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Therapeutic Potential Effect Of Some Medical Plants On Wound : In Vitro Study

A thesis

Submitted to the Council of the College of Medicine, the University of Babylon, as a Partial Fulfillment of the Requirements for the Degree of Master in Pharmacology & Toxicology

By

Doaa Abbas mohammed

B. Pharmacy (2015-2016)

Supervised by

Prof. Dr.	Prof. Dr.
Nisreen J. Mohammed	Rana A. Ghaleb
Ph.D. Pharmacology	Ph.D. Biology

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بسم الله الرحمن الرحيم

﴿ أَفَرَأَيْتُم مَّا تَحْرُثُونَ أَأَنتُمْ تَزْرَعُونَهُ أَمْ نَحْنُ الزَّارِعُونَ ﴾

صدق الله العلي العظيم

سورة الواقعه ايه 63 , 64

Dedication

I dedicate this work to…

To my daughter who left us and lived in heaven Narjis

To my family My father and mother and my lovely husband who encouraged and supported me all the way to continue my MSc. Study

to my supervisors dr nisreen and dr Rana and all who's inspired me

Doaa

Certification

We certify that this thesis entitled "therapeutic potential effect of some plants on wounds (in vitro study) " was prepared by (Doaa abbas aljeml) under our supervision at the department of Pharmacology. College of Medicine, University of Babylon (Iraq) in partial fulfillment of the requirements for the master degree of sciences in pharmacology and toxicology.

Prof. Dr.	Prof. Dr.
Nisreen J. Mohammed	Rana A. Ghaleb
Supervisor	Supervisor

The Recommendation of the Head of Department

In the view of the available recommendation, I forward this thesis for debate by the examining committee

Assist. Prof. Dr .

Selman M. Selman

The Head of Department of Pharmacology

College of Medicine

University of Babylon, Iraq

Examining Committee Certificate

We certify that we have read this thesis entitled "Therapeutic Potential Effect Of Some Medical Plants On Wound : In Vitro Study "doaa abbas mohamed " in its contents and that in our opinion it meets the standard of a thesis for the degree of Master in Pharmacology and Toxicology.

Signature:
Prof. Dr.
Abbas M. Alsaraf
(Chairman)
Date: / / 2022

Signature:
Prof .Dr.
Kaiser N. Madlum
(member)
Date: / / 2022

Signature:
Assist Prof. Dr
Kamal abdul rasool
(Member)
Date: / / 2022

Signature:
Prof. Dr.
Nisreen J. Mohammed Medicine
(Member and Supervisor)
Date: / / 2022

Signature:
Prof. Dr.
Rana A. Ghaleb
(Member and Supervisor)
Date: / / 2022

Signature:
Prof. Dr.
Muhanad abbas alshalah
(Dean of Medicine College)
Date: / / 2022

Aknowledgment

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Summary

Treatment of wounds is essential as the wound can also be lethal at some point in time if not healed properly. Ethnomedicinal plants can treat wounds as they have no side effects, whereas, in the case of chemical drugs, the side effects are on the rise.

So the aim of this study is to determine the effect concentrations of aqueous extract of Tamarix mannifera and Moreingo olifera, and the alcoholic extraction of Propolis . Vitamin C and zinc sulfate on the viability of Madin-Darby Canine Kidney (MDCK) cell lines. Then study the effect of these concentrations on healing of induced wound in MDCK cell line also detect the more active one for wound healing.

1/ cytotoxic assay

Ninety six -well plate were seeded with MDCK cell line in a concentration of $5*10^5$ cells. 3 replicates was considered as a control group, and the three subsequent columns were treated with concentrations of the plants and vitamin c and zinc :

- ✓ T.mannifera aqueous extract: (500, 250, 125, 62.5 ,31.25 and 15.6) µg\ml.
- ✓ M.oleifera aqueous extract : (500, 250, 125, 62.5 ,31.25 and 15.6) µg\ml.
- ✓ Propolis alcoholic extract : (500, 250, 125, 62.5 ,31.25 and 15.6) µg\ml.
- ✓ Vitamin C : (500, 250, 125, 62.5 ,31.25 , 15.6 , 7.8 , 3.9 and 1.95) µg\ml.
- ✓ Zinc : (500, 250, 125, 62.5 ,31.25 , 15.6 , 7.8 , 3.9 and 1.95) µg\ml.

All treated cells were incubated for 24 hours at 37°C after exposure to different concentrations then cells viability measured by MTT assay.

Results showed that there was no significant difference in cell viability except the highest concentrations in all material cause a significant decrease ($p \leq 0.001$) in the viability percentage.

2 / Wound Scratch In Vitro

The second experiment included: expose the scratched cells to the enhancement dose which was obtained from the first experiment.

Tamarix extract (125, 62.5, 31.25, 15.6, 7.8 µ/ml)

Moringa extract (62.5, 31.25, 15.6, 7.8 µ/ml)

Propolis extract (62.5, 31.25, 15.6, 7.8, 3.9, 1.95, 0.97, 0.48, 0.24 µ/ml)

Vitamin C (62.5, 31.25, 15.6, 7.8, 3.9, 1.95 µ/ml)

Zinc (62.5, 31.25, 15.6, 7.8, 3.9, 1.95 µ/ml)

The combination of vitamin C with zinc (62.5, 31.25, 15.6, 7.8, 3.9, 1.95 µ/ml)

Wound was made by using 200µl sterile plastic micropipette tip to press firmly against the cell monolayer of tissue culture plate.

The wound area were digitally photographed every 3 hours .

Results of second experiment showed that were a significant healing ($p<0.001$) in all concentrations at 9 hr in comparison to control group and decrease in wound diameter

The result showed that aqueous extract of Tamarix mannifera , Moringa Oleifera and alcoholic extract of propolis not toxic on MDCK line except at highest concentration while Vitamin C enhance cell viability at lower concentrations while zinc decrease cell viability at highest concentration and the combination of vitamin C and zinc has better effect in wound healing than vitamin c or zinc alone

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List of abbreviations

AA	Ascorbic acid
ADSCs	Adipose-derived stem cells
Art-C	Artepillin C
ATP	Adenosine triphosphate
C	Vitamin c
C TGF	Connective tissue growth factor
Ca	Calcium
CAPE	Caffeic acid phenethyl ester
COX-2	Cyclooxygenase-2
DDW	Deionized distill water
DMSO	Dimethy sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
E	Vitamin E
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EEP	Ethanolic extract of propolis
EGF	Epidermal Growth Factor

eNOS	Endothelial Nitric oxide synthase
EPC	Endothelial progenitor cells
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GSH	Glutathione
GSSH	Glutathione Disulfide
HeLa	Cancer cell line named after Henrietta lacks
HIF 1	Hypoxia inducible factor
IL	Interleukine
INF	Interferon
INOS	Inducible nitric oxide synthase
INOS	Inducible nitric oxide synthase
kDa	Kilo Dalton
KGF	Keratinocyte growth factor
KM	Kaempferol
LPS	Lipopolysaccharide
M.O	Moringa oleifera
MDA	Malondialdehyde
MDCK	Madin-Darby Canine Kidney normal cells
Mg	Magnesium
MIC	Minimum inhibitory concentration
MMP9	Matrix metallopeptidase 9
MSCs	Mesenchymal stem cells
mtRos	Mitochondrial reactive oxygen species
NADH	Nicotinamide adenine dinucleotide phosphate oxidase
NfKb	Nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS	Neuronal nitic oxide synthase
NO	Nitric oxide
PAK 1	P12 activated kinase

PBS	Phosphate buffer saline
PDGF	platelet-derived growth factor
PEG	Polyethylene glycol
PGE2	Prostaglandin E2
PK 15	Pig kidney epithelial cell line
Pk1	protein kinase
ROS	Reactive oxygen species
S	Seconds
T.M	Tamarix Mannifera
TGF-b	Transforming growth factor beta
TNF	Tumor necrosis factor
TRIM	Tripartite motif family
TXNIP	Thioredoxin interacting protein
VEGF	Vascular endothelial growth factor
ZnSO4	Zinc sulfate

1.1 Introduction

Skin, the largest organ of the body serves as the protective barrier against the environment (Kalachaveedu *et al.*, 2020) It performs a variety of roles, including acting as an effective barrier from environmental exposure, thermoregulation, internal homeostasis, immune function, preventing moisture loss from the body and vitamin D metabolism (Mitchell and Hill, 2020) .

The skin is most susceptible to damage from external invasion. Therefore, cutaneous wound treatment and care has become a serious concern in clinics and daily life. Wounds of humans with large or open injuries are unable to close by self contraction, which makes it extremely difficult to heal (Li *et al.*, 2020) .

An improper repair process can cause severe damage, like the loss of skin, initiation of an infection, with consequent harms to the subjacent tissues and even systemic ones. The most common and inevitable impediment to wound healing is the installation of an infection, mostly in the case of chronic wounds (Negut, 2018).

Acute bacterial skin and skin-structure infections pose a significant burden to the healthcare system. The challenge of treating these infections has been lessened by recent development of new antibiotic treatment options , including dalbavancin, oritavancin, tedizolid, and delafloxacin. (Golan, 2019).

Herbal medicines in wound treatment or care include disinfection, debridement, and providing a moist atmosphere which facilitates development of appropriate natural healing climate (Krishnan K and Thomas, 2019).

Aloe vera, commonly known as Kumari, used as an herbal medicine ,if topically applied, significantly increases wound contraction and wound closure. It has a significant influence on the level of collagen, which acts as the precursor protein for wound healing, Turmeric (Curcuma longa) used as herbal medicine for centuries in the treatment of various ailments. Curcumin (chemical compound of turmeric) has been reported of consisting significant wound healing properties . It acts on different stages of wound healing process to fasten the process. Curcumin also has the capacity to enhance granulation tissue formation, collagen deposition, tissue remodeling and wound contraction (Dan *et al.*, 2018).

Aim of Study

1.Study the effect of the medical plant extract (Tamarix Mannifera , Moringa Oleifera) on cell viability of normal MDCK cell line .

2. Study the effect of Propolis , vitamin C and Zinc on cell viability of normal MDCK cell line .

3. Study the effect of medical plant extract (Tamarix Mannifera , Moringa Oleifera) , Propolis , vitamin C and Zinc on wound healing.

1.2 literature review

1.3 Wounds

Wounds have a variety of causes such as surgery, injuries, extrinsic factors (e.g., pressure, burns and cuts), or pathologic conditions such as diabetes or vascular diseases. These types of damage are classified into acute or chronic wounds depending on their underlying causes and consequences (Tottoli *et al.*, 2020).

1.3.1. Classification of wounds

1.3.1.1 According to the depth of damage

Wounds can be divided into:

superficial-not exceeding the subcutaneous tissue , and deep-reaching beyond the subcutaneous tissue (Stojko, et al . 2021).

1.3.1.2 According to time of healing .

1-Acute wounds

Heal between 8–12 weeks and These wounds can be caused by mechanical damage induced by sheer, blunting, and/or stabbing action of hard objects. Acute wounds can also be formed by exposure to extreme heat, irradiation, electrical shock, and/or irritated with corrosive chemicals. (Zahedi P *et al.*, 2009).

An acute wound can become chronic when there is a failure of the wound to progress via sequential phases of healing, which can be attributed to biofilm bacteria in the wound, resulting in the wound remaining in the inflammatory phase over a prolonged period. The bacteria stimulate the production of proinflammatory cytokines that recruits mast cells neutrophils and macrophages in the wound. The inflammatory cells try to kill the bacteria by secreting proteases and reactive oxygen species (ROS) (Aderibigbe and Buyana, 2018).

The signs and symptoms of pain,swelling , erythema, edema, heat, and purulence have been used to identify wound infection, especially in acute wounds (K. Shobha Bhat *et al* ., 2014).

2- Chronic wound

Chronic wounds are defined as ulcers or open wounds that fail to resolve within 3 months and are further classified into three main subgroups: vascular ulcers, pressure ulcers, and diabetic ulcers (Sylakowski et al .,2020).

Damage to all layers of the skin (consisting of the epidermis, dermis, and subcutaneous adipose tissue below) can lead to chronic wounds, Chronic wounds are wounds that have entered a state of pathological inflammation instead of following a healthy healing timeline. While the typical progression of a healthy wound from injury to complete wound closure occurs within 30 day, chronic wounds stall and can remain unresolved indefinitely.(Povolny *et al*., 2019).

Chronic wounds are rarely seen in individuals who are otherwise healthy. In fact, chronic wound patients frequently suffer from ‘‘highly branded’’ diseases such as diabetes and obesity.(Sen *et al.*, 2009).

1.4 Healing of wound

Healing of a skin wound displays an extraordinary mechanism of cascading cellular functions which is unique in nature. Wound healing is a complex series of reactions that involves a number of overlapping processes, including induction of an acute-inflammatory process, regeneration, migration and proliferation of both parenchyma and connective tissue cells, synthesis of extra cellular matrix (ECM) proteins, and remodeling of connective tissue leading to formation of a scar tissue that is in coincidence with the development of wound contraction and epithelialization.(Oryan et al., 2018) .

In adult humans, optimal wound healing involves the following events:

(1) Rapid hemostasis

(2) Inflammation

(3) Mesenchymal cell differentiation, proliferation, and migration to the wound site

(4) Angiogenesis

(5) Re-epithelialization (re-growth of epithelial tissue over the wound surface

(6) Synthesis, cross-linking, and alignment of collagen to provide strength to the healing tissue (Guo and DiPietro, 2010).

Several biopolymers, such as collagen, alginate, hyaluronic acid, chitosan, fucoidan, and Poly-N acetyl glucosamine, are investigated as wound dressing (Moeini *et al.*, 2020) .

1.4.1 Haemostasis

At the time incision, vascular injury occurs on a macro- or microvascular scale. The immediate response of the body is to prevent exsanguination and promote haemostasis. Damaged arterial vessels rapidly constrict through the contraction of smooth muscle in the circular layer of the vessel wall, mediated by increasing cytoplasmic calcium levels. the reduced blood flow mediated by arteriole constriction leads to tissue hypoxia and acidosis. This promotes the production of nitric oxide, adenosine and other vasoactive metabolites to cause a reflex vasodilatation and relaxation of the arterial vessels. (Singh *et al* ., 2017).

Platelets subsequently aggregate and activate on subendothelial collagen, leading to formation of a hemostatic plug through their release of cytokines and growth factors (Ellis *et al.*, 2018).

The coagulation and complement cascades are then initiated. Within the tissue, prothrombin is activated to form thrombin, which then cleaves fibrinogen to generate fibrin. Along with platelets and the plasma fibronectin, fibrin forms the clot (Thiruvoth *et al.*, 2015).

1.4.2 Inflammation

The inflammatory phase initiates with a disruption of blood vessels and extravasation of blood constituents, as well as with the production of provisional extracellular matrix. At the same time, platelets release several vasoactive mediators and chemotactic factors, such as interleukin (IL), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor beta (TGF-b), that recruit inflammatory cells such as neutrophils and macrophages and also fibroblasts and endothelial cells to the site of wound.(Amadeu *et al.*, 2008).

Cells recruited to the site of injury from the blood, work with numerous cell types in and surrounding the injured skin to orchestrate the repair process .This occurs through a combination of the actions of these cells themselves, their signalling pathways , and through the release of their soluble mediators, such as cytokines, chemokines, growth factors, and metabolites that signal other cells to perform specific tasks, to produce the final newly formed tissue (Aitcheson *et al.*, 2021).

Neutrophils, as immune cells, infiltrate the site and usually secrete an appropriate concentration of reactive oxygen species (ROS) and proteases to help eliminate bacteria and foreign pathogens. They also remove the breakdown products of the injured cells and clots, and release various growth factors and cytokines . In addition, other innate and adaptive immune cells, such as macrophages, mast cells, and T and B cells, were shown to participate in the process (Schilrreff and Alexiev, 2022).

One critically important function of wound macrophages is the capacity to facilitate the removal of neutrophils. Neutrophils are abundant in early wounds, and are essential for effective decontamination. Yet a large evidence suggests that neutrophils negatively influence repair, probably because this cell type is capable of destroying normal tissue. Neutrophil proteases, such as elastase can degrade most components of the extracellular matrix as well as proteins (Timothy J. Koh and Luisa Ann DiPietro, 2013).

1.4.3 proliferative phase

The proliferative phase begins around the third to tenth day and encompasses processes that result in re-epithelialization, angiogenesis, collagen deposition, and formation of granulation tissues.The latter process requires a structural framework that is provided by fibroblasts, which secrete elastin and organize the extracellular matrix. Under the stimulation of cytokines, such as interferon-α (IFN-α), and TGF-β, fibroblasts synthesize collagen and fibronectin to facilitate the closure of tissue gaps as well as restoration of mechanical strength (Pang *et al.*, 2020).

Re epithelialization starts 16-24 hours afer injury and continues until the remodeling phase of wound repair . Early after injury, keratinocytes differentiate and migrate between the fibrin clot and the rich collagen dermis while suprabasal keratinocytes located behind the leading edge proliferate to provide more cells to fill the gap. Suprabasal keratinocytes close to the leading edge change shape and migrate on top of basal keratinocytes, becoming leading cells. In the fnal stages of reepithelialization, cells differentiate into epithelial cells that remain firmly attached to the basal membrane (Dorantes and Ayala, 2019).

Angiogenesis is the formation of new blood vessels from preexisting vessels and it is initiated by growth factors such as VEGF, PDGF, and fibroblast growth factor (FGF) while Fibroplasia is the formation of granulation tissue, and its main characteristic is proliferation of fibroblasts in response to PDGF and TNFα. At this time, the production of collagen occurs, and there is a release of growth factors such as keratinocyte growth factor (KGF). Then, the provisional matrix initially formed is replaced by granulation tissue composed of fibroblasts, granulocytes, macrophages, and blood vessels in complex with collagen bundles that form the basis for cell adhesion and migration, growth, and differentiation (Silva *et al.*, 2018).

Remodeling is the last phase of wound healing, and at this stage, the maturation of the wound begins. Extracellular matrix components are partially subject to modifications; in particular, collagen type III is replaced by stronger collagen type I, to form a more organized extracellular structure. Fibroblasts and keratinocytes are two cell lines strictly involved in this process, and their interaction, is essential to the outcome of successful dermal remodeling and the transition from granulation tissue to scar formation (Cucci *et al.*, 2021) .

Overall, the healing process involves numerous cellular and biosynthetic processes, which all require energy in the form of adenosine triphosphate (ATP), as well as amino acids, and other precursor molecules to replace damaged tissue(Ferroni *et al.*, 2020).

1.4.4. Formation of scar

Whereas superficial epidermal skin damage heals efficiently, the healing of deeper dermal wounds may lead to abnormal scar overgrowth and formation of two different types of fibrotic skin disorders: hypertrophic scars or keloids Hypertrophic scars are defined as raised, erythematous, pruritic lesions that do not extend beyond the boundaries of the original wound area grow rapidly during 4 to 12 weeks after wounding and tend to mature and flatten over time (Karppinen *et al.*, 2019) .

Keloids are pathological scars presenting as nodular firm lesions that extend beyond the area of injury. They do not spontaneously regress, often continuing to grow over time , it is results from the lack of control mechanisms regulating cell proliferation and tissue repair (Coppola *et al.*, 2018) .

Keloid scars can appear many years later and beyond the site of injury. Similar to hypertrophic scars, keloids are raised and can be symptomatic (Berman *et al* ., 2017) .

Fibroblasts from hypertrophic behave quite differently than normal fibroblasts. Hypertrophic scar has greater amounts of fibroblasts that exhibit an altered phenotype than normal skin .Hypertrophic fibroblasts show higher expression of TGF-β1 than normal fibroblasts .The increase or prolonged activity of TGF-β1 leads to an overproduction and excess deposition of collagen by fibroblasts that often result in formation of hypertrophic scar (Zhu *et al.*, 2013) .

Hypertrophic scars are similar to keloids and may transform into keloids over time. The standard treatments for these scars are limited by inconsistent efficacy and long treatment/follow-up times (Tosa and Ogawa, 2020).

1.5 The incidence of complications

Wounds do not always follow the expected healing trajectory, because many patients with wounds have comorbidities and underlying long-term conditions. Wound infections, whether they are at surgical sites or in other wound types, can lead to further consequences.(Lindholm and Searle, 2016).

Developing a new, healthy tissue in the wounded area is dependent on cell proliferation, migration, and differentiation. Disorder of mechanisms which regulate these processes at any stage leads to impaired wound healing. Impaired wound healing may be either slow as in diabetes , it is contributes to the greatest number of diagnosed chronic wounds. diabetes is a group of metabolic diseases typified by dysregulation of blood glucose levels that often leads to chronic wound formation through complex pathophysiological mechanisms (Malone-Povolny *et al* ., 2019) , or accelerated (related to hypertrophy and keloid scars) In the case of accelerated healing, there is a large amount of deposition of extracellular matrix, increased cell proliferation, and wound vascularization (Diegelmann and Evans, 2004) .

The formation of hypertrophic scars is a major problem and therapeutic regimens as surgery, injections of corticosteroids, or radiation do not give the expected results. In contrast, high efficacy in reducing the formation of hypertrophic scars was observed in therapies based on mechanical impact on wound environment (Rosińczuk *et al.*, 2016) .

Another form of pathological scarring is keloid changes, whose etiology is quite poorly known. The reasons for pathogens include genetic disorders, apoptosis, dysregulation of mesenchymal-epithelial signaling, and variations of mechanical tension in wound environment (Butler *et al* ., 2008) .

1.6 Factors Influencing Wound Healing

1.6.1 Collagen role in wound healing

Collagen, which is produced by fibroblasts, is the most abundant protein in the human body. A natural structural protein, collagen is involved in all 3 phases of the wound healing cascade. It stimulates cellular migration and contributes to new tissue development. (Fleck and Simman, 2010).

In first phase collagen exposure due to injury activates the clotting cascade, resulting in a fibrin clot that stops the initial bleeding . Fibroblasts contribute to collagen deposition. Simultaneously, collagen degradation releases fragments that promote fibroblast proliferation and synthesis of growth factors that lead to angiogenesis and re-epithelialization (Mathew-Steiner *et al* ., 2021) .

1.6.2 Keratin role in wound healing

Keratins constitute a group of cysteine-rich proteins formed in the epithelial cells of higher vertebrates (e.g. mammals) that can be found in filamentous or hard structures such as nails, horns, wool or hairs (Konop *et al.*, 2014).

Keratin protein materials, divided into trichocyte keratin and epithelial keratin, are intermediate filament proteins and the key components of hair, nail and skin . The two kind keratins contain non-helical end-terminal domains and a highly-conserved, central alpha-helical domain, and trichocytic keratins are similar in composition to the epithelial keratins. (Li *et al.*, 2019).

Keratin have been shown to play a key role in wound healing. Three keratin subtypes have been identified as part of keratinocytes' response to injury: keratins 6, 16 and 17 (KRT6, KRT16 and KRT17) and re epithelialisation of a wound begins after keratinocyte activation (Batzer *et al* ., 2016).

Keratin powder dressings have been shown to release keratin peptides into the wound, provide physical support to cell attachment, proliferation and survival and promote wound healing Migration of keratinocytes is the first step in and is essential to wound re-epithelialization. The loss of continuity between epidermis and dermis upon injury allows the keratinocytes to come in contact with collagens in the dermis and start the repair process migrating across the wound bed(Tang *et al.*, 2012).

1.6.3 Nitric oxide role in wound healing

Nitric oxide is an endogenous gasotransmitter that plays a central role in wound healing. It is generated from L-arginine through a five electron oxidation process catalyzed by a group of three isozymes, called nitric oxide synthases This group of isozymes includes endothelial (eNOS), neuronal (nNOS), and inducible nitric oxide synthase (iNOS). Two of the isozymes, nNOS and eNOS, are constitutively expressed and catalyze low level NO generation through cyclic guanosine monophosphate (cGMP) in vascular endothelial cells and neurons, respectively (Dupont *et al.*, 2014) .

Interestingly, during inflammation associated with wound healing, iNOS coexpresses (Cinelli *et al.*, 2020) .

Nitric oxide (NO) plays a pivotal role in wound healing, including causing the contraction of wound surfaces, dilating blood vessels, participating in inflammation, and promoting collagen synthesis, angiogenesis, and fibroblast proliferation.(Wan *et al.*, 2020).

While normal fibroblasts do not synthesize NO , fibroblasts isolated from wounds have been shown to release NO. Furthermore, decreased collagen synthesis from the cells has been reported when NO production in wound fibroblasts was inhibited in vitro by NOS inhibition .(Nichols *et al.*, 2012).

Diabetes mellitus, which impairs wound healing, is accompanied by a reduction in NO at wound site. Treatment with L-arginine improved healing in diabetic animals accompanying with increases of nitrite/nitrate level in wound fluid. Also, treatment with NO donor to wound area was reversed impaired healing in diabetics with increasing collagen synthesis. it has been established that NO is an important factor.(Obayashi *et al.*, 2006).

Nitric oxide generated by eNOS serves to prevent platelet adhesion to the vessel walls. Reactive oxygen species released by the damaged vasculature react with and inactivate NO, with the resulting NO deficiency leading to platelet aggregation and thrombus formation. Nitric oxide has antimicrobial properties resulting from a combination of nitrosative and oxidative mechanisms.Upon reaction with oxygen and superoxide, nitric oxide forms dinitrogen trioxide and peroxynitrite, respectively. Dinitrogen trioxide causes DNA deamination; peroxynitrite results in lipid peroxidation and membrane damage (Povolny, *et al.*, 2019 *)*.

Nitric oxide has a short half-life (1 – 5 s) in the presence of oxygen and hemoglobin) in vivo, arising from its high reactivity with transition metals, heme-containing proteins, and thiols so its important to give No (Nichols *et al.*, 2012).

Nitric oxide has been implicated as a regulator of all phases of wound healing. Studies demonstrated the ability of NO to modulate chemoattractant cytokines, such as IL-1, IL-6, IL-8, and TGF-b1 (Amadeu *et al.*, 2008).

1.6.4 Hypoxia

Vasoconstriction-induced tissue hypoxia may decrease the strength of the healing wound independently of its ability to reduce resistance to infection. (Allen and Jacofsky, 2017).

Oxygen is a potent signaling molecule for its ability to affect the fundamental characteristics of various cells. A state of hypoxia, may cause an impairment of function. When the cell is unable to extract adequate oxygen, the partial pressure of oxygen within the cell declines, which leads to a reduction in mitochondrial respiration and oxidative metabolism. (Lee *et al.*, 2009).

During the initial inflammatory process, wound sites are often hypoxic. This is due to disruption of vasculature surrounding the wound, leading to impaired oxygen delivery, and exacerbated by a rapid influx of inflammatory cells participating in the healing process with high metabolic demands for oxygen. These inflammatory cells preferentially accumulate in hypoxic areas to play a critical role in granulation, re-epithelialization, and other healing processes Fibroblasts were found to secrete up to nine times more transforming growth factor-β1 (TGF-β1) when exposed to hypoxic conditions, which demonstrates this increased activity. Acute hypoxia thus induces a temporary increase in cellular replication and contributes to initiation of the healing process. (Hong *et al.*, 2014).

Cells sense hypoxia and can alter gene expression changing their metabolism in order to promote cell survival. transcriptional response is mainly mediated by hypoxia-inducible factor 1 (HIF-1) which regulates the transcription of hundreds of genes that promote cell survival in hypoxia. Diferent genes involved in regulation of metabolism, cell proliferation and angiogenesis are modulated by hypoxia.(Alessandro *et al.*, 2019).

1.6.5 Smoking

Smoking impairs wound healing by its effects on chemotaxis, migratory function and oxidative bactericidal mechanisms in the inflammatory phase. In addition, it also reduces fibroblast migration and proliferation. Furthermore, smoking affect immune function, downregulates collagen synthesis and deposition (Singh *et al* ., 2017).

1.6.6 Essential nutrients in wound healing phases.

1.6.6.1 Vitamins

Vitamins C (L-ascorbic acid), A (retinol), and E (tocopherol) show potent anti-oxidant and anti-inflammatory effects.

Vitamin C has many roles in wound healing, and a deficiency in this vitamin has multiple effects on tissue repair. Vitamin C deficiencies result in impaired healing, and have been linked to decreased collagen synthesis and fibroblast proliferation, decreased angiogenesis, and increased capillary fragility. Also, vitamin C deficiency leads to an impaired immune response and increased susceptibility to wound infection (Guo and DiPietro, 2010).

Vitamin C is a naturally occurring potent, water-soluble molecule that has three major roles pertaining to wound healing:

1/ Promoting collagen synthesis .

2/ Modulating immune function .

3/ Acting as an antioxidant.

Vitamin C is a required cofactor for the hydroxylation of proline and lysine residues in procollagen, which is eventually converted into collagen. This hydroxylation is necessary for the stabilisation of the triple-helical structure of collagen. Histologically, vitamin C has also been shown to increase the density of the dermal papillae, which is believed to reflect angiogenesis and stimulation of dermal fibroblasts (Sinno *et al* ., 2011).

Vitamin A is a family of retinoids, it is an essential fat-soluble micronutrient that cannot be synthesized by the human body and must be obtained from the diet. It is available in eggs, fish oils, liver, dairy products it is can promote and enhance various aspects of wound healing via the stimulation of angiogenesis, collagen synthesis, epithelialization, and fibroplasia. Local (topical) and systemic supplementation with vitamin A has been proven to increase dermal collagen deposition (Zinder *et al.*, 2019) .

Vitamin A deficiency leads to delayed epithelialization and wound healing, manifested by impaired wound closure and decreased rates of collagen synthesis and cross-linking of newly formed collagen (Polcz and Barbul, 2019).

1.6.6.2 Protein and amino acids

Proteins provide the main building blocks for tissue growth, cell renewal, and repair after injury. They significantly affect multiple phases of wound healing, hemostasis, inflammation and granulation tissue formation, cell proliferation, tissue reorganization, and normalization by their roles in RNA and DNA synthesis, collagen and elastic tissue formation, nutrition of the immune system, epidermal growth, and keratinization . Collagen formation is particularly affected by proline deficiency (Katherine L. Brown et al, 2010).

1.6.6.3 Metals :

Local supplementation was shown to induce several beneficial effects including acceleration of healing and strengthening of the cicatricial tissue . The dynamic relationship among the different metal elements is extremely complex and two or more of these metals can be competitors and bind to the same protein, inducing direct or indirect regulation of its activity . The binding affinities are responsible for fine regulation of enzymatic or other protein activities and ion availability (Coger *et al.*, 2019) .

A/ Iron

Is a vital co-factor for proteins and enzymes involved in energy metabolism, respiration, DNA synthesis, cell cycle arrest and apoptosis, It has long been known that iron is essential for healthy skin, mucous membranes, hair and nails. Clinical features of iron deficiency include skin pallor, pruritus, and predisposition to skin infection (impetigo, boils and candidiasis), angular chelitis, swollen tongue, fragile nails, kolionychia, and dry brittle hair(Wright *et.al*., 2014) .

B/ ZINC

Zinc stabilizes cell membranes, serves as an essential cofactor for several metallo-enzymes, participates in basal cell mitosis and differentiation . epithelialisation in wound healing, Zinc is also present in a number of zinc-dependent metallo-enzymes in the skin, including matrix metalloproteases (MMPs), superoxide dismutase (SOD), Many of the biochemical and molecular events in wound repair can be expedited by addition of supplementary zinc ion through up-regulation of metallothioneins (Olaifa and Fadason, 2016).

Metallothioneins (MTs) are ubiquitous cysteine-rich proteins that regulate the trafficking of intracellular Zn together with membranous Zn transporters according to physiological demands (Kimura and Kambe, 2016).

Zinc metalloproteins are involved in different skin physiological processes, including augmenting keratinocyte migration during wound repair, stimulating the re -epithelialization of wounds, and promoting the proliferation of endothelial progenitor cells to enhance angiogenesis.

Without zinc, wound healing would be delayed , and patients would suffer severe clinical disorders unsealing wounds . (Han *et al.,* 2020) .

Zinc is ubiquitously found in the body, with 85% stored in muscle and bone, 11% in the skin and liver, and the rest in other tissues. Zinc is located intracellularly and in extracellular matrix in epidermal and dermal tissues in the form of protein complexes where zinc acts as a stabilizer of cell membranes and an essential cofactor (Dalisson and Barralet, 2019).

C/ copper

It is well known that copper ions play an intricate role in cells, regulating the action mechanisms of several cytokines and growth factors in wound repair, and are involved in virtually all stages of the wound healing process. They can reduce the likelihood of wound infection by acting as an antimicrobial agent (Chen *et al.*, 2021).

1.7. Coagulation and reinforcement of the platelet plug

Platelets provide the surface for the assembly and activation of coagulation complexes. The classic coagulation pathways are the intrinsic and extrinsic pathways, both of which are activated by exposure of the subendothelial matrix, and lead to factor X activation. Following activation of factor X by either pathway, prothrombin gets converted into thrombin, which cleaves fibrinogen into fibrin. Factor XIII covalently crosslinks fibrin, which binds the aggregated platelet plug forming a definitive secondary hemostasis plug or the thrombus. The thrombus also serves as the provisional wound matrix for the infiltration of other cells in the subsequent stages of healing (Rodrigues *et al.*, 2019).

1.8 Treatment

1.8.1 Treatment of wounds

A / Antibacterial

One of the topical antibacterial agent used to control wound infection is silver sulfadiazine (Ahmadian et al ., 2020).

Dermal wound inflammation can be healed by the application of an antimicrobial drug, such as sodium fusidate (Mosti et al., 2015). It has a steroid skeleton and is effective against Staphylococcus infections and other gram positive bacteria(Jin *et al.*, 2016).

B / Phytotherapy

sesame, cactus, and beeswax which are thought to improve skin scarring through an enhanced remodelling process and improved microcirculation (Demidova-Rice *et al .,* 2012) , The pharmalogical effects of mebo ointment which also contain beeswax , cactus and sesame are attributable to:

*beta-sitosterol, isolated from phellodendron amurense.

*flavonoids mainly baicalin isolated from scutellaria baicalensis.

*alkaloids mainly berberine, isolated from coptis chinensi.

*Beeswax and sesame oil(Rahman, 2011).

Mebo is a formulation that promotes maturation of keratinocytes, and has been shown to significantly improve the appearance of scars and increase healing times in wounds (Skochdopole *et al.*, 2021).

