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Effect of *Ricinus communis*, phenytoin and metformin and their combination on wound healing. An in vitro study.

A thesis

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Certification

We certify that this thesis entitled "**Effect of *Ricinus communis*, phenytoin and metformin and their combination on wound healing. An in vitro study.**" was prepared by (**Dunia Abdul Hussian Shaker**) 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.

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Dedication

To

My Great Parents…

My Great Sisters…

My Wonderful Brother…

And…

To… All My Teachers …with Deepest Appreciation

Dunia

Acknowledgment

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Summary

Wound is a physical injury of the body that described by disruption of normal structures of the body. Wound may cause damage to superficial skin layers and reach to structures underlying the skin.

Wound healing is an important physiological process to maintain the integrity of skin after trauma, either by accident or by intent procedure. The normal wound healing involves three successive but overlapping phases, including hemostasis/inflammatory phase, proliferative phase, and remodeling phase.

This study aimed to investigate the effect of *Ricinus communis* leaf extracts (ethanolic and aqueous), metformin and phenytoin alone and in combination on healing wounds induced on the normal Madin-Darby Canine Kidney (MDCK) cell line. This study includes two parts:

Part one: in order to determine the safety of tested agents(ethanolic and aqueous plant extracts, metformin and phenytoin),a pilot study was performed to investigate their cytotoxicity on the MDCK normal cell line and accordingly a range of concentrations to be used for wound healing assay was chosen.

In the cytotoxicity assay, the MDCK cell line was seeded in tissue culture 96 well plate and treated with each ethanolic and aqueous *Ricinus communis* leaf extracts with different concentrations (31.25, 62.5, 125, 250,500, 1000 µg/ml).

The same experiment was done but with the use of different concentrations of metformin (3.9, 7.8, 15.6, 31.25, 62.5,125,250 ,500 ,1000, 2000 µg/ml)

or phenytoin (62.5,125,250,500,1000,2000 µg/ml). After that all treated cells were incubated for 24 hours at 37°C, then, the MTT assay was performed.

Part two: a vertical wound was made by using 200µl sterile plastic micropipette tip to press firmly against the cell monolayer of tissue culture plate. Then the wounded cells were exposed to different concentrations of each tested agents that were obtained from part one of this study. Six experiments were done as follows:

Experimental number 1: treatment of the MDCK wound model with (15.6, 31.25,62.5, 125, 250, 500 µg/ml) of ethanolic leaf *Ricinus communis* extract.

Experimental number 2:treatment of the MDCK wound model with (15.6, 31.25,62.5, 125, 250, 500 µg/ml)of aqueous leaf *Ricinus communis* extract.

Experimental number 3: treatment of the MDCK wound model with (3.9, 7.8,15.6,31.25,62.5, 125, 250, 500, 1000 µg/ml) of metformin.

Experimental number 4: treatment of the MDCK wound model with a combination of metformin serial dilution(125,250,500,1000 µg/ml) plus either 250 or 500 µg/ml of *Ricinus communis* leaf ethanol extract.

Experimental number 5: treatment of the MDCK wound model with (1.9, 3.9,7.8,15.6,31.25,62.5 µg/ml) of phenytoin.

Experimental number 6: treatment of the MDCK wound model with a combination of phenytoin serial dilution(1.9,3.9,7.8,15.6,31.25,62.5 µg/ml) plus either 250 or 500 µg/ml of *Ricinus communis* leaf ethanol extract.

After that the wound area were digitally photographed every 3 hours till 12 hours.

The cytotoxicity results showed that the concentration(125 µg/ml) of *Ricinus communis* leaf aqueous extract significantly increased ($p \leq 0.05$) the viability of MDCK cell line, while no significant differences was found($p > 0.05$) after the treatment with the leaf of plant ethanolic extract in comparison to the control group.

Result showed that the viability of MDCK cell line was significantly increased($p \leq 0.05$) after the treatment with the concentration(62.5 µg/ml) of each metformin and phenytoin, while at high concentrations of metformin (2000 µg/ml) and phenytoin(2000,1000,500,250 µg/ml) the viability was high significantly decreased ($p < 0.001$).

Wounds healing results showed that all concentrations except 500 µg/ml of *Ricinus communis* leaf ethanolic extract caused highly significant decreased ($p \leq 0.001$) in the wound diameter, while all concentrations of *Ricinus communis* leaf aqueous extract caused highly significant decreased($p \leq 0.001$) in the diameter of the wound with complete healing in comparison to the control group.

Also results showed that all concentrations of metformin except (15.6, 31.25,62.5 µg/ml) and all concentrations of phenytoin except the lowest one(1.9 µg/ml) respectively caused highly significant decrease ($p < 0.001$) in the diameter of the wound with the complete healing.

Regarding the combined effect of *Ricinus communis* leaf ethanolic extract plus either metformin or phenytoin results showed that both combinations caused highly significant decrease ($p \leq 0.001$) in diameter of wound and the healing of wound results showed incomplete healing of wound that treated with all concentrations of metformin plus *Ricinus communis* leaf ethanolic

extract except 250 µg/ml of plant extract plus 125 and 250 µg/ml of metformin and complete healing with all concentrations of phenytoin plus *Ricinus communis* leaf ethanolic extract except these containing 500 µg/ml of plant extract plus 31.25 and 62.5 µg/ml of phenytoin caused incomplete wound healing.

In conclusion : *Ricinus communis* (ethanolic and aqueous) leaf extracts, metformin and phenytoin have healing effect on the MDCK cell line wound model when used alone better than that of combination between each other except the combination of phenytoin plus 250 µg/ml of plant extract which result in healing at faster time.

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List of Abbreviations

Abbreviation	Meaning
ATP	Adenosine triphosphate
AMPK	Adenosine monophosphate kinase
Bud-8	Human Caucasian skin fibroblast
BM-EPC	Bone marrow -endothelial precursor cells
COX1	Cyclooxygenase 1
C˚	Centigrade
DNA	Deoxyribonucleic Acid
DDW	Deionized distilled water
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylene-diamine tetra acetic acid
ENOS	Endothelial nitric oxide synthase
ECs	Endothelial cells
FBS	Fetal bovine serum
FDA	Food and drug administration
GP Ib/IX/V	Glycoprotein receptor Ib/IX/V
G. officinali	*Galega officinalis* plant
g	gram
HepaRG	Hepatocyte cell line
Hek293	Non-cancerous kidney cells
kg	kilogram
MDCK	Madin-Darby Canine Kidney

MMP	Matrix metalloproteinases
μm	micron membrane
M2	Macrophage-polarized anti-inflammatory
MTT	(3-(4,5-Dimethylthiazole-2-yl)- 2,5-diphenyl-tetrazolium bromide
M1	Pro-inflammatory macrophage
mg	milligram
ml	milliliter
mTOR	mammalian target of rapamycin
μl	microliter
μg	microgram
NADH	Nicotinamide adenine dinucleotide hydrogen
nm	nanometer
NO	Nitric oxide
PBS	Phosphate Buffer Saline
RNA	Ribonucleic acid
R. communis	*Ricinus communis* plant
RCA	*Ricinus communis* agglutinin
RPMI-1640	Roswell Park Memorial Institute-1640
rpm	revolution per minute
SCN	Suprachiasmatic nucleus
TFEB	Transcription factor EB
WHO	World health organization

Chapter One

Introduction and Literature Review

1.1 Introduction

The skin provides a life-protective barrier between the body and the external environment against physical damage, pathogens, fluid loss, and has immune-neuroendocrine functions that contribute to the maintenance of body homeostasis .Structurally the epidermis and dermis are the two layers that make up the skin (Dorantes and Ayala, 2019).

Understanding the normal cell structure (including the skin) and function is essential for the study of cellular responses to injury. Simplistically, cell injury disrupts cellular homeostasis. Cells are injured by numerous etiologic agents from intrinsic and extrinsic sources; however, all of them, activate one or more of four common biochemical mechanisms leading to cell injury. The mechanisms of cell injury include (1) ATP depletion (2) per-meabilization of cell membrane (3) disruption of biochemical pathways, and (4) DNA damage (Miller and Zachary, 2017).

Physiological knowledge of the normal wound healing trajectory through the phases of hemostasis, inflammation, granulation and maturation provides a framework to understand the basic principles of wound healing, in order to develop the skills required for care of wound and the patient can be helped with the complex task of tissue repair (Hadi, 2019).

Many herbal products can be successfully used in the treatment of wounds owing to various mechanisms inducing healing and regeneration of the skin such as those having antimicrobial, anti-inflammatory, and antioxidant activities (Herman and Andrzej, 2020)

Aim of study:

- Study the effects of *Ricinus communis* leaf extracts (ethanolic and aqueous), metformin, and phenytoin on the viability and wound healing in wound model by using MDCK cell line.

- Investigate the combined effect of plant extract (ethanolic) plus either metformin or phenytoin on the wound healing in wound model by using MDCK cell line in comparison to each of them alone.

1.2 Wound:

Wound is defined as damage or disruption to the normal anatomical structure and function. This can range from a simple breach in the epithelial integrity of a tissue such as dermal tissue or it can be deeper, that can extended into subcutaneous tissue with damage to other structures such as tendons, muscles, vessels, nerves, parenchymal organs and even bone (Yadav *et al*., 2017; Kent *et al*., 2018).

Wound produced by any accident or cut with sharp edged things. It may be produced due to physical, chemical, thermal, microbial or immune-logical exploitation to the tissues (Sabale *et al*., 2012).

There are various types of wounds, including an incised wound, lacerated wound, abrasion, contusion, ulcer, and burn wounds . Treatment of the wound usually involves preventing infection because the skin, the body's barrier to infection, is destroyed. Inappropriate caring of the wound may delay its healing, causing the area to become infected and subsequently resulting in chronic wounds (wong *et al*., 2012).

1.3 Classifications of wounds

1.3.1 Classifications of wounds based on duration of healing

1-Acute wounds are tissue injury that normally proceed through the normal wound healing stages, resulting in an expectable and organized tissue repair arrangement (Tottoli *et al*., 2020).

Cuts or surgical incisions are the most common sources of acute wounds, which heal in the anticipated amount of time (Garg and Deep, 2015).

2-Chronic wounds are defined as wounds that fail to proceed through the normal phases of wound healing in an orderly and timely manner, such as diabetic ulcer and burn (Wolcott *et al*., 2010 ; Frykberg and Banks, 2015).

1.3.2 Classifications of wounds based on their nature

1.Closed wounds: in this type of wounds, the skin is unbroken and remains intact, thus the underlying tissue is not directly exposed to the outside environment.

2.Open wounds: in this type of wounds, the skin is broken or removed, and the underlying tissue is exposed to the outside environment. Open wounds are sub classified into abrasion, laceration, puncture and avulsion (Abdell atif and Abou-Taleb, 2018).

A-Abrasion wound

Abrasion wound is a partial thickness wound caused by damage to the skin and can be superficial involving only the epidermis or deep, involving the dermis. Abrasions usually associated with minimal bleeding. Mild abrasions, also known as graze or scrape, does not scar or bleed because the dermis is still intact, but deep abrasions that disrupt the normal dermal structures may lead to the formation of scar tissue (McCurnin and Bassert, 2010).

B- Lacerated wound

Laceration wound is caused by a blunt force result in the tearing of the tissues. It commonly occur as a result of a fall and is often situated on the scalp or over the bony prominences of the face (Young *et al*., 2005).

C- Puncture wound

Puncture wound is a small hole in the soft tissue. caused by nails; however, glass, wood, or other metal objects can be the source of the puncture wound (Haverstock, 2012).

D- Avulsion wound

Avulsion wound is characterized by damage of the subcutaneous layer of the skin (hypodermis).It can result from violent incidents, such as explosions, animal attacks (McCurnin and Bassert, 2010).

1.3.3 Classification of wounds based on contamination

1-Clean wound: An uninfected operative wound in which no inflammation is present. Additionally, this wound does not enter the respiratory, alimentary, genital, or urinary tracts. It often involve the eye, skin, or vascular system.

2-Clean-contaminated wound: An operative wound which enter the respiratory, alimentary, genital, or urinary tracts under controlled conditions and without unusual contamination. Specifically, operation involving the biliary tract, appendix, vagina and oropharynx are included in this category (Ikemefuna *et al*., 2017).

3-Contaminated wound: An open, fresh, accidental wound, operations with a major breaks in the sterile technique (for example, open cardiac massage) or gross spillage from the gastrointestinal tract, and incisions in which acute, non-purulent inflammation is encountered.

4-Dirty-contaminated wound: An old traumatic wound, with retained devitalized tissue or that involves existing clinical infection or perforated viscera (Mioton *et al*., 2013).

1.4 Management of wounds

All wounds should be explored and debrided of devitalized tissue or containments. Because of the risk of litigation, good documentation in this area is extremely important (Gubern *et al*., 2010).

Key components of wound care include such measures as debride-ment, irrigation, and wound cleaning. The debridement of the wound, is a necessary phase because it facilitates the latter stages of healing by removing the sloughy tissue, which causes hypoxia to the wound area, inhibits the development of granulation tissue and slows re-epithelialization (Meaume *et al*., 2012).Appropriate care to removes necrotic tissue and reduces wound bio-burden should be followed in order to enhance the healing of the wound. Wounds may be washed with water, saline, or Ringer's solution or cleaned with active ingredients, such as hydrogen peroxide, acetic acid, alcohol, ionized silver preparations (Wilkins and Unverdorben, 2013).

After a wound has been managed by a given closure technique or left to heal by secondary intention, topical products and dressing materials should be used if necessary. Most commonly, an antibiotic or antiseptic ointment is used. Another option in the care of a healing wound is the use of occlusive dressings. There are several advantages to the use of such products, one major benefit is the creation and maintenance of a moist environment (Rivera and Spencer, 2007).

The process of wound healing is highly affected by the level of moisture at the wound interface. It is well documented that the provision of a moist healing environment results in an increase in the rate of healing via increased re-epithelialization, macrophage and fibroblast activity, rapid debridement, decreased pain (especially during removal), and reduced dressings changes such examples include foam and alginate dressings (Sweeney *et al*., 2012).

1.5 Wound healing process:

Wound healing is defined as a natural physiological process occurring as a reaction to the structural damage of tissues, including skin. Wound healing mechanisms involve sophisticated complimentary interactions between different types of cell, that acting through networks of soluble mediators, including cytokines, chemokine, growth factors, and metabolites. Wound healing consists of four subsequent and overlapping phases (figure 1.1) including hemostasis, inflammation, proliferation (re-epithelialization) and remodeling (scar maturation) (Holl *et al*., 2021; Keskin *et al*., 2021).

1.5.1 Hemostasis phase

Hemostasis is the physiological process that stops bleeding at the site of an injury while maintaining normal blood flow elsewhere in the circulation. The creation of a hemostatic plug halts blood loss. Blood vessels' endothelium maintains an anticoagulant surface that keeps the blood in a fluid state, nonetheless if the blood vessel is damaged, components of the sub-endothelial matrix are exposed to the blood (Gale, 2011).

The blood vessel becomes narrow, restricting the blood flow. Platelets are accumulated to form a clot that seals the ruptured wall of the blood vessel, thereby terminating the bleeding (Alven and Aderibigbe , 2020). Wound healing processes involved in the hemostasis phase include:

A-Vasoconstriction: Vascular spasm occurs whenever there is an injury or damage to the blood vessels. This will trigger a vasoconstriction, which could eventually stop the blood flow. This reaction can be responded within 30 minutes, and is localized to the injured area (Periayah *et al*., 2017). Vasoconstrictors like endothelin cause the vascular smooth muscle to reflexively contract, that is released by the endothelium's damage. Furthermore, circulating catecholamine (epinephrine and norepinephrine) and prostaglandins are liberated from the damaged cells to regulate vasoconstriction. Platelets themselves produce platelet-derived growth factor which preferentially activates mesenchymal cells, especially smooth muscles in the vessel walls causing blood vessel contraction. However, initial reflexive contraction reduce bleeding only temporarily. Because the increasing hypoxia and acidosis of the injured tissue results in passive relaxation of the muscle, that causes bleeding to resume. Therefore subsequent activation of the coagulation cascade is needed for further regulation of vasoconstriction through the mediators serotonin, bradykinin, fibrinopeptide, and thromboxane A_2 to prevent long term bleeding (Rodrigues *et al*., 2018).

B- Platelet plug formation (primary hemostasis): Primary hemostasis occurs as platelets adhere to collagen fibers exposed in the damaged endothelium using specific collagen receptor glycoproteins (GP-Ib/IX/V) to form the primary hemostatic plug. The attachment of platelets to the lesion

site, result in induction of integrin and enhanced the attachment of the platelets to other platelets and the surrounding extracellular matrix(ECM) (Stroncek and Reichert, 2008).

C- Platelet plug coagulation and reinforcement (secondary hemostasis): During secondary hemostasis, the activation of coagulation reinforces the platelet plug through deposition of an insoluble fibrin network. It includes the two main coagulation pathways, intrinsic and extrinsic (Grover and Mackman, 2019) both of which are activated by exposure of the sub-endothelial matrix, and result in the activation of factor X. Following the factor X activation via either pathway, pro-thrombin gets converted into thrombin, which cleaves fibrinogen into fibrin (Rodrigues *et al*., 2018).

1.5.2 Inflammatory phase

Inflammatory phase play a role in clear pathogens as well as foreign material from the wound and to contain the damage to a localized area. Usually, inflammation lasts a few days, but it can last for up to two weeks. Release of the neuropeptide substance P from the peripheral nerves in a wound leads to an increase in the vascular permeability, and various chemo-attractants are released, allowing for an influx of neutrophils and monocytes to localize to the inflammation site, which eventually monocytes differentiate into macrophages. Macrophages of the inflammatory phenotype (M1) work to remove bacteria and debris. Once the wound is free of foreign material, macrophages polarize towards the anti-inflammatory (M2) state to resolve the inflammatory phase (Han and Ceilley, 2017; Berge *et al*., 2021).

1.5.3 Proliferative or granulation phase

This phase is primarily characterized by tissue granulation, formation of new blood vessels (angiogenesis), and epithelialization (Frykberg and Banks, 2015).

Fibroblasts are the key cells involved in the production of ECM. In addition to producing collagen, they produce fibronectin, tenascin and proteoglycans such as hyaluronic acid. Production of ECM is seen clinically as formation of granulation tissue. The combination of new tissue and contraction of surrounding tissues is essential for the healing of ulcers. While new matrix is synthesized, the existing matrix in and around the wound margin is degraded by several enzyme systems such as matrix metalloproteinases (MMP) and plasminogen activators .While some keratinocytes at the wound edge proliferate, others undergo a marked transformation to enable them to phagocyte debris and migrate across the wound bed. Keratinocyte migration coupled with wound contraction results in re-epithelialization and wound closure (Wadinamby , 2013).

1.5.4 Maturational or remodeling phase

Maturation typically begins one week after injury following collagen deposition in the wound and is the longest phase of wound healing, continuing for weeks to months after injury .The main activity happening during this phase is the strengthening and remodeling of the newly formed collagen. In this phase the proliferation and inflammation are reduced, also there is regression of the newly formed capillaries as the nutrient requirements for the wound site is reduced in the wound bed (Mickelson *et al*., 2016).Type III collagen produced by fibroblasts during the proliferative

phase is gradually replaced by type I collagen, through the action of collagenases and (MMP). During remodeling, the collagen becomes more organized and increasingly cross-linked strengthening the scar, fibronectin disappears, and hyaluronic acid and glycosaminoglycan are replaced by proteoglycans. The result is the re-organization of ECM to an architecture more closely resembling normal tissue (Portou *et al*., 2015).

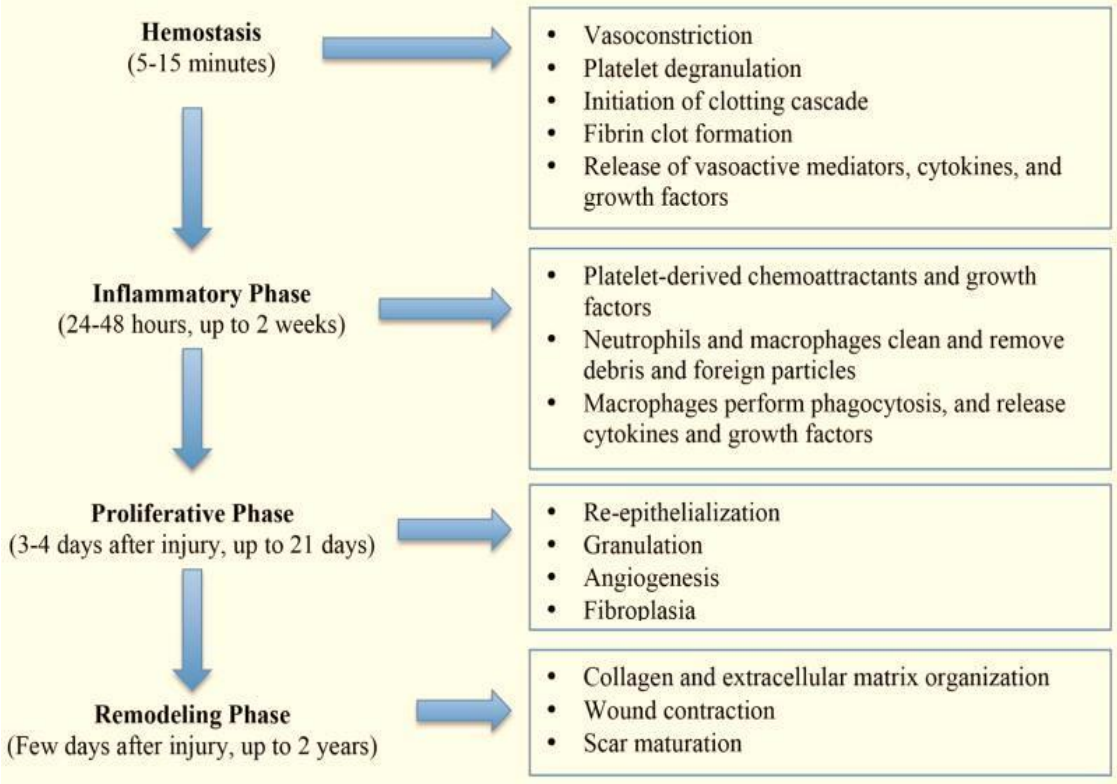


Figure (1.1) Phases of wound healing (Mickelson *et al*., 2016).

1.6 Factor influence wound healing:

1.6.1 Local factor

1.6.1.1 Oxygen

Oxygen is essential for practically all wound-healing procedures as well as cell metabolism, including energy synthesis via adenosine triphosphate. It increases keratinocyte differentiation, migration, and re-epithelialization, protects wounds from infection, stimulates angiogenesis, boosts fibroblast proliferation and collagen synthesis and encourages wound contraction (Guo and Dipietro, 2010).

