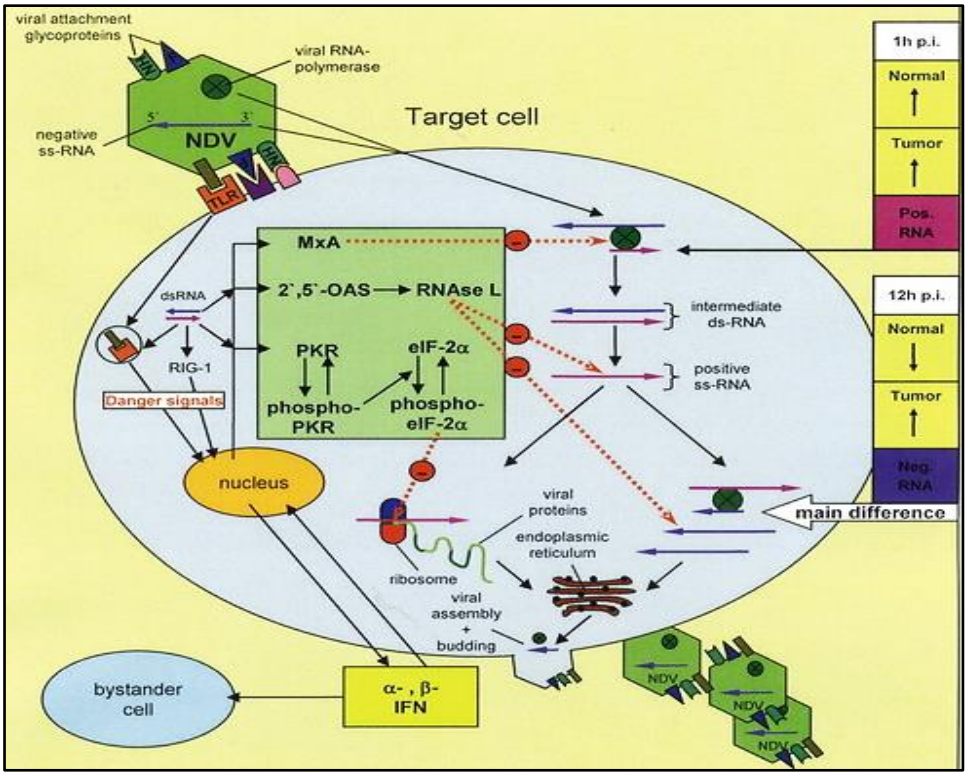


Figure (2-10) Replication of Newcastle Disease virus (Fiola et al., 2006)

2.7.2.4.6 Clinical usage of NDV in cancer therapy

NDV infects the host cells and then replicates itself. Scientists are interested in NDV because it can replicate more quickly in human tumor cells than in normal cells and has oncolytic effects (Lam *et al*., 2011). Newcastle disease virus (NDV) is an oncolytic virus may interact with radiotherapy to enhance treatment against cancer cells. (Vijayakumar *et al.*, 2019). NDV is an avian virus that causes deadly infection to over 250 species of birds. Many reports have demonstrated effect of the oncolytic virus towards human tumor cells. The interesting aspect of NDV is its ability to selectively replicate in cancer cells. NDV strains can be potential therapeutic agent in cancer therapy (Ginting *et al*., 2017). NDV induces both extrinsic and intrinsic apoptosis of malignant cells. NDV infection elicits both innate and adaptive antiviral immunity, resulting in cross-activated anti-tumor immune responses (Schwaiger *et al*., 2017). The majority of tumor cells could be infected by NDV, and the viral

replication was detected into cells by the increase of viral antigens on the cell surface (Fiola et al., 2006). The first report of the application of NDV to treat human cancers was in the early 1950s, when NDV were injected directly into uterine carcinoma, resulting in partial necrosis, but followed by regrowth. Many reports showed the possibility of NDV as a therapeutic agent in cancer treatment, many studies both in mouse models and in human clinical trials which showed favorable results (Lam et al., 2011). Localized therapy with oncolytic NDV induces an inflammatory response which leads to lymphocyte infiltration and an antitumor effect in distant tumors without dissemination of the virus (Meng *et al.*, 2019).

2.7.2.5 Combination therapy

Combination therapy, a treatment modality that combines two or more therapeutic agents, is a cornerstone of cancer therapy. The amalgamation of anti-cancer drugs enhances efficacy compared to the mono-therapy approach because it targets key pathways in a characteristically synergistic or an additive manner. This approach potentially reduces drug resistance, while simultaneously providing therapeutic anti-cancer benefits, such as reducing tumor growth and inducing apoptosis (Mokhtari et al., 2017). Targeting cancer cells metabolism is a promising strategy in inhibiting the progression of cancer cells. The NDV was used as a combination virotherapy to enhance the anti-tumor effect. Human and mouse-breast cancer cells were treated with NDV and/or 2-Deoxy glucose, and the combination treatment showed significant tumor growth inhibition compared to single treatments in vivo (Al-Shammari *et al.*, 2019). The therapeutic challenge is not only going to be maximizing the effectiveness of individual agents, but also to integrate these drugs into the best combination therapy (Mishra *et al.*, 2013). Oncolytic virus has a better level of tumor specificity relative to chemotherapy drugs attributable to each associate innate preference for tumor cells (Ottolino-Perry *et al*., 2010). The theory supported that combination chemotherapy assaulting tumor cells by using different mechanisms of action can prevent tumor cell from having enough time to improve

resistant to treatment (Post *et al*., 2003). Synergistic antitumor combination of NDV and 2-Deoxy glucose had a greater effect on cancer cells, than their individual effects in breast cancer cell lines (Al-Shammari *et al*., 2019). The combination of the 2-targeted drugs was more effective. There is a certain synergistic effect in tumor cell growth inhibition. This will be a theoretical foundation for treatment of cancer in clinical applications (Zhang *et al*., 2013). The apoptosis induction in triple combination had a better effect in comparison with double combination which had a better effect than their individual effect (Dalirsani *et al*., 2012).

The combined use of 2 or more agents is often advantageous as it may permit the lowering of the clinical dosages thereby decreasing the overall toxicity and thus providing the potential for synergistic effects between agents. NDV is the only oncolytic virus that showed no serious side effect in the clinical field (Al-shammari *et al*., 2012). Oncolytic virus will by selection infect, replicate in, and kill tumor cells with least impact on normal tissue. A virus ability to recognize and enter specific receptors on the surface of tumor cells, additionally aids in the disposition of the tumor cell towards viral replication (Pol, 2012).

The strategy of combination therapy is by attacking tumor cells through different mechanisms of action to prevent cancer cells from developing resistance to therapy (Kumar, 2014). The Iraqi oncolytic strain of NDV kills tumor cells by several mechanisms, such as generation of specific immune response against infected tumor cells (Al-Shammari *et al*., 2014). NDV induces apoptosis in the infected cells (Al-Shammari *et al.,* 2015). Phytotherapy has been used to treat a different type of diseases including cancer for a long time, and it was a source for different active anti-tumor agents. NDV-based monotherapeutics have not been very useful to some resistant tumors. Thus, the efficiency of oncolytic NDV must enhance by combining NDV with other novel therapies, the possibility of improving the oncolytic effect induced by NDV. The novel combination recommended for clinical application for cancer therapy (Al-Shammari et al., 2020).

CHAPTER-THREE

3. Material and Methods

Subject:

The research project approval committee was formed by the Council of the College of Medicine / Al-Nahrain University on 4/7/2018. The research project was approved by the scientific committee in the Department of Chemistry and Biochemistry / College of Medicine on 11/7/2018. The approval of the Institutional Review Board (IRB) at the College of Medicine on the research project was obtained on 15/7/2018.

The research project was carried out at the Iraqi Center for Cancer and Medical Genetics Research / Al-Mustansiriyah University, with the support and assistance of the Department of Chemistry and Biochemistry / College of Medicine / Al-Nahrain University and the Department of Medical Chemistry / College of Medicine / University of Al-Qadisiyah. The research project was completed and the thesis submitted on 9/1/2020.

The research project included propagating of Newcastle disease virus (NDV) (as Virotherapy) in Embryonated chicken eggs (ECE) and then it was isolated and purified. The virulence of the Virus (Titer) was measured by Hemagglutination assay (HA). Cell culture of Vero cell line was prepared, then it was treated with NDV to measure $TCID_{50}$ value. Cell culture of AMJ13 and MCF-7 cell lines were prepared, then the effect of Virus and Hexokinase inhibitor (D-Mannoheptulose) (as Phytotherapy) were studied separately on breast cancer cell lines (AMJ13 and MCF-7) and studying their effect together (Synergistically) on cell lines at 72 hr, and comparing their effect with the effect of Docetaxel drug (Positive control) as (Chemotherapy).

The effect of these factors was determined by measuring cytotoxicity ratio by MTT assay. The IC50 and Combination Index (CI) values were measured by Chou-Talalay analysis. The morphological changes and induction of apoptosis were

assessed by using the Crystal violet dye and Acridine Orange dye / Propidium Iodide) respectively to determine dead and viable cells and comparing their effect (Mono and Combination) on normal cell line (Rat Embryo Fibroblast) (REF). Also, the effect of these factors (virus, inhibitor and their combination) was studied on the glycolysis inhibition pathway after treated with cell lines at 72 hr. Hexokinase activity, concentration of (Pyruvate, ATP) and acidity (pH) (which represent lactate acid) were measured. The thesis has been written, designed, directed and performed the statistical analysis by the researcher under the supervision and review of the supervisors.

3.1 Materials

The reagents, stock, working solutions, equipment and apparatus used in the present study are shown in (Table (3-1), (3-2)).

3.1.1 Instruments

Table (3-1): The instruments and glassware used in this study

NO.	Instruments & Equipment	Company	Origin
1	Autoclave	Lab. tech	Korea
2	Beaker 100,50,25 ml	Santa Cruze	USA
3	Biological hood	ESCO	UK
4	Cell Culture plate flat bottom (96) well	Nunc	Denmark
5	Cell Culture plate U bottom (96) well	Nunc	Denmark
6	Centrifuge	Hetttich	Germany
7	Cooled Centrifuge	Hetttich	Germany
8	Cooler box	Coleman	China
9	Cover slips (22*22 mm).	Apel	China
10	Deep Freeze (-86 ^{o}C)	Lab. tech	Korea
11	Disposable syringes 20 ,10,5, 1 ml	Medeco	UAE
12	Disposable Tip (1000,10) µl	Cypress	Belgium
13	Distillation Apparatus	Running waters	USA
14	Elisa micro well system microplate reader	Asyshitech	UK
15	Elisa Plate reader, D-63303, Dreieich	Karl Kolb	Germany
17	Falcon cell culture flask (25,50 and 75) ml	Santa Cruze	USA
18	Fluorescent Microscope	Olympus	Japan

19	Forceps	Putex	India
20	Glass culture bottle	Santa Cruze	USA
21	Graduated cylinder	Santa Cruze	USA
22	Hemocytometer	HBG	Germany
23	Incubator	Memmert	Germany
24	Incubator with CO2	Lab. tech	Korea
25	Inverted Microscope	MEIJI Techno	Japan
26	Lab Spatula	Scienceware	China
27	Laminar air flow Cabinet	K&K	Korea
28	Light Microscope	Olympus	Japan
29	Light Microscope with digital camera	Scopetek	USA
30	Micro-pipette(2-20, 10-100 ,100-1000,) µl	Slamed	Germany
31	Multichannel micropipette (0 -10 µl)	Slamed	Germany
32	Multichannel pipette (10- 250 µl)	Slamed	Germany
33	Nalgene filter syringe (0.22.0.45)	Nalgen	USA
34	Oven	KENWOOD	Turkey
35	pH meter tester	Santec	China
36	Refrigerator	LG.	Korea
37	Refrigerator	Concorde	Korea
38	screw-capped bottles, 100 ml, 250 ml	Brand	India
39	Analytical balance	Stanton	UK
40	Sterile 10 ml centrifuge tubes.	Interlab	China
41	Surgical scissors	Putex	India
42	Surgical tong	Putex	India
43	Tissue culture flasks size (25,50 and 75) ml	Nunc	Denmark
44	Vortex mixer	Stuart	UK
45	Volumetric flask (50-250) ml	Santa Cruze	USA
46	Water bath	Memmert	Germany

3.1.2 Chemical and biological materials

Table (3-2): The chemical and biological materials used in the study

NO.	Chemical and biological materials	Company	Origin
1	Acridine Orange	Sigma –Aldrich	USA
2	AMJ13 human breast cancer cell line	ICCMGR	IQ
3	ATP determination kit	ElabScience	USA
4	crystal violet	Santa Cruz	USA
5	D- Mannoheptulose	Santa Cruz	USA

6	DMSO (Dimethyl Sulphoxide) (99.9%)	Santa Cruz	USA
7	Docetaxel drug	Santa Cruz	USA
8	Ethanol absolute (96%)	Hayman	England
9	Ethylene diaminetetra acetic acid (EDTA)	Merck	USA
10	Fetal bovine serum	Bio west	USA
11	HEPES buffer	Santa Cruz	USA
12	Hexokinase Assay Kit	ElabScience	USA
13	MCF7 human breast cancer cell line	ICCMGR	USA
14	Methyl Thiaozolyl Tetrazolium (MTT)	Bio west	USA
15	Minimum Essential Medium (MEM)	U.S Biological	USA
16	Monkey kidney (Vero) cell line	ICCMGR	USA
17	NDV	ICCMGR	IQ
18	Penicillin - Streptomycin	CAPRICORN	Germany
19	Phosphate buffer saline (PBS)	Sigma –Aldrich	USA
20	Propidium Iodide	Sigma –Aldrich	USA
21	Potassium hydroxide	Santa Cruz	USA
22	Potassium chloride	Santa Cruz	USA
23	Pyruvate determination kit	ElabScience	USA
24	RPMI -1640 media	Gibco	USA
25	REF cell line	ICCMGR	USA
26	Sodium Bicarbonate (99%)	Santa Cruz	USA
27	Trypsin -Versene	Santa Cruz	USA
28	Formaldehyde (37%)	Sigma –Aldrich	USA

3.1.3 Reagents for cell culture

All instruments have been sterilized by Autoclave and all reagents and buffers were prepared according to (Freshney, 2015).

3.1.3.1 Penicillin-Streptomycin (Pen-Strep) (Antibiotics)

Penicillin 500 mg powder and streptomycin 1 g powder were each dissolved in 5 ml Distilled Water (D.W) and stored separately at -20°C as stock solutions. Then, 1ml Penicillin and 0.5 ml streptomycin were removed from their stock solutions and added to each 1 L of culture media after sterilization with 0.22 filter syringe. Also, Penicillin–Streptomycin solution ready for use was bought from Sigma-Aldrich (Freshney, 2015).

3.1.3.2 Sodium Bicarbonate

It was made by dissolving 2.2 g of sodium bicarbonate (99% purity) powder in 1 L of culture media as suggested by the manufacturing company.

3.1.3.3 Phosphate buffer saline (PBS), without Ca^{2+} and Mg^{2+}

It was prepared by mixing Sodium chloride (NaCl) 8 gm, Potassium chloride (KCl) 0.2 gm, disodium hydrogen phosphate (Na_2HPO_4) 0.9 gm, and Potassium dihydrogen phosphate (KH_2PO_4) 0.2 gm. The volume was diluted to the mark (1L) using trebled distilled water (T.D.W).

In addition, dry powdered phosphate-buffered saline was used to prepare 1L of phosphate-buffered Saline: 10.8 g of PBS powder was dissolved in 1000 ml T.D.W, 0.5 ml ampicillin and 0.25 ml streptomycin were added and filtered by Nalgene filter units (pore size, 0.22 μm) in sterilized bottles and stored in the fridge at 4°C until use. The pH was 7.2 (Freshney, 2015).

3.1.3.4 Trypsin

Trypsin solution for primary cultures was prepared by dissolving 2.5 g of trypsin in 100 ml of PBS (25mg/ml) at the pH of 7.2, and stirred continuously on a magnetic stirrer at room temperature. Then they were sterilized by Nalgene filter 0.22 μm, and stored at 4 °C (Freshney, 2015).

3.1.3.5 Stain

3.1.3.5.1 Methyl Thiazolyl Tetrazolium (MTT) Solution

Methyl Thiazolyl Tetrazolium (0.2 g) was dissolved in 100 ml of PBS in order to prepare 2 mg/ml of the dye (Betancur-Galvis *et al*., 2002). The solution was filtered through 0.2 μm syringe filter to remove any blue Formosan product as recommended by (Russel, 1998), and then stored in sterile dark, screw-capped bottles at 4°C. The solution was used within 2 weeks of preparation (Betancur-Galvis *et al*., 2002).

3.1.3.5.2 Crystal Violet stain

It was prepared by dissolving 5 g of crystal violet powder in 200 ml methanol. The solvent was filtered using Whatman No.1 filter paper, 50 ml of formaldehyde (37%) was added, the volume of mixture was completed to 1000 ml by distilled water, and excess solid residue was filtered off using Whatman No.1 filter paper (Yaseen, 1990).

3.1.3.5.3 Acridine Orange-Propidium Iodide (AO/PI)

Acridine Orange/Propidium Iodide Stain is a cell viability dye that causes viable nucleated cells to fluoresce green and nonviable nucleated cells to fluoresce red. It can be used to assess cell viability with the automated fluorescence cell counters. Acridine orange permeates viable cells and binds to nucleic acids. Binding to dsDNA causes Acridine orange to fluoresce green and binding to ssDNA or RNA causes it to fluoresce red.

Propidium iodide binds to nucleic acids. Not being able to permeate intact cell membranes, it is taken up by nonviable cells and cells with compromised membranes. Once bound to nucleic acids, its fluorescence increases 20-30-fold causing the cell to fluoresce red. Here, 1µl of 10 µg/ml Acridine orange/Propidium iodide (AO/PI) by PBS reagent was used (Paramasivam *et al.* 2012).

3.1.3.6 Tissue culture media

All media were prepared as the manufacturer's recommendation.

3.1.3.6. 1 Preparation of Minimum Essential Medium (MEM)

It was prepared by dissolving 11g of MEM (with L-glutamine) (US biological, USA) powder in ~600 ml of TDW and adding Sodium bicarbonate powder (2.2 g), 10 ml of 1 M HEPES buffer, Ampicillin 100 µg/ml, Streptomycin 50 µg/ml, and Fetal Bovine Serum (FBS) 100 ml (10%). The volume was diluted to the mark (1 L) with TDW, and the medium was sterilized using Nalgene 0.2 µm filter unit (Freshney, 2015).

3.1.3.6.2 Preparation of RPMI-1640 Medium

Manufacture protocol according to Gibco Company was used as follow:

A- RPMI-1640 medium powder (with HEPES buffer and L-glutamine)

RPMI-1640 medium powder (10.4 g) was dissolved in ~600 ml of TDW (usbiologi, USA). Sodium bicarbonate powder 2.2 g, 1 ml/L Ampicillin 100µg/ml, 0.5 ml/L Streptomycin 505 µg/ml, and FBS 100 ml. (10%) were added. The volume was completed to 1 L with TDW, and medium was sterilized using Nalgene filter using 0.22 filter unit as recommended by the manufacturing company (Freshney, 2015).

B- RPMI-1640 medium powder (with HEPES buffer, L-glutamine, and Sodium bicarbonate)

1- Preparation of completed culture media (with FBS) (100 ml)

a- RPMI-1640 medium (powder)

100 ml of TDW was added in a scroll bottle sterilized by autoclave and UV. 1.6 gm Media RPMI-1640 (powder) was added. 10 ml Fetal Bovine Serum (FBS) (10%) was added and antibiotic (Penicillin - Streptomycin Solution) (Pen-Strep) 1 ml was added and mixes gently till a yellow color (acidic) appeared. KOH (1N) drops were added to neutral solution (PH=7) to change the color to reddish-orange. The solution was filtered using syringe filter 0.22 to remove debris, and cooled at 4°C, (Appendix (1)).

b- RPMI-1640 medium (solution)

89 ml Media RPMI-1640 solution (ready to use) was added in a scroll bottle sterilized by autoclave and UV, and 10 ml FBS (10%) was added. 1 ml antibiotic (Penicillin - Streptomycin Solution) (Pen-Strep) was added and mixed gently till a yellow color (acidic use solution) appeared. KOH (1N) drops were added to neutralize the solution (pH=7) and the color was changed to reddish-orange, the

solution was filtered using syringe filter 0.22 to remove debris, and cooled at 4°C, (Appendix (2)).

2- Preparation of Serum Free Media (SFM) (without FBS) (100 ml)

A- RPMI-1640 medium (Powder)

100 ml TDW was added in a scroll bottle sterilized by autoclave and UV. 1.6 gm Media RPMI-1640 (powder) was added and 1 ml antibiotic (Penicillin - Streptomycin Solution) (Pen-Strep) was added and mixed gently till a yellow color (acidic) appeared. KOH (1N) drops were added to neutralize the solution (pH=7) and the color was changed to reddish-orange, the solution was filtered by syringe filter 0.22 to remove debris, and cooled at 4°C, (Appendix (3)).

B- RPMI-1640 medium (Solution) ready for use

100 ml Media RPMI-1640 Solution was added in sterilized scroll bottle. 1 ml antibiotic (Penicillin-Streptomycin Solution) (Pen-Strep) was added and mixed gently, (Appendix (4)).

3.1.4 Cell Lines

Four cell lines (AMJ13, MCF-7, Vero, and REF) were supplied from cell bank unit, experimental therapy department, Iraqi Center of Cancer, and Medical Genetics Research (ICCMGR).

3.1.4.1 Ahmed Mortadha Jabria 2013 (AMJ13 cell line)

This new breast cancer cell line (Ahmed Mortadha Jabria 2013) was primarily established by Ahmed Majeed Al-Shammari, at the Iraqi Center for Cancer and Medical Genetic Research at Mustansiriyah University in 2013, from the primary tumor of ductal carcinoma, with a histological diagnosis of infiltrating ductal carcinoma of the breast of a 70-year-old Iraqi woman, mother of 6 children, named Jabria, weighed 70 Kg and 160 cm in height, from the city of Al-Amen in Baghdad. It is considered unique because it is the first breast cancer cell line from an Iraqi

population, and it is expected to be a useful tool in breast cancer research. The cells were morphologically characterized by light and scanning electron microscopy, and found to be elongated multipolar epithelial-like cells with a population doubling time of 22 hrs (Al-Shammari *et al*, 2015).

3.1.4.2 Michigan cancer Foundation-7 (MCF-7 cell line)

MCF-7 is a breast cancer cell line isolated in 1970 from a 69-year-old Caucasian woman. MCF-7 is the acronym of Michigan Cancer Foundation-7, referring to the institute in Detroit where the cell line was established in 1973 by Herbert Soule and co-workers (Lee *et al*., 2015).

Human breast cancer cell line AMJ13 (Iraq) and MCF-7 (USA) were cultured in RPMI-1640 medium (US Biological, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Capricorn-Scientific, Germany), 100 unit/ml penicillin, and 100 µg/ml streptomycin (Capricorn-Scientific, Germany), and incubated at 37°C.

3.1.4.3 Vero cell line

Vero cells are a lineage of cells used in cell cultures. The 'Vero' lineage was isolated from kidney epithelial cells extracted from an African green monkey. The lineage was developed on 27 March 1962 by Yasumura and Kawakita at the Chiba University in Chiba, Japan. The original cell line was named Vero after an abbreviation of Verda Reno, which means green kidney in Esperanto, while Vero itself means truth in Esperanto (Sheets, 2000). These cells are used to calculate virus titer in TCID50 assay.

3.1.4.4 Rat Embryo Fibroblast (REF) cell Line

It is a normal fibroblast endothelial cell of rat embryos. It was established by A. M. Al-Shammari experimental therapy department in Iraqi Center for Cancer and Medical Genetic Research (ICCMGR). The morphological change and apoptosis of REF, MCF-7, and AMJ13 cells were observed with inverted and fluorescent

microscopes, respectively. REF, MCF-7, and AMJ13 cells were seeded in a 96-well plate at 2×10^5 cells per well. The cells were treated with different concentrations of NDV and MH (Ali *et al*., 2019).

3.1.5. Requirements for Virus Propagation and Hemagglutination Test

3.1.5.1 Newcastle Disease Virus

The Iraqi attenuated strain of NDV (Iraq/Najaf/ICCMGR/2013) (Al-Shammari *et al.,* 2014) is supplied by ICCMGR, Experimental Therapy Department, Cell Bank Unit, Mustansiriyah University. A stock of infectious virus is propagated in embryonated chicken eggs (Al-Khaleel Company) for producing embryonated chicken eggs, (Baghdad, Iraq), harvested from allantoic fluid, and purified from debris by centrifugation (3000 rpm, 30 min, 4°C).

NDV was quantified by a hemagglutination test (HA) in which 1 hemagglutination unit (HAU) is defined as the smallest virus concentration leading to visible chicken erythrocyte agglutination. The virus was stored at −86°C. Viral titer was determined by 50% tissue culture infective dose (TCID50) titration on monkey kidney (Vero) cells.

3.1.5.2 Embryonated Chicken Eggs (ECE)

Fertilized eggs (180 eggs) obtained from Al-Khalil hatchery, located in the Abu Ghraib area, were used in propagating the virus and isolating it from a pathological state.

3.1.5.3 Chicken Red Blood Cells (CRBC)

They were collected from brachial veins under the right wing of chicken by using a heparinized syringe to draw about 2 ml of blood which was then used in carrying out hemagglutination test (HA) to determine the titer of the virus.

3.1.6 Colorimetric Assays

3.1.6.1 Hexokinase Assay Kit

Sample: cell lines. Method: Colorimetric method. Specification: 30 tubes/28 samples according to manufacturing protocol (ElabScience Biotechnology Inc.). Application: This kit can measure Hexokinase (HK) activity in serum, plasma, tissue, cells, and other samples. Detection principle: By utilizing the coupling reaction with glucose-6-phosphate dehydrogenase (G-6-PD), and providing a sufficient amount of substrate, HK activity was calculated by measuring the increase value of absorbance at 340 nm.

a- Kit components and preparation of reagents

Reagent 1: liquid, 8 ml x I vial was stored at 4ºC. Reagent 2: Stock solution, 4 ml x I vial was stored at 4ºC. Reagent 2 was prepared with double distilled water at a ratio of 1:9. Fresh solution was prepared before use. Reagent 3: Powder 2 vials was stored at - 20ºC. Preparation of reagent 3: a vial of powder was dissolved with 1 ml double distilled water. Fresh solution was prepared before use. Reagent 4: powder 7 vials were stored at - 20ºC. Preparation of reagent 4: a vial of powder was dissolved with 1 ml double distilled water. Needed amount was prepared before use. Reagent 5: liquid, 330 µl x 1 vial was stored at 4ºC. Appendix (17).

b- Working solution and instruments

Working solution was prepared by the reagent 1, 2, 3, 4, 5 and double distilled water was thoroughly mixed at the ratio of 20:10:5:20:1:40. Fresh solution was prepared before use. Experimental instruments and glassware: Test tube, Micropipette, Vortex mixer, water bath, Centrifuge, ELISA plate reader (340nm).

3.1.6.2 Pyruvate Assay Kit

Sample: cell lines. Method: Colorimetric method. Catalog No: E-BC-K130 according to manufacturing protocol (ElabScience Biotechnology Inc.). Experimental instruments: Test tube, Micropipette, Vortex mixer, 37℃ water

bath/gas bath, and ELISA plate reader (505 nm). Application: This kit measures the pyruvate content of serum, plasma, culture cells, cell culture supernatant, and other samples. Detection principle: Pyruvic acid can react with a chromogenic agent to give a product that is reddish brown in alkaline solution. The depth of the color is directly proportional to the pyruvate content which was calculated by measuring the OD value at 505 nm. Kit components can be seen in (Table (3-3)).

Table (3-3): Kit components for determine pyruvate in sample

	Component	**Specification**	**Storage**
Reagent 1	Clarificant	10 mL × 1 vial	4℃, 6 months
Reagent 2	Chromogenic agent	50 mL ×1 vial	4℃, 6 months, shading light
Reagent 3	Alkaline solution	50 mL × 3 vial	4℃, 6 months
Reagent4	2 μmol/mL Sodium pyruvate standard	1 mL × 1 vial	4℃, 6 months
Preparation of 0.2 μmol/ml sodium pyruvate standard solution: Reagent 4 was diluted with double distilled water for 10 times.			

3.1.6.3 ATP Assay Kit

Sample: cell lines. Method: Colorimetric method. Specification: 100 tubes/48 samples according to manufacture protocol (ElabScience Biotechnology Inc.)**.** Detection principle: Phosphokinase catalyzes the reaction between adenosine triphosphate and creatine to produce creatine phosphate which was then detected by phosphomolybdic acid colorimetry. Experimental instruments and glassware: Test tube, Micropipette, Vortex mixer, water bath, and ELISA plate reader (636nm). This kit measures the ATP content of serum, plasma, cells and cell culture supernatant, and other samples.

a- Kit components and preparation of reagents

Reagent 1: Substrate I solution was dissolved in 10 ml of boiled double distilled water and was allowed to stand in a boiling water bath until it completely dissolved. If it appeared crystal before assay, it was allowed to stand in a boiling water bath until it completely dissolved, and then was stored at 37℃ for assay. Reagent 2: Substrate II, 20 ml × 1 vial at 4℃.

Reagent 3: Accelerant: Powder × 2 vials at -20℃, diluent, 760 µl x 2 vials, 4℃. Reagent 3 application solutions: 1 vial of Reagent 3 diluent was added to 1 vial of Reagent 3 powder and was dissolved fully before use. Reagent 4: Precipitant, 5.5 ml × 1 vial at 4℃. Reagent 5: Developer, A solution, 7 ml × 4 vials at 4℃. B solution, 6 ml × 4 vials 4℃.

Developer application solution: 1 vial of Reagent 5 A solution was added to 1 vial of Reagent 5 B solution, was dissolved fully, and was stored at 4℃. Fresh solution was prepared before use. Reagent 6: Stop Solution, 50 ml ×1 vial at Room Temperature. Reagent 7: ATP Standard, Powder × 2 vials at 4℃. 5 mmol/L ATP standard stock solution: before assay, double distilled water was added into 1vial of ATP standard powder to make a final volume of 1 ml. Fresh solution was prepared before use. 1 mmol/L ATP standard application solution: 5 mmol/L ATP standard stock solutions were diluted with double distilled water 5 times, appendix (18).

3.2. Methods

1. Propagation (injection, collection) of NDV in embryonated chicken eggs (ECE) (180 eggs), purification, filtration, and distribution of NDV.
2. Determination of Titer of NDV by Hemagglutination Test (HA)
3. Cell culture of Vero cell line and preparation of diluted NDV.
4. Determination of Tissue Culture Infective Dose (TCID50) of virus by using Vero cell line to determine the number of NDV in 1 ml of allantoic fluid of embryonated chicken eggs.
5. Determination of the cytotoxicity ratio (CT%) for NDV, D-Mannoheptulose (MH) (HKI) and Docetaxel (DXL) (positive control) by MTT cytotoxicity assay after treatment with NDV, MH and DXL separately by using different drug concentrations (μg/ml), different multiplicity of infection (MOI) for virus depend on TCID50 and different inhibitor concentrations (μg/ml) to normal rat embryo fibroblast (REF), and human breast cancer cell lines (AMJ13 and MCF-7).
6. Determination of IC50 for NDV, MH and DXL through measurements of CT% for NDV, MH and DXL by using GraphPad Prism.
7. Choosing of the most suitable MOI of virus, and concentrations of inhibitor and DXL depending on IC50 values, and then exposure NDV, MH and DXL separately, then NDV and MH synergistically to REF, AMJ13, and MCF-7 cell lines with comparison to the control (untreated cell).
8. Measurement of cytotoxicity ratio (proliferation inhibition), by using the MTT cytotoxicity assay, for REF, AMJ13, and MCF-7 cell lines after their treatment with NDV (MOI 0.3, 1 and 2), MH (62.5, 125 and 250 μg/ml) and DXL (1.25, 2.5 and 5 μg/ml) separately and synergistically for (NDV and MH) at 37ºC for 72 hrs, depending on the MOI of NDV, and concentration of MH and DXL that have been selected.

9. Determination of synergism (combination) between NDV and MH, by combination cytotoxicity assays, Combination Index (CI), or Chou-Talalay equation through Isobologram, GraphPad prism, and CompuSyn program, for REF, AMJ13 and MCF-7 cell lines.

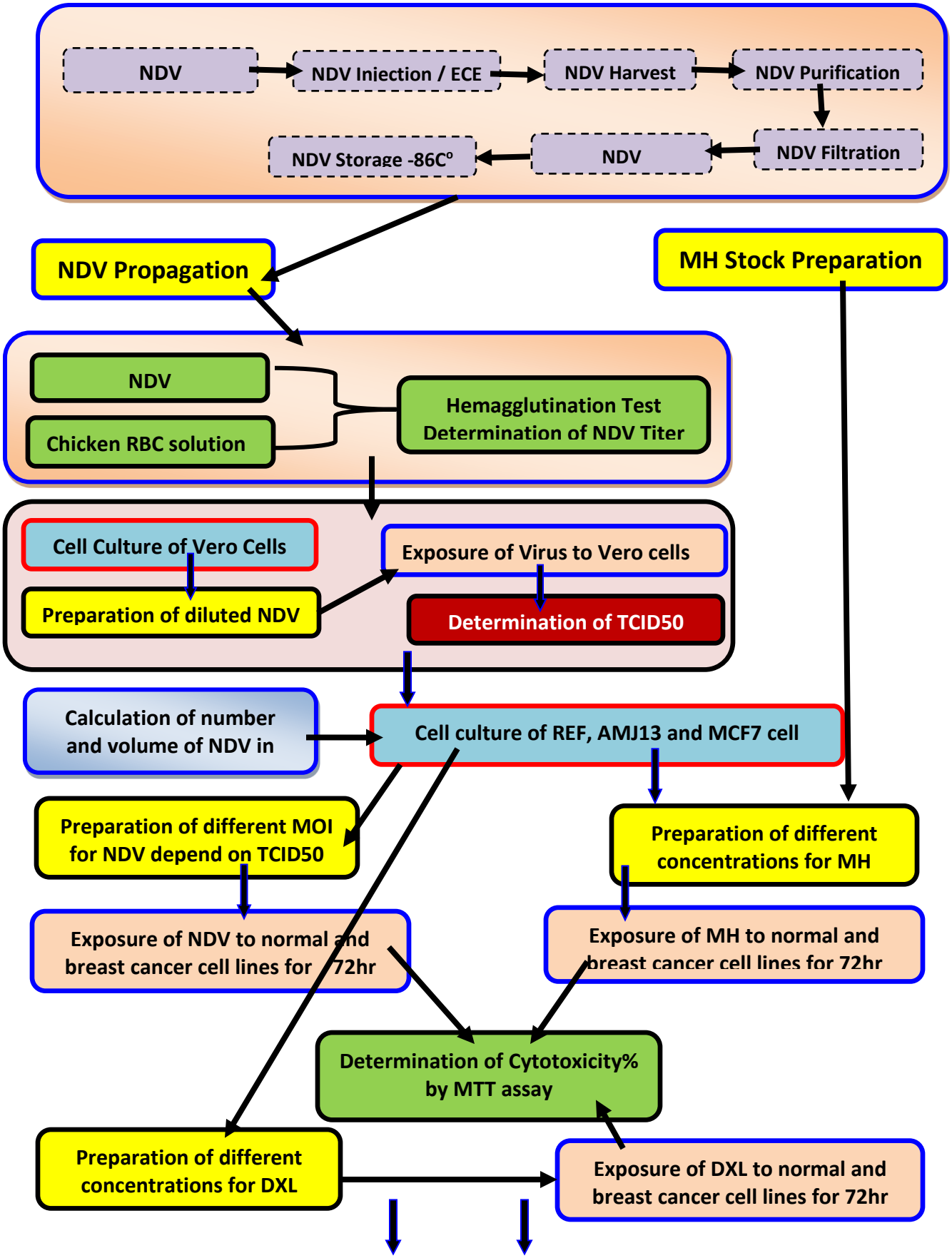
10. Choosing of the most suitable the most suitable MOI of virus and concentration of MH depending on combination index, and then exposure NDV and MH separately and synergistically to REF, AMJ13 and MCF-7 cell lines at 37ºC for 72 hrs with comparison to the control (untreated cell).

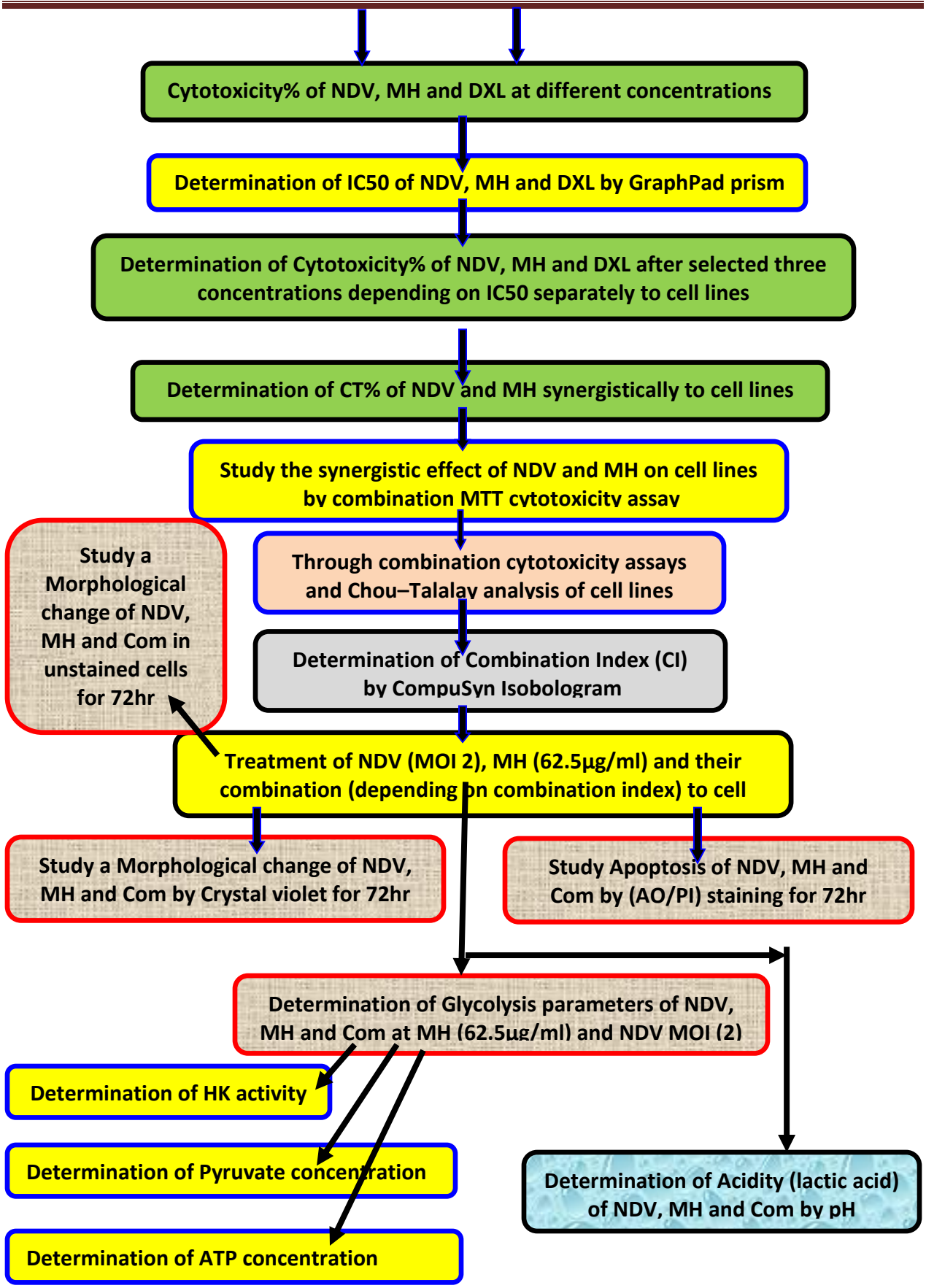
11. Studying of morphological changes using crystal violate after treating REF, AMJ13, and MCF-7 cell lines with NDV (MOI=2) and MH (62.5µg/ml) depends on CI, separately and synergistically at 37ºC for 72 hrs, to explain the morphological changes in treated and untreated control cells.

12. Studying of apoptosis pathway using Propidium Iodide (PI)/ Acridine Orange (AO) after treating REF, AMJ13, and MCF-7 cell lines with NDV (MOI=2) and MH (62.5µg/ml) depends on CI, separately and synergistically at 37ºC for 72 hrs, to explain live and dead cells in treated and untreated control cells.

13. Determination of hexokinase (HK) enzyme activity, Pyruvate, ATP concentration, and acidity (pH values) after treating REF, AMJ13, and MCF-7 cell lines with NDV (MOI=2) and MH (62.5µg/ml) depends on CI, separately and synergistically at 37ºC for 72 hrs, and with comparison to control using colorimetric methods by ELISA plate reader and litmus papers to determine pH values, Scheme (3-1) and (3-2).

Study Design:





Scheme (3-1): Outline of laboratory experiments of the study

3.2.1 The Propagation of the Newcastle Disease Virus (NDV)

3.2.1.1 Virus Preparation

NDV Iraqi strain was provided as a frozen culture by the experimental therapy department at ICCMGR. It was directly thawed and the antibiotics penicillin 100 unit/ml and Streptomycin 100 µg/ml were added to the virus sample. The virus was prepared by the ratio (1:10) with Phosphate buffer saline (PBS) (sterilized and cooled). Then, 6 ml (6000 **µl**) was prepared for 60 eggs by adding (600 **µl** Virus in buffer + 5400 **µl** PBS), or 1ml (1000 **µl**) was prepared for 10 eggs by adding (100 **µl** Virus + 900 **µl** PBS) in cold test tube and placing it on ice.

The sample was centrifuged at 3000 rpm for 30 min at 4^{o}C to remove any debris and large particular matter, then the supernatant was extracted and the virus sample was injected into 9 or 10 days embryonated chicken eggs. Allantoic fluid of infected embryo was harvested in a small tube and stored at -86^{o}C App (22) (Allan *et al*., 1978).

3.2.1.2 The Propagation of NDV in Embryonated Chicken Eggs (ECE)

ECE (9-days-old) (180 eggs) were incubated in a conventional incubator at 37^{o}C and on suitable day humidity appendix (21). The eggs were turned twice daily, and after 1 day of incubation, the eggs were transilluminated with an egg-candling lamp to insure the viability of the embryo appendix (18). All non-fertile eggs and those containing a dead embryo were discarded and live eggs (embryo) were saved in an incubator for the second candling before inoculation, the air sac was marked off and a suitable site of injection was marked on the eggshell.

Where on important blood vessels were running away from the embryo, the egg was rinsed with 70% ethanol to sterilize the outer surface, and then the egg shell was sterilized with iodine at the inoculation site where a small hole was drilled about 0.4 cm above the air sac border (Haas *et al*, 1998). After that, 0.1 ml (100 µl) of prepared virus solution was injected into each (9 or 10-days-old) embryonated chicken egg

with a fine needle (0.3mm), and the puncture holes were covered with paraffin. The eggs were incubated at 37ºC in a humidified incubator for 24 hrs. The embryos were observed daily every 3 hrs for viability. Up to 24-hr dead embryos were discarded and immediately transferred into the refrigerator (4ºC) if 24–48 hrs after their death.

Live embryos were saved in an incubator. Then they were harvested (Collected) after 3-6 hrs in refrigerator at 4ºC., the egg shell was cut by scissors and the allantoic fluid was harvested using sterile syringes, (unclear or bloody allantoic fluids were rejected) purified from debris by cold centrifugation at 3000 rpm for 30 min at 4°C, (Appendix (23)) filtered by syringe filter (0.45μm), and distributed in eppendorf tubes (Appendix (5)). Then, it was measured by HA to find the titer of virus (Figure (3-1)), (Appendix (7)) (Appendix (27)). The $TCID_{50}$ of NDV was calculated (Figure (3-6)), and it was then stored at -86°C. The virus was further propagated in embryonated eggs in which high titers of virus can be recorded from the infected eggs unit (Alexander and Senne, 1998).

3.2.2 Hemagglutination assay (HA)

NDV was quantified using the hemagglutination assay in which 1 hemagglutination unit (HAU) is defined as the smallest virus concentration leading to visible chicken erythrocyte agglutination (Allan et al., 1978).

3.2.2.1 Preparation of chicken RBC solution

The chicken RBC solution was prepared by collecting blood from a healthy bird into a heparinized tube. Chicken blood (2ml) was added into an anticoagulant test tube (heparinized tube) and then transferred into a centrifuge test tube where 7 ml cold PBS was added and mixed using a centrifuge (1000 rpm, 4ºC, and 10 min) to wash the blood, washing it 3 times with PBS.

Supernatant was discarded, the pellet (RBC) was saved, and then taking 0.5 ml of the washed RBCs, which was completed to 50 ml with PBS to achieve 1% RBC solution, or RBC solution (100 ml) was prepared with PBS in a ratio 1:100 (1%): 1

ml RBC + 99 ml PBS, 250 µl RBC + 25 ml PBS or 100 µl RBC + 10 ml PBS for the Hemagglutination test (Versteeg, 1985), (appendix (6)).

3.2.2.2 Titration of NDV

PBS (50 µl) was added to all wells in a 96-well microplate u-shape (7 lines for 7 eggs from A-G and H was the control). Virus allantoic fluids (VAF) (50 µl) was taken from each tube and added to the tissue culture plate (U-shape), it was added to $well_1$ and mixed with PBS, then 50 µl was taken from the mixture ($well_1$) and added to $well_2$ and also mixed with PBS.

50 µl was taken from the mixture ($well_2$) and added to $well_3$ and so on till $well_{12}$ (2 fold serial dilution was done) where 50 µl was discarded from it (The tips were replaced between each addition). Adding was limited to 7 lines because the 8 line was the control (Appendix (7)). RBC solution (50 µl) (1%) was added to all wells in the 96-well micro plate U-shape (8 lines) and mixed by inverting, not by vortex.

The 96-well micro plate was saved in an incubator for 30 min to get the virus titer for all lines. NDV titer was calculated for all virus allantoic fluids tubes by HA test (Appendix (7)) (Allan *et al*., 1978)

The VAF tubes (Known titer) were separated by cooling, centrifuged and collected, those with high titer values (7-12) were collected together and those with titer values (5-6) were also collected together in tubes (25 ml). VAF tubes with titer values (7-12) and (5-6) were filtrated using syringe filters (0.45 µm) and distributed to several eppendorf tubes and were stored at deep freeze (-86 ^{o}C) (Appendix (5)).

Agglutination is a positive result while no agglutination is a negative result**,** and the titer value was determined by the number of positive results as compared to the control (Figure (3-1)), (Appendix (25)) and (Appendix (27)) (Haas, et al., 1988).

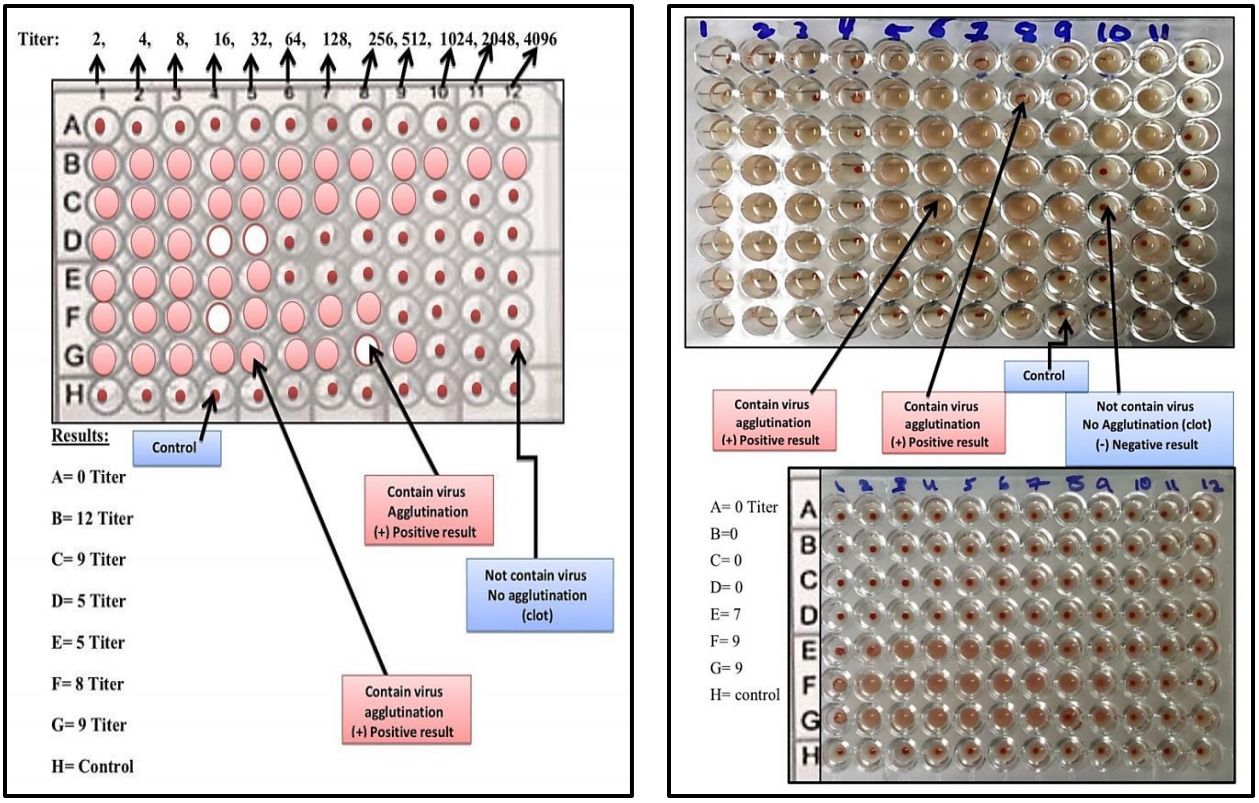


Figure (3-1): Calculation of titer of NDV by HA test in 96-well microplate

3.2.3 Cell lines

The AMJ13 human breast cancer cell line (Al-Shammari *et al*., 2015), MCF7 human breast cancer cell line and REF cell line were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), 100 unit's/ml penicillin, and 100 µg/ml streptomycin, and incubated at 37°C. Monkey kidney Vero cell line was cultured in MEM medium with 10% fetal bovine serum (FBS), 100 unit's/ml penicillin, and 100 µg/ml streptomycin, and incubated at 37°C.

Different cell lines (Vero, REF, AMJ13 and MCF-7) were detached from their flasks when they reached the subconfluent monolayer by trypsinization. Culture medium (20 ml) and 10% serum were added to the falcons and mixed gently with cells to prepare cell suspension. The cell suspension in the culture flask was poured aseptically into a sterile beaker. Using a multi micropipette, 200µ1 of the cell suspension was transferred into each well in a 96-well microplate, and the plate was covered with a sterile adhesive film, lid placed on and rocked gently, and incubated

in a 5% CO2 incubator at 37ºC for 24 hrs to allow cell attachment, proliferation, and confluent monolayer achievement. These cells were regularly assessed for standard growth characteristics and regularly authenticated (Freshney, 2015).

3.2.3.1. Maintenance of cell lines in Vitro

Cell lines were subcultured when they were confluence monolayers. The growth medium was decanted off and the cell culture was washed once with 2 ml trypsin-versene solution. Trypsin (3 ml) was added to the cell culture, the flask rocked gently, and part of it was decanted again to obtain about 1 ml of trypsin solution covering the cell surface. Then, the cells were incubated at 37°C until they were detached from the flask. The cells were dispensed in 5 ml growth medium and then redistributed at the required concentration into culture. The cells were incubated at 37ºC under a humidified atmosphere containing 5% CO_2 (Al-Shammari and Yaseen, 2012).