Silicone sheeting is an adhesive, soft and semi-occlusive dressing that has been a popular topical treatment for hypertrophic and keloid scars for decades (Song *et al* ., 2018).

1.8.2 Treatment of scar (keloid)

First-line therapy for keloids include intralesional corticosteroid injection such as triamcinolone acetonide, dexamethasone, methylprednisolone and hydrocortisone, silicone elastomer sheeting, cryotherapy, or pressure dressing. These treatments are reasonable, simple to perform and useful for small lesions. However, un favorable impacts of these modalities are hyperpigmentation, pain, telangiectasia, or skin atrophy me of the other approaches used as a second-line treatment are surgical excision of keloid, combined corticosteroid and cryotherapy, or a triple therapy including surgery followed by corticosteroid injection and silicone sheeting; however, this method is time taking and expensive (Naik, 2022).

1.9 Role of antioxidant in wound healing

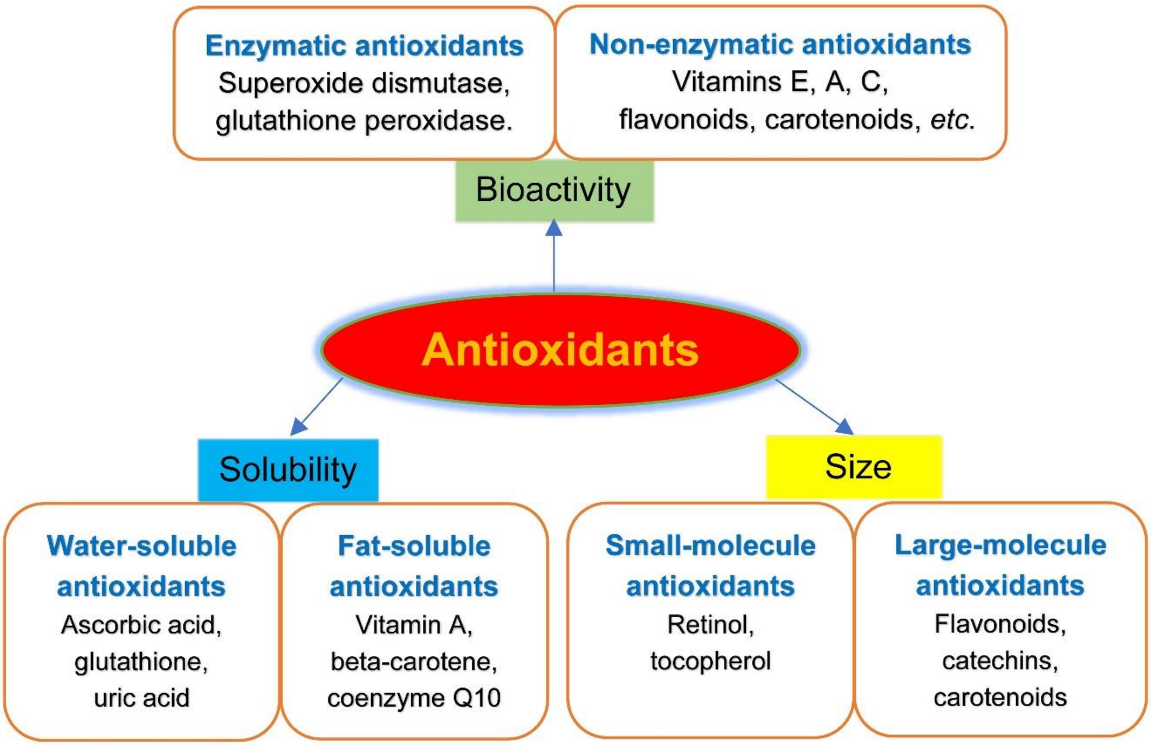
Free radicals are highly unstable molecules, and ROS are a form of free radicals that include the oxygen atom as well as reactive molecules such as superoxides and peroxides. Although normally formed as a byproduct of metabolism and reactive to invading organisms, overproduction leads to an increased load of free radicals and ROS known as oxidative stress (Fitzmaurice *et al* ., 2011).

Appropriate amounts of ROS can prevent the wound from infection and promote wound healing. However, in chronic wounds, redox homeostasis is destroyed and oxygen radicle , a strong oxidizing substance, is excessively generated, resulting in a great loss of antioxidants, leading to cell oxidative damage and inhibited wound healing (Kurahashi and Fujii, 2015).

It has also been noted that many plants or plant-derived compounds possessing high levels of antioxidant properties also show wound-healing activities (Süntar *et al.*, 2012).

Polyphenols ,an antioxidant found in many plants can be subdivided into 3 main classes, flavonoids, stilbenoids, and phenolic acids . Flavonoids are the most prevalent of these. The effectiveness of polyphenol compounds in the inhibition of oxidative processes is related to their reactive species (RS) scavenging activity (Papuc *et al.*, 2017).

Normal endothelial progenitor cells have been shown to express intrinsically high levels of the antioxidant enzyme manganese superoxide dismutase (MnSOD), which plays a key role in EPC resistance to oxidative stress via scavenging mitochondrial ROS (mt ROS) , Recent studies have shown that high glucose elevates oxidative stress and decreases EPC survival by inhibiting cell proliferation, NO production, MMP-9 activity, and migration . These observations indicate that a loss of tolerance to hyperglycemia-induced excessive oxidative stress in EPCs may lead to their dysfunction in diabetes (Marrotte *et al.*, 2010).



Fig(1.1) Different classes of antioxidants (Ayoka TO, Ezema BO, Eze CN, 2022)

Glutathione (GSH) is an antioxidant that acts as a free radical scavenger and a detoxifying agent in cells. It is useful in a multitude of processes, cellular proliferation, cell division, and differentiation Under oxidative stress, GSH is converted by GSH dependent peroxidases into GSSG (GSH disulfide) oxidizes form upon its reaction with ROS. GSH is synthesized in the cytosol and further distributed to different organelles (Bansal *et al* ., 2018).

ß -carotene, are provitamins A, a precursor of immunomodulary vitamin A. Vitamin A functions in proliferation regulation and differentiation of various cell types including skin cells (Zerres and Stahl, 2020).

1.10 Cell culture

Cell culture is the technique in which cells are removed from an organism and placed in a fluid medium. Under proper conditions, the cells can live and even grow. The growth can be characterized by cell division (mitosis) or by other processes, such as differentiation, during which the cells can change into specific types that are capable of functions analogous to tissues or organs in the whole organism (Lynn, 2009).

Cell culture refers to laboratory methods that enable the growth of eukaryotic or prokaryotic cells in physiological conditions. Its origin can be found in the early 20th century when it was introduced to study tissue growth and maturation, virus biology and vaccine development, the role of genes in disease and health, and the use of large-scale hybrid cell lines to generate biopharmaceuticals. In a clinical context, cell culture is most commonly linked to creating model systems that study basic cell biology, replicate disease mechanisms, or investigate the toxicity of novel drug compounds (Segeritz and Vallier, 2017).

1.10.1 Primary culture

Cells that are cultured directly from a tissue are known as primary cells. A primary culture may be produced either by allowing cells to migrate out from the tissue after sterile dissection, which is adhering to a substrate, or by disaggregating the tissue mechanically or enzymatically to produce a suspension of cells. These cells are, therefore, more representative of the cell types in the tissue from which they were isolated from, although many cells are unable to attach and survive in vitro. Most primary cultures are usually heterogeneous, have a low growth fraction, and have a limited lifespan, with the exception of some derived from tumours. Cells that have attached are trypsinised and reseeded in a fresh flask and become a secondary culture (Christina Philippeos *et al* ., 2012) .

The most popular types of primary cells used in research are epithelial cells, fibroblasts, keratinocytes, melanocytes, endothelial cells, muscle cells, hematopoietic and mesenchymal stem cells (Sigma Aldrich, 2020).

1.10.2 Secondary culture

Secondary cell culture When primary cell cultures are passaged or subcultured and grown for a long period of time in fresh medium, they form secondary cultures and are longlasting (unlike cells of primary cell cultures) due to the availability of fresh nutrients at regular intervals. The passaging or subculturing is carried out by enzymatic digestion of adherent cells. This is followed by washing and re-suspending of the required amount of cells in appropriate volumes of growth media. Secondary cell cultures are preferred as these are easy to grow and are readily available; they have been useful in virological, immunological, and toxicological research (Verma *et al* , 2020) .

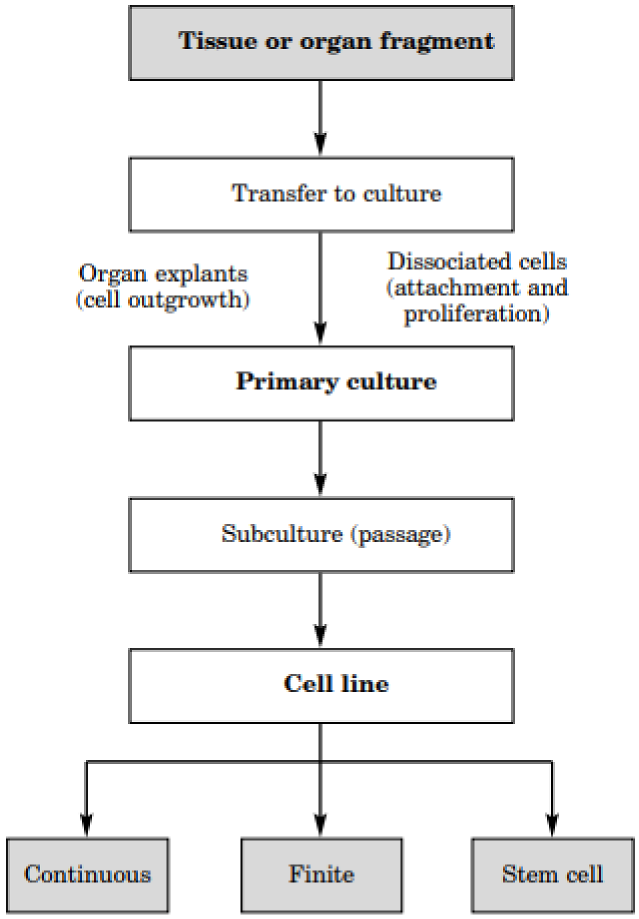


Fig (1.2) : Relationships between the main types of in vitro systems (Coecke *et al.*, 2005)

1.10.3 Advantage of cell culture technique

1. Physiochemical and physiological condition: The role and impact of the culture's pH, temperature, O2/CO2 concentration, and osmotic pressure.

2 Cell metabolism: The study of cell metabolism as well as the physiology and biochemistry of cells.

3. Cytotoxic assay: The effect of different substances or medications on certain cell types, such as liver cells, can be investigated.

4. Homogenous cultures: These cultures help study the biology and origin of the cells.

5. Valuable biological data from large-scale cell cultures: Specific proteins can be synthesized in large quantities from genetically modified cells in large-scale cultures.

6. Consistency of outcomes: The results achieved utilizing a single type/clonal population can be reproduced.

7. Identification of cell type: The presence of markers such as molecules or karyotyping can be used to identify specific cell types.

8. Ethics: Ethical, moral, and legal questions for utilizing animals in experiments can be avoided. (Freshney, 2010) .

1.10.4 cell line

Cell lines are usually named by the researcher who developed them and till recently were lacking a standard nomenclature protocol This had led to cell line misidentification. In addition, cell lines also suffer from cross-contamination from other sources including other cell lines. All these factors affect overall scientific reproducibility. Common contaminants include Mycoplasma . Cell line contamination is regarded as one of the most prevalent problems in biological research. (Mohammad *et al.*, 2019) .

The HeLa cell line was developed in the 1950s from a particularly aggressive strain of cervical cancer cells taken during a routine biopsy from a 30-year-old mother of five, Henrietta Lacks. She was treated for the disease by Dr. George Gey in the colored ward of The Johns Hopkins Hospital. As the head of tissue research, Dr. George Gey was at that time attempting to establish immortal cell lines that could be used in medical research. Taking tissue samples from poor and/or American Negro cancer patients being treated in the colored ward without informed written consent was not unusual and neither doctors nor their patients were aware of any of the ethical issues involved. Henrietta Lacks was diagnosed in 1950, and died within a year at the age of 31. But her cells lived on and became the first human cell line to be established (Minden, 2012) .

A few years after the establishment of the HeLa cell line, in 1963, at Ibadan University in Nigeria, Robert James Valentine Pulvertaft established, from a Nigerian patient affected by Burkitt's lymphoma, the RAJI cell line, the first human continuous hematopoietic cell line . Although the RAJI cell line was successively proven to be a model system that is generated by Epstein–Barr virus infection, the definition of the culture conditions that are necessary for its growth in vitro paved the way for the stabilization of new cell lines growing in suspension .

1.10.5 Types of cell lines

Normal cells usually divide only a limited number of times before losing their ability to proliferate, which is a genetically determined event known as senescence; these cell lines are known as finite. However, some cell lines become immortal through a process called transformation, which can occur spontaneously or can be chemically or virally induced. When a finite cell line undergoes transformation and acquires the ability to divide indefinitely, it becomes a continuous cell line (Hadi, 2010) .

1.10.6 Madin-Darby Canine Kidney (MDCK) cells

Model mammalian cell line used in medical research. The MDCK cell line was developed by Madin and Darby in 1958 from kidney tissue from a Cocker Spaniel. MDCK cells are epithelioid cells and usually exhibit adhesive growth when cultured in vitro. Due to their high viral production efficiency, rapid proliferation, and low mutation rate, MDCK cells are considered an efficient host for influenza virus production (Ye *et al.*, 2021).

MDCK cells are used for a wide variety of cell biology studies including , cell-cell adhesions, collective cell motility, as well as responses to growth factors. It is one of few cell culture models that is suited for 3 D cell culture and multicellular rearrangements known as branching morphogenesis (O'Brien *et al* .,2002) .

MDCK cells were initially used in virology, they are permissive and support the growth of influenza virus making them suitable for virology research, diagnostics, and vaccine production (Matrosovich *et al.*, 2003) .

1.10.7 Cell strain

Cell line is a permanently established cell culture which will proliferate forever if a suitable fresh medium is provided continuously, whereas cell strains have been adapted to culture but, unlike cell lines, have a finite division potential. A cell strain is obtained either from a primary culture or a cell line. This is done by selection or cloning of those particular cells having specific properties or characteristics (e.g. specific function or karyotype) which must be defined (Bhatia, 2019) .

1.11 Herbal medicines

Medicinal plants have been reported to show wound healing potential via angiogenesis, activation of NF- κB, favoring pro-inflammatory cytokines, increased expression of inducible nitric oxide synthase (iNOS) and alpha 1 type 1 collagen, and anti-oxidant activity (Firdous and Sautya, 2018) .

Most plants or parts contain phytoconstituents that have wide ranging properties like antimicrobial, anti-inflammatory, antioxidant, antipruritic, proliferative, and analgesic that are often key in wound management or healing (Marume *et al.*, 2017) .

1.11.1 *Tamarix mannifera*

Tamaricaceae (Caryophyllales) include about 80 halo- phyte, rheophyte and xerophyte species occurring in arid and semi-arid zones of Asia, Africa and Europe . These plants are characterized with needlelike leaves which are covered with salt, secreted from the salt glands (Samadi *et al* ., 2013).

Tamarix species are developed in dry atmospheres to fix the sand ridges; while their development in wet atmospheres are not wanted since they go about as obtrusive plants which forestalls the development of different species Tamarix spp. is traditionally used for gastrointestinal disorders, wounds, diabetes, and dental problems. Phenolic acids, flavonoids, and tannins constitute the main phytochemicals of these plants. Preclinical pharmacological evaluations have demonstrated several biological activities for Tamarix spp. including antidiabetic, hepatoprotective, wound healing, and anti-inflammatory The reported phytochemical work on Tamarix species has shown that the main chemical constituents are polyphenolic constituents such as flavonoids, tannins, and phenolic acids , 100 and 300 mg/ kg considered as toxic dose. (Bahramsoltani *et al.*, 2020).

Tamarix is rich in antioxidant, that could accelerate wound healing, enhance hydroxyproline content, decrease malondialdehyde/nitric oxide, and elevate the level of reduced glutathione in wound bed (Deng *et al.*, 2021) .

1.11.2 *Moringa oleifera*

(Fig 1.3)Different vegetative and reproductive parts of *M. oleifera* tree; i field grown tree, ii bundle of foliage, iii flowers, and iv fruit (pod) (Saini *et al* ., 2016)

Moringa Oleifera also known as drumstick tree or miracle tree, is widely cultivated in tropical and subtropical areas of Asia, Africa, and Central America. Its leaves are rich in amino acids, vitamins, proteins, minerals, and other nutrients, and often are used as food to improve nutrition and health care (Chen *et al*., 2020) .

The dried leaves of *Moringa Oleifera* MO are a great source of polyphenol compounds, such as flavonoids and phenolic acids. Flavonoids, which are synthesized in the plant as a response to microbial infections, have a benzo-γ-pyrone ring as a common structure. Intake of flavonoids has been shown to protect against chronic diseases associated with oxidative stress, including cardiovascular disease and cancer.(Vergara-jimenez *et al* ., 2017) .

The lethal dose of 50% LD50 was estimated to be 1585 mg/kg (Sidney and Michael ., 2015)

1.11.2.1 Phytochemistry

Table 1.1 Phytometabolites reported in different parts of MO taken from Iraq AL Hilla .(Singh *et al*., 2020)

part of Plant	**Phytometabolites**	**Group of the phytometabolites**
Seeds, leaves, stem and roots	Kaempferol	Flavonoid
Seeds, leaves, stem and roots	Quercetin	Flavonoid
Leaves	Myrecytin	Flavonoid
Leaves , flowers and seeds	Gallic acid Ellagic acid Chlorogenic acid Ferulic acid	Phenolic acid

A / Kaempferol

Kaempferol, which has anticancer, antiproliferative, antiinflammatory, antibacterial, and antioxidant activities, Lipophilic molecules with fewer hydroxyl groups like KM contribute the formation of collagen fibrils also it is increase the Contraction and epithelialization which are important factors in wound healing (Özay *et al.*, 2019) .

B / Quercetin

It is one of the strongest antioxidants amongst different flavonoids, and some of its pharmacological activities include antioxidant, anti-inflammatory, angiogenic, antibacterial, immunomodulatory, antiviral, enhanced myofibroblast activity, proliferation of epithelial cells and fibroblast hese properties makes quercetin as a promising wound healing agent Quercetin increased the expressions of IL-10, VEGF, TGF-b1, CD31 and decreased the expressions of TNF-a. also improve collagen deposition (Kant *et al.*, 2020) .

Quercetin which can prevent free radicals from impairing low -density lipoproteins. Therefore, Quercetin (lipid peroxidation inhibitor) useful against cancer cells In addition to the chemoprevention feature of Quercetin in free radical scavenging, it also shows anti -cancer property by the growth inhibition and pro -apoptosis effect against cancer cell models and tumors (Ezzati *et al.*, 2020) .

C / gallic acid

A naturally occurring gallic acid , is highly antioxidant and may play a protective role in healthy individuals by inhibiting apoptosis.

Pharmacological agents containing gallic acid and of diverse therapeutic categories as antioxidants, anticancer, antimicrobial, chondro-protective effect, carbonic anhydrase inhibitors, antidiabetic activity, anti-ulcerogenic, (Zahrani *et al* ., 2020),The attention on gallic acid is due to its medicinal efficacy as antioxidants, Because antioxidant providing favorable environment for tissue healing (Singh *et al* ., 2020) .

D / Others constituent

The MO leaves are also established as a rich source of omega-3 and omega-6 polyunsaturated fatty acids (PUFAs), in the form of a-linolenic acid , and linoleic acid . Palmitic acid is recorded in the major saturated fatty acid, accounting for 16–18 % of the total fatty acids in the Moringa leaves . (Idris Maizuwo, 2017) . Calories, proteins, fats, carbohydrates, but especially vitamins and fiber, are greatly concentrated in dried M.O leaves (Trigo *et al.*, 2021).

Extract of leaf showed a concentration-dependent increase in glutathione level and a decrease in malondialdehyde level (Ayon Bhattacharya *et al* ., 2018).

1.11.2.2 Therapeutic activities

1-Antibacterial activity

Numerous investigations have recommended that different extracts from various tissues of MO showed antibacterial properties against both gram-negative and gram-positive bacteria ,fresh leaf juice against Pseudomonas aeruginosa and Staphylococcus aureus aqueous leaf extract against S. aureus, Vibrio cholerae, and Escherichia coli (Dhakad *et al.*, 2019).

2-Antifungal activity

Leaf extract of MO has the ability to inhibit the growth of Saprophytic Fungi (Ayanbimpe *et al.*, 2009).

3- The aqueous extract of MO seeds and leaves has massive potential to inhibit the tumor progression without affecting the normal physiology and functioning of the experimental animal (mice) body and thus can be used as a cancer therapeutic agent (Barhoi *et al.*, 2021) .

4-Every part of plant M.O has been reported to exhibit pharmacological properties such as antioxidant, antidiabetic, anti-obesity, anticancer, hepatoprotective, nephroprotective, neuroprotective, antibacterial, and antiviral activities (Singh *et al.*, 2020).

1.11.2.3 Effects on the reproductive system

Leaf extract showed a significant increase in the weight of testis, enhances seminiferous tubule, epididymis, testis and seminal vesicle the antioxidants present in the leaves of the plant, acting in concert with the antioxidant system present in the epididymis further preserved and enhanced the process of spermatogenesis also it is proved that the sperm cytoplasm contained very low concentrations of scavenging enzymes therefore an increase in the antioxidant enzyme system levels by Moringa treatment can favor the reproductive process. (Cajuday and Pocsidio, 2010) .

1.11.3 Propolis

Resinous material collected by bees from plants exudates and buds and mixed with wax and bee enzymes. It consisted of 30% beeswax, 50% resins and vegetable balsams, 10% essential oils, 5% pollen, and 5% other substances. Its color varies from green and red to dark brown. Propolis has a characteristic smell and shows adhesive properties because it strongly interacts with oils and proteins of the skin.(Abu-Seida, 2015) .

Indeed, it is a wax-like, resinous substance used by honeybees as building and sealing material as well as a defensive agent coating the inner walls of the beehive. Propolis is produced by a small number of worker bees by mixing plant resins (collected mainly in the afternoon on sunny days) with wax (Rojczyk et al., 2020) .

Propolis has a large spectrum of potential therapeutic bioactivities, including antioxidant, antibacterial, anti-inflammatory, neuroprotective, immunomodulatory, anticancer, and antiviral properties. As an antiviral agent, propolis and various constituents have shown promising preclinical efficacy against adenoviruses, influenza viruses, respiratory tract viruses, herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), human immunodeficiency virus (HIV), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) . Lethal Dose 50 of more than 300 mg/kg . (Yosri et al., 2021).

1.11.3.1 Chemical composition of propolis

Until now, more than 300 chemical constituents have been identified in propolis from different regions. The main chemical classes present in propolis are flavonoids, phenolics, and aromatic compounds. Propolis also contains some volatile oils, terpenes, and bee wax (Toreti et al., 2013) .

The percentage of diverse material present in propolis depends upon the time of its collection and also on the geographical origin (Anjum *et al.*, 2019) .

Table 1.2 chemical composition of propolis taken from Iraq AL Hilla (Georgeta Balica *et al* ., 2021)

Flavonoids	Kaempferol , chrysin, pinocembrin, galangin, quercetin, myricetin, rutin, rhamnetin
Terpenoids	terpineol, camphor, squalene, copaene, calarene, calamenene,
Amino acids	aspartic acid, glutamic acid, serine, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, tryptophane, asparagine
Vitamins	B1, B2, B3, B5, B6, C, E

1.11.3.2 Therapeutic application of propolis

1 - Role in wound healing

Propolis, which is well tolerated with rare incidents of allergy and no toxicity, is referred to as an excellent candidate for burn management, enhancing skin cell proliferation, activation, and growth capacity .Some results confirm the propolis therapeutic efficacy, throughout quantitative and qualitative analyses of collagen types I and III expression and degradation in wounds matrix, indicating that propolis could have favorable biochemical environment supporting re-epithelization Its suggested that propolis is able to quench free radicals in skin. This outcome of propolis on free radicals in the epidermis is the source of safety of its application in the therapy of burn wounds.Other findings reveal that propolis speed up the burned tissue repair by stimulation of the wound bed matrix remodeling, proposing that the observed changes in extracellular matrix content after propolis application may be connected with the ability of its flavonoid compounds to reduce lipid peroxidation and to prevent necrosis of cells (Martinotti and Ranzato, 2015) .

2- Anti-bacterial activity of propolis

Propolis has a significant effect against bacteria such as Enterococcus spp., Escherichia coli and Staphylococcus aureus The mode of action of propolis is due to the interaction between phenolic with other compounds such as pinocembrin, galangin, and pinobanksin It is show antibacterial activity against some aerobic bacteria such as, Bacillus cereus, B. subtilis, Enterococcus faecalis, Micrococcus luteus, Nocardia asteroids, Rhodococcus equi, Staphylococcus auricularis, S. epidermidis, S. capitis, S. warnerii, S. mutans, S. hominis, Streptococcus cricetus, St. faecalis, (Anjum *et al.*, 2019) .

Propolis contains diverse compounds with biological activity, in particular anthocyanins, flavonoids, tannins, saponins, terpenoids, polypeptides and lecithins. Among the biological activities attributed to propolis are antibacterial, antifungal, antioxidant and anti-inflammatory capacity (Ramón-sierra *et al.*, 2019) .

3-Hepatoprotective activity of propolis

Flavonoids have the ability to capture free radicals and inhibit lipid peroxidation , The biological activity of propolis is often associated with the presence of flavonoids , Phytochemical analysis of extracts from 40 active compounds in various kinds of propolis based on their region showed the presence of general constituents such as phenol, tannins , and flavonoids, These antioxidant compounds can be used to neutralize free radicals.(Sahlan *et al.*, 2021) .

4 – uses in covid 19

Caffeic acid phenethyl ester (CAPE), the major constituent of the Egyptian propolis, is one of PAK1 inhibitors which acts via the down regulation of RAC (a signaling protein in human cells). In other words, CAPE is capable of blocking viral infection including corona virus and preventing coronavirus-induced lung fibrosis (Hesham Refaat *et al* ., 2020) .

1.11.4. Vitamin C

Known as ascorbic acid or L-ascorbic acid, is a water-soluble vitamin with antioxidant properties that make it essential for skin health. Skin contains high concentrations of vitamin C (in total skin it ranges from 0.4 to 1 mg/100 g of wet-tissue weight) concentrated in the intracellular compartments, transported by blood vessels of dermal layer (Dattola *et al.*, 2020).

The body requires vitamin C for normal physiological functions. It helps in the synthesis and metabolism of tyrosine, folic acid and tryptophan, hydroxylation of glycine, proline, lysine carnitine and catecholamine. As an antioxidant, it protects the body from various deleterious effects of free radicals, pollutants and toxins (Chambial *et al.*, 2013).

Many infections lead to the activation of phagocytes, which release oxidizing agents referred to as reactive oxygen species (ROS). These play a role in the processes that lead to the deactivation of viruses and the killing of bacteria . However, many of the ROS appear to be harmful to the host cells, and in some cases they seem to play a role in the pathogenesis of infections . Vitamin C is an efficient water-soluble antioxidant and protect host cells against the actions of ROS released by phagocytes (Hemilä, 2017).

Vitamin C is a well-known natural antioxidant involved in all phases of wound healing, mainly in collagen formation phase . some studies report the participation of vitamin C in the immune system modulation so it has a role in respiratory system infection (Voss *et al.*, 2018) .

1.11.5. zinc

Zinc has a key role as a catalyst in a wide range of reactions . it is regulates gene expression; stabilizes cell membranes, helping to strengthen their defense against oxidative stress (Chasapis et al .,2020). it is participates in the synthesis, storage, and release of insulin; interacts with platelets in blood clotting; and influences thyroid hormone function. It is necessary for visual pigments; sperm production; fetal development; and behavior and learning performance. (Read *et al* ., 2019) .

Zinc directly protects the lipids and proteins of cellular membranes and thiol-dependent macromolecules (enzymes, tubulin, and microtubules) from oxidative damage. Zinc depletion results in an abnormal thiol redox state in cellular membranes producing increased osmotic fragility of RBC membranes and inactive membrane calcium channel proteins. (Cummings and Kovacic, 2009) .

There are multiple potential pathophysiologic pathways through which zinc deficiency may contribute to the development or worsening of heart failure, including increased oxidative stress, derangements of the cardiomyocyte extracellular matrix, and loss of cardiomyocytes. (Rosenblum *et al.*, 2020).

Zinc is hydrophilic and cannot permeate across the cytoplasmic plasma membrane and the membranes of intracellular compartments. Cellular and whole-body zinc levels are controlled by Zn2+ transporters (Lee, 2018).

study design

Part 1
Cytotoxicity assay

stock solution preparation :

plant extract | supplement

Tamarix mannifera 2000µg/ml	Moreingo oleifera 2000 µg/ml	Propolis 2000 µg/ml	Vitamin C 2000 µg/ml	Zinc Sulfate 2000 µg/ml

serial dilution was made

Tamarix mannifera 500-15.6µg/ml	Moreingo oleifera 500-15.6µg/ml	Propolis 500-15.6µg/ml	Vitamin C 500-1.95µg/ml	Zinc Sulfate 500-1.95 µg/ml

Treatment of 96 well plate cultured with MDCK cells line

Incubation 24 hr

MTT assay determine inhancement or inhibition

2- wound experiment

Cell seeded in 48 well plate

surface scratch

Treat and incubated with suitable concentration of

Tamarix manifera , moringa oleifera, propolis , vitamin C , zinc sulfate, combination vitamin c and zinc

Imaging of the wound healing process every 3hr Measurement of wound diameter by image software.

2. Materials&Methods

The experimental work was performed in the research laboratory at College of Medicine\ University of Babylon during the period from September 2021-April 2022.

2.1. Materials

2.1.1. Equipement

The equipement employed in the present work are listed in table (2.1).

Table (2.1) Equipment and Instruments Used In Study.

Equipment	Manufacturers/country(Origin)
Autoclave	Prestige medical, England.
Centrifuge	Hettich, Germany.
Cell culture plate (96- wells)	SPL Korea.
Cell culture flask (25ml)	SPL Korea.
Distiller	Griffin, England.
Electric oven	Memmert – germany
Eppendorf centrifuge 5702 RH	Eppendorf , Germany.
ELISA Reader	Human , Germany.
Freezer -20 °C	Mettler, Switzerland.
Incubator	Memmert, Germany.
Inverted microscope	T.C Meiji techno, Japan.
Laminar air flow cabinet	Labtech, Korea.
Magnatic stirrer	Scotech, Germany.
Micropipettes (different size)	Dragon-Med India.

Refrigerator	Arcelik, Turky.
Shaker	Denely, England.
Sensitive Balance	` Labtech, Korea.
Syringe 5 ml	MED , China.
Ultrasonic	Binder, Germany.
Vortex	Kottermann, Germany.
Water bath	Minilyotrap, England.
Warring Blender	National, Japan.
0.45 and 0.22 µM Millipore Nalgene filters	Biofilm , Australia.

2.1.2.Chemicals

Table (2.2) lists the chemicals used in the present study together with its origin and company.

Table (2.2)Chemicals Materials

Chemical materials	Manufacturers/ country (Origin)
De-ionized water	GFL, Germany
Dimethyl sulfoxid (DMSO)	GCC, England
Ethanol	Scharlau, Spain
Fetal bovine serum (FBS)	Capricorn Germany
Gentamycin (80 mg vial)	The Arab pharm. Salat, Jordan
Hepes buffer	Sigma ,USA

Liqud nitrogen	Clever, USA
MTT(3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) dye powder	Roth , Germany
Phosphate buffer saline (PBS)	USA
RPMI 1640 medium w/L-glutamine, 25mM HEPES (powder**)**	US Biological life science USA
Sodium bicarbonate	BDH, England
Trypsin EDTA	USA
Vitamin c	USA
Zinc sulfate	Germany

2.1.3. Cell line and Maintenance

The Madin-Darby Canine Kidney (MDCK) cells are a model mammalian cell line were purchased from LONZA Biologics (Slough, UK).

2.1.3.1 Cell thawing

One cryotube of the frozen cells was taken from the liquid nitrogen tank and thawed in a 37 ºC water bath. The vial was removed from the water before the ice floccule dissolved completely, wiped with 70% alcohol. Without delay, the cell suspension content of the vial was pipetted under laminar flow cabinet into a 15 ml sterile plastic centrifuge tube containing

10 ml of pre-warmed growth medium. Centrifugation was done at 800 rpm for 10 min and the supernatant was aspirated and decanted. The cells pellet was re-suspended into 5ml warm fresh growth media with 10% FBS and transferred into 25 ml size cell culture flask incubated at 37C° and the growth media replaced on the next day (Way and Bc, 2015).

2.1.3.2 Subculture of cells line

After the cells became confluent as monolayer, subculture was done according to the protocol mentioned by (Meleady and O'Connor, 2006).

1-Cultures were viewed using an inverted microscope to assess the degree of confluence and confirm the absence of bacterial and fungal contamination.

2. Spent medium was removed.

3. The cell monolayer was washed with PBS without Ca2+, Mg2+ using a volume equivalent to half the volume of culture medium. This wash step was repeated if the cells were known to adhere strongly.

4. Trypsin- EDTA (Ethylene diamine tetra acetic acid) was pipetted into the washed cell monolayer using 1ml per 25cm^2 of surface area. Flask was rotated to cover the monolayer with trypsin. The excess trypsin was decanted.

5. Flask returned to the incubator and left for 2-10 minutes.

6. The cells were examined using an inverted microscope to ensure that all the cells are detached and floating. The side of the flasks may be gently tapped to release any remaining attached cells.