1.6.1.2 Infection

Endotoxins and bacteria can both cause persistently elevated in level of pro-inflammatory cytokines such as interleukin-1 and tumor necrosis factor-alpha and elongate the inflammatory phase. Prolonged inflammation in turn increases the level of (MMPs), a family of proteases that can degrade the ECM (Giri, 2018).

1.6.2 Systemic factor

1.6.2.1 Age

In older patients wound may heal more slowly than those in younger patients, mainly because of ages associated comorbidities. Additionally older patients may intake inadequate nutritional, and may have altered hormonal responses, poor hydration, and compromised immune, circulatory, and respiratory systems, any of which can increase the risk of skin break-down and delay wound healing.

1.6.2.2 Immunosuppression and radiation therapy

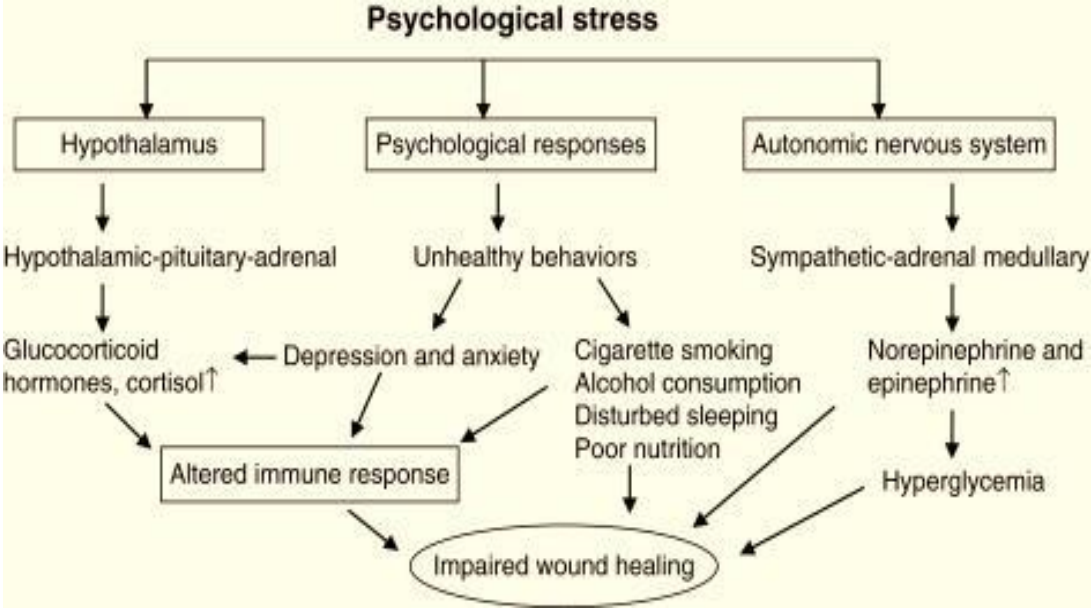
Immune system suppression by either diseases, age, or medications can delay wound healing through the inhibition of cellular metabolism, rapid cell division, and angiogenesis and thus inhibit many pathways that are critical to appropriate wound repair. Radiation therapy can cause ulceration or change in the skin, either immediately after the initiation of a treatment or then after the termination of the treatment (Cathy, 2011).

1.6.2.3 Medications

Use of anti-inflammatory medications, such as glucocorticoids , non-steroidal anti-inflammatory drugs, and chemotherapeutics have been shown to slow wound healing, mostly as a result of interference with platelet function or inflammatory processes (Pence and Woods , 2014).

1.6.2.4 Stress

Psychological stress leads to the activation of the hypothalamic-pituitary-adrenal and the sympathetic-adrenal-medullary axes, enhancing glucocorticoids and catechol-amines production which can directly influence several components of the healing process. Substantial evidence from animals and humans studies indicate that physiological stress responses can retard the initial inflammatory phase of wound healing (figure1.2) (Gouin, and Glaser, 2011).



Figure(1.2) Effects of stress on wound healing (Giri, 2018).

1.6.2.5 Diabetes mellitus

Vascular changes in people with diabetes mellitus account for some of the problems with wound healing. Reducing the blood supply to the area and directly to the wound bed can have a huge impact on the speed of healing and thus the ability to prevent further complications e.g. infection.

In the diabetic patient the wound healing inflammatory phase is impaired due to the thickening of the blood vessels, therefore reducing the leucocytes numbers and speed in which reach the site of injury. Furthermore there seems to be some evidence to suggest that even if leucocytes are present their phagocytosis ability is significantly impaired (Sharp and Clark, 2011).

1.6.2.6 Obesity

Obesity increase wound complications and as have been shown by several studies the postulate mechanisms is due to poor vascularity of

adipose tissue, that can decrease oxygen tension, which in turn may suppress collagen synthesis, capacity to fight infection, and ability to support the necessary mechanisms for the healing cascade (Pierpont *et al*., 2014).

1.6.2.7 Nutrition

1.6.2.7.1 Protein and amino acids

Proteins provide the main building blocks for tissue growth, cell renewal, and repair throughout the wound healing process. Proteins significantly affect the entire process of wound healing through their roles in RNA and DNA synthesis, collagen and elastic tissue formation, immune system function, epidermal growth, and keratinization therefore, it is vital to provide proteins for wound healing (Wang *et al*., 2022).

1.6.2.7.2 Lipids and essential fatty acids

Fats serve as the building blocks for epidermal and dermal tissues and offer the energy required for proliferation. They are important for cell membrane synthesis, epidermal phospholipids, inflammatory reactions, and intracellular matrix synthesis (Brown and Phillips, 2010).

1.6.2.7.3 Vitamins

Vitamin A deficiency impairs the function of B and T cells and antibody production during the inflammatory phase. It also decreases epithelialization, collagen synthesis, and development of granulation tissue in the proliferative and remodeling phases (Barchitta *et al*., 2019).

Vitamin C has numerous functions in wound healing, and it is deficiency has multiple effects on tissue repair. It is necessary for collagen

formation, proper immune function, and as a tissue antioxidant (Douglas and Alan, 2003).

Vitamin E as an anti-oxidant, it maintains and stabilizes cellular membrane integrity by providing protection against destruction by oxidation. Vitamin E also has anti-inflammatory properties and has been suggested to have a role in decreasing excess scar formation in chronic wounds (Guo and Dipietro, 2010).

1.6.2.7.4 Minerals

Zinc: play important roles in enzymatic regulation. Hundreds of zinc-containing enzymes are involved in wound healing and are required in tissue repair, growth, antioxidant capacity, and immune function. Zinc is specifically critical for collagen, DNA, RNA, and protein synthesis, as well as cellular proliferation (Shields , 2021).

Magnesium: is an important trace element that functions as a cofactor for enzymes necessary for protein and collagen formation and tissue growth. During wound healing magnesium interacts with adenosine triphosphate to support the processes for collagen synthesis (Stechmiller, 2010).

1.7 Herbal Medicine and wound healing potential

The use of medicinal plants and herbs has recently been increased throughout the world for the maintenance and improvement of health and for the treatment of various human conditions and diseases(Abu-Al-Basal,2010)

The importance of plant-based products for disease treatment is growing exponentially due to the increased incidence of adverse drug

reactions and the development of microbial resistance to the available antimicrobials (Elkousy *et al*., 2021).

Different herbal formulations have been reported to hasten/accelerate the process of wound healing by enhancing epitheliazation, neo-vascularization, formation of granulation tissue, collagen synthesis, wound contraction and tensile strength (Nagoba and Davane , 2019).

The exudate of *Commiphora mukul* is an effective treatment for various wound kinds. A mouthwash made from the exudate of this plant was discovered in a clinical research to be efficient in treating intra-oral mucosal wounds. Its ability to reduce inflammation has been linked to COX1 inhibition. *Aloe vera* (*L*) leaf hydro-alcoholic extract speeds up burn healing. The gel increases wound contraction, epithelialization, alignment, and organization of the regenerated scar tissue, it also decreases scar tissue size and have antimicrobial and antifungal properties. Aqueous extract of *Adiantum capillus-veneris L* has angiogenic effects. Aqueous, methanol, and ethanol extracts of this plant showed significant antibacterial and antifungal activities against most of the multidrug-resistant bacterial and fungal strains (Hosseinkhani *et al*.,2017).

Medicinal plants contain biologically active chemical substances such as saponins, tannins, essential oils, flavonoids, alkaloids and other chemical compounds ,which have curative properties. Most of these phytochemical constituents are potent bioactive compounds found in medicinal plant parts which are precursors for the synthesis of useful drugs (Okugbi *et al*., 2015).

More than 80% of people throughout the world still use traditional medicines to treat a variety of diseases, according to a survey by the world health organization (WHO). In the developed countries 25 percent of the medical drugs are based on plants and their derivatives and the use of medicinal plants is well known among the indigenous people in rural areas of many developing countries (Alam *et al*., 2011). For example, about 25% of the prescriptions of drugs dispensed in the United States contain at least one active ingredient derived from plant material. Some are made from plant extracts; others are synthesized to mimic a natural plant compound (Tebeje, 2021).

1.8 *Ricinus communis*

Ricinus communis belongs to the Euphorbia Family (Euphorbiaceae), a diverse and economically-important family of flowering plants (Prakash and Gupta, 2014).

R. communis is the native plant in Africa, central America, India and in most of the tropical and subtropical areas of the world. By the Egyptians 6000 years ago, it was mainly cultivated and used as fuel for burn the lamps. It is usually a small soft wooded tree that grows up to 6 meters with varying stem pigmentation; the leaves which is usually 15 – 45cm long may be green or reddish in color made of about 5-12 coarsely toothed lobes; the fruits which is usually a three-celled thorny capsule covered with soft spins encloses the seeds (figure 1.3) (Ahmad *et al*., 2016).

The name "castor" was originally applied to the plant in Jamaica where it is called "Agnus- castus". Because the Castor plant’s leaves look

alike the palm of Christ hence many years ago the plant was referred to as "Palma Christe" (Singh *et al*., 2021).

The plant has proven its medical potential as the extracts from different parts of the plant have recently displayed remarkable bioactivities against several ailments, including pain, paralysis, diabetes, constipation and wound infections. Also, it is effective as an antioxidant and anti-inflammatory, hepato-protective, and anticancer agent, these medicinal applications are due to the presence of a wide range of phyto-constituents, including terpenoids, alkaloids, poly-uronides, anthrax-quinones, flavonoids, tannins, saponins , glycosides, steroids, and reducing sugars (Khalid *et al*., 2022).

Figure (1.3): *Ricinus communis* L.: A. Shoot of the plant, B. Flowers, C. Fruit (capsules) and D. Seeds (Marwat *et al*., 2017).

1.8.1 Toxicity

The castor contains many compounds that are poisonous to human beings, animals, insects and microorganisms. The major toxic protein, ricin is used as a biological weapon as a single milligram can kill an adult human being. It is mainly present in seeds and in small amount it is found in other parts of the plant. Ricin irreversibly inactivates ribosomes, leading to irreversible inhibition of protein synthesis and eventually resulting in cell death. Ricinine is an alkaloid toxin belong to a piperidine alkaloid. It is found in small amounts in all parts of the plant, including the leaves and seed. Unlike ricin, ricinine cannot be inactivated by conventional heat treatment due to its high temperature resistance. Since ricinine can be co-extracted with ricin, it can be used as a marker for the exposure to ricin. The symptoms of human poisoning begin within a few hours of ingestion and they are vomiting, abdominal pain, diarrhea, and even bloody diarrhea appear. Within several days' severe dehydration, a decrease in urine and a decrease in blood pressure occur (Franke *et al*., 2019). The less toxic protein *Ricinus communis* agglutinin(RCA) is another toxic protein in the castor bean, RCA is not significantly absorbed from the gut, thus causing clinically significant hemolysis only after intravenous administration, which if injected in the blood stream of a person the blood is coagulated(Marwat *et al*., 2017).

1.8.2 Pharmacological activities of *Ricinus communis*

A-Antioxidant activity

The antioxidant property of *R. communis* varies significantly depending upon the method of extraction and the extracted part of the plant. Leaves and seeds showed higher antioxidant activity than other plant parts

(Swarnakar *et al*., 2021). The antioxidant of *R.communis* is attributed to presence of different chemical constituent including methyl ricinoleate, ricinoleic acid, 12-octadecadienoic acid and methyl ester. Some studies revealed that gallic acid, quercetin, gastisic acid, rutin, epicatechin and ellagic acid are the major phenolic compounds responsible for the antioxidant activity of the *R. communis* dry leaves (Kumar, 2017).

B-Antibacterial activity

Ricinus communis and its phytochemicals have antimicrobial properties against various microorganisms. Some of the reported antimicrobial activity of the crude leaves extract of *R.communis* includes inhibition of various bacteria such as Staphylococcus aureus, Escherichia coli, Streptococcus mutans, Enterococcus faecalis and methicillin-resistant Staphylococcus aureus. The antimicrobial activity of various aqueous and solvent of *R. communis* extracts has been tested. The ethanolic extract of *R.communis* was found to be more effective in a large number of the cases, and the highest activity was shown to be against Staphylococcus aureus (Abdul *et al*., 2018).

C-Anti-inflammatory activity

Inflammation is a pathophysiological response of mammalian tissues to a variety of noxious agents including infectious organisms, toxic chemical substances, physical injury *etc.* resulting in local accumulation of plasma fluid and blood cells (Saini *et al*., 2010).

Plant constituents are responsible for both free radicals scavenging and anti-inflammatory activity, including terpenoids , phenolic compounds (flavonoids, quinones, phenolic acids, lignans, coumarins, stilbenes, tannins)

and nitrogen compounds (alkaloids, betalains, and amines) and carotenoids, these compounds had potent anti-oxidant and anti-inflammatory (Masoko and Nemudzivhadi's, 2014).

D-Anti-asthmatic activity

Ricinus communis L. roots is effective in treatment of asthma because of its anti-allergic and mast cell stabilizing potential effect. *R.communis* displayed the mast cell stabilizing effect due to saponin content which is present in the roots. Flavonoids play a major role in bronchodilator and smooth muscle relaxant activity. Additionally, the apigenin and luteolin like flavonoids were generally inhibit basophil histamine release and neutrophils beta glucuronidase release, and finally shows in-vivo anti-allergic activity (Jena and Gupta, 2012; Swarnakar *et al*., 2021).

E-Wound healing activity:

Ricinus communis possess wound healing activity which is related to the active constituent of castor oil that produces antioxidant activity through the inhibition of lipid peroxidation. Those agents whose inhibits lipid per oxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibers, increasing the circulation, preventing the cell damage and by promoting the DNA synthesis. The wound healing activity of castor oil (% closure of scar area and epithelialization) in excision wound model. Is due to astringent and antimicrobial properties of tannins, flavonoids, tri-terpenoids and sesquiterpenes present in the castor oil, which can promote the wound healing process, as they are responsible for wound contraction and increased rate of epithelialization. Thus reducing the scar area and the

epithelialization time in excision wound model (Jena and Gupta,2012; Bhakta and Kumar , 2015).

1.9 Drugs used in wound healing

1.9.1 Hormone replacement therapy

Estrogen deficiency amplifies inflammatory responses and delays angiogenesis, whereas exogenous estrogen promotes re-epithelialization and matrix deposition by fibroblasts and increases local vascularization (Levine, 2017).

1.9.2 Metformin

Metformin have various actions like anti-inflammatory, antioxidant and effect on hemostatic mechanisms can prove to be promising for the wound healing especially in diabetics (Patil and Limaye, 2017).

1.9.3 Phenytoin

phenytoin promotes wound healing by inhibiting the enzyme collagenase. It is effective in some low grade pressure ulcers and trophic ulcers due to leprosy. The possibility of systemic absorption and toxicity has limited its use (Enoch *et al.,* 2006).

1.9.4 Antimicrobials

The use of topical antimicrobials is beneficial for infection control in wound care because wound infection is the major cause of delayed healing. Topical antimicrobial agents divided into three groups: disinfectants, antiseptics, and antibiotics. Disinfectants are agents that can eradicate all

microorganisms, including spores; however, these agents cannot be applied on living tissue because of their toxicity (Punjataewakupt *et al*., 2019).

Antiseptics are chemical substances that can be used on intact skin. they have broad spectrum activity which can kill or inhibit bacteria, fungus, protozoa, viruses, and prions , but some antiseptic agent often show dose-dependent cytotoxicity to the host cells including keratinocytes, fibroblasts, and leukocytes (Sarheed *et al*., 2016).

Antibiotics, which are either naturally or synthetically produced, are chemical substances that have the ability to kill or inhibit microorganisms with specific cell targeting action. Antibiotics are relatively non-cytotoxic but bacterial resistance to antibiotics is more common(Punjataewakupt *et al*., 2019).

1-10-Drug used in this study for wound healing

Metformin

Metformin (1,1-dimethylbiguanide hydrochloride) is the first-line drug indicated for the clinical treatment of type 2 diabetes, discovered in 1922, and introduced as a therapeutic agent in 1957. Metformin, initially extracted from the plant *Galega officinalis* (French lilac) (Die *et al*., 2021). Chemical analyses of *G. officinalis* found the plant to be rich in guanidine and related compounds, especially the immature seed pods. In 1918, guanidine was reported to reduce blood glucose in animals (figure 1.4) (Bailey, 2017).

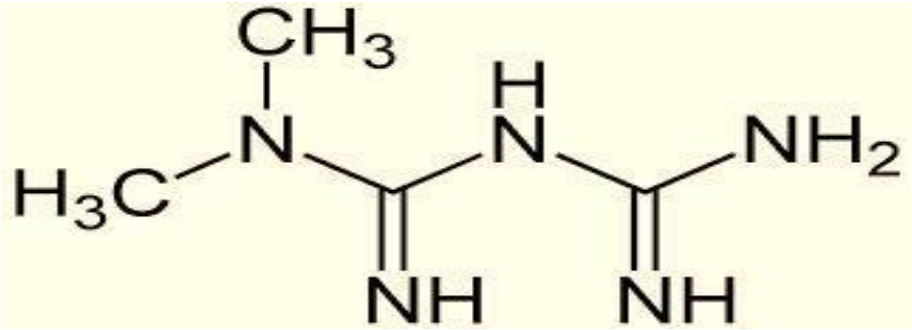


Figure (1.4):Structure of metformin, N, N dimethyl imidodi car-bonimidic diamide (Danish *et al*., 2015)

A-Mechanism of action

Metformin reduces serum glucose level by several different mechanisms, notably through non-pancreatic mechanisms without increasing insulin secretion. It increases the effects of insulin; hence, it is termed "insulin sensitizer". Metformin also suppresses the endogenous glucose production by the liver, which is mainly due to a reduction in the rate of gluconeogenesis and to lesser extent of glycolgenolysis. Moreover, metformin activates the enzyme adenosine monophosphate kinase(AMPK) resulting in an inhibition to key enzymes involved in the gluconeogenesis and glycogen synthesis in the liver while stimulating insulin signaling and glucose transport in the muscles (Nasri and Kopaei, 2014).

B- Adverse effects

The most common adverse effects of metformin being gastrointestinal symptoms, including nausea and vomiting and/or abdominal discomfort, and the most serious adverse effects being lactic acidosis, mainly in diabetic patients with liver and kidney dysfunction. Lactic acidosis has been ascribed to the promotion of anaerobic metabolism through interference with

mitochondrial respiration, resulting in an increase in lactate generation. Vitamin B12 mal-absorption is another potential side effect of metformin (Wang *et al*., 2017; Rangel and Inzucchi , 2017).

C- Contraindication

Metformin is contraindicated in patient with renal dysfunction due to the risk of lactic acidosis. It should be discontinued in cases of acute myocardial infarction, exacerbation of heart failure, sepsis, or other disorders that can cause acute renal failure. Metformin should be used with caution in patients older than 80 years and in those with heart failure or alcohol abuse (Whalen *et al***.,** 2019).

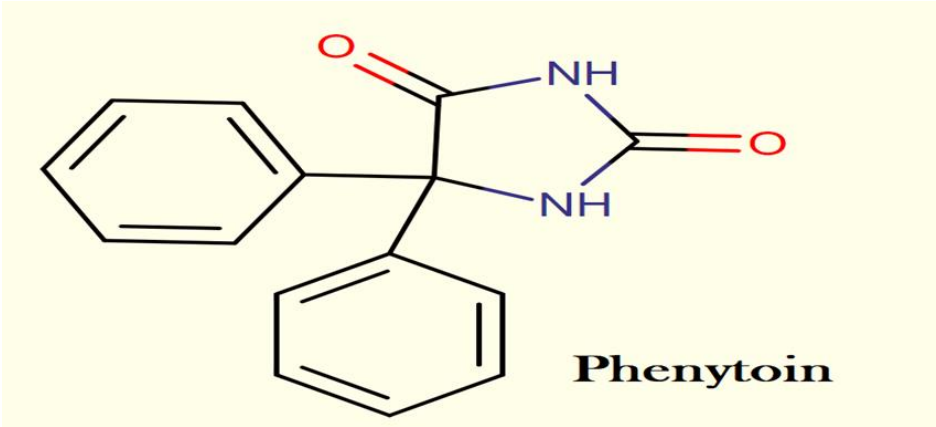
D- Metformin and wound healing

Metformin modulates the adenosine monophosphate-activated protein kinase(AMPK) –mammalian target of rapamycin (mTOR) –transcription factor EB (Transcription factor EB is a protein that in humans is encoded by the TFEB gene)pathway, which is known to regulate the autophagy process. The activation of autophagy results in the inhibition of apoptosis, stimulation of angiogenesis, and reduction of oxidative stress. By increasing the protein synthesis rate, decreasing insulin resistance and controlling hyperglycemia, metformin creates the metabolic premises for better and faster healing of the wounds and facilitates the skin graft take (Miricescu *et al*., 2021).

Metformin had various actions like anti-inflammatory , antioxidant and effect on hemostatic mechanisms can prove promising for the wound healing especially in diabetics. These effects of metformin are independent of its anti-diabetic action (Patil and Limaye, 2017).

Phenytoin

Phenytoin (5-5dipenyl 2-4 imidazolidione sodium) is barbiturate derivative, was synthesized in 1908 and is clinical use as a potent anti-epileptic drug since 1937. Structurally phenytoin has two phenyl rings at the C5 position of the hydantoin molecule (figure 1.5), and these ring seem to be responsible for its activity as anticonvulsant. Other substituents, such as alkyl groups at C5, increase its sedative properties, as in classical barbiturates (Hesselink and Kopsky, 2017).



Figure(1.5)The structure of phenytoin, 5,5-diphenylimidazolidine-2,4-dione (Ehmann *et al*., 2014).