3.2.3.2 Cell culture of Vero cell line

Tissue culture falcon (TCF) contains complete media (CM) and Vero cell line saved in an incubator. After the cell became a monolayer (Figure (3-3)), complete media was discarded from TCF, this means the Vero cells remained adhesive on the walls. Also, 3 ml trypsin 0.25% was added to TCF to detach the cells from the wall or surface (Trypsinization), after which they were incubated for 3 min (Appendix (8)). A change of solution from pure to turbid is indicated by indicators on the detached cells. Detached cells should be round shaped and free floating in the trypsin solution as soon they detach (Figure (3-2)). Complete media was added to TCF (contains Vero cells + Trypsin). Trypsin

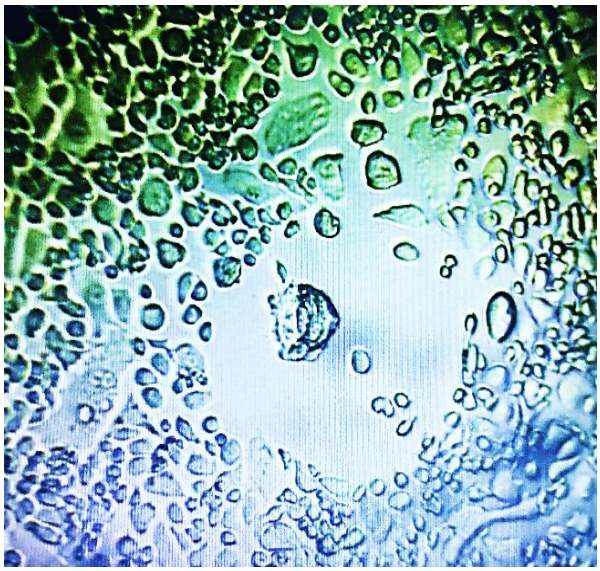


Figure (3-2): Trypsinization to detach cells from the wall in cell culture.

was inhibited by FBS to remove or to end trypsin. Solution (10 µl) was taken from TCF (Vero cells + CM) solution to a hemocytometer to calculate number of the cells by light microscope**,** must be at least 40 cells in 4 squares (Figure (3-4)).

A. Calculation of cells in 1ml and 1 well

The number of cells in each square (4 squares) was calculated, and then the average was calculated. The average was multiplied by 10^4 to find the number of cells in 1 ml (1000 µl). For example, if the average number of cells is 10 cells, 10 cells × 10^4 = 100000 cells, implying that 1 ml contain 10^5 cells. The cell suspension (Vero cells + CM solution) (X) volume in each well was calculated (at least 7000 cells in a well) through number of cells in 1 ml as follow:

No. of cells 100000 1000 µl cell suspension

No. of cells in well 7000 X

X= 7000 cell × 1000 µl / 100000 cell = 70 µl cell suspension

Each well must contain 100 µl cell suspension; therefore, 70 µl was completed to 100 µl by adding 30 µl CM for each well.

70 µl cells + 30 µl CM …… 100 µl for one well

7000 µl cells + 3000µl CM …….. 10000 µl (10ml) for 100 wells

This implies 10 ml is required to complete the 100 µl cell suspension for all wells in 96-well microplate. Then, 10 ml (cell suspension 7 ml + CM 3 ml) was added into a petri dish and distributed into all wells in 96-well microplate to complete the 100 µl cell suspension, after which 96- well microplate was incubated for (24-48) hrs. The cell culture was examined by an inverted microscope to confirm that the cells were monolayer, after which the media was discarded leaving the Vero cell ready for use. (Figure (3-4)). Then $TCID_{50}$ was calculated.

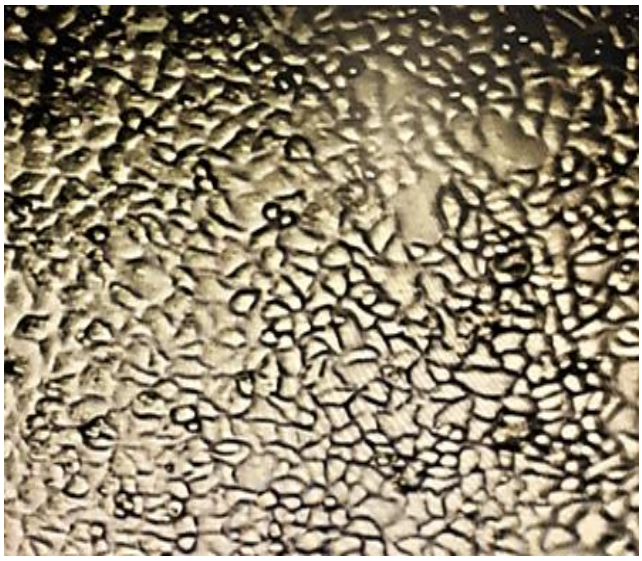


Figure (3-3): Vero cells confluent monolayer after storage at -86°C for 4 months

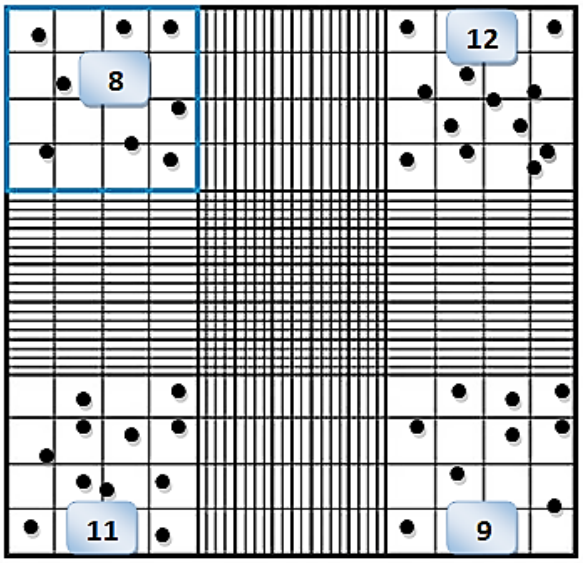


Figure (3-4): Calculation number of cells in sample by hemocytometer under microscope

3.2.4 Preparation of diluted NDV

Ten sterile eppendorf tubes (1.5ml) were formatted and numbered in sequence, 50 ml of SFM was prepared, and 900 µl SFM was added in sequence to all eppendorf tubes, 100µl stock virus suspension was added serially tenfold diluted after replacing the tip each time. Diluted allantoic virus suspension was prepared (10^{-1}-10^{-10}). (Appendix (9)) (Al-Shammari, 2003).

3.2.5 Exposure of NDV to Vero cells line

SFM (100 µl) was added to column 1 and 12 in 96- well microplate as a control, and then 50 µl SFM and 50 µl diluted virus were added to column 2-11, taking into account the eppendorf tubes sequence when adding, after which the 96- well microplate was wrapped with parafilm (Appendix (10)).

Each column has the same concentration, but each row has different concentrations. Any well with syncytia or cytopathic effect is considered a positive result. After complete addition of diluted virus, 96- well microplate was incubated at 37°C for 5 days, after which the titer value of the virus was read (TCID50) (Figure (3-5)).

3.2.6 Determination of Tissue Culture Infective Dose 50% ($TCID_{50}$) of NDV

$TCID_{50}$ which means the amount of a pathogenic agent that will cause infection in 50 % of cell cultures inoculated. Vero cell lines were seeded at 10^4 cell/well in 96-well microplate flat bottom, and after 24 hr or when confluent monolayer was achieved, growth media was discarded and virus suspension (ten-fold serial dilution) was prepared, 1/10, $1/10^2$, $1/10^3$, $1/10^4$, $1/10^5$, $1/10^6$, $1/10^7$, $1/10^8$, $1/10^9$, and $1/10^{10}$. Then, the cells were inoculated with 50 µl/well of NDV with 8 replicates for each dilution, control treated with SFM (100 µl) only. The plate was covered with a sterile adhesive paper and incubated for 2 hrs at room temperature to allow virus penetration, (Appendix (10)).

After that, the cells were washed with PBS and 200 µl of serum free medium was added into each well. The plate was covered again and incubated at 37°C for 5 days. NDV titer was calculated on Vero cell lines in comparison with the control to determine infections dose of 50% in tissue cultures as described by (Flint *et al*., 2009), in which the titer was determined as the dilution that cause 50% cytopathic effect in the incubated well. The following formula is used to calculate the $TCID_{50}$ used (Table (3-4) and (Figure (3-5)).

$$\log_{10} (TCID_{50}/ml) = L + d (S - 0.5) + \log (1/v)$$

L = negative $\log_{10}$ of the most concentrated virus dilution tested, in which all wells are positive (no. of positive columns completely). L = 5

d = $\log_{10}$ of dilution factor. d = log 10= 1 (1:10 dilution)

S= sum of individual proportions (pi)

Pi = calculated proportion of an individual dilution

v = volume of inoculum (ml/well)

Dilution Factor = V_{total} / V_{Sample}

Pi = Amount of positive wells / total amount of wells per dilution

V= volume of inoculum (ml/well) = 50 µl /ml = 50 µl / 1000µl = 0.05

Table (3-4): Calculation method of TCID50 of the virus after treating Vero cell line with NDV

$\log_{10}$ ($TCID_{50}$ /ml) = L + d (S - 0.5) + log (1/v)

S =1+ 0.75+ 0.375+ 0.125 + 0 = **2.25**,

d= 1, **L**= 5, **v** = 50 µl = 0.05ml

$\log_{10}$ ($TCID_{50}$/ml) = 5+1(2.25 - 0.5) + $\log_{10}$ (1/0.05)

$\log_{10}$ ($TCID_{50}$/ml) = 5+1 (2.25 - 0.5) +1.3

$\log_{10}$ ($TCID_{50}$/ml) = 5+2.25-0.5+1.3 = 8.05

$TCID_{50}$/ml = $10^{8.05}$ = 1.12 x 10^{8}

$TCID_{50}$/ml =1.12 x 10^{8}/ml

$TCID_{50}$/ml =112.2 x 10^{6}/ml

$\log_{10}$ virus dilution	ratio of infection	Proportion (pi)
-1	8/8	1
-2	8/8	1
-3	8/8	1
-4	8/8	1
-5	8/8	1
-6	6/8	0.75
-7	3/8	0.375
-8	1/8	0.125
-9	0/8	0.0
-10	0/8	0.0

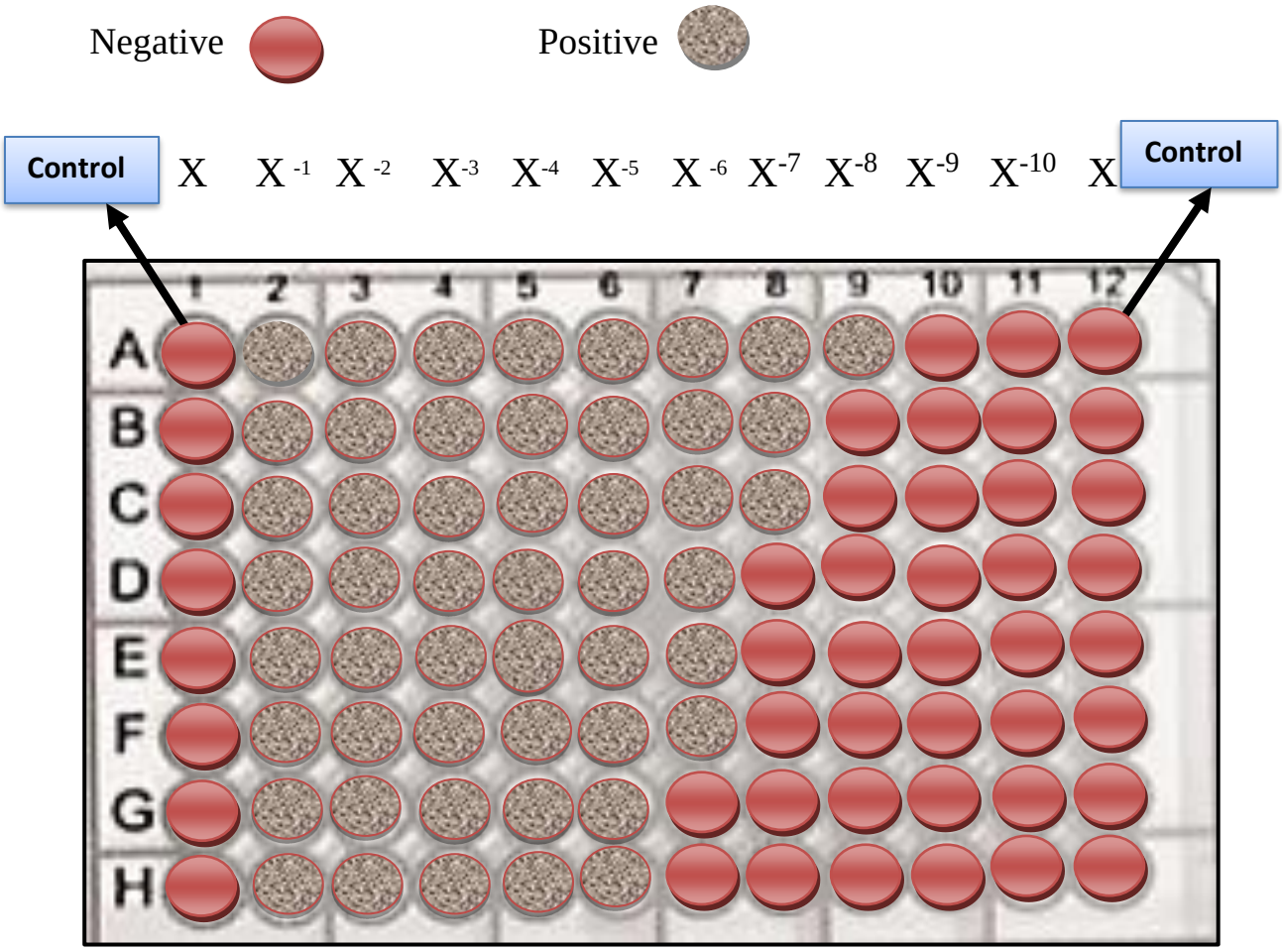


Figure (3-5): Exposure of NDV to Vero cells line and calculation of TCID50

3.2.7 Exposure stages of the cell lines

In the present study, cell lines were exposed to 3 different agents of exposure:

1- NDV as an agent (Virotherapy) to induce apoptosis

2- D-Mannoheptulose as an agent (phytotherapy) to inhibit of glycolysis

3- Docetaxel drug as an agent (Chemotherapy) as positive control

In addition, cell lines were treated with combination between (NDV+MH) (Synergistically).

3.2.7.1 Treatment of cell lines with NDV, MH, and DXL

3.2.7.1.1 Preparation of stock and diluted solutions

Sterile eppendorf tubes (1.5 ml) were formatted and numbered in sequence. NDV stock solution ($TCID_{50}$=124.6 × 10^6 virus/ml), 25 ml SFM, and 250 µl antibiotic (Pen-Streptomycin) (1%) were used to prepare diluted NDV solutions (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 MOI) after adding 500µl cold SFM and NDV stock solution (0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, and 5.12 µl, respectively), in accordance with calculations of MOI NDV (Appendix (11)) and (Table (3-5)).

MH Stock solution (14.87 ml) was prepared by dissolving 50 mg MH in 14.87ml PBS, and then used in preparing diluted MH solution [(8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 mM) or (1680, 840, 420, 210, 105, 52.5, 26.25, and 13.125 µg/ml)] after adding 300 µl SFM and 300 µl MH stock solution (3360 µg/ml)(16mM), serially eightfold dilution (Appendix (12)).

DMSO (dimethyl sulfoxide) (1%) 25 ml was prepared. Docetaxel Stock Solution (DXLSS) [2000 µg/ml or (2 mg/ml, 2475 µM, and 2.475 mM)] was prepared by dissolving 20 mg docetaxel in 10 ml DMSO solution. Diluted stock docetaxel solution (DSDXLS) (20µg/ml) was prepared using 50µl DXLSS in 5ml DMSO (1%) which was used to prepare diluted docetaxel solutions [(10, 5, 2.5, 1.25, 0.625, and 0.313 µg/ml) or (12.378, 6.189, 3.09, 1.547, 0.773, and 0.386 µM)] after

adding 600 µl cold SFM and 600µl DSDXLS (20µg/ml), 6-fold serial dilution in sterile tubes (Appendix (13)) (Freshney, 2015).

3.2.7.1.2 Exposure of NDV, MH, and DXL to cell lines

Cell culture of REF, AMJ13, and MCF-7 cell lines were done in the same way as that of Vero cell line (3.2.3.2), with the replacement of Vero cell line with others cell lines. 96- well microplate cell line after incubation, the media was aspirated from 96-well microplate using a multipipette and 100µl diluted MOI NDV solution or diluted MH concentration or diluted docetaxel drug solution was added to wells in each column in accordance with the number of eppendorf tubes, after which 100 µl SFM was added to another wells as control. Three replicates were used for each concentration. The plate was covered with a sterile adhesive parafilm and incubated at 37°C for 72 hrs (Appendix (11, 14 and 15)), after which the optical density (OD) was read by MTT assay (Appendix (16)) and cytotoxicity ratio was calculated (Freshney, 2015).

3.2.7.1.3 Calculation of the number and the volume of virus in a well in accordance with TCID50 of NDV:

1- Calculation of the number of virus in a well:

Each MOI of virus was calculated as follow:

0.1MOI ……1 virus to each 10 cells or 1000 virus to each 10000 cells or 1000 virus to 1 well (1 well = 10000 cells).

2- Calculation of the volume of virus solution in a well

TCID50 =124.6 × 10^6 virus/ml = 124.6 × 10^6 virus/1000 µl (stock solution)

If MOI= 0.1

Stock: 124.6 × 10^6 virus …… 1000 µl

Diluted: 1000 virus …… ……. X (volume of virus solution in 1 well)

X= (1000 virus ×1000 µl)/ (124,600,000 virus) =0.008 µl (volume of virus solution in 1 well) or 0.04 µl (volume of virus solution for 5 wells) was taken from stock NDV solution + 500 µl SFM= 500 µl diluted NDV solution (0.1 MOI).

If MOI = 12.8

12.8 MOI ……12.8 virus to each 1 cell or 128000 virus to each 10000 cells or 128000 virus to 1 well.

Stock: 124.6×10^6 virus ……..…… 1000 µl

Diluted: 128000 virus ……. X (volume of virus solution in 1 well)

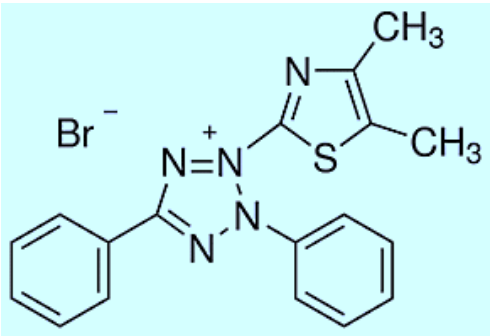
X= (128000 virus ×1000 µl) / (124,600,000 virus) = 1.027 µl (volume of virus solution in 1 well) or 5.135µl (volume of virus solution for 5 wells) + 500 µl SFM = 500 µl diluted NDV solution (12.8 MOI). Different MOI NDV was prepared (Table (3-5)).

Table (3-5) Preparation of different MOI for NDV depend on TCID50

MOI/NDV	No. of NDV/10 cells	No. of NDV/10^4 cells (1well)	Volume of NDV µl /1 well	Volume of NDV µl /5 well
0.1	1	1000	0.008	0.04
0.2	2	2000	0.016	0.08
0.4	4	4000	0.032	0.16
0.8	8	8000	0.064	0.32
1.6	16	16000	0.128	0.64
3.2	32	32000	0.256	1.28
6.4	64	64000	0.512	2.56
12.8	128	128000	1.024	5.12

3.2.8. MTT Cytotoxicity Assay

Cell culture of REF, AMJ13, and MCF-7 cell lines were done as in the same way as that of cell line (3.2.3.2). The cytotoxicity was investigated using MTT



MTT, Methyl thiazolyl diphenyl-tetrazolium. Sigma Aldrich

[3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. After cell lines were treatment with NDV, MH, and Docetaxel, the cytotoxicity ratio was determined to measure the concentration of inhibition that kills 50% of cells (IC50). The IC50 was calculated using GraphPad Prism (version 7) for NDV, MH and DXL.

The CompuSyn computer software compared the IC50 of MH and NDV separately and the IC50 of them combined, on different cell lines (Gao et al., 2003). After IC50 was measured, the cells were treated with selected different concentrations of D-Mannoheptulose (Santa Cruz, USA) (250,125, and 62.5 µg/ml) and Docetaxel (1.25, 2.5 and 5 µg/ml), and MOI of NDV (0.3, 1, and 2) depending on the IC50 values, after which they were incubated at 37°C for 72 hrs. Triplicates were used for each concentration of each treatment modality. (Al-shammari *et al*., 2016)

After that, the media in 96- well microplate was discarded and 50 µl of serum free media (SFM) and 50 µl of MTT dye (yellow solution 2 mg/ml) were added into each well and incubated at 37°C for 3 hrs. MTT dye solution was discarded, the crystals remaining in the wells were solubilized by the addition of 100 µl of Dimethyl Sulphoxide (DMSO) (1%) (Santa Cruz Biotechnology, USA) into each well to dissolve the MTT-Formosan crystals, and the plate was wrapped with parafilm, rocked, and incubated for 15 min.

The optical density value for treated and untreated cells was measured at 492 nm using the ELISA plate reader (Freshney, 2015), (Appendix (16)). The endpoint parameter that was calculated for each cell line included percentage of cell growth, or percentage of cell proliferation (PR) = mean of treatment / mean of control (Van Meerloo *et al*., 2011), and the inhibition of cell growth (G.I) or percentage of cytotoxicity (CT %) which was calculated as:

$$\textbf{Cytotoxicity \%} = (OD_{Control} - OD_{sample}) / OD_{Control} \times 100$$

Where $OD_{control}$ is the mean optical density of untreated wells, and OD_{Sample} is the optical density of treated wells (Jabir *et al*., 2019). MTT was used to determine the effect of NDV, MH, and their combination in a study on the viability of tumor cells. This assay is based on metabolic reduction of colorless tetrazolium salt by the mitochondrial enzyme activity in viable cells. It is particularly useful for assaying cell suspensions because of its specificity for living cells (Mosmann, 1983).

3.2.9. Synergistic cytotoxicity assay and Chou-Talalay analysis

Cell lines were seeded at 1×10^4 cells/well into 96-well microplates and incubated overnight. NDV was exposed first at MOI of 2, 1 and 0.3, then MH was exposed at the indicated concentration (250,125 and 62.5 μg/ml) as serial dilutions to test growth inhibition. After 72 hrs of incubation, cell cytotoxicity assay, or growth inhibition was performed to assess the cytotoxicity rate or inhibition rate of cells by MTT cytotoxicity assay. For synergism determination, NDV and MH were studied as non-constant ratios. To analyze the combination of NDV and MH, Chou–Talalay combination index (CI) was calculated using the CompuSyn software (CombuSyn Inc., Paramus, NJ, USA). Unfixed ratios of NDV and MH and mutually exclusive equations were used to determine the CI. A combination index between 0.9 and 1.1 is considered additive, whereas CI< 0.9 and > 1.1 indicates synergism and antagonism, respectively (Al-shammari *et al*., 2016). The results were also analyzed by isobologram technique using CombuSyn software. The axes on Isobologram represented the doses of each treated. Two points on the x and y axes were chosen that corresponded to the doses of each agent necessary to generate that particular combination index (CI) called the fraction affected (Fa) value. The straight line or hypotenuse drawn between these 2 points on the x and y axes corresponded to a strictly additive interaction between 2 therapeutic agents. If the point was located on the lower left of the hypotenuse, the effect was synergistic and if the point was located on the upper right of the hypotenuse, the effect was antagonistic (Tallarida, 2011).

3.2.10. Morphological analysis

Cell culture was done as described in (3.2.3.2). To visualize the shape of the cells under an inverted microscope, 100 µl of cell suspension for cell lines were seeded in 96- well microplate at a density of $1x10^4$ cells, and incubated for 24-48 hrs at 37°C.

After confluence of cells to become monolayered, the media was aspirated, and then the cells were exposed to MH (62.5µg/ml) and NDV MOI 2 in accordance with combination index separately and synergistically at 37°C for 72 hrs. The media was discarded, the plates were stained with 50 µl crystal violet and incubated at 37°C for 15 min, and the stain was washed off gently with tap water until the dye was removed. The cell was observed under an inverted microscope at 40x magnification and photographed with a digital camera (Ali *et al.*, 2019).

3.2.11 Assessment of apoptosis (PI/AO staining)

The Propidium iodide/ Acridine Orange (PI/AO) dual staining method was used to measure apoptosis of the REF, AMJ13 and MCF-7 cell lines. Cell culture was done as described in (3.2.3.2). It was seeded 10000 cells/well, 100 µl of cell suspension is transferred to a 96-well microplate for modified PI/AO staining. Cells were treated with MH (62.5µg/ml) and NDV MOI (2) in the 37°C incubator for 72 hrs before they were subjected to PI/AO staining. Then, 1 ml of cell suspension was used for conventional PI/AO staining, (Ribble *et al*., 2005).

Acridine orange (AO) (10 µg/ml) prepared using PBS (10 µl AO + 1ml PBS) and Propidium iodide (PI) (10 µg/ml) prepared using PBS (10 µl PI + 1ml PBS) were used after mixing them at a ratio of 1:1. The stain mixture (AO/PI) was used to add 50 µl to all 96-well microplate, after which it was allowed for 2 min at room temperature (RT) before it was discarded. PBS (100 µl) was added to all 96-well microplate for washing, it was discarded, and photographs were taken directly by fluorescent microscope 40X magnification (Jabir et al., 2019; Ali *et al.*, 2019).

3.2.12 Glycolysis parameters determination

Hexokinase Assay Kit: Catalog No: E-BC-K121, Pyruvate Assay Kit: Catalog No: E-BC-K130, and ATP Assay Kit: Catalog No: E-BC-K157, Method: Colorimetric methods are supplied from ElabScience, USA. ELISA plate reader. Operation procedures according to manufacturing protocol (ElabScience Biotechnology Inc.).

3.2.12.1 Determination of Hexokinase

Operation procedures: Cells culture samples:

Cell culture was done as described in (3.2.3.2) but by using REF, AMJ13 and MCF-7 cell lines in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin, and incubated in a humidified atmosphere of 5% CO2 at 37ºC. The cell lines were treated with MH (62.5 µg/ml) and NDV (MOI 2) separately and synergistically, incubator at 37℃ for 72 hrs. Cell suspension was collected by thawing to prepare cells samples.

Operation steps to determine hexokinase in sample:

The Spectrophotometer or ELISA plate reader was preheated for 30 min, the wavelength was adjusted at 340 nm, and it was set to zero with distilled water. The prepared working solution was incubated at 37℃ for 10 min before detection. Operation table as follow:

The solution was fully mixed and the time was recorded immediately, the tube or plate was incubated in 37ºC, and the absorbance was measured at 340 nm at 30 s (A1) and 150 s (A2), respectively by using ELISA plate reader. ΔA=A2 - A1

Calculation of results for cell sample:

Operation steps	Sample tube
Cell sample (µl)	50
Working solution (µl)	960

$$\text{HK activity (U/ml)} = \frac{\Delta A}{mmole\ Extinction\ coefficient} \times \frac{1}{Colorimetric\ diameter \times Reaction\ time} \times Dilution\ multiple\ of\ sample\ in\ react\ system\ \left(\frac{1.01}{0.05}\right)$$

$$\text{HK activity (U/ml)} = \frac{\Delta A}{6.22} \times \frac{1}{0.5 \times 2} \times \frac{1.01}{0.05}$$

3.2.12.2 Determination of Pyruvate

Operation steps to determine Pyruvate in sample: Cells culture samples:

Cell culture was done as described in (3.2.3.2) but by using REF, AMJ13 and MCF-7 cell lines. The cell lines were treated with MH (62.5 µg/ml) and NDV (MOI 2) separately and synergistically, incubator at 37℃ for 72 hrs. Cell suspension was collected by thawing to prepare cells samples. Blank tube: 0.1 ml of double-distilled water (DDW), 0.1 ml of Reagent (R) 1 and 0.5 ml of R 2 were added. Standard tube: 0.1 ml of 0.2 µmol/ml Sodium pyruvate standard solution R4, 0.1 ml of R 1 and 0.5 ml of R 2 were added. Sample tube: 0.1 ml of sample, 0.1 ml of R 1 and 0.5 ml of R 2 were added. The content of each tube was mixed fully using a vortex mixer for 5s, the tubes were incubated at 37℃ for 10 min, R 3 (2.5 ml) was added into each tube and mixed fully, the tubes were incubated at room temperature for 5 min, ELISA plate reader was set to zero with DDW. The OD value was measured of each tube or well at 505 nm (Table (3-6)).

Table (3-6): Operation steps to determine pyruvate in sample

Operation steps	Blank tube	Standard tube	Sample tube
Double-distilled water (ml)	0.1		
0.2 µmol/ml Sodium pyruvate standard solution (ml) (R4)		0.1	
Cells sample (ml)			0.1

Reagent 1 (ml)	0.1	0.1	0.1
Reagent 2 (ml)	0.5	0.5	0.5
The solutions were fully mixed and incubated at 37℃ for 10 min			
Reagent 3 (ml)	2.5	2.5	2.5

Calculation of results for cell sample:

$$\text{Pyruvate Concentration } (\mu mol/ml) = \frac{OD\ Sample - OD\ Blank}{OD\ Standard - OD\ Blank} \times \text{Concentration of Standard (0.2 µmol/ml)}$$

3.2.12.3 Determination of ATP

a. Sample preparation: Cells culture samples:

Cell culture was done as described in (3.2.3.2) but by using REF, AMJ13 and MCF-7 cell lines. The cell lines were treated with MH (62.5 µg/ml) and NDV (MOI 2) separately and their combination, incubator at 37℃ for 72 hrs. Cells suspension was collected by thawing to prepare cells samples.

b. Operation steps to determine ATP in sample:

Blank tube: 30 µl standard solution, 100 µl R1, 200 µl R2, and 30 µl double distilled water were added, standard tube: 30 µl standard solution, 100 µl R1, 200 µl R2, and 30 µl R3 were added, sample tube: 30 µl sample, 100 µl R1, 200 µl R2, and 30 µl R3 were added, control tube: 30 µl sample, 100 µl R1, 200 µl R2, and 30 µl double distilled water were added, the tubes were mixed and place in a water bath at 37℃ for 30 min, 50 µl R4 was added to all tubes and mixed fully, the content of each tube was centrifuge at 4000 r/m for 5 minutes. 300 µl of supernatant samples were taken and were added to another tubes, 500 µl R5 was added to all new tubes, mixed and left at room temperature for 2 min, after which 500 µl R6 was added to all tubes. The solutions were mixed and left at room temperature for 5 min, and their OD or absorbance values were measured at 636 nm using ELISA plate reader (Table (3-7)).

Table (3-7) Operation steps to determine ATP in sample

Operation steps	Blank	Standard	Sample	Control
1 mmol/l ATP standard application solution (µl)	30	30		
Cells sample(µl)			30	30
Reagent 1: substrate I (µl)	100	100	100	100
Reagent 2: substrate II(µl)	200	200	200	200
Reagent 3: accelerant (µl)		30	30	
Double distilled water (µl)	30			30
The solutions were mixed and incubated for 30 min in water bath at 37℃				
Reagent 4: precipitant (µl)	50	50	50	50

Centrifuge for 5 minutes at 4000 rpm after mixing fully

The solution was mixed, still for 5 minutes at room temperature. The OD values were

Supernatant sample (µl)	300	300	300	300
Reagent 5: developer (µl)	500	500	500	500
The solutions were mixed and still for 2 minutes at room temperature				
Reagent 6: stop solution (µl)	500	500	500	500

measured of each tube or microplate at 636nm wavelength using ELISA plate reader.

c. Calculation of results

Computational formula for cell:

$$\text{Concentration of ATP (µmol /L)} = \frac{OD\ Sample - OD\ Control}{OD\ Standard - OD\ Blank} \times \text{Concentration of Standard (1000 µmol/L)}$$

3.2.13. pH measurements

The cell lines (REF, AMJ13 and MCF-7) were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin, and incubated in a humidified atmosphere of 5% CO2 at 37ºC, overnight before the treatment. A of 96-well microplate was used to seed 1×10^4 cell lines, and then incubated at 37ºC for 24-48 hrs until monolayer was achieved as observed by an inverted microscope (Cell culture was done as described in (3.2.3.2)).

The cell lines were treated with D-Mannoheptulose (62.5 µg/ml) and NDV (MOI 2) separately and synergistically, incubated at 37℃ for 72 hr, cell suspension was collected by thawing, and then the pH was measured using litmus papers for AMJ13, MCF7, and REF cell lines with comparison to the control (Al-Shammari *et al*., 2015).

3.2.14. Statistical Analysis.

All results were presented as, means ± SD, t-test was carried out and analyzed using the statistical software Excel version 19, SoftMax program 6.4, SPSS version 16 and GraphPad prism version 7. Isobologram version 1 were performed to compare the difference between groups under different conditions, P values <0.05 were considered significant. CompuSyn software program algorithm assessed the combination index CI. Combined dose-response curves were fitted on Chou-Talalay lines. CI <1 indicates synergistic interaction, whereas CI >1.1 was antagonistic and CI between 1 and 1.1 was considered additive (Chou, 2010).

CHAPTER-FOUR

4. Results

4.1. Virus propagation in Embryonated Chicken Eggs (ECE)

4.1.1. Propagation of virus (NDV) in chicken embryo

Embryonated chicken eggs (ECE) were used in virus propagation. The infected embryos were characterized with hemorrhage in comparison to control embryos (Figure (4-1)). The results showed that NDV Iraqi virulent strain was able to kill all embryos (9 or 10 days old) up to 48 hrs of inoculation in the ECE. Some embryonated chicken eggs were killed before 24 hrs, this type of ECE were canceled and save after 24 hrs to aggregate the virus.

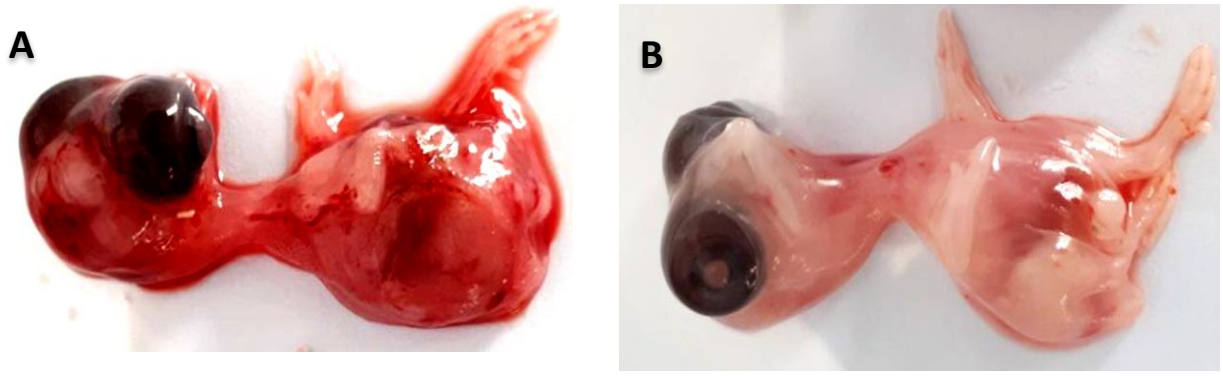
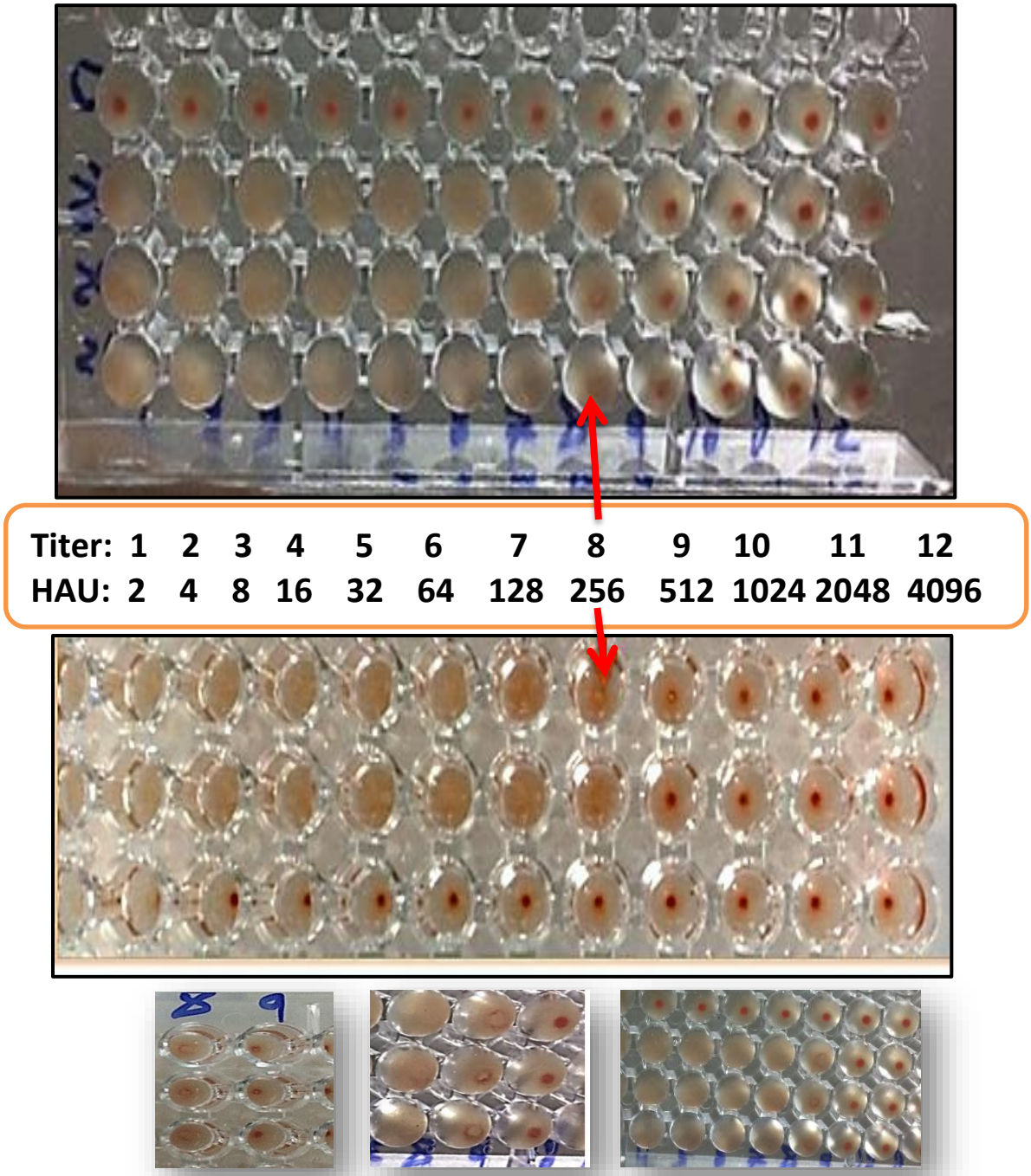


Figure: (4-1) Inoculation of NDV in ECE: (A) Skin hemorrhage in ECE after inoculated with NDV (B) ECE control.

4.1.2. Harvest of NDV and measurement of Hemagglutination Unit (HAU)

The results showed that harvested Iraqi strain of NDV has the ability to kill all the chicken embryos through 24-72 hrs after the inoculation of the chicken embryonated eggs with marked hemorrhage in the infected embryos as compared to the control uninfected embryos. Virus was aggregated, purified, tittered, and quantified by hemagglutination test. Titer and HAU of NDV was measured (Titer = 8), (HAU = 256). It showed a positive result as a typical hemagglutination mesh pattern of chicken red blood cell at 256 HAU (Figure (4-2)).



Figures (4-2) Hemagglutination test, show the agglutination (positive result) of RBC forms a distinctive mesh at the bottom (titer 8). The negative hemagglutination is characterized by sharp red dot in comparison to the control.

4.1.3. Tissue Culture Infectious Dose (TCID $_{50}$)

The NDV titer was measured on Vero cell line to determine the $TCID_{50}$ of the virus. $TCID_{50}$ was measured (124.6×10^6 virus/ ml) (Table (4-2)). It showed that NDV formed plaques on infected Vero cells line compared with uninfected control cells (Figure (4-3)).

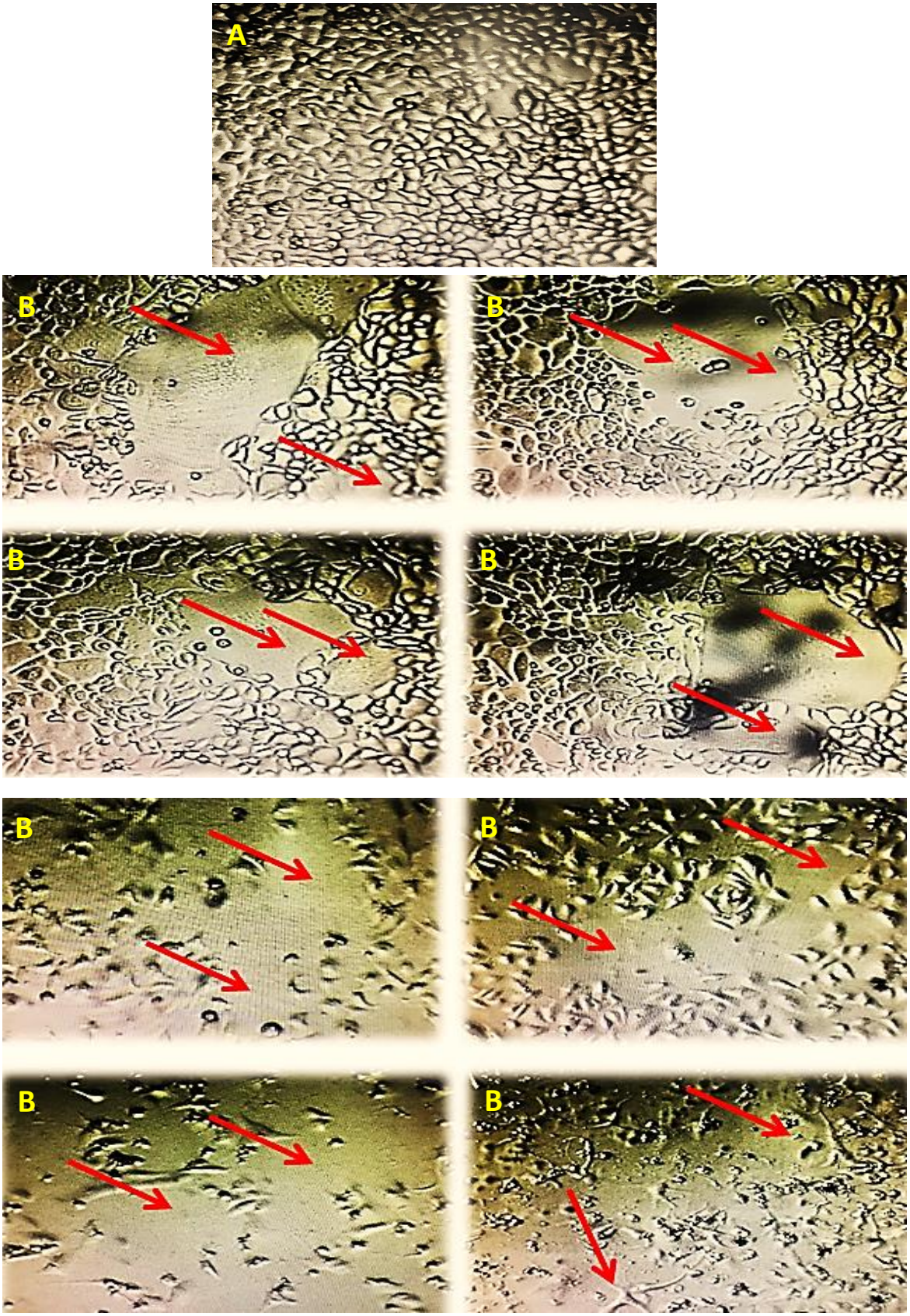


Figure (4-3) Vero cells treated with NDV: A- Vero cells untreated as control, B- Vero cells treated with NDV showed hemolysis of cells (apoptosis) and plaques formation between cells, (Red arrows refer to plaques formation).

Table (4-1) Results of Vero cells were treated with NDV to determine TCID50

A			B			C		
log_{10} virus dilution	Ratio of infection	Proportion (pi)	log_{10} virus dilution	Ratio of infection	Proportion (pi)	log_{10} virus dilution	Ratio of infection	Proportion (pi)
-1	8/8	1	-1	8/8	1	-1	8/8	1
-2	8/8	1	-2	8/8	1	-2	8/8	1
-3	8/8	1	-3	8/8	1	-3	8/8	1
-4	8/8	1	-4	8/8	1	-4	8/8	1
-5	8/8	1	-5	8/8	1	-5	8/8	1
-6	6/8	0.750	-6	5/8	0.625	-6	5/8	0.625
-7	3/8	0.375	-7	3/8	0.375	-7	4/8	0.500
-8	1/8	0.125	-8	1/8	0.125	-8	2/8	0.250
-9	0/8	0.0	-9	1/8	0.125	-9	0/8	0.0
-10	0/8	0.0	-10	0/8	0.0	-10	0/8	0.0

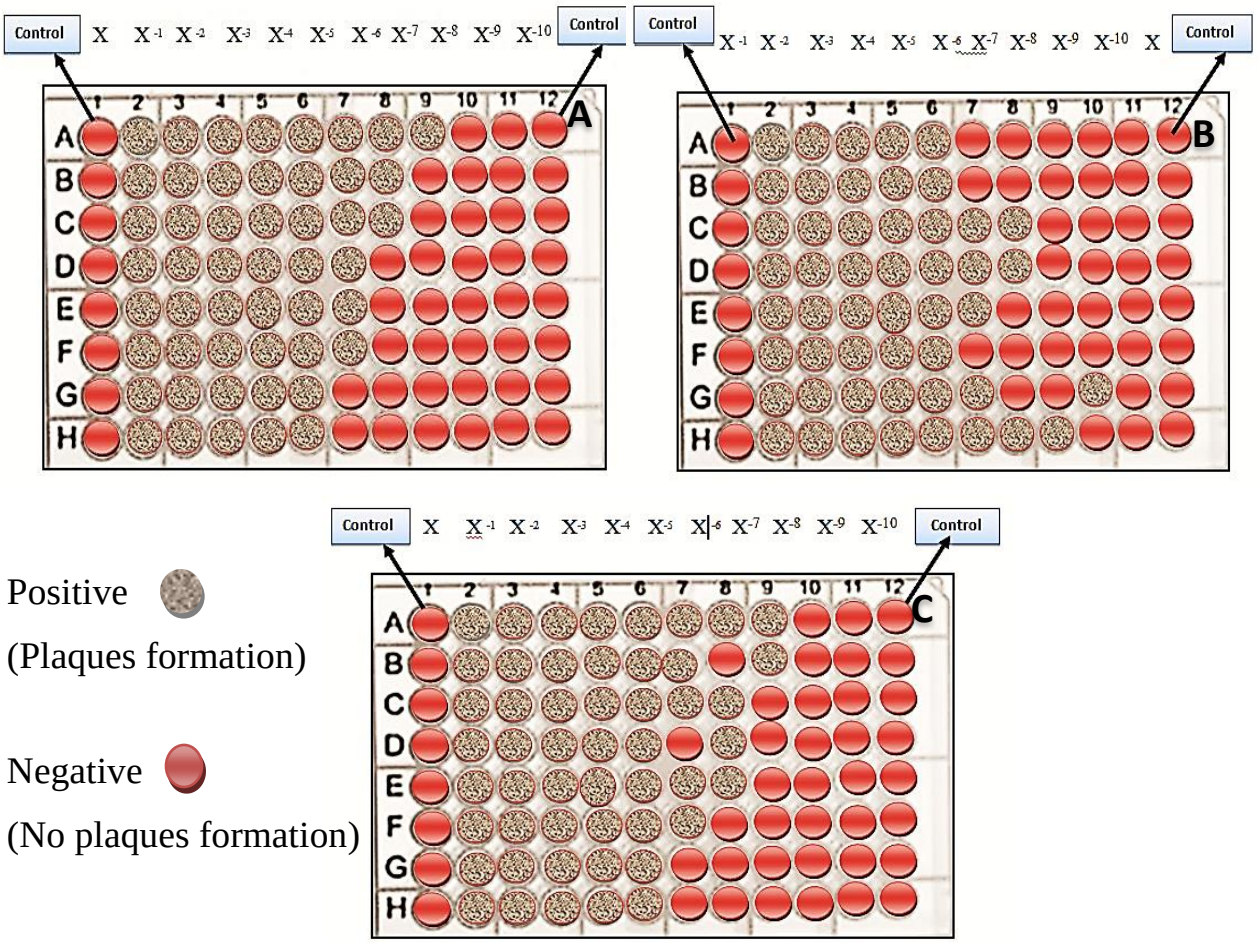


Figure (4-4) Exposure of NDV to Vero cell line to measure TCDI50: A- exp 1, B- exp 2, and C- exp 3

Vero cell lines were treated with the virus showed plaques formation. The positive result represents plaques formation between cells were explained in the first experiment (Table (4-1-A)) and (Figure (4-4-A)), the second experiment (Table (4-1-B)) and figure (4-B), and the third experiment (Table (4-1-C)) and (Figure (4-4-C)). The final values for TCID50/ml for exposure of NDV to Vero cell line in 96-well microplate which represents the number of viruses for each 1ml are shown in (Table (4-2)).

Table (4-2) Final value for TCID50/ml for exposure of NDV to Vero cells

No. of Exp	$TCID_{50}$/ml	Average $TCID_{50}$/ml
1	1.122×10^8/ml	1.246×10^8 /ml
2	1.122×10^8/ml	
3	1.496×10^8/ ml	124.6×10^6/ml

4.2 Cytotoxicity assay

The effect of cytotoxicity was evaluated using different MOI of NDV (Table (4-3)) by MTT cytotoxicity assay. Through our findings, increased MOI of the virus was shown to increase cytotoxicity or improve growth inhibition. There is a statistically significant difference between inhibition of the virus for breast cancer cell lines compared to normal REF cell line, where effect of NDV seen in breast cancer cells was more than that seen in normal cells (Figure (4-5 and 6)).