8. The required number of cells was transferred to a new labeled flask containing pre-warmed medium.

9. The cell line was incubated at 37C°.

10. This process was repeated as demanded by the growth characteristics of the cell line(Verma *et al* ., 2014).

2.1.3.3 Harvesting of cells

Harvesting is a technique that uses the proteolytic enzyme trypsin, to detach and disaggregate the adherent monolayer cells from the bottom of the culture vessel. This procedure was performed whenever the cells need to be harvested for passage and cell count And it include the following steps:

1. When the cell growth reaches a monolayer and before the exponential phase, the medium was aspirated and discarded.
2. The cells were washed with 3ml of warm PBS solution.
3. About 1ml of warm trypsin EDTA solution was added to cover the monolayer, and with gentle rocking of the flask dish 4-5 times to flood the monolayer.
4. The flask dish was incubated at 37°C until the monolayer was detached.
5. Once the cells detached, the flask was removed from incubator and complete separation of the cells was done by rocking the flask from side to side.
6. The cells were gently pipetted up and down to disrupt cell clumps into single cells.
7. Cells were counted and re-suspended into the desired number in a growth medium with 5-10% FBS, which was used to inactivate trypsin.
8. The cells were sub cultured into two flasks or cultured on culture plate.

2.1.3.4 Freezing of cells line

Cell lines source were kept frozen at -196 C^0 in Nitrogen tank according to the following protocol:

1. Tissue culture flask with a monolayer near the exponential phase was taken and washed twice with 5 ml of PBS, then 3 ml of warm trypsin was added. Halve of the trypsin volume was decant.
2. The flask was incubated at 37 C° until the cell layer detached and the cells was aided to disaggregate into single cells by gentile rocking on the flask sides.
3. The flask content was transferred into 15 ml sterile plastic centrifuge tube. Centrifugation was done at 800 rpm for 10 minutes.
4. The supernatant was decanted and the cell pellet was re-suspended with 1 ml of the freezing media and transferred into 1.5 ml sterile freezing vial.
5. The vial kept for 10 minutes at room temperature and transferred to –80C° deep freezer for 24 h and then stored for a long time in the liquid nitrogen tanked after one minute (Yang *et al.*, 2019) .

2.2 Methods

2.2.1.Preparation of Reagents and Solutions

2.2.1.1. Phosphate Buffer Saline (PBS):

According to the Gibco manufacturer manual, the PBS was prepared by dissolving only one tablet in 500 ml of Deionized Distilled Water (DDW) with continuous stirring by a magnetic stirrer at room temperature resulting in a PH value of 7.45 without need for adjustment. Autoclaving is required for complete sterilization and stored in a closed bottle until use to keep sterile .

2.2.1.2 MTT Solution:

Principle The general purpose of the MTT assay is to measure viable cells in relatively high throughput (96-well plates) without the need for elaborate cell counting. Therefore, the most common use is to determine the cytotoxicity of several drugs at different concentrations. The principle of the MTT assay is to detect the cellular mitochondrial activity of the viable and thereby an increase or decrease in the number of viable cells is linearly related to the mitochondrial activity. The mitochondrial activity of the cells is reflected by the conversion of the pale-yellow tetrazolium salt (MTT dye) into dark purple formazan crystals by NADH (Figure 2.1) which can be solubilized for homogenous measurement. Thus, any increase or decrease in viable cell number can be detected by measuring formazan concentration that reflected in the measurements of optical density (absorbance) using a plate reader at 570 nm. The darker the solution, the greater the number of viable and metabolically active cells.

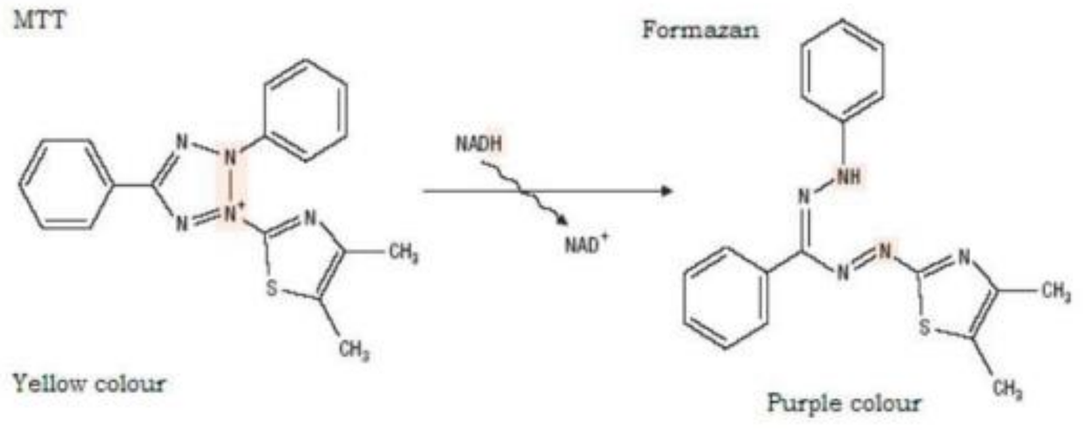


Figure 2.1: Principle of MTT Assay (Sukhramani et al., 2011) .

MTT powder (0.5 gm) was dissolved in PBS (100 ml) to achieve 5 mg/ml concentration. A 0.2 μm millipore filter was utilized to sterilize the MTT solution and stored in a sterile and light protected bottle. The solution was stored at 4°C of temperature for multiple uses or at -20°C of temperature for long storage(Johan van Meerloo and Gertjan J.L. Kaspers, 2003) .

Procedure (Meerloo et al; 2011):

1- At the end of the drug exposure period, the medium was removed from the

wells and then the cells were washed with PBS. A blank control was carried to

assess unspecific formazan conversion.

2- A volume of 1.2 ml of MTT solution (5 mg/ ml) was added to 10.8 ml medium to obtain final concentration of 0.5 mg/mL. Then, 200 µl of the resulting solution was added in each well.

3- The plate was incubated for 3 hours at 37°C until intracellular purple formazan crystals were visible under the inverted microscope.

4- The supernatant was removed and 100 µl DMSO was added in each well to

dissolve the resultant formazan crystals.

5- The plate was incubated at room temperature for 30 minutes until the cells have lysed and purple crystals have dissolved.

6- Absorbance was measured by a microplate reader at 570 nm.

The absorbance reading of the blank must be subtracted from all samples.

Absorbance readings from test samples must then be divided by those of the control and multiplied by 100 to give percentage cell viability or proliferation.

Absorbance values greater than the control indicate cell proliferation, while lower values suggest cell death or inhibition of proliferation. Percent of cell viability or percent of inhibition was calculated by the following formula:

$$\% \text{ viability} = (AT - AB) / (AC - AB) \times 100\%$$

Where, AT = Absorbance of treated cells (drug).

AB = Absorbance of blank (only medium).

AC = Absorbance of control (untreated).

% Inhibition = 100 – % viability

2.2.1.3. Gentamycin Stock Solution:

A gentamycin vial of 40 mg/ ml solution was considered as a stock solution and stored at a temperature of 4 C° until use.

2.2.1.4. Trypsin-(EDTA) Solution:

As indicated by US Biological headings, a weight of 10.1 gm of trypsin-EDTA powder and dissolving in 0.9 Liter of double distilled water (DDW) with continuous mixing at room temperature. 7.2 of PH value should be reached and complete the volume to 1 Liter by DDW, the solution was sterilized through using Millipore filters of 0.45 and 0.22 µm respectively, after that, the solution was kept at (- 20C°) of temperature (Roth, 2009).

2.2.2 Preparation of Tissue Culture Medium:

2.2.2.1: Preparation of Serum-Free Medium:

Liquid RPMI-1640 medium was prepared from RPMI-1640 powder medium according to Gibco product manual as following:

From the RPMI-1640 powder medium, 10.43 g was dissolved in approximately 900 ml of DDW in a volumetric flask with addition of The other components include: 2 g sodium bicarbonate powder or according to need and 1.25 ml from gentamycin stock solution with continuous stirring. The volume was completed by DDW to one liter and the pH of the medium adjusted to 7.4. Sterilization was done by 0.4 and 0.2 μm millipore filters subsequently.

2.2.2.2. Preparation of Serum-Medium:

Medium with serum was prepared as described in the preparation of serum-free medium in (2.2.2.1) in addition to 10 percent of fetal bovine serum.

2.2.2.3. Preparation of Freezing Medium:

The freezing medium was prepared from the following compositions: 6 ml serum-free medium, 3 ml FBS, and 1 ml DMSO. The solution was stored at (- 20) C° temperature between uses (Meleady and O'Connor, 2006)

2.2.3 Collection and preparation of plant sample

2.2.3.1 Preparation of Tamarix Mannifera extracts and stock solution

Plant under study was collected from different places in Hilla governorate, as leaves of Tamarix mannifera, which diagnosed by Dr. Beyda Rasheed Hilo/College of Agriculture/AL-QASIM Green University, the plant washed with distilled water and dried under shadow to prevent effect of sun on phytochemicals , then was grounded and the powder was kept in a container until use. By using sensitive digital weighing balance, weight 20gm from the powder of the plant and put it in Pyrex beaker with 100ml of boiling deionized water, then left for 30 min (Oreopoulou *et al* ., 2019) Then the beaker was put on the heater over night at 40 °C with continuous magnetic stirring, after that the mixture was centrifuged at 2500 rpm for 10 min. The supernatant was passed through a Whatman filter paper and left over night, after that we put the extract in the oven at 40-45°C **.**Extract has been weighed and dissolved in suitable amount of deionized water in order to obtain the final stock concentration (2000µg\ml), which was sterilized by using Millipore filter 0.22µm (Misganaw *et al* ., 2019).

2.2.3.2 Preparation of Moringa Oleifera extracts and stock solutions

Fresh leaves of Moringa oleifera plant were collected from small plant nursery, in Hilla city- Iraq. The plant leaves were washed with deionized water thoroughly and left to dry for 14 days in dark place at room temperature, then grinded to fine powder and stored in tight dry container for further use.

The plant leaves then soaked and macerated in concentration of 100 gm of leaf powder in 1 liter of DDW and left for extraction at room temperature for 48 hours.

Aqueous extract is filtered many times using medical gauze and the separated extract is then filtered using Whitman filter paper and then centrifuged at 1000 rpm for 10 min to dispose the settled waste and obtain clear liquid, left to dry to obtain solid extract.

Preparation of stock solution for aqueous extract was made by dissolving 60 mg of aqueous extract in 30 ml DDW to obtain final concentration of 2000 µg / ml then filtered using milipore filter syringe to discard any impurities .

2.2.3.3 Preparation of propolis extracts

Propolis sample was collected from honey bees hives in Hilla City during the spring season of 2020. The propolis sample was cleaned and cut into small pieces and stored in a clean container for preparation of (EEP) ethanol extracted propolis .

To prepare ethanol extracted propolis, 10 grams of propolis mixed with 100 ml of 96% ethanol alcohol in dark-bottle and left for 10 days with shaking (4-5) times per day at 37°C. Then the supernatant was collected and the insoluble fractions were separated by filtration using filter paper and evaporate the ethanol by an oven at 45-50ºC. After that, the EEP ethanolic extract propolis was weighted and stored in a dark container and made a concentration of 2000 µg/ml of EEP to be use in this research after filtration by milipore filter paper of (0.22µm).

2.2.3.4. Preparation of Zinc Sulfate and vitamin C stock solution

Seventy mg of pure powder of each one was solubilized in 35 ml deionized water for the preparation of stock solution in small flask to get a stock solution with a concentration of 2000 µg/ml .

2.3.The Experiments

2.3.1. Cytotoxicity assay :

2.3.1.1. Study the effect of Tamarix mannifera on cell viability of MDCK cell line .

Pilot study was done inorder to choose the appropriate concentrations Then Ninety six -well plate were seeded with MDCK cell line in a concentration of $5*10^5$ cells. 3 replicates was considered as a control group, and the three subsequent columns were treated with Tamarix mannifera aqueous extract in serial dilutions of (500, 250,125, 62.5, 31.25, 15.6 µg/ml), three replicate for each concentration. Then the plate was covered with a self-plastic lid and incubated once for 24 hours. then the wells were washed with 200 µl of sterile PBS. The effect of the tammarix mannifera on the growth of the MDCK line was assessed by MTT assay.

2.3.1.2.Study Effect of Moringa oleifera on cell viability of MDCK cell line .

Same procedure done but treated with serial dilutions of Moringa oleifera aqueous extract (500, 250,125, 62.5, 31.25, 15.6 µg/ml) three replicate for each concentration. Then the plate was covered with a self-plastic lid and incubated once for 24 hours the wells were washed with 200 µl of sterile PBS. The effect of the Moringa oleifera on the growth of the MDCK line was assessed by MTT assay.

2.3.1.3. Study Effect of propolis on cell viability of MDCK cell line

Same procedure done but treated with propolis in serial dilutions of (500, 250, 125, 62.5, 31.25, 15.6 µg/ml) used in same procedure and cell viability assessed with MTT assay.

2.3.1.4. Study Effect of zinc sulfate and vitamin c on cell viability of MDCK cell line in different concentrations .

The 96-well plate were seeded with MDCK cell line in a concentration of $5*10^5$ cells , and treated with zinc and other for vitamin C in serial dilutions of (500, 250, 125, 62.5, 31.25, 15.6 , 7.8 , 3.9 , 1.95 µg/ml) 3 replicate for each concentration. The effect of the zinc and vitamin c on the growth of the MDCK line was assessed by MTT assay.

2.3.2.Cell Culture Preparations and Wound Scratch In Vitro

1- The required number of cells to form a confluent monolayer were placed in a 48-well plate for 100% confluence in 24hours.

2- Place the culture plate inside the incubator until a confluent monolayer was formed.

3-In a sterile environment (typically a biosafety cabinet) a scratch was made by using 200µl sterile plastic micropipette tip to press firmly against the cell monolayer of tissue culture plate and swiftly make a vertical wound down through the cell monolayer(Grada et. al.,2017).

4- After creating the scratch, the cells were washed with phosphate buffer saline solution to remove cell debris, before treating with different concentrations of plants extraction .

5- The wound was treated with a serial dilution of each substance at 200µl final volume in three replicate.

6- Two hundred microliter cultured media was also used to treat wound and considered as positive control.

7- Following the generation and inspection of the wound an initial picture was taken. The tissue culture plate was placed in an incubator set at the appropriate temperature (37C°).

8-after 3hours, the plate was removed from the incubator and placed under an inverted microscope to take a snapshot picture and to check for wound closure.

2.3.3.Measurement of wound

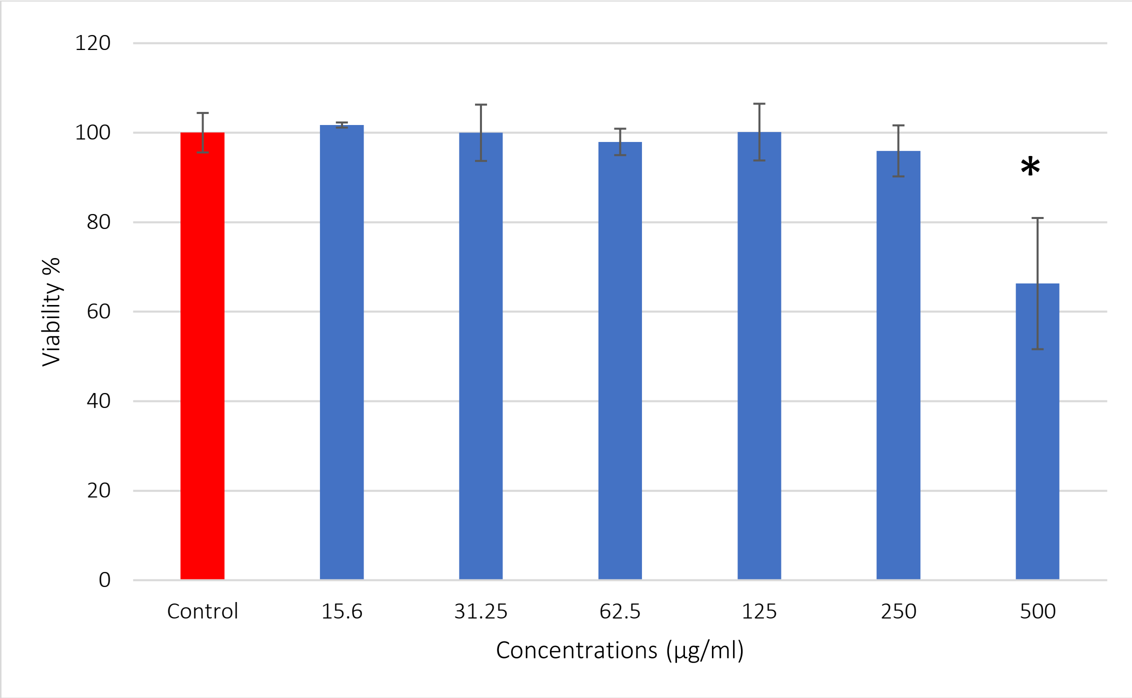
Snapshot method was used to document the cell migration by taking sequential digital photograph of the gap using camera (Lenovo q30). Fixing the plate under inverted microscope and the wound area were digitally photographed in morning at 0 hr ,3h,6h,9h,and. Measured by using software “image J” (National Institutes of Health, Maryland, USA) (Schneider et. al., 2012).

2.4 Statistical Analysis

All data were collected and analyzed by Microsoft Office Excel 2016 and Sigma plot version 12.5 software. ANOVA one way test used to assess significant differences among the means of data of cells viability where the p-values ≤ 0.001 and ≤ 0.05 considered statistically highly significant and significant respectively.

3.1 Effect of Tamarix Mannifera aqueous extract on the viability of MDCK cell line :

Result showed that there were no significant difference in cells viability percent between all concentrations of T. mannifera extract in comparism to control group , except the highest one 500 μg/ml which cause a significant decrease ($p \leq 0.001$) in the viability percentage as shown in (Fig3.1):



Figure(3.1): Cells viability percentage of MDCK cell line measured by MTT assay at different concentrations of Tamarix mannifera aqueous extract after incubation for 24 hours

3.2 Effect of Tamarix Mannifera aqueous extract on wound healing

The result showed there were a significant healing ($p \leq 0.001$) in all concentration at 6 , 9 hr in comparison to control group and decrease wound diameter , also complete healing after 3 hr at 7.8 µg / ml as shown in (Fig3.2A) and documented in fig (3. 2 B), (3.2C) , (3.2D) , (3.2E), (3.2F) .

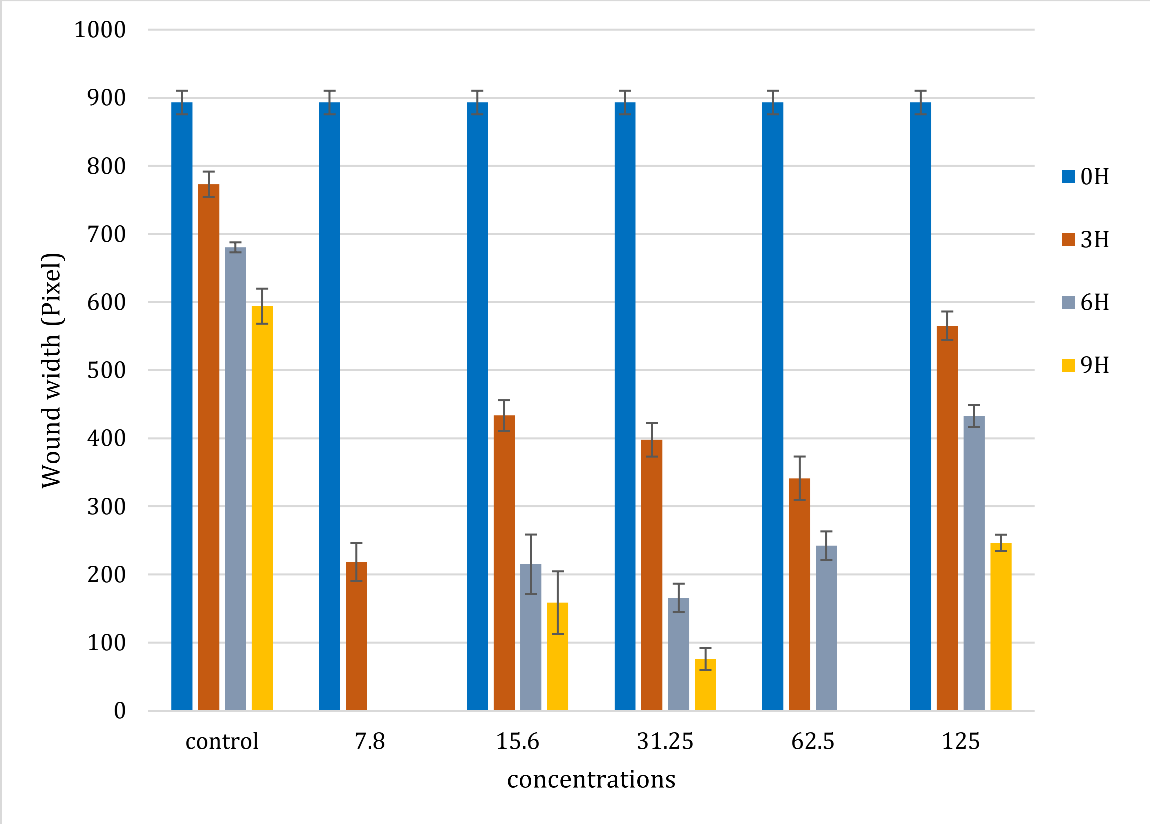
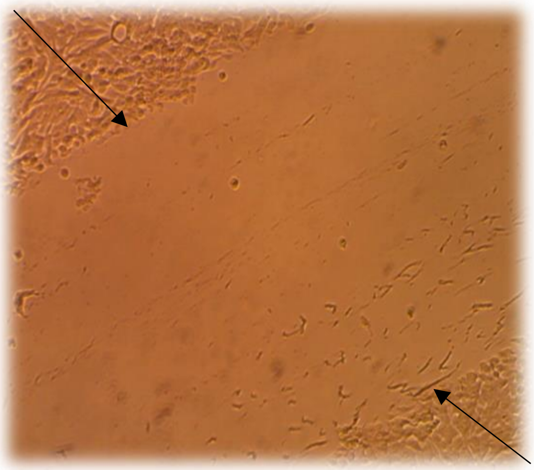


Figure (3.2A) : Effect of Tamarix mannifera aqueous extract at different concentration on wound diameter after incubation for different periods.



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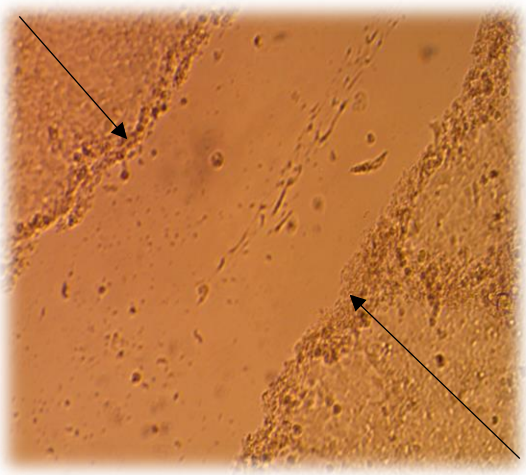

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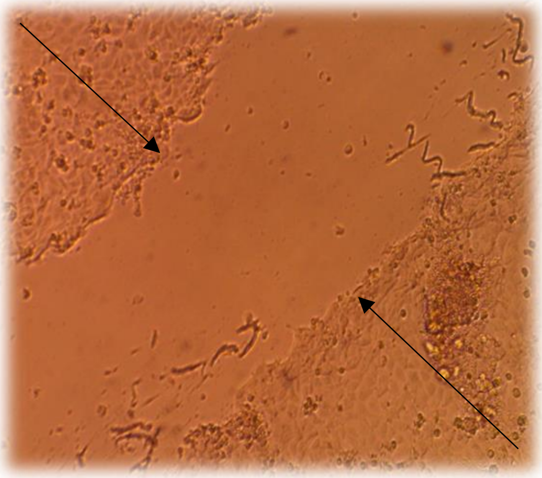

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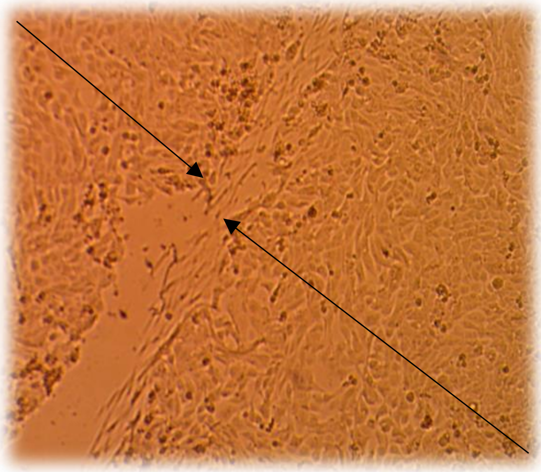

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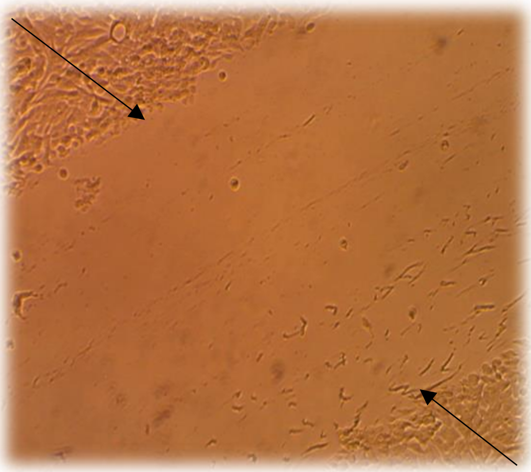
Figure (3.2B): Inverted Microscope (10XLence) Image of MDCK cell line Treated with Tamarix Mannifera (125µg/ml) after incubation for different periods.



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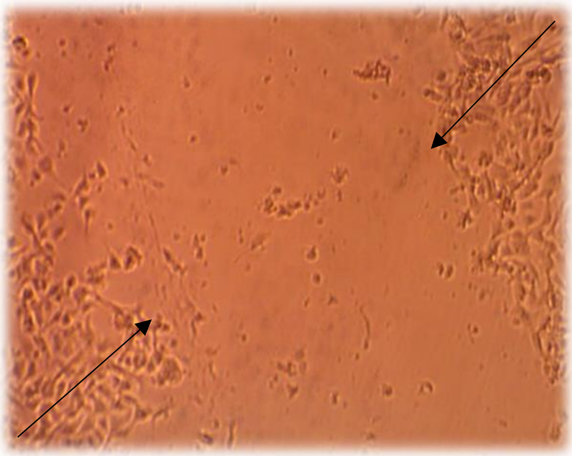

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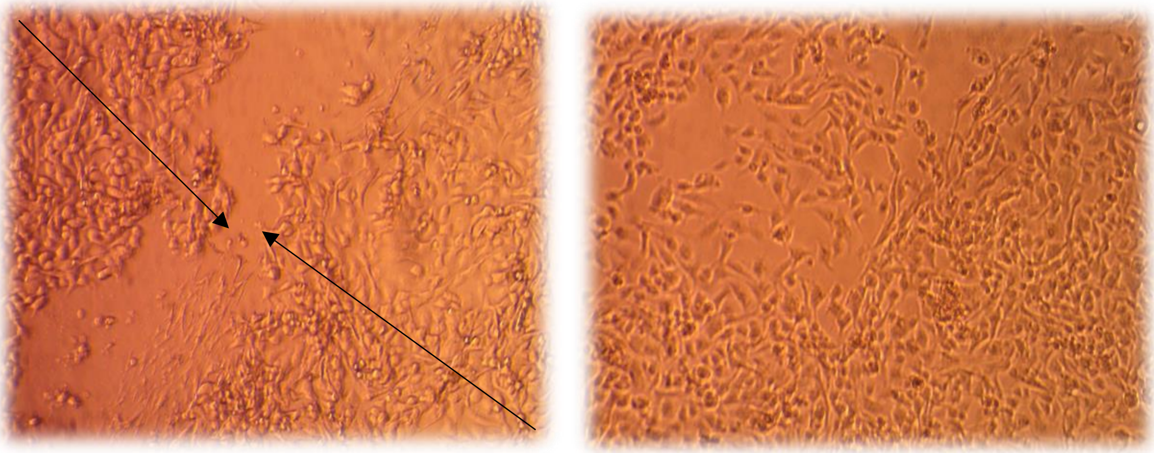
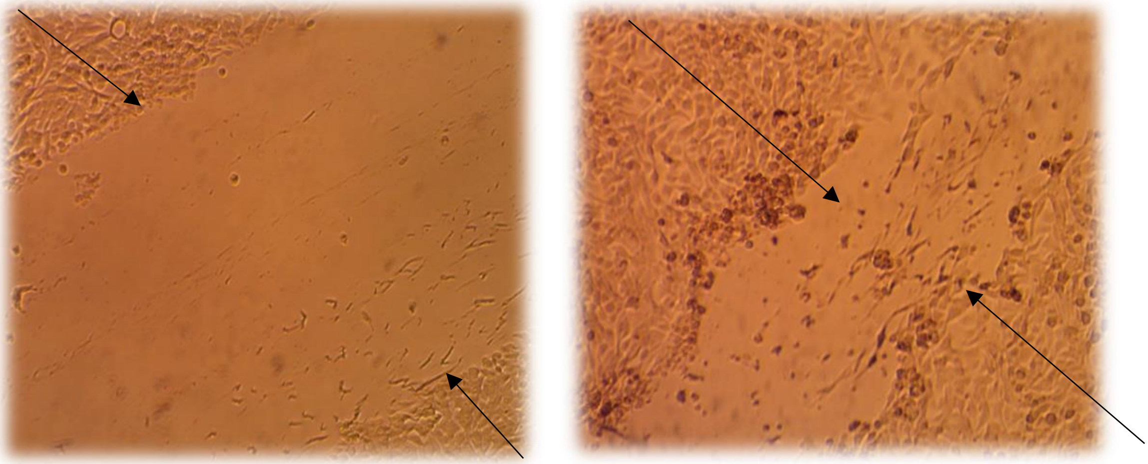
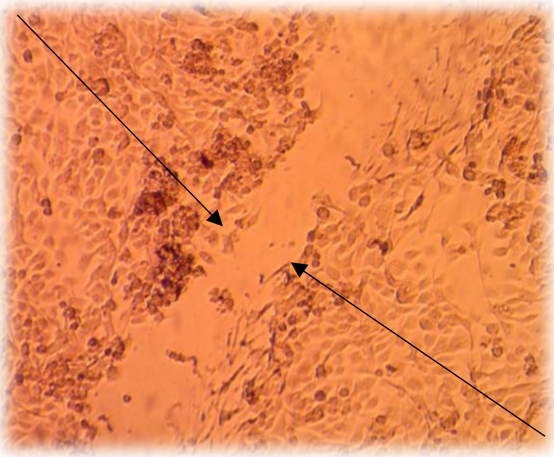


6hr **9hr**

Figure (3.2C): Inverted Microscope (10XLence) Image of MDCK cell line Treated with Tamarix Mannifera (62.5 µg/ml) after incubation for different periods.



0hr **3hr**



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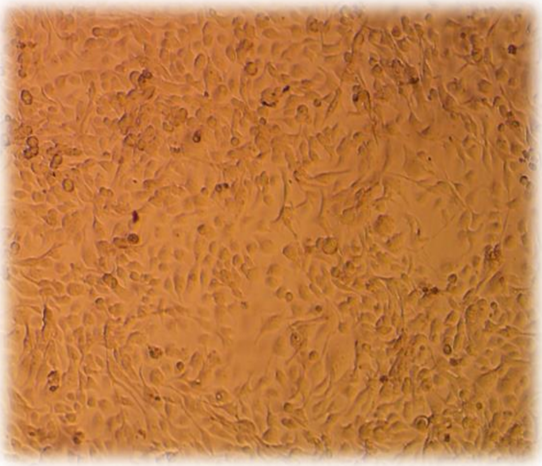

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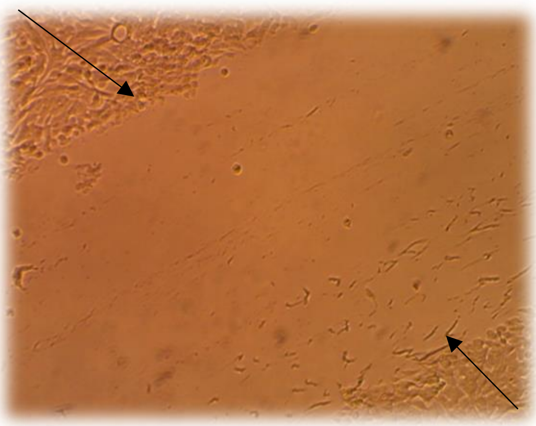
Figure (3.2D) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with Tamarix Mannifera (31.25 µg/ml) after incubation for different periods.



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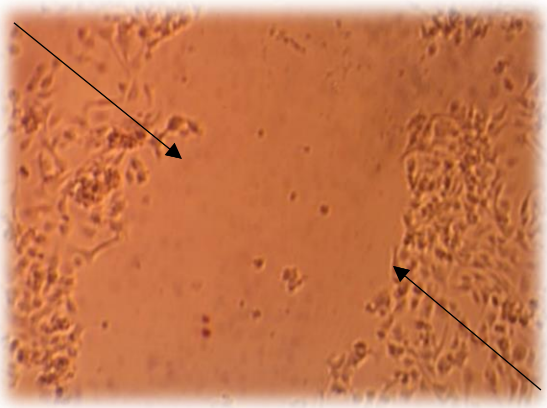

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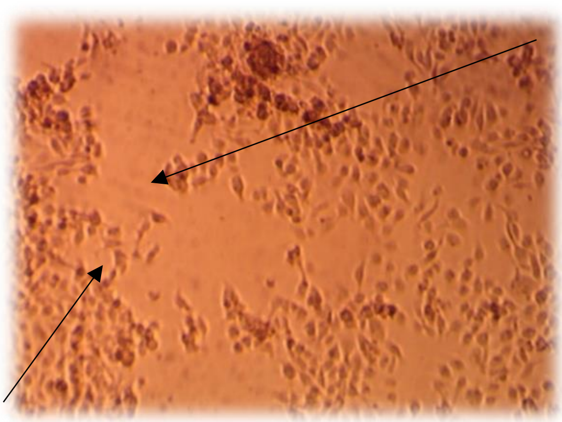

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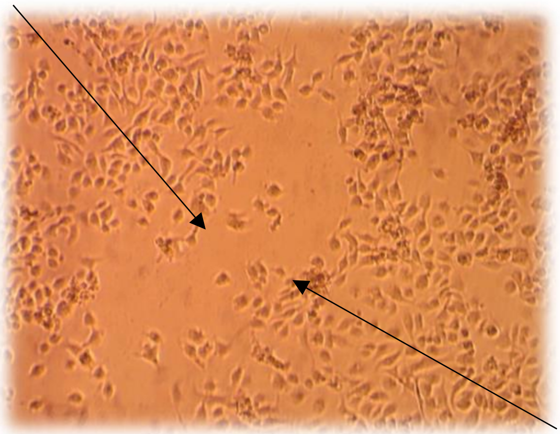

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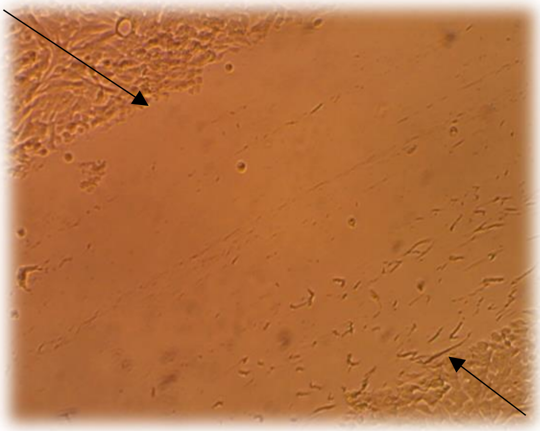
Figure (3.2E): Inverted Microscope (10XLence) Image of MDCK cell line Treated with Tamarix Mannifera (15.6 µg/ml) after incubation for different periods.