A- Mechanism of action

Phenytoin blocks voltage-gated sodium channels in the brain. The voltage-gated sodium channels are coded for by the suprachiasmatic nucleus (SCN), a family of genes, which has members expressed in the heart and skeletal muscle as well as the peripheral and central nervous systems. The genes SCN1A, SCN2A, and SCN3A code for the α subunits expressed in the

brain. Mutations in SCN1A are associated with epilepsy. Epilepsy-associated variants have also been reported in SCN2A but few variants in SCN3A have been documented.

Phenytoin binds the SCN2A channel preferentially in the open formation. It is thought that phenytoin blocks sodium channels poorly at slow firing rates allowing normal brain activity but suppresses the high-frequency repetitive firing characteristic of seizures (Thorn *et al*., 2012).

B- Adverse effect of phenytoin

Common adverse effects of phenytoin which occurs due to long term use are gingival hyperplasia, coarsening of the facie, enlargement of the lips and thickening of the scalp and face. Phenytoin can also cause hirsutism in about 12% of children who are receiving phenytoin, this usually occurs within 3 months of initiating the therapy. Some patients who are treated with phenytoin have a transient burning sensation and rash (Sivarajah, 2017).

C- Contraindication

Phenytoin should not be used during pregnancy because maternal exposure to phenytoin causes fetal hydantoin syndrome, which is characterized by microcephaly, facial dysmorphism, heart defects, hypo-plastic nails and fingers, and growth or mental retardation (Yoshioka *et al*., 2021).

D- Phenytoin and wound healing

It has been suggested that phenytoin increases selectively the proliferation of fibroblasts, through the release of cytokines from

keratinocytes. Phenytoin also stimulates the formation of granular tissue, reduces collagenase activity, and promotes the production and deposition of collagen thus enhancing the strength of the wounded area. It also exerts antibacterial activity by reducing the bacterial contamination of the wound. Accordingly, biopsies of wound tissue treated with phenytoin exhibit signs of increased collagenization, neovascularization, and reduced infiltration of circulating inflammatory cells (Spampinato *et al*., 2020).

Chapter Two

Materials and Methods

2- Materials and Methods

2.1 Materials

This experimental study was performed in postgraduate lab /department of pharmacology at college of medicine\ university of Babylon from October 2021 – January 2022.

2.1.1 Instruments and Tools

The instruments and tools used in the study with their suppliers are listed in (Table 2.1)

Table (2. 1) List of instruments and tools used in the study

Equipment	**Manufacturers/country(Origin)**
Autoclave	Prestige medical, England
Centrifuge	Hettich, Germany
Distiller	Griffin, England
Electric oven	Lab tech, Korea
Incubator	Memmert, Germany
Inverted microscope	T.C Meiji techno, Japan
Laminar air flow cabinet	Lab tech, Korea
Magnatic stirrer	Scotech, Germany
Refrigerator	Arcelik, Turky
Shaker	Denely, England
Sensitive Balance	` Lab tech, Korea
Ultrasonic	Binder, Germany

Water bath	Minilyotrap, England
Micropipettes (different size)	Dragon-Med India
Cell culture flask (25ml)	SPL Korea
Cell culture plate (96-wells)	SPL Korea
ELISA Reader	Human Germany
Whatman filter paper	Merck Germany
Syringe 5 ml	MED China
0.45 and 0.22 μM Millipore Nalgene filters	Biofil Australia
pH Meter	WTW Germany
Freezer -20 °C	Mettler, Switzerland

2.1.2 Chemicals

The chemicals used in this study with their suppliers are listed in (Table 2.2).

Table 2.2 List of chemicals materials used in the study

Chemical materials	**Manufacturers/ country (Origin)**
Alcohol spray (ethanol 70%)	Aljoud Iraq
Fetal bovine serum (FBS)	Capricorn Germany
Phosphate buffer saline tablet	Gibco UK
MTT(3-(4,5- Dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide) dye powder	Roth Germany
RPMI 1640 medium w/L-glutamine,	US Biological life science USA

25mM HEPES (powder**)**	
Alcohol liquid 99.9% ethanol solution	France Al-cools France
Penicillin- streptomycin solution	Capricorn Germany
Dimethyl sulfoxide (DMSO)	Sigma Aldrich Germany
Trypsin ethylene diamine tetra-acetic acid(EDTA) powder	US biological USA
Metformin	US biological USA
Sodium bicarbonate powder	Ludeco Belgium
De-ionized water	Promega ,USA
Liquid nitrogen	Clever, USA
Phenytoin	Germany
HEPES buffer	Sigma ,USA
L-glutamine	Sigma ,USA

2.2 Methods

2.2.1 Preparation of reagents and solutions

2.2.1.1 Phosphate buffer saline (PBS):

Phosphate buffer saline was prepared according to Gibco manufacturer manual by dissolving one tablet of PBS in 500 ml deionized distilled water (DDW) with stirring constantly on a magnetic stirrer at room temperature, the pH will be 7.45 and requires no adjustment. Sterilization was done by autoclaving and kept sterile in a closed bottle until use.

2.2.1.2 Trypsin-(EDTA) solution:

As indicated by US Biological headings, a weight of a 10.1 g of trypsin-EDTA powder was dissolved in 0.9 liter of double distilled water (DDW) with continuous mixing at room temperature. 7.2 of PH value should be reached the volume was completed 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.

2.2.1.3 MTT solution:

A weight of 0.5 g of MTT powder was dissolved in 100 ml PBS to obtain a concentration of 5 mg/ml. Then the MTT solution was sterilized through a 0.2 μm Millipore filter, then the solution was kept in a sterile and light protected container and stored at 4°C for frequent use or at (-20 C˚) for long term storage (Meerloo *et al*., 2011).

2.2.2 Preparation of culture media

2.2.2.1 Preparation of Serum-Free Medium:

Liquid RPMI-1640 medium was prepared according to US Biologics from RPMI-1640 medium powder as the following:

Weighing RPMI-1640 powder to obtain 16.353 grams and dissolved in 900ml of DDW without heating, 2 g of sodium bicarbonate can be added if required with gentle stirring to adjust pH, additional water was added to obtain 1 liter**,** and Penicillin-Streptomycin at 1% was added then filter-sterilized using 0.22-micron membrane filter. The mixture contains heat labile compounds that can be damaged with autoclaving. The prepared media should be kept at (4°C) and used within a short period of time.

2.2.2.2 Preparation of Serum-Medium :

Serum-medium was prepared as described in (2.2.2.1) with the addition of 10% FBS.

2.2.2.3 Freezing media

Ten ml of stock solution was prepared from the following constituents: 6 ml serum free media, 3 ml fetal bovine serum, 1ml dimethyl sulfoxide (DMSO) was added drop by drop with mixing. The stock was stored at (- 20 C˚) (Kielberg, 2010).

2.2.3 Preparation of cell line

The Madin-Darby Canine Kidney (MDCK) cell lines in frozen vials were obtained from tissue culture laboratory in the college of medicine / university of Babylon.

2.2.3.1 Cell thawing

The frozen cell line vial was removed from liquid nitrogen container with caution and directly placed into a beaker containing pre-warmed (37°C) sterile DDW. The vial was removed from the water before the ice floccule dissolved completely, then it was wiped with 70% ethanol. 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 serum-free medium. Centrifugation was done at 1000 rpm for 5 minutes and the supernatant was aspirated and decanted. The cells pellet was re-suspended into 5ml warm (37C˚) serum-medium and transferred into 25 ml size cell culture flask, incubated at 37C˚ and the serum medium replaced on the next day (Phelan and May, 2017).

2.2.3.2 Subculture of cell line

1- The cells are checked and examined using the inverted microscope with phase contrast capabilities to ensure that the cells are healthy and sub-confluent and free of contamination.

2- The laminar flow is sanitized by wiping off the surface of working area with 70% ethanol.

3- The growth medium is removed from the flask using a pipette and wash the monolayer with a sufficient volume of PBS to ensure the removal of all media from the flask.

4- An appropriate volume of the trypsin/EDTA solution is added to the flask and incubate at 37 c° to allow the cells to detach from the inside surface of the flask (within 2-10 min).

5- The cells were examined using an inverted microscope to ensure that all the cells are detached and in suspension. Gently tap the flask with the palm of the hand a couple of times to release any remaining detached cells.

6- The trypsin is inactivated by adding an equal volume of serum-containing media to the flask.

7- Then the cell suspension is divided into two flask and label each flask with cell line name, passage number, and date.

8- The cell line was incubated at 37C°for 24 hr.

(Meleady and Connor, 2006).

2.2.3.3 Harvesting of cells

Harvesting is a technique that uses proteolytic enzymes to detach adherent cells from the surface of a cell culture flask. First, the growth medium in the vessel was aspirated and discarded. PBS was used to wash the cells twice. Afterward, the enzymatic harvesting solution was added to the vessel. After 15 minutes, the proteolytic reaction was neutralized by adding the serum-containing culture medium. The cells in the tissue culture flasks were harvested by using different enzymatic solutions composed of

different concentrations of trypsin and Ethylenediaminetetraacetic acid (EDTA) (Viazzi *et al*., 2015).

2.2.3.4 Freezing of cell line

Cell lines source were kept frozen at (-180°C) 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 37C° 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 hour and then stored for a long time in the liquid nitrogen tank (Kielberg, 2010).

2.2.4 Preparation MTT assay 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 for MTT assay 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 cells 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) by the use of a plate reader at 570 nm. The darker the solution, the greater the number of viable and metabolically active cells (Meerloo *et al*., 2011).

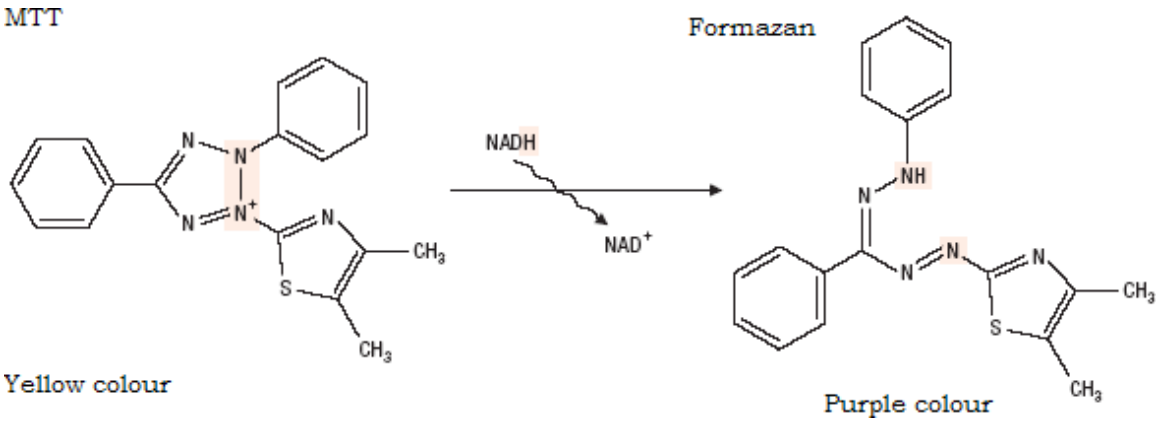


Figure 2.1: Principle of MTT Assay (Sukhramani *et al*., 2011)

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

% viability = (AT – AB) / (AC – AB) × 100%

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

AB = Absorbance of blank (only medium).

AC = Absorbance of control (untreated).

% Inhibition = 100 – % viability

2.2.5 Preparation of *Ricinus communis* leaf extract

Fresh leaves of *R. communis* plant were collected from Al-bakarly, in the city of Hilla - Iraq, then they were identified and approved by Al-qassim Green University / College of Agriculture.

The plant leaves then were washed thoroughly with distilled water and left to dry for 14 days in dark place at room temperature, then grinded to fine powder using electrical grinder and kept in a tightly sealed dry container for future use (Suurbaar *et al*., 2017). Leaf powder then soaked and macerated in both alcoholic (ethanol 100%) and aqueous in concentration of 50g of leaf powder in 500mL of ethanol and 20g of leaf powder in 200ml of DW and left for extraction at room temperature for 48 hours.

Solution of both ethanolic and aqueous extract were filtered many times using medical gauze and the separated solution is then filtered using Whitman filter paper no.1 and then left to dry in incubator to obtain semi solid products.

Preparation of stock solution for ethanolic and aqueous extracts of *R. communis* was made by dissolving 70 mg of ethanolic extract in 1ml ethanol 100% and complete to 35 ml RPMI 1640 free of serum to obtain final concentration of 2000 µg/ml, for ethanolic stock solution and then centrifuged at 1270 rpm for 5 min to dispose the settled waste and obtain clear liquid, then filtered using Millipore filter syringe to discard any impurities. While the aqueous extract of *R. communis* was made by dissolving 60 mg of aqueous extract in 30 ml medium serum free to obtain final concentration of 2,000 µg/ml for aqueous stock solution, then filtered using Millipore filter syringe to discard any Impurities.

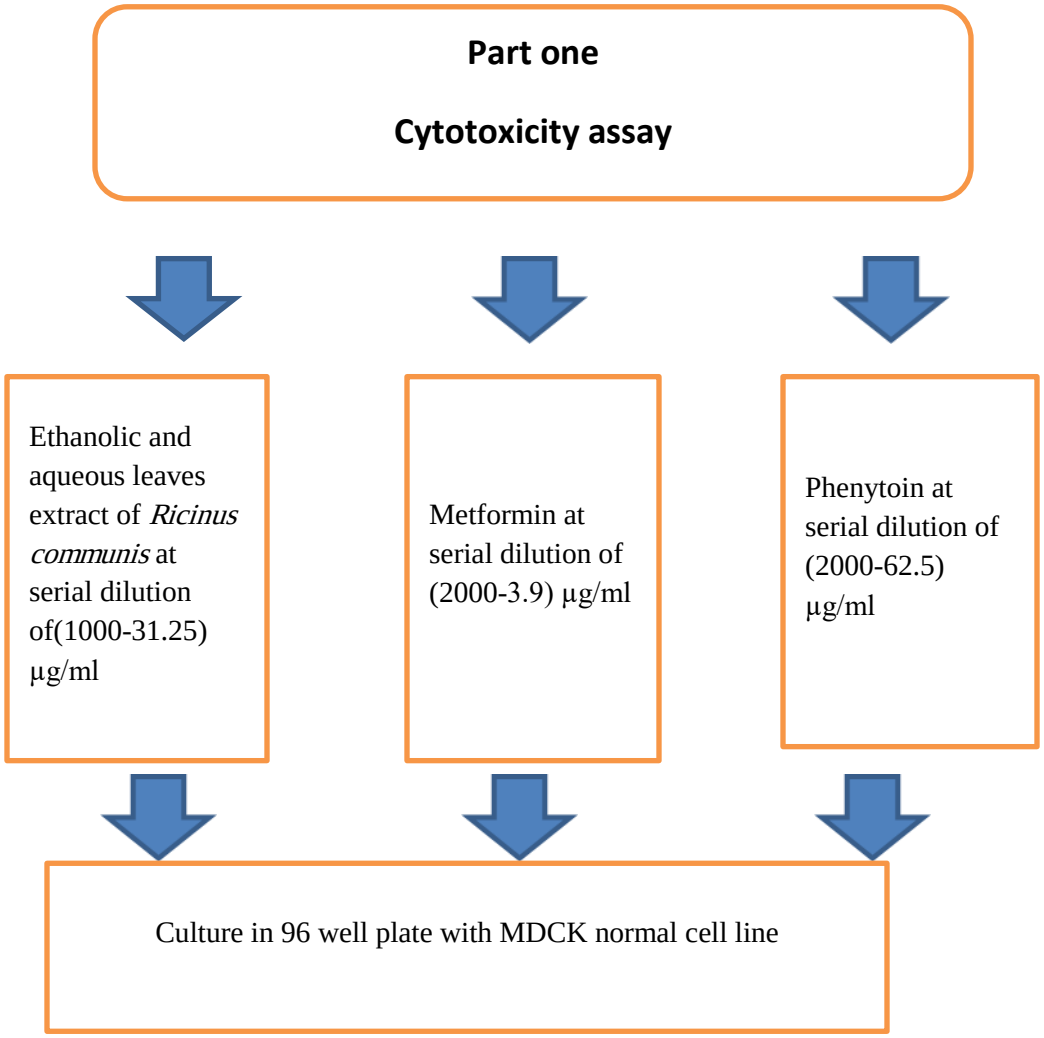
2.2.6 Preparation of metformin stock solution

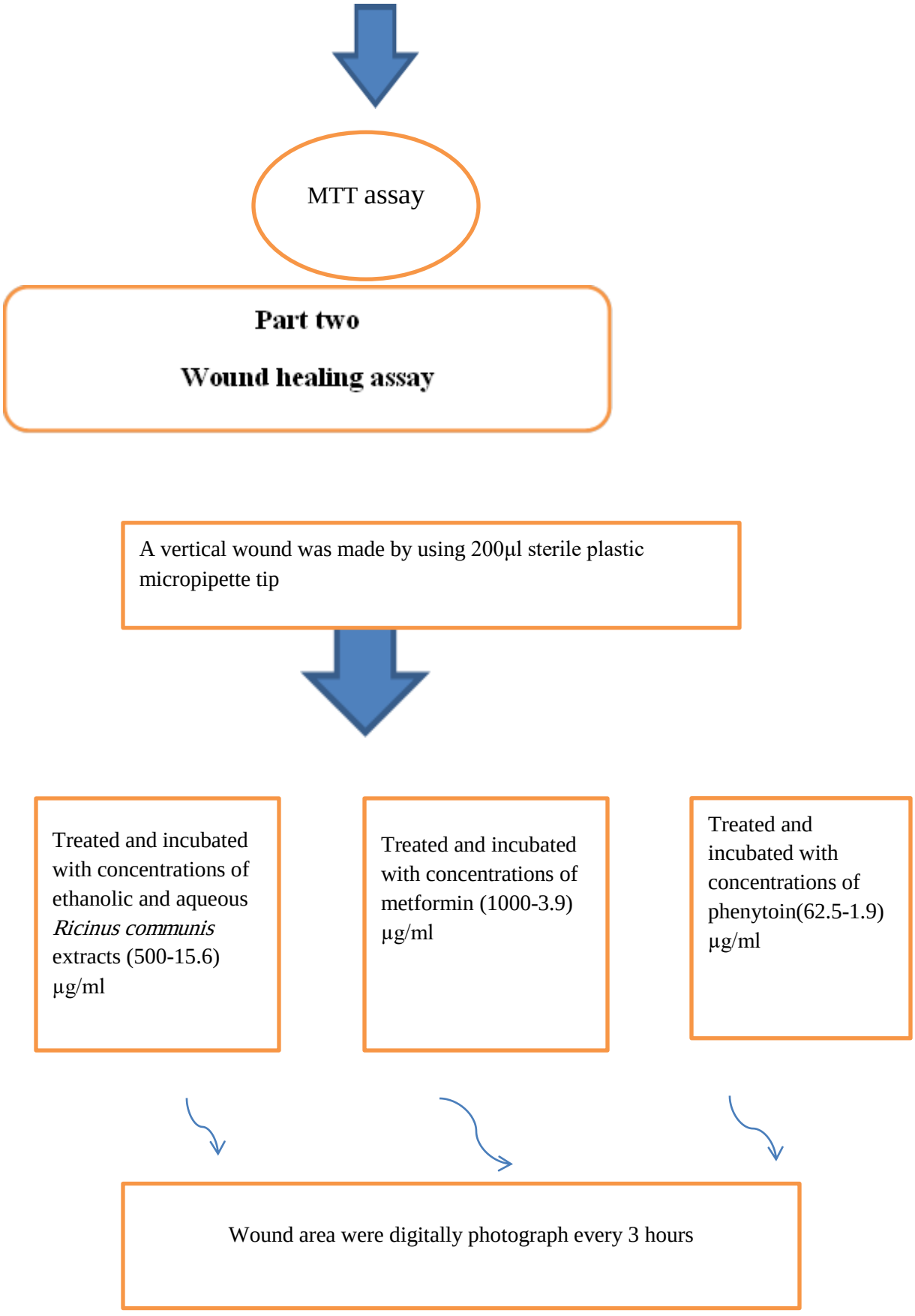
Metformin (100 mg pure drug powder) was solubilized with serum-free medium 25 ml, to obtain a stock solution of the drug (4000 µg/ml) and from this stock a serial dilution was made.

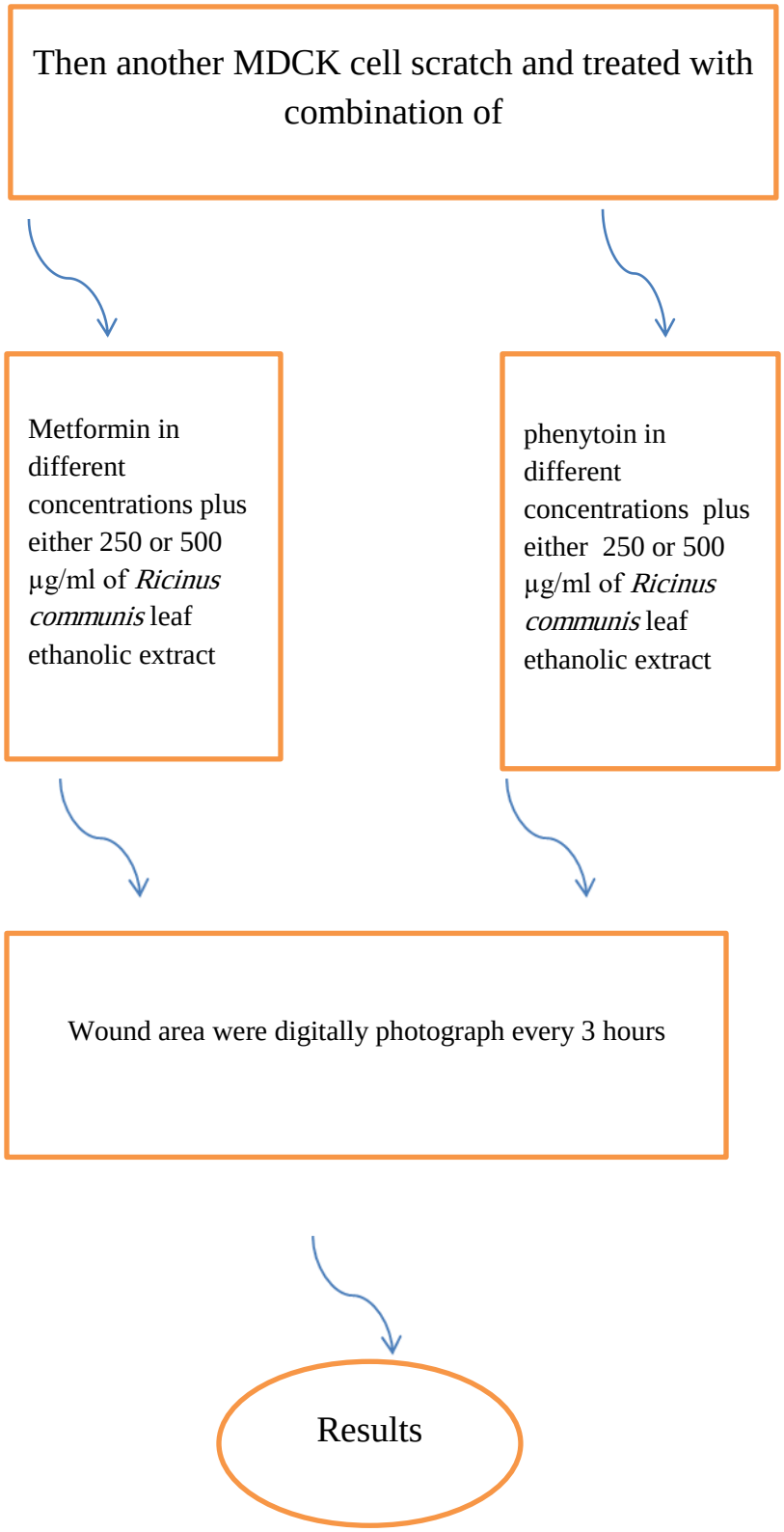
2.2.7 Preparation of phenytoin stock solution

Phenytoin ampule of 50 mg/ml solution was considered as stock solution from this stock a serial dilution was made.