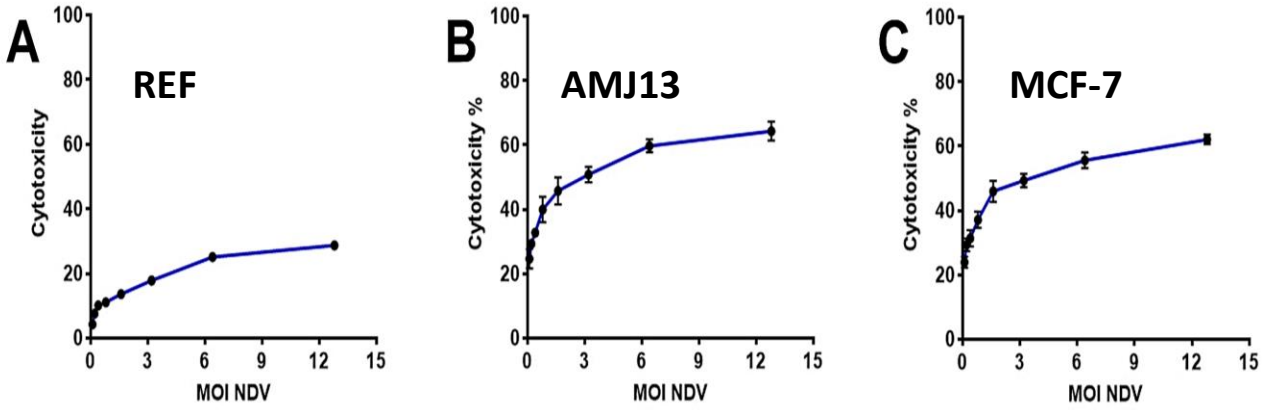
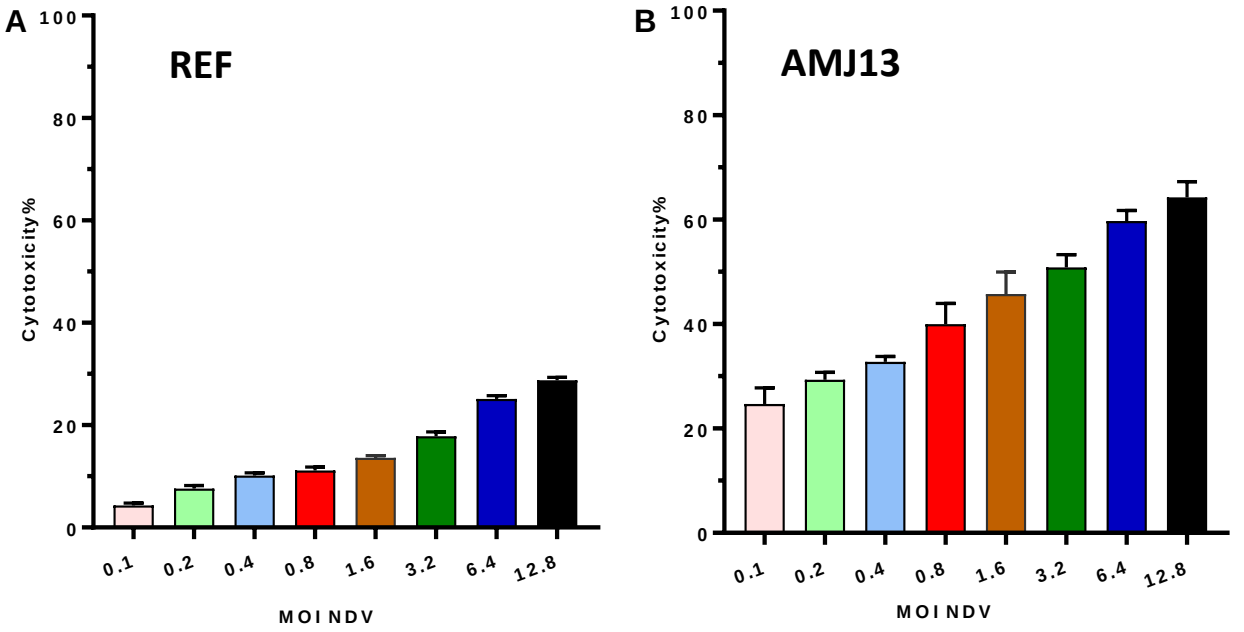


Figure (4-5) Cytotoxicity effect (CT %) after treatment of cell lines with NDV: A- NDV to REF, B- NDV to AMJ13, and C- NDV to MCF7 by linear regression.



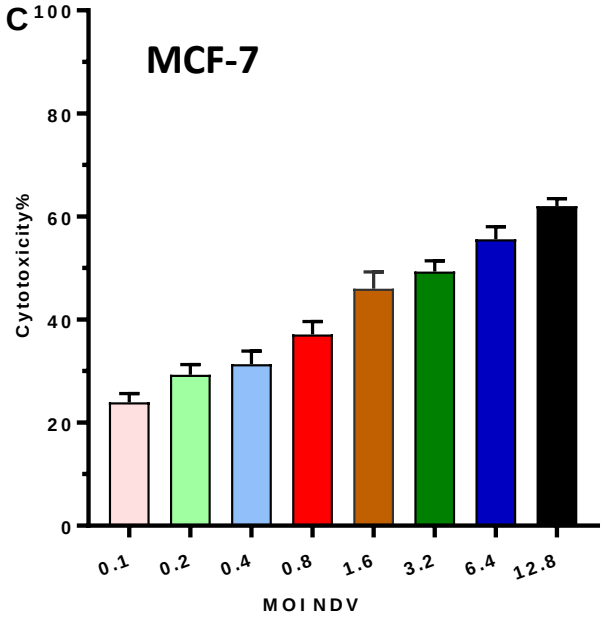


Table (4-3) CT% after treatment of cell lines with NDV

MOI NDV	CT% REF	CT% AMJ13	CT% MCF7
0.1	4.31	24.69	23.95
0.2	7.60	29.33	29.29
0.4	10.14	32.77	31.35
0.8	11.11	39.96	37.12
1.6	13.61	45.74	45.99
3.2	17.81	50.84	49.31
6.4	25.10	59.70	55.56
12.8	28.75	64.26	62.02

Figure (4-6) Comparison of cytotoxicity between different MOI after treatment of cell lines with NDV: A- CT % - NDV to REF, B- CT % - NDV to AMJ13, and C- CT % - NDV to MCF7 by column bar graph

The effect of cytotoxicity was evaluated using different concentrations of MH (µg/ml) (Table (4-4)) and by MTT cytotoxicity assay. From these results, increased concentration of the inhibitor has been shown to increase cytotoxicity, or enhance growth inhibition. There is a statistically significant difference between inhibition

by MH for breast cancer cell lines compared to normal REF cell line, where a greater effect of MH was seen in breast cancer cells than in normal cells (Figure (4-7 and 8)).

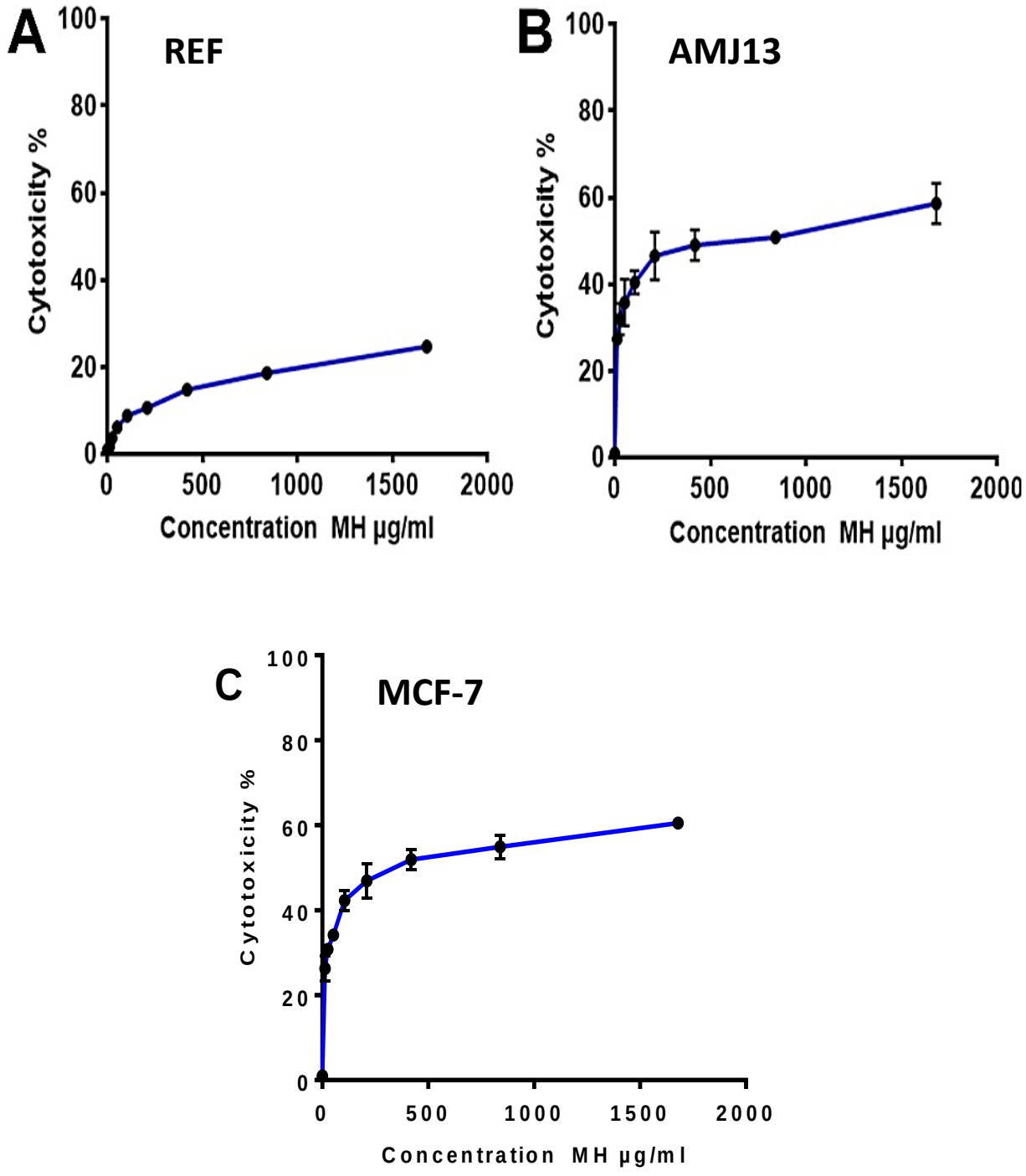


Figure (4-7) Cytotoxicity effect (CT %) after treating cell lines with MH: A - MH to REF, B - MH to AMJ13, and C - MH to MCF7 by linear regression.

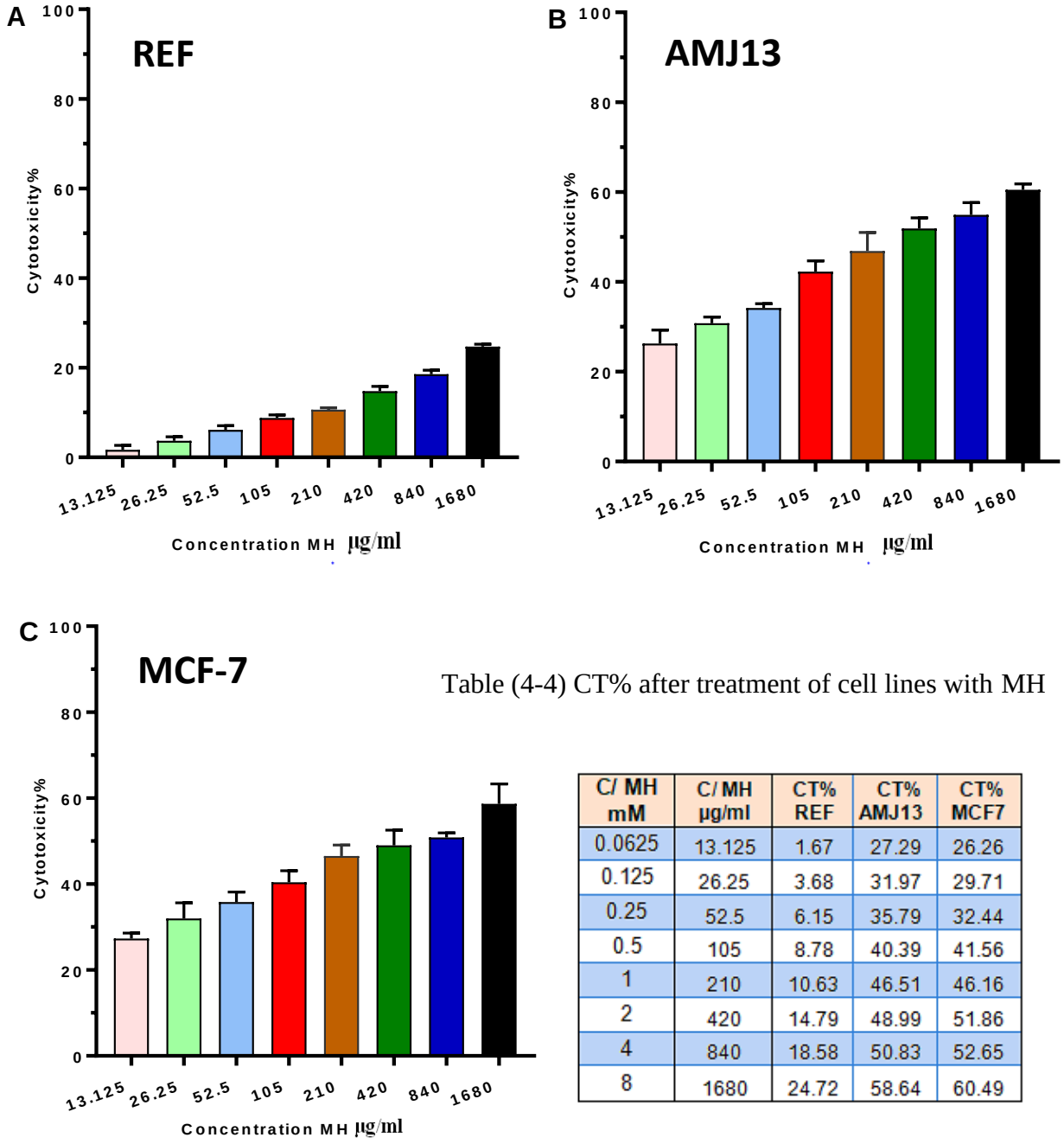


Table (4-4) CT% after treatment of cell lines with MH

C/ MH mM	C/ MH µg/ml	CT% REF	CT% AMJ13	CT% MCF7
0.0625	13.125	1.67	27.29	26.26
0.125	26.25	3.68	31.97	29.71
0.25	52.5	6.15	35.79	32.44
0.5	105	8.78	40.39	41.56
1	210	10.63	46.51	46.16
2	420	14.79	48.99	51.86
4	840	18.58	50.83	52.65
8	1680	24.72	58.64	60.49

Figure (4-8) Comparison of cytotoxicity between different concentrations (µg/ml) after cell lines were treated with MH: A- CT % - MH to REF, B- CT % - MH to AMJ13, and C- CT % - MH to MCF7 by column bar graph

The effect of cytotoxicity was evaluated using different concentrations of DXL (µg/ml) (Table (4-5)) and by MTT cytotoxicity assay. From these results, increased concentration of the drug has been shown to increase cytotoxicity or

induce proliferation inhibition. There is a significant difference between inhibition by drug for breast cancer cell lines compared to normal REF cell line, where a greater effect of the drug was seen in breast cancer cells than normal cells (Figure (4-9 and 10-I)).

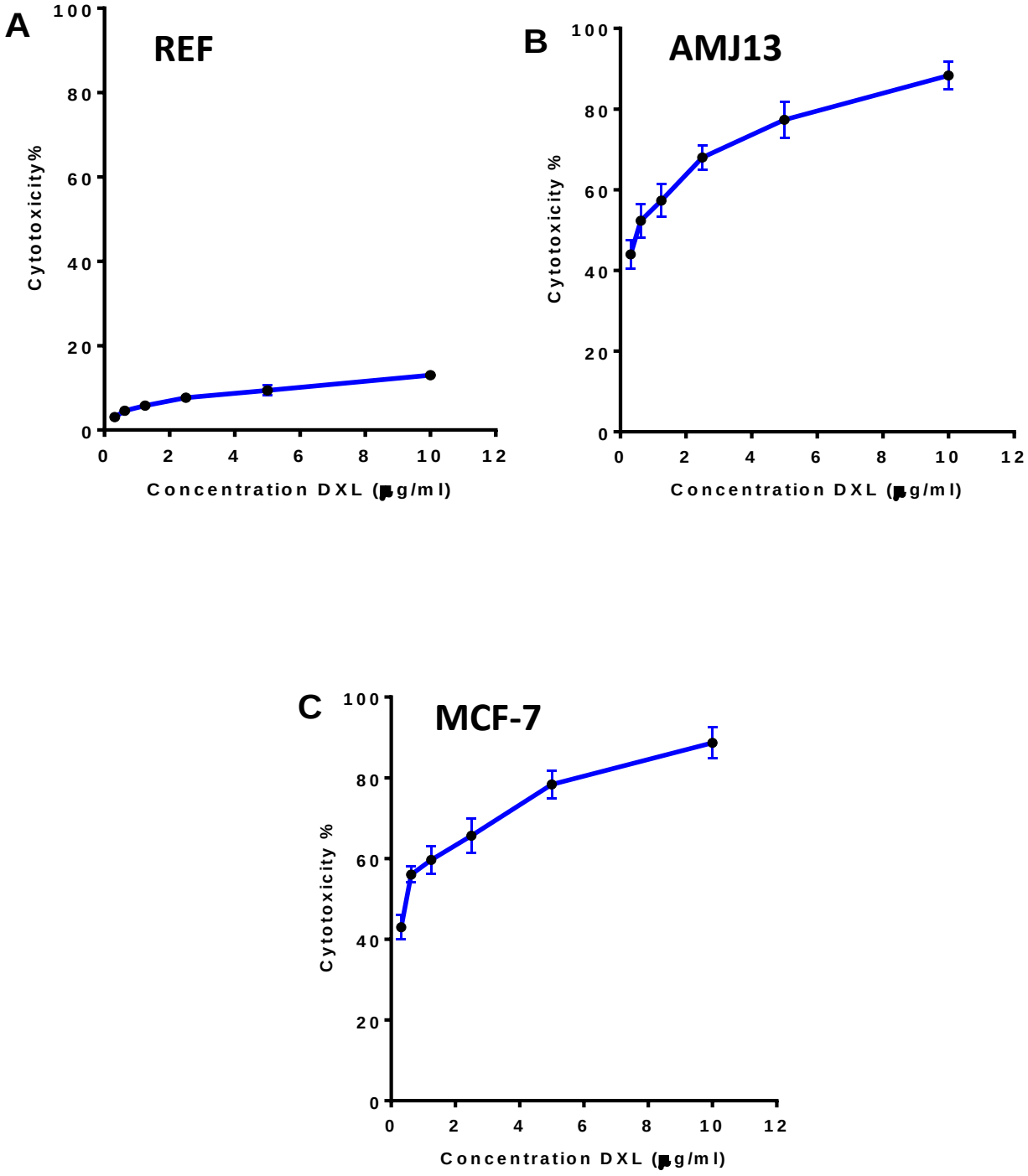
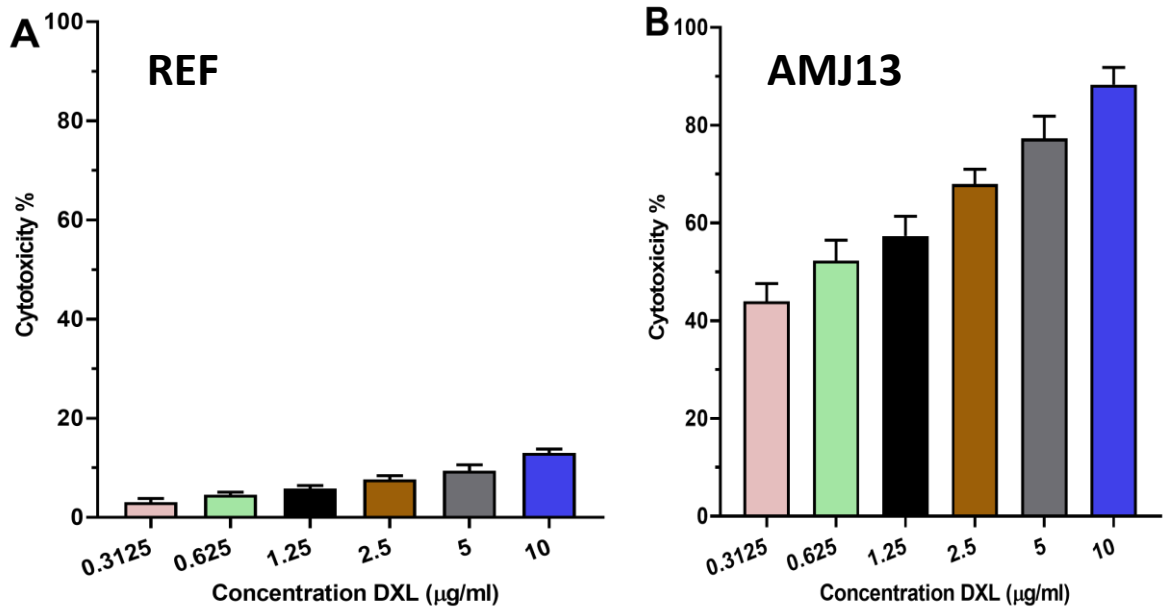


Figure (4-9) Cytotoxicity effect (CT %) after cell lines were treated with Docetaxel (DXL) (µg/ml): A- DXL to REF, B- DXL to AMJ13, and C- DXL to MCF7 by linear regression.



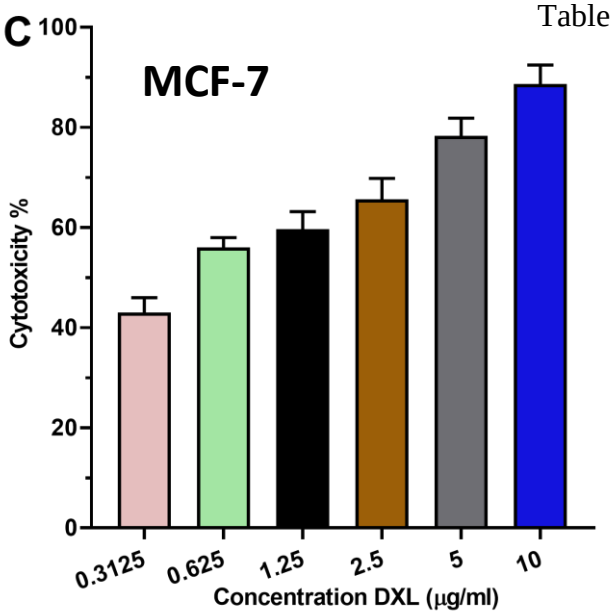


Table (4-5) CT% after treatment of cell lines with DXL

C/DXL µM	µg/ml	CT% REF	CT% AMJ13	CT% MCF7
12.37	10	13	88.33	88.67
6.18	5	9.4	77.33	78.33
3.09	2.5	7.67	68.00	65.67
1.54	1.25	5.80	57.33	59.67
0.77	0.625	4.57	52.33	56.00
0.38	0.313	3.07	44.00	43.00

Figure (4-10-I) Comparison of cytotoxicity between different concentrations (µg/ml) after cell lines were treated with DXL: A- CT % - DXL to REF, B- CT % - DXL to AMJ13, and C- CT % - DXL to MCF7 by column bar graph. DXL

Docetaxel is a chemotherapy drug that was used as a positive control for comparison with the effect of D-Mannoheptulose as a phytotherapeutic, NDV as a virotherapy, and their combination on normal and breast cancer cell lines.

4.2.1 Cytotoxicity of NDV and MH against breast cancer and normal cell lines

MTT cytotoxicity assay was used to evaluate the effect against cancer and normal cells of different concentrations of MH and over a range of MOI of NDV (Figure 4-10-II). There were no noticeable percentages of cytotoxicity (CT%) for MH against normal REF cells as the CT% ranged from 1.67%, to 24.72% at higher concentrations. While there was higher cytotoxicity against breast cancer cells ranged from 27.29% to 58.64% for AMJ13 cells; and 26.26% to 60.49% for MCF-7 after MH treatment (Figures 4-10-II A, B, and C). NDV virotherapy did not induce cytotoxic effect against normal embryonic REF cells as the CT% ranged from 4.31% to 28.75% for REF (Figure 4-10-II F). Breast cancer cells were more sensitive to NDV virotherapy as the CT% ranged from 24.69% to 64.26% for AMJ13; and 23.95% to 62.02% for MCF-7 cell line after NDV treatment (Figures 4-10-II D and E).

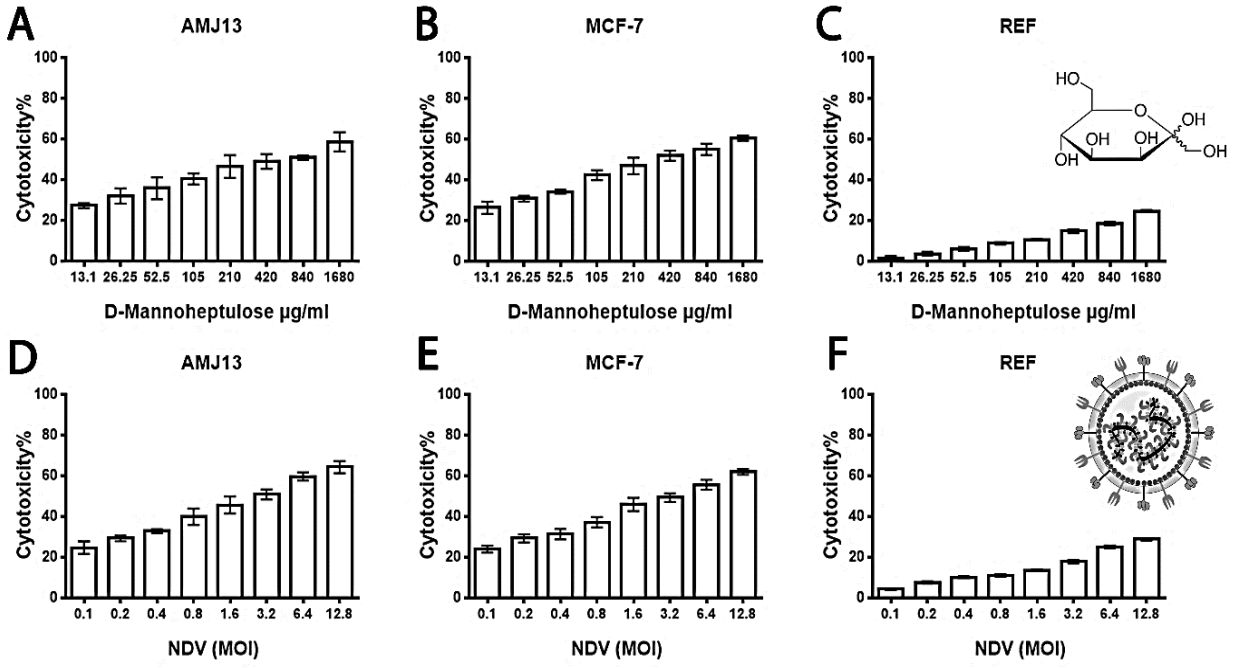


Figure (4-10- II): MH and oncolytic AMHA1 NDV are cytotoxic against human AMJ13 and MCF-7 breast cancer cells, but not cytotoxic to normal embryonic REF cells. The cells were treated with (A, B, C) D-Mannoheptulose (MH) (13.125, 26.25, 52.5, 105, 210, 420, 840, and 1680 µg/ml) or (D, E, F) NDV (MOI 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8) for 72h. cytotoxicity was investigated using MTT assay. All data shown are means ± SD from three independent experiments.

4.3. Determination of IC50

To evaluate the effect of each treatment on cell proliferation, IC50 value was measured in normal and breast cancer cell lines. IC50 was measured for NDV and it showed that 57.5 MOI infectious virus were needed to kill REF normal cells, while only about 2 virus (1.648 MOI) were needed to kill AMJ13 Iraqi breast cancer cells and (1.561 MOI) MCF7 breast cancer cells (Figure 4-11 A, B, and C) with different MOI of NDV (Table (4-3)).

IC50 value for the hexokinase inhibitor MH in normal REF cells was 486.9 µg/ml while for breast cancer cells is was 75% less required to kill them, (124.7 µg/ml for AMJ13 and 122.6 µg/ml for MCF-7), (Figure 4-12 A, B, and C) with different concentrations of MH (Table (4-4)).

IC50 of DXL was twice higher in normal cells (6.072 µg/ml) than in cancer cells, (3.118 µg/ml for AMJ13 and 3.221 µg/ml for MCF-7), (figure 13- A, B, and C) with different concentrations of DXL (Table (4-5)).

Statistical data of IC50 for comparison of cell lines after treatment with NDV, MH, and DXL can be seen in (Table (4-6)). IC50 is a measure of the potency of a virus, drug, and inhibitor in inhibiting breast cancer cell lines. It is a quantitative measure that indicates the amount of a particular inhibitory substance (DXL, NDV, and MH) needed to inhibit proliferation of breast cancer. It expresses the drug concentration at which 50% of cells are inhibited which explains why the value of IC50 that appeared in the breast cancer cells was less than that in the normal cell lines.

The results showed that there are significant differences in IC50 between breast cancer cell lines and REF cell line after treatment with virus, inhibitor, and drug, while no significant differences were found between MCF-7 and AMJ13 cell lines (Figure (4-14)). Cytotoxicity was measured between REF, AMJ13, and MCF-7 cell lines at specific concentrations after treatment with 2 MOI NDV, 62.5 µg/ml

MH, and, 1.25 µg/ml DXL depend on combination index for 72 hrs. The effect of NDV, MH, and DXL on breast cancer cell lines was very clear compared to normal REF cell line where a significant difference was found between them (Figure (4-15)) and (Table (4-7)).

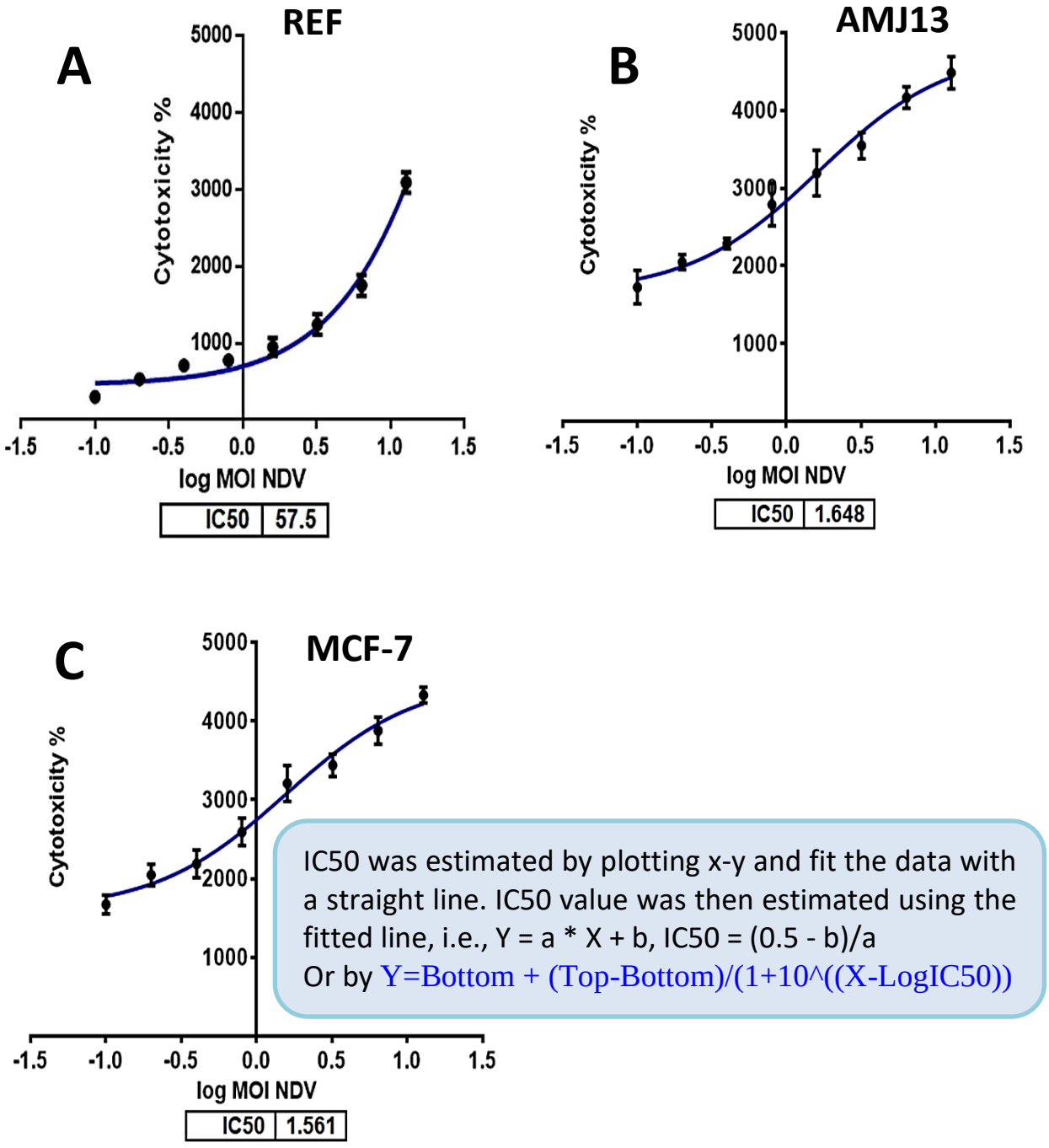


Figure (4-11): IC50 to exposure of NDV to cell lines: IC50 values after REF, AMJ13, and MCF-7 cell lines were treated with NDV, by GraphPad Prism software. A- IC50 - NDV to REF, B- IC50 - NDV to AMJ13, and C - IC50 - NDV to MCF-7.

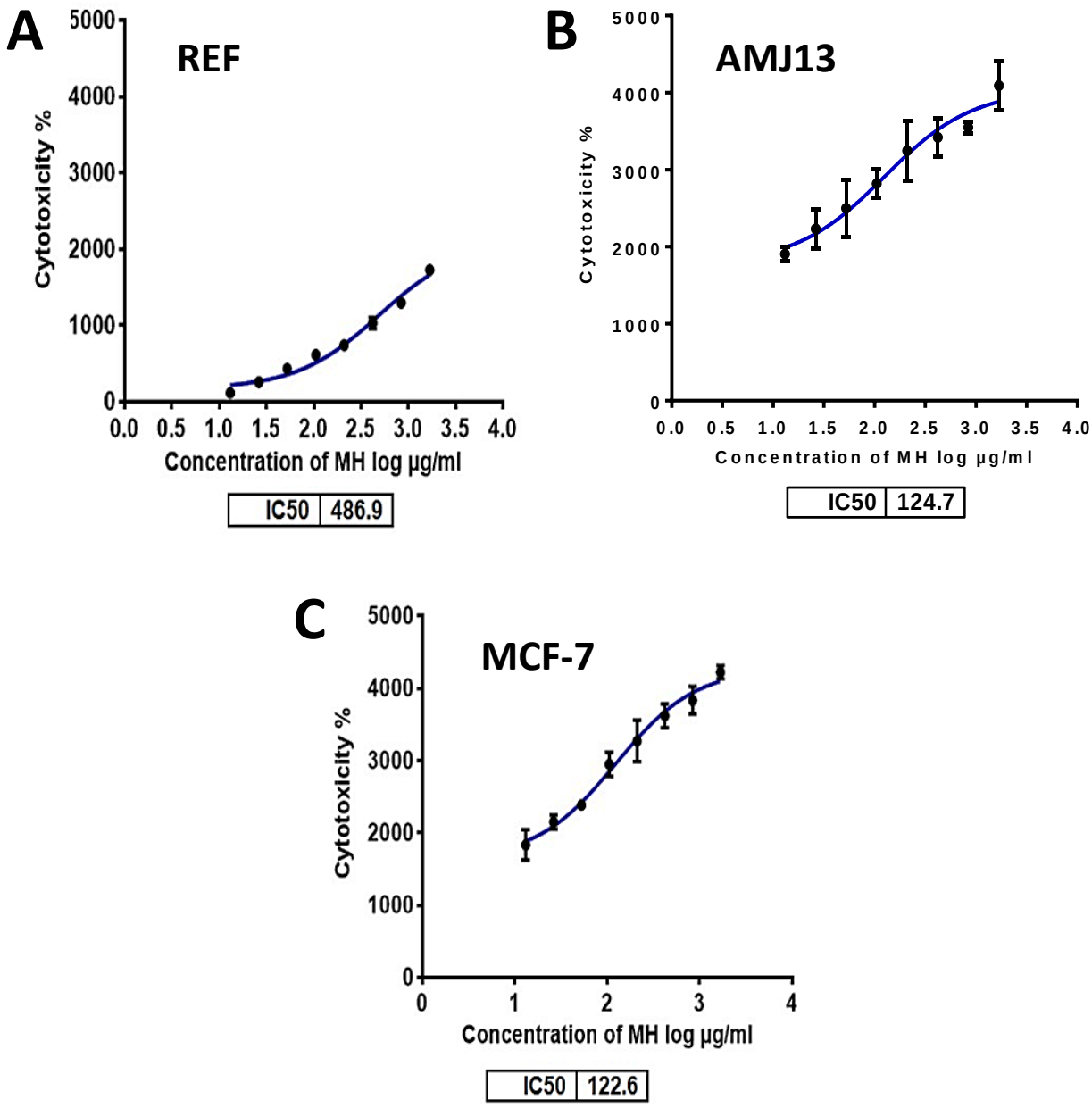


Figure (4-12): IC50 to exposure of MH to cell lines: IC50 values after REF, AMJ13, and MCF-7 cell lines were treated with MH, by GraphPad Prism software. A- IC50 - MH to REF, B- IC50 - MH to AMJ13, and C - IC50 - MH to MCF-7.

The cytotoxicity assay analysis showed that IC50 values for NDV MOI (57.5 REF, 1.648 AMJ13 and 1.561 MCF-7), IC50 values of MH (486.9µg REF, 124.7µg AMJ13, and 122.6µg MCF7). IC50 values of DXL (6.072µg REF, 3.118µg AMJ13, and 3.221µg MCF7). Therefore, it was chosen IC50 related doses of the MH and NDV for the combination study, (0.3, 1, 2 MOI) for NDV and (62.5, 125, and 250 µg/ml) for MH. (1.25, 2.5 and 5 µg/ml) for DXL as positive control.

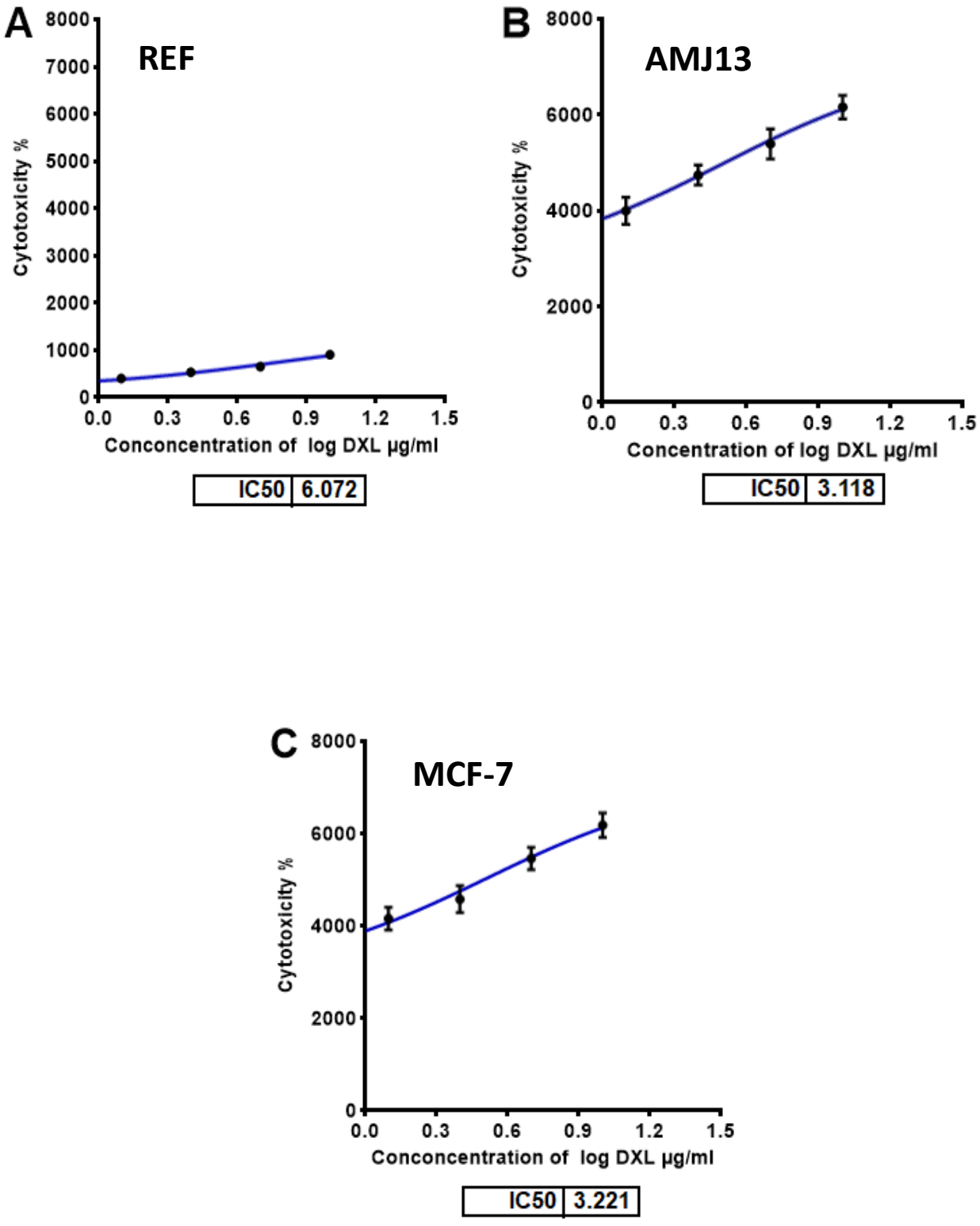


Figure (4-13): IC50 to exposure of DXL (µg/ml) to cell lines: IC50 values after REF, AMJ13, and MCF-7 cell lines were treated with DXL by GraphPad Prism software. A- IC50 - DXL to REF, B- IC50 - DXL to AMJ13, and C - IC50 - DXL to MCF-7.

Table (4-6): Statistical data for comparison of IC50 in cell lines after treated with NDV, MH, and DXL.

IC50 – NDV		Mean ± SD
Cell Lines	REF	57.5 ± 3.7
	AMJ13	1.65 ± 0.06
	MCF-7	1.56 ± 0.06
P value (T-test)		
REF	MCF7	
****S, P < 0.0001		
AMJ13	MCF7	
ns, P = 0.162 (P >0.05)		
REF	AMJ13	
****S, P < 0.0001		

IC50 – MH		Mean ± SD
Cell Lines	REF	487 ± 5.8
	AMJ13	125 ± 5.9
	MCF-7	123 ± 5.5
P value (T-test)		
REF	MCF7	
****S, P < 0.0001		
AMJ13	MCF7	
ns, P = 0.675 (P >0.05)		
REF	AMJ13	
****S, P < 0.0001		

IC50 – DXL		Mean ± SD
Cell Lines	REF	6.1 ± 0.11
	AMJ13	3.1 ± 0.23
	MCF-7	3.2 ± 0.09
P value (T-test)		
REF	MCF7	
****S, P < 0.0001		
AMJ13	MCF7	
ns, P = 0.515 (P >0.05)		
REF	AMJ13	
****S, P < 0.0001		

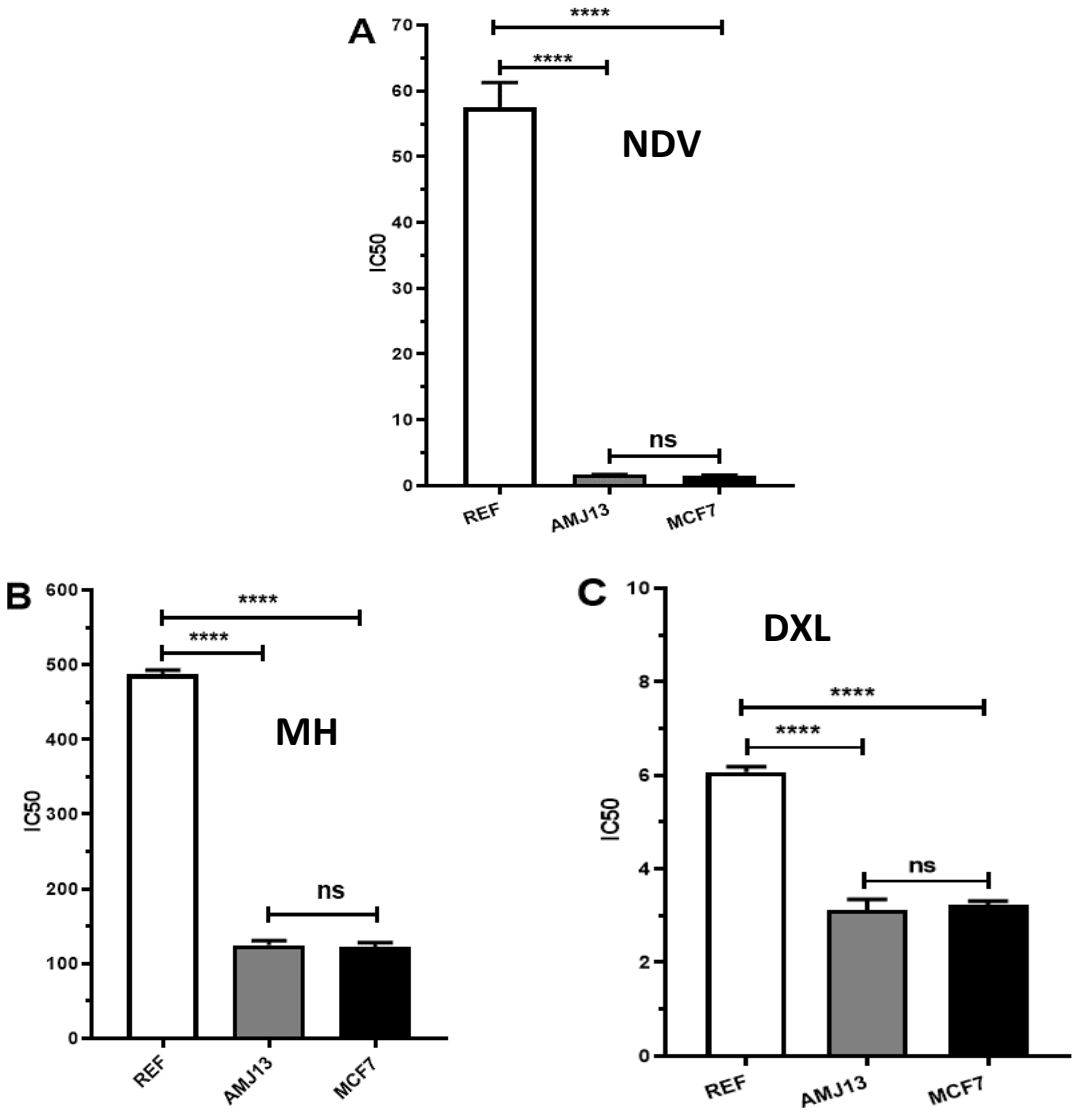


Figure (4-14): Comparison of IC50 between REF, AMJ13, and MCF-7 cell lines after treatment with NDV, MH, and DXL for 72 hrs, A-IC50-NDV, B- IC50 –MH, and C- IC50-DXL.

Table (4-7): Statistical data for comparison of CT% in cell lines after treated with NDV, MH, and DXL.

CT% – NDV		Mean ± SD
Cell Lines	REF	16 ± 1.8
	AMJ13	58 ± 2.8
	MCF-7	67 ± 2.5
P value (T-test)		
REF	MCF7	
****S, P < 0.0001		
AMJ13	MCF7	
*s, P = 0.014 (P < 0.05)		
REF	AMJ13	
****S, P < 0.0001		

CT% – MH		Mean ± SD
Cell Lines	REF	7.3 ± 0.71
	AMJ13	43 ± 3.2
	MCF-7	40 ± 1.0
P value (T-test)		
REF	MCF7	
****S, P < 0.0001		
AMJ13	MCF7	
ns, P = 0.208 (P > 0.05)		
REF	AMJ13	
****S, P < 0.0001		

CT% – DXL		Mean ± SD
Cell Lines	REF	5.8 ± 0.62
	AMJ13	57.3 ± 4.04
	MCF-7	59.6 ± 3.31
P value (T-test)		
REF	MCF7	
****S, P < 0.0001		
AMJ13	MCF7	
ns, P = 0.492 (P > 0.05)		
REF	AMJ13	
****S, P < 0.0001		

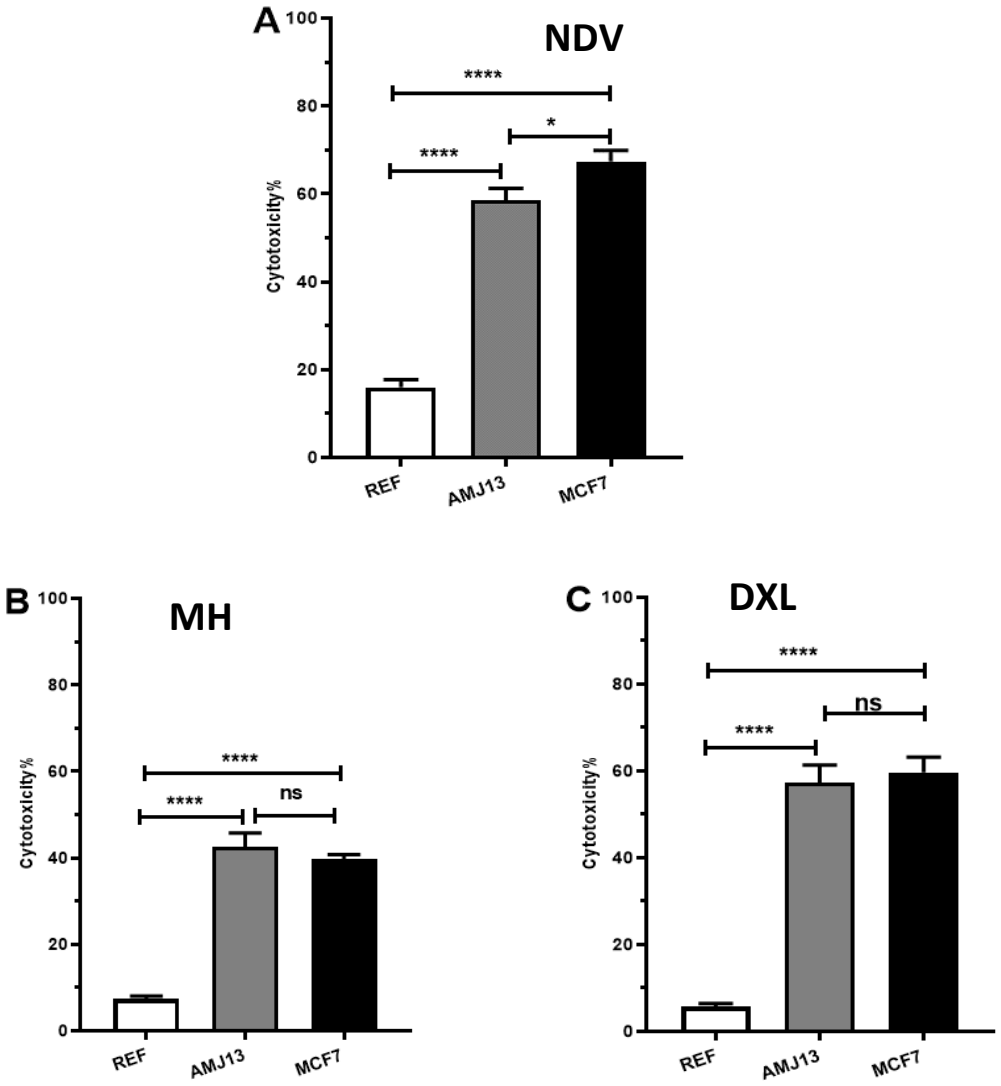


Figure (4-15): Comparison of CT% between REF, AMJ13, and MCF-7 cell lines after treatment with specific concentrations of NDV, MH, and DXL, at 2 MOI NDV, 62.5 µg/ml MH, and 1.25 µg/ml DXL, for 72 hrs. A-CT% -NDV, B- CT% –MH and C- CT%-DXL. Values represent the (mean ± SD). *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

4.4. Study the synergistic effect of NDV and D-Mannoheptulose

Three specific MOI based on IC50 values (0.3, 1, and 2 MOI) of NDV with three specific concentrations also based on IC50 value (250,125, and 62.5 µg/ml) of MH were combined in MTT cytotoxicity assay to study the synergism in cancer cells (Figure 4-16, 17 and 18). The results of cytotoxicity of NDV, MH, and their combination, in REF, AMJ13 and MCF-7 cell line were explained in (Figure 4-16, 17, and 18). The virus (0.3, 1, and 2 MOI) and inhibitor (250, 125, and 62.5 µg/ml) induced cytotoxicity (growth inhibition) significantly when compared between them, but insignificantly as shown in (Figure 4-17 A, E and I), (Figure 4-18, C), and significantly with combination in breast cancer cell line. The combination of MH and virus was a significantly better promoter and enhancer of growth inhibition when compared with their separate treatment in breast cancer cell lines (Figure 4-17 and 18)).