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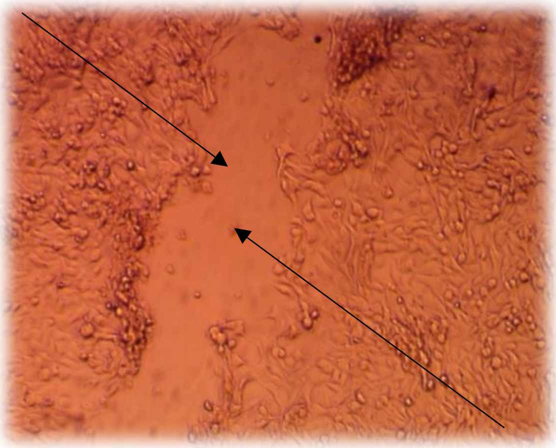

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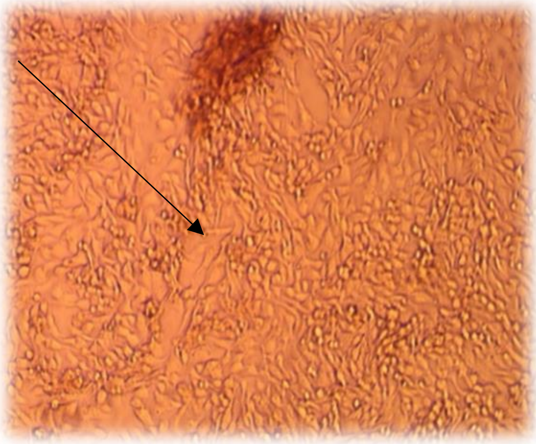

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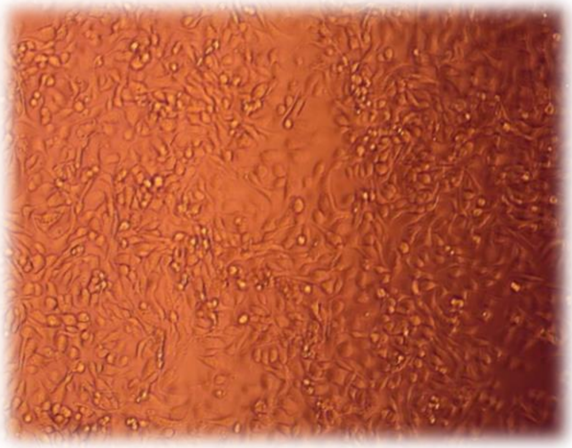
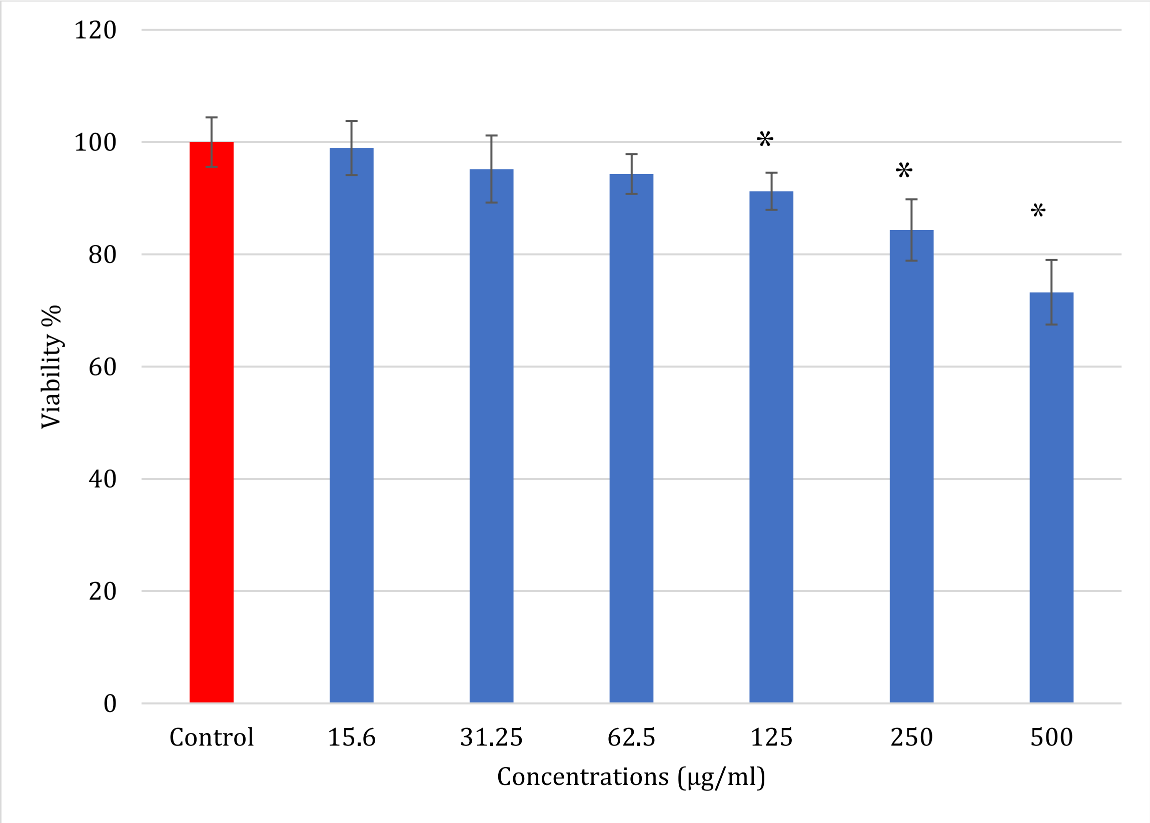


9hr

Figure (3.2F) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with Tamarix Mannifera (7.8 µg/ml) after incubation for different periods.

3.3 Effect of Moringa Oleifera aqueous extract on viability of MDCK cell line :

the concentrations (500 , 250 , 125 µg/ml) of Moringa Oleifera extract showed significant decrease ($p \leq 0.001$) in the cell viability percentage in comparism to control group as shown in (Fig 3.3) after incubation period 24 hr



Figure(3.3): Cells viability percentage of MDCK cell line measured by MTT assay at different concentrations of Moringa Oleifera aqueous extract after incubation for 24 hours

3.4 Effect of Moringa Oleifera aqueous extract on wound healing.

The result showed there were a significant healing ($p \leq 0.001$) in all concentration at 9 hr in comparison to control group and decrease wound diameter after 3hr as in (fig 3.4A) and documented in fig (3.4B) , (3.4C) , (3.4D) and (3.4E)

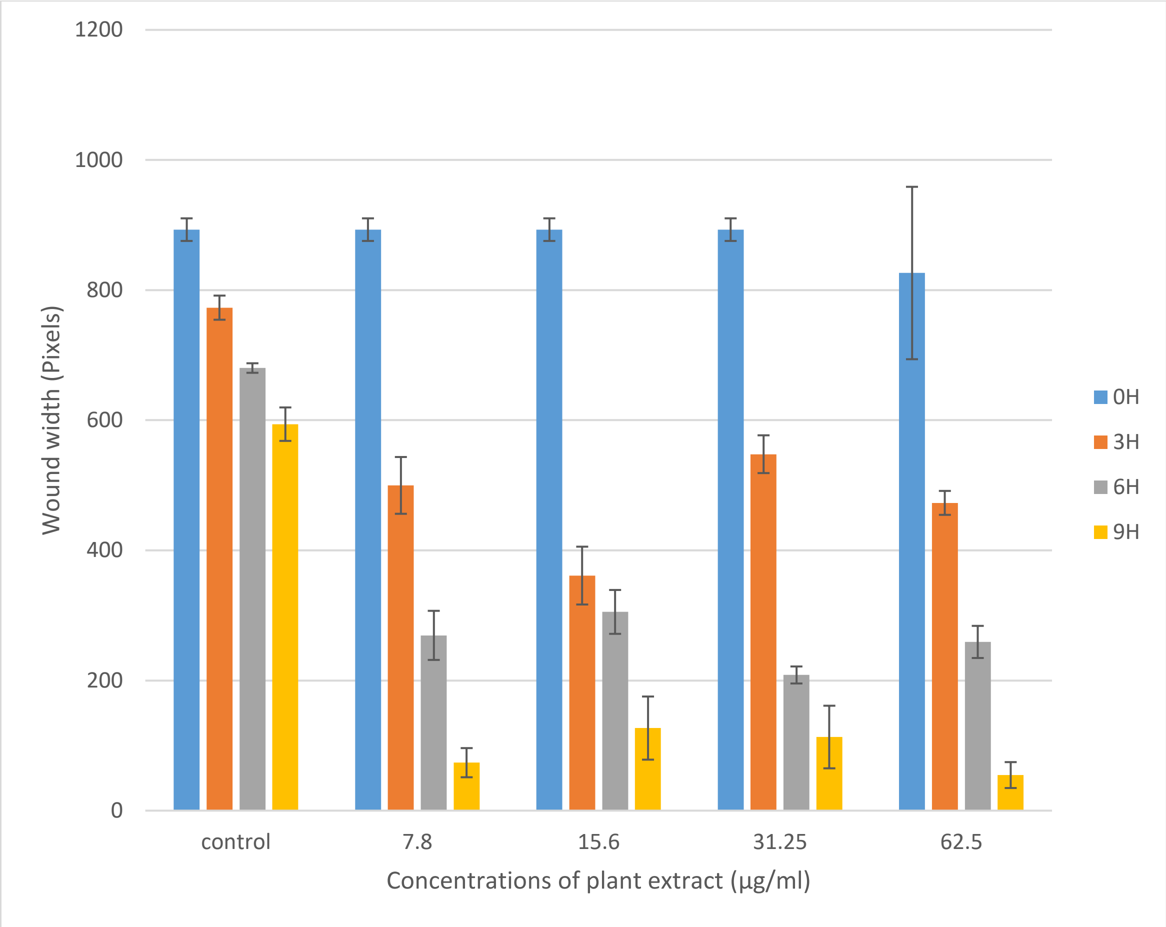
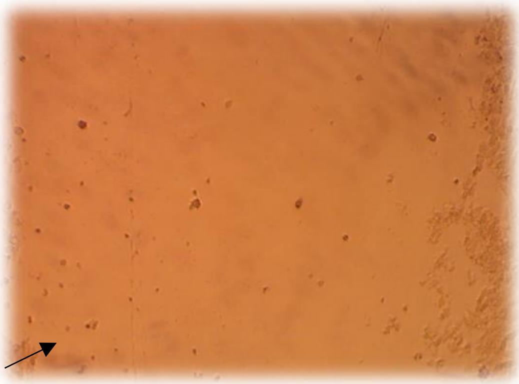


Figure (3.4A) : Effect of Moringa Oleifera aqueous extract at different concentration on wound diameter after incubation for different times.



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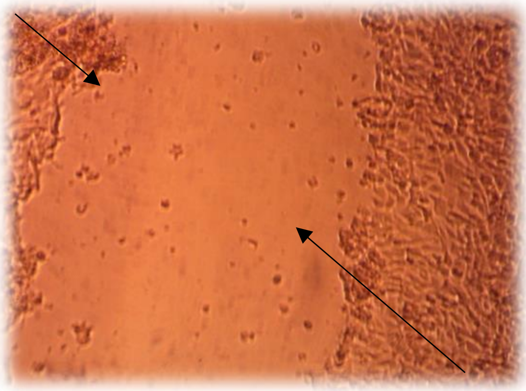

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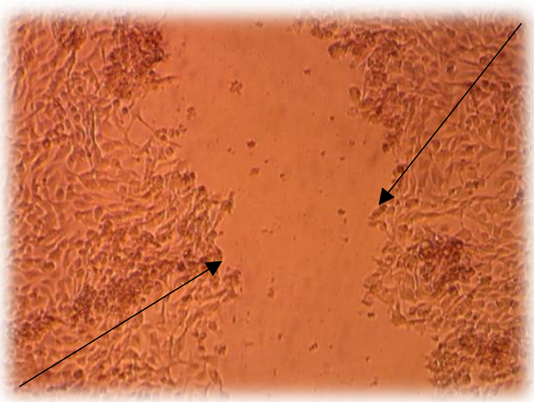

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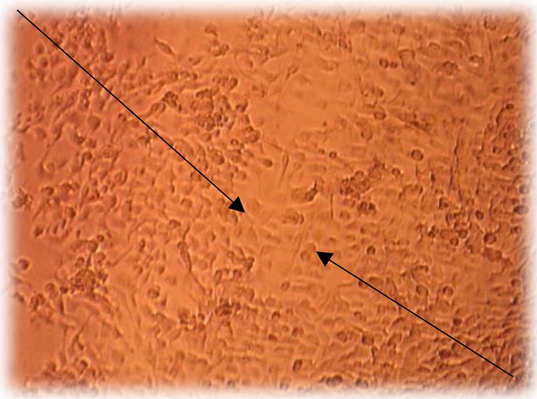
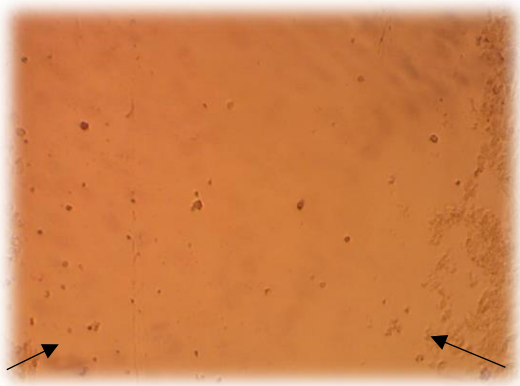


9hr

Figure (3.4 B) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with Moringa oleifera (62.5µg/ml) after incubation for different periods.



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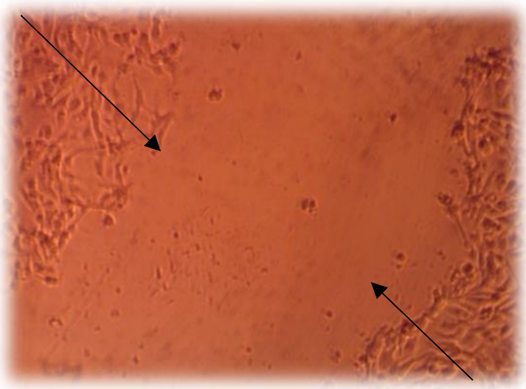

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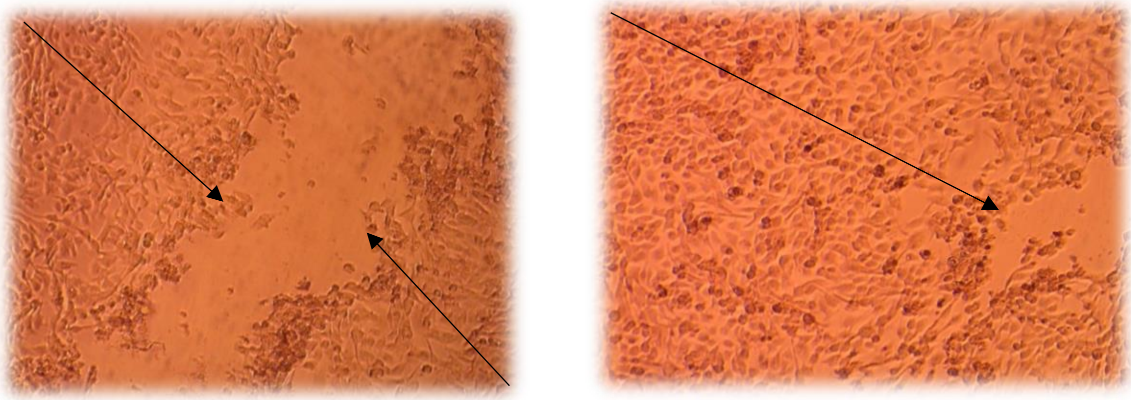
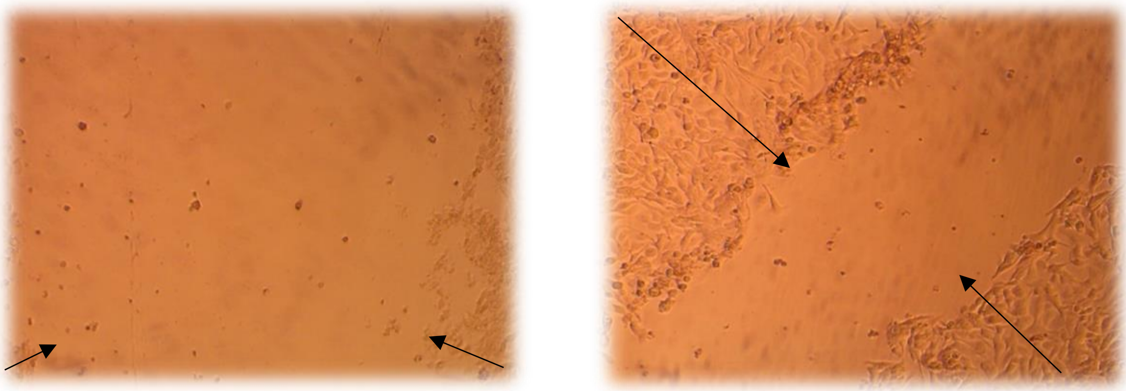


Figure (3.4 C) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with Moringa oleifera (31.25µg/ml) after incubation for different periods.



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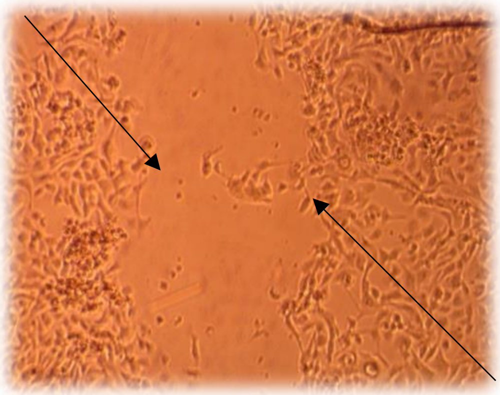

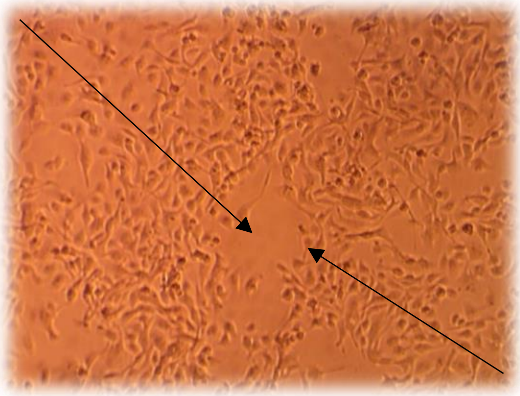

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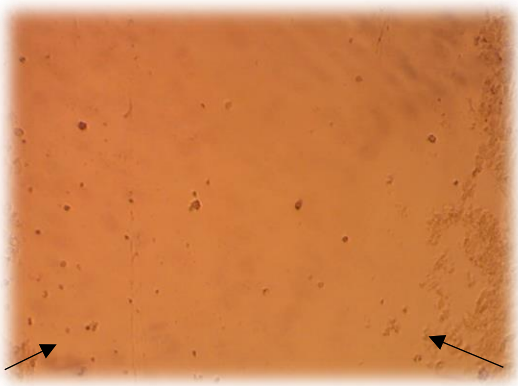
Figure (3.4 D) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with Moringa oleifera (15.6 µg/ml) after incubation for different periods.



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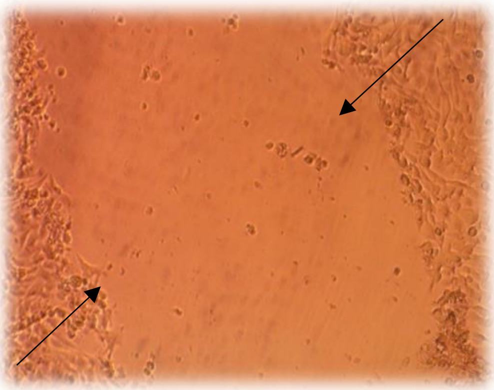

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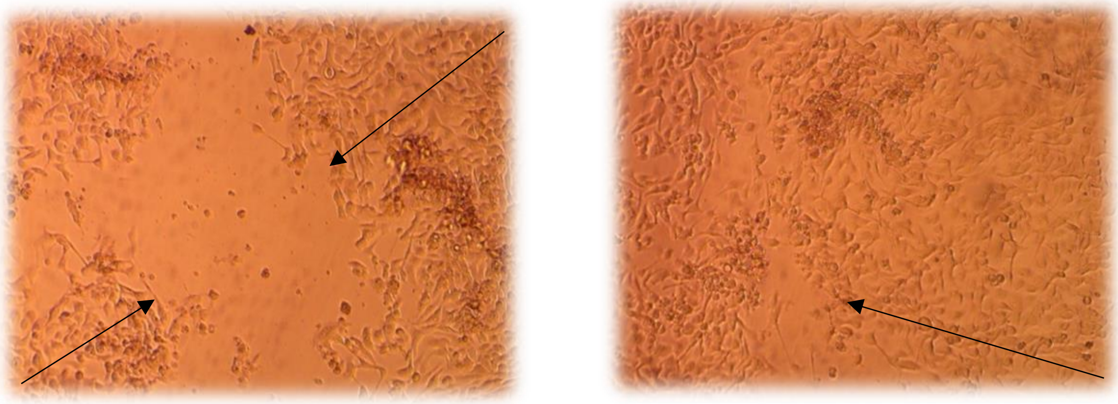

Figure (3.4 E) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with Moringa oleifera (7.8 µg/ml) only after incubation for different periodS.

3.5 Effect of Propolis alcholic extract on viability Of MDCK cell line:

There were no significant difference in cells viability after using of propolis between concentrations of propolis alcholic extract except the concentrations (500, 250, 125µg/ml) which cause a significant decrease ($p \leq 0.001$) in the viability percentage in comparism to control group as shown in (Fig 3.5)

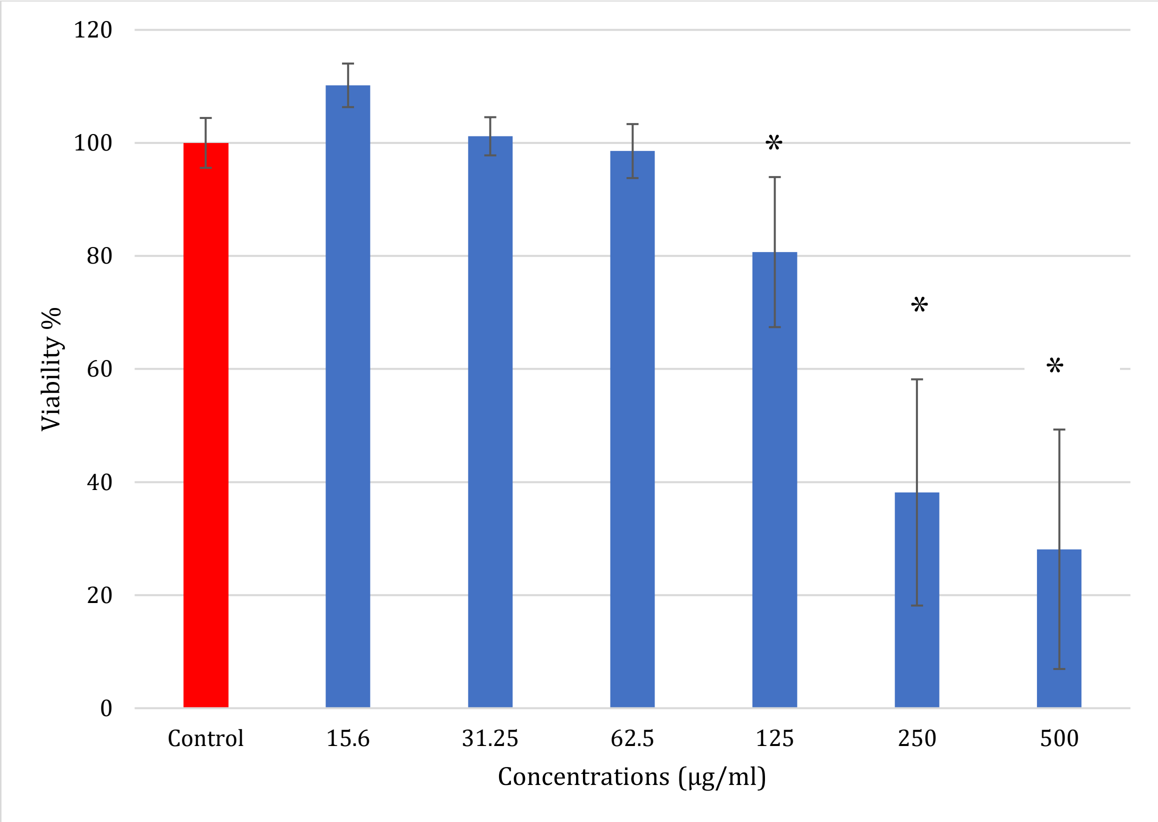

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Figure (3.5): **Cells viability percentage of MDCK cell line measured by MTT assay at different concentrations of propolis alcholic extract after incubation for 24 hours**

3.6 Effect of propolis alcholic extract on wound healing

The result showed there were a significant healing ($p \leq 0.001$) in all concentration at 9 hr in comparison to control group and decrease wound diameter after 3 hrs at low concentrations as showen in (fig3.6A) and documented in fig (3.6B) , (3.6C) , (3.6D), (3.6E) , (3.6F) ,(3.6G), (3.6H) , (3.6I) and (3.6J)

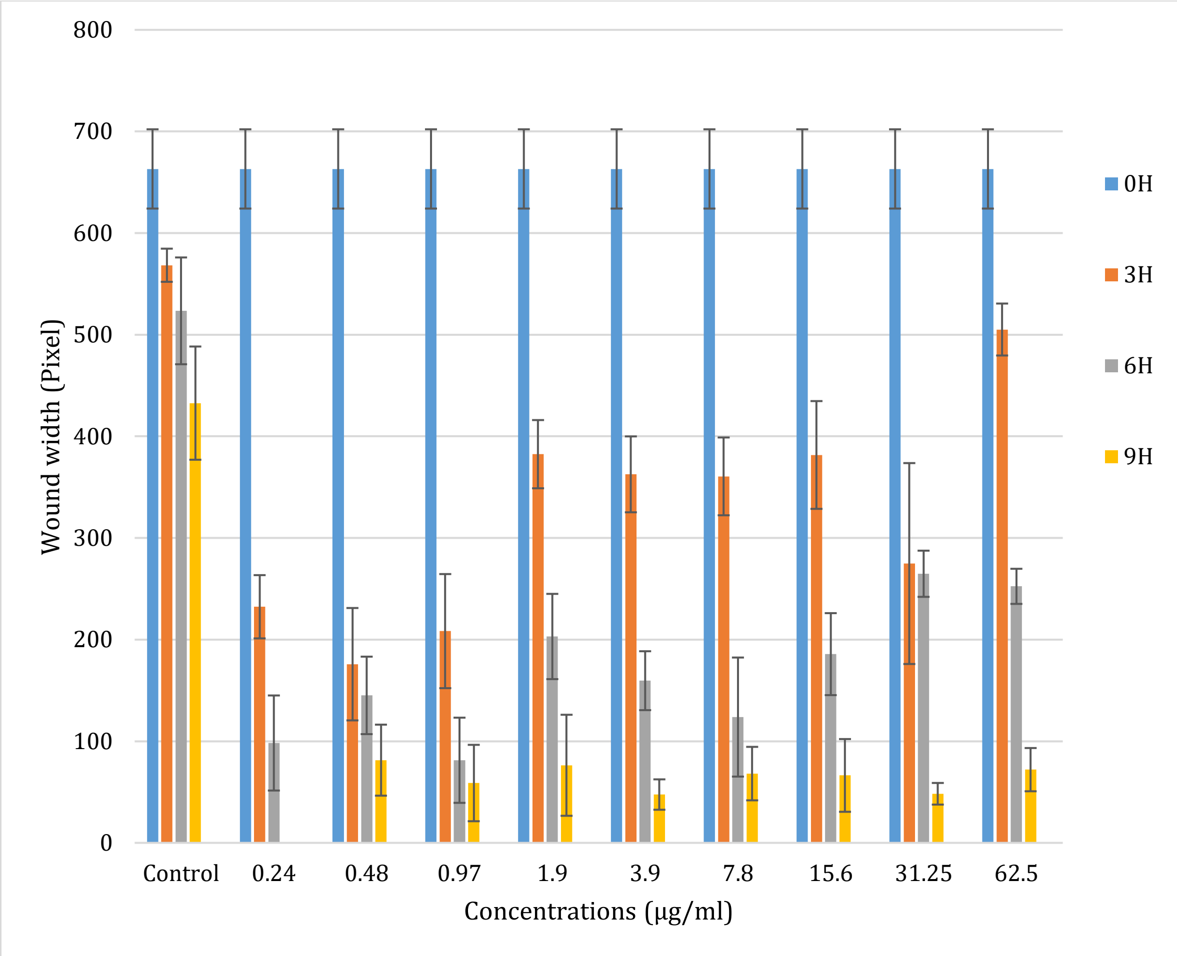
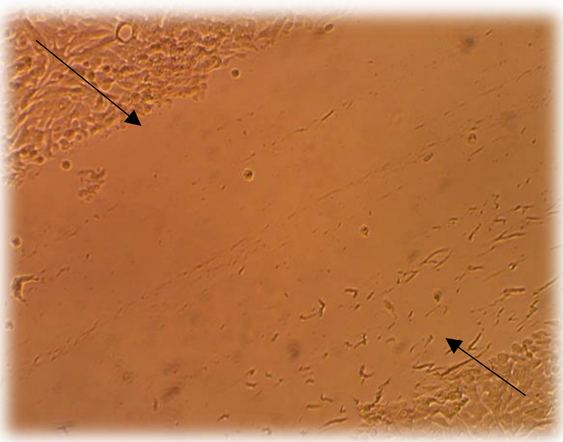


Figure (3.6A) :Effect of Propolis alcholic extract at different concentration on wound diameter after incubation for different periods.



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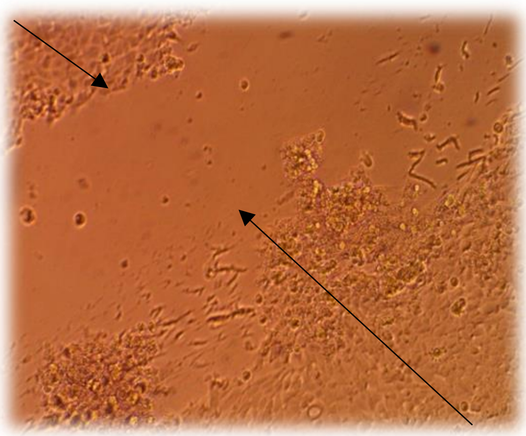

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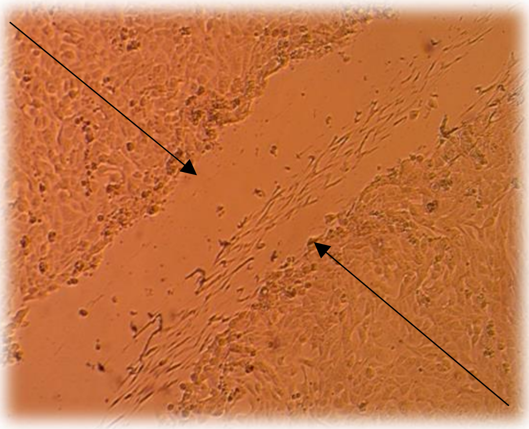

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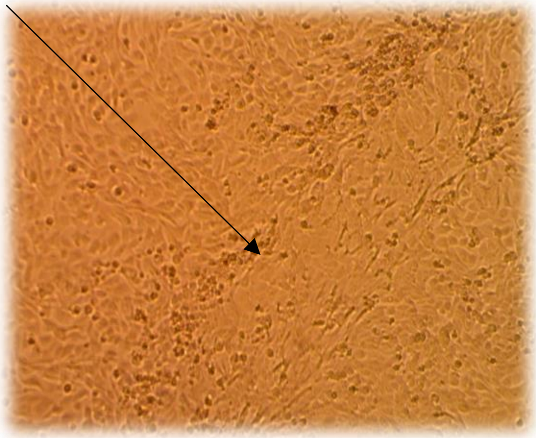

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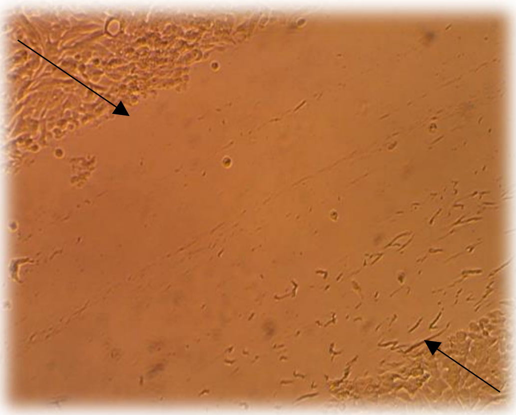
Figure (3.6B): Inverted Microscope (10XLence) Image of MDCK cell line Treated with propolis (62.5µg/ml) after incubation for different periods.