Study Design




MTT assay
Part two
Wound healing assay
A vertical wound was made by using 200µl sterile plastic micropipette tip
Treated and incubated with concentrations of ethanolic and aqueous *Ricinus communis* extracts (500-15.6) µg/ml
Treated and incubated with concentrations of metformin (1000-3.9) µg/ml
Treated and incubated with concentrations of phenytoin(62.5-1.9) µg/ml
Wound area were digitally photograph every 3 hours


Then another MDCK cell scratch and treated with combination of
Metformin in different concentrations plus either 250 or 500 µg/ml of *Ricinus communis* leaf ethanolic extract
phenytoin in different concentrations plus either 250 or 500 µg/ml of *Ricinus communis* leaf ethanolic extract
Wound area were digitally photograph every 3 hours
Results

2.2.8 Experimental Design:

Part one: cytotoxicity assay

The MDCK cell line was seeded in 96 tissue culture plates. All cells were treated with different concentrations of plant extracts (ethanolic and aqueous) at serial dilutions ranged from 31.25 to 1000 µg/ml (Three replicates were used for each concentration of both ethanolic and aqueous extract) along with three replicates as a control group. The same experiment was repeated with metformin at serial dilution from 3.9 to 2000 µg/ml and phenytoin from 62.5 to 2000 µg/ml. After that all treated cells were incubated for 24 hours, at the end of the exposure period, the cell lines growth was assessed by MTT assay.

Part two: wound healing assay

Experiment number.1: Effect of *Ricinus communis* ethanol leaf extract on the MDCK cell line

1- The required number of cells to form a confluent monolayer were placed in a 96-well plate for 100% confluence in 24hours.

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

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

4-After creating the scratch, the cells were washed with PBS solution to remove cell debris, before treating with different concentrations of *R.communis* ethanolic leaf extract.

5-Three replicates wells of 96-well plate in column No.1 was considered as a control group(untreated), and each one of the remaining columns three wells replicates were exposed to 200 µl of each of the serial dilution of *R.communis* ethanolic extract (15.6,31.25,62.5, 125, 250, 500 µg/ml).

6-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°).

7-At several points e.g. every 3hours, the plate was removed from the incubator and placed under an inverted microscope to take a snapshot picture in order to check wound closure.

Experiment number.2: Effect of *Ricinus communis* aqueous leaf extract on MDCK cell line

As in experiment number.1 but with the use of *R.communis* aqueous leaf extract instead of ethanol leaf extract.

Experiment number.3: Effect of metformin on MDCK cell line

As in experiment number.1 but, the MDCK cells were treated with a serial dilution of metformin (3.9,7.8,15.6,31.25,62.5, 125, 250, 500, 1000 µg/ml)

Experiment number. 4 and 5: Effect of metformin plus 250 or 500 µg /ml of *R.communis* ethanol leaf extract combination on MDCK cell line

As in experiment number.1 but, the MDCK cells were treated with (250 µg /ml and 500 µg /ml)of *R.communis* leaf extract in combination with a serial dilution of metformin (125,250,500,1000 µg/ml)

Experiment number.6: Effect of phenytoin on MDCK cell line

As in experiment number.1 but, the MDCK cells were treated with a serial dilution of phenytoin (1.9,3.9,7.8,15.6,31.25,62.5 µg/ml)

Experiment number. 7and 8: Effect of phenytoin plus 250 or 500 µg /ml of *R.communis* ethanol leaf extract combination on MDCK cell line

As in experiment number.1 but, the MDCK cells were treated with (250 µg /ml and 500 µg /ml)of *R.communis* leaf extract in combination with a serial dilution of phenytoin (1.9,3.9,7.8,15.6,31.25,62.5 µg/ml)

2.2.9 Measurement of wound healing

Snapshot method was used to document the cell migration by taking sequential digital photograph of the gap using camera (Lenovo q30). The plate was fixed under inverted microscope and the wound area were digitally photographed every 3 hours. By using software “image J” (National Institutes of Health, Maryland, USA).

2.2.10 Statistical Analysis

All data were collected and analyzed by Microsoft Office Excel 2010 and Sigma plot version 12 software. ANOVA one way test was used to assess significant differences among the means of data. the p-value ($p \leq 0.001$, $p \leq 0.05$) were considered statistically significant.

Chapter Three
Results

3.1-Effect of *Ricinus communis* ethanolic leaf extract on the viability of MDCK normal cell line

In comparison to the control group result showed insignificant difference ($p>0.05$) in cell viability percentage of the MDCK normal cell line group treated with different concentrations of *R.communis* ethanolic leaf extract (figure 3.1)

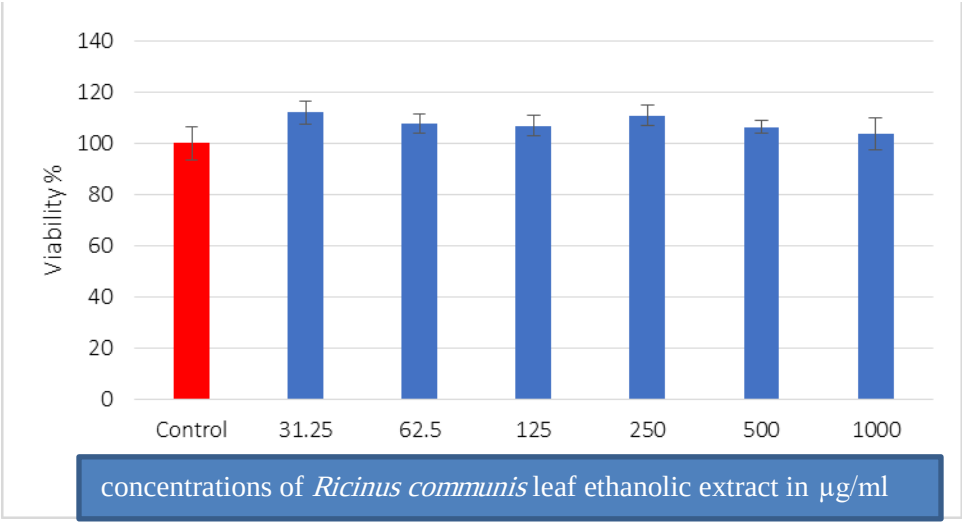


Figure 3.1: Effect of *Ricinus communis* ethanolic leaf extract on the viability of MDCK normal cell line.

3.2-Effect of *Ricinus communis* aqueous leaf extract on the viability of MDCK normal cell line

In comparison to the control group result showed insignificant difference ($p>0.05$) in cell viability percentage of the MDCK normal cell line treated with all concentrations of *R.communis* aqueous extract, except the concentration 125 µg/ml which caused significant increase in cell viability percentage ($p\leq0.05$) of the MDCK normal cell line (figure 3.2).

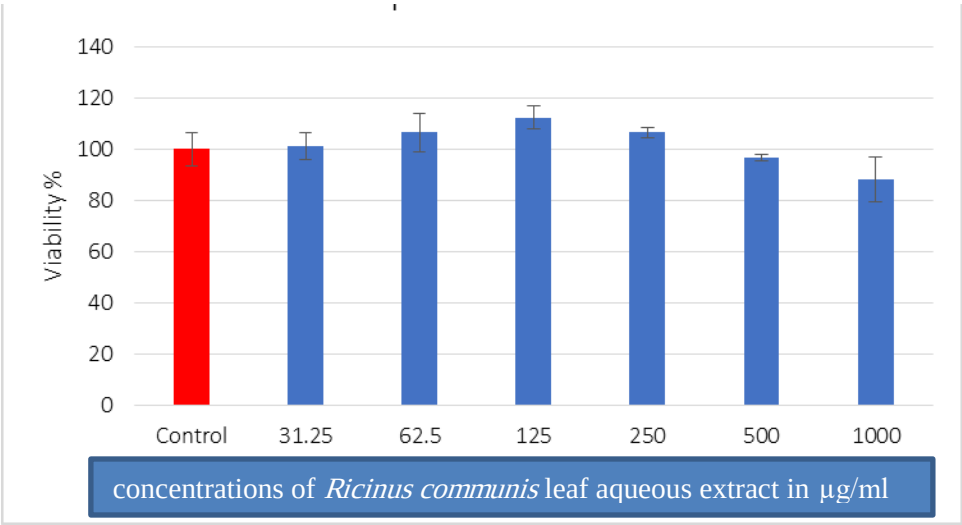


Figure 3.2: Effect of *Ricinus communis* aqueous leaf extract on the viability of MDCK normal cell line

3.3-Effect of metformin on the viability of MDCK normal cell line

In comparison to the control group result showed insignificant difference ($p>0.05$) in cell viability percentage of the MDCK normal cell line treated with all concentrations of metformin, except the concentration 62.5 µg/ml which caused significant increase in cell viability percentage ($p\leq0.05$), while the concentration 2000 µg/ml caused highly significant decrease in cell viability($p<0.001$) of the MDCK normal cell line (figure 3.3).

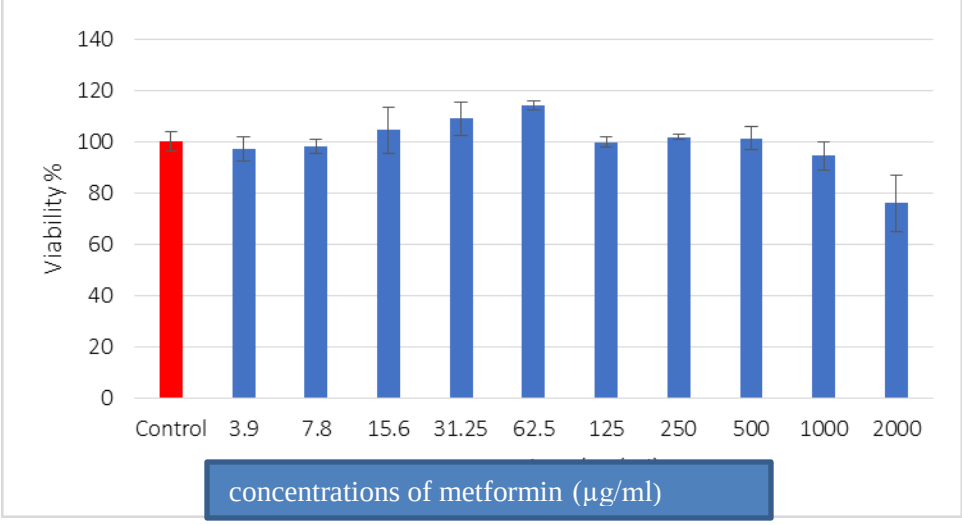


Figure 3.3: Effect of metformin on the viability of MDCK normal cell line

3.4-Effect of phenytoin on the viability of MDCK normal cell line

In comparison to the control group results showed insignificant difference ($p>0.05$) in cell viability percentage of the MDCK normal cell line group treated with the concentration 125 µg/ml of phenytoin ,while the concentration 62.5 µg/ml of phenytoin caused significant increase($p\leq0.05$) in viability of these cells. Result also showed that the high concentrations (2000,1000,500,250) µg/ml of phenytoin caused high significant decrease ($p<0.001$) in the viability of MDCK normal cell line (figure 3.4).

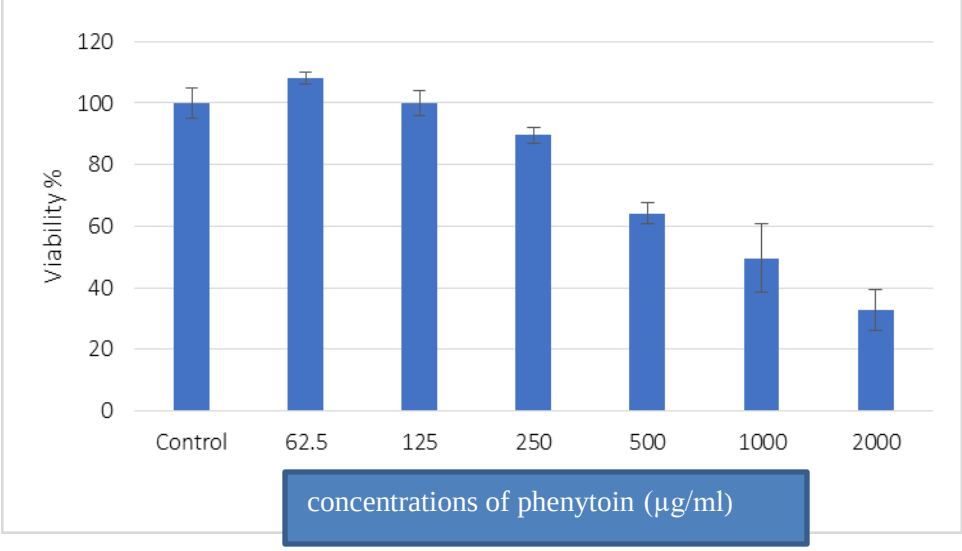


Figure 3.4: Effect of phenytoin on the viability of MDCK normal cell line

3.5-Effect of *Ricinus communis* ethanolic leaf extract on the healing of MDCK normal cell line- wound model

In comparison to the control group results showed that after 12 hour of incubation all the concentrations of *R. communis* ethanolic leaf extract except the concentration 500 µg/ml decreased wound diameter high significantly ($p<0.001$). While the concentration (500) µg/ml caused high significant increase in the diameter of wound (figure 3.5)

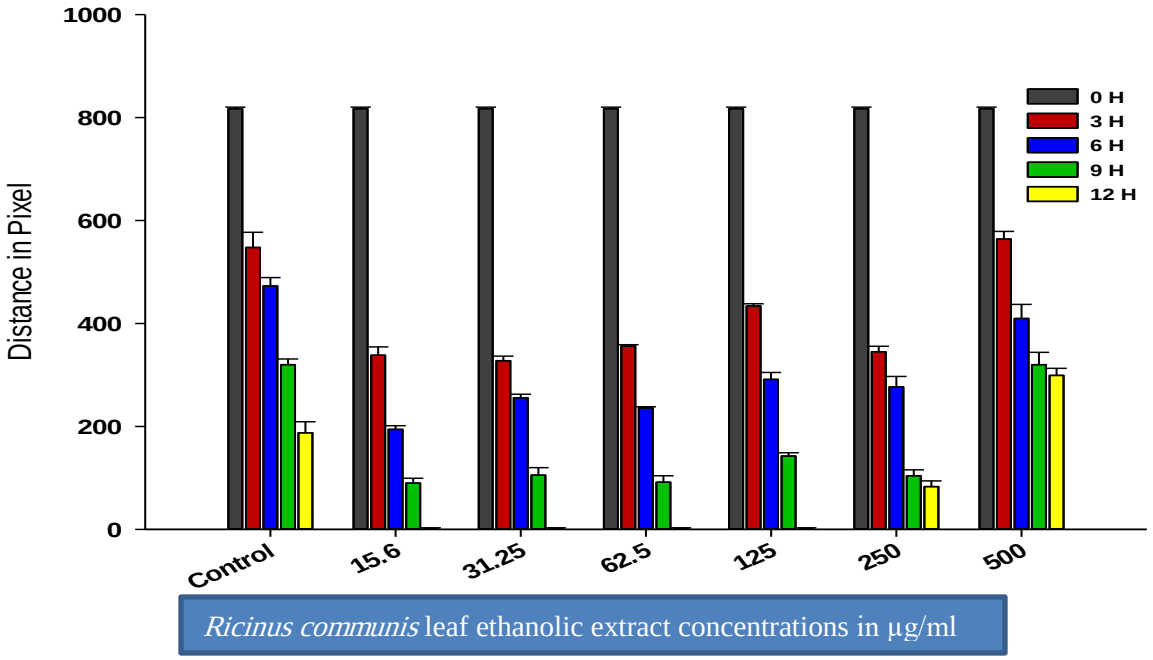


Figure 3.5: Effect of *Ricinus communis* ethanolic leaf extract on the healing of MDCK normal cell line- wound model

3.6-Effect of *Ricinus communis* aqueous leaf extract on the healing of MDCK normal cell line- wound model

In comparison to the control group results showed that all concentrations of *R. communis* aqueous leaf extract caused highly significant decrease ($p<0.001$) in the diameter of the MDCK normal cell line induced wound after 3,6,9 and 12 hours of incubation and wound healing was completed after 12 hours of incubation (Figure 3.6).

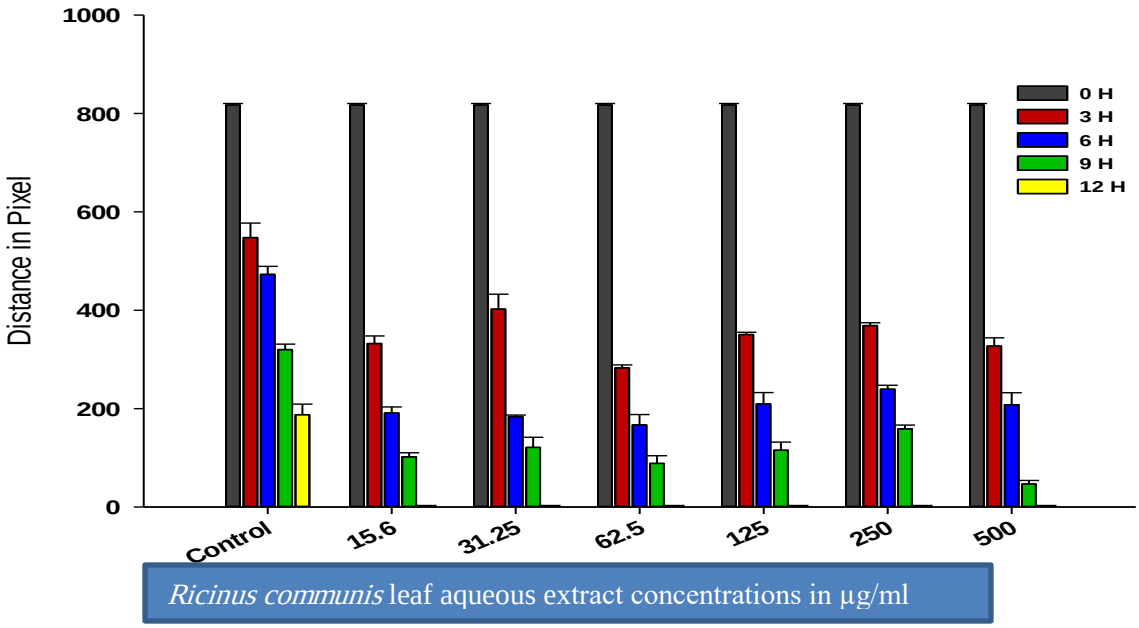


Figure 3.6: Effect of *Ricinus communis* aqueous leaf extract on the healing of MDCK normal cell line- wound model

3.7-Effect of metformin on the healing of MDCK normal cell line- wound model

In comparison to the control group results showed that all concentrations of metformin except the concentrations (15.6,31.25,62.5) µg/ml decreased wound diameter high significant($p<0.001$) resulting in complete wound healing. While the concentrations (15.6,31.25,62.5) µg/ml caused no complete healing of the MDCK normal cell line induced wound after 12 hours of incubation (Figure 3.7)

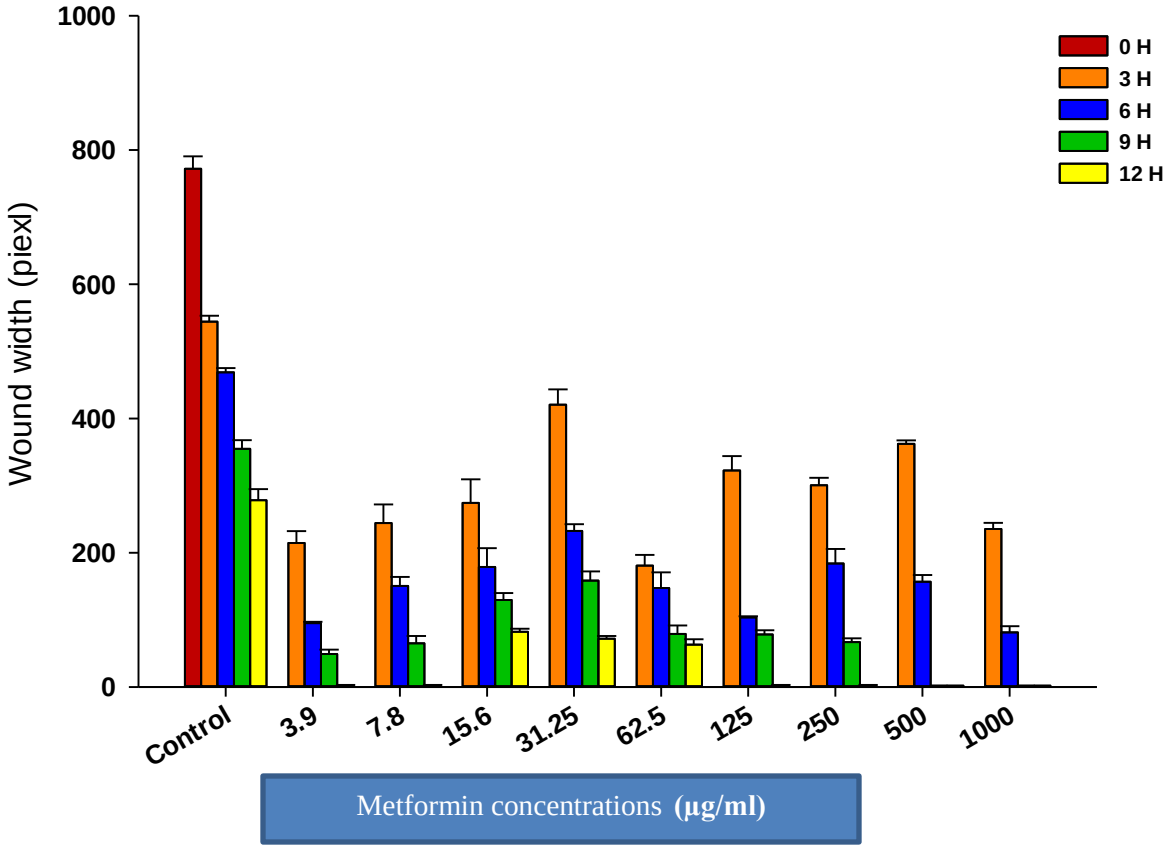


Figure 3.7: Effect of metformin on the healing of MDCK normal cell line-wound model.