While in normal REF cell line, the effect of virus, inhibitor, and their combination was insignificant and induced cytotoxicity less than in breast cancer cell (Figure (4-16)). A significant induction was identified in growth inhibition in all the treatment modalities in the breast cancer cell lines but not in the normal cells line. (Figure (4-19)) showed in vitro inhibition of growth induced by high MOI of virus, also treatment with NDV showed enhancement of the growth inhibition properties in breast cancer cell lines more than normal cell line. Dose response evaluation of treatments differed significantly between REF and breast cancer cell lines. Statistical data for CT% of NDV for comparison of cell lines can be seen in (Table (4-8)). Figure (20) showed in vitro inhibition of growth induced by high concentrations of MH, as well as treatment with MH showed enhancement of the growth inhibition properties in breast cancer cell lines more than normal cell line. Dose response evaluation of treatments differed significantly between REF and breast cancer cell lines. Statistical data for CT% of MH for comparison of cell lines can be seen in (Table (4-9)).

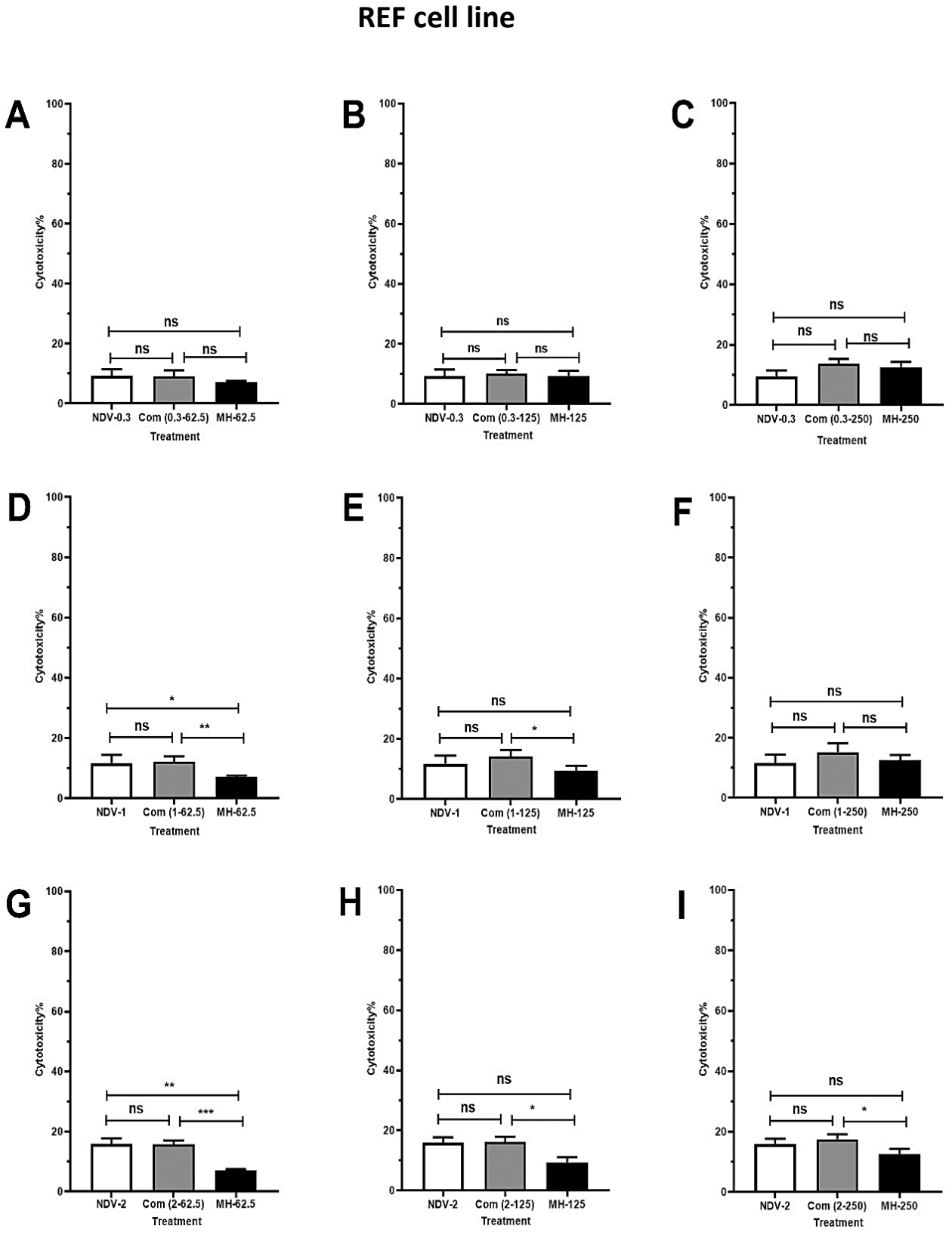


Figure (4-16): Cytotoxicity ratio of normal (REF) cell line after treatment with MH (250, 125, and 62.5 µg/ml), NDV (0.3, 1, and 2 MOI), and combination between them. Values represent the (mean ± SD). *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

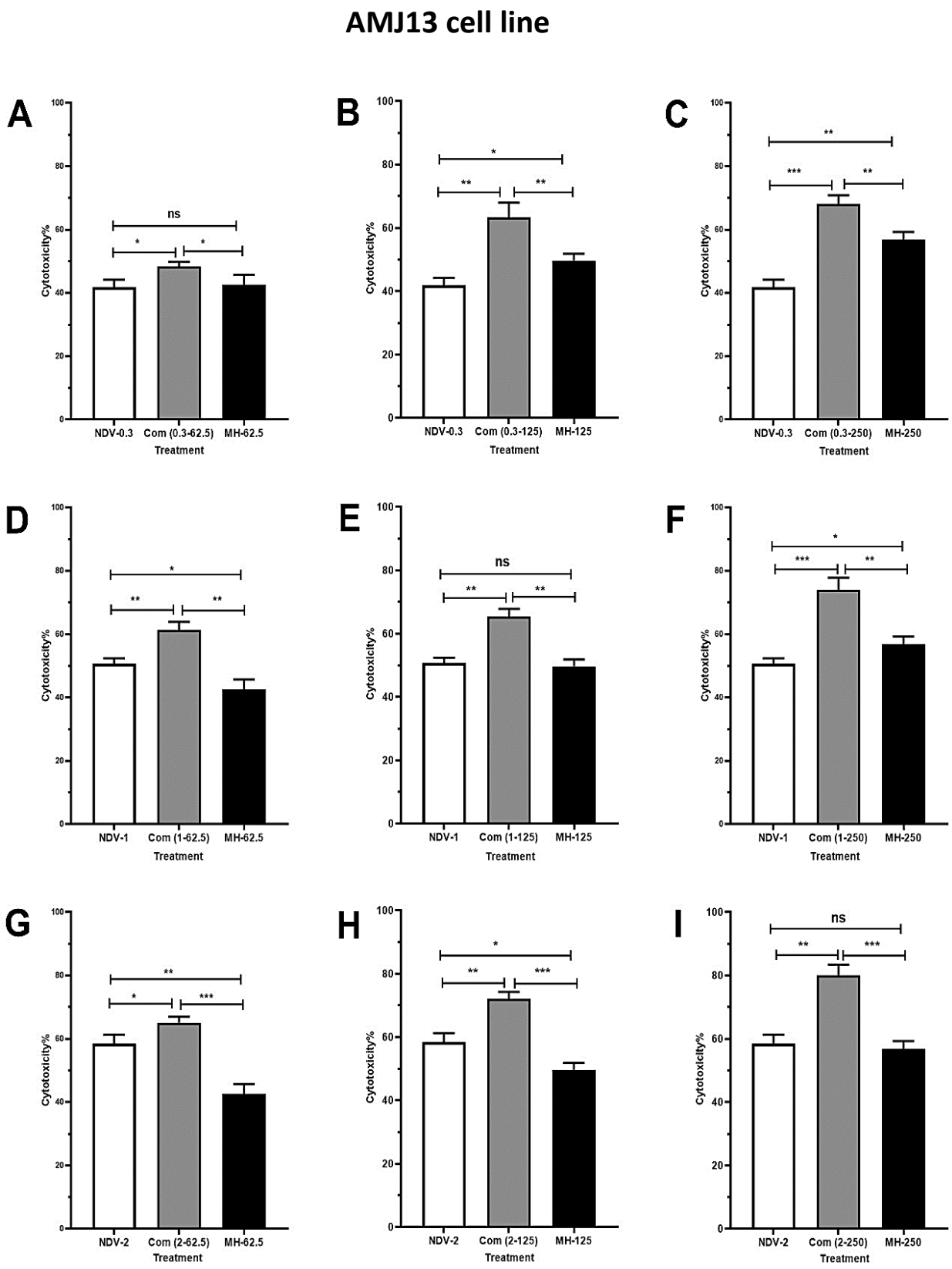


Figure (4-17): Cytotoxicity ratio of breast cancer (AMJ13) cell line after treatment with MH (250, 125, and 62.5 µg/ml), NDV (0.3, 1, and 2 MOI), and combination between them. Values represent the (mean ± SD). *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

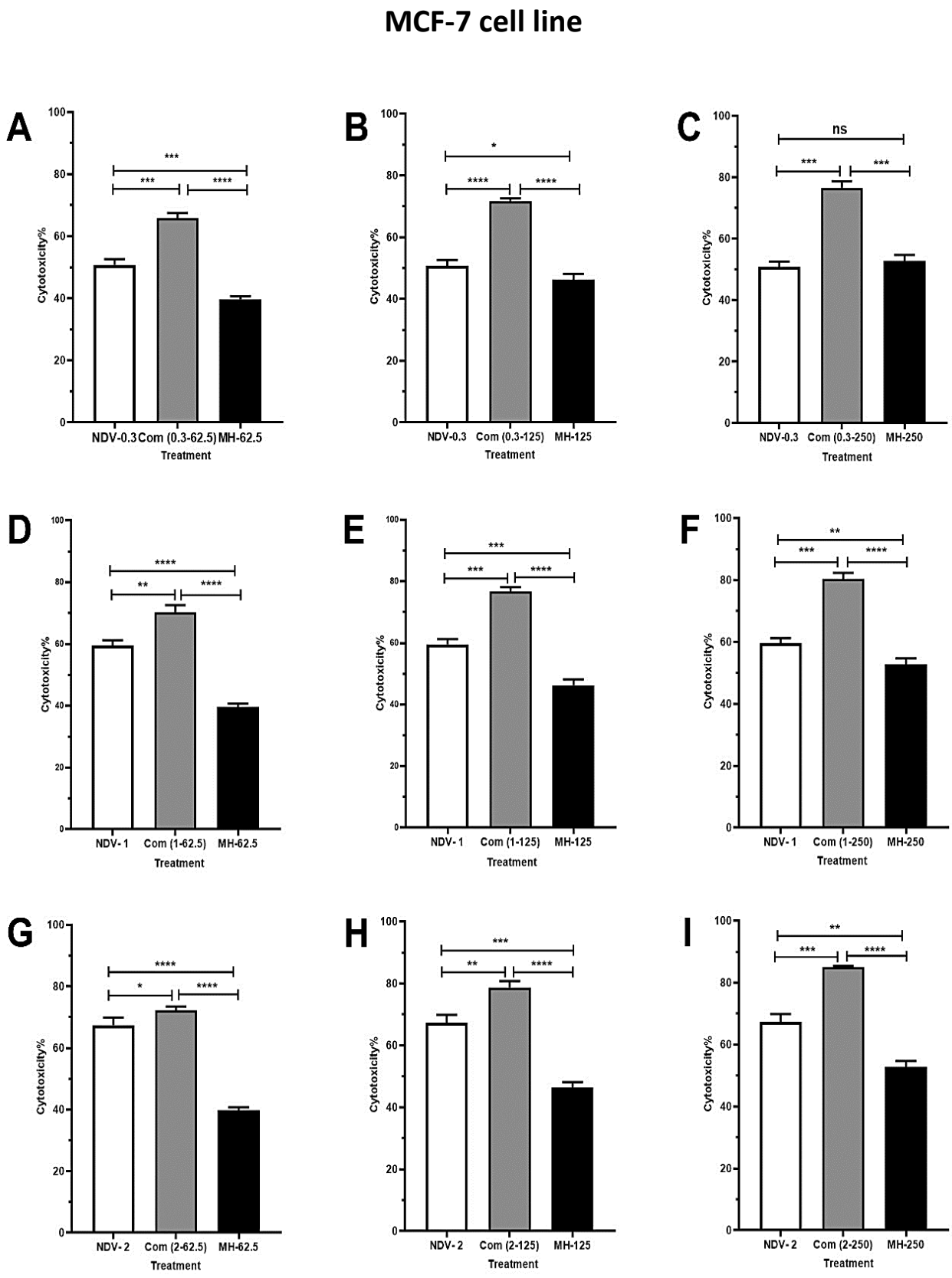


Figure (4-18): Cytotoxicity ratio of breast cancer (MCF-7) cell line after treatment with MH (250, 125, and 62.5 µg/ml), NDV (0.3, 1, and 2 MOI), and combination between them. Values represent the (mean ± SD). *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

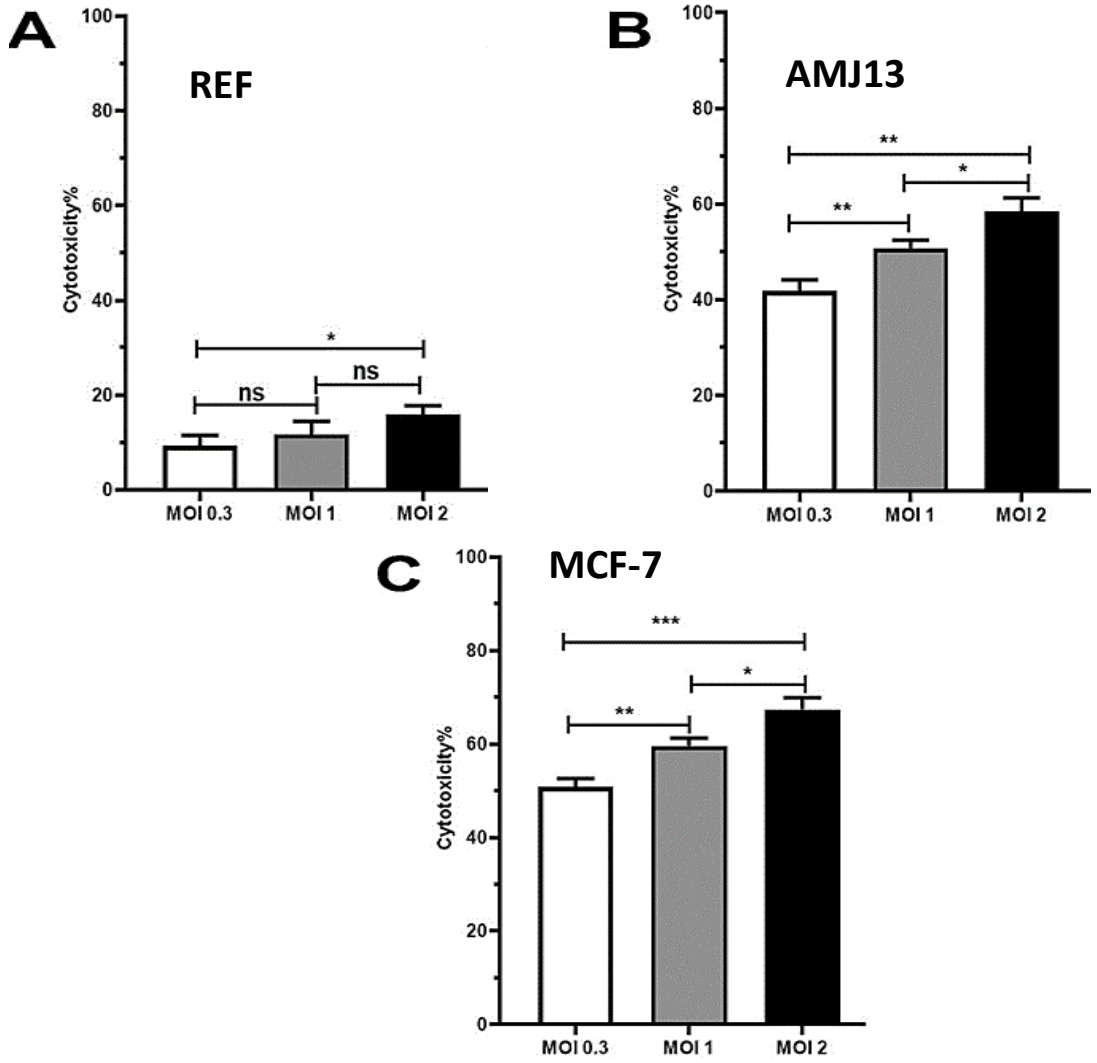


Figure (4-19): Cytotoxicity% of NDV against normal REF and breast cancer cell lines by using 3 different exposure doses (0.3, 1, and 2 MOI) of NDV depends on the IC50 value, A- CT % of REF, B- CT % of AMJ13, and C-CT % of MCF-7 after treatment with NDV. The values represent the (Mean ± SD) at *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

Table (4-8): Statistical data for cytotoxicity% of NDV against cell lines

CT% – NDV-REF		Mean ± SD
MOI	0.3	9.3 ± 2.1
	1	12 ± 2.8
	2	16 ± 1.8
P value (T-test)		
MOI 0.3	MOI 1	
ns, P > 0.05		
MOI 0.3	MOI 2	
*s, P = (P < 0.05)		
MOI 1	MOI 2	
ns, P > 0.05		

CT% – NDV - AMJ13		Mean ± SD
MOI	0.3	42 ± 2.3
	1	51 ± 1.7
	2	58 ± 2.8
P value (T-test)		
MOI 0.3	MOI 1	
**S, P < 0.01		
MOI 0.3	MOI 2	
**S, P < 0.01		
MOI 1	MOI 2	
*s, P = (P < 0.05)		

CT% – NDV-MCF-7		Mean ± SD
MOI	0.3	51 ± 1.8
	1	60 ± 1.6
	2	67 ± 2.5
P value (T-test)		
MOI 0.3	MOI 1	
**S, P < 0.01		
MOI 0.3	MOI 2	
***S, P < 0.001		
MOI 1	MOI 2	
*s, P = (P < 0.05)		

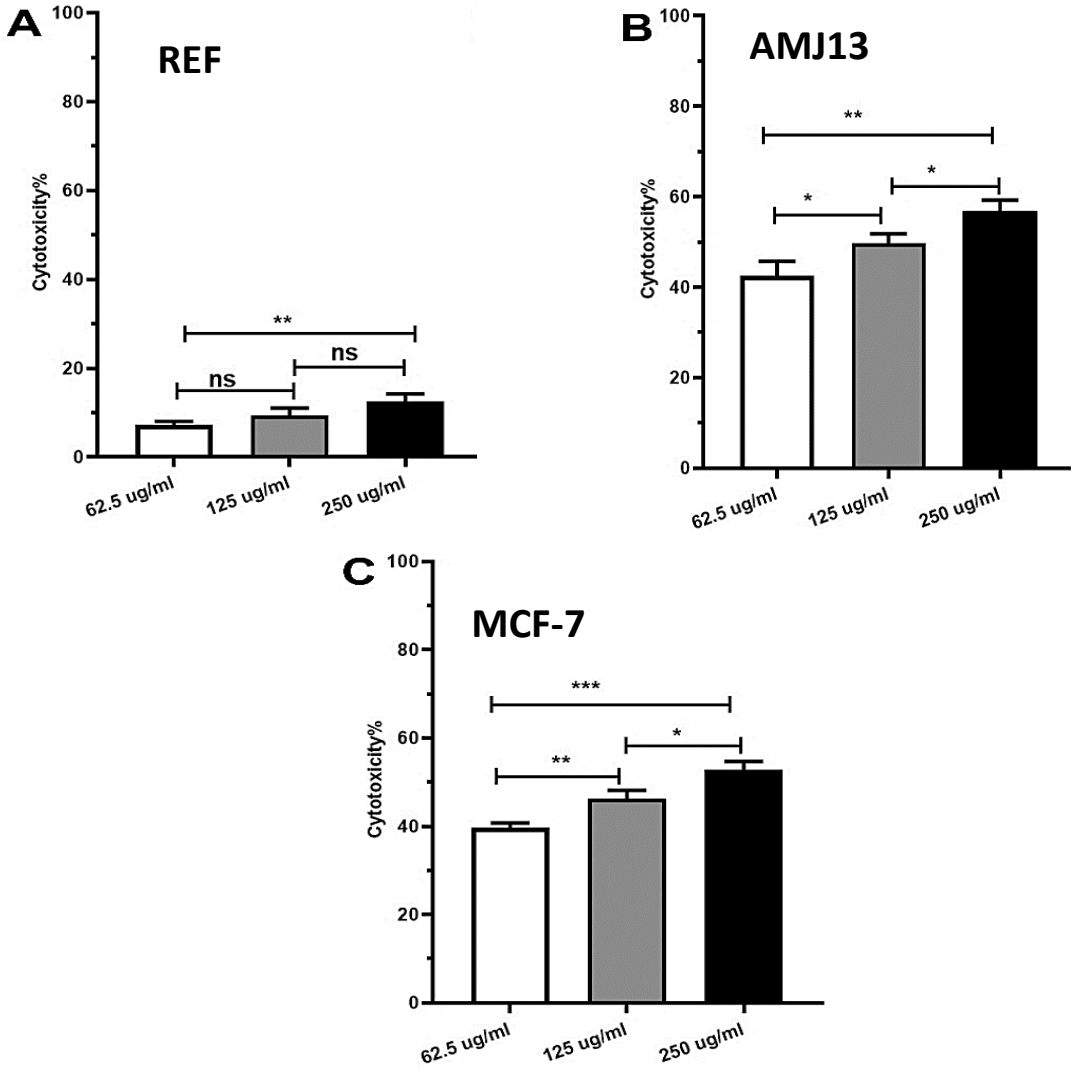


Figure (4-20): Cytotoxicity% of MH against normal REF and breast cancer cell lines by using 3 different exposure doses (250,125, and 62.5 µg/ml) of MH depends on the IC50 value, A-CT % of REF, B- CT % of AMJ13, and C-CT % of MCF-7 after treatment with MH. The values represent the (Mean ± SD) at *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

Table (4-9): Statistical data for cytotoxicity% of MH against cell lines

CT% – MH-REF		Mean ± SD
Conc. MH µg/ml	62.5	7.3 ± 0.7
	125	9.4 ± 1.7
	250	13 ± 1.7
P value (T-test)		
MOI 0.3	MOI 1	
ns, P > 0.05		
MOI 0.3	MOI 2	
*s, P = (P < 0.05)		
MOI 1	MOI 2	
ns, P > 0.05		

CT% – MH - AMJ13		Mean ± SD
Conc. MH µg/ml	62.5	43 ± 3.2
	125	50 ± 2.1
	250	57 ± 2.4
P value (T-test)		
MOI 0.3	MOI 1	
**S, P < 0.01		
MOI 0.3	MOI 2	
**S, P < 0.01		
MOI 1	MOI 2	
*s, P = (P < 0.05)		

CT% – MH-MCF-7		Mean ± SD
Conc. MH µg/ml	62.5	40 ± 1.0
	125	46 ± 1.8
	250	53 ± 1.9
P value (T-test)		
MOI 0.3	MOI 1	
**S, P < 0.01		
MOI 0.3	MOI 2	
***S, P < 0.001		
MOI 1	MOI 2	
*s, P = (P < 0.05)		

Cytotoxicity was determined by MTT cytotoxicity assay in normal REF and breast cancer (AMJ13 and MCF-7) cell lines, in accordance with specific concentrations (5, 2.5, and 1.25 µg/ml) of DXL (Figure (4-21)). The best effect was noticed at 5µg/ml of DXL. Inhibition of growth induced by high concentrations of DXL was seen, and also, treatment with DXL showed enhancement of the growth inhibition properties in breast cancer cell lines more than normal cell line.

Dose response evaluation of treatments differed significantly between REF and breast cancer cell lines (Figure (4-21)). There is a significant difference between the effect of DXL at 1.25 µg/ml and combination at (2 MOI-NDV and 62.5 µg/ml MH), to growth inhibition (cytotoxicity) for normal and breast cancer cell lines (Figure (4-22)).

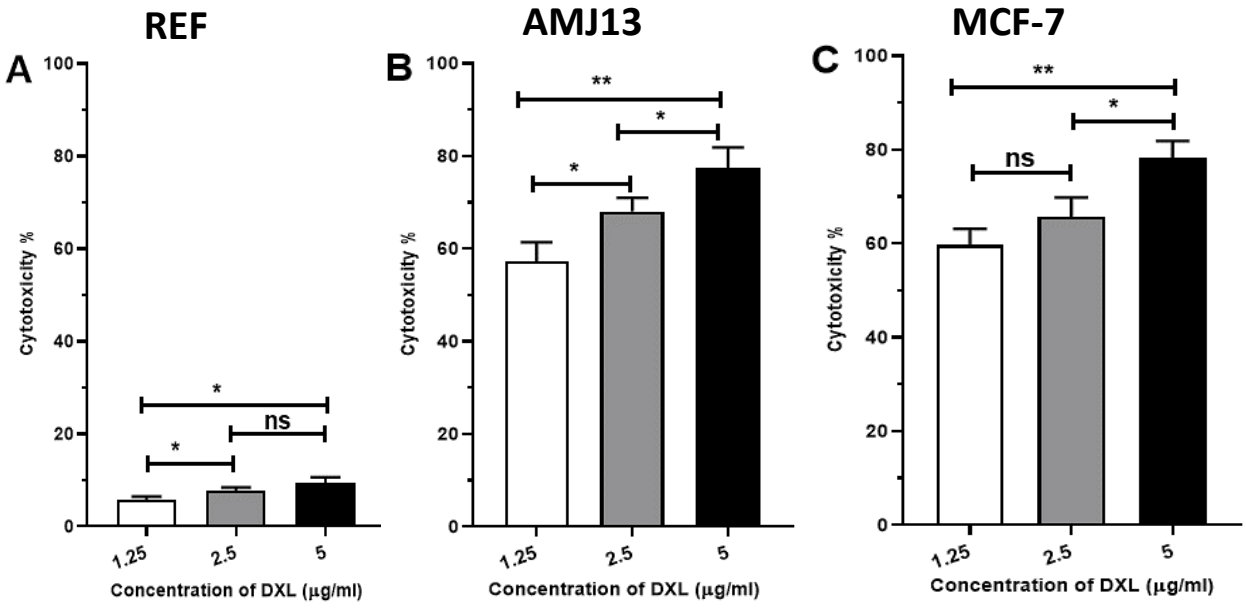


Figure (4-21): Cytotoxicity% of DXL against normal REF and breast cancer cell lines by using 3 different exposure doses (1.25,2.5, and 5 µg/ml) of DXL depends on the IC50 value, A- CT % of REF, B- CT % of AMJ13, and C-CT % of MCF-7 after treatment with DXL. The values represent the (Mean ± SD) at *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

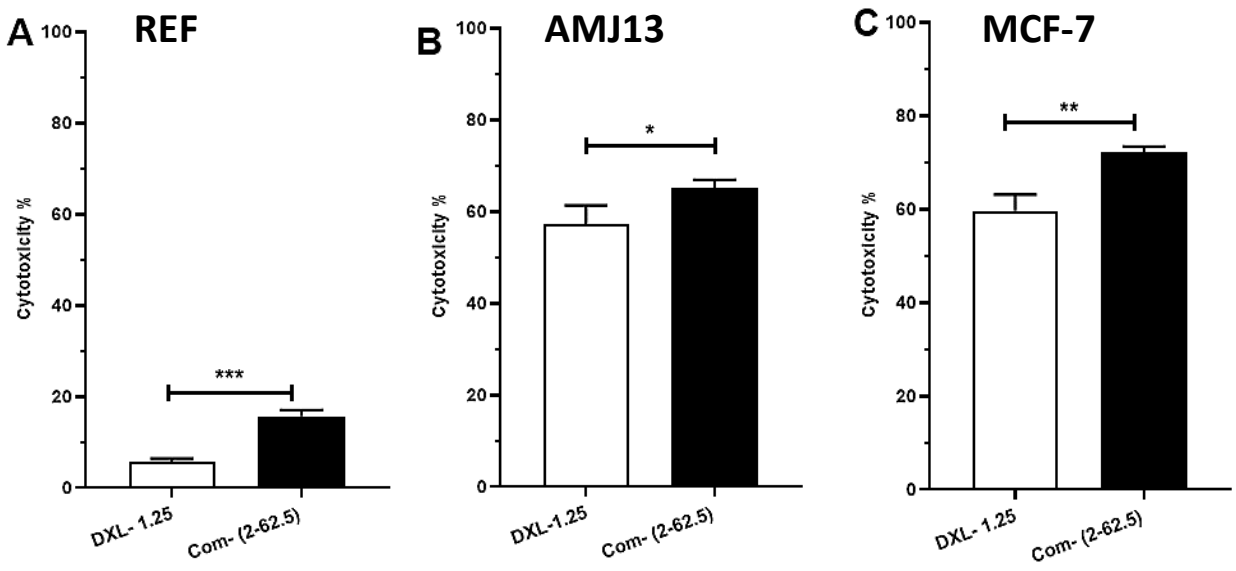


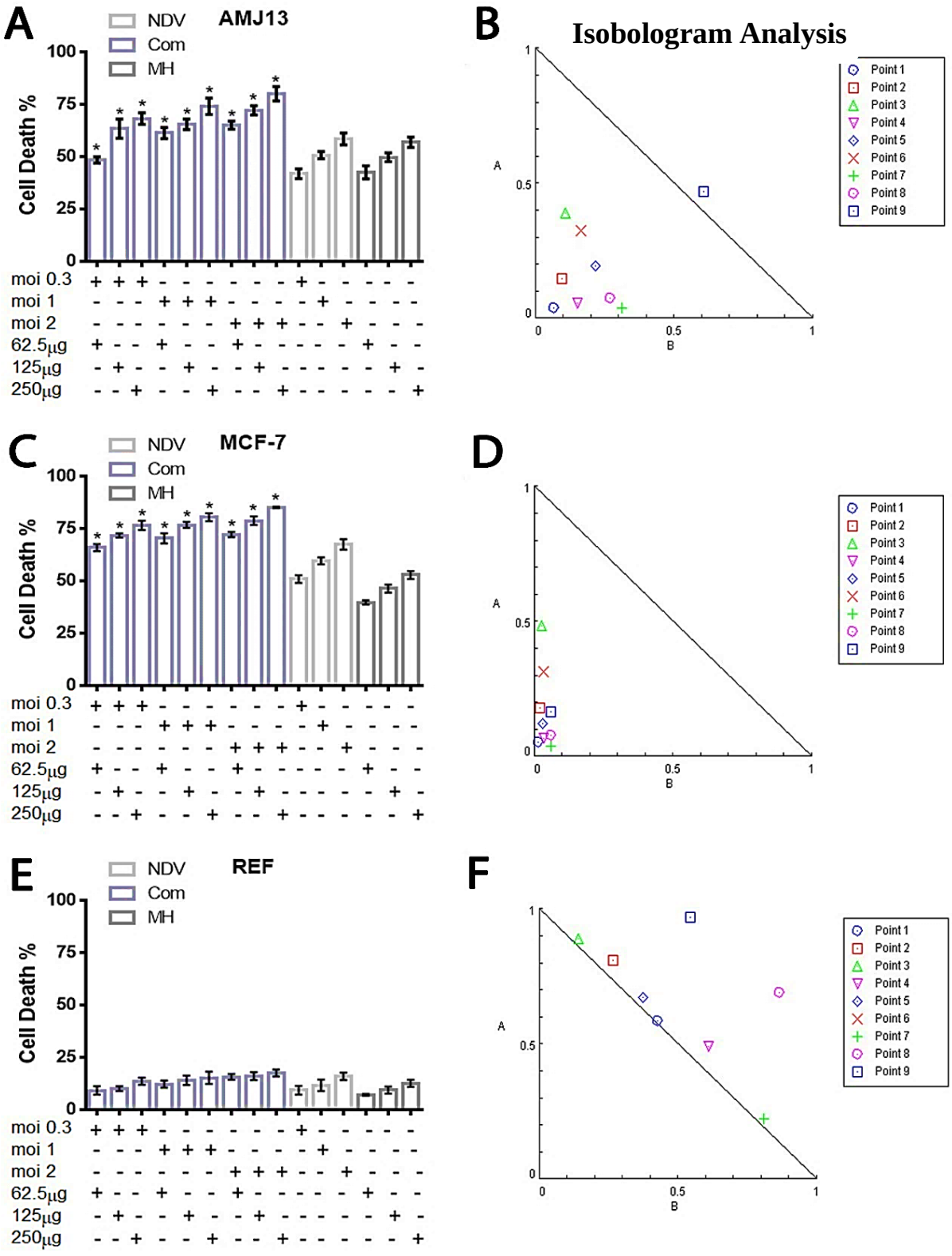
Figure (4-22) Comparison between the effect of DXL and Combination therapy in cell lines. A- in REF, B- in AMJ13 and C- in MCF-7

4.5 Combination cytotoxicity assays and Chou–Talalay analysis of cell lines

In order to investigate the effects of oncolytic NDV and MH combination therapy, it was examined the cytotoxicity ratio of the NDV (0.3, 1, and 2 MOI) and for the MH (62.5, 125, and 250 μg/ml). Synergism were observed in all combined doses against both breast cancer (AMJ13 and MCF7) cell lines (Figure 4-23 A, B, G, H and C, D, I, J), but with the exception of the ninth point which indicated additive as shown in (Table (4-10 A)) (Figure (4-23 A)). Whereas no synergism relationships were observed among treatments against the non-cancerous REF cell line (Table (4-10 C)) (Figure (4-23 E, F, K and L)).

The CI was estimated from the dose-effect data of single and combined treatments by using CompuSyn Isobologram. CI < 1 indicates synergism; CI = 1 to 1.1 indicates an additive effect; and CI > 1.1 indicates antagonism. The AMJ13 cell line had CI < 1 in eight combination points, indicating a synergistic effect or interaction between NDV and MH. Additive was observed in one combination point (Table (4-10 A) and (Figure (4-23 A and B)). The combination points for the combined treatment to MCF-7 cell line indicated a synergistic effect or interaction between NDV and MH at all points (Table (4-10 B)) and (Figure (4-23 C and D)).

In the normal REF cell line, all combination points were CI > 1, indicating antagonism and additive effect which is neglected effect as there was no killing effect reached 50% at all tested concentrations (Table (4-10 C) and (Figure (4-23 E and F)).



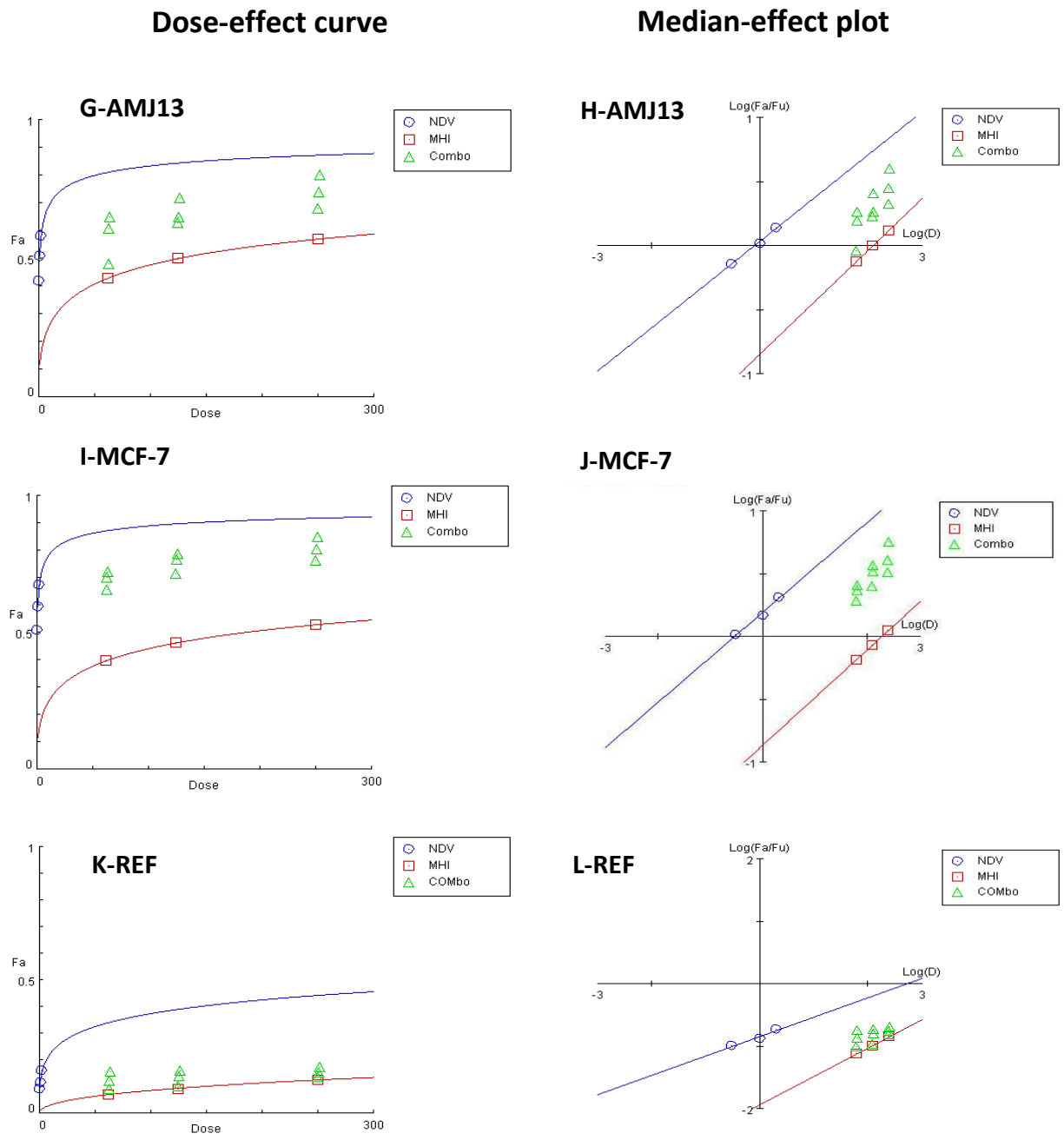


Figure (4-23) Combination of NDV and MH showed superior anticancer activity in compare to monotherapies in both AMJ13 and MCF-7 breast cancer cells. However, there was no enhanced toxicity against non-cancerous REF cells. (A, C and E) AMJ13, MCF-7 and REF cells were treated with NDV (0.3, 1, and 2 MOI) and with MH (62.5, 125, and 250 µg/ml), then cell viability was measured by MTT assay. Illustrations of normalized isobologram of non-constant combination ratios (B, D and F), Dose-effect curve (G, I and K) and Median-effect plot (H, J and L) for AMJ13, MCF-7 and REF respectively were measured by Chou-Talalay method where CI value quantitatively defines synergism. (CI < 1), additive effect (CI = 1-1.1) and antagonism (CI > 1.1). All data shown are means ± SEM (*P < 0.05 compared to mono-treatments) data from three different experiments. The red line represents phytotherapy (MH) single treatment, blue line represents the single treatment of NDV and green triangle is the combination treatment using ComboSyn software. The ratio of the fraction affected (fa) vs the fraction unaffected (fu) is equal to the dose (D) vs the median-effect dose.

Table (4-10): Cytotoxicity of NDV and MH combination or alone against AMJ13, MCF-7 and REF cells. Combination index (CI) was measured by CompuSyn software. CI data for non-constant combination (NDV+MH) of cell lines showing synergism effect between NDV and MH in breast cancer and normal cell lines. (A) CI values from treatment of AMJ13 cancer cells; (B) CI values from treatment in MCF-7 cancer cells; (C) CI values from treatment of non-cancer cells (REF).

A- AMJ13

Points	NDV (MOI)	MH (µg/ml)	Effect CT	CI	Effect
1	2	250	0.80	0.10595	Synergism
2	2	125	0.72	0.24635	Synergism
3	2	62.5	0.65	0.49989	Synergism
4	1	250	0.74	0.20755	Synergism
5	1	125	0.65	0.41359	Synergism
6	1	62.5	0.61	0.49220	Synergism
7	0.3	250	0.68	0.35256	Synergism
8	0.3	125	0.63	0.34603	Synergism
9	0.3	62.5	0.48	1.07844	Additive

B- MCF-7

Points	NDV (MOI)	MH (µg/ml)	Effect CT	CI	Effect
1	2	250	0.85	0.06841	Synergism
2	2	125	0.79	0.20042	Synergism
3	2	62.5	0.72	0.51245	Synergism
4	1	250	0.80	0.10016	Synergism
5	1	125	0.77	0.15299	Synergism
6	1	62.5	0.70	0.34948	Synergism
7	0.3	250	0.77	0.09957	Synergism
8	0.3	125	0.72	0.13597	Synergism
9	0.3	62.5	0.69	0.22648	Synergism

C- REF

Points	NDV (MOI)	MH (µg/ml)	Effect CT	CI	Effect
1	2	250	0.17	1.01291	Additive
2	2	125	0.16	1.07549	Additive
3	2	62.5	0.16	1.03328	Additive
4	1	250	0.15	1.10258	Antagonism
5	1	125	0.14	1.04811	Additive
6	1	62.5	0.12	1.38593	Antagonism
7	0.3	250	0.14	1.03584	Additive
8	0.3	125	0.10	1.56003	Antagonism
9	0.3	62.5	0.09	1.51414	Antagonism

4.6. Morphological changes

Morphological changes were showed after treatment with MH (62.5µg/ml) and NDV (MOI = 2) to depend on combination index that has been measured for NDV and MH for 72hrs. In unstained cells and stained cells, by crystal violate, was noticed significant cytopathic effect, a large number of cramped cells with granulation, formation of syncytial, and shrinkage of cells which results in opaque foci of cells which leads to separation and floating of the treated cells in the cultured media compared with control.

It was revealed that synergism between the inhibitor and the virus increased morphological changes in unstained AMJ13 cell line and the following were observed: cytopathic effect, large number of cramped cells, and empty plaque spaces between cells compared with the control with granulation and shrinkage. It was noticed differences in morphological changes after treated with MH, NDV and their combination (Figure (4-24)), the yellow arrows in (Figure (4-24)) refer to cytopathic effect areas. It was noticed that synergism between inhibitor and virus increased morphological changes in unstained MCF-7 cell line and it was showed cytopathic effect, and large number of cramped cells with granulation and shrinkage. Differences in morphological changes were seen after treatment with MH, NDV, and their combination in comparison with the control (Figure (4-25)), The yellow arrows in (Figure (4-25)) refer to cytopathic effect areas. But there were no differences in morphological changes or it was showed insignificant cytopathic effect in unstained REF cell line (Figure (4-26)).

In AMJ13 and MCF-7 cells lines, proliferation of tumor cells has been inhibited by MH and NDV. NDV and MH effectively induced morphological changes in AMJ13 and MCF-7 treated cells compared with untreated cells (control) in unstained cells (Figure (4-24 and 25)) and stained cells (Figure (4-27 and 28)). Morphological changes were observed more in cells after treatment with NDV and MH in comparison with cells treated with NDV and MH separately, and in

comparison with control, morphological changes in treated cells showed significant cytopathic effect within 72 hrs, also similar to incomplete confluence of cell culture (Figure (4-27, 28)), but in stained REF cell lines, minimal morphological changes showed insignificant cytopathic effect (Figure (4-29)).

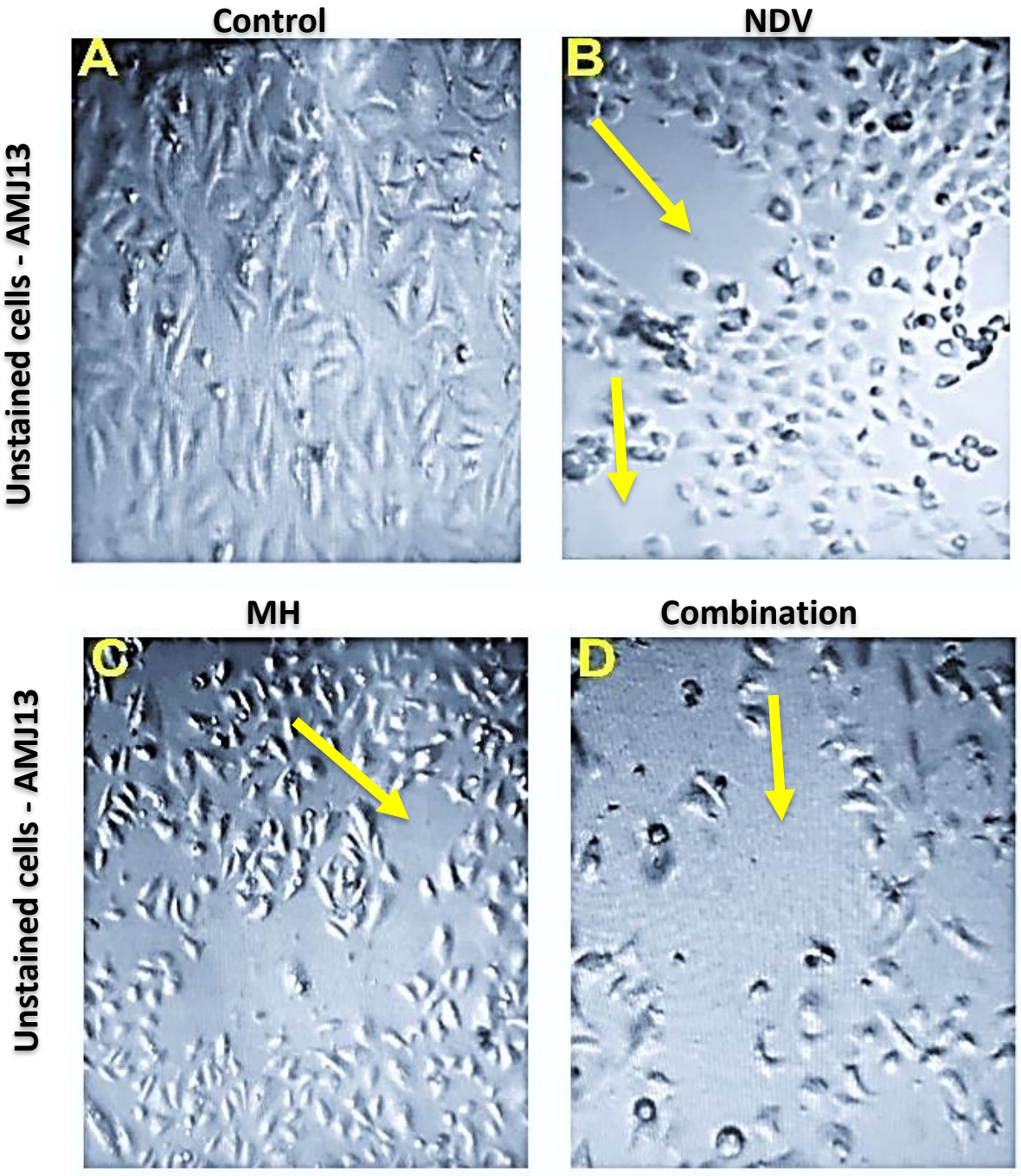


Figure (4-24): Effect of NDV (MOI = 2), MH (62.5µg/ml), and their combination on unstained AMJ13 cell lines to explain proliferation inhibition after treatment with different doses for 72 hrs by MTT cytotoxicity assay and by using inverted microscope 40X. The yellow arrows refer to cytopathic effect areas.

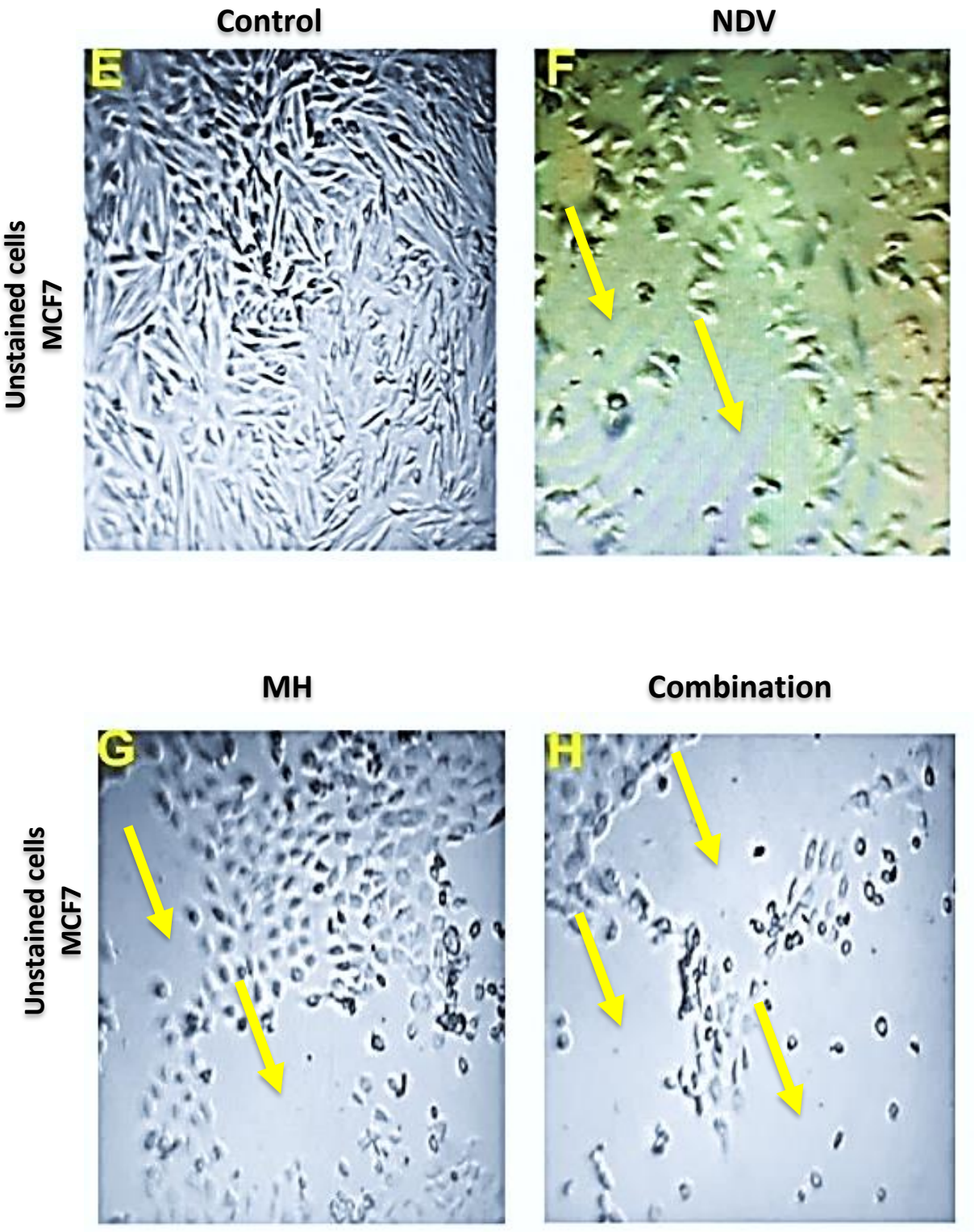


Figure (4-25): Effect of NDV (MOI = 2), MH (62.5µg/ml), and their combination on unstained MCF-7 cell lines to explain proliferation inhibition after treatment with different doses for 72 hrs by MTT cytotoxicity assay and by using inverted microscope 40X. The yellow arrows refer to cytopathic effect areas.