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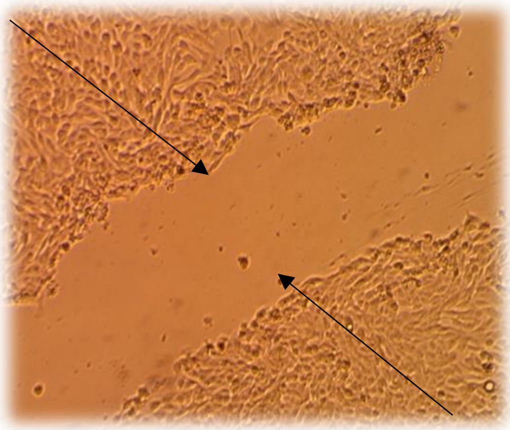

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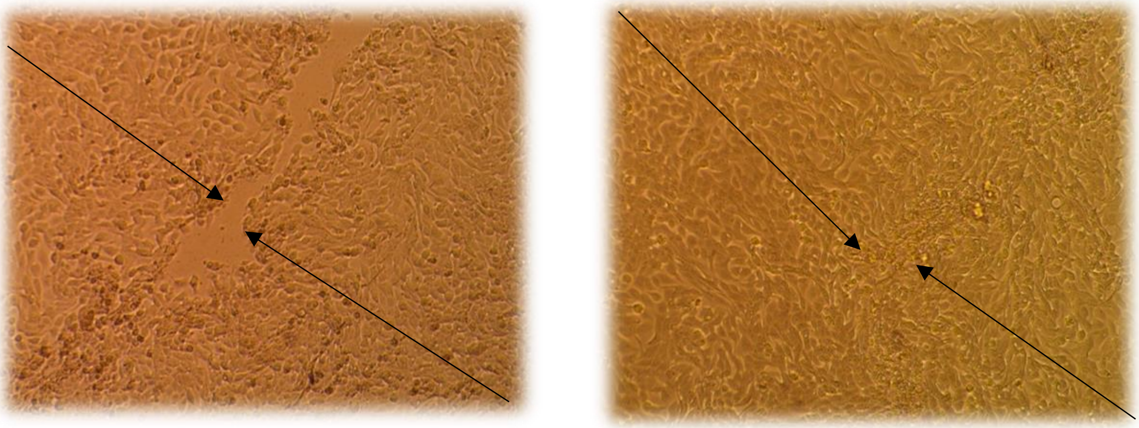
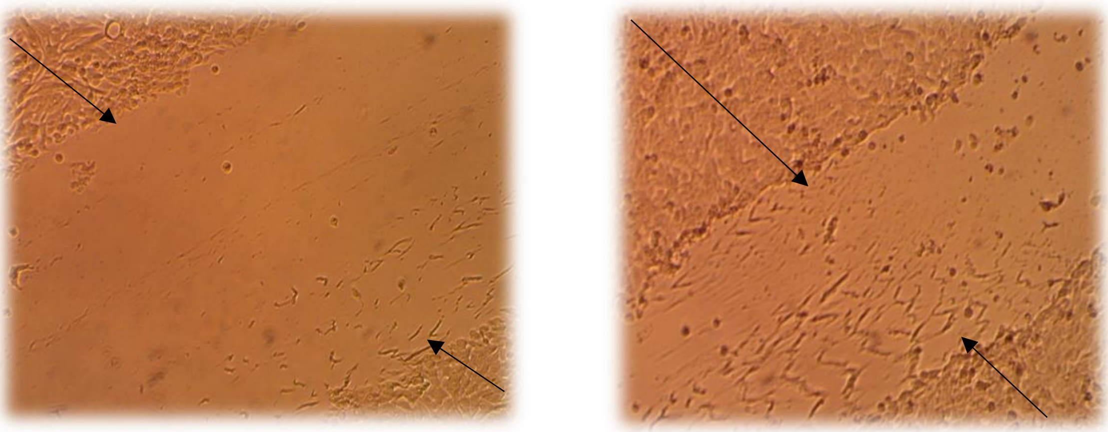
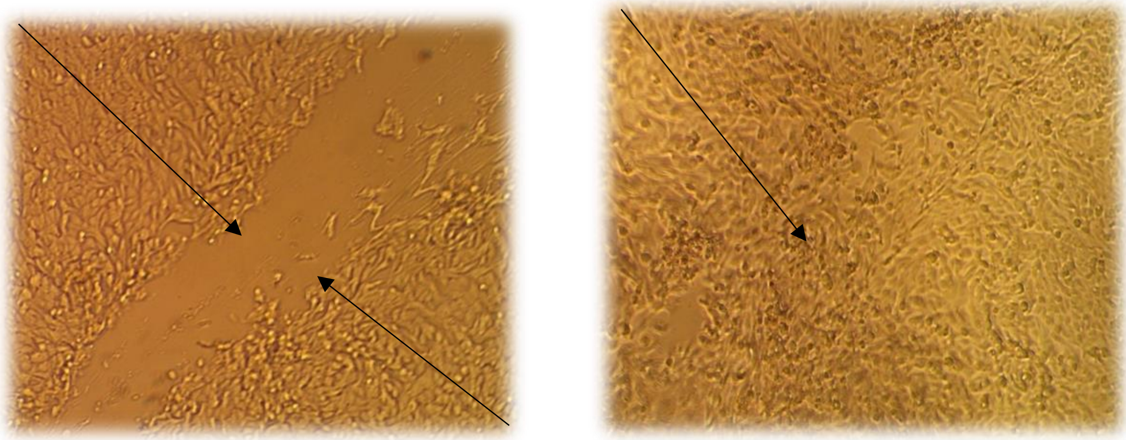


6hr **9hr**

Figure (3.6C): Inverted Microscope (10XLence) Image of MDCK cell line Treated with propolis (31.25 µg/ml) after incubation for different periods.

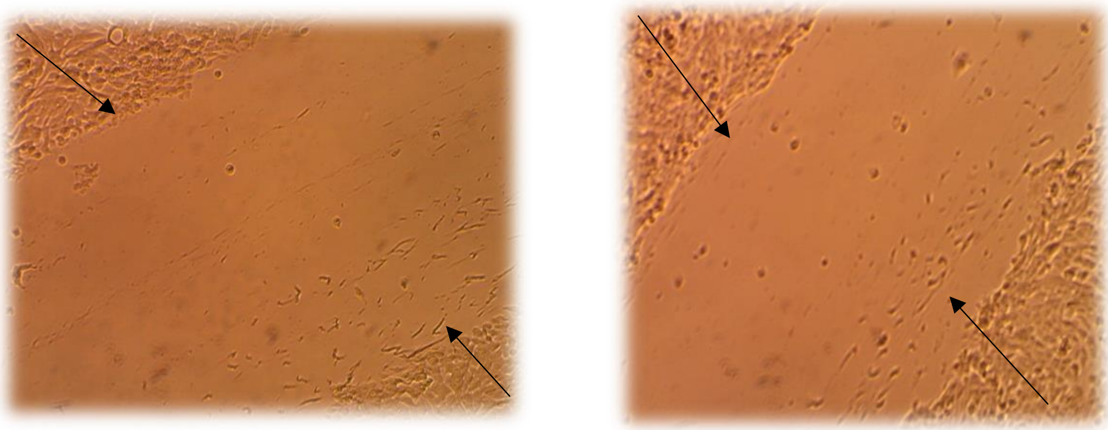


0hr **3hr**



6hr **9hr**

Figure (3.6D) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with propolis (15.6 µg/ml) after incubation for different periods.



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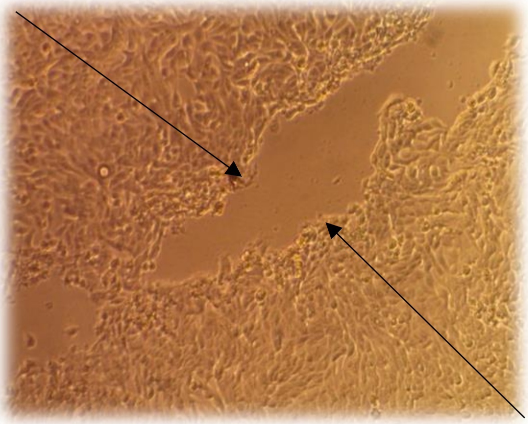
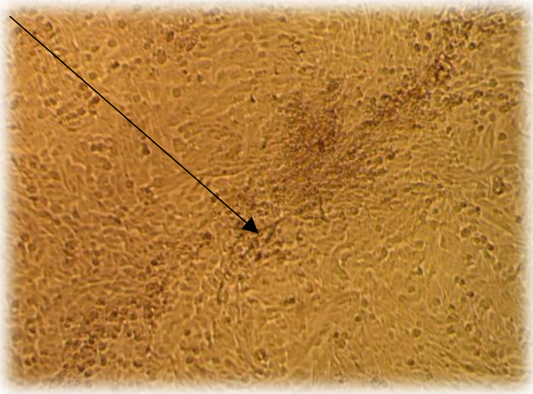
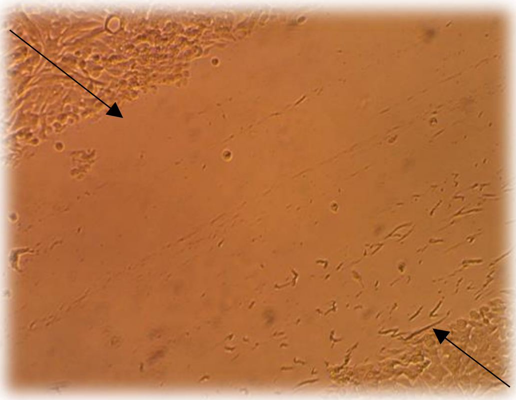
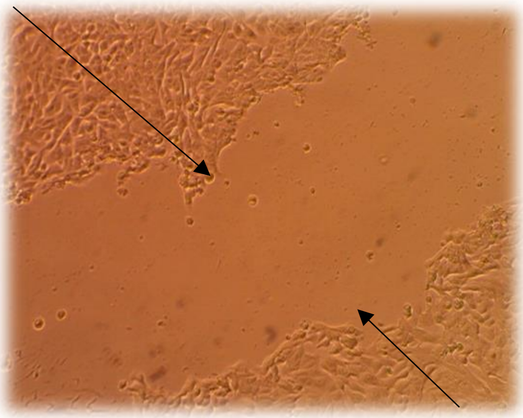




6hr **9hr**

Figure (3.6E) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with propolis (7.8 µg/ml) after incubation for different periods.





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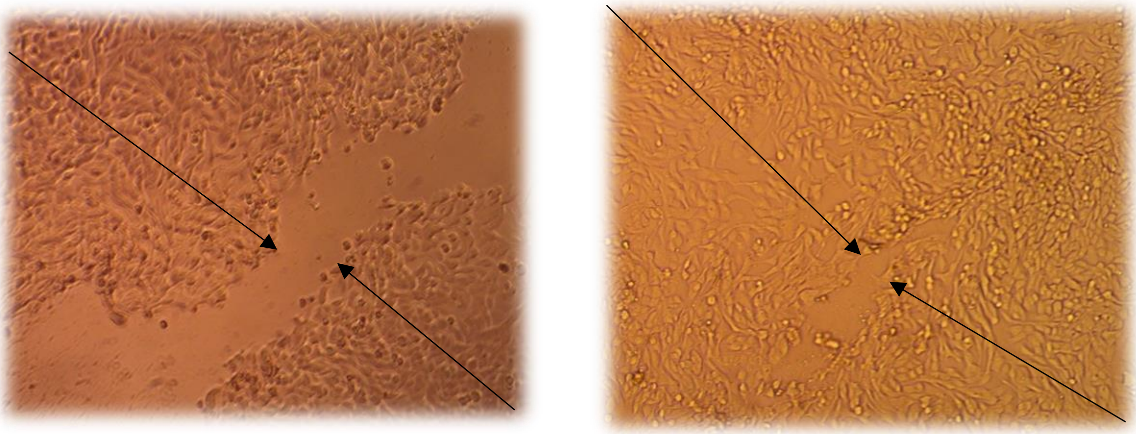
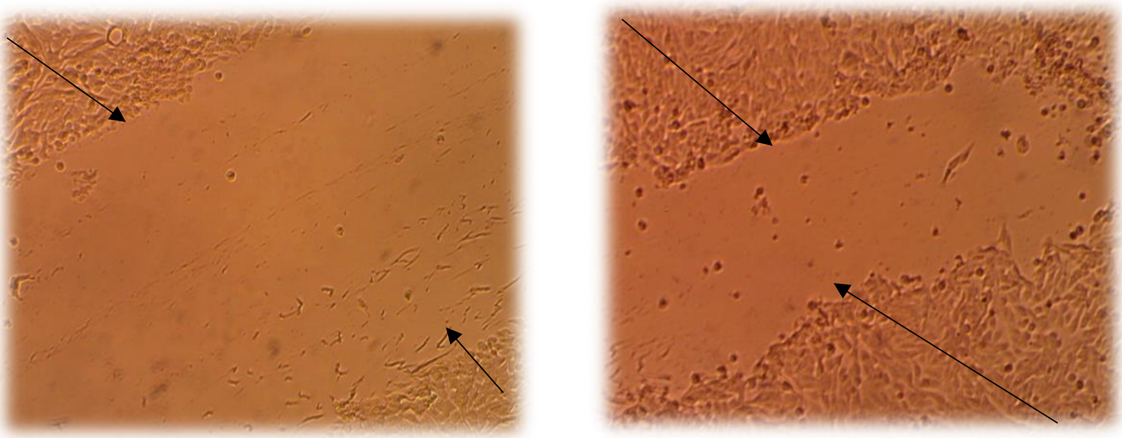
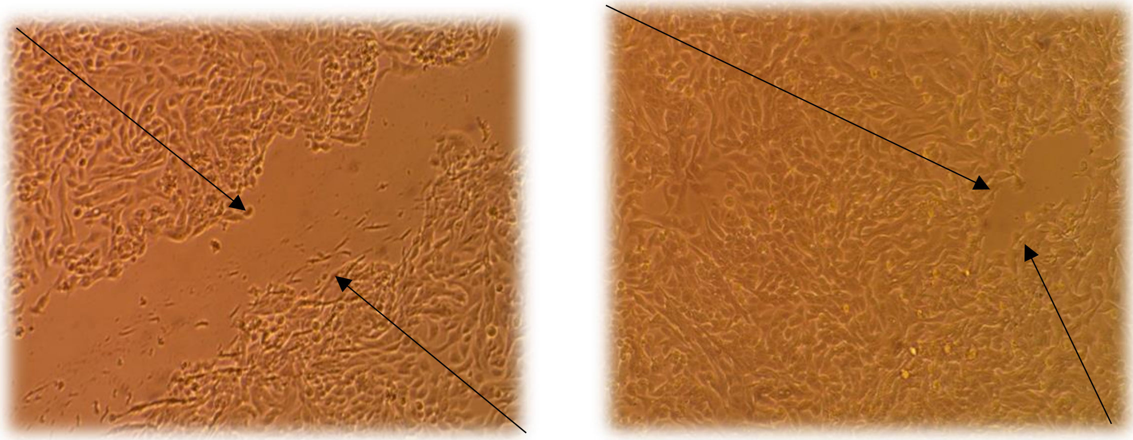


6hr **9hr**

Figure(3.6F) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with propolis (3.9µg/ml) after incubation for different periods.

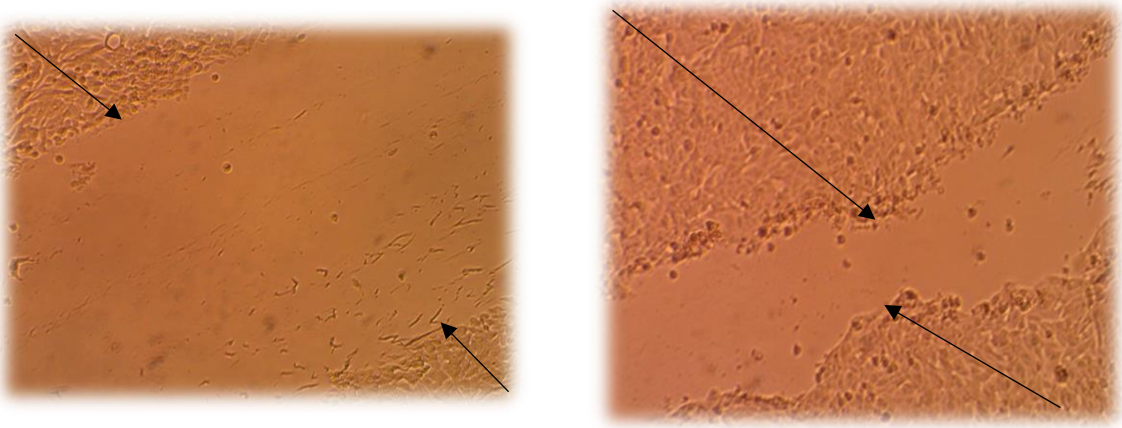


0hr **3hr**



6hr 9hr

Figure (3.6G) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with propolis (1.95µg/ml) after incubation for different periods.



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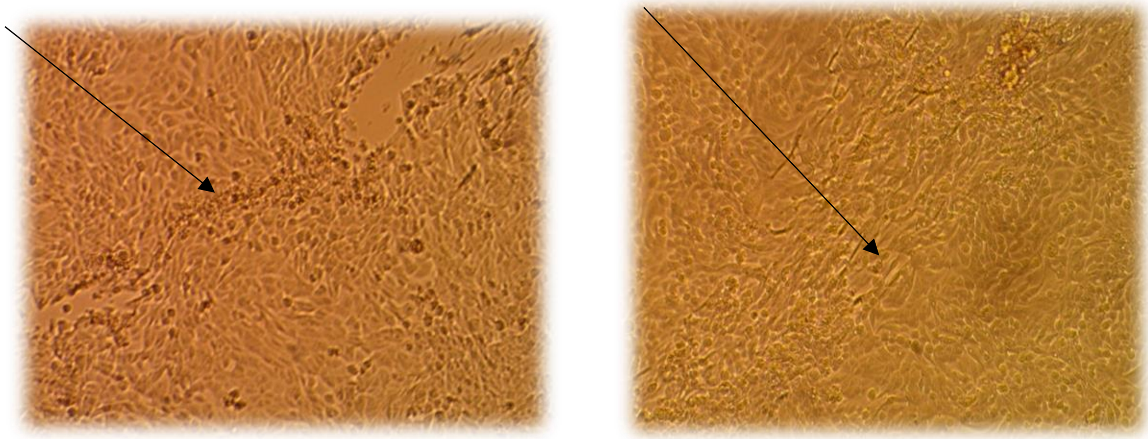
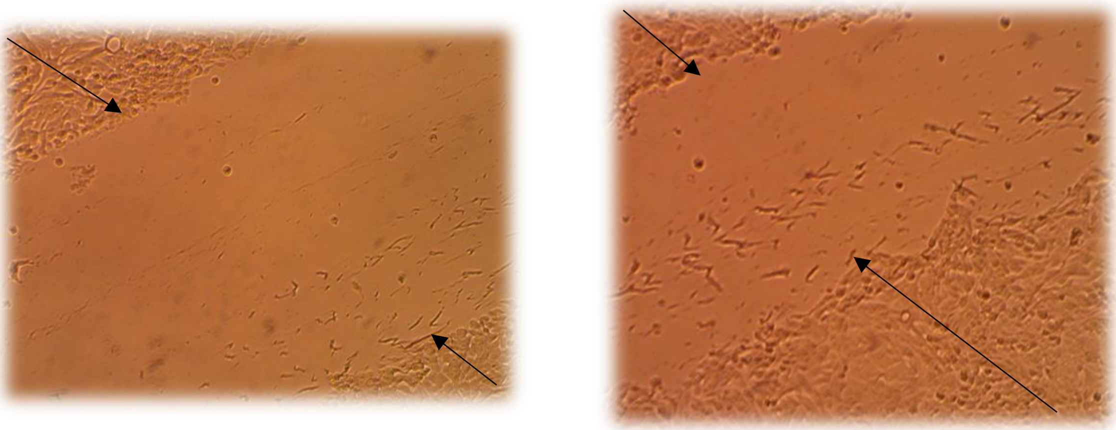


Figure (3.6H): Inverted Microscope (10XLence) Image of MDCK cell line Treated with propolis (0.97µg/ml) after incubation for different periods.



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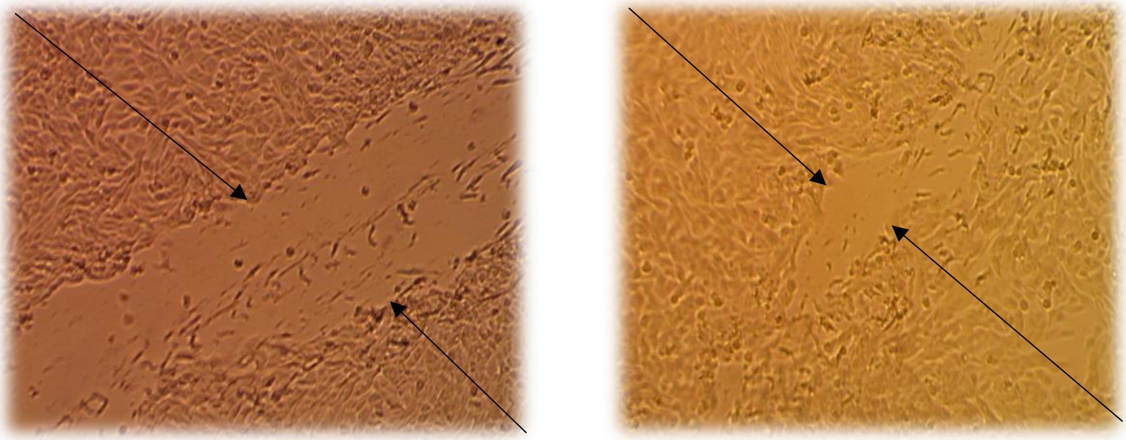
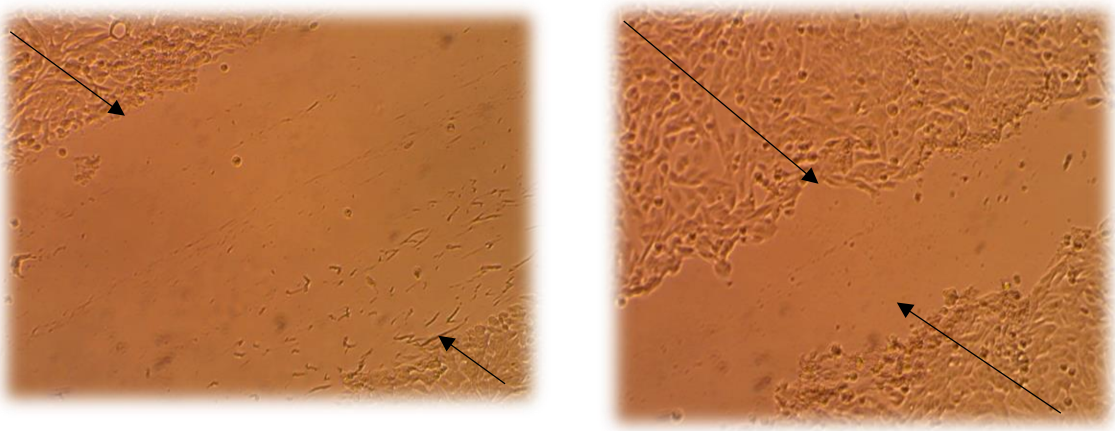


6hr 9hr

Figure (3.6I) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with propolis (0.48µg/ml) after incubation for different periods.



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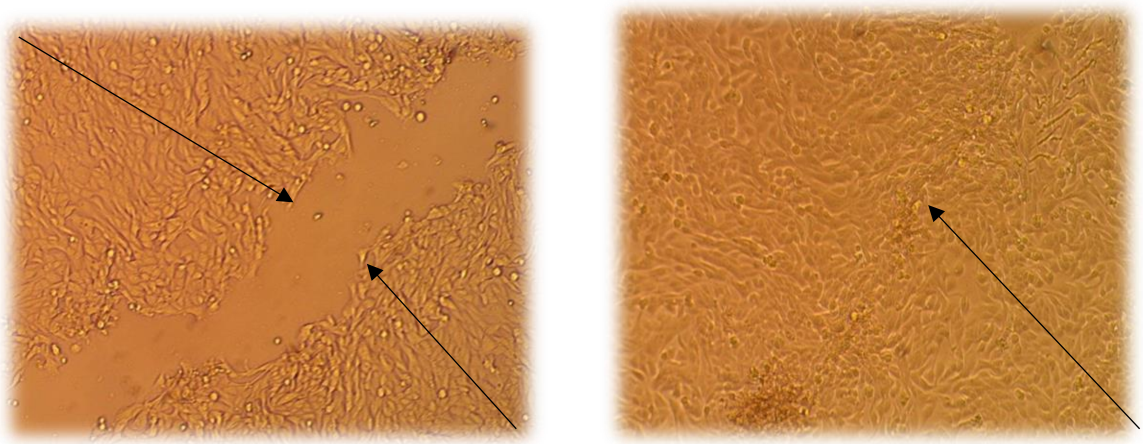


6hr **9hr**

Figure (3.6J) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with propolis (0.24µg/ml) after incubation for different periods.

3.7 Effect of vitamin C extract on cell viability Of MDCK line

vitamin C has significant difference in cell viability in concentrations (15.9 , 7.8 , 3.9 µg/ml) which show significant increase ($p \leq 0.001$) in cell viability in comparism to control group as shown in (Fig 3.7)

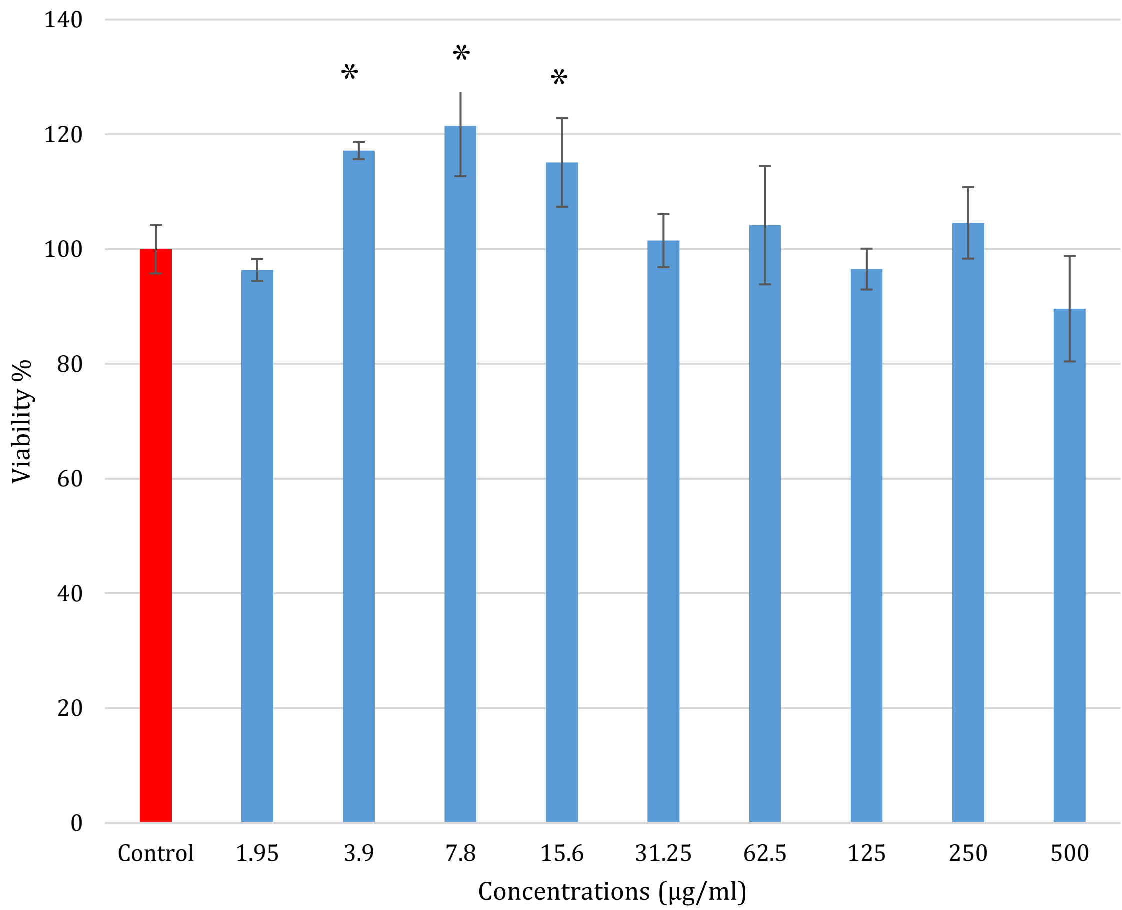


Figure (3.7): Cells viability percentage of MDCK cell line measured by MTT assay at different concentrations of vitamin C after incubation for 24 hours

3.8 Effect of vitamin C on wound healing

the result showed there were a significant healing ($p \leq 0.001$) in all concentration at 9 hr in comparison to control group and decrease wound diameter after 3hr at low concentrations as shown in (Fig3.8A) and documented in fig (3.8B, 3.8C , 3.8D , 3.8E , 3.8F and 3.8G)

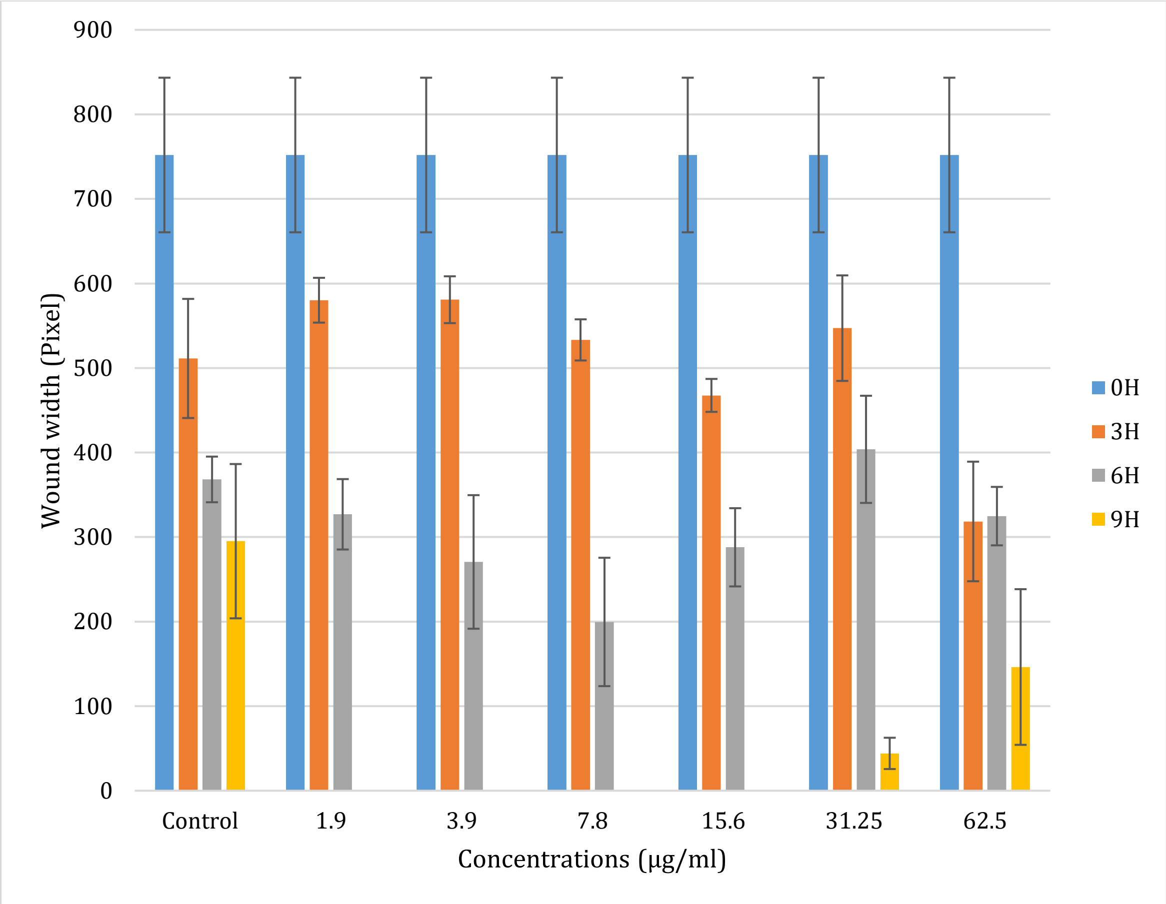
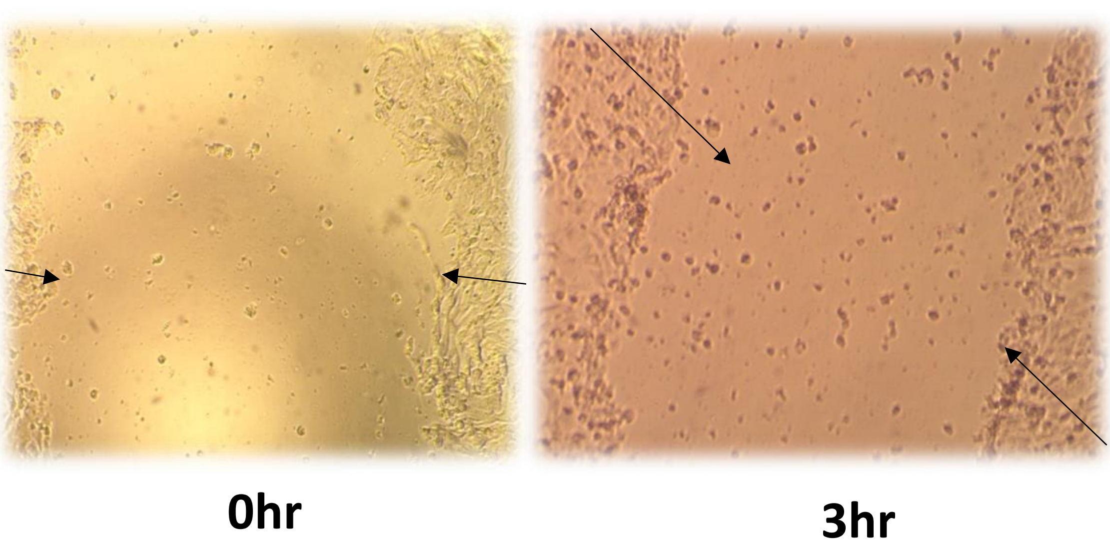
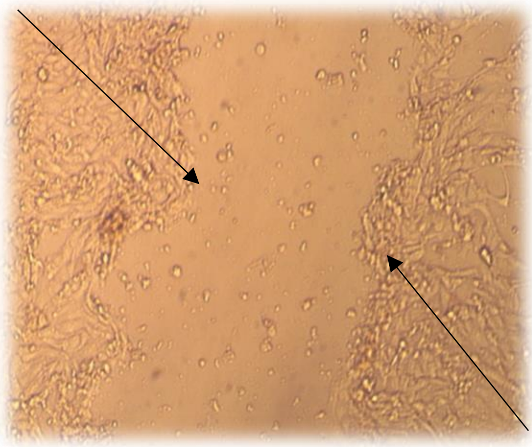


Figure (3.8A) : Effect of Vitamin C at different concentration on wound diameter after incubation for different periods.