3.8 and 3.9-Effects of metformin plus 250 or 500 µg/ml *Ricinus communis* leaf ethanolic extract combination on the healing of MDCK normal cell line- wound model

In comparison to the control group results showed that the concentrations of combination(250 of *R. communis* leaf ethanolic extract + (125 and 250 of metformin) µg/ml decreased wound diameter high significant($p \leq 0.001$) resulting in complete wound healing occurred after 12

hours of treatment. While the concentrations(250 of *R. communis* leaf ethanolic extract + (500 and1000 of metformin) µg/ml caused decreased wound diameter high significant($p \leq 0.001$) but no complete wound healing occurred of the MDCK normal cell line induced wound after 12 hours of incubation (figure 3.8).

While the result of combination 500 µg/ml of *R. communis* leaf ethanolic extract plus metformin showed that all concentrations of combination caused highly significant decrease ($p \leq 0.001$) in the diameter of the MDCK normal cell line induced wound after 3,6,9 and 12 hours of incubation, except the concentration (500 of *R. communis* leaf ethanolic extract +1000 of metformin) µg/ml caused high significant increase in the diameter of wound after 12 hours of incubation (figure 3.9).

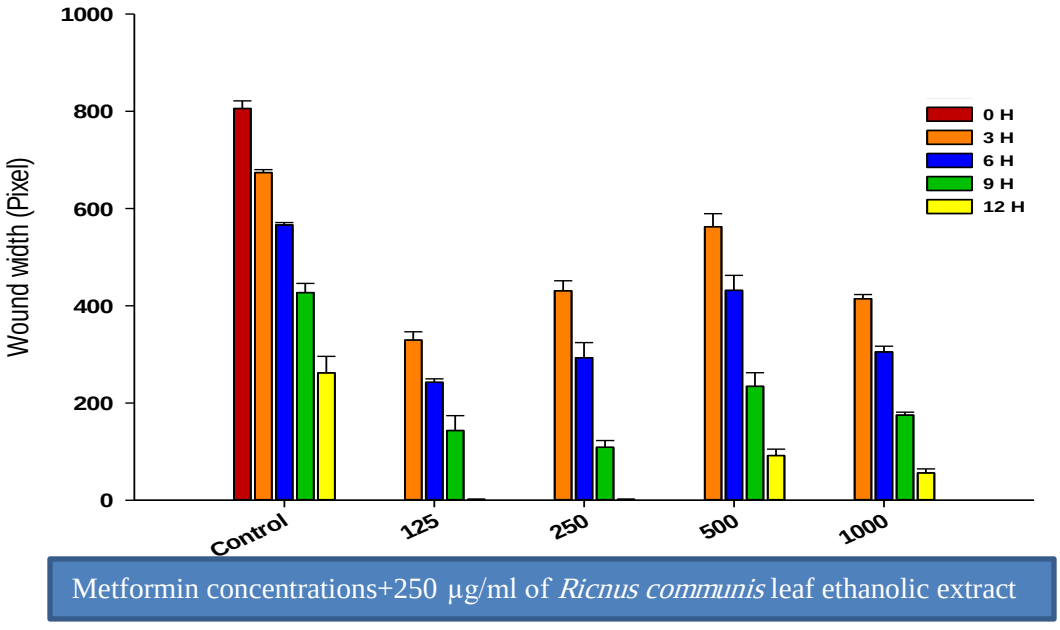


Figure 3.8: Effects of metformin plus 250 µg/ml *Ricinus communis* leaf ethanolic extract combination on the healing of MDCK normal cell line-wound model.

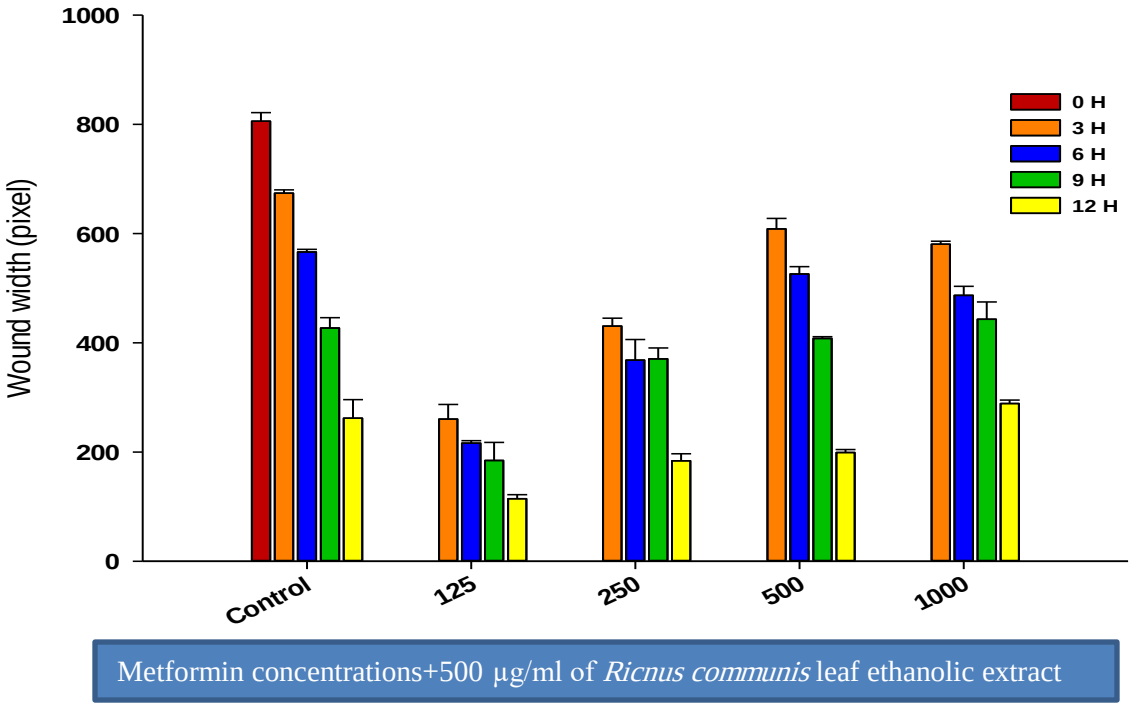


Figure 3.9: Effects of metformin plus 500 µg/ml *Ricinus communis* leaf ethanolic extract combination on the healing of MDCK normal cell line-wound model.

3.10-Effect of phenytoin on the healing of MDCK normal cell line- wound model

In comparison to the control group results showed that all concentrations of phenytoin caused highly significant decrease($p \leq 0.001$) in the diameter of MDCK normal cell line- induced wound after 3,6,9 hours of incubation and wound healing was completed after 12 hours of incubation except low concentration (1.9) µg/ml (Figure 3.10)

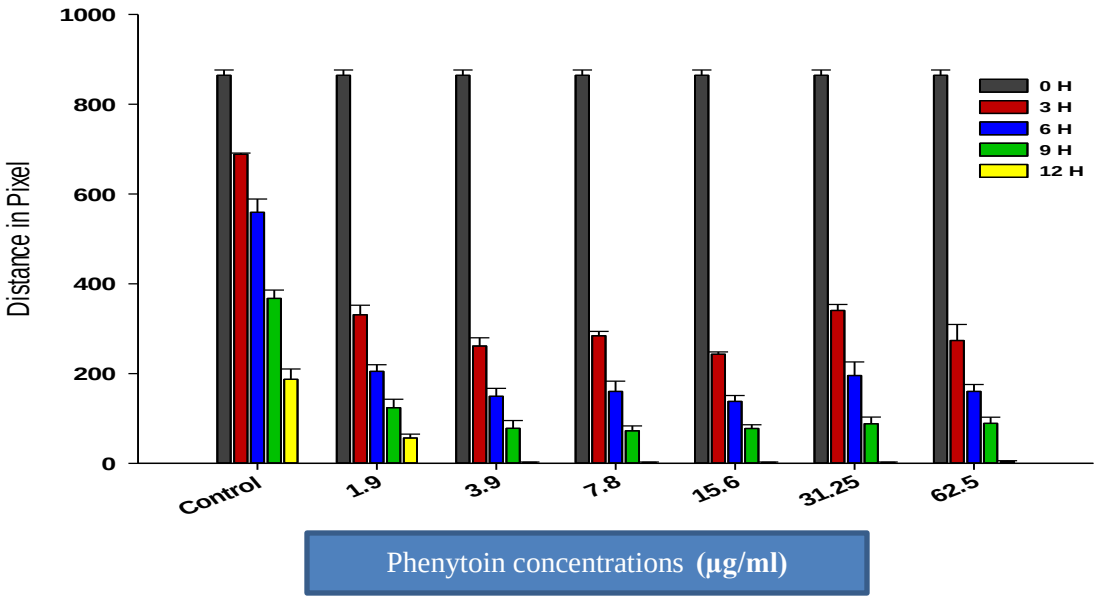


Figure 3.10: Effect of phenytoin on the healing of MDCK normal cell line-wound model

3.11and 3.12-Effects of phenytoin plus 250 or 500 µg/ml *Ricinus communis* leaf ethanolic extract combination on the healing of MDCK normal cell line- wound model

In comparison to the control group results showed that all concentrations of combination phenytoin plus 250 µg/ml *Ricinus communis* leaf ethanolic extract caused highly significant decrease ($p<0.001$) in the diameter of the MDCK normal cell line induced wound after 3,6,9 and 12 hours of incubation and wound healing was completed after 12 hours of incubation (Figure 3.11)

While the result of combination 500 µg/ml of *R. communis* leaf ethanolic extract plus phenytoin showed that all concentrations of combination caused

highly significant decrease($p<0.001$) in the diameter of MDCK normal cell line- induced wound after 3,6,9 hours of incubation and wound healing was completed after 12 hours of incubation except high concentrations (500 of *R. communis* leaf ethanolic extract+(31.25,62.5 of phenytoin) µg/ml (figure 3.12)

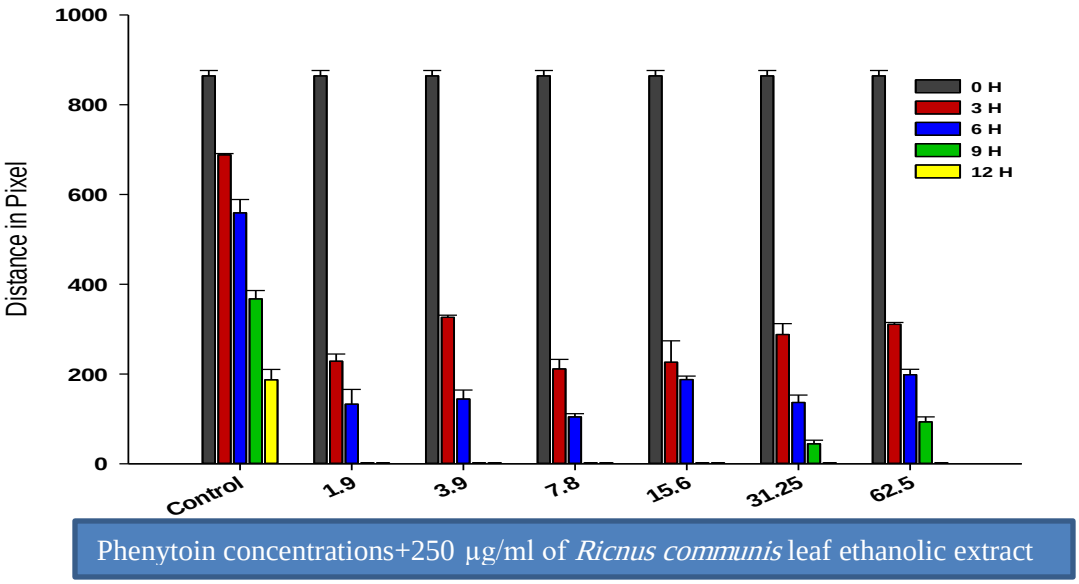


Figure 3.11: Effects of phenytoin plus 250 µg/ml *Ricinus communis* leaf ethanolic extract combination on the healing of MDCK normal cell line-wound model.

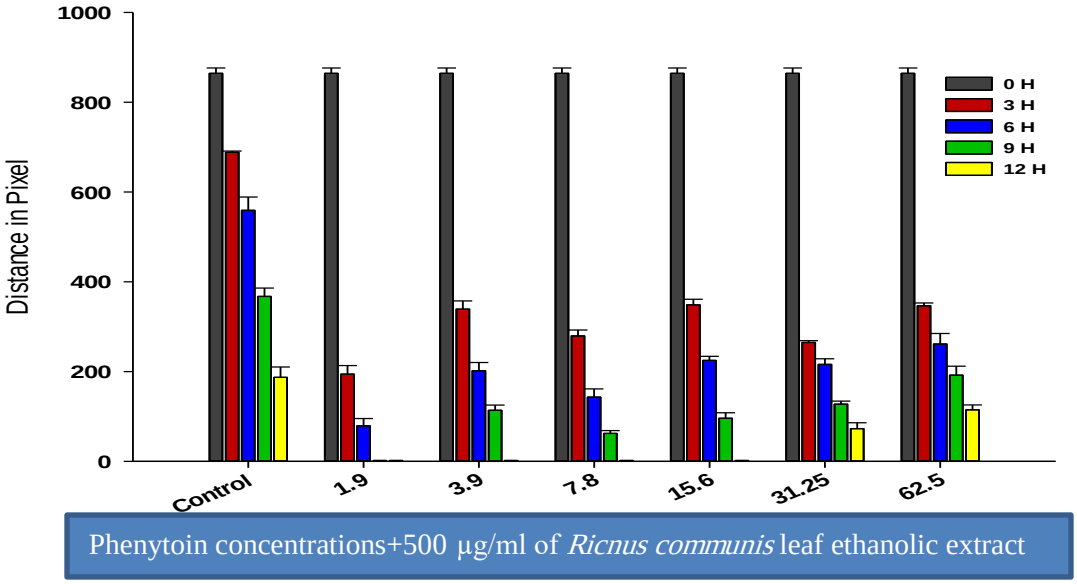


Figure 3.12: Effects of phenytoin plus 500 µg/ml *Ricinus communis* leaf ethanolic extract combination on the healing of MDCK normal cell line-wound model.

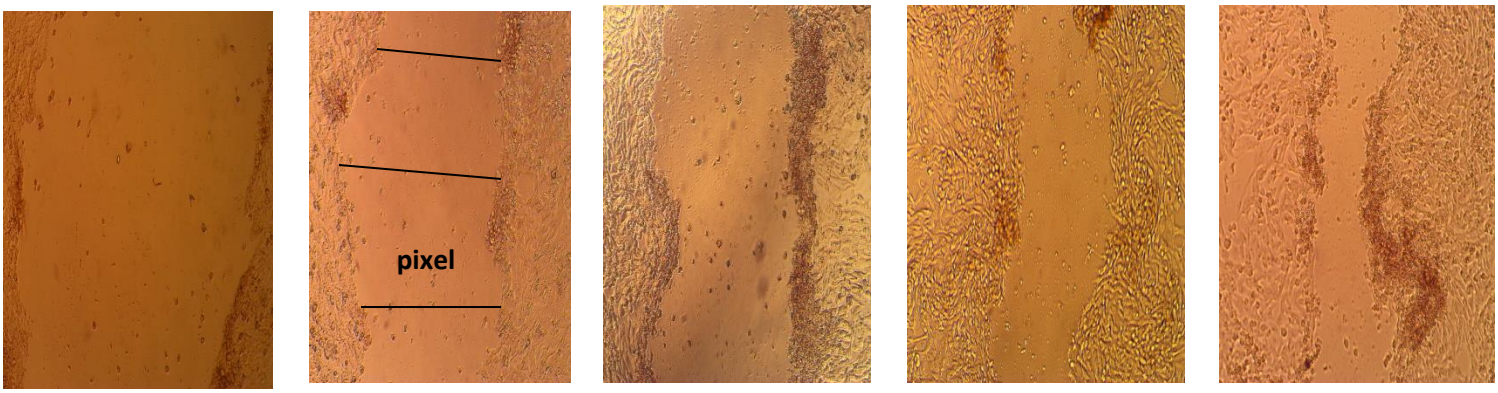


Figure 3.13: Photomicrograph(10XLence) of MDCK cell line without treatment of *Ricinus communis* (ethanolic and aqueous)leaf extracts (control group) at different period of incubation.

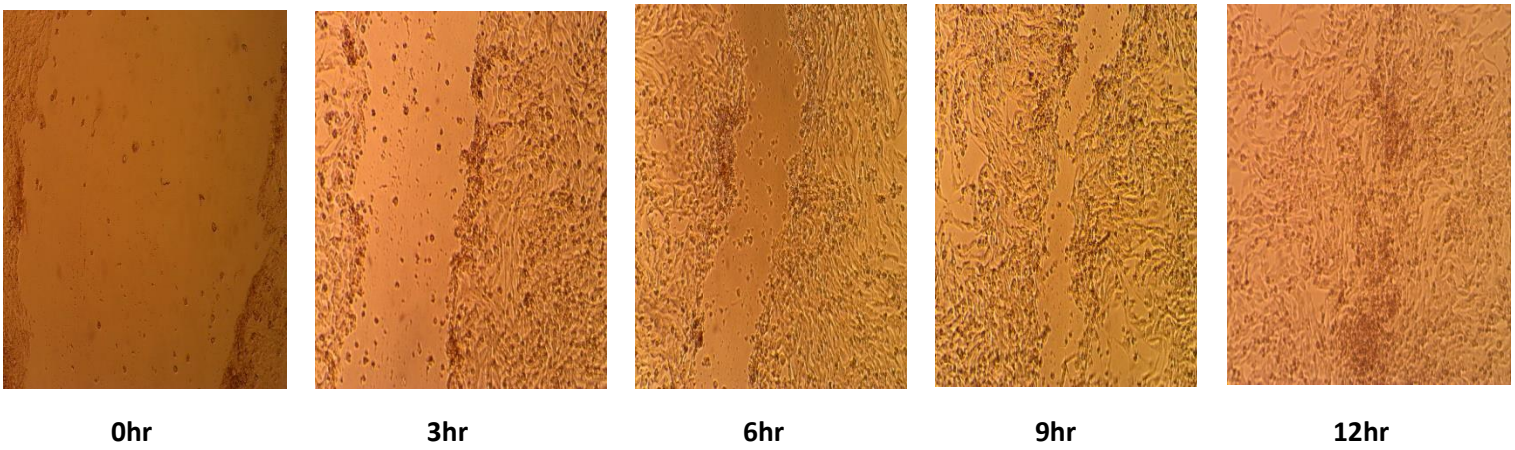


Figure 3.14: Photomicrograph (10XLence) of MDCK cell line treated with *Ricinus communis* ethanolic leaf extract (15.6 µg/ml) at different period of incubation.

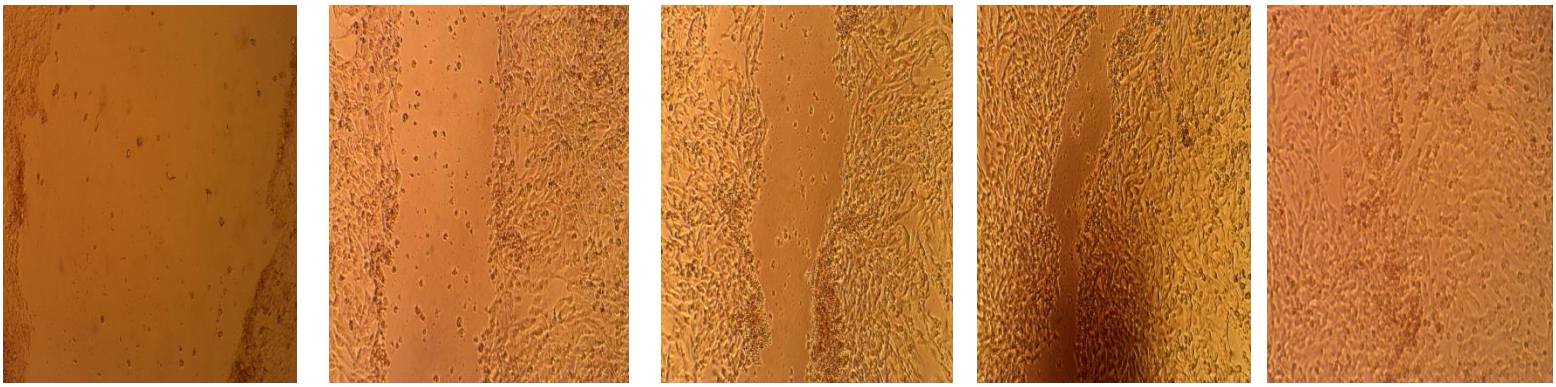


Figure 3.15: Photomicrograph (10XLence) of MDCK cell line treated with *Ricinus communis* ethanolic leaf extract (31.25 µg/ml) at different period of incubation.

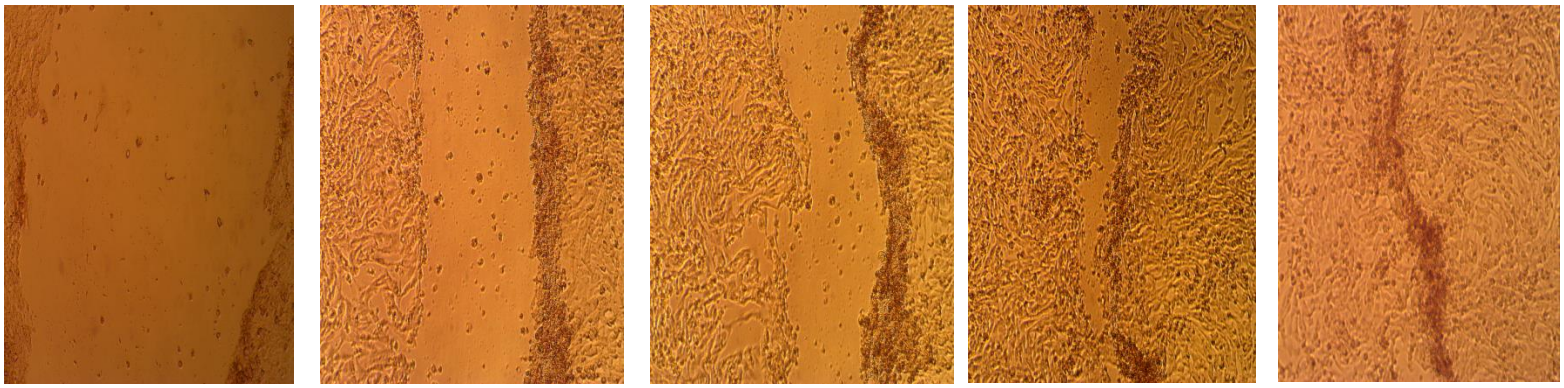


Figure 3.16: Photomicrograph (10XLence) of MDCK cell line treated with *Ricinus communis* ethanolic leaf extract (62.5 µg/ml) at different period of incubation.