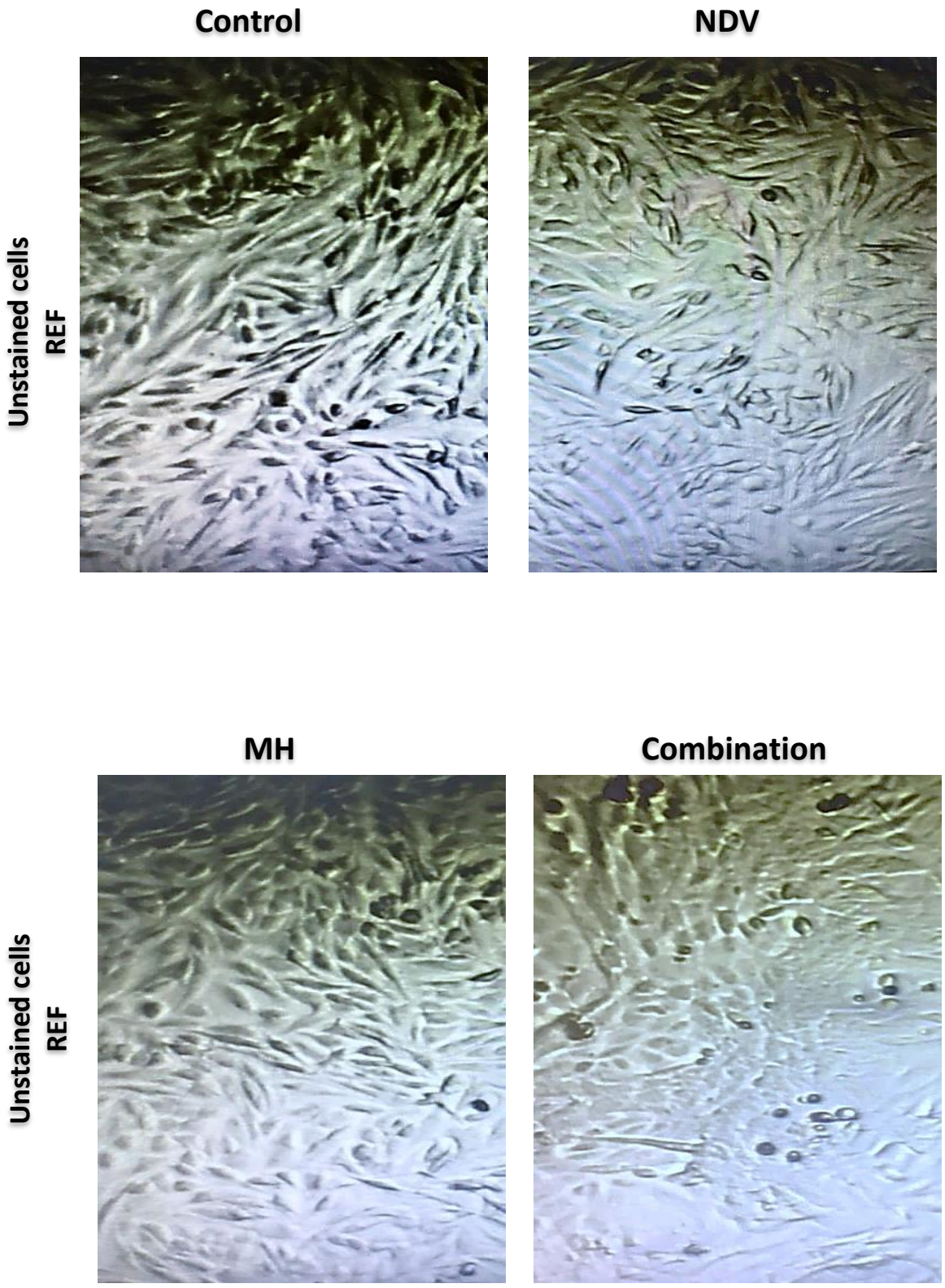


Figure (4-26): Effect of NDV (MOI = 2), MH (62.5µg/ml), and their combination on unstained REF cell lines to explain proliferation inhibition after treatment with different doses for 72 hrs by MTT cytotoxicity assay and by using inverted microscope 40X. It was noticed insignificant cytopathic effect in unstained REF cell line.

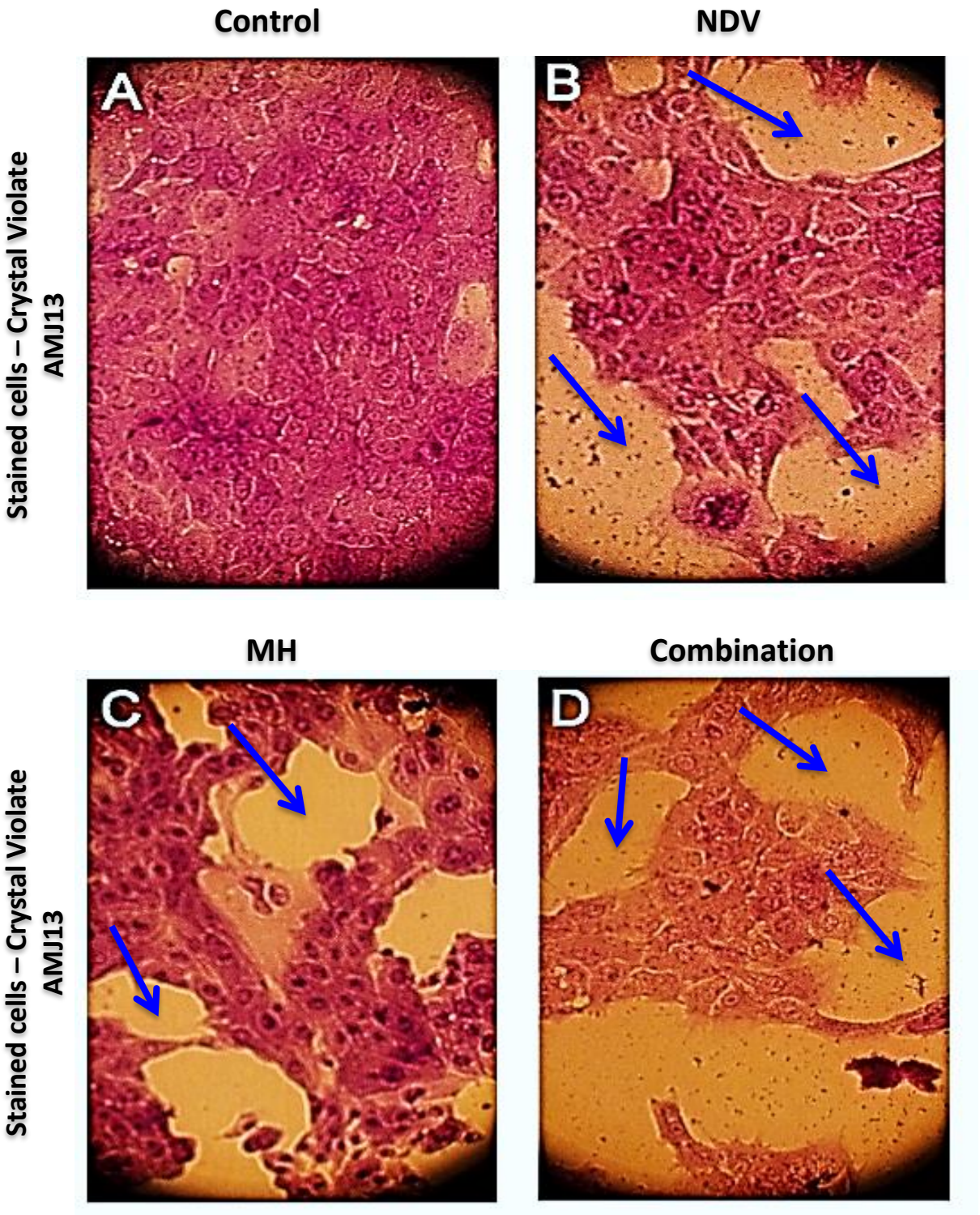


Figure (4-27): Morphological changes in AMJ13 cell line after treatment with NDV (MOI = 2), MH (62.5µg/ml), and their combination: untreated cells (control), treated cells with NDV, MH, and combination. All cells were stained by crystal violate and used inverted microscope 40X to explain morphological changes. A-Control, B- NDV, C- MH and D-Combination. The blue arrows refer to cytopathic effect areas.

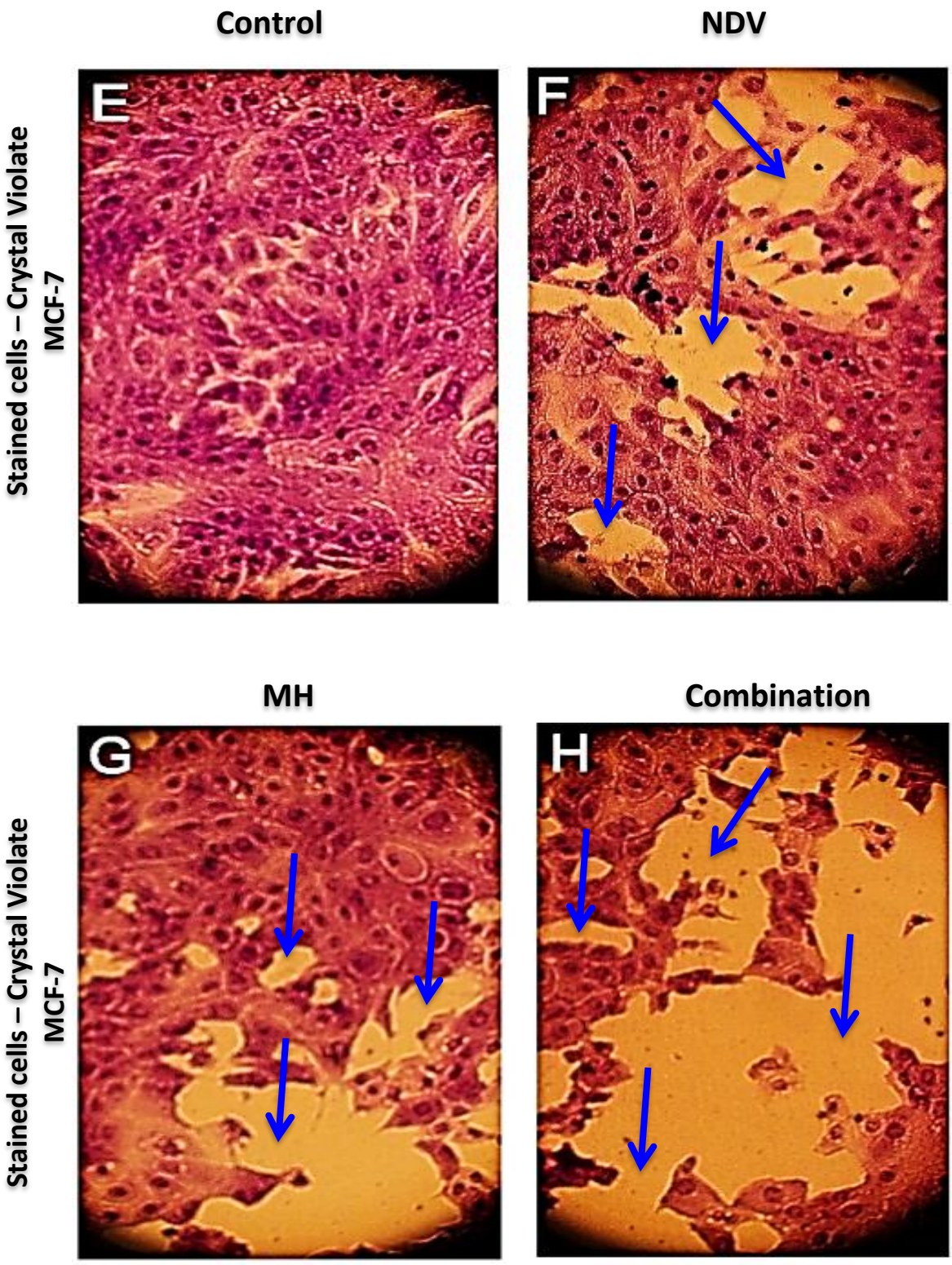


Figure (4-28): Morphological changes in MCF-7 cell line after treatment with NDV (MOI = 2), MH (62.5µg/ml), and their combination: untreated cells (control), treated cells with NDV, MH, and combination. All cells were stained by crystal violate and used inverted microscope 40X to explain morphological changes. E-Control, F- NDV, G- MH and H-Combination. The blue arrows refer to cytopathic effect areas.

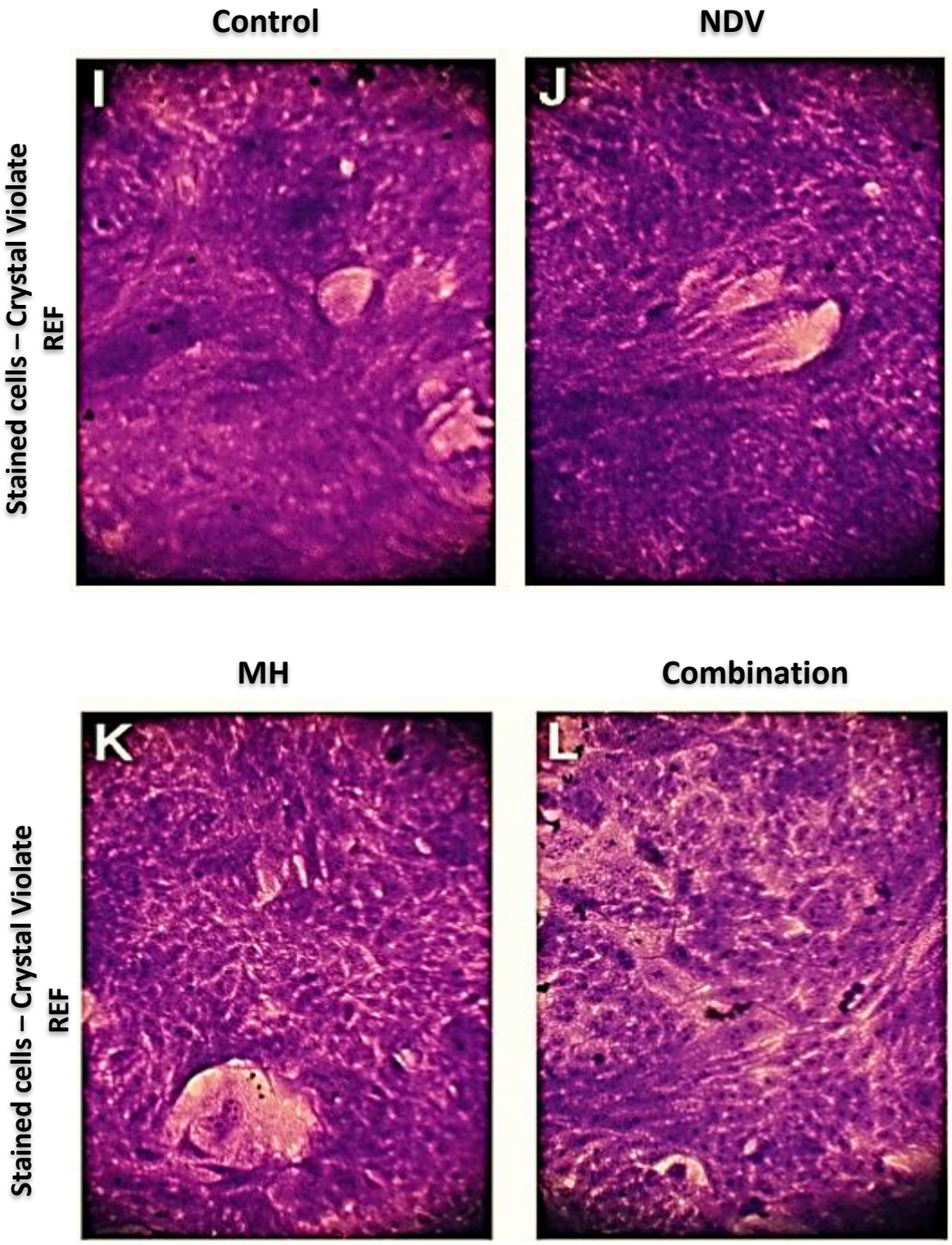


Figure (4-29): Morphological changes in REF cell line after treatment with NDV (MOI = 2), MH (62.5µg/ml), and combination: untreated cells (control), treated cells with NDV, MH, and combination. All cells were stained by crystal violate and used inverted microscope 40X to explain morphological changes. It was noticed insignificant cytopathic effect in stained REF cell line.

4.7. Apoptosis assay

An apoptosis assay detects and quantifies the cellular events associated with programmed cell death, during apoptosis, the cell shrinks and pulls away from its neighbors. Apoptosis was measured using acridine orange/propidium iodide assay, the control untreated viable cells appear green, and apoptotic cells in MH and NDV treated cells appear stain yellow, or orange cells (dead cells) as shown by fluorescent microscope (Figures (4-30 and 31)).

When the cells were exposed to the virus and MH, the results revealed synergism between inhibitor and virus which increased apoptosis. It was noticed more effect from combination than from NDV and MH alone.

In the figure (Figures (4-30 and 31)), red arrows explain viable cells (control) (green cells) and blue arrows show dead cells (yellow or orange cells) after treatment with dose of MH and NDV (MH (62.5µg/ml) and NDV (MOI = 2)) for 72 hrs (Figure (4-30 and 31)).

In AMJ13 and MCF-7 cell lines, yellow cells were seen on exposure to NDV and MH separately while orange cells were seen on exposure to synergism between them, in comparison to the control which showed green cells that are considered untreated cells.

NDV induced apoptosis in the infected cells, the results showed that NDV induced apoptosis in MCF-7 and AMJ13 cells. Based on current results in two different cell lines (MCF-7 and AMJ13), NDV exhibited oncolytic activity on two tumor cell lines and to a lower degree on normal cell line (REF).

Synergistic activity for the combination of MH with NDV batter than MH and NDV in AMJ13 and MCF-7 cell lines with compared to normal REF cell line. NDV may augment the anti-tumor activity of MH by increasing the cellular sensitivity to phytotherapy agent (inhibitor) (MH), and this enhanced sensitivity is partially caused by the induction of apoptosis in response to virulent NDV strain.

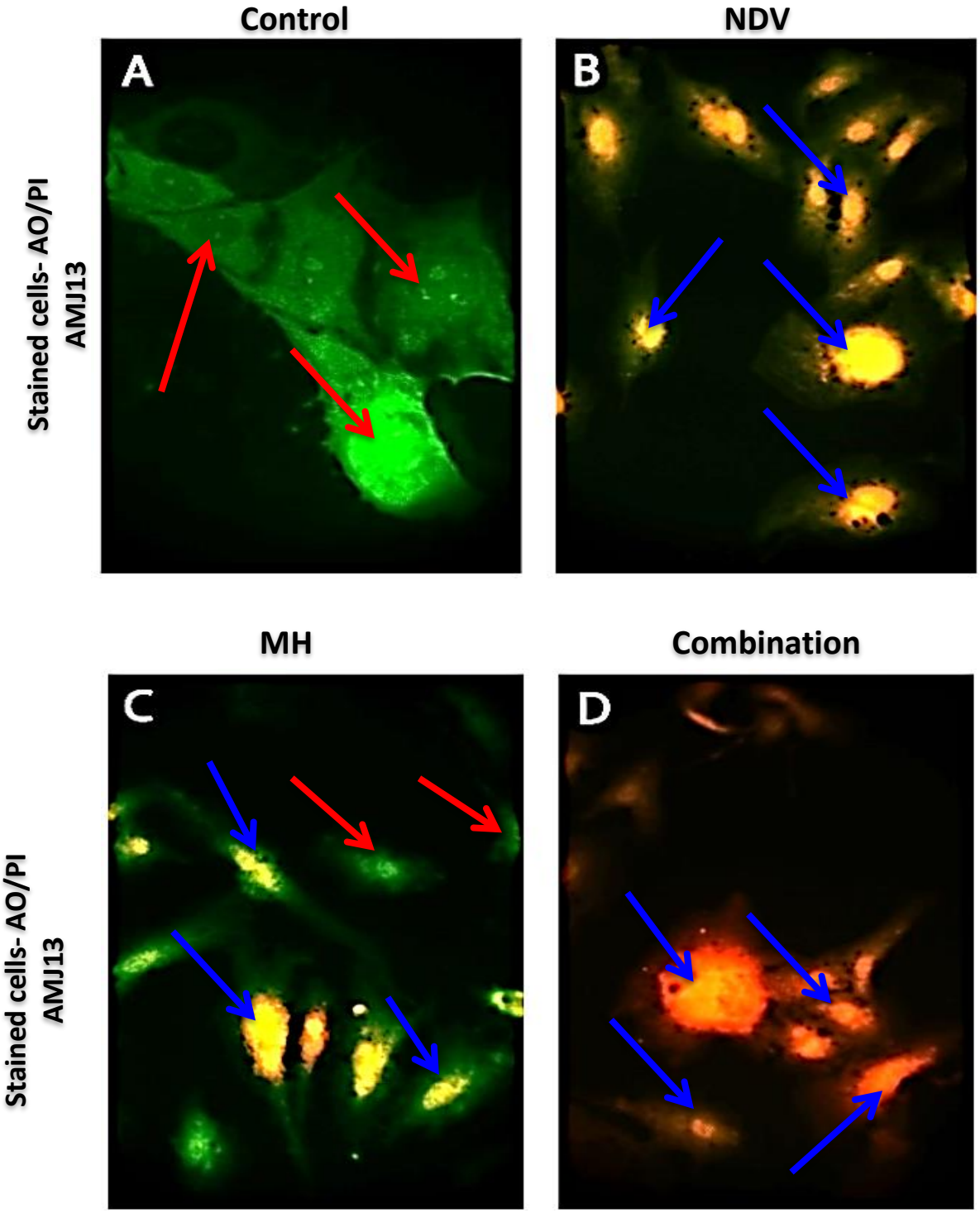


Figure (4-30): Apoptosis effect in AMJ13 cell lines after treated with NDV (MOI = 2), MH (62.5µg/ml), and their combination: (B, C and D) treated cells and A untreated cells (control) in AMJ13. All cells (A-D) were stained by AO/PI and used fluorescent microscope 40X to explain apoptosis effects. The red arrows explain viable cells and blue arrows show dead cells after treatment with a dose of MH and NDV for 72 hrs.

NDV and MH induced apoptosis which was viewed under a fluorescence microscope 40X (Figure (4-30 and 31)). Fluorescent microscope, as shown in

(Figure (4-30 and 31)), revealed that apoptosis occurred in cells after treatment with NDV, MH, and their combination, especially cell shrinkage and condensation of cytoplasm in a dose-dependent manner within 72 hrs of treatment. In stained REF cell lines, minimal apoptosis showed and insignificant cytopathic effect (Figure (4-32)).

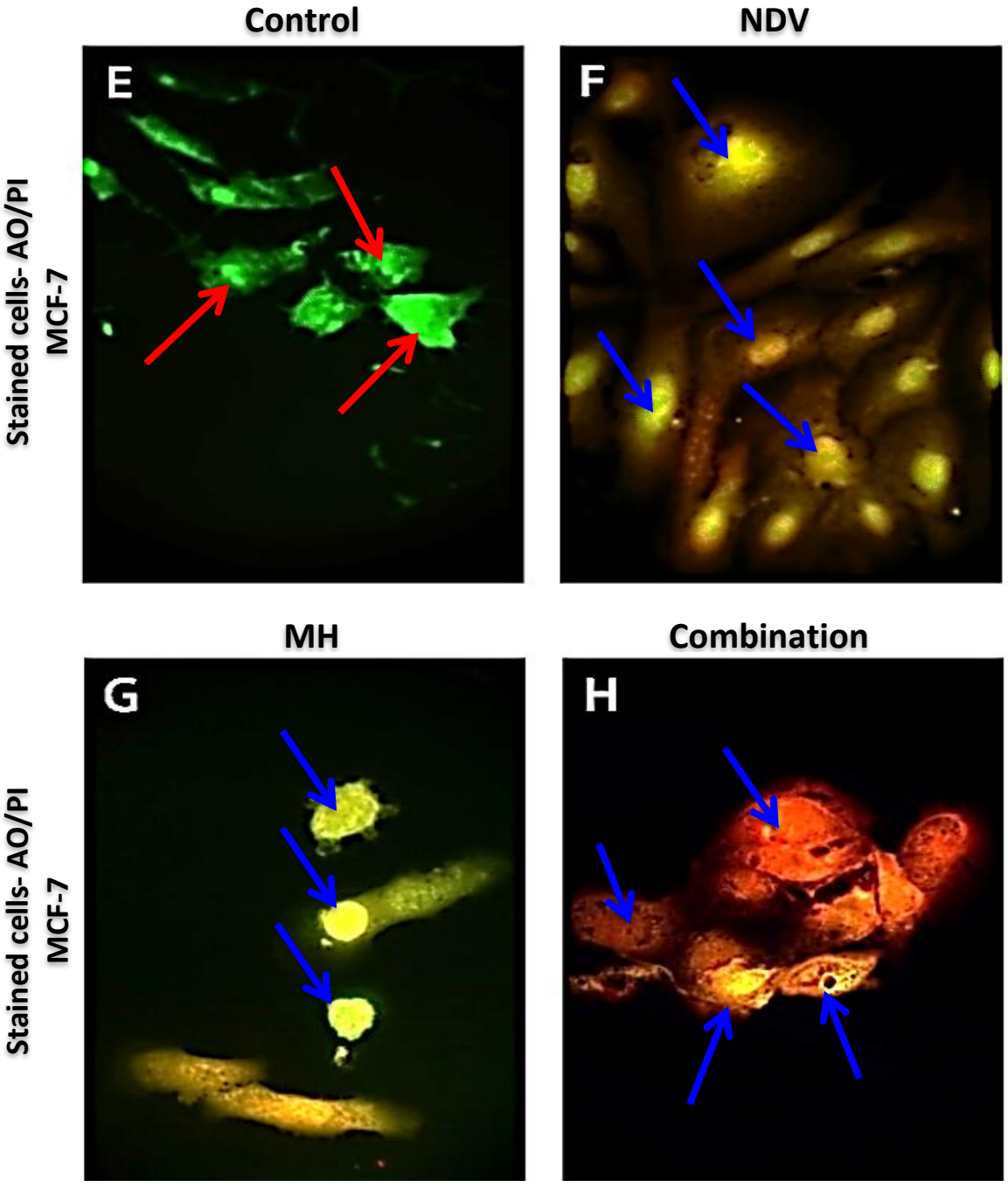


Figure (4-31): Apoptosis effect in MCF-7 cell lines after treatment with NDV (MOI = 2), MH (62.5µg/ml), and their combination: (F, G and H) treated cells and E untreated cells (control) in MCF-7. All cells (E-H) were stained by AO/PI and used fluorescent microscope 40X to explain apoptosis effects. The red arrows explain viable cells and blue arrows show dead cells after treatment with a dose of MH and NDV for 72 hrs.

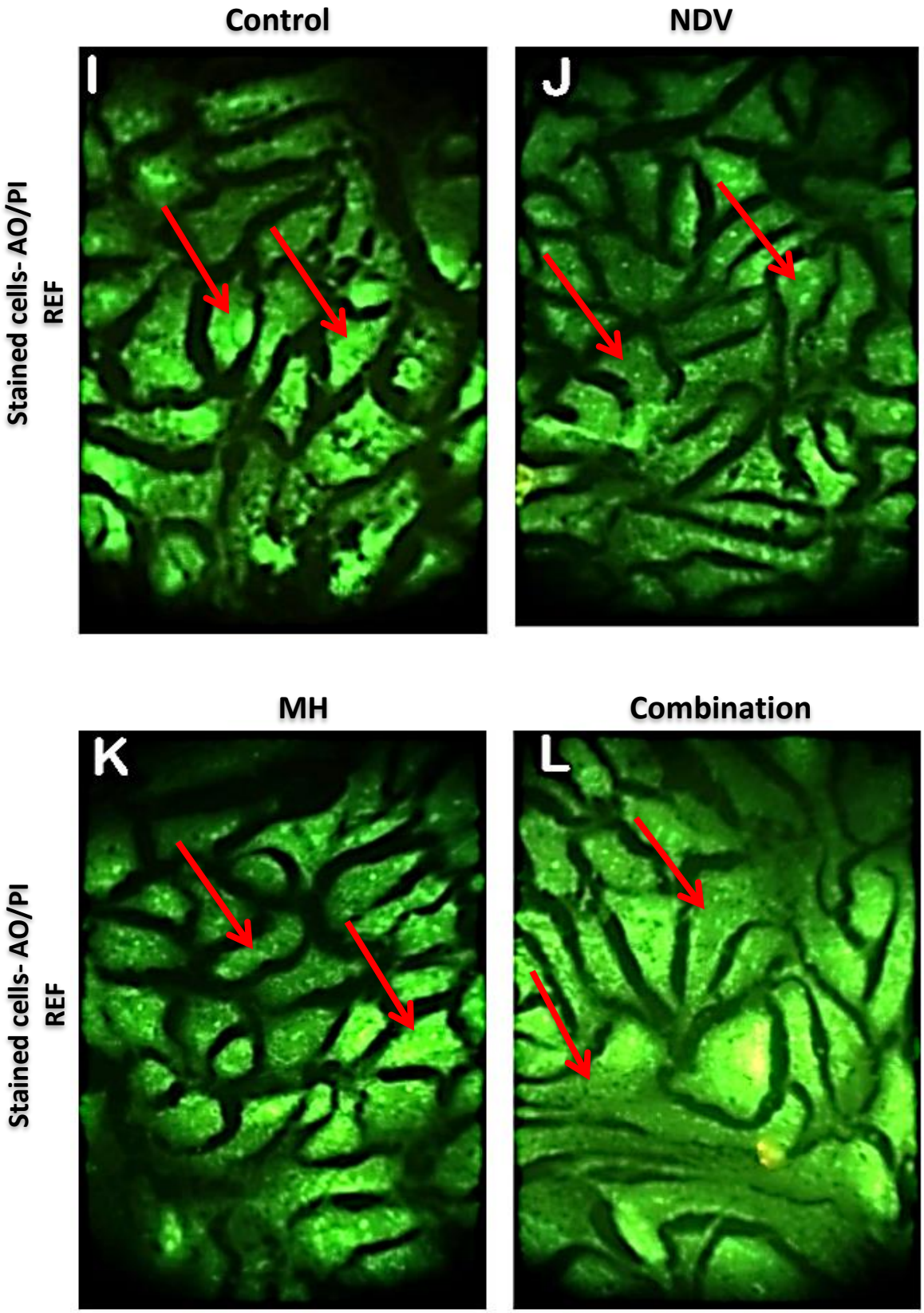


Figure (4-32): Apoptosis effect in REF cell lines after treatment with NDV (MOI = 2), MH (62.5µg/ml), and their combination: (J, K, and L) treated cells and I untreated cells (control) in REF. All cells (I-L) were stained by AO/PI and used fluorescent microscope 40X to explain apoptosis effects. The red arrows explain viable cells after treatment with a dose of MH and NDV for 72 hrs. It was noticed insignificant cytopathic effect in stained REF cell.

4.7.1 Relationship of apoptosis and morphological changes of cell lines

The morphological changes exhibited by treated cells after 72h of treatment were attributed to an intense cytopathic effect for the combination therapy. Morphological changes and apoptosis were more intense in the cells treated by combined NDV and MH than in those treated with NDV or MH alone. This result indicated that the synergism between the inhibitor and virus enhanced the morphological changes and percentage of apoptosis in cancer cells (Figure (4-32. a diagram J and K)). It was noticed weaker or less intense morphological changes and apoptosis in the normal REF cell line (Figure (4-32. a M)) than in the breast cancer cell lines treated with MH, NDV, and their combination.

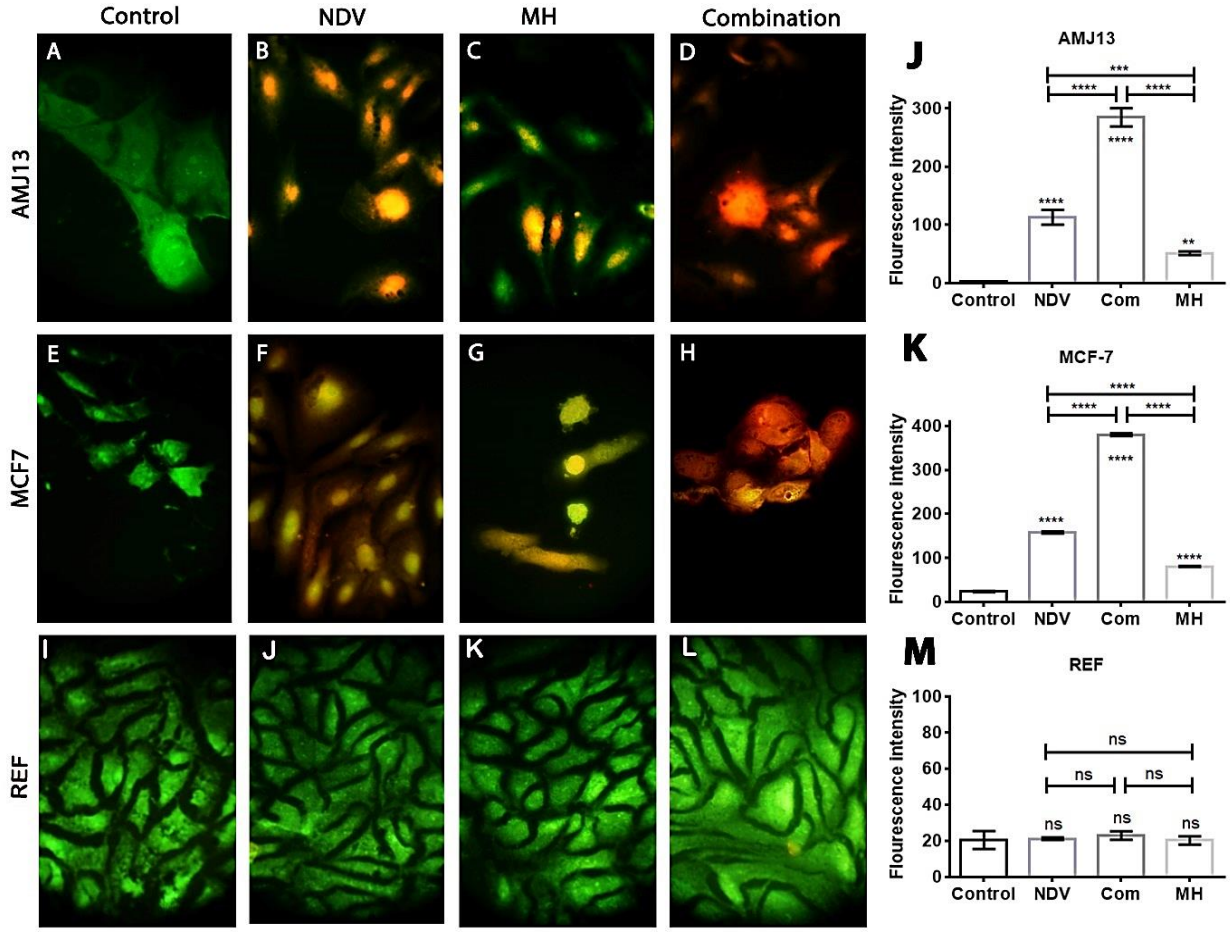


Figure (4-32.a). Fluorescent intensity: Investigation the MH-NDV combined therapy to induce apoptosis in treated cells using AO/PI: (A-D) AMJ13 cancer cells indicated that MH-NDV induce apoptosis as evidenced red stained cells, Untreated control cells emitting green fluorescence (A, E and I). (E-H) MCF-7 cells showing that number apoptotic cells are higher in the combination treatment. (I – L) There were no effect against REF cells by combination therapy using MH-NDV. AMJ13 and MCF-7 cells (J, K) expressed significant apoptotic cell death in compare to monotherapies and the control untreated cells. REF (M) no significant changes in all treatment modalities. Values represent the (mean ± SD). *P < 0.05, **P < 0.01 and ***P < 0.001.

4.8. Glycolysis pathway and its products inhibition

4.8.1. Hexokinase assay

The results revealed deficiency of hexokinase activity due to the effect of MH (62.5µg/ml) and NDV (MOI = 2). The concentration of MH and MOI of NDV were selected depending on the values of the combination index (CI). The statistical analysis data of HK inhibition is shown in (Table (4-11)). The HK enzyme was evaluated for the comparison of treated and untreated cells at 72 hrs.

Table (4-11): Statistical data of inhibition of hexokinase enzyme after treatment of cell lines with NDV, MH, and their combination, in comparison with control.

HK (U/ml) - REF		Mean ± SD
The group	Control	3.3 ± 0.24
	NDV	3 ± 0.11
	MH	3 ± 0.09
	Com	2.9 ± 0.21

P value (T-test)			
Control	NDV	Control	MH
ns, P= 0.08 (P >0.05)		ns, P= 0.051 (P >0.05)	
Control	Com	NDV	Com
ns, P= 0.057 (P >0.05)		ns, P= 0.438 (P >0.05)	
MH	Com	NDV	MH
ns, P= 0.623 (P >0.05)		ns, P= 0.614 (P >0.05)	

HK (U/ml) – AMJ13		Mean ± SD
The group	Control	3.2 ± 0.24
	NDV	2 ± 0.16
	MH	1.4 ± 0.01
	Com	1.1 ± 0.05

P value (T-test)			
Control	NDV	Control	MH
***S, P= 0.0004 (P <0.0005)		***S, P= 0.0002 (P <0.0005)	
Control	Com	NDV	Com
***S, P= 0.0003 (P <0.0005)		***S, P= 0.001 (P < 0.005)	
MH	Com	NDV	MH
*S, P= 0.01 (P <0.05)		**S, P= 0.001 (P <0.005)	

HK (U/ml) – MCF-7		Mean ± SD
The group	Control	3.0 ± 0.27
	NDV	1.9 ± 0.25
	MH	1.4 ± 0.09
	Com	1.2 ± 0.10

P value (T-test)			
Control	NDV	Control	MH
**S, P= 0.004 (P <0.01		***S, P= 0.0002 (P <0.001)	
Control	Com	NDV	Com
***S, P= 0.0002 (P <0.001)		**S, P= 0.003 (P < 0.01)	
MH	Com	NDV	MH
*S, P= 0.0057 (P <0.05)		*S, P= 0.005 (P <0.05)	

The inhibition of HK's effectiveness by the virus was significant when compared with the control, or less than MH. MH also inhibited HK significantly in comparison with the control or more than the virus. The combination of MH and

virus was a significantly better inhibitor and enhancer of growth inhibition when compared with separate treatment or with control of breast cancer cell lines, while the effect was insignificant in normal cell line. Results showed insignificant differences between (NDV - control), (NDV - Com), (NDV - MH), (MH - control), (Com - control) and (Com - MH) in normal REF cell line whereas the difference between them was significant in breast cancer cell lines (Figure (4-33)). It was identified a significant reduction in the HK enzyme activity in all the treatment modalities in the cancer cells but not in the normal cells. MH–NDV combination efficiently inhibits glycolysis products in the treated breast cancer cells but not in normal cells.

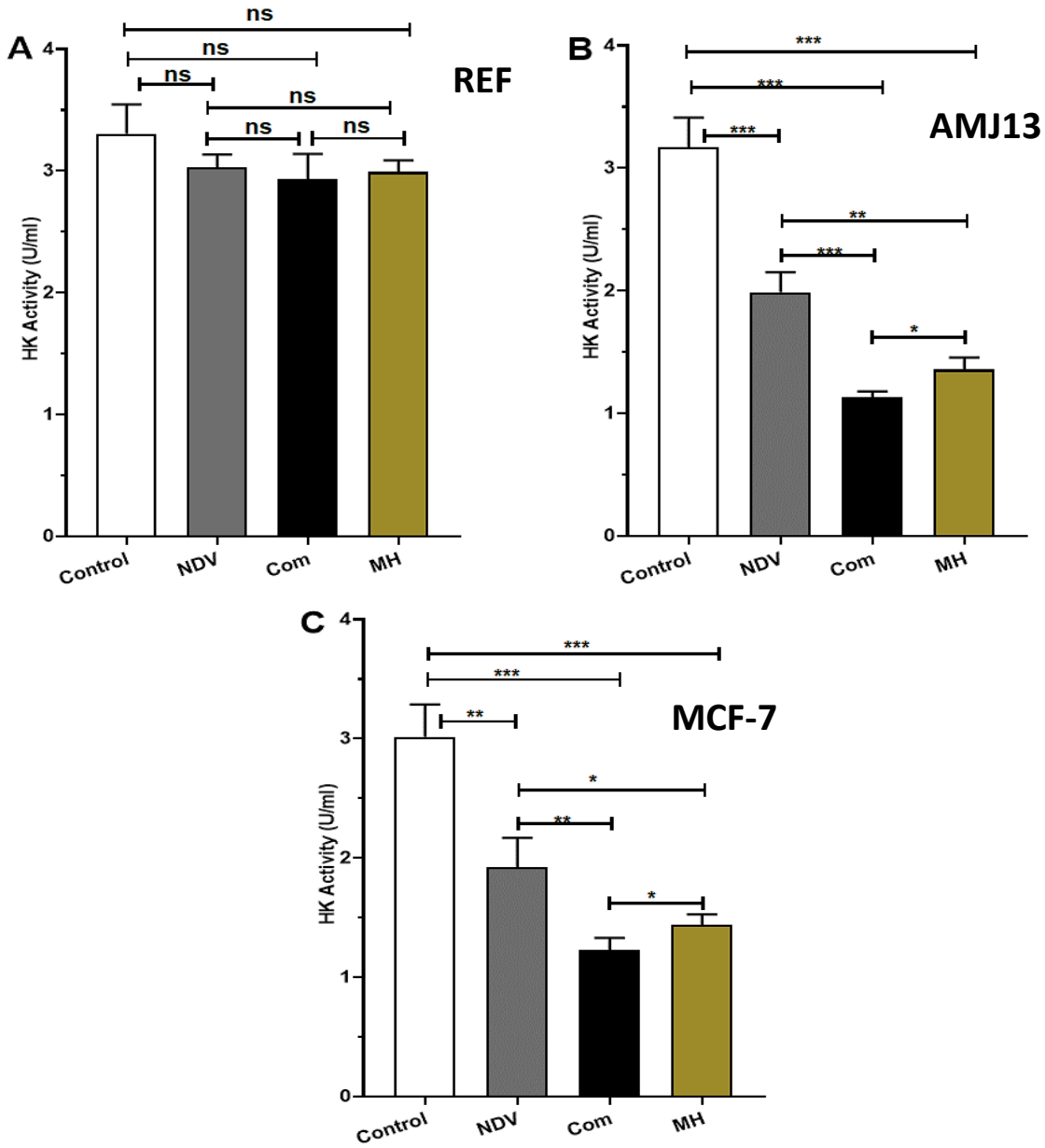


Figure (4-33) Effect of NDV and MH on the Activity of hexokinase (HK) in glycolysis inhibition represented in (U/ml). A) in REF, B) AMJ13 and C) MCF7 cell lines after treatment with NDV at MOI 2, MH at 62.5 µg/ml, and their combination, and comparison with control at *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

4.8.2. Pyruvate assay

From the results shown in (Figure (4-34)), it was observed reduction of pyruvate concentration after 72 hrs of treatment with MH (62.5µg/ml) and NDV (MOI = 2). The statistical analysis data of pyruvate inhibition is shown in (Table (4-12)). The virus reduced the concentration of pyruvate significantly when compared with control and less than MH. MH also reduced pyruvate significantly in comparison with control and more than the virus in AMJ13 and almost equal to the virus in MCF-7. The combination of MH and virus was a significantly better inhibitor and enhancer of growth inhibition when compared with separate treatment or with control of breast cancer cell lines.

Table (4-12): Statistical data of inhibition of pyruvate after treatment of cell lines with NDV, MH, and their combination, and comparison with control.

Pyruvate (µmol/ml) - REF		Mean ± SD
The group	Control	0.45 ± 0.013
	NDV	0.42 ± 0.027
	MH	0.43 ± 0.016
	Com	0.40 ± 0.034
P value (T-test)		
Control	NDV	Control MH
ns, P= 0.2 (P >0.05)		ns, P= 0.175 (P >0.05)
Control	Com	NDV Com
ns, P= 0.0825 (P >0.05)		ns, P= 0.436 (P >0.05)
MH	Com	NDV MH
ns, P= 0.26 (P >0.05)		ns, P= 0.735 (P >0.05)

Pyruvate (µmol/ml) –AMJ13		Mean ± SD
The group	Control	0.48 ± 0.03
	NDV	0.28 ± 0.016
	MH	0.25 ± 0.011
	Com	0.14 ± 0.022
P value (T-test)		
Control	NDV	Control MH
***S, P= 0.0005 (P <0.001)		***S, P= 0.0002 (P <0.001)
Control	Com	NDV Com
***S, P= 0.0005 (P <0.001)		***S, P= 0.003 (P <0.001)
MH	Com	NDV MH
**S, P= 0.019 (P <0.01)		*S, P= 0.046 (P <0.05)

Pyruvate (µmol/ml) – MCF-7		Mean ± SD
The group	Control	0.5 ± 0.035
	NDV	0.23 ± 0.36
	MH	0.23 ± 0.01
	Com	0.15 ± 0.01

P value (T-test)		
Control	NDV	Control MH
***S, P= 0.0008 (P <0.001)		***S, P= 0.0003 (P <0.001)
Control	Com	NDV Com
****S, (P <0.0001)		*S, P= 0.017 (P < 0.05)
MH	Com	NDV MH
**S, P= 0.003 (P <0.01)		ns, P= 0.893 (P >0.05)

While, the effect was insignificant in normal REF cell line. The results showed insignificant differences between (NDV -control), (NDV - Com), (NDV - MH), (MH - control), (Com - control) and (Com -MH) in normal REF cell line, and (NDV - MH) in MCF-7 cell line, whereas the difference between them was significant in breast cancer cell lines (Figure (4-34)). It was identified a significant reduction in the pyruvate concentration in all the treatment modalities in the cancer cells but not in the normal cells.

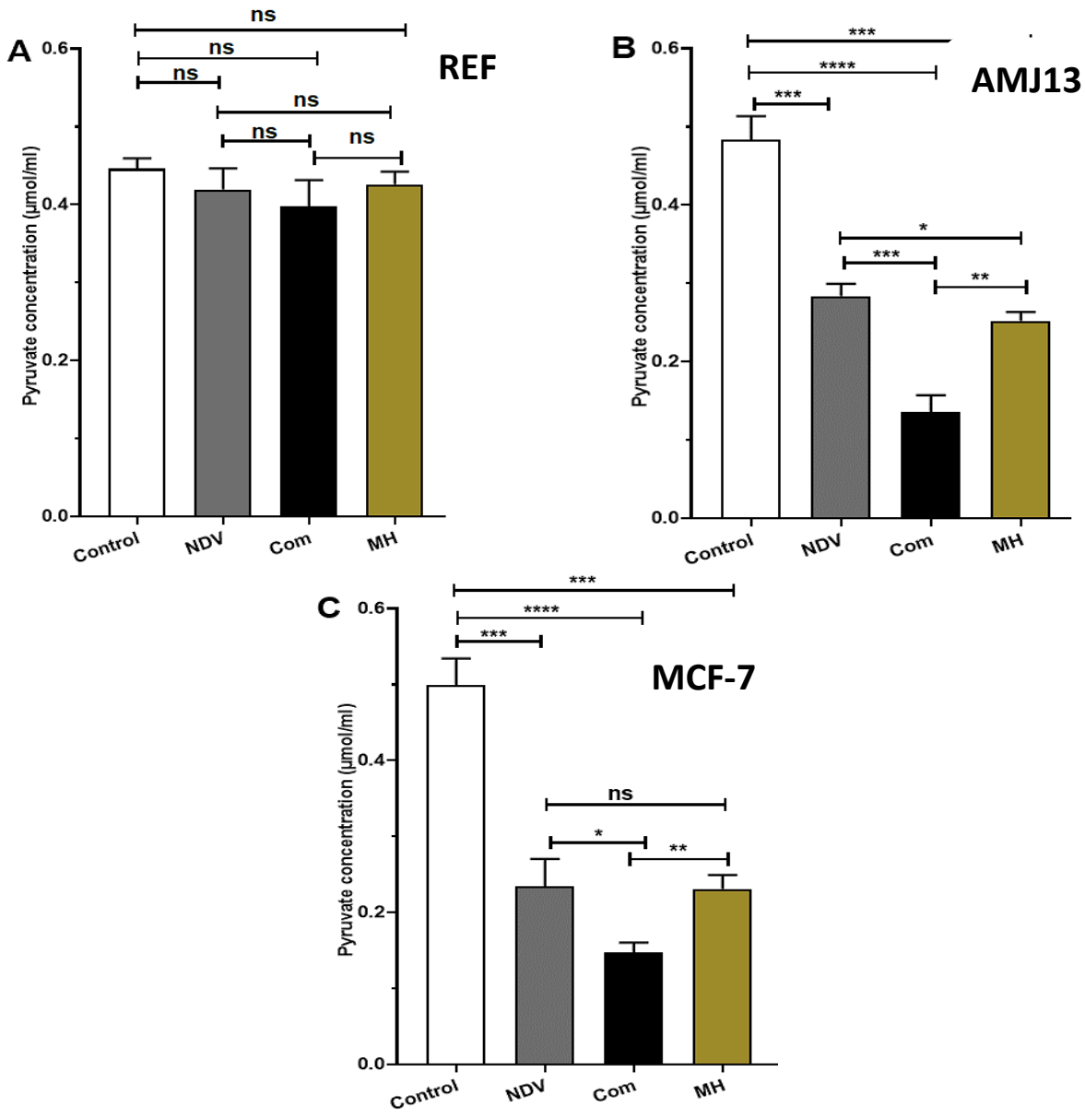


Figure (4-34) Effect of NDV and MH on the Pyruvate concentration in glycolysis inhibition represented in (µmol/ml). A) in REF, B) AMJ13 and C) MCF7 cell lines after treatment with NDV at MOI 2, MH at 62.5 µg/ml, and their combination, and comparison with control at *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

4.8.3 ATP assay

From the results shown in (Figure (4-35)), it has been noticed that ATP concentration decreased after treatment with MH (62.5µg/ml), NDV (MOI = 2), and their combination for 72 hrs. (Table (4-13)) shows the statistical analysis. The virus reduced the concentration of ATP significantly when compared with control or more than MH. MH also reduced ATP significantly in comparison with control or less than the virus. The combination of MH and virus was a significantly better inhibitor and enhancer of growth inhibition when compared with separate treatment or with control of breast cancer cell lines. Whereas, the effect was insignificant in normal REF cell line.