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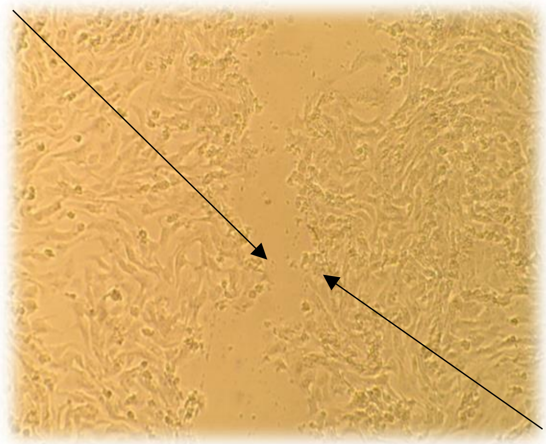

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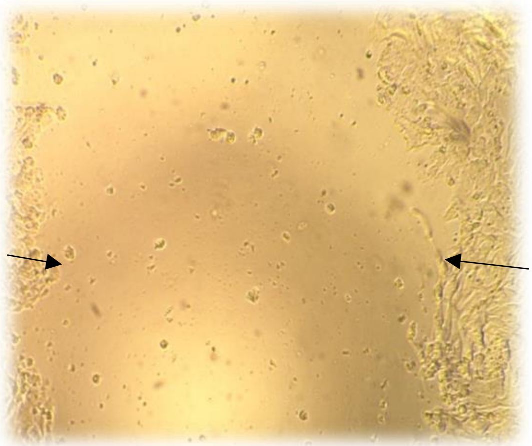
Figure (3.8B) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with vitamin C (62.5 µg/ml) after incubation for different periods.



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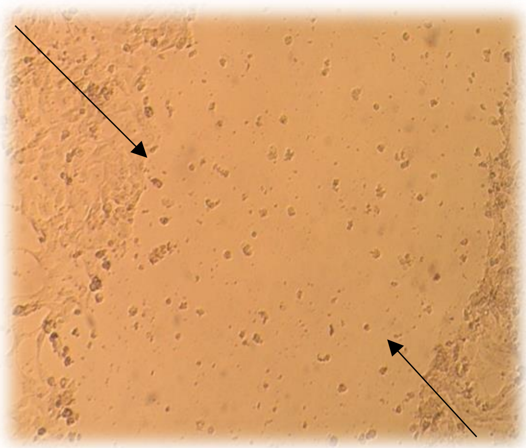

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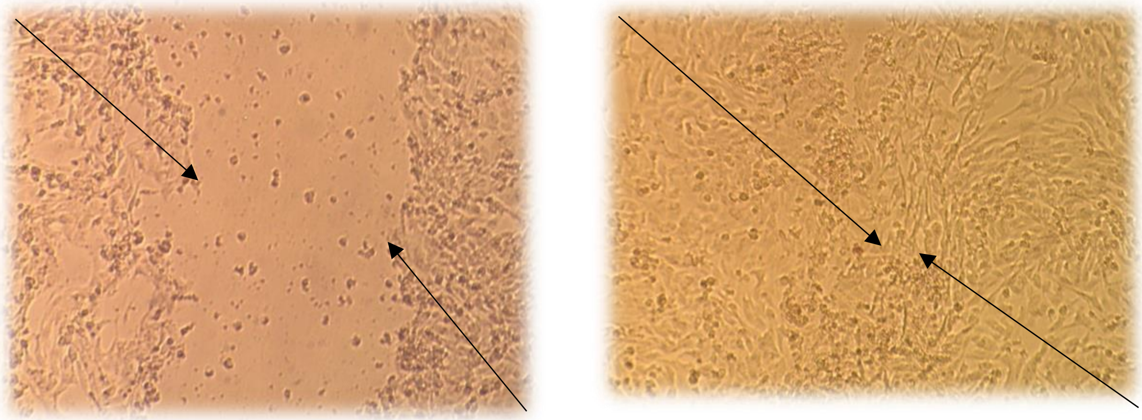
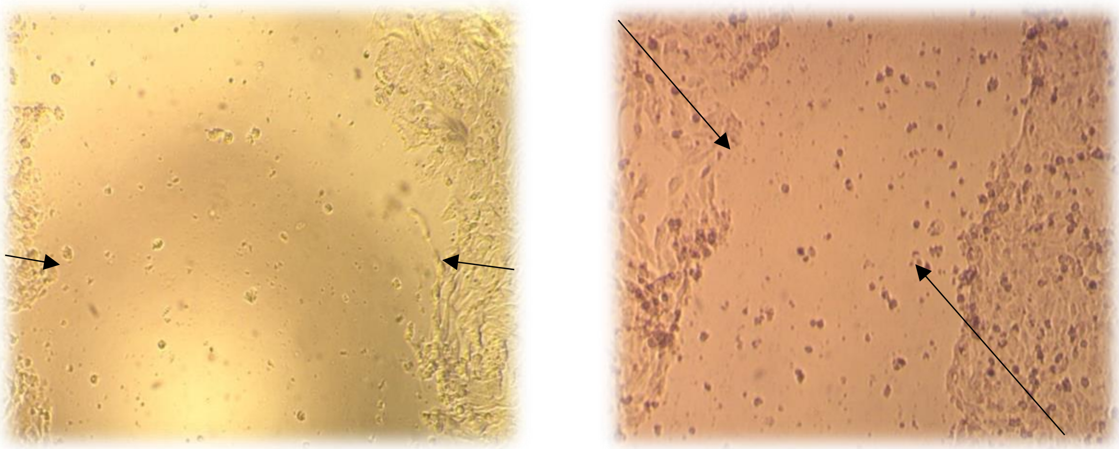


6hr 9hr

Figure (3.8C) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with vitamin C (31.25 µg/ml) after incubation for different periods.



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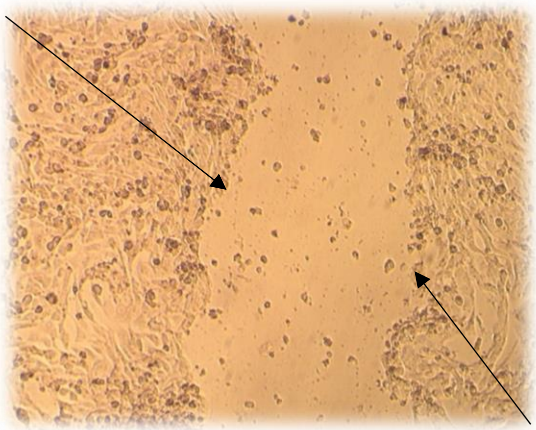

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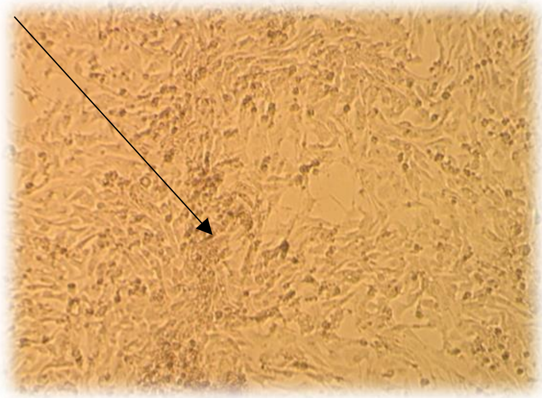
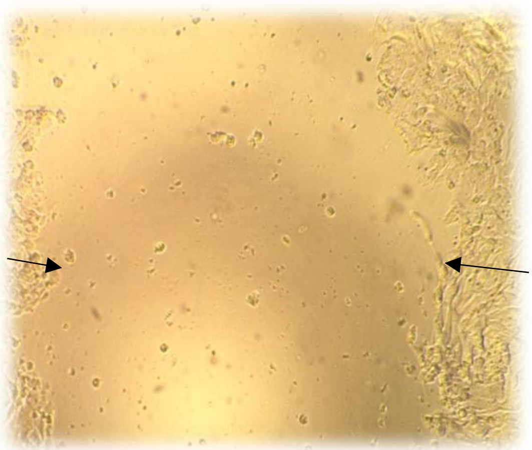


9hr

Figure (3.8D) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with vitamin C (15.6 µg/ml) after incubation for different periods.



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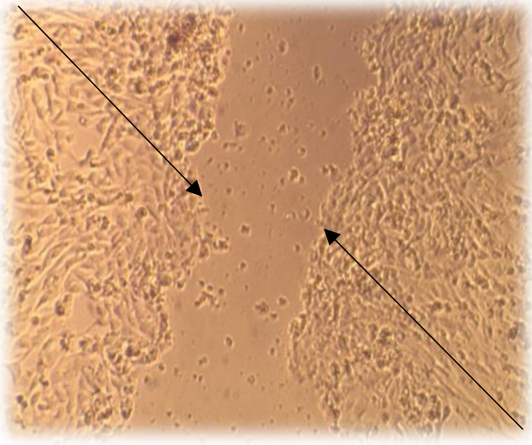

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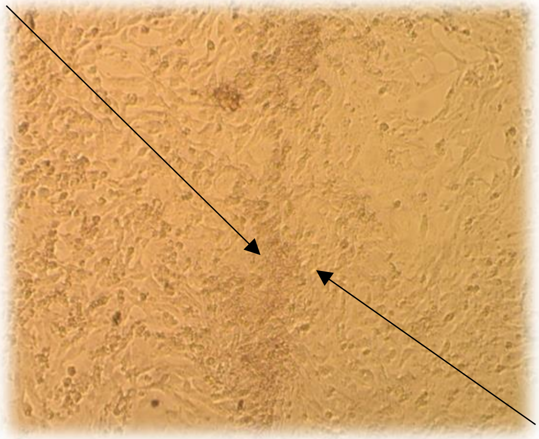

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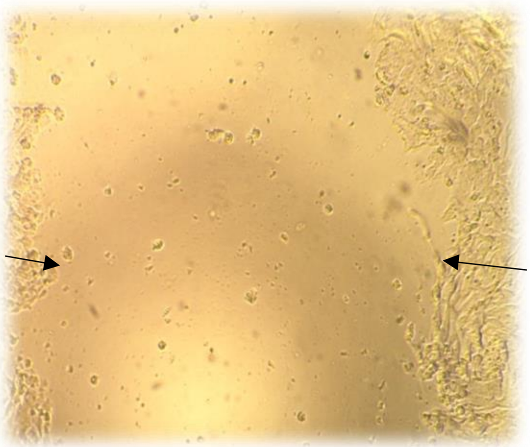
Figure (3.8E): Inverted Microscope (10XLence) Image of MDCK cell line Treated with vitamin C (7.8 µg/ml) after incubation for different periods.



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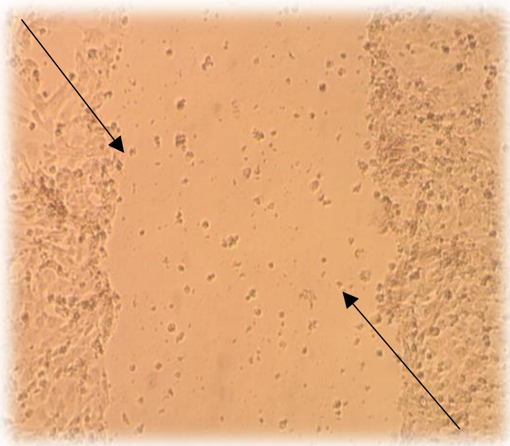

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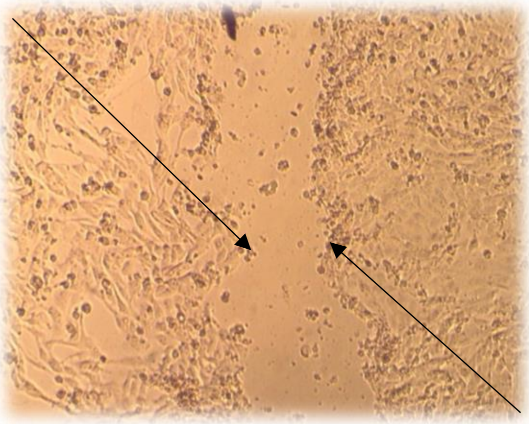

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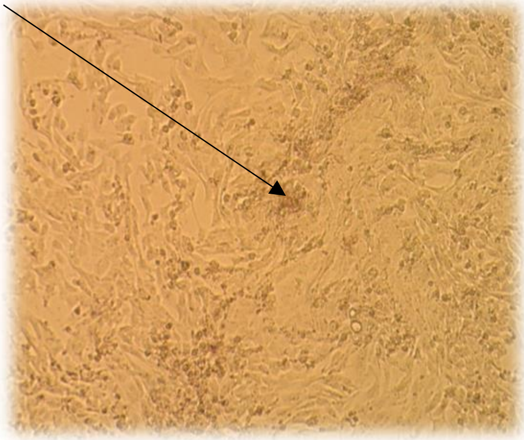

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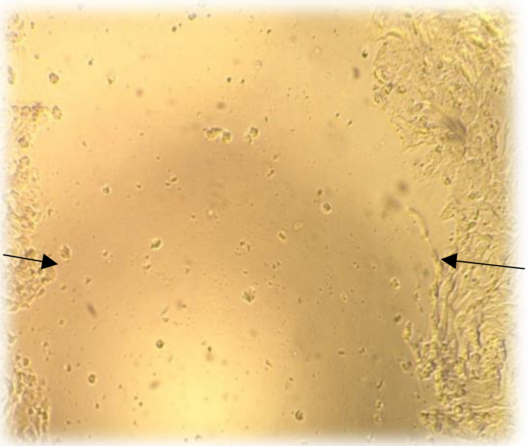
Figure (3.8F) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with vitamin C (3.9 μg/ml) after incubation for different periods.



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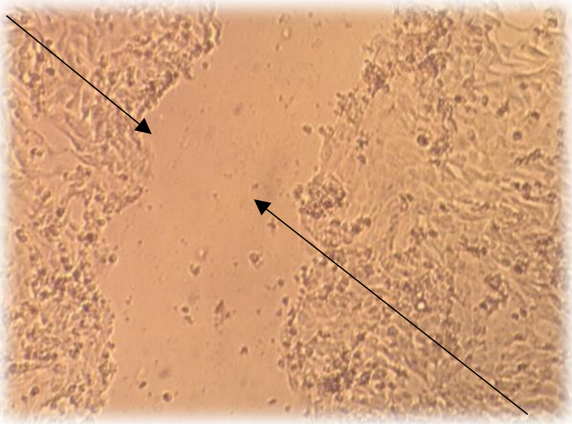

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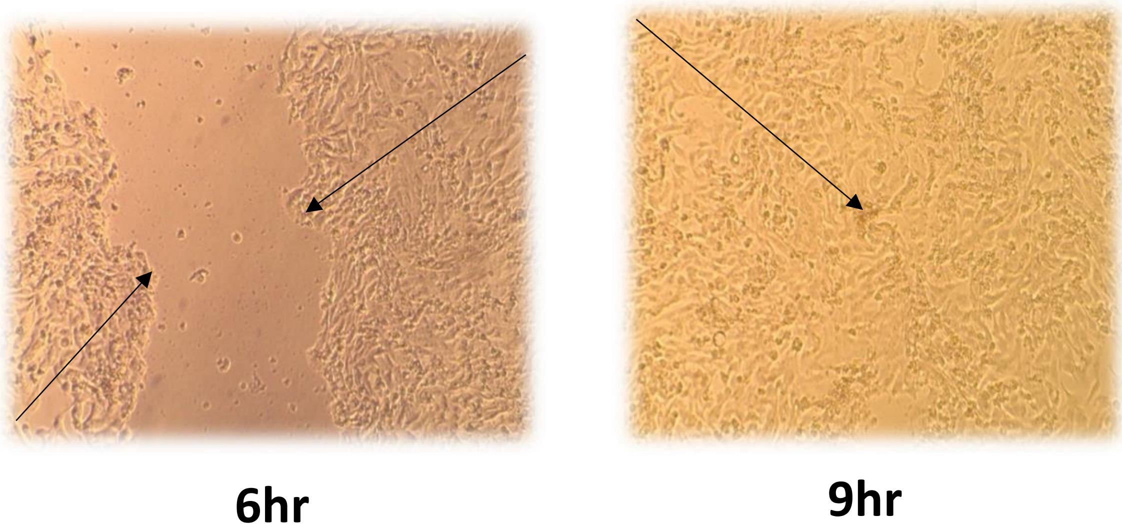


Figure (3.8G) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with vitamin C (1.95 µg/ml) after incubation for different periods.

3.9 Effect of Zinc on cell viability OF MDCK cell line

Result showed that there were no significant difference in cells viability percentage in low concentrations of zinc except the highest concentrations (500,250,125 µg/ ml) which cause a significant decrease ($p \leq 0.001$) in the viability percentage in comparison to control group as shown in(Fig3.9)

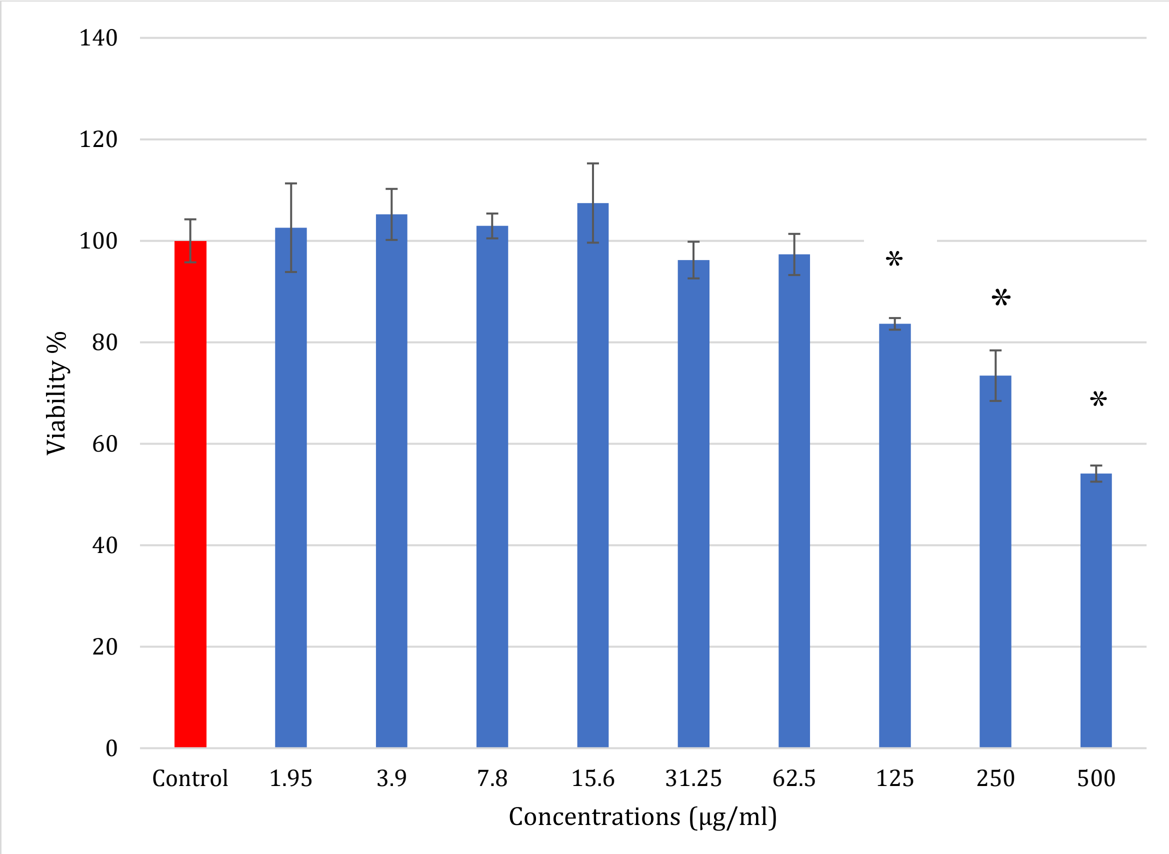


Figure (3.9): Cells viability percentage of MDCK cell line measured by MTT assay at different concentrations of Zinc after incubation for 24 hours

3.10 Effect of zinc on wound healing

the result showed there were a significant healing ($p \leq 0.001$) in all concentrations at 9 hr in comparison to control group and decrease wound diameter after 6 hrs as in (Fig 3.10A) and documented in fig (3.10B , 3.10C , 3.10D , 3.10E , 3.10F and 3.10G)

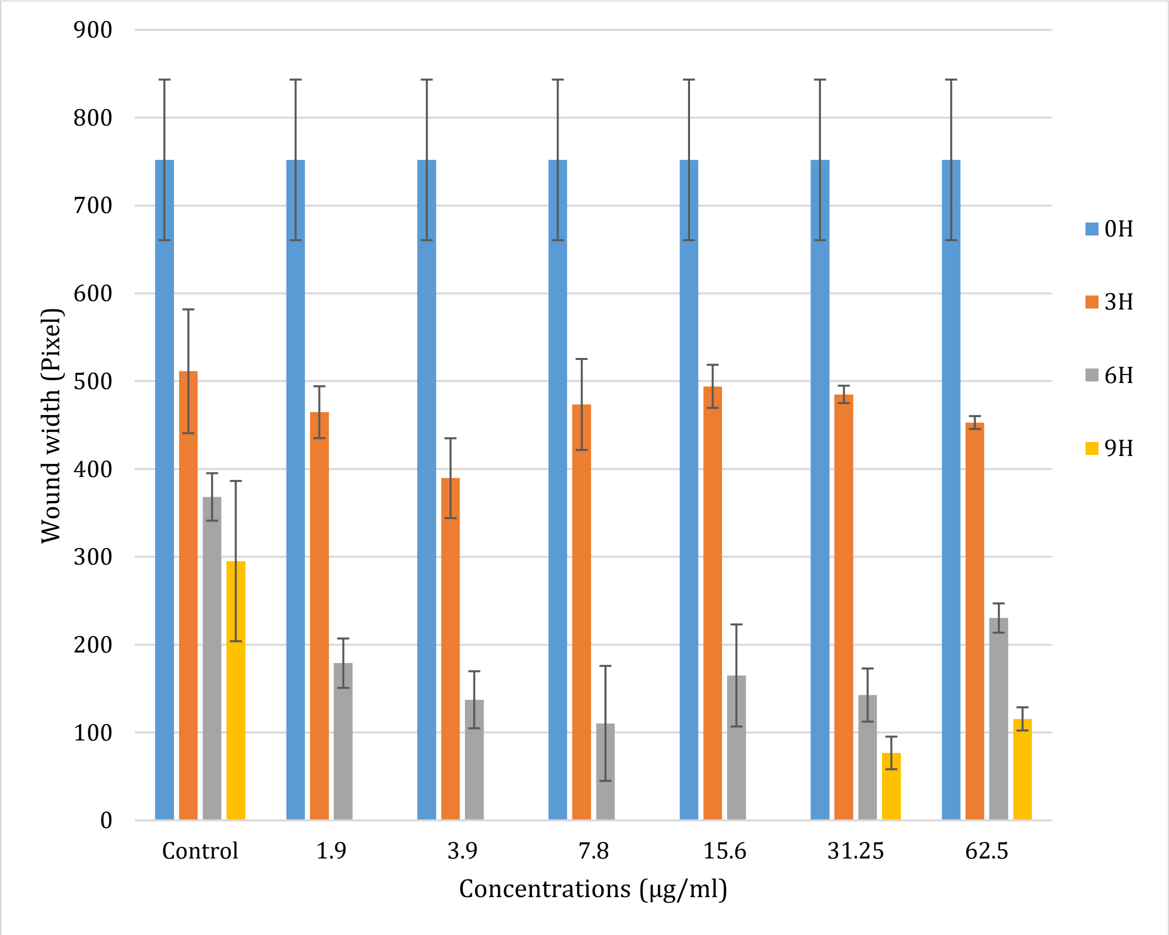


Figure (3.10A) : Effect Of Zinc At Different Concentration On Wound Diameter After Incubation For different times

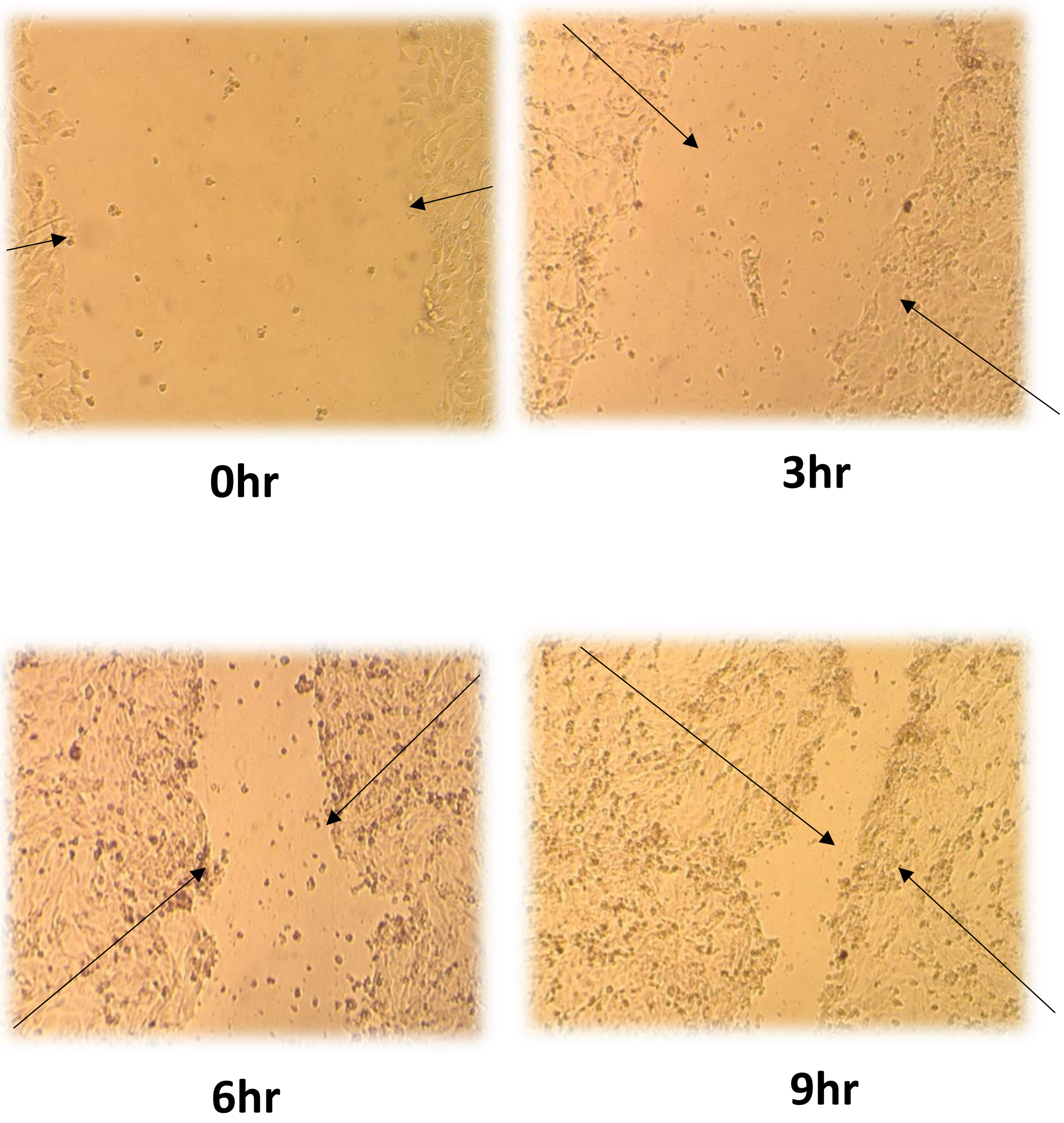


Figure (3.10B) : Inverted Microscope (10Xlence) Image Of MDCK Cell Line Treated With Zinc (62.5 µg/ml) After Incubation for different periods.

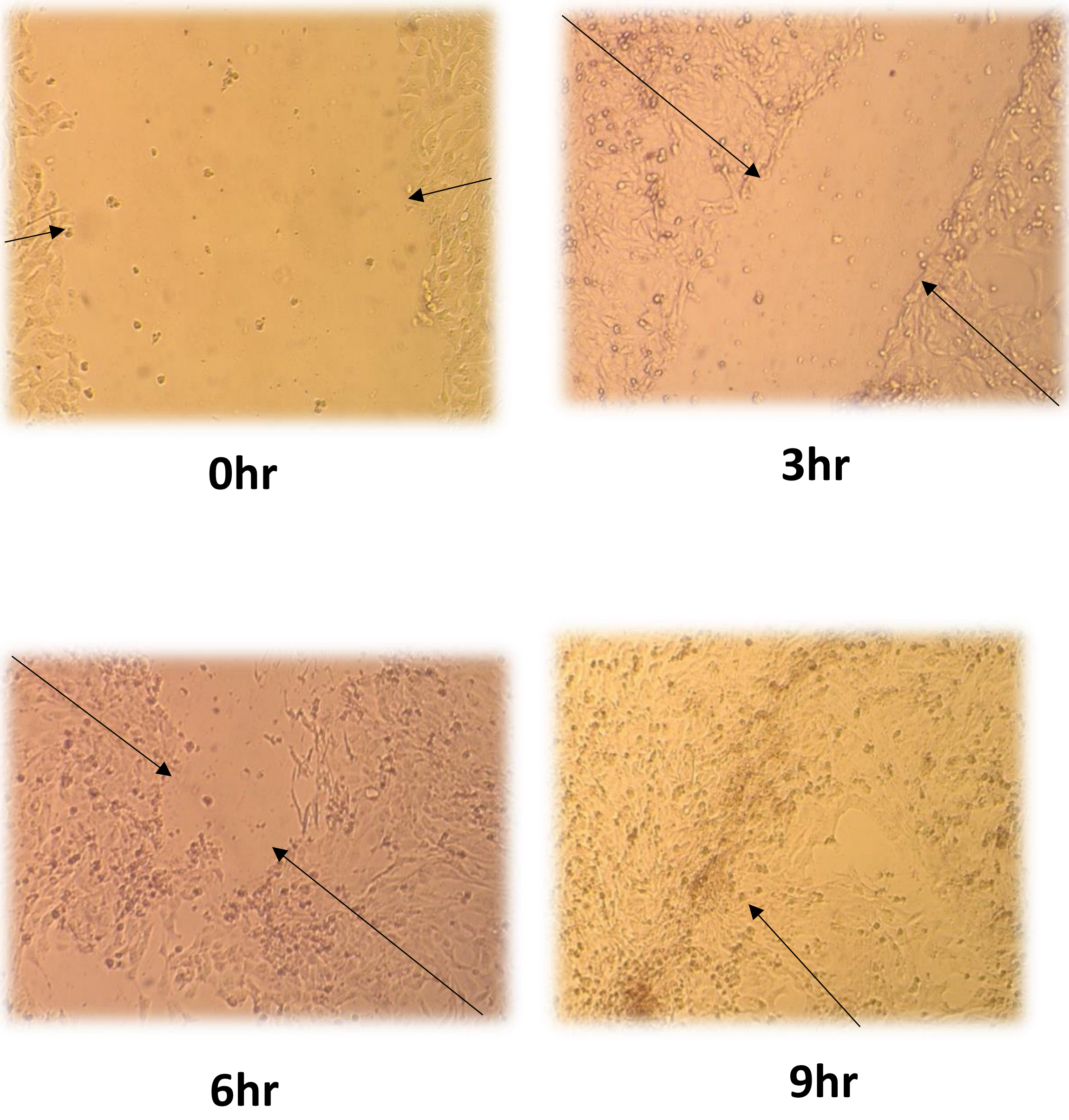
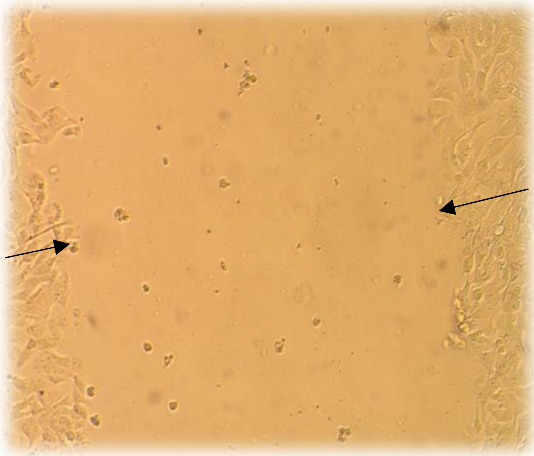


Figure (3.10 C) : Inverted Microscope (10Xlence) Image Of MDCK Cell Line Treated With Zinc (31.25 μg/ml) Only After Incubation For different periods.