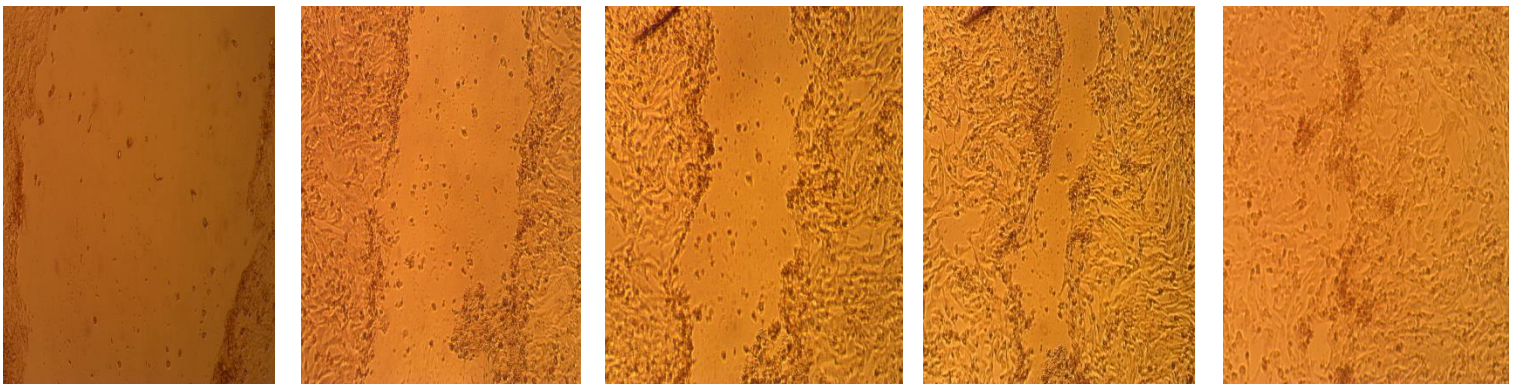


Figure 3.17: Photomicrograph (10XLence) of MDCK cell line treated with *Ricinus communis* ethanolic leaf extract (125 µg/ml) at different period of incubation.

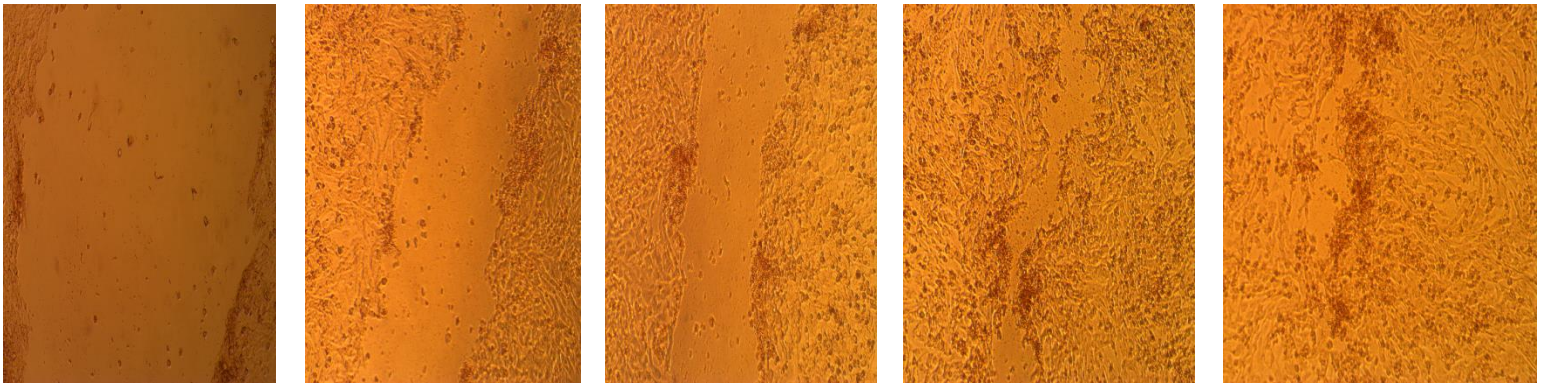


Figure 3.18: Photomicrograph (10XLence) of MDCK cell line treated with *Ricinus communis* ethanolic leaf extract (250 µg/ml) at different period of incubation.

Figure 3.19: Photomicrograph (10XLence) of MDCK cell line treated with *Ricinus communis* ethanolic leaf extract (500 µg/ml) at different period of incubation.

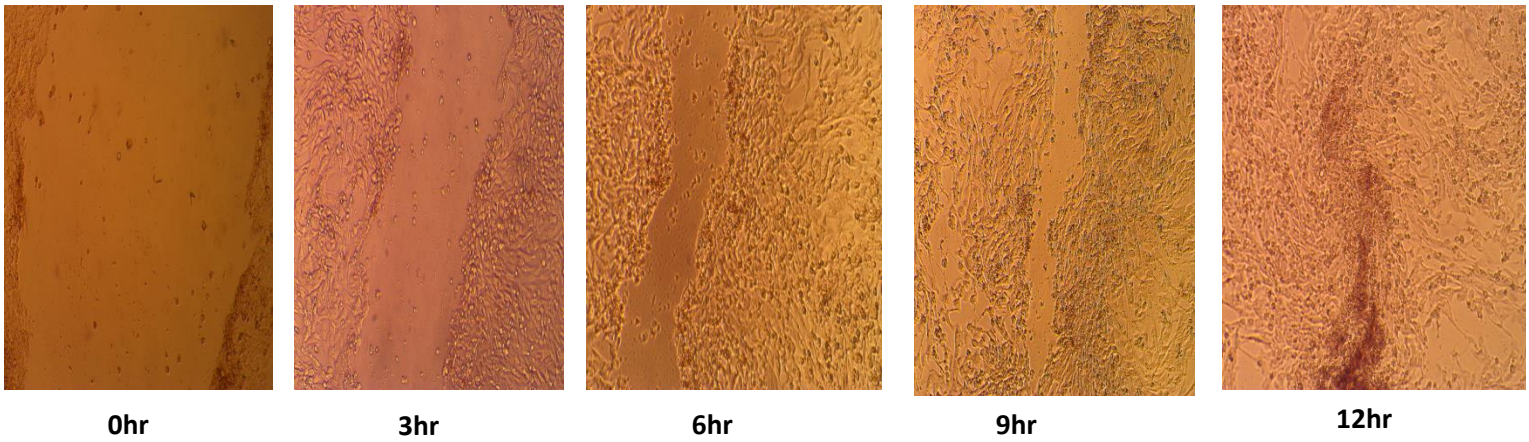


Figure 3.20: Photomicrograph (10XLence) of MDCK cell line treated with *Ricinus communis* aqueous leaf extract (15.6 µg/ml) at different period of incubation.

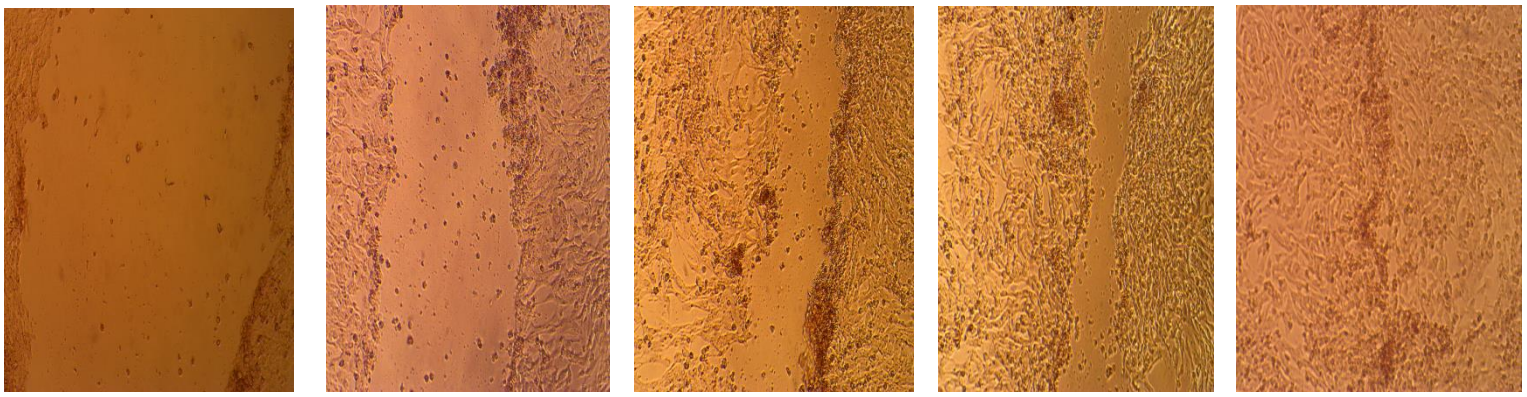


Figure3.21: Photomicrograph (10XLence) of MDCK cell line treated with *Ricinus communis* aqueous leaf extract (31.25 µg/ml) at different period of incubation.

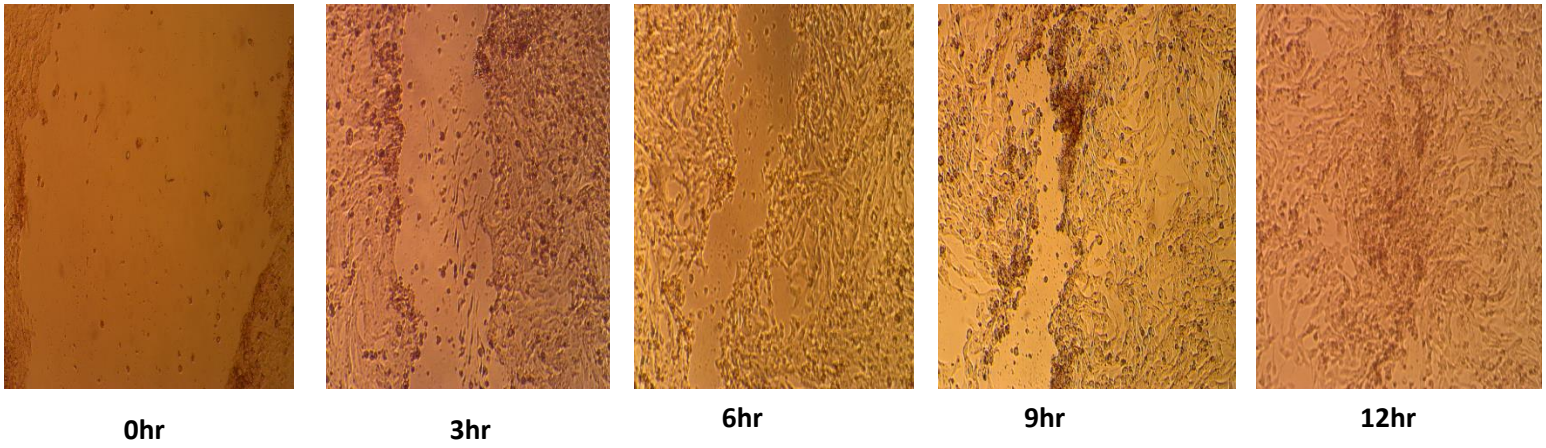


Figure 3.22: Photomicrograph (10XLence) of MDCK cell line treated with *Ricinus communis* aqueous leaf extract (62.5 µg/ml) at different period of incubation.

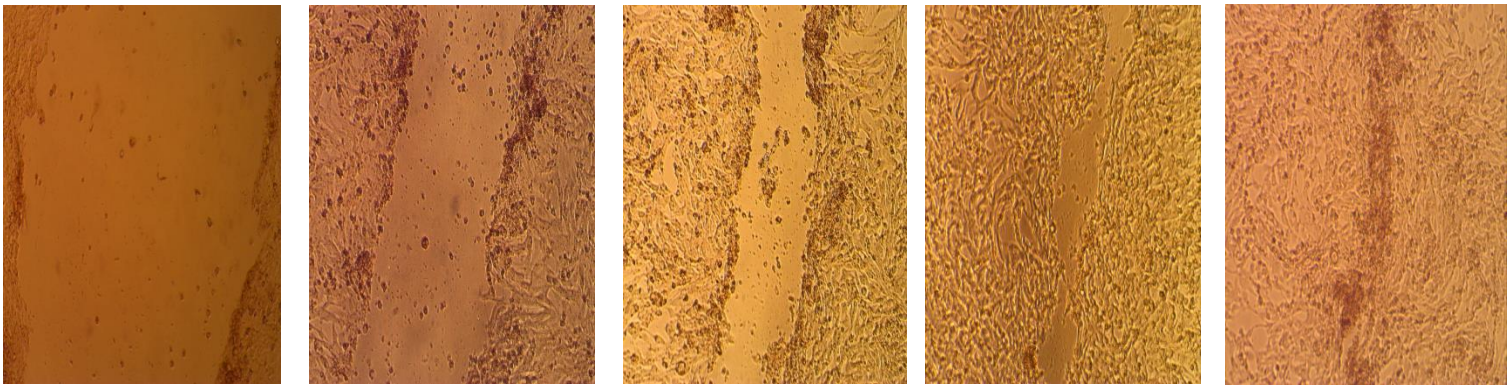


Figure 3.23: Photomicrograph (10XLence) of MDCK cell line treated with *Ricinus communis* aqueous leaf extract (125 µg/ml) at different period of incubation.

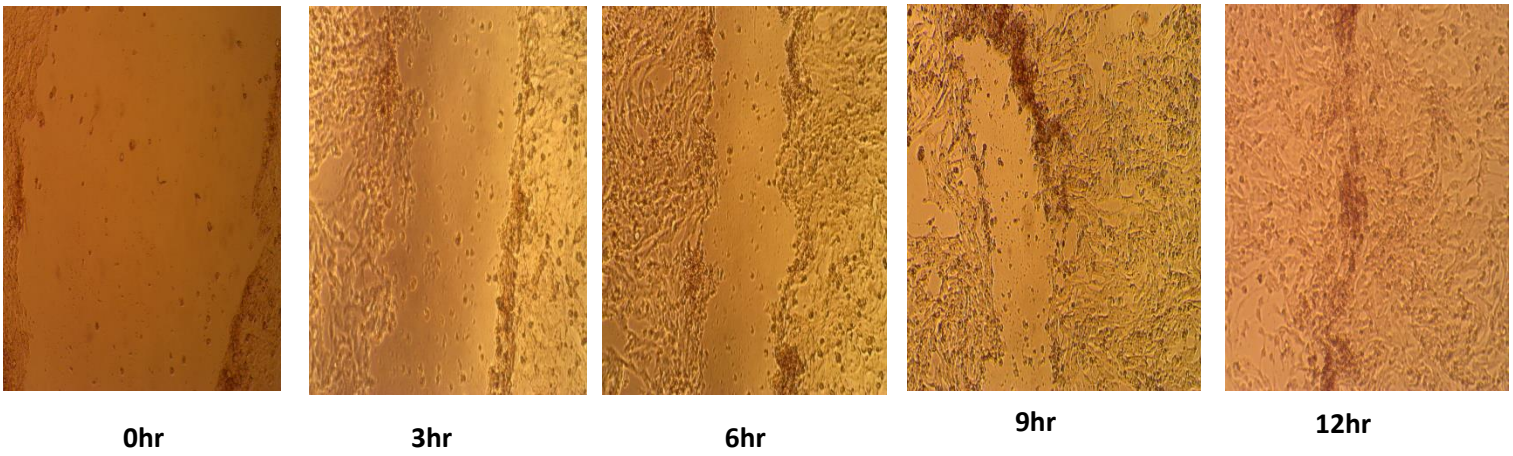
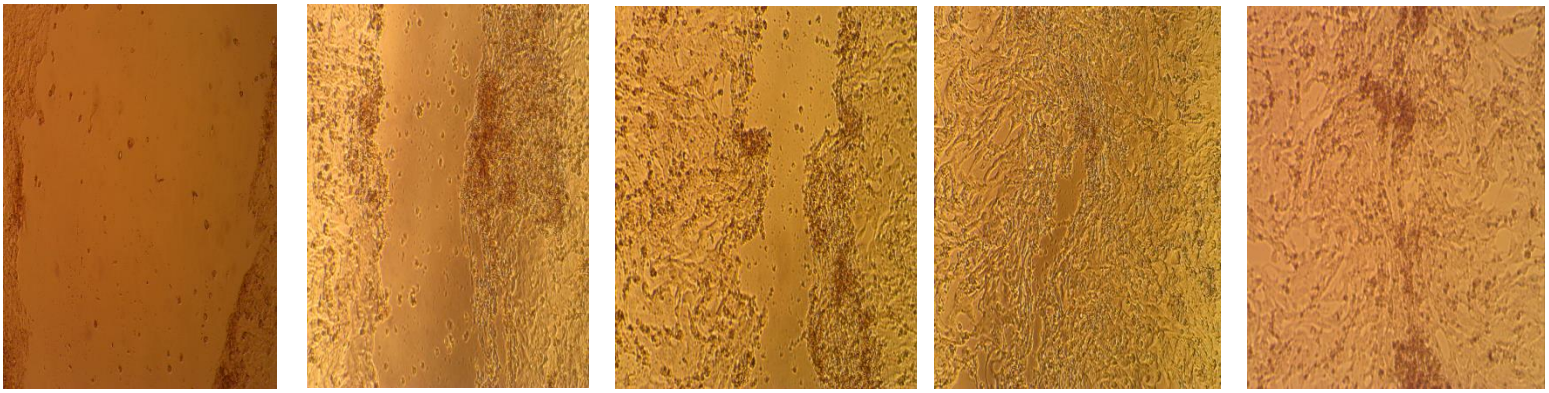
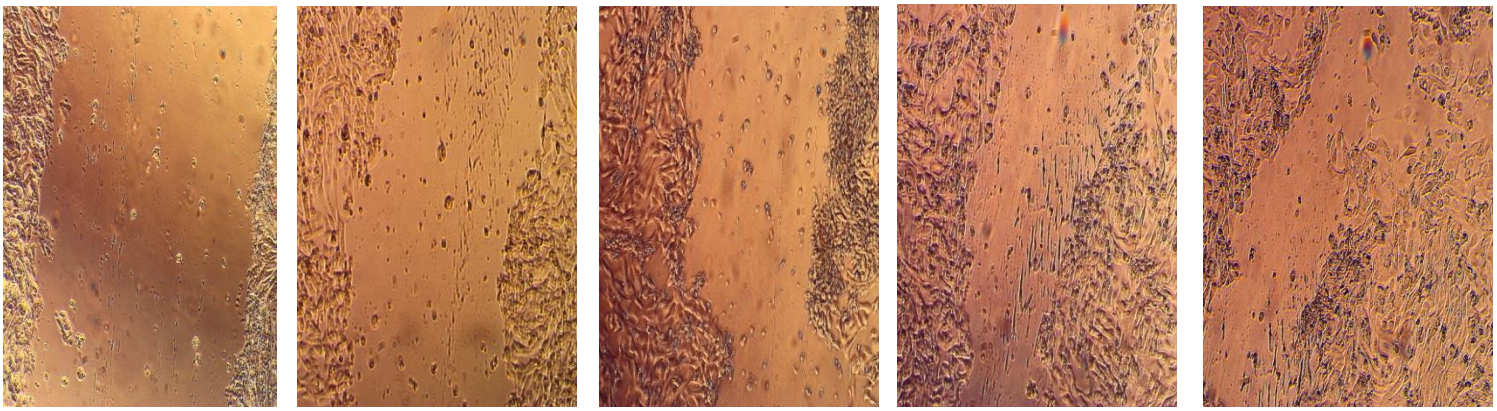


Figure 3.24: Photomicrograph (10XLence) of MDCK cell line treated with *Ricinus communis* aqueous leaf extract (250 µg/ml) at different period of incubation.



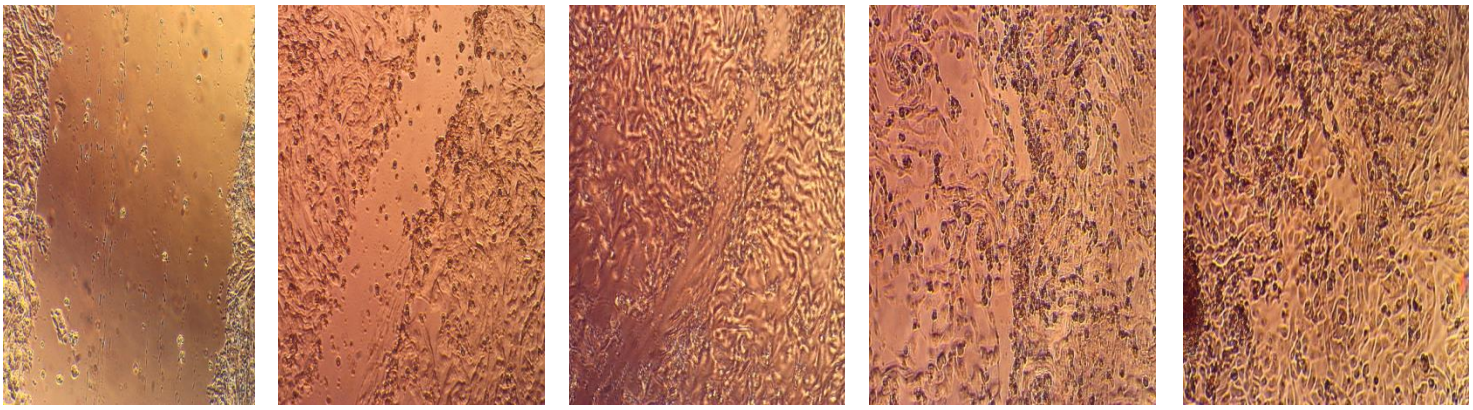
0hr 3hr 6hr 9hr 12hr

Figure 3.25: Photomicrograph (10XLence) of MDCK cell line treated with *Ricinus communis* aqueous leaf extract (500 µg/ml) at different period of incubation.