Table (4-13): Statistical data of inhibition of ATP production after treatment of cell lines with NDV, MH, and their combination, in comparison with the control.

ATP (mmol/L) - REF		Mean ± SD
The group	Control	1.1 ± 0.05
	NDV	1 ± 0.11
	MH	1 ± 0.04
	Com	1 ± 0.08

P value (T-test)			
Control	NDV	Control	MH
ns, P= 0.33 (P >0.05)		ns, P= 0.06 (P >0.05)	
Control	Com	NDV	Com
ns, P= 0.09 (P >0.05)		ns, P= 0.658 (P >0.05)	
MH	Com	NDV	MH
ns, P= 0.64 (P >0.05)		ns, P= 0.874 (P >0.05)	

ATP (mmol/L) – AMJ13		Mean ± SD
The group	Control	1.2 ± 0.057
	NDV	0.58 ± 0.056
	MH	0.61 ± 0.077
	Com	0.39 ± 0.059

P value (T-test)			
Control	NDV	Control	MH
****S, (P <0.0001)		****S, (P <0.0001)	
Control	Com	NDV	Com
****S, (P <0.0001)		**S, P= 0.0032 (P < 0.01)	
MH	Com	NDV	MH
**S, P= 0.0036 (P <0.01)		ns, P= 0.512 (P >0.05)	

ATP (mmol/L) – MCF7		Mean ± SD
The group	Control	1.3 ± 0.074
	NDV	0.65 ± 0.061
	MH	0.69 ± 0.065
	Com	0.35 ± 0.04

P value (T-test)			
Control	NDV	Control	MH
****S, (P <0.0001)		****S, (P <0.0001)	
Control	Com	NDV	Com
****S, (P <0.0001)		***S, P= 0.0002 (P < 0.001)	
MH	Com	NDV	MH
***S, P= 0.0001 (P <0.001)		ns, P= 0.397 (P >0.05)	

The results showed insignificant differences between (NDV -control), (NDV - Com), (NDV - MH), (MH - control), (Com - control) and (Com -MH) in normal REF cell line, and (NDV - MH) in AMJ13 and MCF-7 cell line, whereas the difference between them was significant in breast cancer cell lines (Figure (4-35)). It was identified a significant reduction in the ATP concentration in all the treatment modalities in the cancer cells but not in the normal cells. MH–NDV combination efficiently inhibits glycolysis and inhibit ATP production in the treated breast cancer cells but not in normal cells.

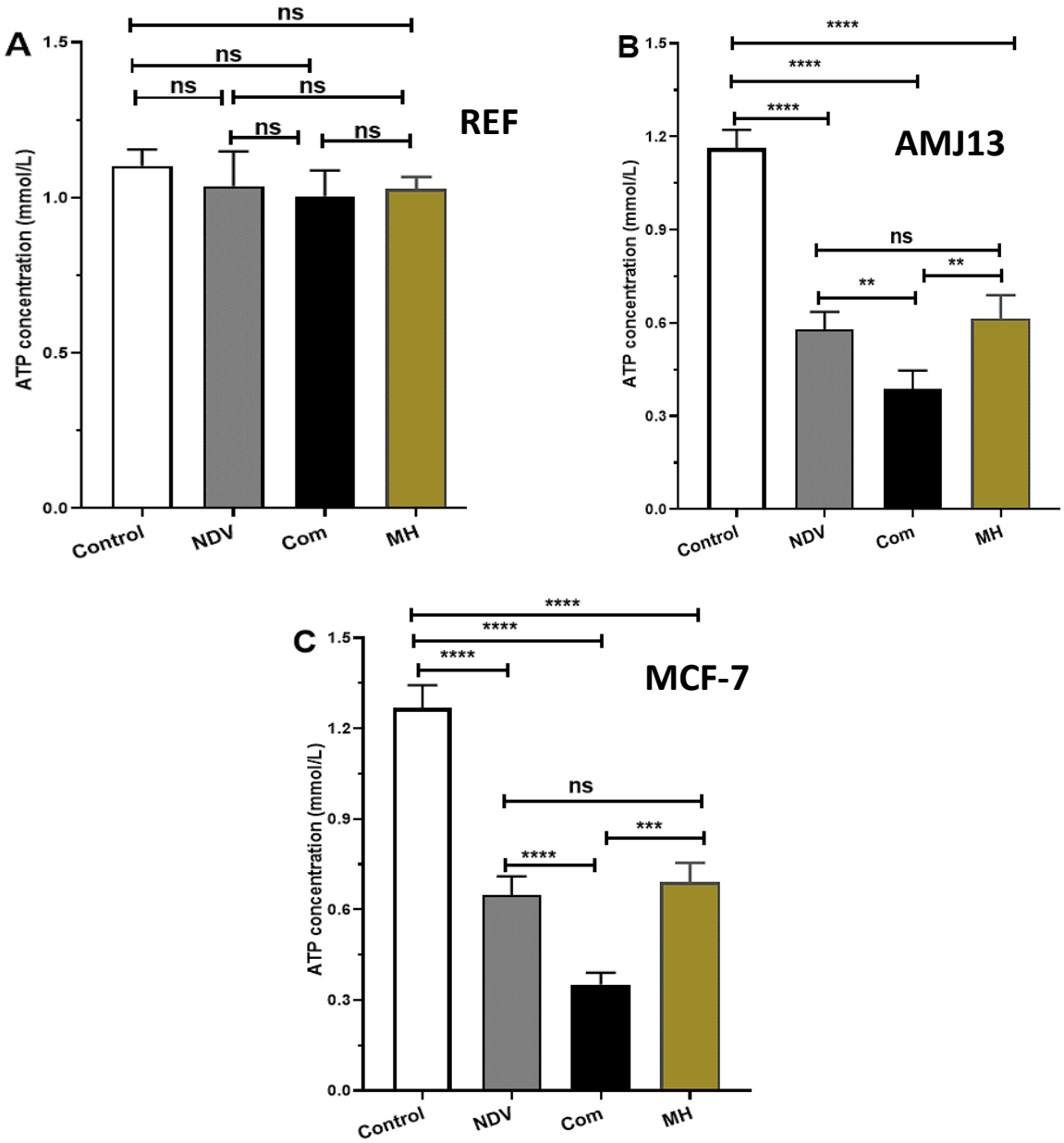


Figure (4-35) Effect of NDV and MH on the ATP concentration in glycolysis inhibition represented in (mmol/L). A) in REF, B) AMJ13 and C) MCF7 cell lines after treatment with NDV at MOI 2, MH at 62.5 µg/ml, and their combination, and comparison with control at *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

4.8.4 pH measurements

Measurements of the pH scales of the treated and control cell lines are shown in (Figure (4-36)). Elevation was noticed in the pH scale after treatment of MCF-7 and AMJ13 cell lines with 62.5µg/ml MH, 2 MOI NDV, and their combination for 72 hrs. The reduction of acidity (lactic acid) was caused by the reduced of lactate concentration in comparison with the control. When used separately, the virus and MH inhibited the acidity significantly when compared with the control. The combination of MH and virus was a significantly better inhibitor and induced proliferation inhibition when compared with control of breast cancer cell lines, whereas the effect of NDV, MH, and their combination was less in normal REF cell line (Figure (4-36)). The statistical analysis data of acidity is shown in (Table (4-14)).

Normalization measurements revealed that, when used separately, the virus and MH inhibited the acidity significantly in breast cancer cell lines when compared with normal REF cell line. The combination of MH and virus was a significantly better inhibitor when compared with normal REF cell line (Figure (4-37)).

Table (4-14): Statistical data of acidity after treatment of cell lines with NDV, MH, and their combination, and comparison with control.

pH - REF		Mean ± SD
The group	Control	7.3 ± 0.1
	NDV	7.7 ± 0.15
	MH	7.6 ± 0.1
	Com	8.2 ± 0.15

pH – AMJ13		Mean ± SD
The group	Control	6.3 ± 0.12
	NDV	7.3 ± 0.1
	MH	7.3 ± 0.1
	Com	8.2 ± 0.2

pH – MCF-7		Mean ± SD
The group	Control	6.3 ± 0.15
	NDV	7.4 ± 0.1
	MH	7.4 ± 0.15
	Com	8.2 ± 0.15

Results showed that normalization measurements have significant differences between (AMJ13 and REF) and (MCF7 and REF) after treatment with NDV, MH, and their combination. MH–NDV efficiently decrease cancer cell glycolysis products pyruvate, ATP, and acidity (represent lactic acid) compared to monotherapies.

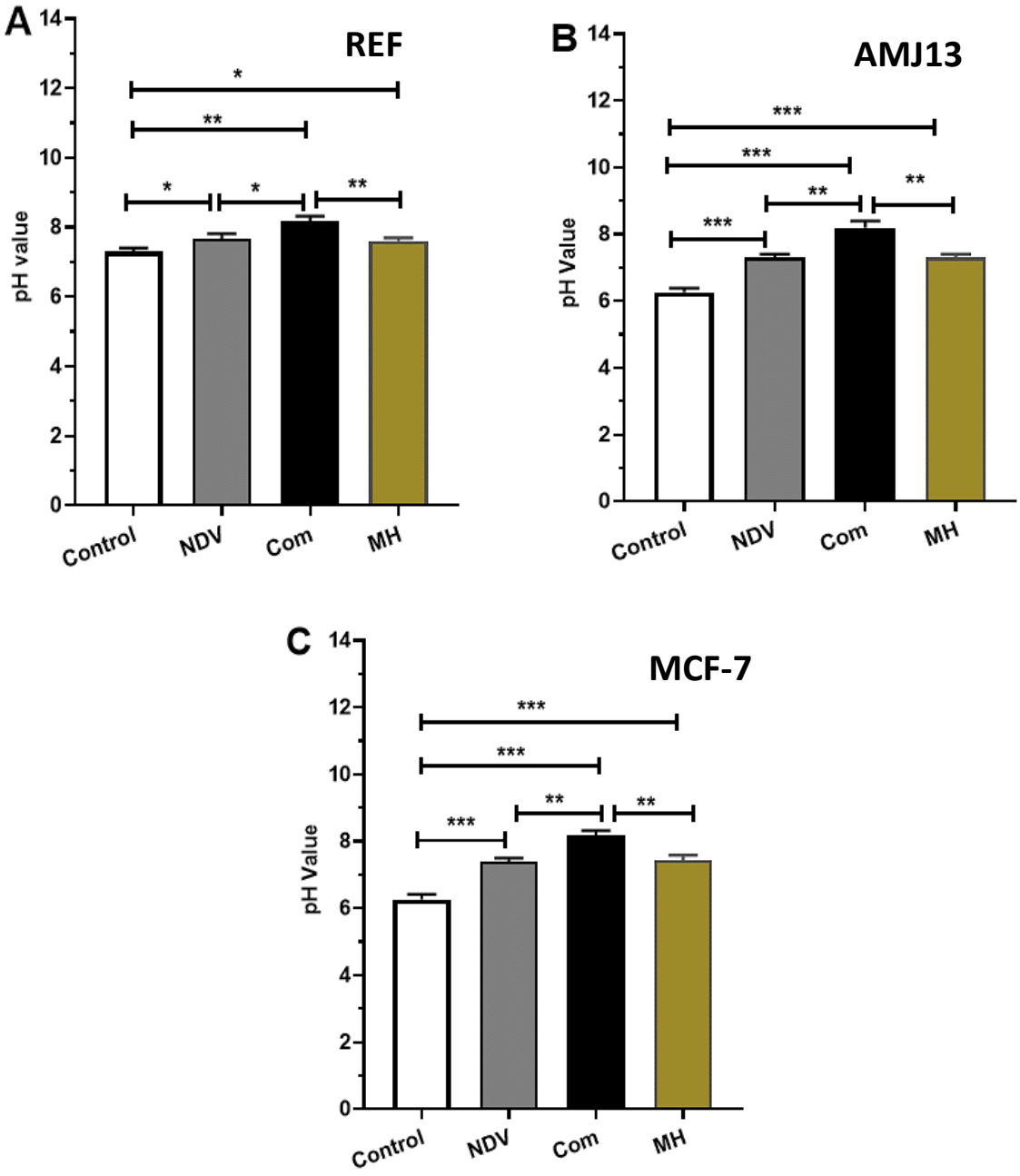


Figure (4-36) Effect of NDV and MH on the acidity of REF, AMJ13, and MCF7 cell lines based on pH values: pH values of A- the REF cell line, B- the AMJ13 cell line, and C- the MCF7 cell line after treatment with MH at 62.5 µg/ml, NDV at MOI 2, and their combination, and comparison with control. at *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

MH–NDV induce significant reduction on the level of acidity in both breast cancer cell lines but not in normal embryonic REF cells, see (Figure (4-36)). A significant reduction was identified in the acidity in all the treatment modalities in the cancer cells but not in the normal cells. MH–NDV combination efficiently inhibits glycolysis products in the treated breast cancer cells but not in normal cells.

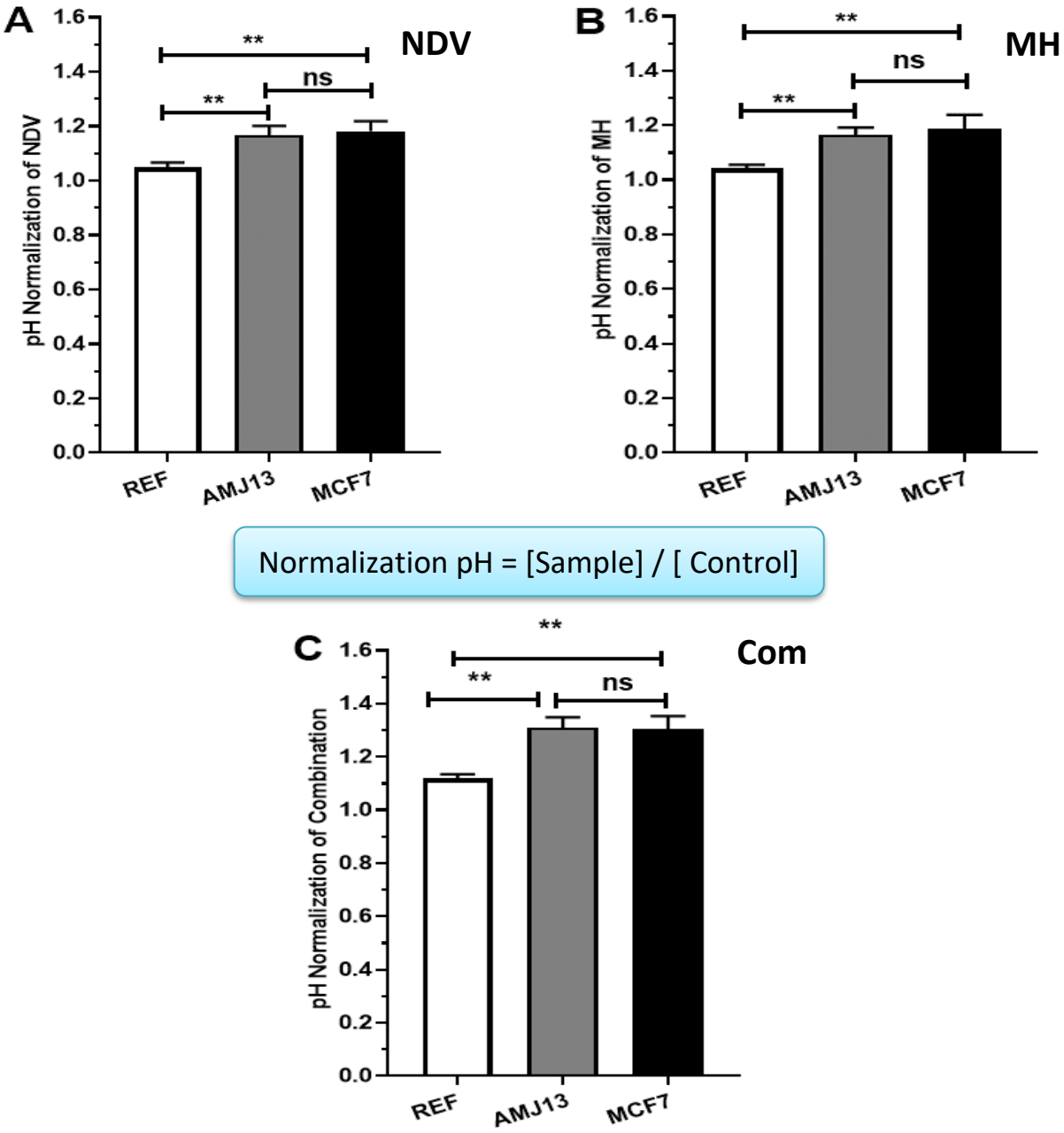


Figure (4-37) Normalization (Nor): effect of treatment with MH at 62.5 µg/ml, NDV at MOI 2, and their combination (Com) on the acidity of cell lines based on pH values. (A) Normalization of NDV–cell lines, (B) Normalization of MH–cell lines, (C) Normalization of Com–cell lines. at *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

Further investigation to the mechanism by which MH–NDV inhibit breast cancer cell proliferation was done through examination whether MH–NDV efficiently decrease cancer cell glycolysis production of pyruvate, ATP and acidity (represent lactic acid) compared to monotherapies as shown in comparison between tests (Figure (4-38)).

Remarkably, MH–NDV combination induce significant reduction of the levels of pyruvate, ATP and acidity in both breast cancer cell lines but not in normal embryonic REF cells.

4.8.5 Comparison between tests

MH–NDV combination efficiently inhibits glycolysis products in the treated breast cancer cells but not in normal cells in compare to monotherapies (Figure (4-38 A)). MH–NDV combined therapy induce significant decrease in the activity of hexokinase in AMJ13 and MCF-7 cancer cell lines, while there is no significant reduction in normal REF cells.

(B and C) measurement of pyruvate and ATP levels in cancer cells significantly reduced in MH-NDV combination therapy in compare to both monotherapies and untreated control cells. However, the reduction in the normal REF cells was not significant.

(D) Measurements of pH levels in AMJ13 and MCF-7 breast cancer cells indicate that MH-NDV combination treated cells supernatant was more alkaline in compare to both monotherapies significantly, while untreated control cells in breast cancer cells showing acidity, as well as the control and treated non-cancerous REF cells was alkaline. All data shown are means ± SD (*$P < 0.05$) data from three different experiments.

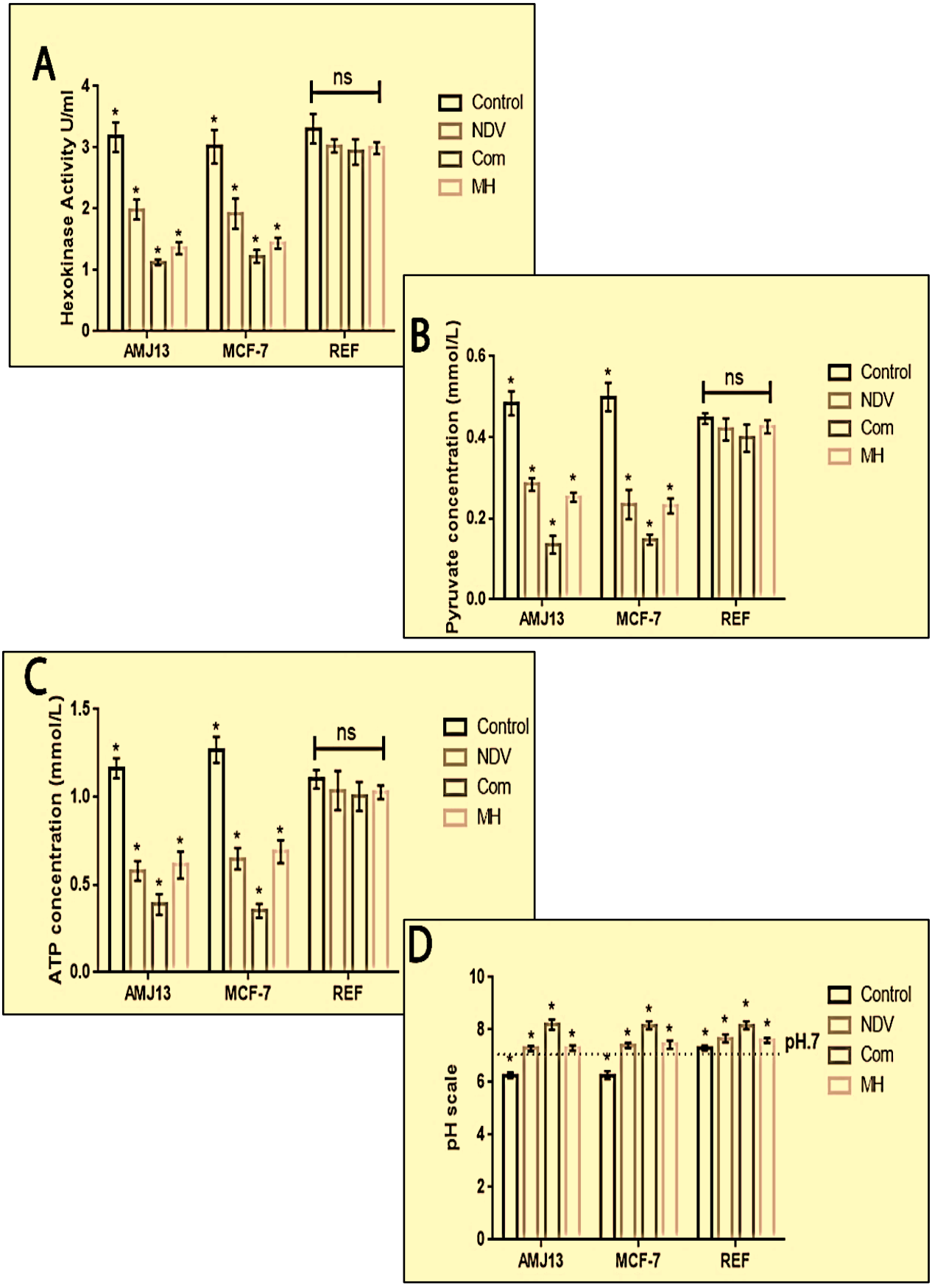


Figure (4-38). Comparison between tests (HK, Pyruvate, ATP and pH)

CHAPTER-FIVE

5. Discussion

5.1. Newcastle disease Virus Preparation and Collection

In tumor pathogenesis and metastasis, defects in apoptosis are essential. They allow tumor cells to overcome nutrient deprivation, absence of growth-stimulating signals and presence of growth-inhibitory signals. Newcastle disease virus Iraqi Strain is interesting oncolytic agent with promising anti-tumor properties. One of the major anti-tumor properties is apoptotic induction (Al-Shammari, 2018). (Al-Shammari, 2018) revealed that NDV had powerful effect on inducing apoptosis in AMN3 mammary adenocarcinoma in vitro, this study is similar to the current study that revealed that NDV increase apoptosis induction in breast cancer cell lines.

The results showed that Iraqi attenuated strain of NDV has the ability to kill most chicken embryos within 48 hrs as described by (Alexande, 2001). The isolated virus showed Syncytia formation which is a hallmark of NDV infection in host cells. It was reported by (Qin *et al*., 2008) that Syncytia are a typical cytopathic effect caused by the virus and can lead to tissue necrosis, and that it might also be a mechanism of virus spread. NDV strains can be categorized as velogenic (highly virulent), mesogenic (intermediate virulence), or lentogenic (non-virulent). Mesogenic strains in 60–90 hrs and lentogenic strains in more than 90 hrs. (Alexander and Senne, 1998).

Newcastle disease virus was quantified by the hemagglutination assay. Hemagglutination (adhesion of RBC) test showed a positive result as a typical hemagglutination mesh pattern of chicken red blood cell 256 HAU, the virus is able to agglutinate human, or animal red blood cells (Wood *et al*., 2014). The virus induces syncytia by fusing infected cells with neighboring cells leading to the formation of multinucleated enlarged cells. This event is induced by surface expression of viral fusion protein that is fusogenic directly at the host cell

membrane. Syncytia is an ideal cytopathic effect caused by the virus, which leads to tissue necrosis. Usually these syncytia are the result of expression of a viral fusion protein at the host cell membrane during viral replication, it is the mark of the mechanism of virus spread (Freeman *et al*., 2014).

The family Paramyxoviridae includes many viruses such as NDV, that significantly affect animal health. An essential step in the paramyxovirus life cycle is viral entry into host cells, mediated by virus-cell membrane fusion. Upon viral entry, infection results in expression of the paramyxoviral glycoproteins on the infected cell surface. This can lead to cell-cell fusion (syncytia formation), often linked to pathogenesis. Thus, membrane fusion is essential for both viral entry and cell-cell fusion, and it is an attractive target for therapeutic development (Aguilar *et al*., 2016).

NDV Iraqi strain is a broad-spectrum oncolytic agent that can destroy tumor cells (Al-Shamery, 2003) demonstrated that NDV Iraqi strain exhibits oncolytic activity to wide range of tumor cells such as mammary adenocarcinoma, Rhabdomyosarcoma (malignant tumor involving striated muscle tissue), and freshly cultured melanoma cell lines, where the virus showed the characterized features of NDV cytopathic effects and syncytia formation in the tumor cells, this were also confirmed by other researchers (Bar-Eli *et al*., 1996).

(Wu *et al*. 2016) demonstrated that NDV Anhinga strain could not only directly kill tumor, but could also elicit immune reaction and a potent immunological memory in vivo which could become an effectual vector for tumor treatment.

(Al-Shammari *et al*., 2016) revealed that NDV is a very promising anti-malignancy agent when combined with other antitumor agents and when the combinations of NDV and Doxorubicin were tested on normal rat embryo fibroblasts, there seemed to be no favorable interactions at the same low doses; but,

at higher doses there was toxicity on the normal cells, this similar to current study that revealed no effect on the normal cell line at low doses. NDV has been shown to be selective in replicating in tumor cells and not replicating on normal rat embryo fibroblast cell lines (Reichard et al., 1992), This proves the increased effect of the virus on cancer cell lines and its non-effect on normal REF cells in the present study.

5.2. Tissue Culture Infective Dose 50 ($TCID_{50}$) of NDV in Vero cell line

Tissue culture infective dose fifty percent ($TCID_{50}$) is an assay used to determine the titer of virus (Eliyana and Redhuan, 2008). According to (Florence *et al*., 1992), TCID50 refers to the ability of a certain virus dilution to infect 50% of the cell cultures inoculated. Therefore, a titer is defined as a number of infectious virus units per unit volume.

The TCID50 titer provides qualitative information on dose, or dilution that causes cytopathic effect (CPE) in 50% of the sample, instead of giving the quantitative amount of virus infection that is present in a sample. Newcastle disease virus formed plaques on Vero cell line and normal tissue bird such as chick embryo fibroblast in vitro. It did not form plaques on any normal fibroblast cell line from other sources (Cuadrado - Castanoa *et al.*, 2015).

The gated of $TCID_{50}$ (124.6×10^6) used in the present study ensure the high virulent of NDV. When each infected cell produces virus and eventually lyses, only the immediately adjacent cells become infected. Each group of infected cells is referred to as a plaque that is surrounded by uninfected cells. After several infection cycles, the infected cells in the center of the plaques begin to lyse, and then the virus moves to the uninfected cells gradually (Fu *et al*., 2011). (Ravindra *et al.* 2008) showed that NDV can induce apoptosis in Vero cells after exposure and replication for 48 hrs.

5.3 Cytotoxicity and IC_{50} of NDV, MH, and DXL in cancer cell lines

The in vitro results of this study revealed that an increased concentration of MH, DXL, and MOI of NDV increased in cytotoxicity and antiproliferation effect against breast cancer cell lines, because MH caused inhibition of glycolysis and this effect on glycolysis pathway reduced HK activity, [Pyruvate], [ATP], and acidity (lactic acid). The results are similar to those related to inhibition of glycolysis for 3-bromopyruvate and 2-deoxyglucose, results in mitochondrial pathway-induced apoptosis (Danos *et al*., 2008); (Kim *et al*., 2008).

Docetaxel, a chemotherapy drug, was used as a positive control for comparison with the effect of D-Mannoheptulose as phytotherapy, NDV as virotherapy, and their combination on normal and breast cancer cell lines. The effect of MH and NDV had a significant killing level in breast cancer cells, while it had a non-significant killing level in normal cells. This was caused by differences between normal and breast cancer cells in receptors, cell environment, pH, apoptosis, and the effect of morphological changes. These results support the study (Mikirova *et al*., 2017) as glycolysis increased in tumor cells in comparison with normal cells. D-Mannoheptulose has been found to inhibit glucokinase and hexokinase of rat liver (Coore and Randle, 1964).

The half maximal inhibitory concentration (IC50) is a measure of the potency of a virus, drug, and inhibitor in inhibiting breast cancer cell lines. IC50 is a quantitative measure that indicates the amount of a particular inhibitory substance (DXL, NDV, and MH) needed to inhibit proliferation of breast cancer. The results showed that there are significant differences in IC50 between breast cancer cell lines in comparison to REF cell line after treatment with virus, inhibitor, and drug. This is because their effect in cells differs according to their type as virotherapy, phytotherapy, and chemotherapy, and it is due to the difference between the nature of normal cells and cancer cells. Insignificant difference was found between MCF-

7 and AMJ13 cell lines. Cytotoxicity was measured between REF, AMJ13, and MCF-7 cell lines at specific concentrations after treatment with NDV, MH, and DXL. It was found that, the effect of NDV, MH, and DXL on breast cancer cell lines was very clear compared to normal REF cell line where a significant difference was found between them.

5.4 Synergistic effect of NDV and MH on cells lines

Inhibition of growth induced by high concentrations of the virus, inhibitor, and their combination is the best. Also, treatment of NDV and MH showed enhancement to the growth inhibition properties in breast cancer cell lines more than normal cell line. The combination of MH and virus was a significantly better promoter and enhancer of growth inhibition when compared with separate treatment in breast cancer cell lines.

While in normal REF cell line, the effect of virus, inhibitor, and their combination was insignificant and induced cytotoxicity less than in breast cancer cell lines. From the results, a significant induction was identified in growth inhibition in all the treatment modalities in the breast cancer cell lines but not in the normal cells line. Dose response evaluation of treatments differed significantly between REF and breast cancer cell lines. Combination between NDV and MH in cell lines showed high synergism in MCF-7 at all interactions between MH and NDV, and also in AMJ13 with the exception one value which was considered additive.

On the other hand, all REF cell line values indicated antagonism and additive effect which is neglected effect as there was no killing effect reached 50% at all tested concentrations. The results implied that synergism between inhibitor and virus had more effect in inhibition of proliferation, anticancer growth action, and caused increase in cytotoxicity, inhibition of HK, pyruvate, ATP, and acidity which

lead to induced morphological changes and apoptosis. Isobologram analysis, or Chou–Talalay equation (Combination index) showing the synergistic effect between NDV and MH in 9 different doses used on normal and breast cancer cell lines. Isobologram analyses in AMJ13 and MCF-7 cell line revealed synergism between NDV and MH, indicating that the effect is synergistic, while1 point in AMJ13 indicated that the effect is antagonism. Also, isobologram analyses in REF cell line revealed antagonism between NDV and MH, indicating the effect is antagonistic, or less interaction in combination between virus and inhibitor.

Combination therapies give great advantages represented by better effectiveness, decreased cancer toxicity, and reduced development of drug resistance. Therefore, these advantages became a standard for the treatment of several diseases and are a promising approach in indications of unmet medical need (Foucquier and Gued, 2015). There are many methods to determine the synergy of combination of two, or more chemotherapy combined for each other to treat a lot of diseases and cancers, these methods were applied by CompuSyn program (Chou, 2010; Rodea-Palomares *et al*., 2010; W. Humphrey *et al*., 2011). The selective nature of NDV makes it an ideal virotherapy agent. Its ability to replicate in tumor tissue allows massive amplification of the input dose at the tumor site, and at the same time, its lack of replication in normal tissues allows efficient clearance and reduction of toxicity (Ravindra *et al*., 2009).

The results of cytotoxicity activity of combination (NDV and MH) on cell lines showed the inhibitory rates. These results reported a synergistic effect of 3 combination concentrations that were used on MCF-7 and AMJ13 cell lines. The results of the present study reflects the great importance of combination therapy in the treatment of breast cancer and it turned out that the high rates from the combination of MH and NDV that were obtained in the two cell lines tumors AMJ13 and MCF-7, may also be useful in treating other types of cancer, even if

they have different genetic makeups (AL-Shammari *et al*., 2016). The synergistic effect was a concept that NDV may increase anti-tumor activity by rising cellular sensitivity to the therapeutic agents due to enhanced susceptibility that is partially caused by the induction of apoptosis which was previously confirmed to be induced by NDV (Naik *et al*., 2011).

The use of MH with NDV decreased the proliferation activity of cancer cell culture, decrease signaling via the major pathways that promote cancer cell culture progression as in the study of (Applegate and Lane, 2015). This interpretation outcome of combining MH with NDV increased apoptosis of cancer cells.

A synergistic interaction allows the use of lower doses of the combination constituents, a situation that may reduce adverse reactions. Most previous study results, which relate to the effect of Newcastle disease virus and MH separately on some cancers, showed the effects and results closely. Combination of NDV and MH synergistically enhanced the cytotoxic effect on cancer cells than their separate effect. Another study showed that synergistic antitumor combination of NDV and 2-Deoxy glucose had a synergistic and greater effect on cancer cells than their separate use in breast cancer cell lines (human AMJ13 and murine AMN3) cell line (Salim, 2016).

From the results of cytotoxicity of NDV, MH, and their combination in AMJ13, MCF7, and REF cell lines, which have already been explained. It can be concluded that increased MOI of NDV and concentration of MH increased cytotoxicity, while their combination caused a better result by inducing cytotoxicity, decreasing proliferation of cancer cells, and elevating growth inhibition as the virus induced apoptosis and MH inhibited hexokinase which lead to inhibition of glycolysis. A significant difference was found in cytotoxicity between normal REF cell line and breast cancer cell lines of the virus, inhibitor, and drug, while insignificant difference was found between them in MCF-7 and

AMJ13 cell lines. There is a significant difference between effect the DXL at 1.25 µg/ml and combination at (2MOI-NDV and 62.5 µg/ml MH) on the treatment of normal and breast cancer cell lines. This shows that combined therapy is better than chemotherapy alone. The results of some studies such as (Aghi *et al*., 2006) showed antagonism when they used a combination of NDV with other chemotherapies. Also, this type of analysis determines synergy and it is an acceptable method of assessing the efficacy of chemotherapy combination therapies as well as combination chemotherapy and viral therapy (Mullerad *et al*., 2005).

The findings of the present study showed that the combination of NDV and MH gave a high percentage of inhibition rates in three concentrations with cancer cell lines such as MCF-7. Therefore, the present study suggests that, the result of additive in one concentration of the AMJ13 cell line is possibly due to low concentrations of the combination.

On the other hand, the remaining concentrations showed a synergistic activity on AMJ13 cell line, which means that the synergism in the combined effect observed, is significantly greater than the expected activity. Other studies have used different methods, one by halving the administered dose of chemotherapeutic agent (rituximab, and doxorubicin) and completing the eliminated dose with NDV in order to reduce the toxicity of chemotherapy (Al shammari *et al*., 2016). One investigator has hypothesized that combination will act synergistically (McCart *et al*., 2000).

The theory supported that combination chemotherapy in assaulting tumor cells using different mechanisms of action can prevent tumor cells from having enough time to improve resistant to treatment (Post *et al*., 2003). The strategy of combination therapy is by attacking tumor cells through different mechanisms of action to prevent cancer cells from developing resistance to therapy (Kumar *et al*., 2014).

The mechanism of synergistic activity for the combination of MH with NDV is unknown, but it can be proposed a few hypotheses. First, NDV may augment the anti-tumor activity of MH by increasing the cellular sensitivity to phytotherapy agent (inhibitor) (MH), and this enhanced sensitivity is partially caused by the induction of apoptosis in response to virulent NDV strain. Second, a synergistic dose of MH may augment the viral replication. Each agent may work independently on different cell populations. In addition, virotherapy with NDV may complement the anti-tumor activity of MH which selectively targets tumor cell populations that are resistant to chemotherapy or other conventional treatments. This may be of important value because most human tumors consist of a mixture of cells that have a different genetic makeup. Heterogeneity in the tumor cell populations may be the major reason most monotherapies fail to achieve complete tumor remission (Henderson and Yu. 2002).

Moreover, one of the objectives of this study was to reduce the toxic side effects of chemotherapy or other conventional treatments by using combination therapy (virotherapy and phytotherapy) in breast cancer cell lines. Also, this was clear by comparison with Docetaxel drug (DXL) as positive control and a chemotherapy. This can be achieved by reducing the administered dose while maintaining the same or stronger for anti-tumor activity when using chemotherapy with combination therapy.

5.5 Study of morphological changes and apoptosis in cell lines

The morphological changes showed the presence of cytopathic effect, which was detected in treated cells, a large number of cramped cells with granulation, formation of syncytial, and shrinkage of cells were noticed when compared with the control. Morphological changes and apoptosis showed after treatment with MH (62.5µg/ml) and NDV (MOI = 2) depends on the combination index that has been

measured for NDV and MH for 72 hrs. Apoptosis was seen in stained cells by AO/PI and in treated cells as red cells, while viable cells were green.

Apoptosis is a spontaneous process of programmed cell death, which can be induced by a variety of physical and chemical factors, and it is precisely regulated by an organism. Although there are three predominant signaling pathways in apoptosis (the mitochondrion, the death receptor, and the endoplasmic reticulum signaling pathways), the integration and amplification of apoptotic signaling usually occurs at the mitochondrial level (Guo *et. al*, 2016). Cancer cells continue to divide uncontrollably as they can ignore signals that normally tell cells to stop dividing or that begin a process known as programmed cell death, or apoptosis (Gatenby and Gillies, 2004).

Also study of apoptosis using AO/PI staining by fluorescent microscopy was similar with the study (Danos *et al.*, 2008) (Kim *et al*., 2008) which revealed that inhibition of glycolysis, for specific inhibitors, 3-bromopyruvate and 2-deoxyglucose, results in mitochondrial pathway-induced apoptosis. Also, a compatible research study confirmed that over-expression of LDH-B by panepoxydone inhibitor in cancer cell lines leads to enhanced apoptosis and mitochondrial damage (Arora *et al*., 2015).

NDV induced apoptosis in various cancerous cells, the critical relationship between apoptosis and cancer signifies that any therapeutic strategy aimed at specifically triggering apoptosis in cancer cells will have a potential therapeutic effect (Ravindra *et al*., 2008). NDV induced apoptosis in the infected cells (Al-Shammari *et al*., 2015).

NDV stimulated specific anti-tumor immune response and was able to induce endoplasmic reticulum stress; all these mechanisms beside the direct cytolytic effect are secondary to virus replication (Al-Shammari and Yaseen, 2012). NDV

has been demonstrated to induce apoptosis in various cancerous cells (Ravindra *et al*., 2008), and human breast carcinoma cells (Washburn *et al*., 2003).

The results showed that NDV induced apoptosis in MCF-7 and AMJ13 cells by inducing mitochondrial apoptosis. (Al-Shammari and Yaseen, 2012) revealed that NDV has powerful effects on inducing apoptosis in AMN3 mammary adenocarcinoma in vitro. Based on current results in two different cell lines (MCF-7 and AMJ13), NDV exhibited oncolytic activity on two tumor cell lines and to a lower degree on normal cell line (REF). Previous studies have shown that a virulent NDV strain is oncolytic (Al-Shammari *et al*., 2016). This strain showed antitumor activity both in vitro and in vivo in a clinical trial (Csatary *et al*., 1999).

In current study was found alternative or supportive treatment for chemotherapy or other conventional treatments, by using the inhibitor (MH) or virus (NDV) or their combination (MH-NDV). Also, it was obtained an anti-tumor efficacy and proliferation inhibition of cancer cells through glycolysis inhibition and induction of apoptosis. This can be an alternative treatment as combination therapy that can be used to reduce the dose of chemotherapy or other conventional treatments with the maintaining the same or more anti-proliferative activity and overcoming chemotherapy resistance or other treatments.

5.7 Glycolysis inhibition

Malignant cells use glycolysis as the main source for energy, meanwhile, a mass of lactate and pyruvate is generated during the process. However, lactate, the end product of glycolysis, is exported to the extracellular medium and contributes to the acidification of tumor microenvironment, which favors tumor development, invasion, chemotherapy and radiotherapy resistance, recurrence of tumor, and suppresses the immune defense of anticancer (Goetze *et al*., 2011). D-Mannoheptulose has been found to inhibit glucokinase and hexokinase of rat

liver and other tissues (Coore and Randle, 1964), and it competitively inhibits the phosphorylation of glucose in homogenates of isolated islets of Langerhans (Malaise *et al*., 1968). Cancer cells derive most of their energy from glycolysis that is, glucose is converted to lactate for energy followed by lactate fermentation, even when oxygen is available. This is termed the Warburg effect. This produces far less energy than oxidative phosphorylation and as cancer cells require a lot of energy to grow this seems paradoxical.

The Warburg effect may be beneficial to cancer cells because it provides precursors for many biosynthetic pathways. These precursors include amino acid precursors and NADPH and ribose sugars for DNA and RNA synthesis. The Warburg effect may be caused by impaired oxygen sensing in cancer cells. Glycolytic enzymes such as GLUT1, lactate dehydrogenase, pyruvate kinase and the lactate exporter are unregulated in cancer cells, whilst pyruvate dehydrogenase is inhibited leading to increased glycolytic flux and impaired ability of pyruvate to enter oxidative phosphorylation (Fadaka *et al*., 2017).

The results mentioned demonstrated that MH could have inhibited glycolytic flux in MCF-7 and AMJ13 cell lines by suppressing the activity of hexokinase (HK), resulting in ATP depletion and decreasing lactate generation, which induced insufficiency of energy supplement to support cancer cells mitosis, proliferation, and invasion. The acidic microenvironment, which favors cancer cells resistance to chemotherapy, was disrupted by decreasing production of lactate. This agrees with (Lu *et al*., 2011).

The effect of MH in inhibition of hexokinase and effect of NDV in induction of apoptosis in addition to synergism between them led to the decrease in HK activity, pyruvate, ATP concentration, and acidity in breast cancer cell lines. The virus, MH, and their combination inhibited the effectiveness of HK and pyruvate, ATP concentration, and acidity significantly when compared with the control.

Therefore, it led to prevention of proliferation of cancer cells and induced growth inhibition, this agrees with (Mathupala *et al*., 2006) which mentioned that HK via its mitochondrial location suppresses the death of cancer cells. For these reasons, targeting key enzyme by inhibitors led to inhibition of glycolysis, therefore suppressing proliferation of cancer cells. The combination of MH and virus was a significantly better inhibitor and enhancer of growth inhibition when compared with separate treatment or with control of breast cancer cell lines, while the effect was insignificant in normal REF cell line.

A significant reduction in the ATP concentration was identified in all the treatment modalities in the cancer cells but not in the normal cells. MH–NDV combination efficiently inhibits glycolysis products in the treated breast cancer cells but not in normal cells. Because the concentration of pyruvate depends on the action of hexokinase in the glycolysis pathway, the decrease in hexokinase activity led to deficiency in pyruvate concentration. The results in this study have shown that MH and NDV could prominently suppress the generation of intercellular ATP by inhibition of glycolysis and induction of apoptosis. A decrease of ATP related to another study revealed that, mitochondrial membrane potential ($\Delta\psi m$) is necessary for the activity of ATP synthase in normal cells, which generates ATP. The loss of $\Delta\psi m$ and damage of the mitochondrial inhibited the activity of ATP synthase, leading to ATP deficiency (Vander Heiden *et al*., 2009).

In another study, decreased ATP level in MCF-7 and MDA-MB-231 cells was identified to be effective in inhibiting energy metabolism in breast cancer cells (Cheng *et al*., 2013); (Ding *et al*., 2012). To obtain sufficient energy for growth and proliferation, cancer cells require an increased glucose uptake. In hypoxic conditions, glucose via glycolysis is catabolized to lactate to produce ATP. In hypoxic conditions, the monocarboxylate transporters MCT4 are up regulated, enabling cells to rapidly dispose the accumulated lactic acid (Ullah *et al.,* 2006).

(Fadaka *et al*., 2017) mentioned that glycolysis generates ATP more rapidly than OXPHOS, and this offers a selective advantage to rapidly growing tumor cells. When glycolysis is inhibited, lactate production is completely stopped and there is an abrupt decrease of intracellular ATP concentration (Gonin-Giraud *et al*., 2002), this is consistent with the present study. This study is similar to findings (Arora et al., 2015) by using panepoxydone inhibitor significantly reduces lactate production. The results have also supported an increased glycolysis in transformed cells (cancer cells) in comparison with normal cells (Mikirova *et al.,* 2017).

Also, the decrease of ATP that was proved in the current study is in agreement with the study (Lu *et al*., 2011) which proved that ATP depletion induced insufficiency of energy supplement to support cancer cells mitosis, proliferation, and invasion. Through the results, an increase in pH measurements (less acidity) was noticed in breast cancer cell lines in comparison to the control, and less effect in normal REF cell line after treatment with MH and NDV for 72 hrs, which lead to a decrease of acidity.

This decrease of acidity results from the reduction of lactate concentration that resulted from the decrease in pyruvate concentration which lead to growth inhibition and anti-proliferation of cancer cells. This result is compatible with the study (Arora *et al*., 2015) which proved that deficiency in lactate concentration leads to reduction in the acidity of the cell environment, which is considered favorable in preventing proliferation of cancer cells as they grow in acidic environments. Release of lactate from the cell is one of the main causes of extracellular acidosis (Fadaka *et al*., 2017).

One important difference is that cancer cells are less specialized than normal cells (Elstrom *et al.,* 2004*).* In the presence of MH, glycolysis is inhibited because there is no production of glucose-6-P, hence no increase in ATP concentration which is necessary for cancer cells. Warburg originally proposed that the aerobic

glycolysis in cancer cells was due to a permanent impairment of mitochondrial oxidative phosphorylation. Warburg observed that cancer cells tend to convert most glucose to lactate (Kroemer and Pouyssegur, 2008).

Warburg originally hypothesized that cancer cells develop a defect in the mitochondria that leads to impaired aerobic respiration and a subsequent reliance on glycolytic metabolism (Vander Heiden *et al*., 2009). Each reaction in the glycolytic pathway is catalyzed by a specific enzyme or enzyme complex (Pelicano *et al*., 2006). This effect means higher glucose uptake of cancer cells compared to normal cells, which can be used as a target for selective therapy by targeting glucose metabolism (Aft *et al*., 2002).

Through the current results, a decrease in the ATP concentration was observed by the effect of the inhibitor and the virus or their combination, and this led to treatment of breast cancer, based on the study (Xu *et al*., 2005), which showed that cellular ATP depletion is the main event for cancer treatment. It has also been reported that the Warburg effect is generally attributed to a "breathing injury" or a defect in the mitochondrial oxidative phosphorylation process, forcing cells to use the glycolic pathway to generate ATP, which affects ATP concentration later. It should be noted that previous studies have suggested that apoptosis is an ATP-dependent process, and depletion of ATP tends to cause cell death by necrosis (McConkey, 1998).

(Khalifa *et al*., 2013) mentioned that the methanolic extracts of Avocado fruit and leaves are highly promising to be used in cancer therapy and antioxidant treatments, this corresponds with the current study in which the inhibitor (MH) was used, which is originally extracted from the avocado plant. D-mannoheptulose is a seven-carbon sugar commonly found in avocado fruits that inhibits HK, thus reducing glycolysis. According to (Granchi & Minutolo, 2012) study, D-mannoheptulose is considered safe to use as a hexokinase inhibitor to treat breast

cancer cell lines and thus does not cause side effects on human health. The phenomenon of aerobic glycolysis increase in cancer cells was first described by Otto Warburg.

Otto Warburg showed that compared to normal cells, malignant cells exhibit significantly elevated glycolytic activity even in the presence of sufficient oxygen, and considered this phenomenon as the most fundamental metabolic alteration in malignant transformation, or 'the origin of cancer cells' (Warburg, 1956), This is agree with the current study, where an increase in the concentration of pyruvate, ATP and acidity was observed in cancer cells compared to normal cells as control. also, it was observed by increasing the effectiveness of treatment by NDV and MH and their combination in breast cancer cell lines compared to normal cells. Cancer cells consume far more glucose than normal cells to maintain sufficient ATP supply for their active metabolism and proliferation. As such, maintaining a high level of glycolytic activity is essential for cancer cells to survive and growth.

This metabolic feature has led to the hypothesis that inhibition of glycolysis may severely abolish ATP generation in cancer cells and thus may preferentially kill the malignant cells (Munoz-Pinedo *et al*., 2003; Izyumov et al., 2004; Xu *et al*., 2005), and this is agree with the present study, where an increase in the concentration of ATP was observed in cancer cells compared to normal cells as control. Also, A decrease in ATP concentration was also observed after treatment with NDV, MH and their combination, and thus agree with the hypothesis.

Inhibition of glycolysis alone may not be sufficient to effectively kill the malignant cells. It has been suggested that ATP depletion should reach certain thresholds in order to trigger cell death by apoptosis or necrosis processes, with a depletion of 25–70% ATP leading to apoptosis (shrinkage, active process), and an over 85% ATP depletion causing necrosis (swelling , passive process, inflammation and cell lysis) (Lieberthal *et al*., 1998).

In current study was used the combination therapy by synergism between NDV and MH to get ATP depletion. Where it was observed a significant decrease in ATP concentration after combination therapy in AMJ13 and MCF-7 cell lines. One way to achieve a high level of ATP depletion and improve therapeutic activity is to combine multiple ATP-depleting agents with different mechanisms of action (Martin *et al*., 2001). This is what happened in present study through the use of two agents (NDV and MH) to depletion of ATP. Where a significant decrease in ATP concentration was also observed after treated with combination therapy in comparison with virus and inhibitor separately.

Other studies have provided compelling evidence showing that cancer cells with mitochondrial defects or under hypoxia are highly sensitive to glycolysis inhibition (Pelicano and Martin, 2006). It should be noted that previous studies have suggested that apoptosis is an ATP-dependent process and that depletion of ATP tends to cause cell death (Xu *et al*., 2005).

Through this clarification, it was concluded that there is a relationship between ATP depletion and apoptosis and in this study, it has been proven that ATP depletion led to an increase in cell death, its mean that it promoted programmed cell death. In the current study was suggested that inhibition of glycolysis is an effective strategy to kill cancer cells. This novel approach may have broad applications in cancer treatment, considering the prevalent Warburg effect observed in a wide spectrum of human cancers.

(Wang *et al*., 2016) described HK2- targeting modulate Warburg effect to stimulate cancer cell apoptosis. HK inhibition can cause ATP depletion, thus resulting in insufficient energy supply for cancer cell mitosis, proliferation, and invasion as previously described (Coore and Randle, 1964). HK activity in breast cancer cell lines was lower than that in normal cells, and a non-significant reduction in HK activity was observed in the REF normal cell line.

(Patra *et al.,* 2013) discovered that deletion of HK2 gene in adult mice does not considerably disturb normal tissues. Moreover, MH is a specific inhibitor for HK II (Dakubo, 2010) and normal cells rely more on HK I (Xu *et al.,* 2018) this is explained why REF cells showed mild non-significant reduction in HK activity. There are several studies described the role of HK2 as essential in tumor initiation and development in breast cancer; therefore, HK2 deletion as cancer treatment have therapeutic value with no adverse physiological side effects (Patra *et al*., 2013).