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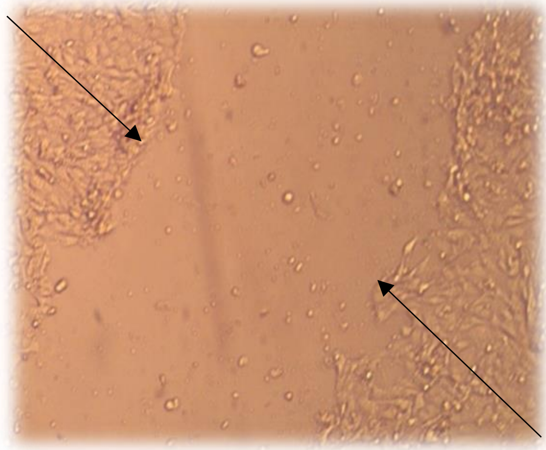

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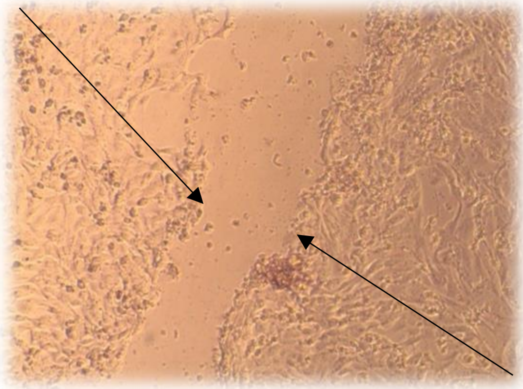

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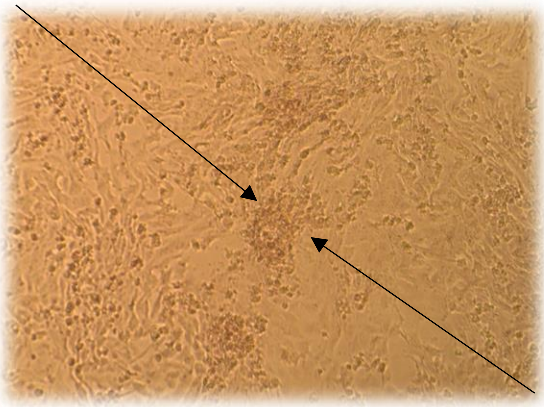


9hr

Figure(3.10 D) : Inverted Microscope (10Xlence) Image Of MDCK Cell Line Treated With Zinc (15.6 μg/ml) After Incubation For different periods.

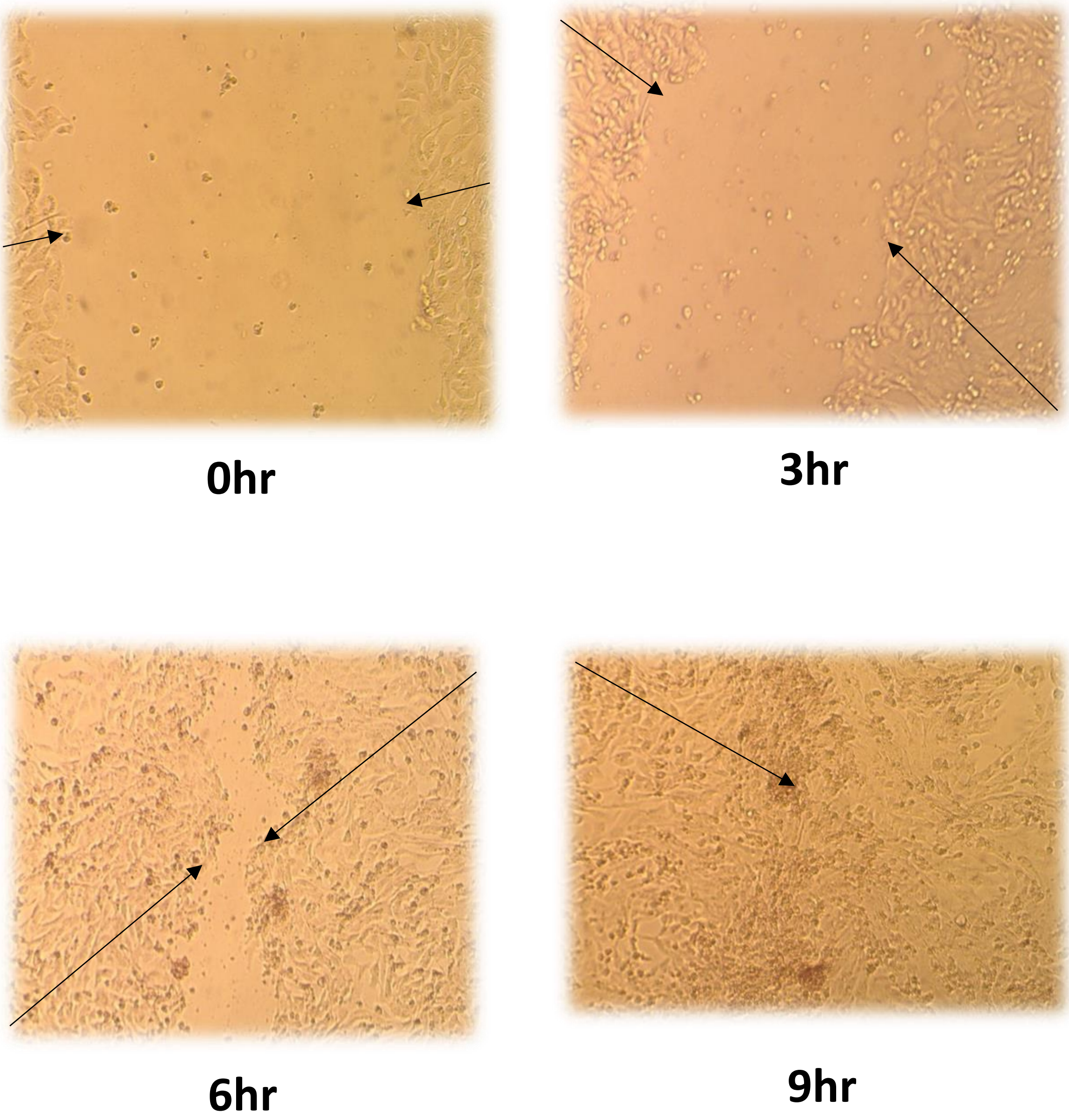


Figure (3.10 E): Inverted Microscope (10Xlence) Image Of MDCK Cell Line Treated With Zinc (7.8 µg/ml) After Incubation For different periods.

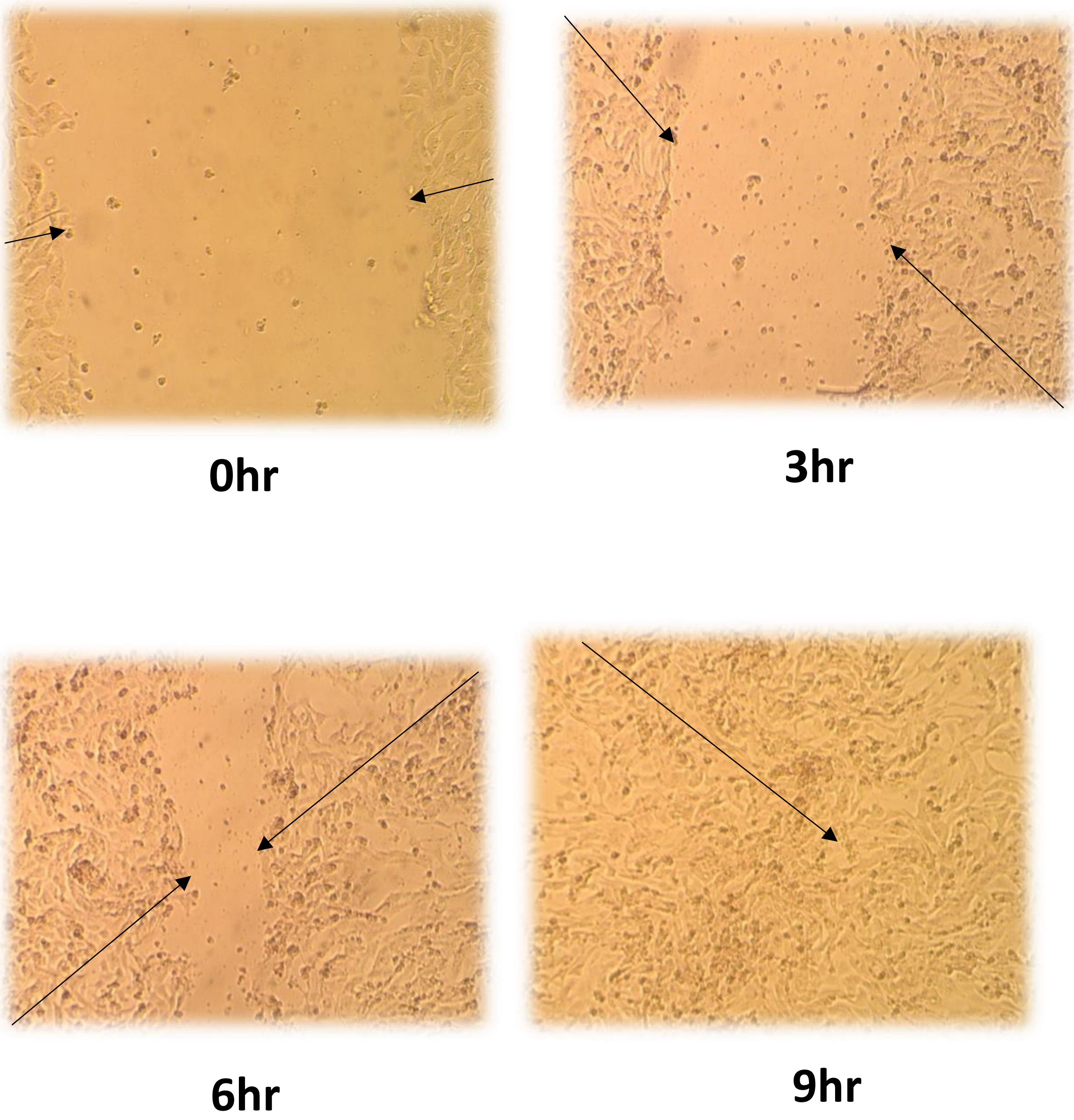


Figure (3.10 F) : Inverted Microscope (10Xlence) Image Of MDCK Cell Line Treated With Zinc (3.9 µg/ml) After Incubation For different periods.

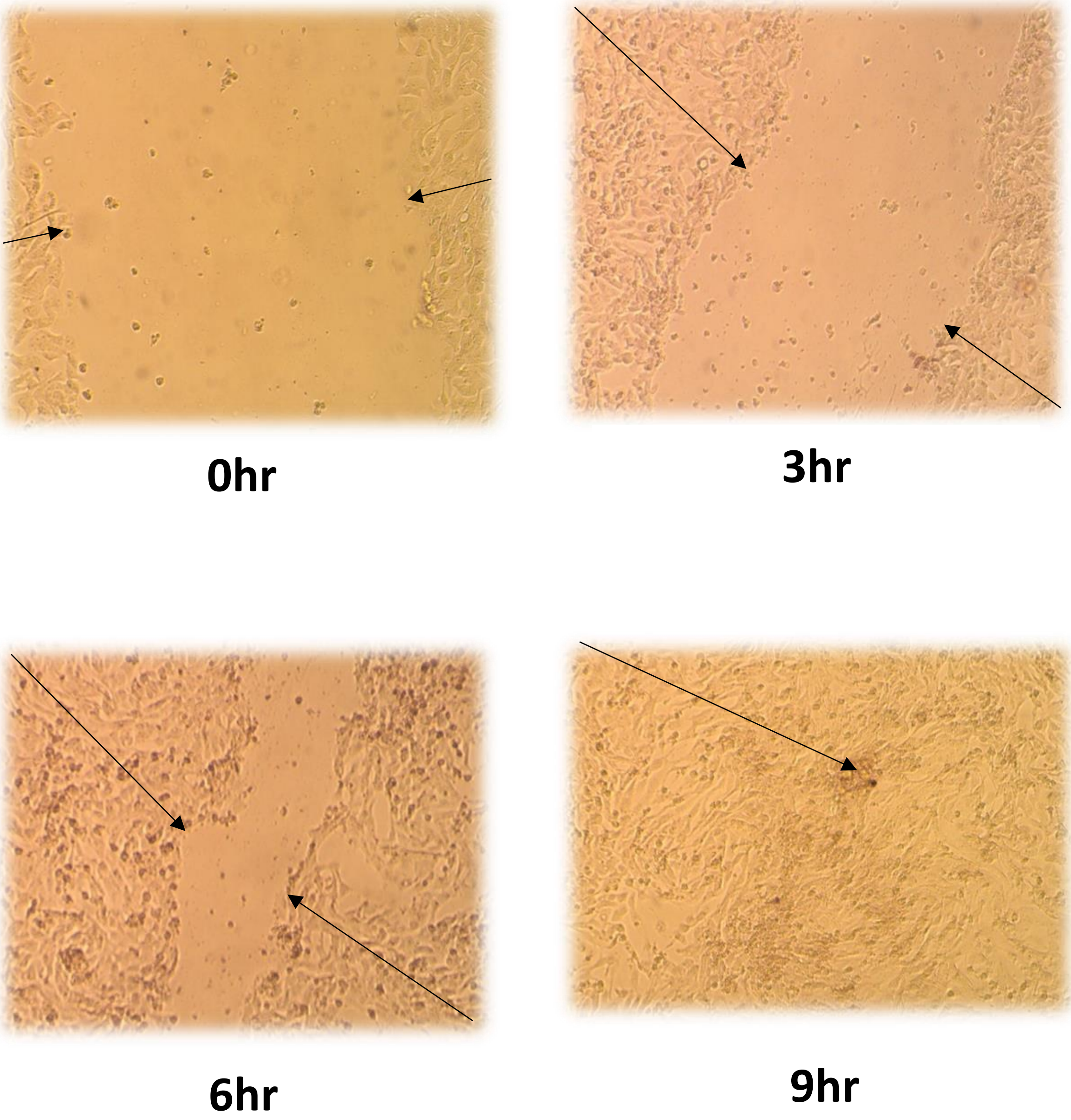


Figure (3.10 G) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with zinc (1.95 µg/ml) after incubation for different periods.

3.11 Effect of combination vitamin C and zinc on wound healing

the result showed there were a significant healing ($p \leq 0.001$) in all concentration at 9 hr in comparison to control group and complete healing after 3 hr at 1.9 concentrations as shown in (Fig 3.11 A) and documented in fig (3.11 B , 3.11 C , 3.11 D, 3.11E ,3.11F and 3.11G)

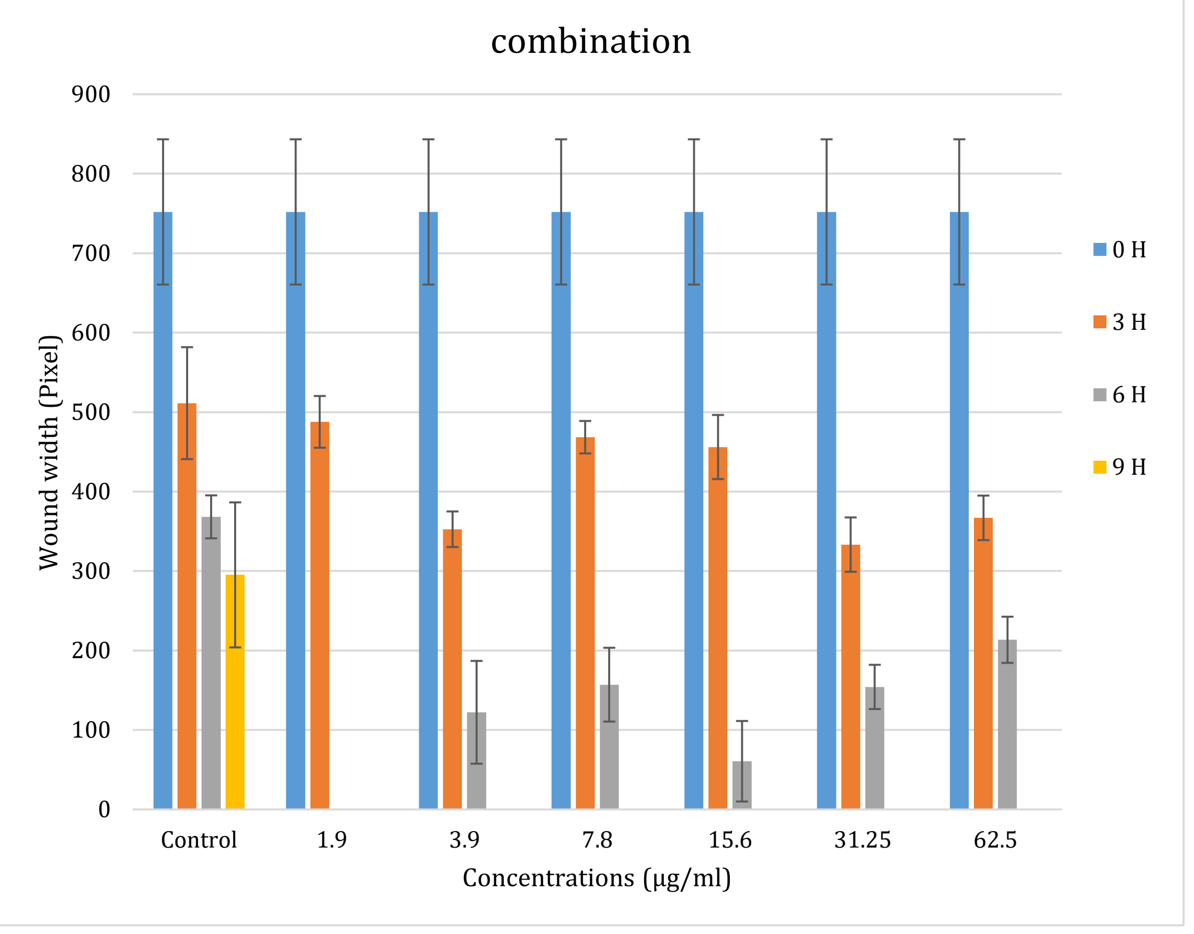


Figure (3.11A) : Effect of vitamin c and zinc at different concentration on wound diameter

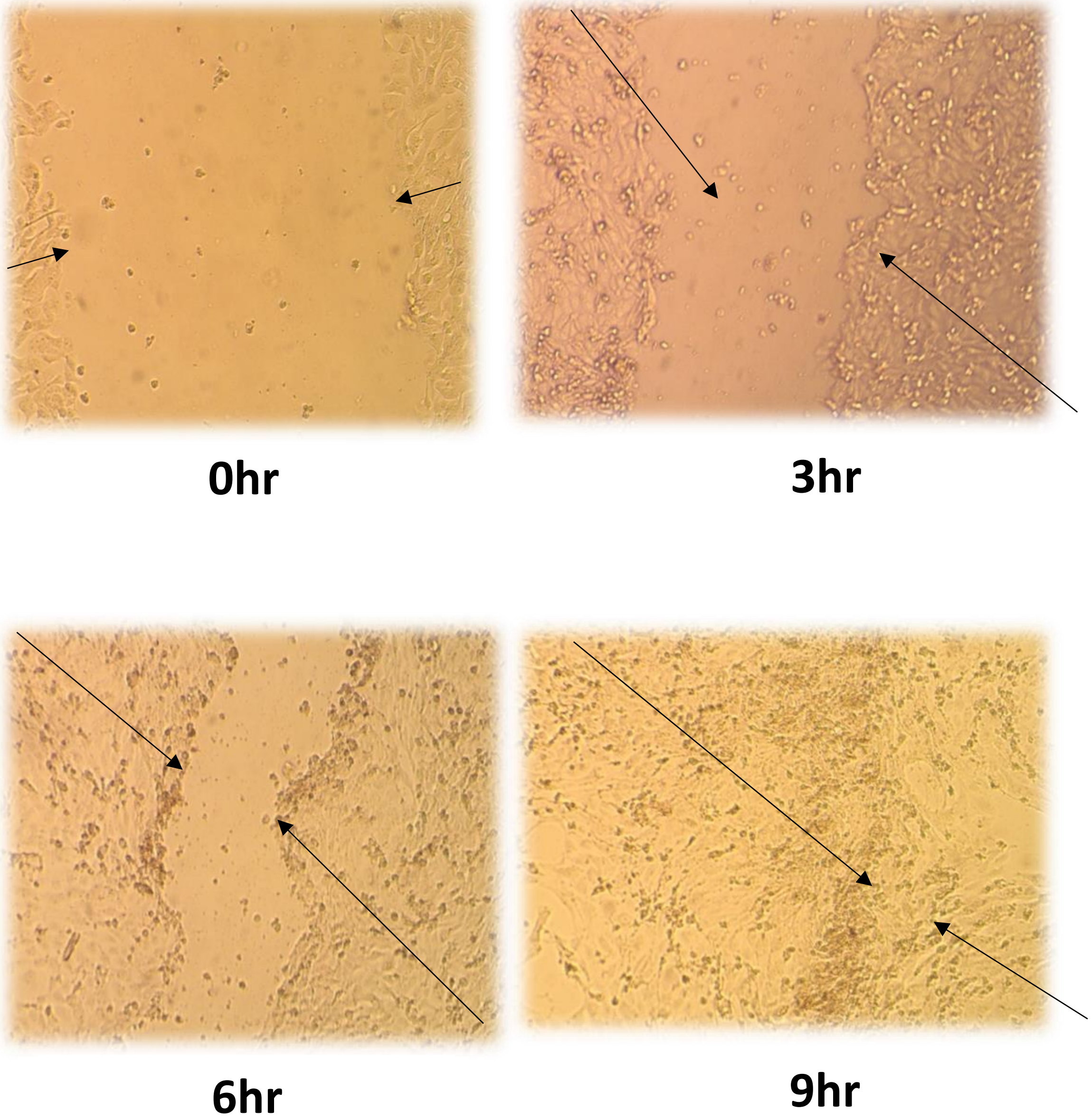


Figure (3.11B) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with vitamin C and zinc (62.5 µg/ml) after incubation for different periods.

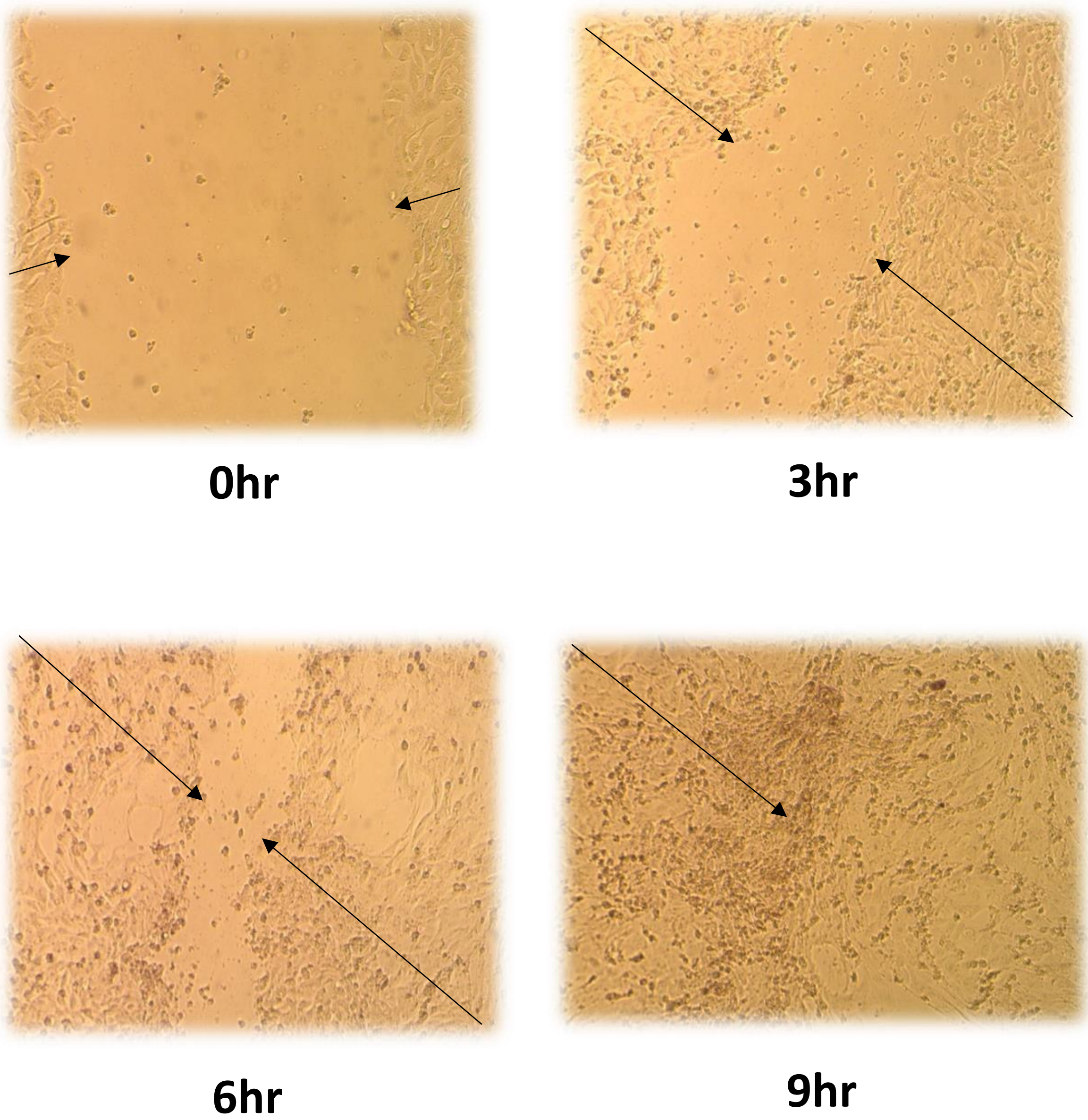


Figure (3.11C): Inverted Microscope (10XLence) Image of MDCK cell line Treated with vitamin C and zinc (31.25 µg/ml)after incubation for different periods.

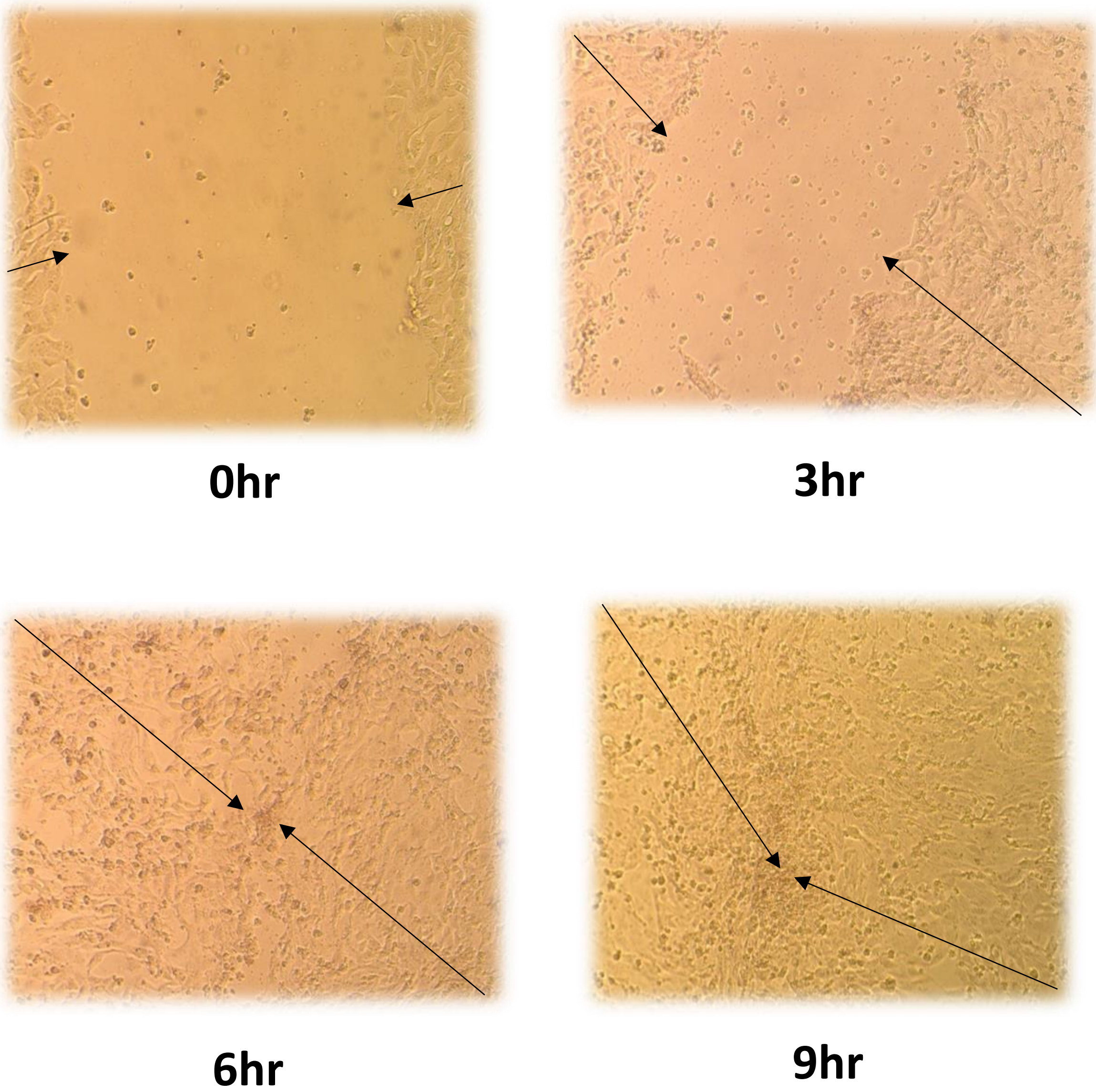


Figure (3.11 D) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with vitamin C and zinc (15.6 µg/ml) after incubation for different periods.

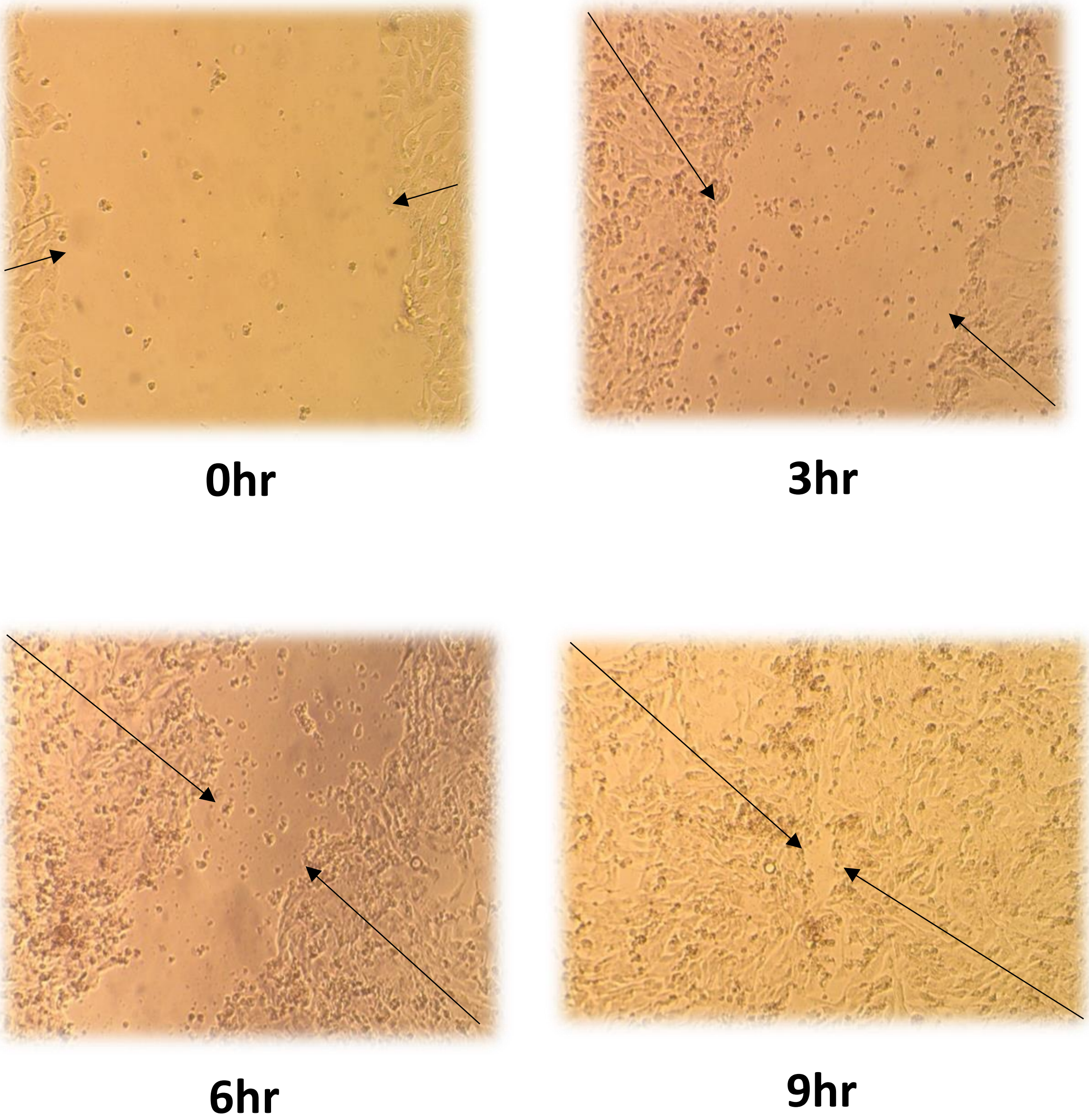


Figure (3.11 E): Inverted Microscope (10XLence) Image of MDCK cell line Treated with vitamin C and zinc (7.8 µg/ml) after incubation different periods..