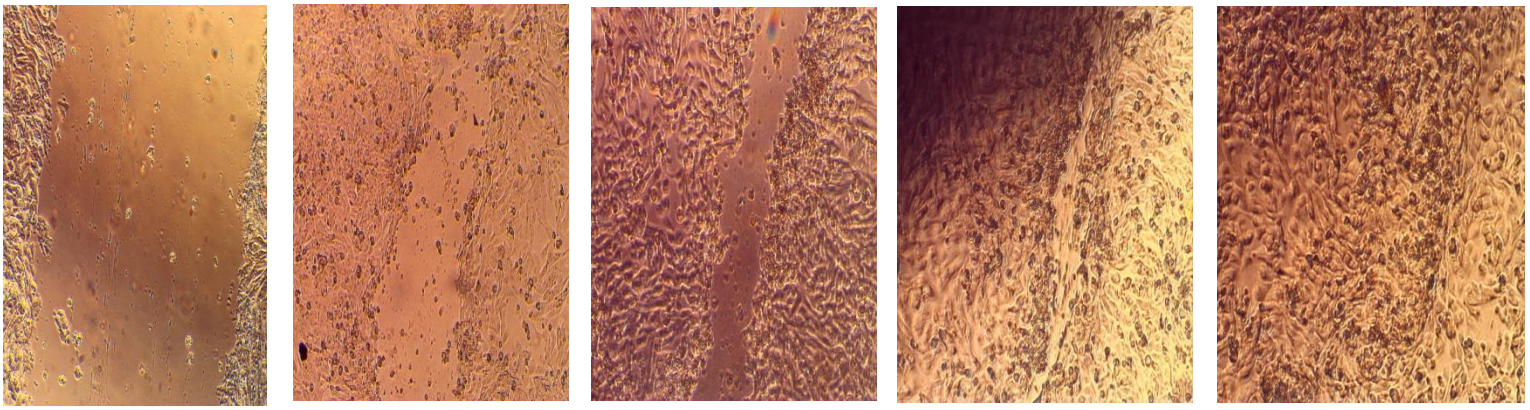
0hr 3hr 6hr 9hr 12hr

Figure 3.26: Photomicrograph(10XLence) of MDCK cell line without treatment of metformin (control group) at different period of incubation.



0hr 3hr 6hr 9hr 12hr

Figure 3.27: Photomicrograph (10XLence) of MDCK cell line treated with metformin (3.9 µg/ml) at different period of incubation.



0hr 3hr 6hr 9hr 12hr

Figure 3.28: Photomicrograph (10XLence) of MDCK cell line treated with metformin (7.8 µg/ml) at different period of incubation.

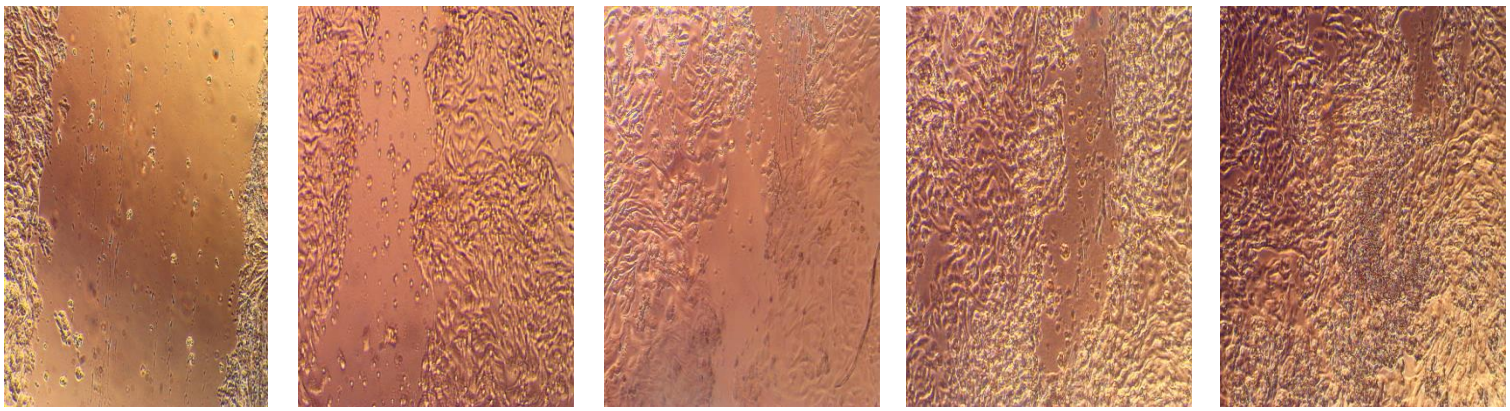


Figure 3.29: Photomicrograph (10XLence) of MDCK cell line treated with metformin (15.6 µg/ml) at different period of incubation.

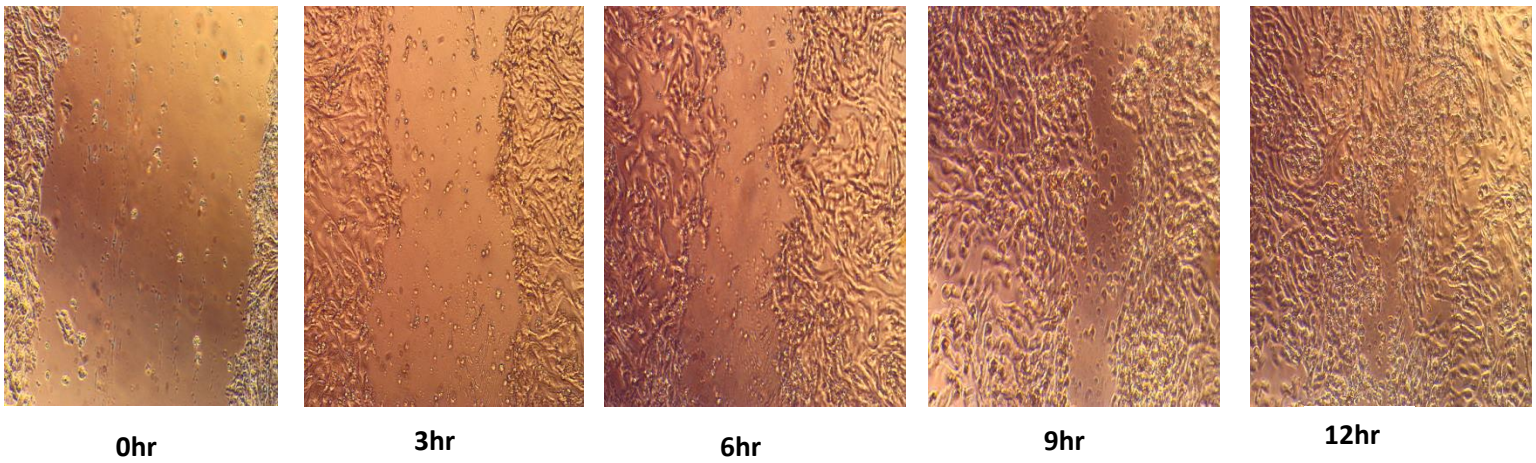
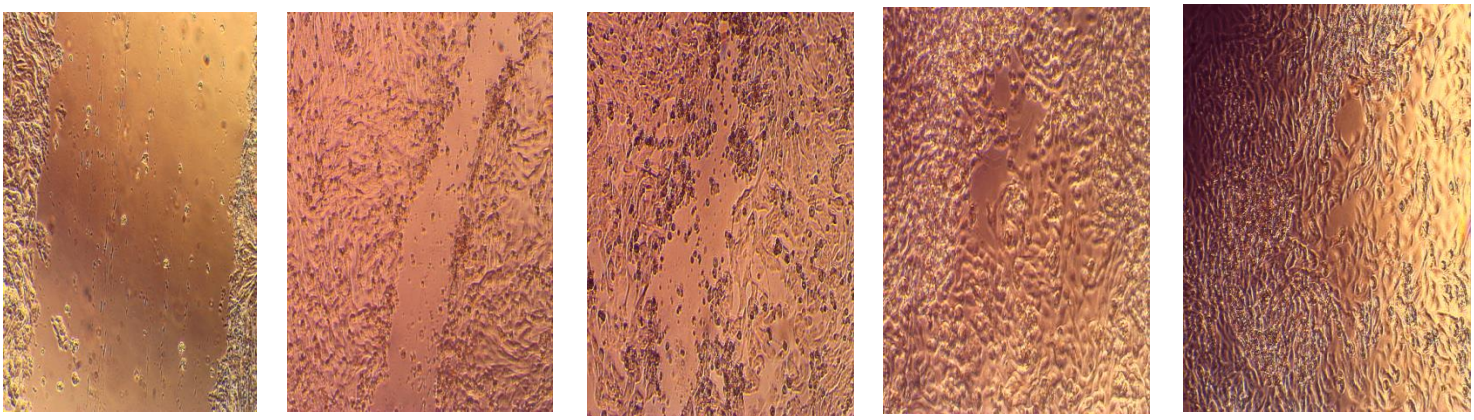
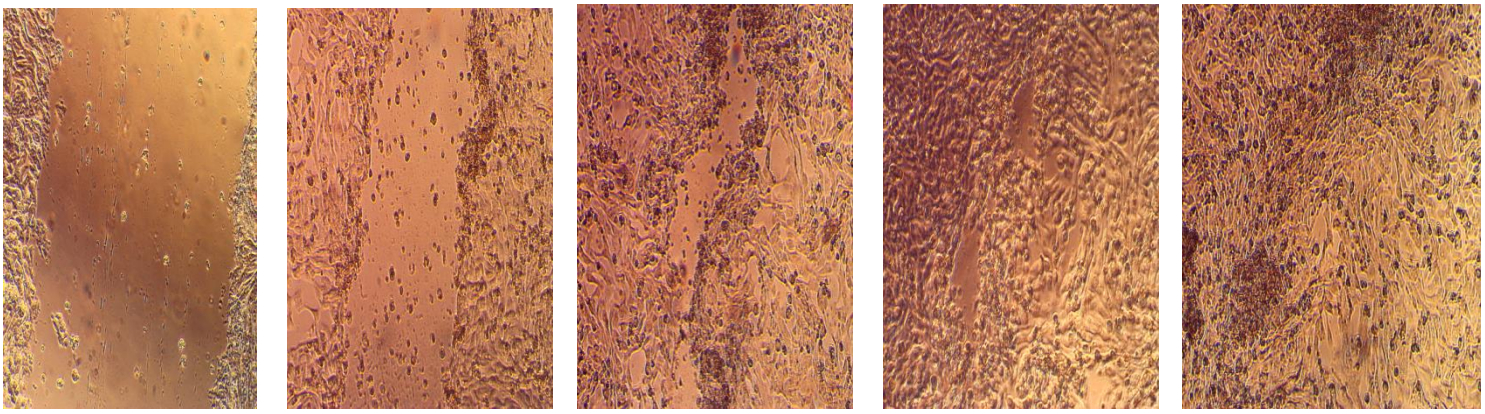


Figure 3.30: Photomicrograph (10XLence) of MDCK cell line treated with metformin (31.25 µg/ml) at different period of incubation.



0hr 3hr 6hr 9hr 12hr

Figure 3.31: Photomicrograph (10XLence) of MDCK cell line treated with metformin (62.5 µg/ml) at different period of incubation.



0hr 3hr 6hr 9hr 12hr

Figure 3.32: Photomicrograph (10XLence) of MDCK cell line treated with metformin (125 µg/ml) at different period of incubation.

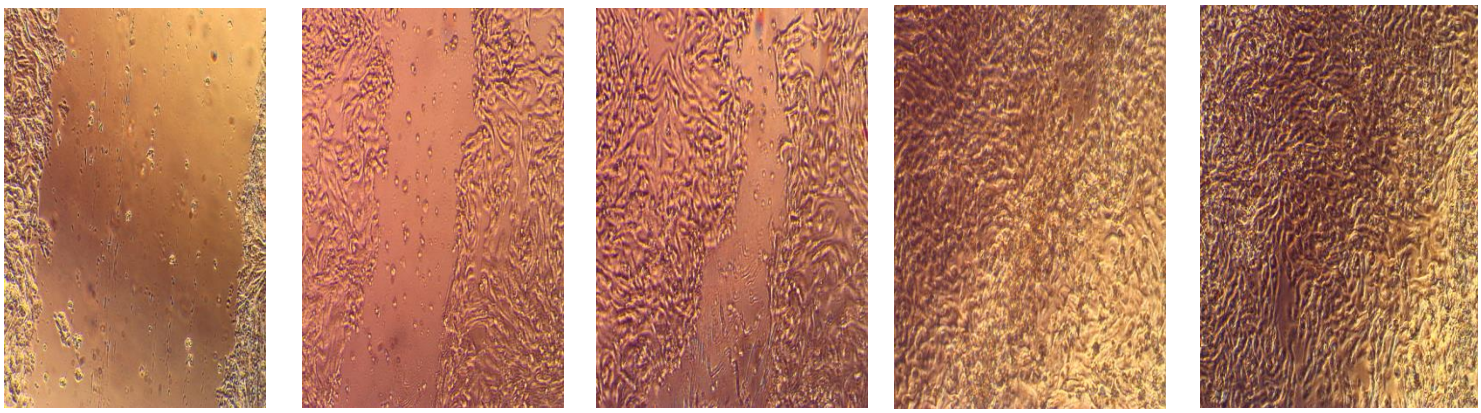


Figure 3.33: Photomicrograph (10XLence) of MDCK cell line treated with metformin (250 µg/ml) at different period of incubation.

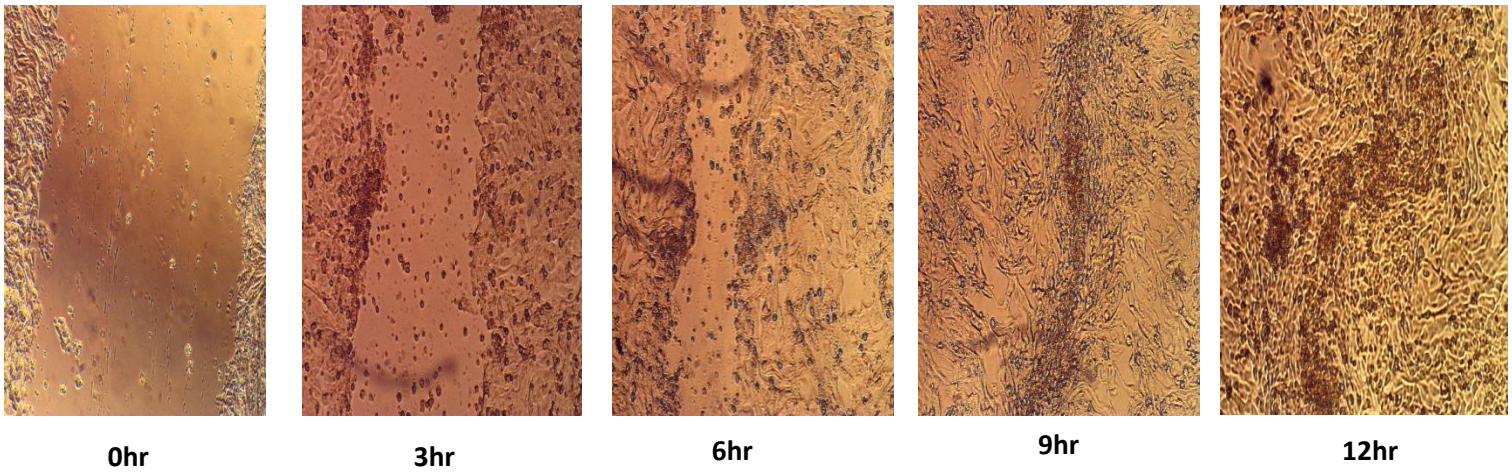


Figure 3.34: Photomicrograph (10XLence) of MDCK cell line treated with metformin (500 µg/ml) at different period of incubation.

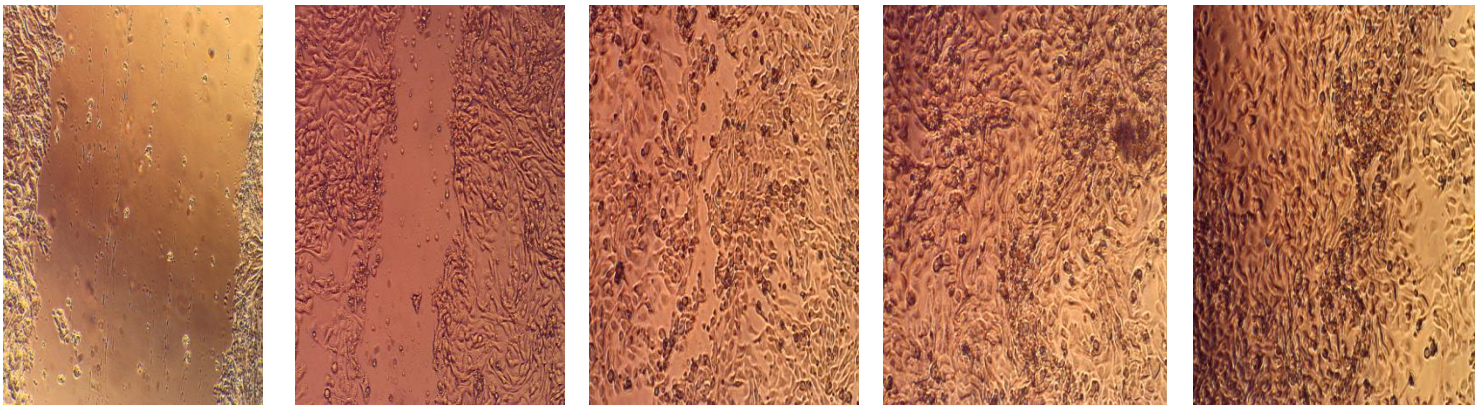


Figure 3.35: Photomicrograph (10XLence) of MDCK cell line treated with metformin (1000 µg/ml) at different period of incubation.

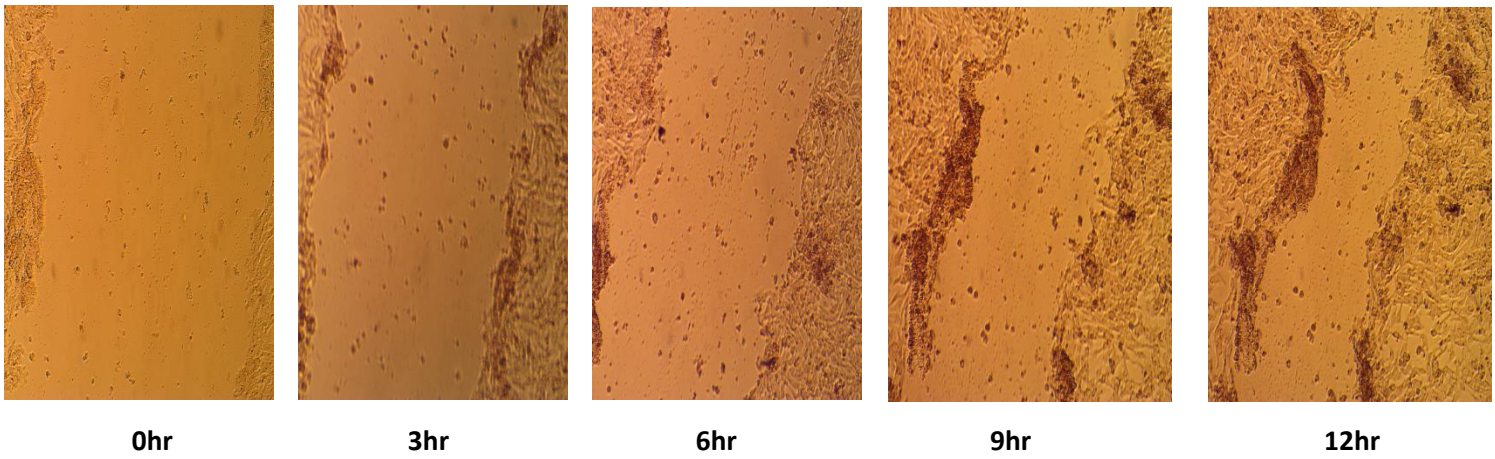


Figure 3.36: Photomicrograph (10XLence) of MDCK cell line without treatment of metformin plus 250 or 500 µg/ml of *Ricinus communis* leaf ethanolic extract (control group) at different period of incubation.