It was noticed a significant reduction in pyruvate concentration, after treatment with combination in breast cancer cell lines, and a non-significant difference in the REF normal cell line after 72 h of treatment. Given that pyruvate concentration depends on the action of HK in the glycolysis pathway (Ding *et al*., 2012), the decrease in HK resulted in a deficiency in pyruvate concentration which confirmed by the study. In correlation with previous experiments results, it was noticed that MH-NDV combination suppress the generation of intercellular ATP. It was found that the ATP concentrations in AMJ13 and MCF-7 cell lines treated with MH and NDV were significantly lower than to those of the control. Reducing ATP levels effectively inhibits energy metabolism in MCF-7 and AMJ13 cells.

The pH values of treated breast cancer cell lines secretions were higher than those of the control. A non-significant effect was observed in the normal REF cell line after treatment with MH and NDV. Acidity decreased because lactate concentration decreased as a result of reduced pyruvate concentration and inhibited the growth and proliferation of cancer cells. It is proved that lactate deficiency reduces the acidity of the cell environment. This effect is favorable for preventing cancer cell proliferation because tumor or cancer cells grow in acidic environments. Elevated glucose metabolism decreases the pH in the microenvironment due to lactate secretion (Estrella *et al.,* 2013).


CONCLUSION &
RECOMMENDATION

CONCLUSION

The following conclusions were made from the present study:

1- Combination index analysis (CI) showed presence of synergistic inhibitory effect between NDV and MH against breast cancer cells. While, it showed presence of antagonistic and additive effect for NDV and MH combination in normal REF cell line.

2- Increase concentration of MH and MOI of NDV induce cytotoxicity, morphological changes, and apoptosis. The combined MH and NDV treatment had potent cytotoxic effects against breast cancer cell lines but not against the normal REF cell line that proved safety.

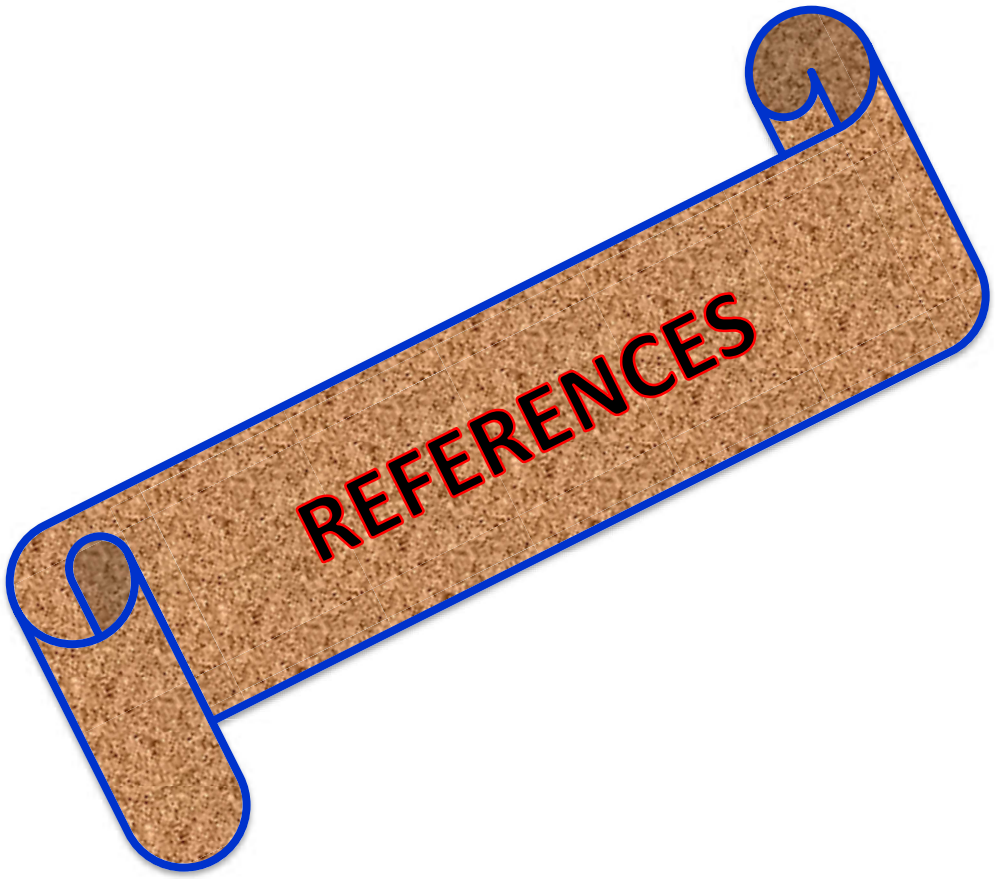
3- The combination of MH and NDV was a significantly better inhibitor and enhancer of growth inhibition and glycolysis inhibition.

4- A significant reduction was identified in the HK activity, [Pyruvate], [ATP], and acidity in all the treatment modalities in the breast cancer cells but not in the normal cells, especially with the combination therapy.

5- Synergism of NDV and MH can be considered as a future treatment of cancer cells and a new strategic way to be used as an effective treatment. This strategy has potential applications as an effective cancer treatment.

RECOMMENDATIONS

- Study the combination treatment of MH and NDV in vivo using mouse tumor models.
- Study the internal organs histology to confirm the safety of the combination therapy.
- Testing the combination of NDV with MH in vitro against more cancer cell lines.
- Study more glycolysis parameters such as Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) enzyme in the treated cells.
- Increase the efficacy of the treatment by combining more anti-glycolysis agent such as pyruvate kinase inhibitor with NDV and MH.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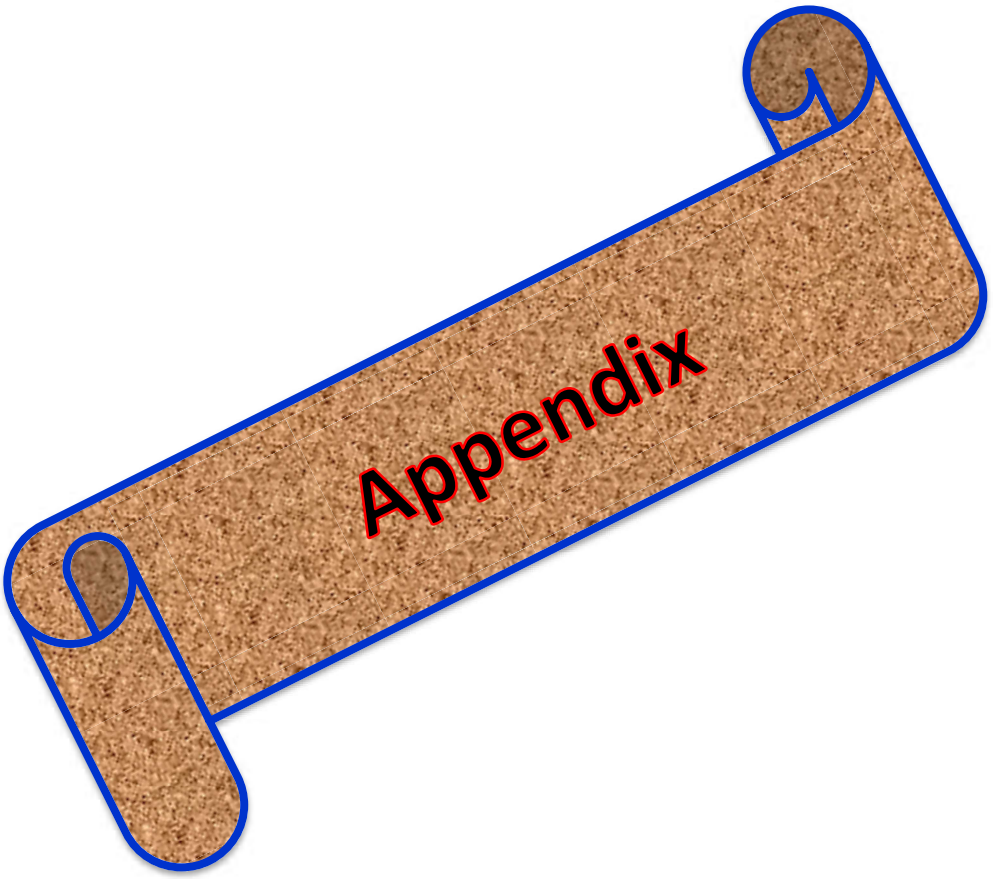
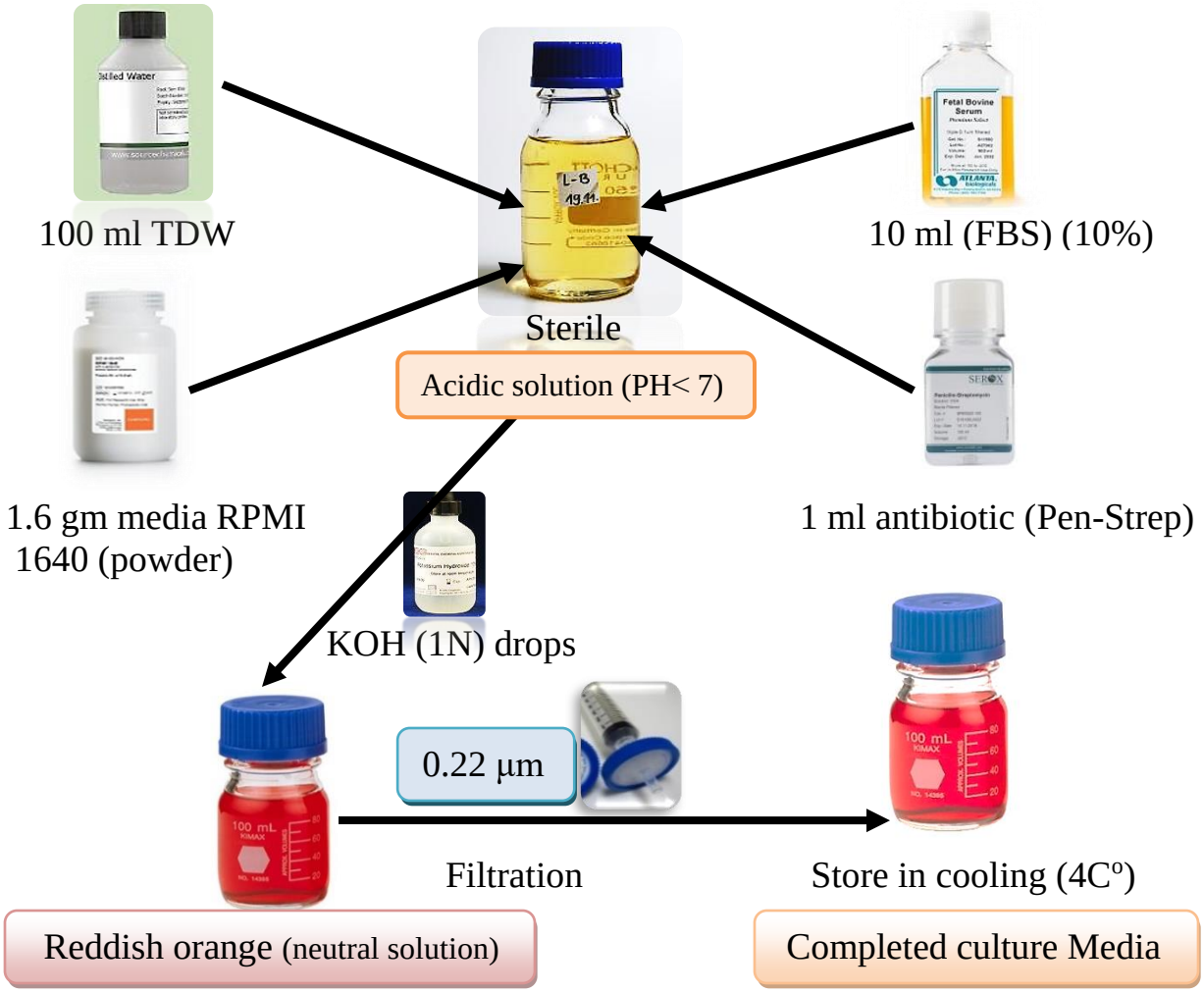
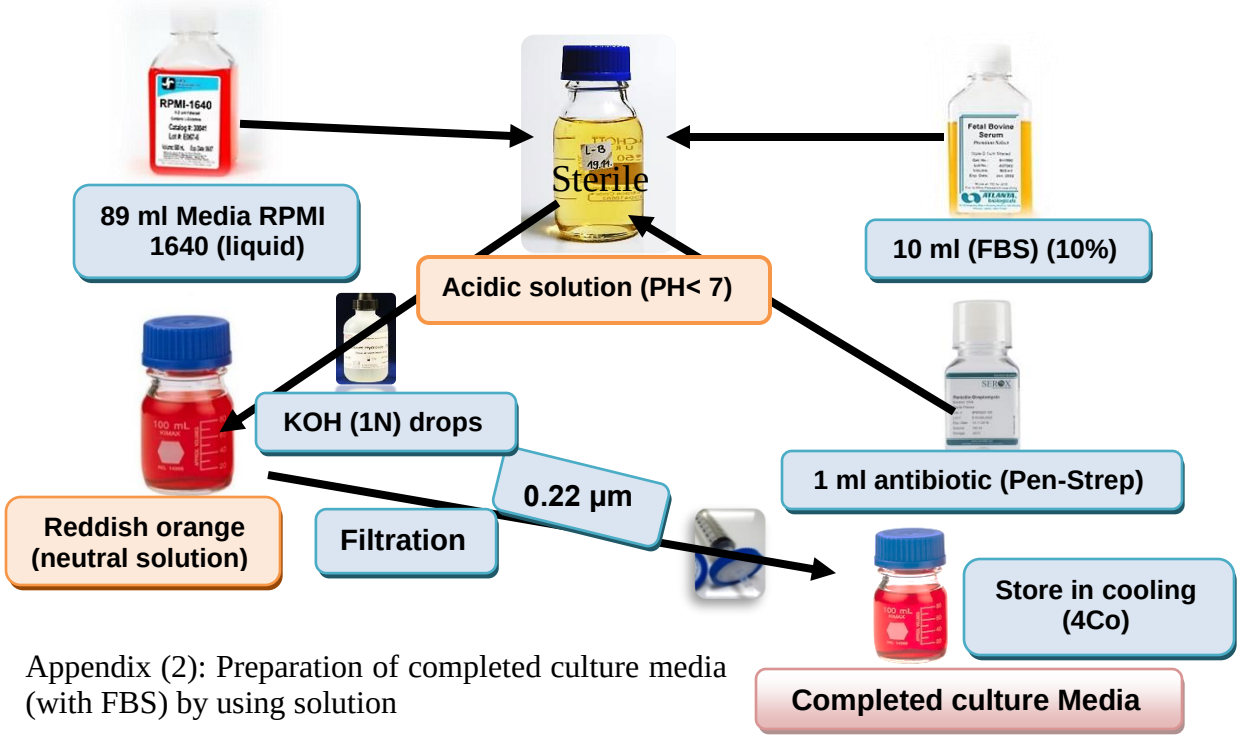
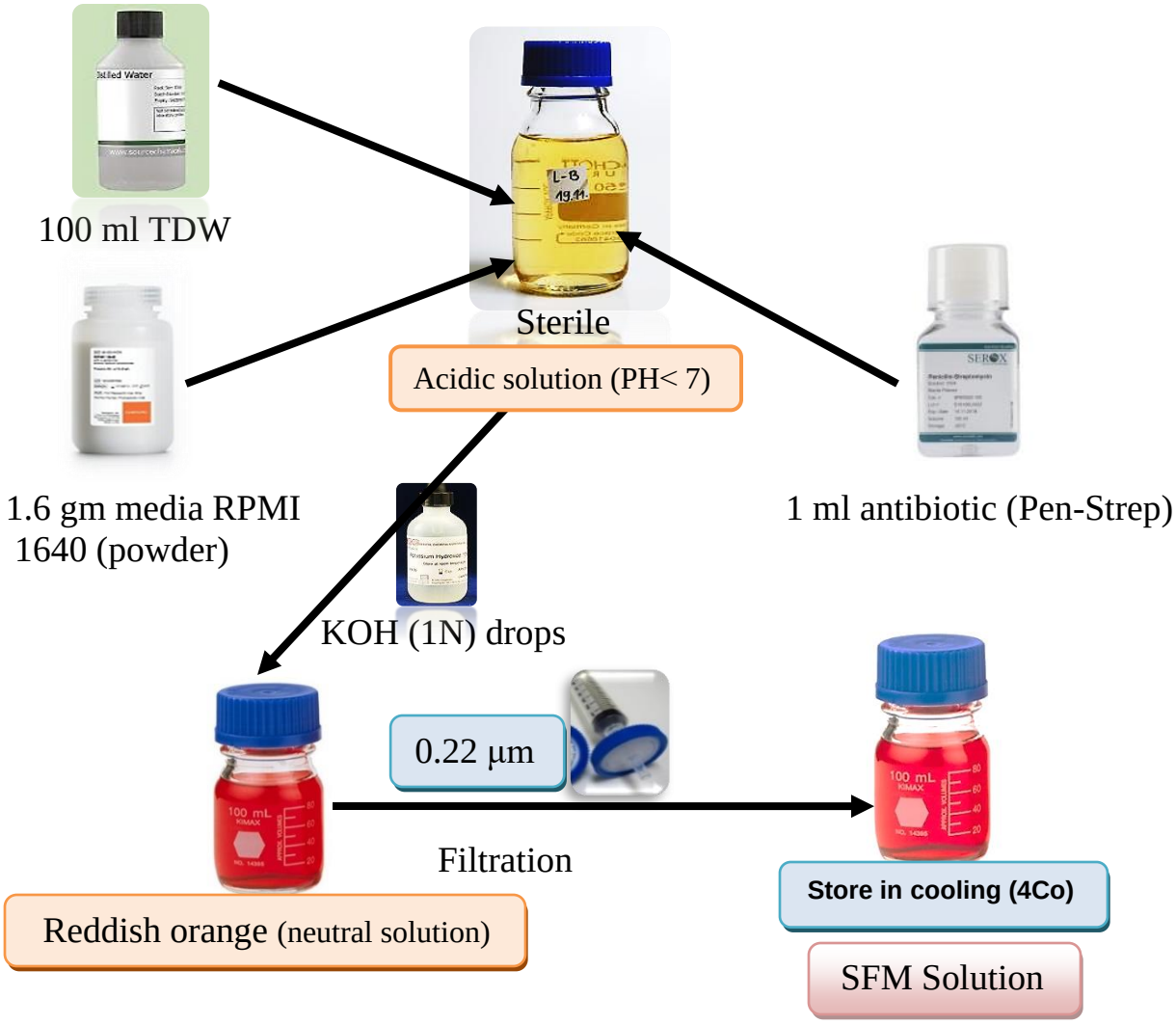

Appendix



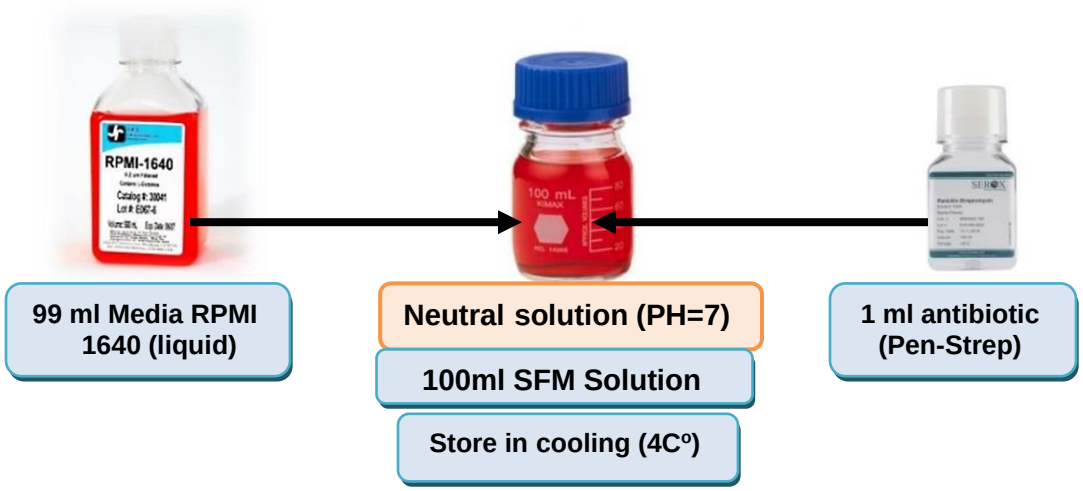
Appendix (1): Preparation of completed culture media (with FBS) by using powder



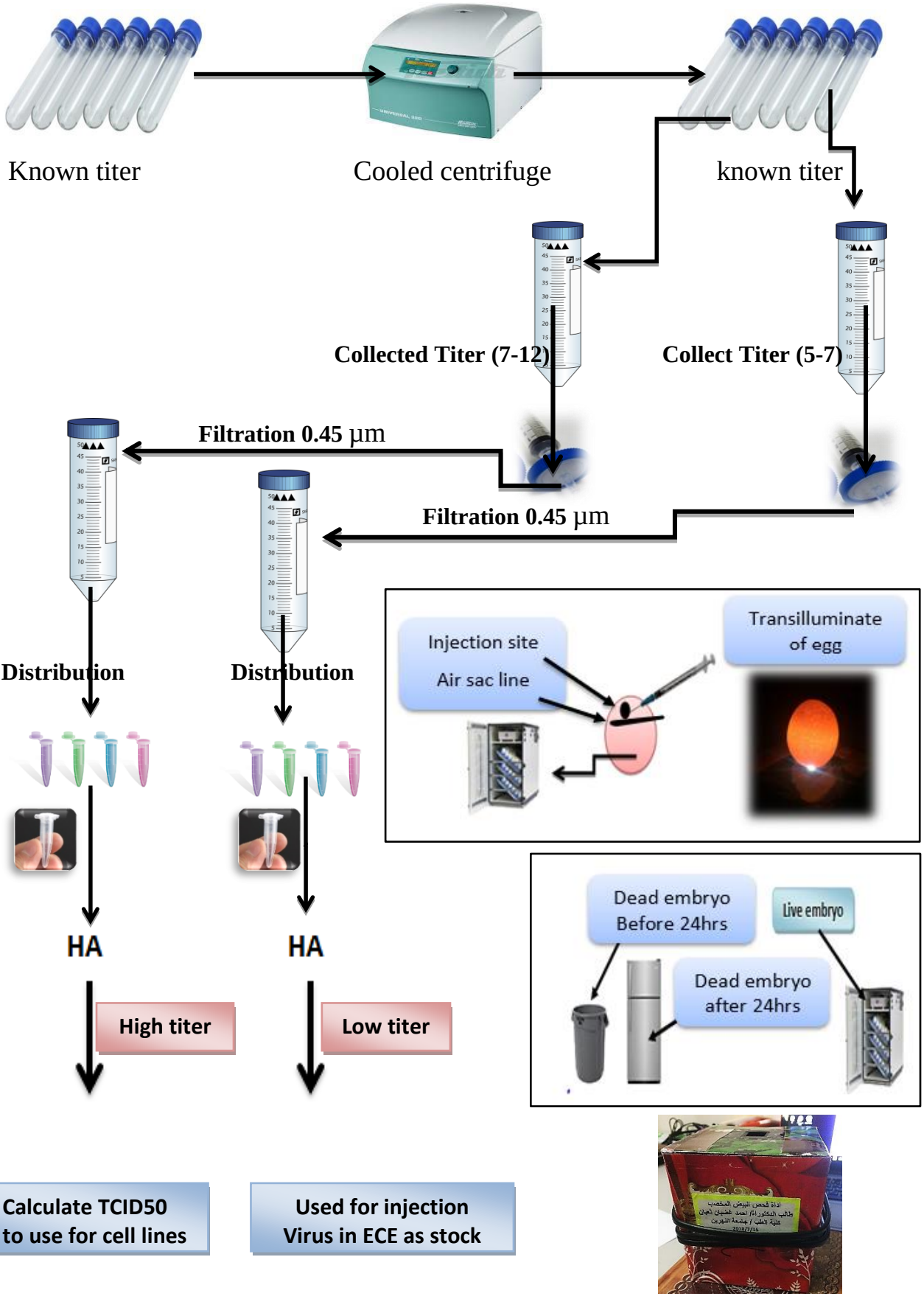
Appendix (2): Preparation of completed culture media (with FBS) by using solution



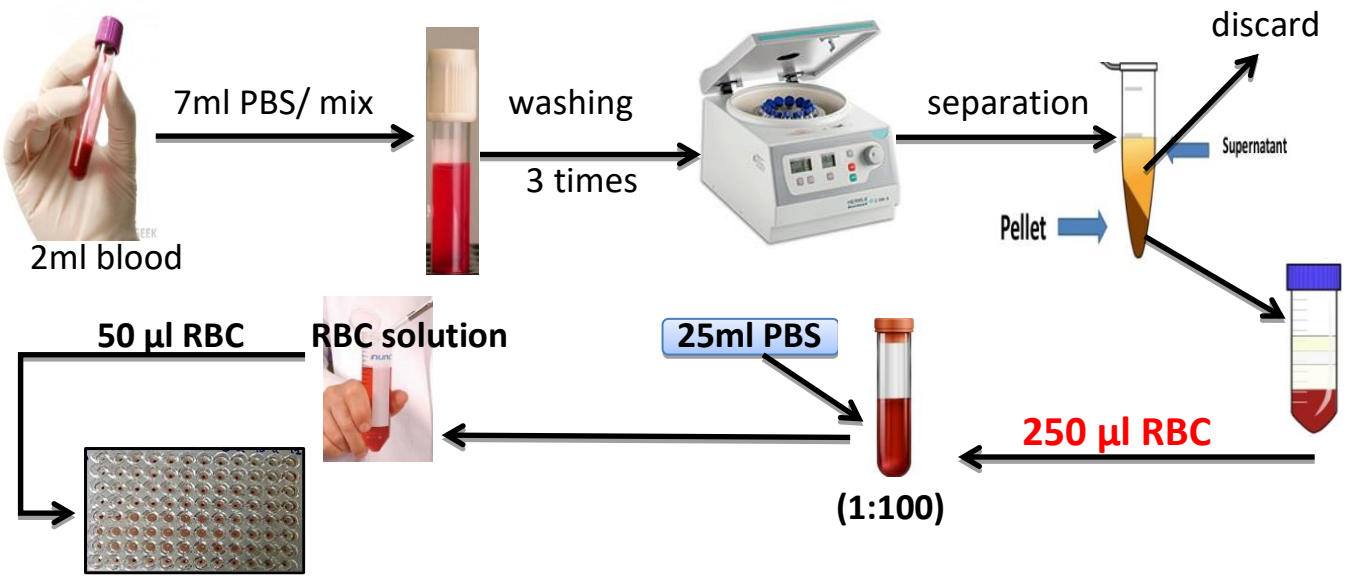
Appendix (3): Preparation of Serum Free Media (SFM) (without FBS) by using powder



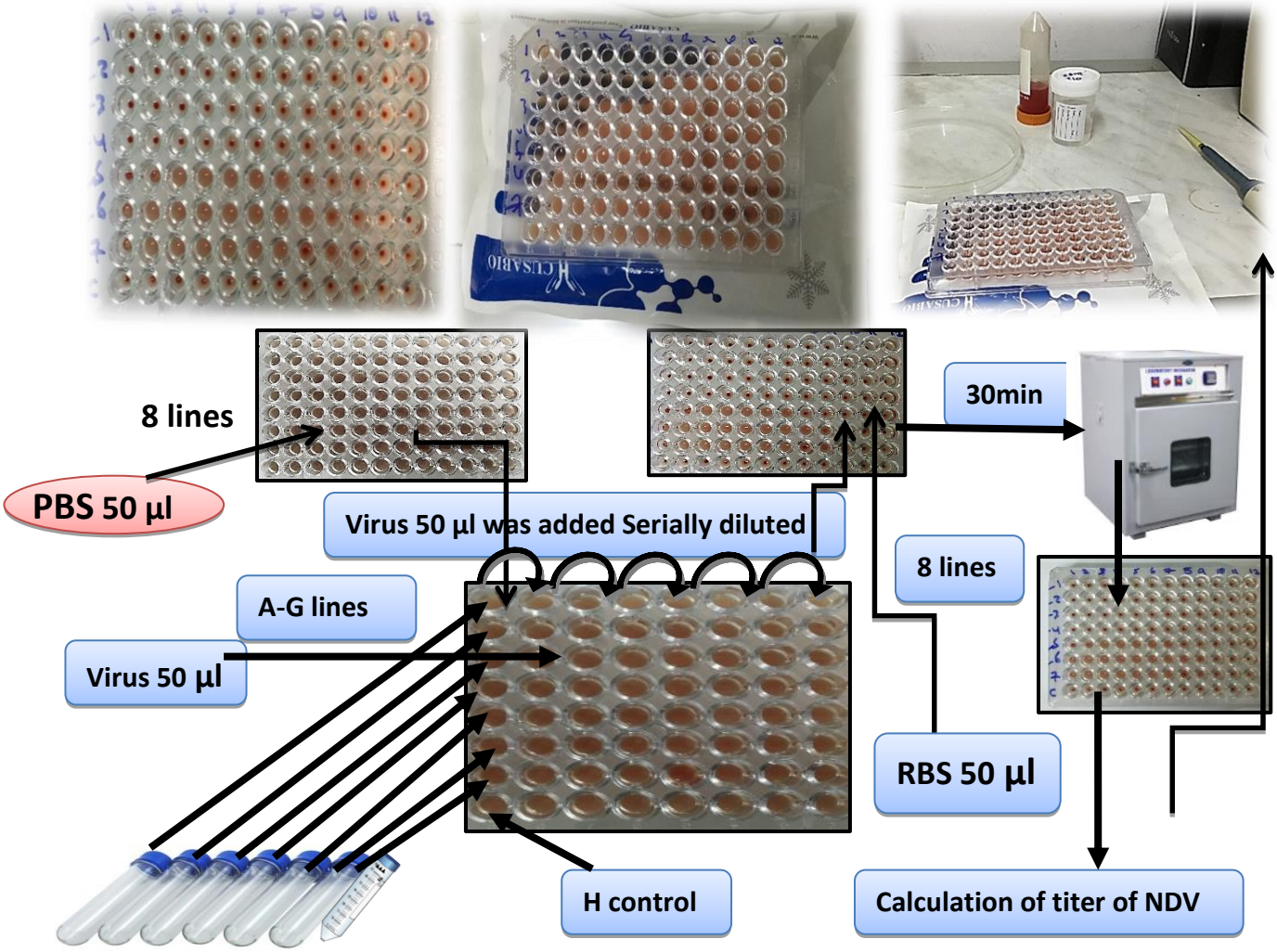
Appendix (4): Preparation of SFM (without FBS) by using solution



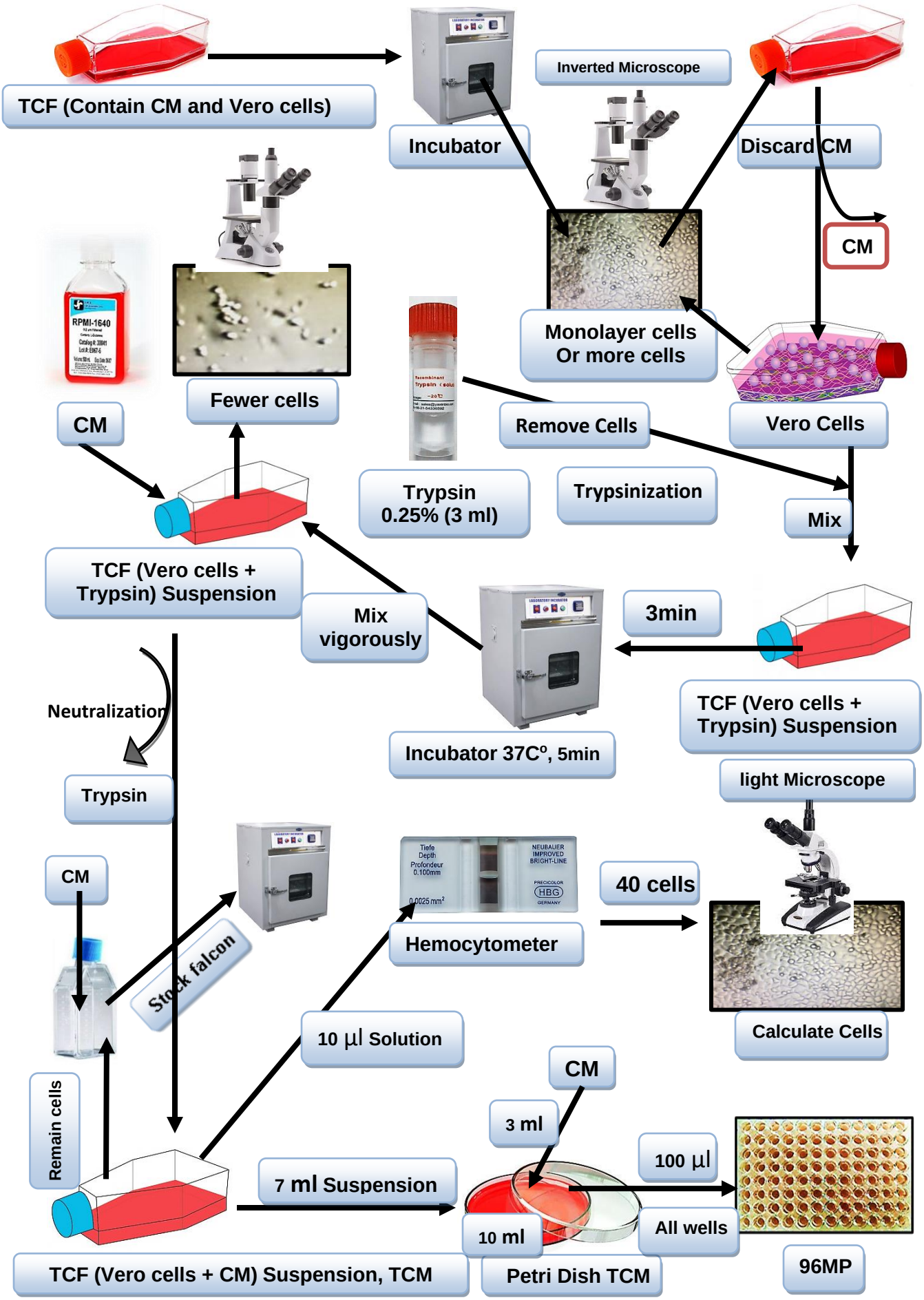
Appendix (5): Propagation of NDV in Chicken Embryo eggs: Check eggs, Injection, collection, purification, filtration, and distribution of NDV

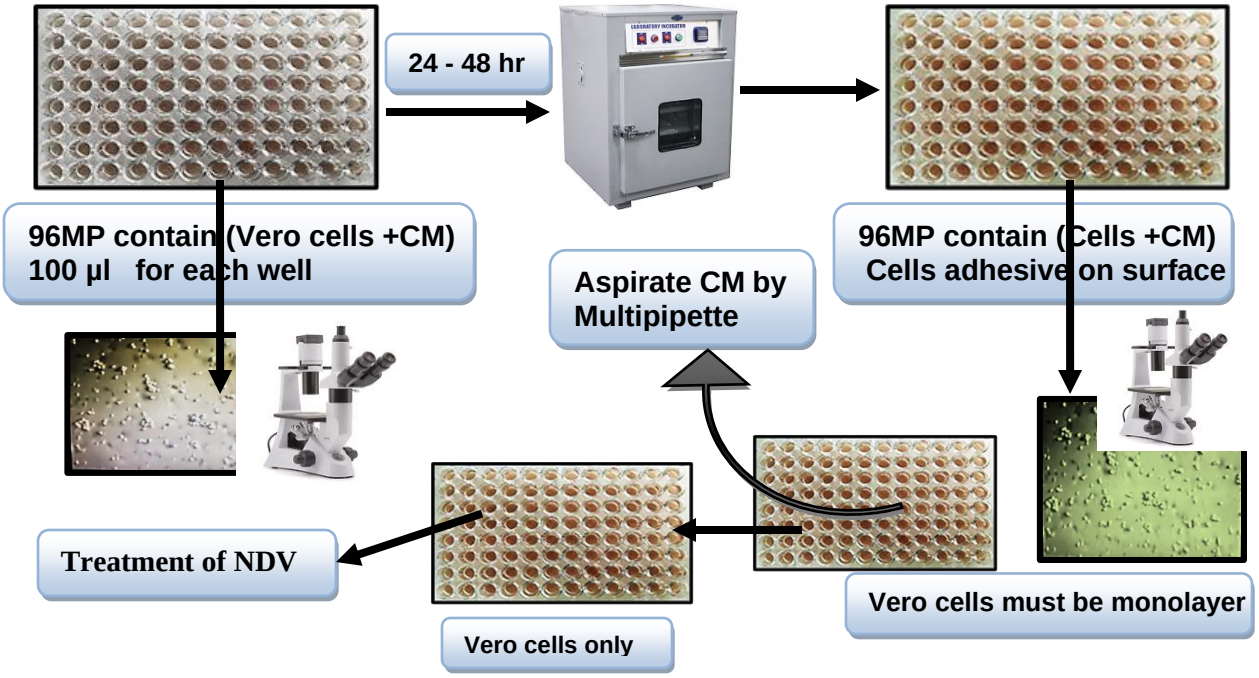


Appendix (6): Preparation of RBC Solution

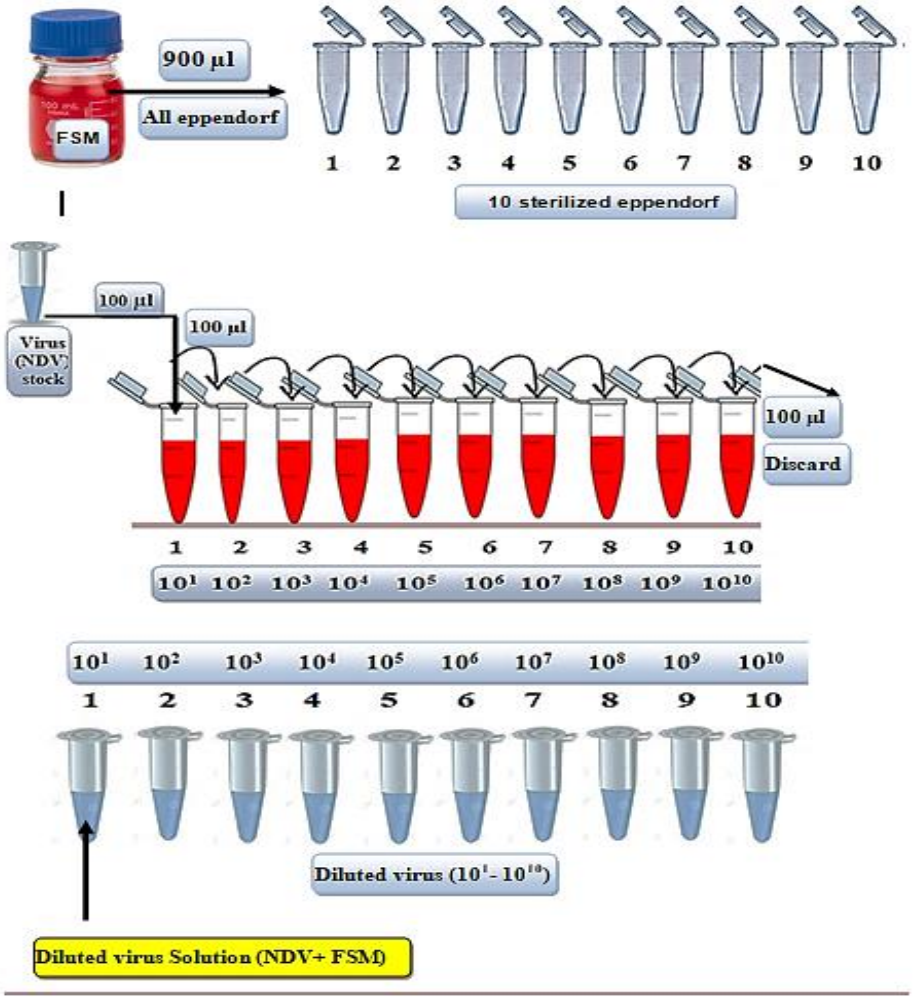


Appendix (7) Determination of Titer of NDV by Hemagglutination Test (HA)

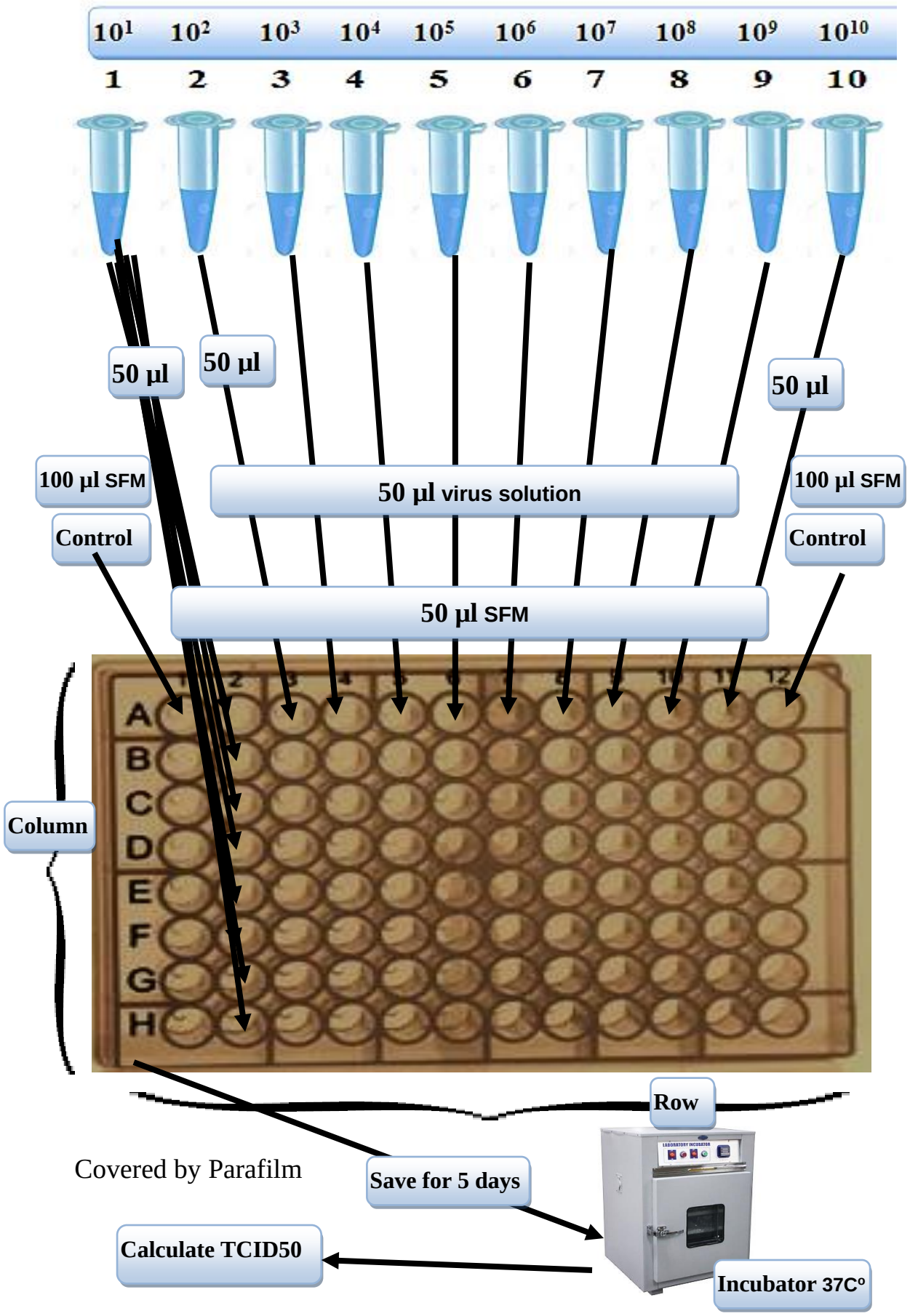

TCF (Contain CM and Vero cells)
Incubator
Inverted Microscope
Discard CM
CM
Monolayer cells
Or more cells
Vero Cells
Remove Cells
Trypsinization
Trypsin
0.25% (3 ml)
Mix
3min
TCF (Vero cells +
Trypsin) Suspension
Incubator 37C°, 5min
light Microscope
Calculate Cells
40 cells
Hemocytometer
Mix
vigorously
TCF (Vero cells +
Trypsin) Suspension
Fewer cells
CM
Neutralization
Trypsin
CM
Stock falcon
10 µl Solution
Remain cells
7 ml Suspension
TCF (Vero cells + CM) Suspension, TCM
CM
3 ml
10 ml
Petri Dish TCM
100 µl
All wells
96MP



Appendix (8): cell culture of Vero cell line

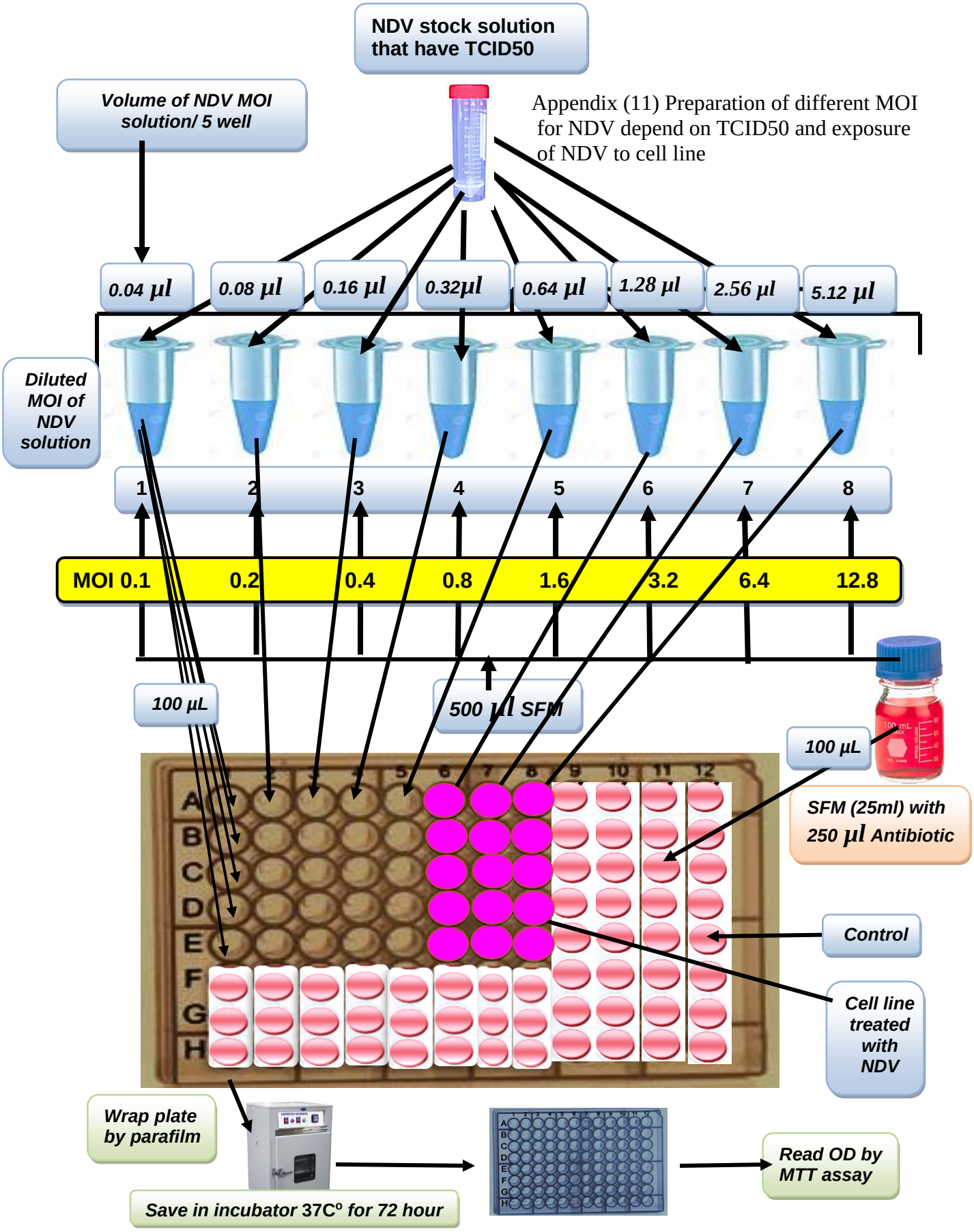


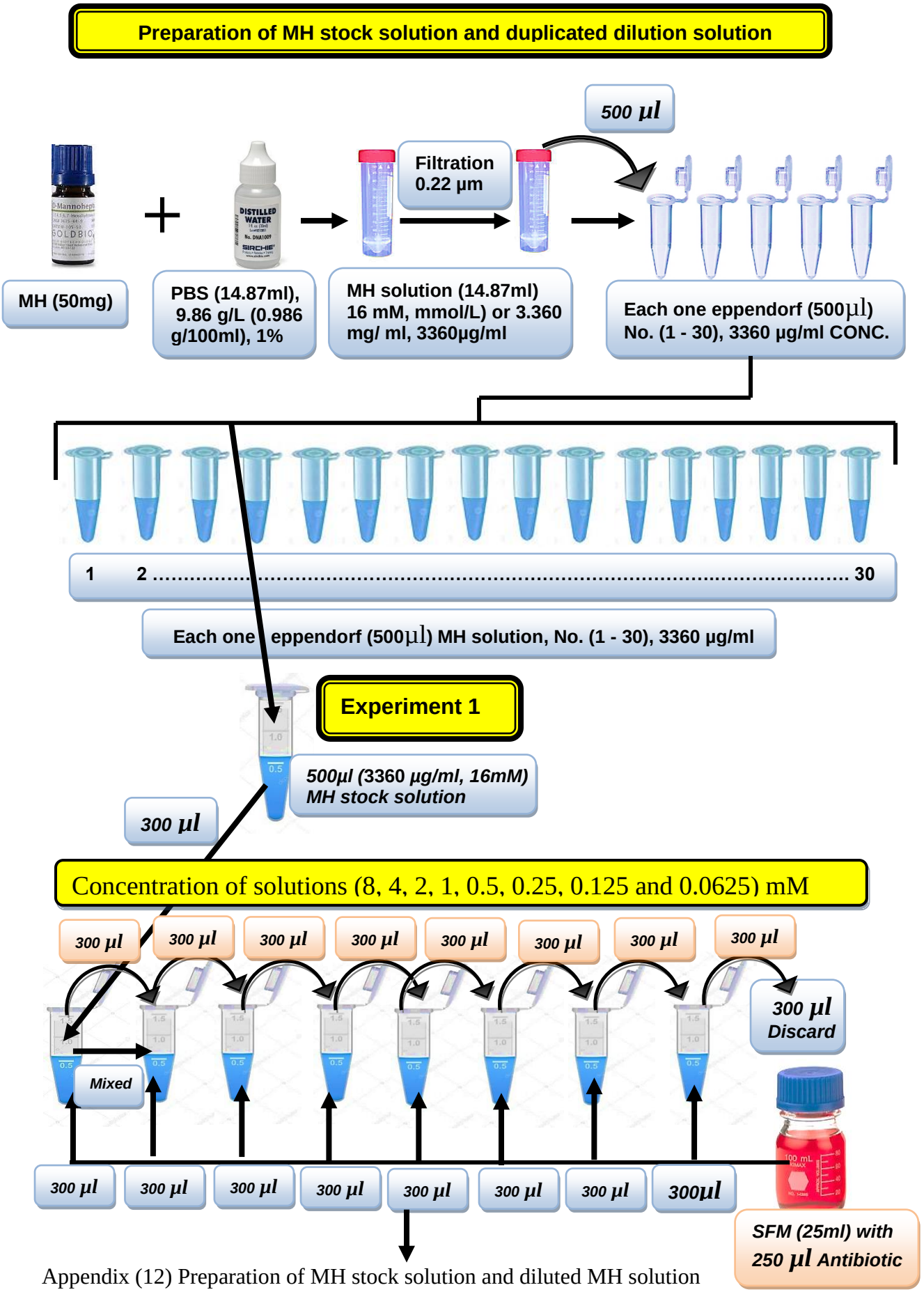
Appendix (9) Preparation of the diluted virus solution from stock virus solution