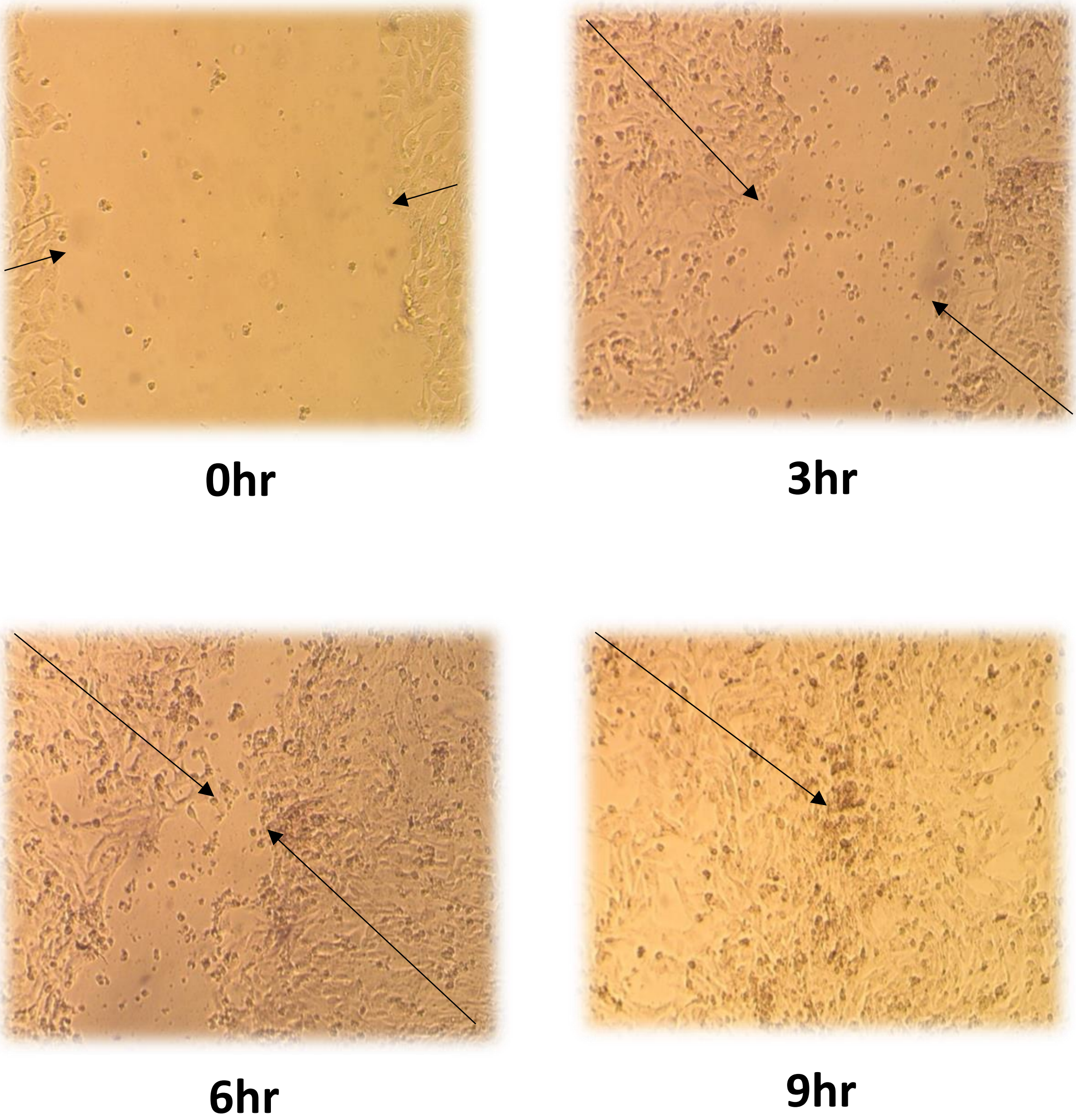
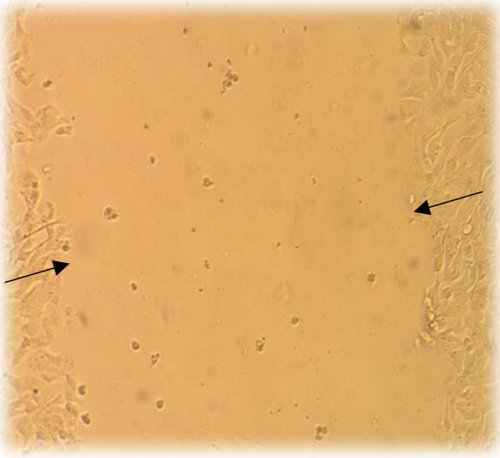


Figure (3.11 F) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with vitamin C and zinc (3.9 μg/ml) after incubation for different periods.



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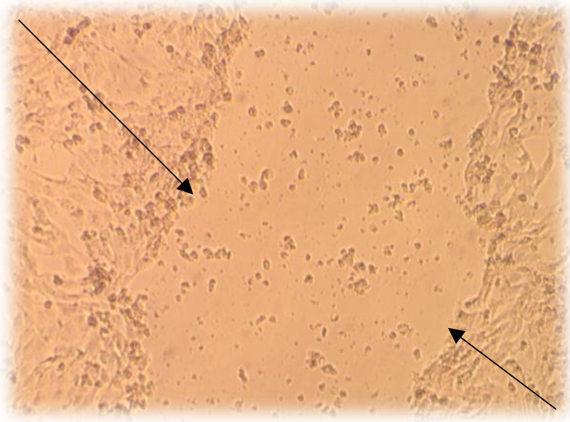
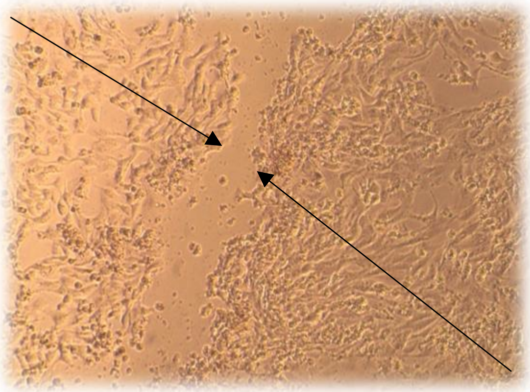
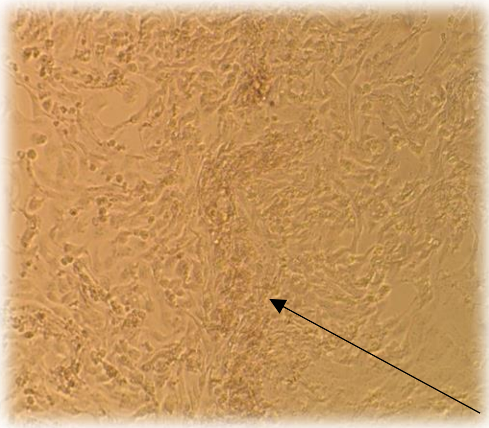


3hr



6hr



9hr

Figure (3.11 G) : Inverted Microscope (10XLence) Image of MDCK cell line Treated with vitamin C and zinc (1.9µg/ml) after incubation for different periods.

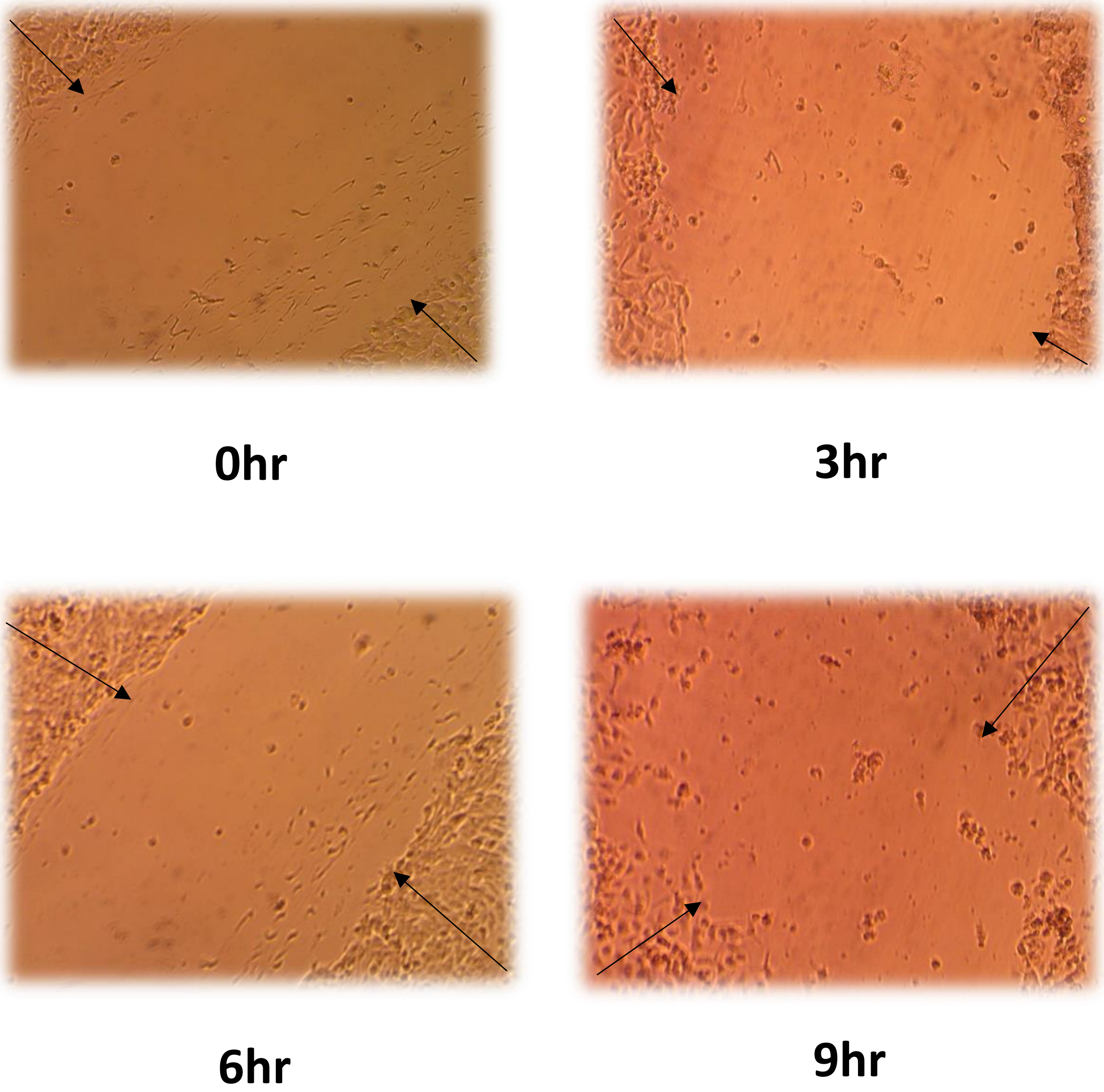


Figure (3.12) : Inverted Microscope (10XLence) Image of MDCK cell line without Treated (control) after incubation for different periods.

4. Discussion

4.1 Effect of Tamarix mannifera aqueous extract on cell viability

Cell viability was measured after incubation period for 24 hr at 37 C Result showed that there was no significant difference in cells viability between all concentrations of T. mannifera extract except the highest one 500 µg/ml , which cause a significant decrease ($p \leq 0.001$) in the viability percentage So its non toxic at low concentration .

Deionized water was used as a solvent for the extraction of total polyphenols, tannins, flavonoids, carotenoids and ascorbic acid contents of T.mannifera, because it's an effective solvent that can dissolve many compounds including polyphenols, tannins, flavonoids, carotenoids and ascorbic acid (Zuorro *et al* .,2019) .

Polyphenol did not exhibit pronounced toxicity to normal cells as examined by MTT analysis of human primary skin fibroblasts and not inducing any cytotoxic side-effects to normal cells (Kondo *et al.*, 2009).

Kaempferol is a polyphenol is much less toxic to normal cells. Administration of kaempferol actually increases oxidative stress in glioblastoma cells, increasing production of reactive oxygen species (ROS) in these cancerous cells noted that these cytotoxic effects seem to target cancer cells specifically. In fact, kaempferol appears to serve a protective role with respect to normal body cells. (Chen and Chen, 2013).

(Khan *et al*., 2013) reported that there is no cytotoxic activities of various extracts in vitro and Tamarix deserves more attention due to its biological activities .

4.2 Effect of Tamarix mannifera aqueous extract on wound healing

Many polyphenols extracted from the plant can be used as natural antioxidants. Many studies have shown the content and species of phenolic compounds to have close correlations with antioxidant capacity (Piechocka *et al.*, 2020).

Several investigations have revealed the presence of many bioactive components in all varieties, such as carotenoids, flavonoids, phenolic acids, alkaloids, vitamins, fats (mainly linoleic acid, palmitic acid, and oleic acid), sugars, and minerals. It is known that many bioactivities, such as antioxidant effects, are linked to the presence of phenolics . It was reported that the type of solvent used had a significant influence on the content of phenolic acids in the extracts (Piechocka *et al.*, 2020) .

The activity of T.mannifera aqueous extract in wound healing related to:

Gallic acid which improved wound healing by decreasing wound area, increasing fibroblast cells, reducing inflammatory cell infiltration, and decreasing inflammation, acting on redox balance and oxidative stress in healing process (Pressi *et al.*, 2022).

The tamaricaceous plants produce a unique class of ellagitannins (tannins) with diverse structures in addition to phenolic acids and flavonoids This tannins effect is related to their antioxidant activity tannin extract was shown to reduce wound area and shortening epithelization and scar formation. Due to its antimicrobial activity animals with wounds infected by Staphylococcus aureus healed (Pizzi, 2021).

Tannins do not function solely as primary antioxidants, (i.e., they donate hydrogen atom or electrons) they also function as secondary antioxidants. Tannins have the ability to chelate metal ions such as Fe(II) and interfere with one of the reaction steps in the Fenton reaction and thereby retard oxidation (Amarowicz, 2007).

Also its contain Arginine (ARG), an essential amino acid. It promotes wound healing, increasing breaking strength and scar collagen deposition..Circulating arginine is a substrate for protein and collagen metabolism in extrahepatic tissues . Arginine stimulates protein synthesis, the function of T-lymphocytes and regulates nitric oxide (NO) activity. It has been suggested that the presence of NO produced from arginine aids the transition of a wound from the acute inflammatory phase to the proliferative phase of wound healing (Palmieri and 1 MD, 2019).

Flavonoids as mitochondrial ROS Scavengers, such beneficial effects on health have long been attributed to their antioxidant properties, which lead to a reduction in ROS, regardless of their source (endogenous or exogenous). The ability to scavenge ROS and reactive nitrogenous species RNS is determined by the hydroxyl configuration of the flavonoid B-ring, as it donates hydrogen and an electron to free radicals, relatively stable flavonoid radicals are formed in this process (Kicinska *et al.*, 2020).

4.3 Effect of Moringa Oleifera aqueous extract on cell viability

The toxicity at higher concentrations toward human normal cells. May be because that they are incorporated into cells, increase intracellular ROS levels, and then exert cytotoxicity (Matsuo *et al.*, 2005).

It can be due to the availability of more nutrition and some certain compounds for cells and may because of antioxidant and nutritionally important active compounds of M. oleifera leaves. (Mag *et al.*, 2015).

(Gothai *et al.*, 2016) disagree with our study and show that The cytotoxic effect of MO was determined by MTT assay using different gradient concentration (15.62, 31.25, 62.5, 125, 250, and 500 μg/mL concentration). Cell viability scrutiny showed that MO had no toxic effect normal cells .

MO had broad range of pharmacological attribute is probably due to unique combination of potentially bioactive compounds such as rhamnosyloxy benzyl isothiocyanate and its derivatives, niaziminins, niazinins, β-sitosterol, niacin, phenolic acids, glucosinolate, flavonoids, gallic acid, coumarin, and caffeic acids in *M. oleifera*. (Stohs and Hartman, 2015).

(Muhammad *et al.*, 2013) reported aqueous MO extract significantly enhanced proliferation and viability of fibroblast cells when compared to untreated control which may be justified by the fact that bioactive compounds responsible for the enhancement of proliferation and migration of fibroblast cells and show that aqueous fraction was nontoxic to human dermal fibroblast cells even at 72 hrs since it did not affect the cellular activity of fibroblast cells even at high concentration of 800 μg/mL .

Some biocompounds in the extracts which selectively target cancer cells with little efect on normal cells. Particularly, d-allose, a rare monosaccharide found in Moringa oleifera leaves, induces the expression of thioredoxin interacting protein (TXNIP), which functions as tumor suppressor and metastasis suppressor. d-allose inhibits the growth of cancer cells at G1 phase without exerting appreciable efects on normal cells (Do *et al.*, 2020) .

4.4 Effect of Moringa Oleifera aqueous extract on wound healing.

Extract from Moringa oleifera leaves showed that MO is rich in quercetin-O-glucoside and quercetin-O-malonyl glucoside, responsible for the antioxidant, radical scavenging and antibacterial activities (Pagano *et al.*, 2020).

The high phenolic content of Moringa species contributes to their high antioxidant activity. Phenolic compounds stabilize radicals produced in cells by donating or accepting electrons, hence acting as antioxidants . quercetin , kaempferol and myricetin showed antioxidant activity by increasing ferric reducing activity and inhibiting 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals (Marume *et al.*, 2017).

It was found that *M. oleifera* from different cultivars had different antioxidant, phytochemical, and antimicrobial profiles. in addition to its antioxidant activity. The extract reduced plasma malondialdehyde (MDA) levels, increased Trolox equivalent antioxidant activity, increased the ferric reducing ability of the plasma (Ngamukote *et al.*, 2016).

Mechanism underlying the anti-inflammatory activity may be attributed to the regulation of neutrophils and c-Jun N-terminal kinase pathway. Active ingredients contributing to anti-inflammatory property are tannins, phenols, alkaloids, flavonoids, carotenoids, β-sitosterol (Bhattacharya *et al.*, 2018).

M. oleifera has anti-inflammatory action related to its inhibition of NO, Prostaglandin E2 (PGE2), proinflammatory cytokines, and inflammatory mediator's production in Lipopolysaccharide (LPS) stimulated macrophages through preventing degradation of Nuclear factor kappa b (NF-*K*b) signaling pathway (Tan *et al.*, 2015).

Other study showed that the essential oils of *M. oleifera* and their components show a better antimicrobial effectiveness against Gram-positive bacteria, The molecular mechanism of action of the essential oil of MO is unknown, but the essential oil can probably inhibit the generation of adenosine triphosphate from dextrose and disrupt the cell membrane (Das *et al.*, 2017).

Studies conducted on the effect of wound healing of leaf extract showed improved tissue regeneration, decreased wound size, downregulated inflammatory mediators, and upregulated vascular endothelial growth factor in wound tissues, and remarkable antiproliferative and anti-migratory effects on normal human dermal fibroblasts (Gothai *et al.*, 2016).

4.5 Effect of Propolis alcoholic extract on cell viability

Kilicoglu *et al.* observed that fibroblast proliferation, activation and synthesis capabilities were better in the presence of propolis than in its absence.

Reasons for enhancement of cells proliferation may be due to the presence of antioxidant compound in the plant under the research study such as flavonoids (are powerful antioxidants, and have been shown to be capable of scavenging free radicals and thereby protecting lipids and other compounds such as Vitamin C from being oxidised or destroyed) , phenolic acids ,Phenols possess biological properties including ability in scavenging reactive oxygen species(Woźniak et al, 2019).

(Najafi *et al.*, 2007) show that if two groups of cells (cancer cells and normal cells) lay beside each other, treatment of the cells by EEP helps to kill the cancer cells and stimulate proliferation of normal cells during the treatment.

The comparison of the responses of normal and malignant cells to propolis extracts revealed that cancerous cells were more sensitive in terms of growth inhibition than normal cells, which could be explained by selective toxicity of the chemical constituents of propolis, especially phenolic acids and flavonoids, against cells depending on the type of cells (Valente *et al.*, 2011).

4.6 Effect of propolis alcoholic extract on wound healing

Propolis speed up the burned tissue repair by stimulation of the wound bed matrix remodeling, proposing that the observed changes in extracellular matrix content after propolis application may be connected with the ability of its flavonoid compounds to reduce lipid peroxidation and to prevent necrosis of cells (Olczyk *et al.*, 2013) .

Propolis treatment stimulates significant increases in ECM components during the initial phase of wound repair, followed by a reduction in the ECM molecules. It is postulated that this biological effect of propolis is associated with its ability to stimulate the expression of transforming growth factor-β (TGF-β) that participates in the early phases of wound repair such as hemostasis and inflammation (Farooqui and A. Farooqui, 2010).

There is significant difference compared to untreated wounds . The center of the treated wounds became a scar and the total wound size appeared lesser than those of control group along the duration of the study. There was a significant reduction in the wound surface area in the propolis treated wound The wound reepithelization, contraction, and total wound healing were faster in propolis treated group than in control group , this is agree with (Abu-Seida, 2015).

Propolis helps the wound healing in a time-dependent manner. This could be attributed to immunomodulatory , antioxidant , analgesic, and anti-inflammatory effects of the propolis (Abdelrazeg *et al.*, 2020).

Its anti-inflammatory effects by the inhibition of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), TNF-α and IL-6 expression , propolis is also shown to inhibit Matrix metallopeptidase 9(MMP9), which may help reduce the degradation of ECM in chronic wounds The inhibition of MMP9 is of particular interest because there is increased expression of collagenases and gelatinases in chronic wounds (Shah and Amini-Nik, 2017).

Caffeic acid phenethyl ester (CAPE) derived from the propolis is a potent inhibitor of T cell receptor-mediated T cell proliferation. This inhibition is via suppression of both IL-2 gene transcription and IL-2 synthesis in stimulated T cells so it has immunosuppressive activity and play a key role in the onset of several inflammatory diseases (Chan *et al.*, 2013) .

Data suggest an increase in the fungicidal activity of macrophages by propolis. In addition, propolis inhibits bacterial growth by preventing cell division, disorganizing the cytoplasm, the cytoplasmic membrane, and the cell wall, causing a bacteriolysis and inhibition of protein synthesis (Ramadoss and Subha, 2015) .

Oliveira *et al.* shared a similar view that propolis speeds up the healing process not only through its anti-inflammatory effect, but also by direct action on fibroblast proliferation. (Marquele-Oliveira *et al.*, 2019).

(Aparecida *et al.*, 2011) (Szliszka *et al.*, 2013) have reported the anti-inflammatory potential of Art-C (Artepillin C) through a decrease in the cytokine synthesis (TNF-α, IL-1β, IL-6, IL-8, IFN-γ and others) and pro-inflammatory molecules (nitric oxide, histamine, leukotrienes, prostaglandins, prostacyclins and thromboxanes); inhibition of vasodilatation; migration of new immune cells; and prevention of the symptoms of inflammation such as pain, oedema and erythema.

The concentration of 250 μg/mL was that promoted the highest percentage (92.4%) of antioxidant activity in ethanolic extract of propolis, the concentration of 5 μg/mL promoted the lower potential (22.1%) of antioxidant activity propolis showed the highest antimicrobial activity against *Enterococcus* sp., *Staphylococcus aureus*, and *Klebsiella* sp. with MIC Minimum inhibitory concentration values of 31.3, 62.5, and 31.3 μg/mL (Silva *et al.*, 2019).

4.7 Effect of vitamin C on MDCK cell line viability

Higher concentrations of vitamin C (40 mg/L) displayed a remarkably negative influence on proliferation, it showed cytotoxic effects on fibroblasts and keratinocytes after 24 hours of incubation time (Rembe *et al* ., 2018).

The reason for this effect could be the function of vitamin C as a cofactor in collagen synthesis (staudte *et al*., 2010).

The cell viability of l-ascorbic acid was stronger than the control cells . Significant cell growth in l-ascorbic acid-treated human epithelial cells was observed when compared with untreated control. Since cell proliferation and wound repair are closely related, it is believed that l-ascorbic acid would effectively increase the potential effect of wound healing activity in human (lam P.-L.*et al* ., 2014) .

Topical vitamin C protect the skin from UV-induced damage by reducing free radicals; it is required for collagen synthesis and may reduce wrinkles. Even though vitamin C is a promising agent for cell proliferation, skin protection, and wound healing support. (Chiricozzi, 2013).

4.8 Effect of vitamin C on wound healing

Result showed there were a significant healing ($p<0.001$) in all concentration at 9 hr in comparison to control group and decrease wound diameter .

Hypothesised that Vitamin C could promote wound healing by altering the inflammatory, proliferative and remodelling phases of wound healing The proliferation stage of wound healing is typically characterised by increased expression of the pro-reparative growth factor cytokines such as TGF-β, Connective tissue growth factor (CTGF) and VEGF . TGF-β plays an important role at all stages of wound healing. In the initial stages, it serves as a chemotactic factor for pro-inflammatory cells as well as fibroblasts. In later stages, TGF-β provides a strong mitogenic signal for fibroblasts its showed vitamin c increase TGF-β levels (Bassem M Mohammed et al , 2016).

Vitamin C could promote wound healing through a variety of mechanisms. For example, vitamin C protects the function of vascular endothelium, increasing the expression of vascular endothelial growth factor (VEGF), which promotes cell division and secretion of matrix proteins. VEGF also promotes angiogenesis, which is an essential element for the regeneration of damaged tissues (Li *et al.*, 2018).

Collagen is the major protein component of connective tissue and is composed primarily of glycine, proline, and hydroxyproline. Collagen synthesis requires hydroxylation of lysine and proline, and co-factors such as ferrous iron and vitamin C. Impaired wound healing results from deficiencies in any of these co-factors(Guo and DiPietro, 2010).

4.9 Effect of Zinc on MDCK cell line viability

This finding confirmed previous reports of it being biocompatible with normal cells at low concentration , Zn sulfate has no cytotoxicity on normal cells (Kanagamani *et al.*, 2019).

(Saranya *et al.*, 2017) showed improved cell viability at lower concentration (10 µg/100 µl) in all type of cells (Vero, PK 15 and MDCK cells) Viability of the cells was assessed by the ability of living cells to reduce the yellow dye MTT to a blue formazan crystal. This may be due to the capability of reactive. oxygen species (ROS) generation . Elevated

ROS levels induce significant damage to the DNA of the cells, resulting in the arrest of cell-cycle and subsequently cell death .

Other study shows that Zn did not affect normal Madin-Darby Canine Kidney (MDCK) cells which showed 95 % cell viability at a concentration of 0.06 mg/mL. Did not show any toxicity towards normal even at 200 ug/mL concentration (Singh, Das and Sil, 2020).

4.10 Effect of zinc sulfate on wound healing

Zinc is an essential trace element (micronutrient) which plays important roles in human physiology. Zinc is a cofactor for many metalloenzymes required for cell membrane repair, cell proliferation, growth and immune system function topical Zn sulphate ($ZnSO_4$) dampens inflammation and reduces bacterial growth in epidermal wounds (Larsen et al., 2017).

Zinc ability to heal wounds related to owning the antimicrobial property as well as their role in fibroblast proliferation , Antioxidant properties of natural products is evaluated these as it correlates with oxidation inhibition mechanism of biomolecules and therefore, confer amazing protection against stress related disorders .Antioxidants in a moderate concentration can significantly improve healing of wounds (Ahmed *et al.*, 2018) .

Zinc was shown to increase keratinocyte migration and participate in re-epithelialization of the epidermis .Concurrent with re-epithelialization, endothelial cells migrate and proliferate into wound sites to establish new blood vessels in a process called neovascularization, or angiogenesis, thus supplying essential oxygen and nutrients for the growth of cells in the wound bed (Lin *et al.*, 2018).

Tripartite motif family (TRIM) proteins and an N-terminal ring zinc finger domain play important biochemical roles in regulating biochemical processes associated with wound healing and normal physiological processes. TRIM protein, Mitsugumin 53, and TRIM72 are implicated in tissue repair after injury. Vascular endothelial and transforming growth factors facilitate wound healing, and these growth factors require zinc for normal physiological functions. (Dennis et al ,2020).

4.11 Effect of combination vitamin C and Zinc Sulfate on wound healing

Vitamin C and zinc are essential nutrients Both have profound effects on cellular growth and differentiation, and are vital for the optimal functioning of the immune system. Inadequacy and clinical deficiency of vitamin C and/or zinc lead to impaired immune response with altered resistance to infections, impaired growth, and weakened collagenous structures with delayed wound healing (wintergrest et al ,2006).

Vitamin C seems to be involved in wound healing with several roles in cell migration and transformation , antioxidant response, and angiogenesis. In the inflammatory phase, it participates in the recruitment of cells to the wound and their transformation into macrophages . During collagen synthesis, vitamin C forms extra-bounds between collagen fibers that increase stability and strength of collagen matrix . Vitamin C is essential to counteract the production of free radicals in damaged cells, while its deficiency might increase the fragility of new vessels However, vitamin C supplementation seems to have a beneficial effect only in combination with zinc (Barchitta *et al.*, 2019).

Supplementation of vitamin C in doses of about 500mg, combined with at least 17mg zinc is helpful for wound healing. experimental evidence supports nutritional supplementation for promoting the healing of wounds, although the exact genetic mechanism of action is not well understood and lack of clinical validated studies. To support this hypothesis there is also the evidence that malnutrition negatively interfere with wound closure (Palmieri and 1 MD, 2019) .

Vitamin C contribute to the proper functioning of Zn, which act promoting antibody production and contribute to the proper proliferation and maintenance of immunocompetent cells (Ribeiro *et al.*, 2019).

Conclusion:

1 - Aqueous extract of Tamarix mannifera , Moreingo Oleifera and alcoholic extract of propolis not toxic on MDCK line except at highest concentration while Vitamin C enhance cell viability at lower concentrations while zinc decrease cell viability at highest concentration.

2- All agents cause good wound healing effect .

3- Active constituent play an important role in cell viability and then of wound healing by different role depending on their constituent.

4- Combination of vitamin C and zinc has better effect in wound healing than vitamin c or zinc alone.

Recommendation :

1- Separation and purification of the active ingrediant that recorded through the qualitative analysis of Tamarix mannifera and Moreingo oleifera leaves extracts, to investigate about their wound healing effect

2- Use the obtained optimal concentration obtained in this research study for combination of plant extract and Vitamin c and zinc for wound healing on cell line.

3- Applied this study on lab animal (in vivo study)

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الخلاصه

معالجة الجروح ضروري لأن الجرح يمكن أن يكون مميتا إذا لم يلتئم بشكل صحيح. يمكن لنباتات طب الاعشاب أن تعالج الجروح لأنها لا تسبب أي آثار جانبية ، بينما في حالة الأدوية الكيميائية ، فإن الآثار الجانبية آخذة في الازدياد.

هدفنا في هذه الدراسة هو تحديد تراكيز المستخلص المائي لمادة التماريكس مانيفيرا والمورينجو اوليفيرا و المستخلص الكحولي من البروبوليس وكذلك فيتامين سي والزنك على حيوية خطوط خلايا الكلى مادين داربي (MDCK). ثم قمنا بدراسة تأثير هذه التراكيزا على التئام الجروح واكتشاف أكثرها فعاليه في التئام الجروح

تضمنت التجربة الأولى: تعريض الخلايا لتركيزات مختلفة من النباتات والزنك وفيتامين سي وقد تم انباتها في طبق زرعي في 96 مكان زرعي .

في جميع التجارب كانت التركيزات المخففه المستخدمة من النباتات وفيتامين سي والزنك كما يلي:

✓ مستخلص التامريكس المائية: (500 ، 250 ، 125 ، 62.5 ، 31.25 ، 15.6) ميكروغرام / مل.

مستخلص مائي مورينجو اوليفيرا: (500 ، 250 ، 125 ، 62.5 ، 31.25 ، 15.6) ميكروغرام / مل.

✓ مستخلص العكبر الكحولي: (500 ، 250 ، 125 ، 62.5 ، 31.25 و 15.6) ميكروغرام / مل.

فيتامين سي : (500 ، 250 ، 125 ، 62.5 ، 31.25 ، 15.6 ، 7.8 ، 3.9 ، 1.95) ميكروغرام / مل.

✓ الزنك: (500 ، 250 ، 125 ، 62.5 ، 31.25 ، 15.6 ، 7.8 ، 3.9 و 1.95) ميكروغرام / مل.

تم تحضين جميع الخلايا لمدة 24 ساعة عند 37 درجة مئويه

قابلية بقاء الخلايا تقاس بمقايس MTTو أظهرت النتائج عدم وجود فرق معنوي في حيوية الخلايا ولكن تسببت المستخلصات والزنك بأعلى تراكيز في انخفاض معنوي ($P<0.001$) في نسبة الحيوية.

تضمنت التجربة الثانية: تعريض الخلايا المخدوشة لتراكيز معينه التي تم الحصول عليها من التجربة الأولى.

مستخلص التماريكس (125 , 62.5, 31.25 , 15.6 , 7.8 µ/مل)

مستخلص المورينجو (62.5, 31.25 , 15.6 , 7.8 µ/مل)

مستخلص العكبر (62.5, 31.25 , 15.6 , 7.8 , 3.9 , 1.95 , 0.97 , 0.48 , 0.24 µ/مل)

فيتامين سي (62.5, 31.25 , 15.6 , 7.8 , 3.9 , 1.95 µ /مل)

زنك (62.5, 31.25 , 15.6 , 7.8 , 3.9 , 1.95 µ /مل)

اتحاد فيتامين سي مع الزنك (62.5, 31.25 , 15.6 , 7.8 , 3.9 , 1.95 µ /مل)

تم عمل الجرح باستخدام طرف قطاره بلاستيكية معقمة سعة 200 ميكرولتر للضغط بقوة على الطبقة أحادية الخلية في الطبق الزرعي .

تم تصوير منطقة الجرح رقميًا كل 3 ساعات بدءًا من الساعة 9 صباحًا (0 ساعة) و 3 ساعات و 6 ساعات و 9 مساءً أي بعد 9 ساعات

أظهرت نتائج التجربة الثانية وجود شفاء معنوي في جميع التراكيز عند 9 ساعات مقارنة بالمجموعة الخاليه من الدواء وصغر قطر الجرح

اظهرت النتائج أن المستخلص المائي من التماركس والمورينجو والمستخلص الكحولي للعكبر غير سام على خط الخلايا إلا عند أعلى تركيز بينما يعزز فيتامين سي بقاء الخلية بتركيزات منخفضة بينما يقلل الزنك من حيوية الخلية عند أعلى تركيز وان المزيج من فيتامين سي و الزنك له تأثير أفضل في التئام الجروح من فيتامين سي أو الزنك وحده .

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامــعة بابــل

كليـة الطب

تأثير مستخلص بعض النباتات العشبيه على الجروح:دراسه في الزجاج

رســالــــــة

مقدمــــة إلــى مجلس كليــة الطــب / جامعــة بابــل

كجزء من متطلبــات نيـل درجــة الماجستيـر في الأدويه و السموم

من قبل

دعاء عباس محمد الجمل

بكالوريوس صيدلة

2016-2015

اشراف

أ.د نسرين جلال محمد
دكتوراه ادويه
بايولوجي

أ.د رنا اياد غالب
دكتوراه

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