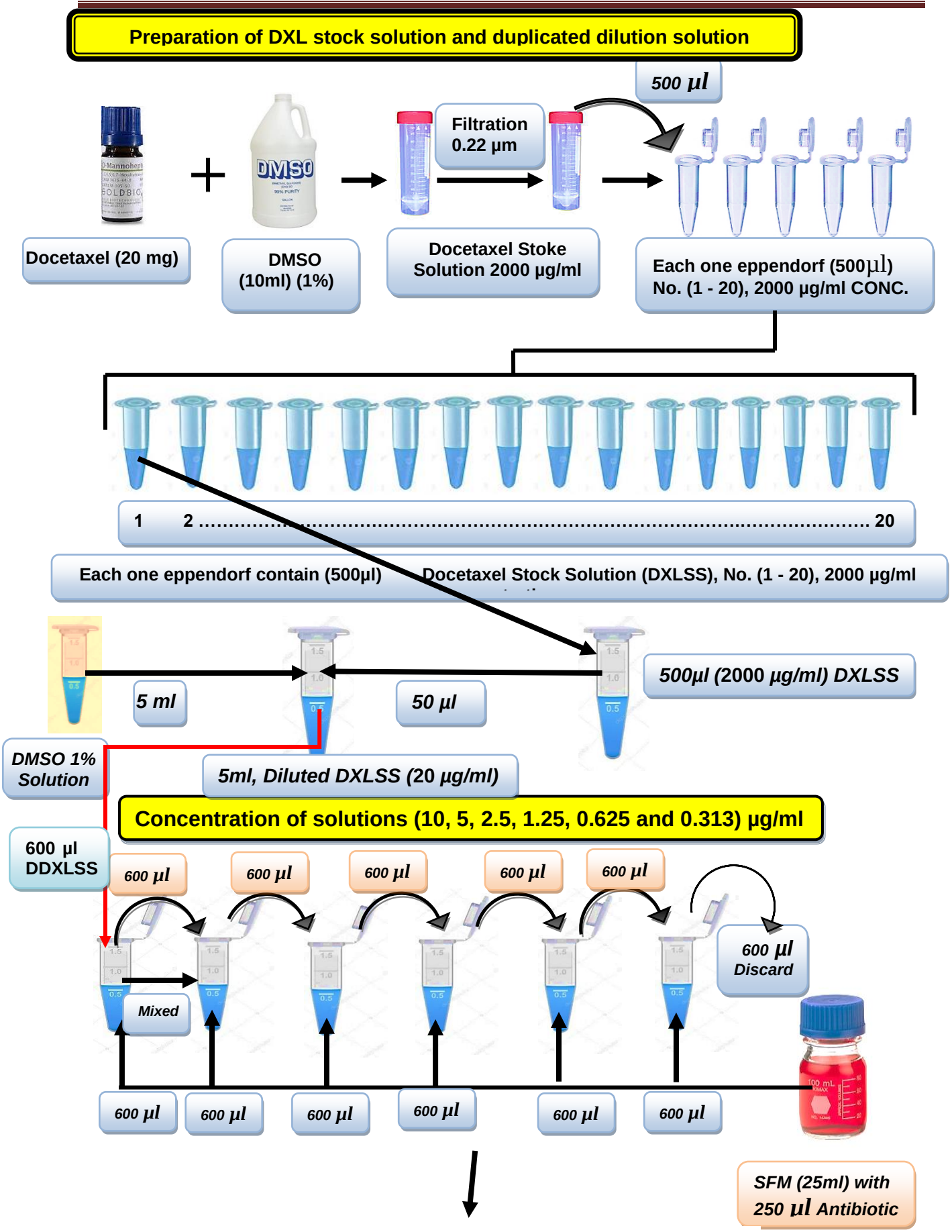
Appendix (10) Exposure NDV to Vero cell line to measure TCID50

Appendix (11) Preparation of different MOI for NDV depend on TCID50 and exposure of NDV to cell line

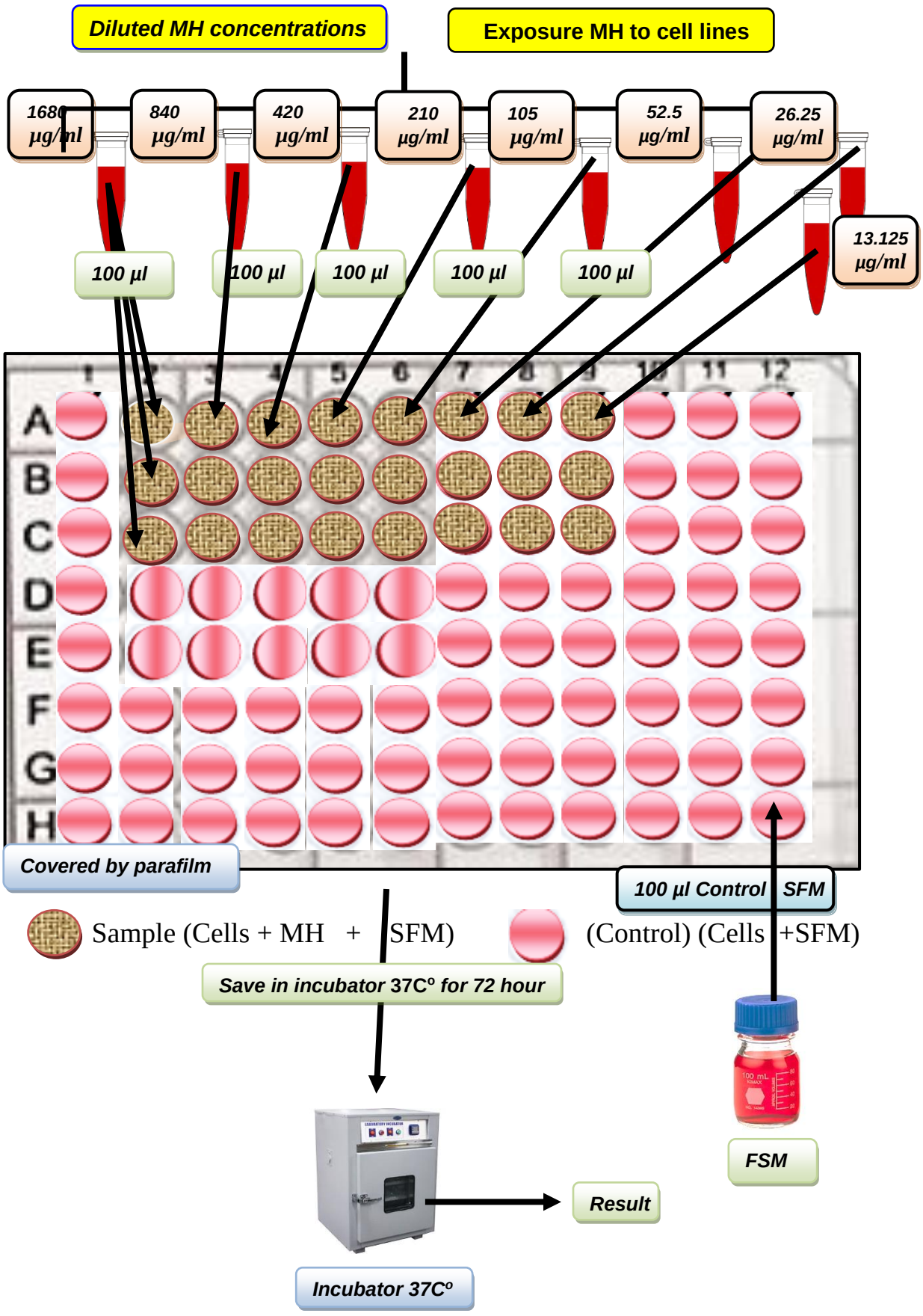




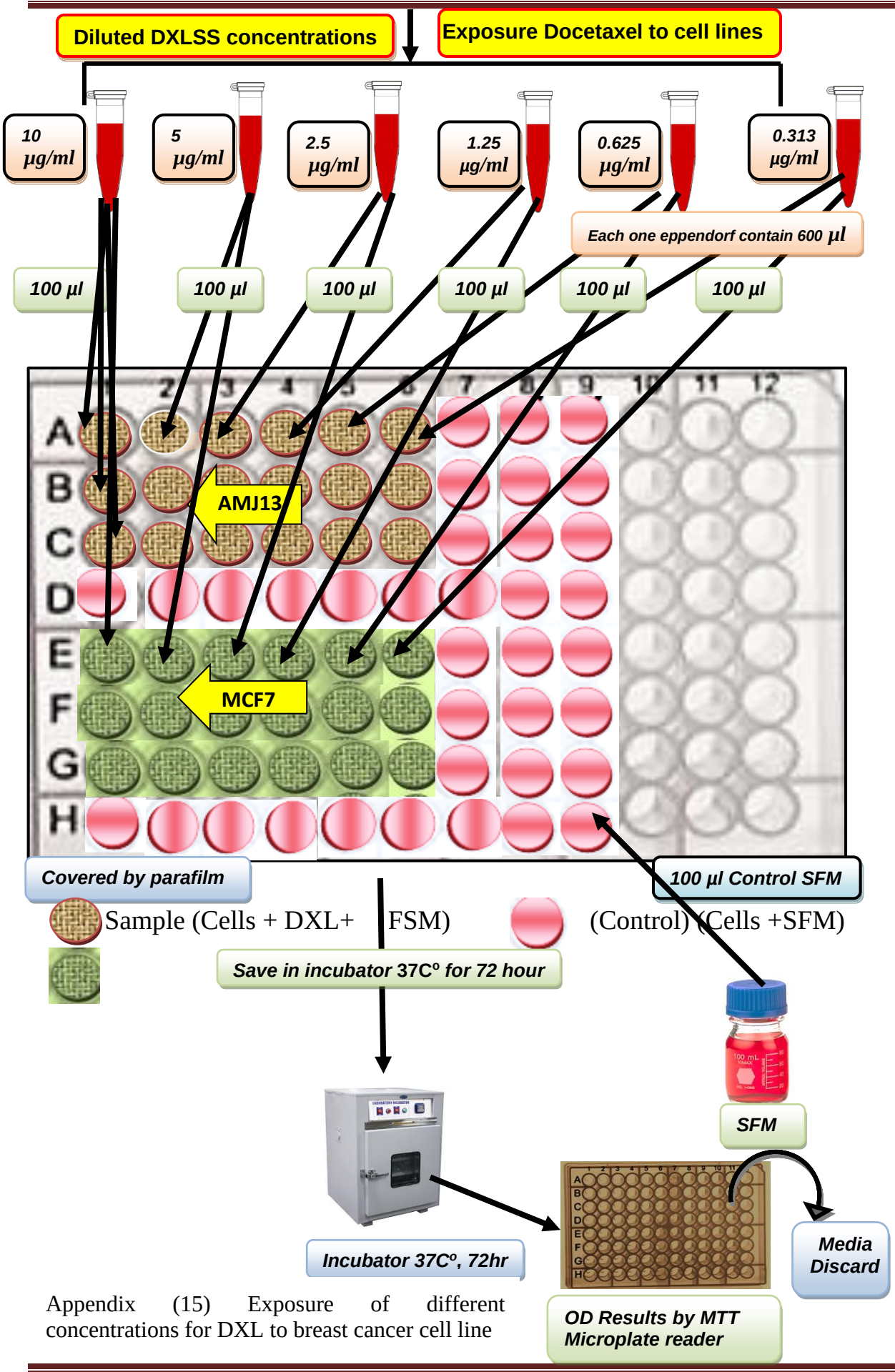
Appendix (12) Preparation of MH stock solution and diluted MH solution



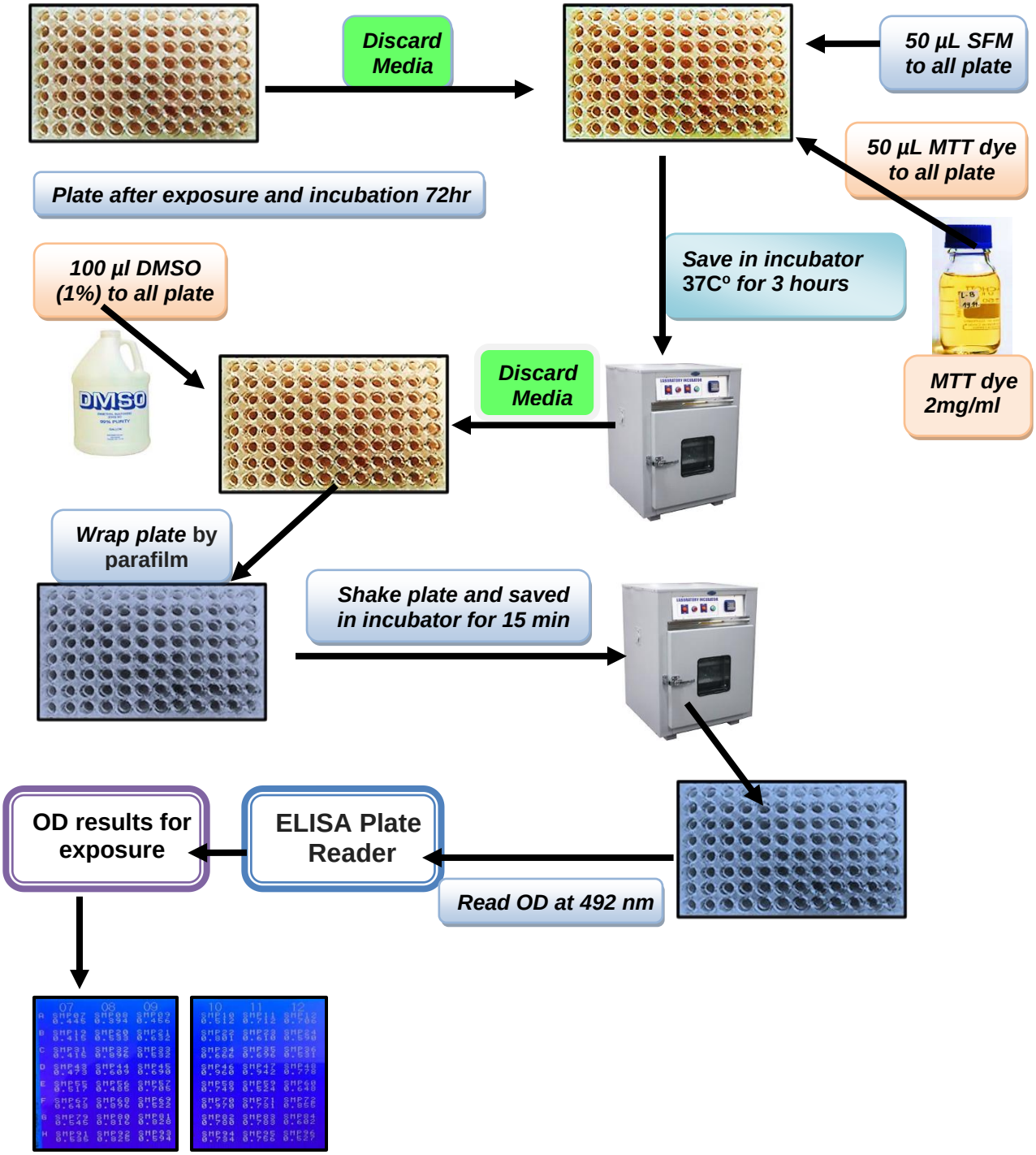
Appendix (13) Preparation of stock and diluted DXL drug solution



Appendix (14) Exposure of MH to cell lines



Appendix (15) Exposure of different concentrations for DXL to breast cancer cell line



Appendix (16) MTT Cytotoxicity Assay

Kit components

	Components	Specifications	Storage
Reagent 1	Liquid	8 mL × 1 vial	4℃, 3 months
Reagent 2	Stock solution	4 mL × 1 vial	4℃, 3 months
Preparation of Reagent 2: Dilute the Reagent 2 with double distilled water at a ratio of 1:9. Prepare the fresh solution before use.			
Reagent 3	Powder	2 vials	-20℃, 3 months
Preparation of Reagent 3: Dissolve a vial of powder with 1 mL double distilled water. Prepare the fresh solution before use.			
Reagent 4	Powder	7 vials	-20℃, 3 months
Preparation of Reagent 4: Dissolve a vial of powder with 1 mL double distilled water. Prepare the needed amount before use.			
Reagent 5	Liquid	330 µL × 1 vial	4℃, 3 months. **Do not freeze.**
Preparation of working solution: Mix the Reagent 1, Reagent 2, Reagent 3, Reagent 4, Reagent 5 and double distilled water at the ratio of 20:10:5:20:1:40 thoroughly. Prepare the fresh solution before use.			

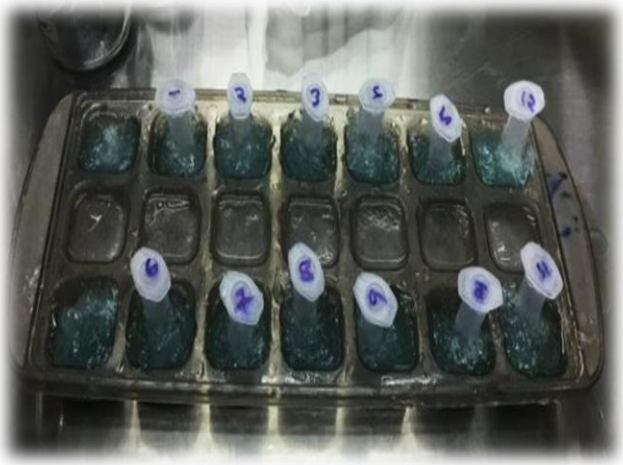
Appendix (17) HK Kit components and preparation of reagents

Appendix (18) Transillumination of eggs by candling lamp to ensure the viability of the embryo.

Kit components and preparation of reagents

Item		Specifications	Storage
Reagent 1	Substrate Ⅰ	Powder × 1 vial	Room Temperature
Substrate Ⅰ solution: Dissolve with 10 mL of boiled double distilled water before use, and stand it in boiling water bath until completely dissolved. If it appear crystal before assay, please stand it in boiling water bath to make it completely dissolved, then store at 37℃ for assay.			
Reagent 2	Substrate Ⅱ	20 mL × 1 vial	4℃
Reagent 3	Accelerant	Powder × 2 vials	-20℃
		Diluent, 760 μL x 2 vials	4℃
Reagent 3 application solution: add 1 vial of Reagent 3 diluent to 1 vial of Reagent 3 powder, dissolve fully before use.			
Reagent 4	Precipitant	5.5 mL × 1 vial	4℃
Reagent 5	Developer	A solution, 7 mL × 4 vials	4℃
		B solution, 6 mL × 4 vials	4℃
Developer application solution: add 1 vial of Reagent 5 A solution to 1 vial of Reagent 5 B solution, dissolve fully and store at 4℃. Prepare fresh solution before use.			
Reagent 6	Stop Solution	50 mL ×1 vial	Room Temperature
Reagent 7	ATP Standard	Powder × 2 vials	4℃
5 mmol/L ATP standard stock solution: before assay, add double distilled water into 1 vial of ATP standard powder to make a final volume of 1 mL. Prepare fresh solution before use.			
1 mmol/L ATP standard application solution: dilute 5 mmol/L ATP standard stock solution with double distilled water 5 times.			

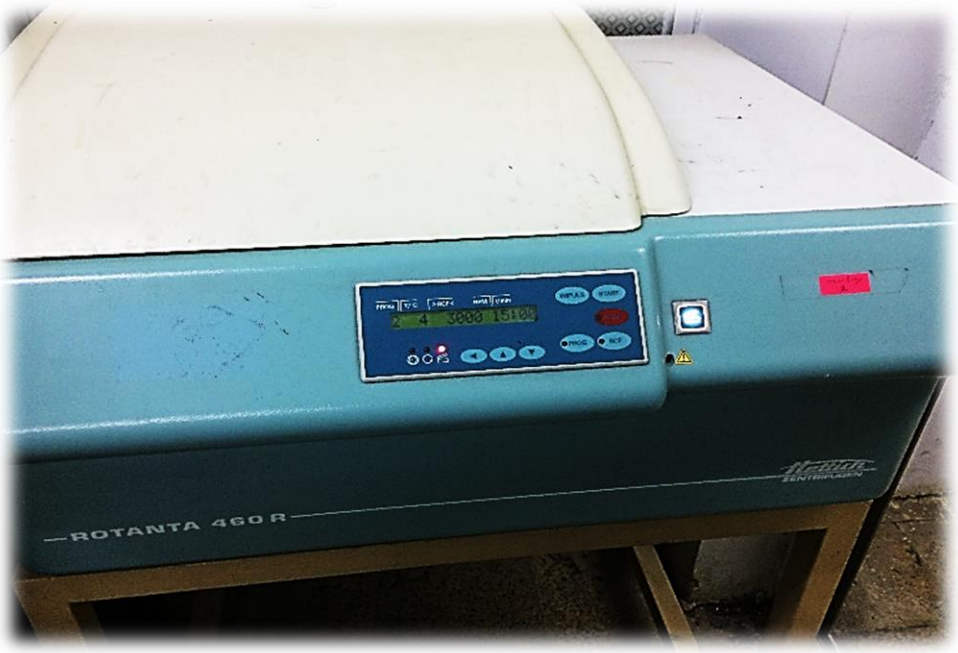
Appendix (19) ATP Kit components and preparation of reagent



Appendix (20) Save Eppendorf tubes in ice

Appendix (21) Embryonated chicken eggs (9-days-old) were incubated in a conventional incubator at 37ºC

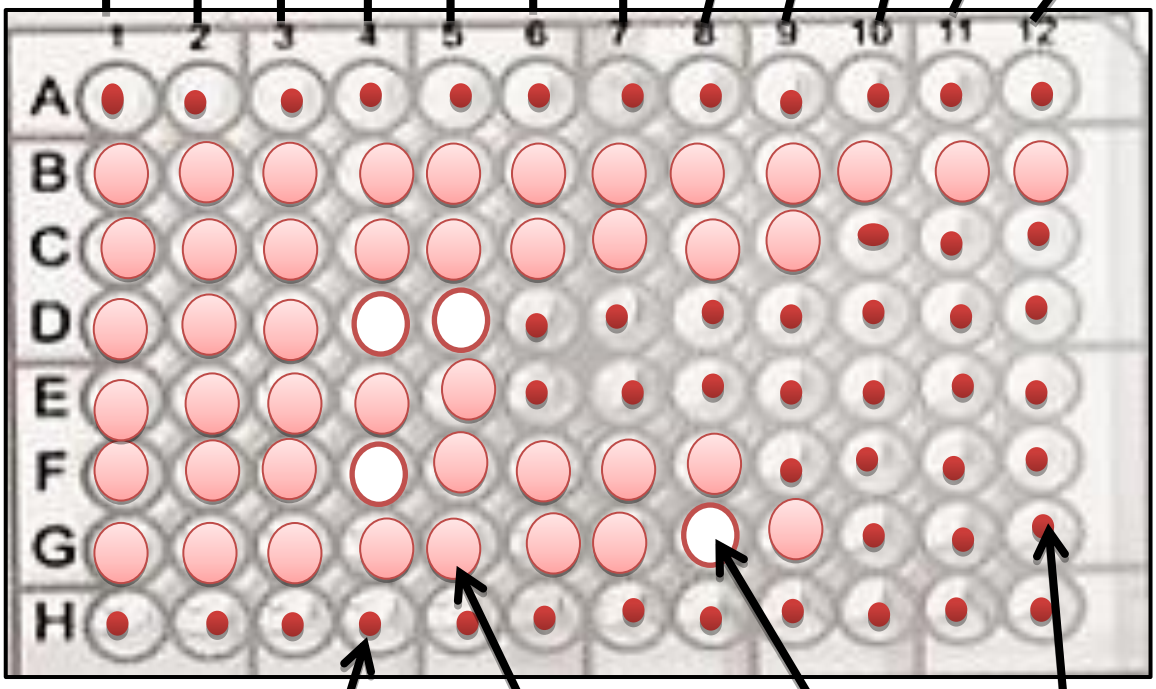
Appendix (22) Allantoic fluid of infected embryo was harvested in Eppendorf tubes and stored at -86ºC into deep freeze after calculating TCID50.



Appendix (24) Inverted and fluorescent Microscope

To calculate titer for all allantoic fluid in plate culture: (2^X) (2^1, 2^2, 2^3, 2^4….2^{12})

Titer: HAU 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096



Results:

Control

A= 0 Titer / not contain virus 0.0%

B= 12 Titer / 4096 HAU contain virus 100%

C= 9 Titer / 512 HAU contain virus 75%

D= 5 Titer / 32 HAU contain virus 41.7%

E= 5 Titer / contain virus 41.7%

F= 8 Titer / 256 HAU contain virus 66.7 %

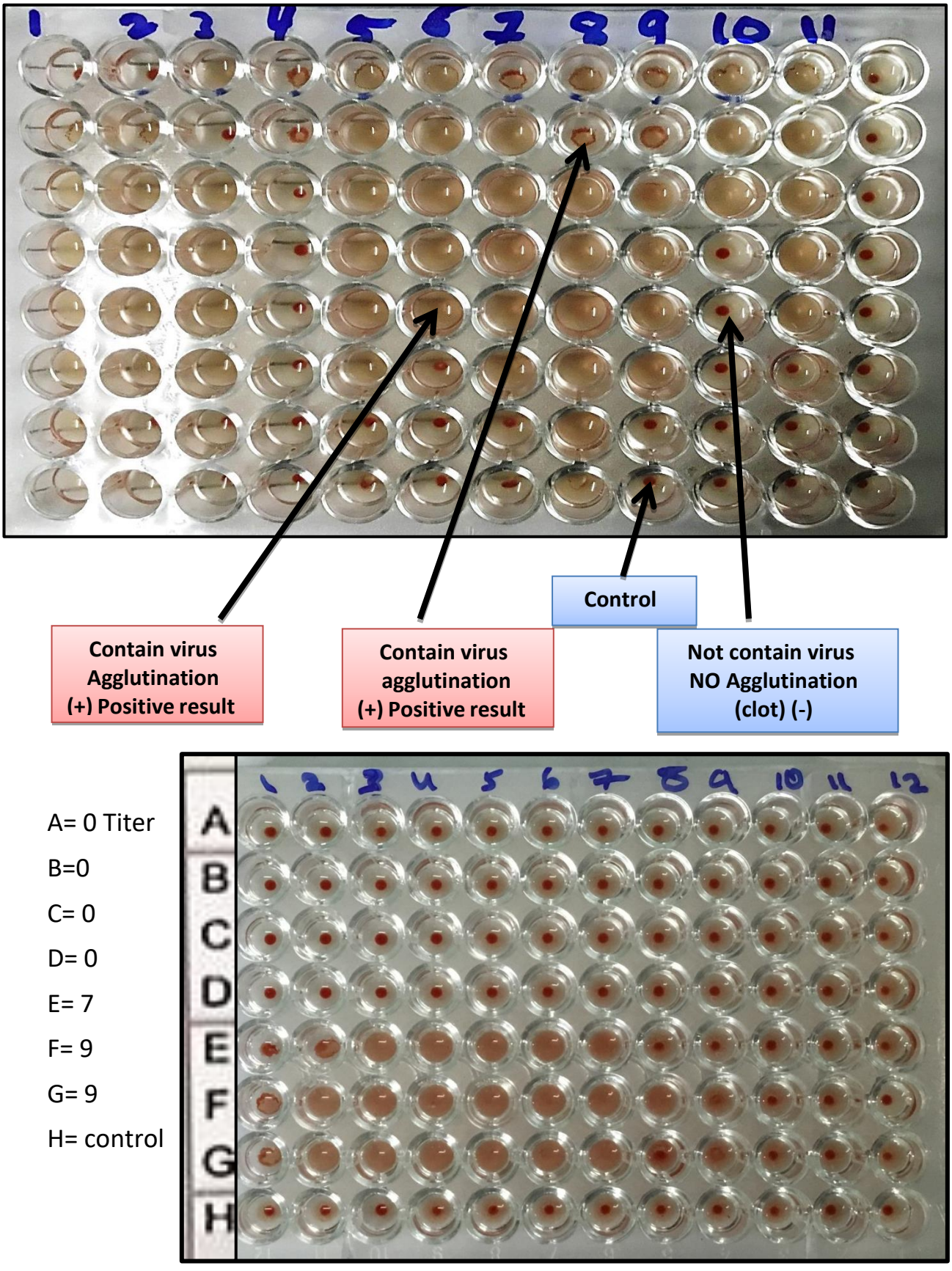
G= 9 Titer / contain virus 75%

H= Control / not contain virus

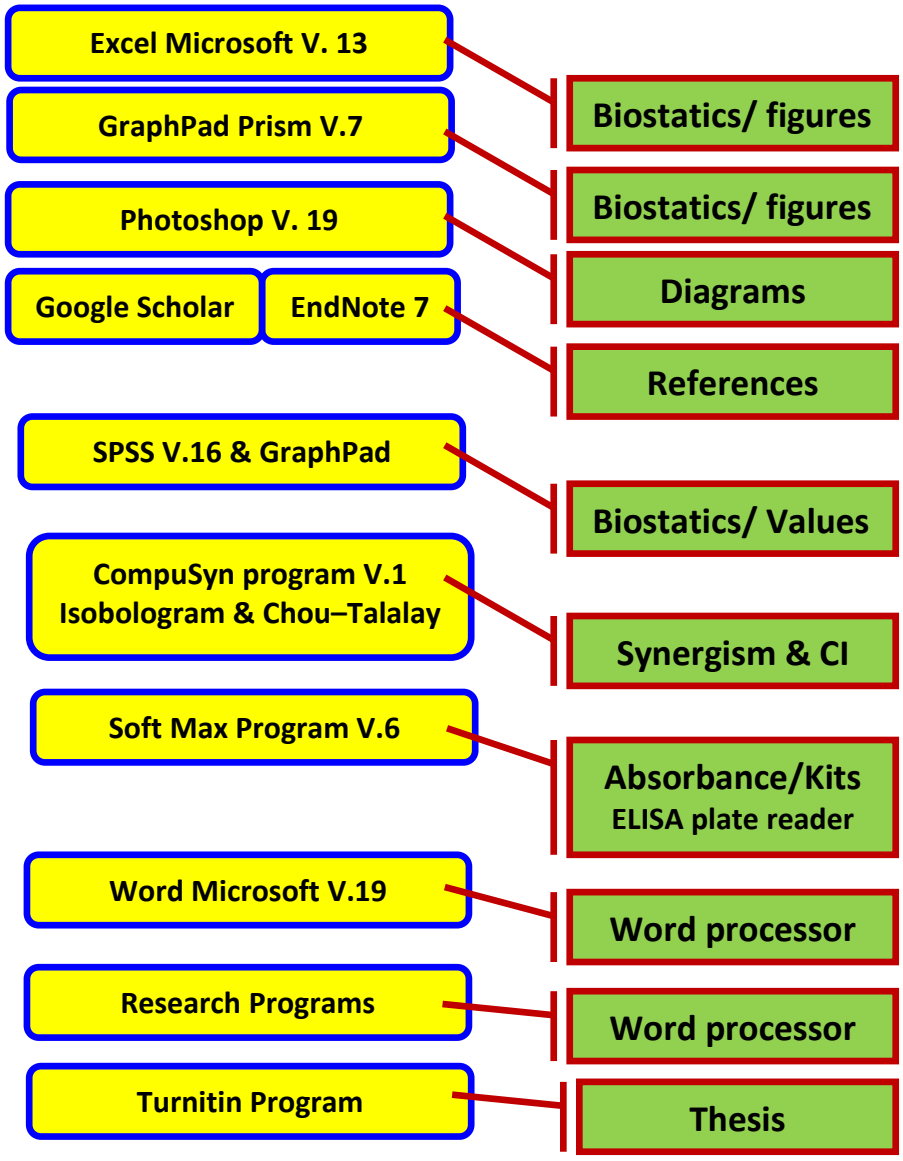
Contain virus agglutination (+) Positive result

Not contain virus NO Agglutination (clot) (-)

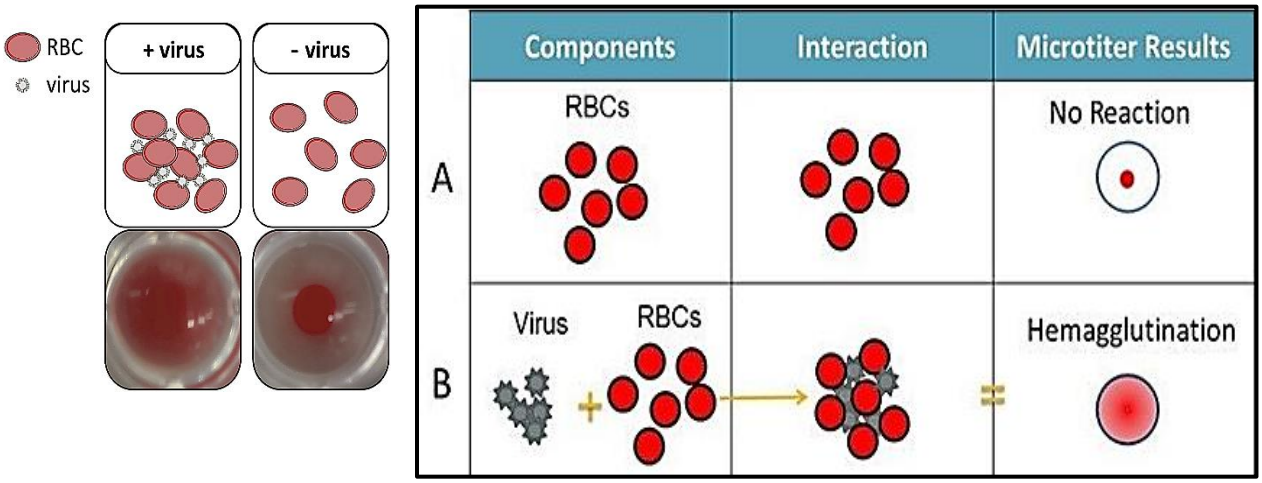
Contain virus Agglutination (+) Positive result



Appendix (25) Calculation of titer of NDV by HA test in 96-well microplate



Appendix (26): Outline of the programs were used in the study



Appendix (27): Hemagglutination (the clumping together of red blood cells) by NDV (Karakus, 2018)

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Acceptance letter

LPMA-Conference Special Issue

Received: May 9-11, 2020

Accepted: June 20, 2020

Dear Authors;

Ahmed Ghdhban Al-Ziaydi [1] **, Ahmed Majeed Al-Shammari** [2] **, Mohammed I. Hamzah** [3] **, Haider Sabah kadhim** [4] **, Majid Sakhi Jabir** [5]

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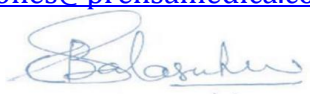
*Corresponding author, E-mail: Ahmed.Alshammari@iccmgr.org

On the behalf of La Prensa Medica Argentina, we are so glad to inform you that the manuscript, entitled '**Newcastle disease virus kills breast cancer cells by inhibiting the glycolysis pathway and inducing apoptosis**', has been accepted for publication in A conference special issue of LPMA. Please let us inform you that the special issue at the date June 20, 2020 under the rules, Ethics, strategy and protocol of the journal and the publisher.

Thank you for submitting your work to us, and once more congratulations.

Best regards,
Please do not hesitate to contact the main office
Editorial main office
ediciones@prensamedica.com.ar



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Acceptance Letter

TMREES Conference Series
Technologies and Materials for Renewable Energy, Environment and Sustainability
TMREES20-Greece Int'l Conf. Athens - Greece, June 25-27, 2020.

Sunday, May 10, 2020

Speaker: …Ahmed Majeed Al-Shammari…. / Oral-Visio /ID: #.58../SN: 859…
Authors: …Ahmed Ghdhban Al-Ziaydi, Mohammed I. Hamzah, Ahmed Majeed Al-Shammari, Haider Sabah Kadhim and Majid Jabir……..
Paper Title: …The Anti-proliferative Activity of D- Mannoheptulose against Breast Cancer Cell line through Glycolysis Inhibition…….
Country: …Iraq……..// **Affiliation:** …Iraqi Center for Cancer and Medical Genetic Research, Mustansiriyah University…….

Publishers: Accepted for publication in a special volume with the American Institute of Physics (APC)
Indexed: Scopus, Web of Science (WOS), International Scientific Indexing (ISI)
Metrics: H-Index: 60, ISSN: 0094-243X, E-ISSN: 1551-7616

Dear **Ahmed Majeed Al-Shammari**,

We are pleased to inform you that your submission has been reviewed and received a positive recommendation by the Program Committee.
Your paper has been Accepted for publication in a special volume with the American Institute of Physics (APC)!
And thank you for your interest in the Tmrees20-Greece International Conference (www.tmrees.org).

On behalf of the Conference Chairs Committee, we would like to formally invite you to participate the Tmrees20-Greece International Conference on Technologies and Materials for Renewable Energy, Environment and Sustainability to present your paper by **Remote Video Presentation**.

We would like to inform you that presentations will be realized, thus you are kindly invited to register in advance to book a slot for your presentation:
https://zoom.us/meeting/register/tJMqd-muqzItH9LSDsO5xIDx1T7WOb3C-TC7
Meeting ID: 977 0065 9756
Password: 002020
Date Time: Jun 25, 26 and 27, 2020, 10:00 AM to 06:00 PM (Athens time)

We look forward to meeting you in **TMREES20-Greece Int'l Conf. Athens - Greece, June 25-27, 2020.**

Best Regards,
On behalf of the General Chairs
Prof. Panagiotis Papageorgas
Department of Electrical
And Electronics Engineering
University of West Attica
Athens, Greece

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College of Science, University of Al-Qadisiyah

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Date: **8/06/2020**

Acceptance Letter

We are pleased to inform you that your manuscript entitled

Propagation of Newcastle Disease Virus in Embryonated Chicken Eggs and its Research Applications in Cell lines

has been accepted for online publication in the 1st International Virtual Conference on Pure Science that will held in College of Science, University of Al-Qadisiyah, Iraq, at the IOP publishing, Journal of Physics: Conference Series, June, 2020.

Authors and Affiliation Details:

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[2]*Ahmed M. Al-Shammari*, [2]*Iraqi Center of Cancer and Medical Genetics Research (ICCMGR) Al-Mustansiria University, Iraq*

[3]*Mohammed I. Hamzah*, [3]*College of Medicine, University of Al-Nahrain, Iraq.*

Thank you for your contribution to 1st International Virtual Conference on Pure Science

Sincerely

Assist Prof. Dr. Salwan Ali Abed
Conference Chairman

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ORIGINAL ARTICLE

Newcastle disease virus suppress glycolysis pathway and induce breast cancer cells death

Ahmed Ghdhban Al-Ziaydi[1] · Ahmed Majeed Al-Shammari[2] · Mohammed I. Hamzah[3] · Haider Sabah Kadhim[4] · Majid Sakhi Jabir[5]

Received: 17 January 2020 / Accepted: 18 June 2020

Abstract Newcastle disease virus (NDV) can modulate cancer cell signaling pathway and induce apoptosis in cancer cells. Cancer cells increase their glycolysis rates to meet the energy demands for their survival and generate ATP as the primary energy source for cell growth and proliferation. Interfering the glycolysis pathway may be a valuable antitumor strategy. This study aimed to assess the effect of NDV on the glycolysis pathway in infected breast cancer cells. Oncolytic NDV attenuated AMHA1 strain was used in this study. AMJ13 and MCF7 breast cancer cell lines and a normal embryonic REF cell line were infected with NDV with different multiplicity of infections (moi) to determine the IC50 of NDV through MTT assay. Crystal violet staining was done to study the morphological changes. NDV apoptosis induction was assessed using AO/PI assay. NDV interference with the glycolysis pathway was examined through measuring hexokinase (HK) activity, pyruvate, and ATP concentrations, and pH levels in NDV infected and non-infected breast cancer cells and in normal embryonic cells. The results showed that NDV replicates efficiently in cancer cells and spare normal cells and induce morphological changes and apoptosis in breast cancer cells but not in normal cells. NDV infected cancer cells showed decreased in the HK activity, pyruvate and ATP concentrations, and acidity, which reflect a significant decrease in the glycolysis activity of the NDV infected tumor cells. No effects on the normal cells were observed. In conclusion, oncolytic NDV ability to reduce glycolysis pathway activity in cancer cells can be an exciting module to improve antitumor therapeutics.

Keywords Warburg effect · Pyruvate · Virotherapy · Cancer metabolism · Oncolytics

Introduction

Newcastle disease virus (NDV) is belonging to the genus Avulavirus of the family Paramyxoviridae. Viruses from this family are enveloped, nonsegmented, negative-sense RNA viruses that cause the inflammation of the respiratory and gastrointestinal tracts in a wide variety of poultry species [28]. The virulent NDV strain still inducing outbreaks in different countries, including Iraq, leading to severe economic damages in the poultry industry [11]; yet, attenuated and lentogenic NDV has promising antitumor activity and excellent safety in laboratory animals [26]. The Iraqi NDV strain AMHA1 is an oncolytic virus that is attenuated strain isolated originally from an outbreak [7]. The NDV induces apoptosis in cancer cells through both intrinsic and extrinsic pathways [8], causing DNA fragmentation and FAS ligand expression [13]. Furthermore, AMHA1 NDV strain found to induce apoptosis in cancer cells in caspase-dependent and independent pathways [29]. Oncolytic NDV strain found to interfere with biological

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Published online: 27 June 2020

الخلاصة

حديثا، كانت هناك عدة طرق جديدة تهدف إلى علاج السرطان، وسرطان الثدي على وجه الخصوص، من خلال الحد من الآثار الجانبية للعلاج التقليدي، وغيرها من الأساليب الجديدة مثل العلاجات المستهدفة ، العلاج الفيروسي ومجموعة من هذه العلاجات الجديدة. يقلل علاج التآزر من الآثار الجانبية والسمية، ويتغلب على مقاومة الخلايا السرطانية للعلاج.

تظهر معظم الخلايا السرطانية زيادة انحلال السكر حيث تستخدم هذا المسار الأيضي لتوليد الأدينوسين ثلاثي الفوسفات لنمو الخلايا والانتشار. يوضح تأثير واربورغ أن تثبيط انحلال السكر قد يكون ذا قيمة علاجية في العلاج المضاد للأورام. يمكن لفيروس مرض نيوكاسل (السلالة العراقية) ومثبطات الإنزيم أن تعدل التمايز وتحفز موت الخلايا المبرمج في خطوط سرطان الثدي. يعمل الـ د- مانوهيبتولوز بايلوجياً عن طريق تثبيط انحلال السكر، وبالتالي يثبط نمو الورم.

الهدف: لتقييم الآثار التآزرية لفايروس مرض نيوكاسل كعلاج فيروسي ود- مانوهيبتولوز كمثبط للهيكسوكاينيز في تثبيط مسار انحلال السكر وبالتالي التمثيل الغذائي، كذلك لمنع التكاثر وتحفيز موت الخلايا المبرمج في خطوط خلايا سرطان الثدي بالمقارنة مع خطوط الخلايا اللمفية في جنين الفأر الطبيعي.

الطريقة: يتم تكاثر فايروس مرض نيوكاسل في بيض الدجاج الجنيني ويقدر كميا باستخدام اختبار التراص الدموي ومتوسط الجرعة المصابة بالزراعة النسيجية على خط خلايا فيرو. تم استخدام خط سرطان الثدي البشري أحمد مرتضى جابرية ٢٠١٣، ومؤسسة ميشيغان للسرطان -٧، وخط الخلايا الليفية الجنينية الطبيعية للفئران. ثم تم قياس نسبة السمية الخلوية للفيروس والمثبط ضد خطوط أحمد مرتضى جابريا ٢٠١٣ ، ومؤسسة ميشيغان للسرطان ٧ ، والخلايا الليفية الجنينية للفئران لمدة ٧٢ ساعة باستخدام فحص ميثيل ثيازوليل تترازوليوم، لتحديد نصف اقصى تركيز للمثبط عند إصابات متعددة مختلفة تتراوح بين (٠,١-١٢,٨) وتراكيز مخففة تتراوح بين (١٣,١٢٥-١٦٨٠ ميكروغرام / مل) للفيروس والمثبط ، على التوالي ، تم استخدام نصف اقصى تركيز للمثبط لدراسة الجمع او التأزر بينهما، باستخدام برنامج كومبوساين ، تم قياس مؤشر الاقتران لتحديد الجرعات الفعالة للفيروس والمثبط. بعدها تم قياس التغيرات الشكلية / باستخدام صبغة الكريستال البنفسجية، وموت الخلايا المبرمج باستخدام مزيج من صبغة الأكريدين البرتقالية / يوديد البروبيديوم، وفعالية الهيكسوكاينيز، وتركيز البيروفات، وتركيز الأدينوسين ثلاثي الفوسفات، والرقم

الهيدروجيني (الذي يعبر عن الحموضة) في سرطان الثدي المعالج وغير المعالج وخطوط الخلايا الطبيعية، لفحص التأثير على مسار التحلل السكري.

النتائج: كشفت هذه الدراسة أن الفايروس والمثبط بشكل فردي يثبط الانتشار والنمو، ويخفض قابلية الحياة والنمو للخلية، ويحث على التغيرات الشكلية وموت الخلايا المبرمج في خطوط الخلايا السرطانية للثدي. من خلال تثبيط تحلل السكر وتحريض موت الخلايا المبرمج، لم تكن هناك نسب ملحوظة من السمية الخلوية للـ د- مانوهيبتولوز ضد الخلايا الليفية الجنينية للفئران حيث تراوحت نسبة السمية الخلوية من (١,٦٧٪ إلى ٢٤,٧٢٪) عند التراكيز العالية. بينما كان هناك ارتفاع في السمية الخلوية ضد خلايا سرطان الثدي تراوحت بين (٢٧,٢٩٪ إلى ٥٨,٦٤٪) لخط خلايا أحمد مرتضى جابرية ٢٠١٣. و (٢٦,٢٦٪ إلى ٦٠,٤٩٪) لخط خلايا مؤسسة ميشيغان للسرطان -٧ بعد علاج المثبط. لم يسبب الفيروس تأثيرًا سامًا للخلايا على خط الخلايا الطبيعي حيث تراوحت نسبة السمية الخلوية من (٤,٣١٪ إلى ٢٨,٧٥٪) للخلايا الليفية الجنينية للفئران. كانت خلايا سرطان الثدي أكثر حساسية للفيروس حيث تراوحت نسبة السمية الخلوية من (٢٤,٦٩٪ إلى ٦٤,٢٦٪) لخط خلايا أحمد مرتضى جابرية ٢٠١٣. و (٢٣,٩٥٪ إلى ٦٢,٠٢٪) لخط خلايا مؤسسة ميشيغان للسرطان -٧ بعد علاج الفيروس.

لوحظ تثبيط تحلل السكر من خلال انخفاض فعالية الهيكسوكاينيز من (٣,٢ وحدة / مل كسيطرة إلى ٢,٠ وحدة / مل مع فيروس و ١,٤ وحدة / مل مع مثبط و ١,١ وحدة / مل مع الجمع بينهما في خط خلايا أحمد مرتضى جابرية ٢٠١٣ ومن ٣,٠ وحدة / مل كسيطرة إلى ١,٩ وحدة / مل مع فيروس ، ١,٤ وحدة / مل مع مثبط و ١,٢ وحدة / مل مع الجمع بينهما في خط خلايا مؤسسة ميشيغان للسرطان -٧) ، وتركيز البيروفات من (٠,٤٨ ميكرو مول / مل كسيطرة إلى ٠,٢٨ ميكرو مول / مل بالفيروس ، ٠,٢٥ ميكرو مول / مل مع مثبط و ٠,١٤ ميكرومول / مل مع الجمع بينهما في خط خلايا أحمد مرتضى جابرية ٢٠١٣ ومن ٠,٥ ميكرومول / مل كسيطرة إلى ٠,٢٣ ميكرومول / مل مع فيروس ، ٠,٢٣ ميكرومول / مل مع مثبط و ٠,١٥ ميكرومول / مل مع الجمع بينهما في خط خلايا مؤسسة ميشيغان للسرطان -٧) وتركيز الأدينوسين ثلاثي الفوسفات بتراكيز تتراوح من (١,٢ مليمول / لتر كسيطرة إلى ٠,٥٨ مليمول / لتر مع فيروس ، ٠,٦١ مليمول / لتر مع مثبط و ٠,٣٩ مليمول / لتر مع الجمع بينهما في خط خلايا أحمد مرتضى جابرية ٢٠١٣ ومن ١,٣ مليمول / لتر كسيطرة إلى ٠,٦٥ مليمول / لتر مع فيروس ، ٠,٦٩ مليمول / لتر مع مثبط و ٠,٣٥ مليمول / لتر مع الجمع بينهما في خط خلايا مؤسسة ميشيغان للسرطان -٧. لوحظ انخفاض الحموضة من خلال زيادة الرقم الهيدروجيني من (٦,٣ كسيطرة إلى ٧,٣ مع فيروس و٧,٣ مع مثبط و٨,٢

مع الجمع بينهما في خط خلايا أحمد مرتضى جابرية ٢٠١٣ ومن ٦,٣ كسيطرة إلى ٧,٤ مع فيروس و٧,٤ مع مثبط و٨,٢ مع الجمع بينهما في خط خلايا مؤسسة ميشيغان للسرطان -٧.

كشفت نتائج العلاج المركب أن التآزر بين الفايروس والمثبط أنتج تأثير أقوى مضاد للورم من تأثير العلاج الأحادي مع أي منهما. نتج عن الجمع بينهما زيادة تثبيط الخلايا الحية، وزيادة التغيرات الشكلية الخلوية، وارتفاع تحريض موت الخلايا المبرمج في خطوط الخلايا السرطانية المعالجة عن طريق تثبيط مسار تحلل السكر، كما يتضح من خلال انخفاض فعالية الهيكسوكاينيز، وتركيز البيروفات، والأدينوسين ثلاثي الفوسفات والحموضة البيئية التي تعزى الى انخفاض تركيز اللاكتات (حامض اللاكتيك)، مقارنة مع خطوط الخلايا الطبيعية. علاوة على ذلك، كان هذا العلاج آمنًا نسبيًا في الخلايا الطبيعية.

الاستنتاج: التآزر بين الفيروس والمثبط يزيد من السمية الخلوية ويسبب تثبيط نمو كبير للخلايا عن طريق تثبيط تحلل السكر وتحفيز موت الخلايا المبرمج في خلايا سرطان الثدي ولكن ليس في الخلايا الطبيعية في المختبر. كما أنه أفضل من العلاج الفردي. تم تحديد انخفاض كبير في فعالية الهيكسوكاينيز، وتركيز البيروفات، والأدينوسين ثلاثي الفوسفات، والحموضة في جميع طرق العلاج في خلايا سرطان الثدي ولكن ليس في الخلايا الطبيعية. يمكن اعتبار التآزر بين الفيروس والمثبط علاجًا مستقبليًا للخلايا السرطانية وطريقة استراتيجية جديدة لاستخدامها كعلاج فعال.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كليـة الطـب

الفعالية الأحادية والمختلطة المضادة للتكاثر للمانوهيبتولوز وفايروس مرض نيوكاسل ضد خطوط خلايا سرطان الثدي التي تستهدف تثبيط تحلل الكلوكوز

أطروحة

مقدمة إلى مجلس كلية الطب/جامعة النهرين
وهي جزء من متطلبات نيل درجة الدكتوراة في فلسفة الكيمياء الطبية

من قبل

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