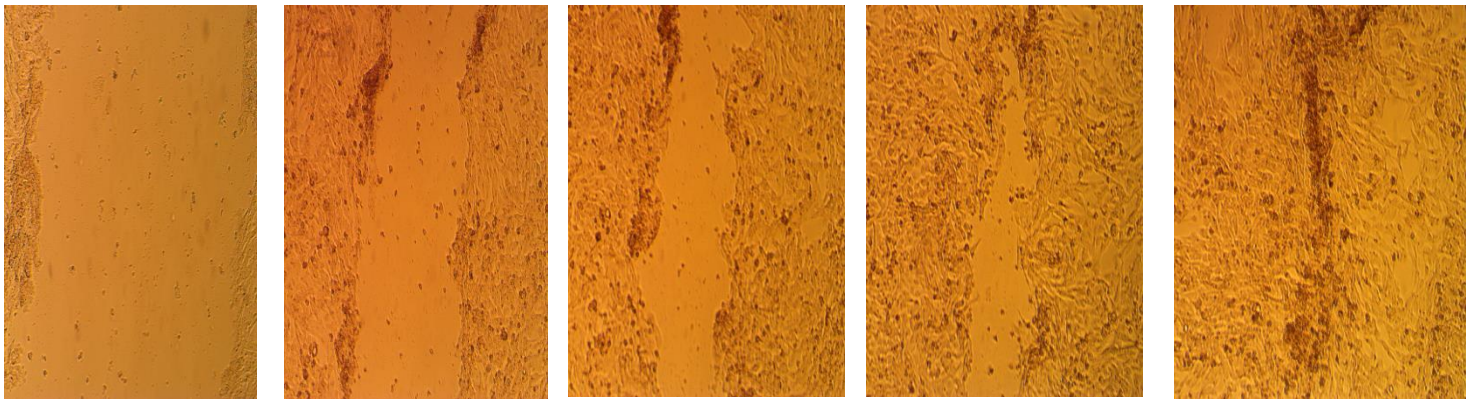


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Figure 3.37: Photomicrograph (10XLence) of MDCK cell line treated with metformin 125µg/ml plus 250µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

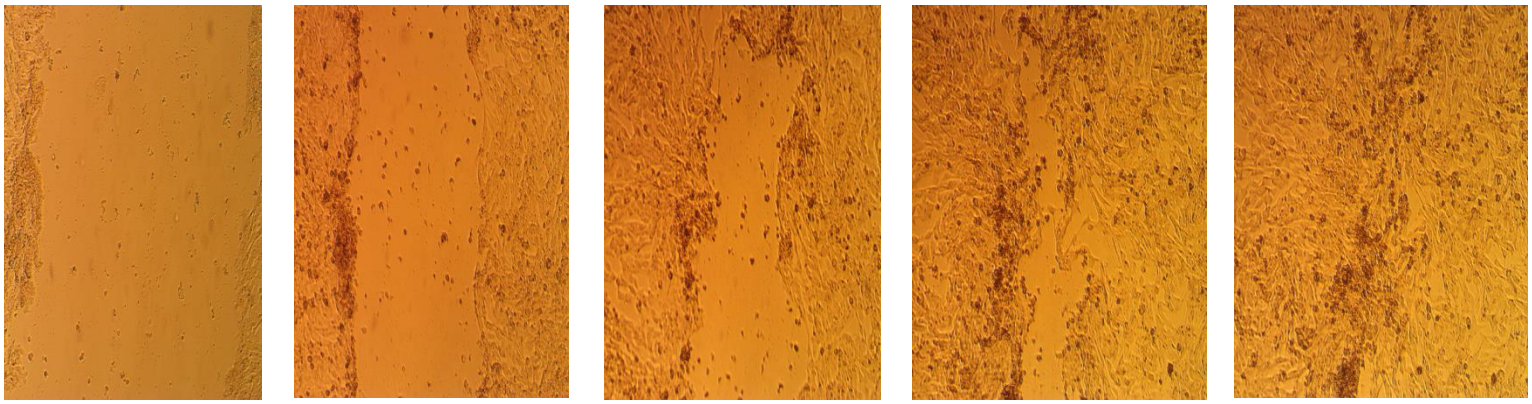

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Figure 3.38: Photomicrograph (10XLence) of MDCK cell line treated with metformin 250µg/ml plus 250µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

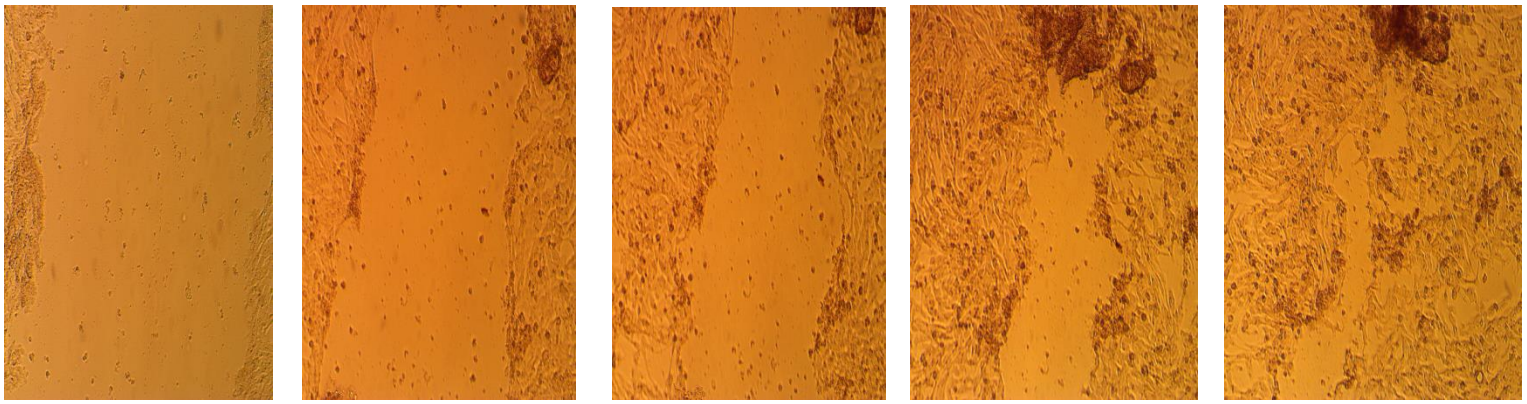

Figure 3.39: Photomicrograph (10XLence) of MDCK cell line treated with metformin 500µg/ml plus 250µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

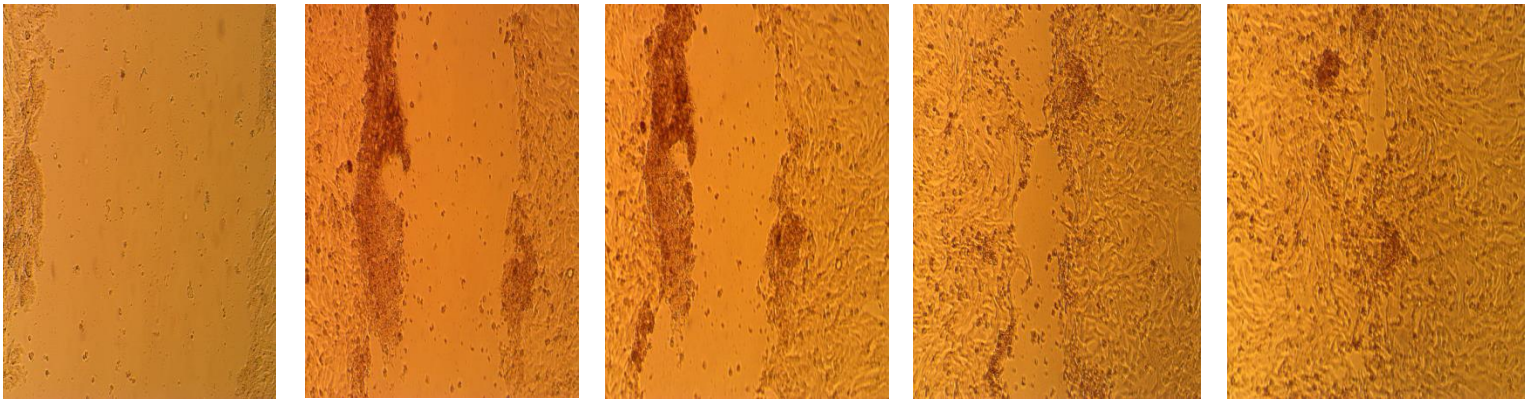

Figure 3.40: Photomicrograph (10XLence) of MDCK cell line treated with metformin 1000µg/ml plus 250µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

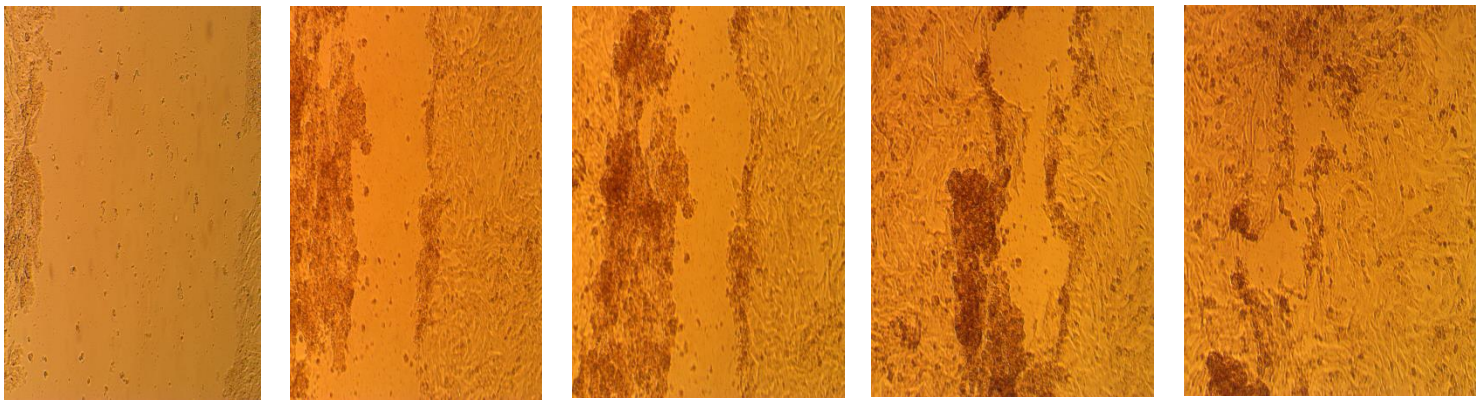

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Figure 3.41: Photomicrograph (10XLence) of MDCK cell line treated with metformin 125µg/ml plus 500µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

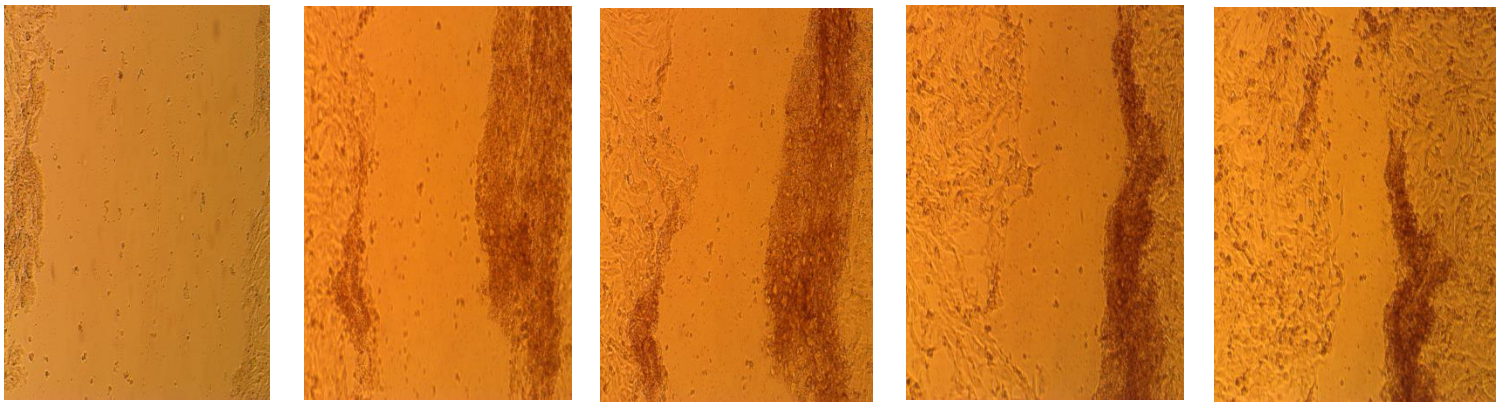

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Figure 3.42: Photomicrograph (10XLence) of MDCK cell line treated with metformin 250µg/ml plus 500µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

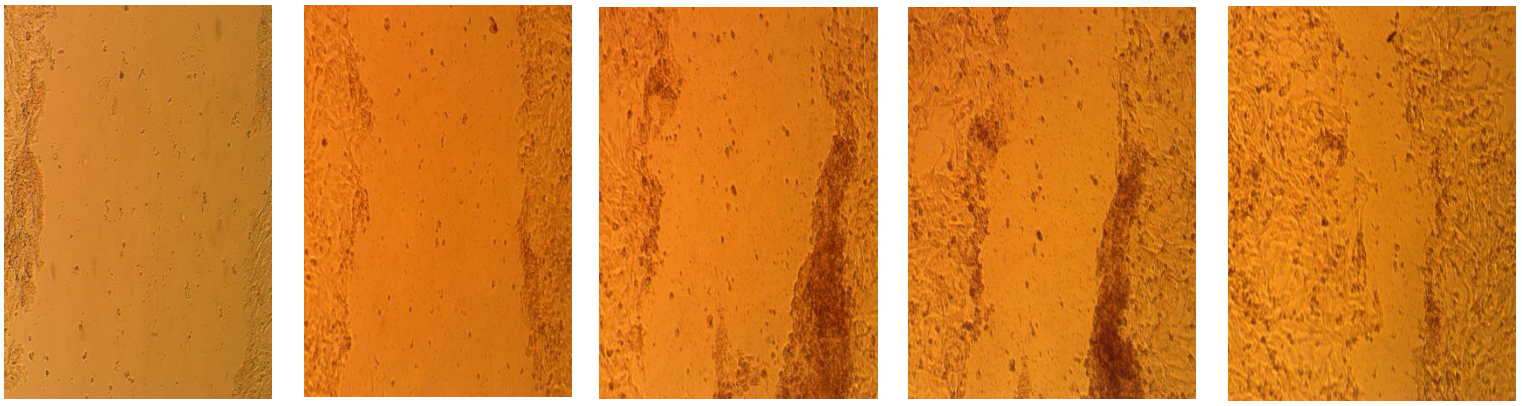

Figure 3.43: Photomicrograph (10XLence) of MDCK cell line treated with metformin 500µg/ml plus 500µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

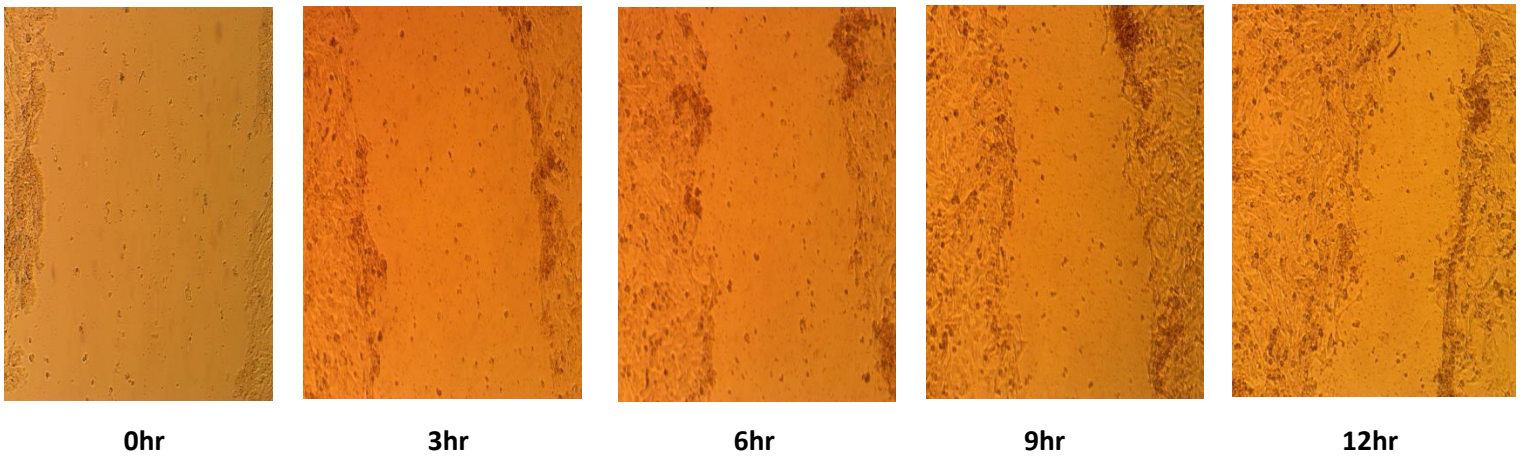

Figure 3.44: Photomicrograph (10XLence) of MDCK cell line treated with metformin 1000µg/ml plus 500µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

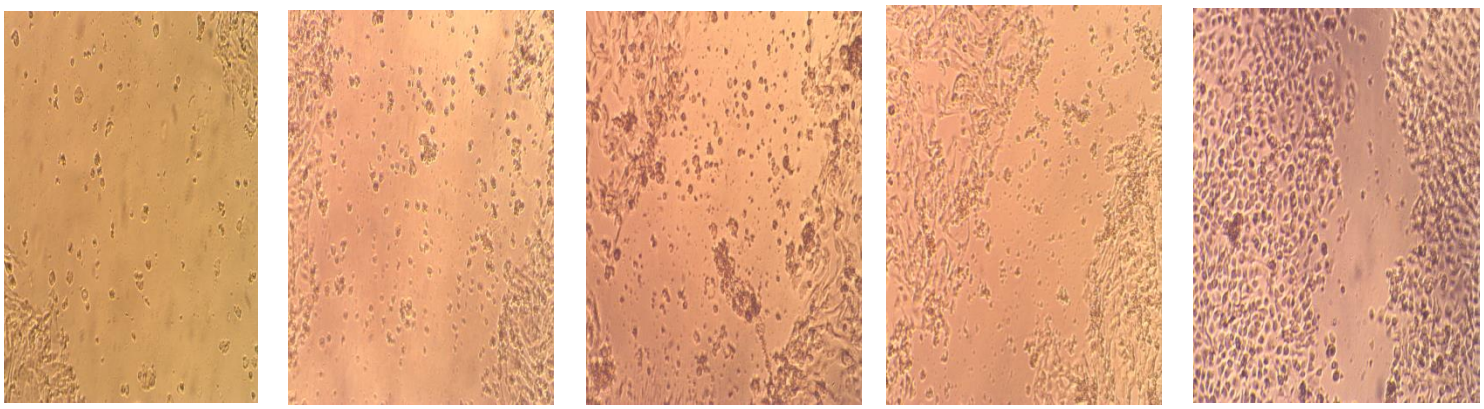

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Figure 3.45: Photomicrograph (10XLence) of MDCK cell line without treatment of phenytoin (control group) at different period of incubation.

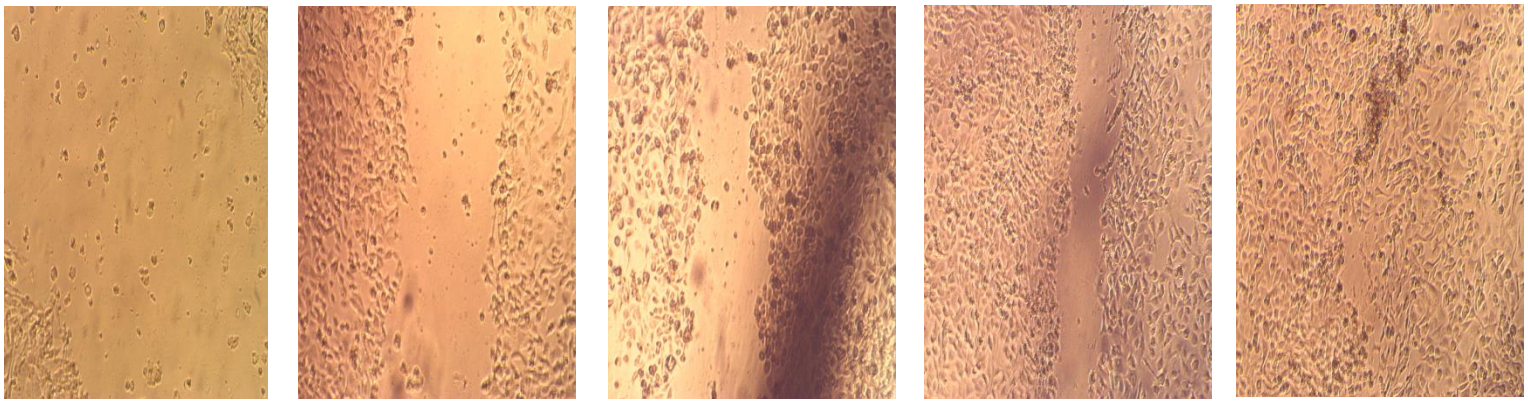

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Figure 3.46: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin(1.9 µg/ml) at different period of incubation.

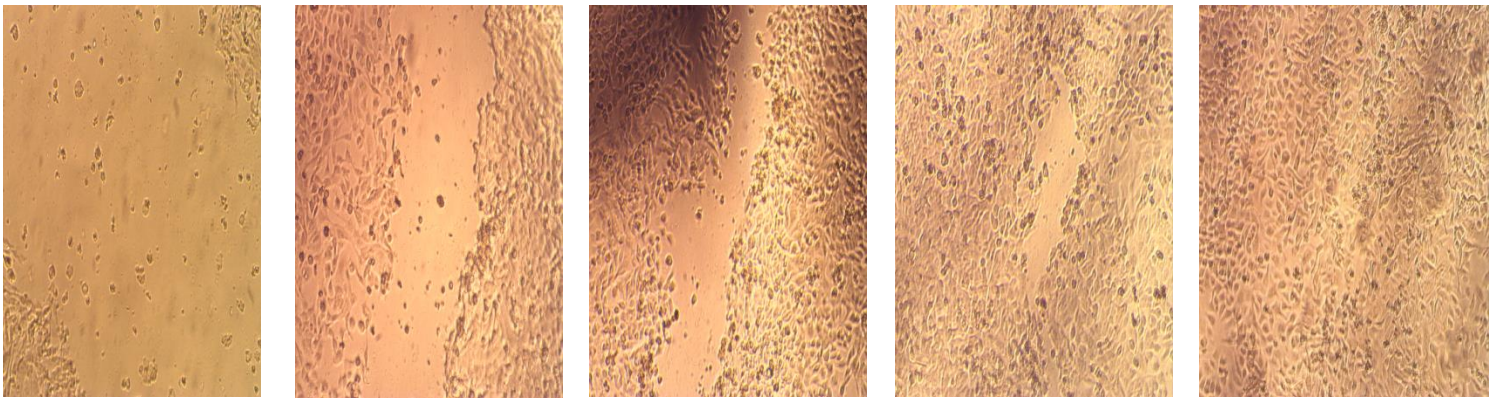

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Figure 3.47: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin(3.9 µg/ml) at different period of incubation.

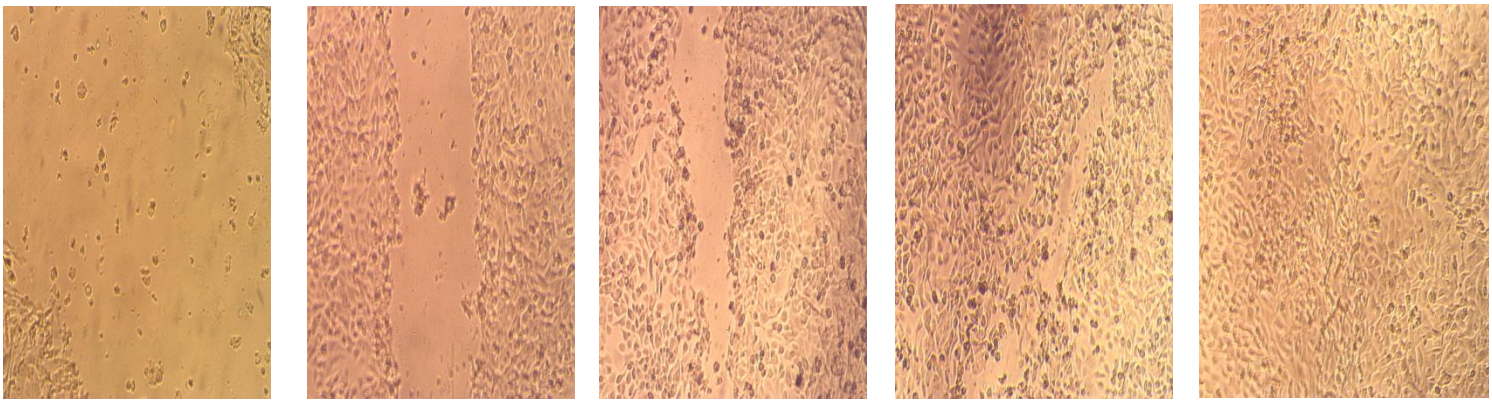

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Figure 3.48: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin(7.8 µg/ml) at different period of incubation.

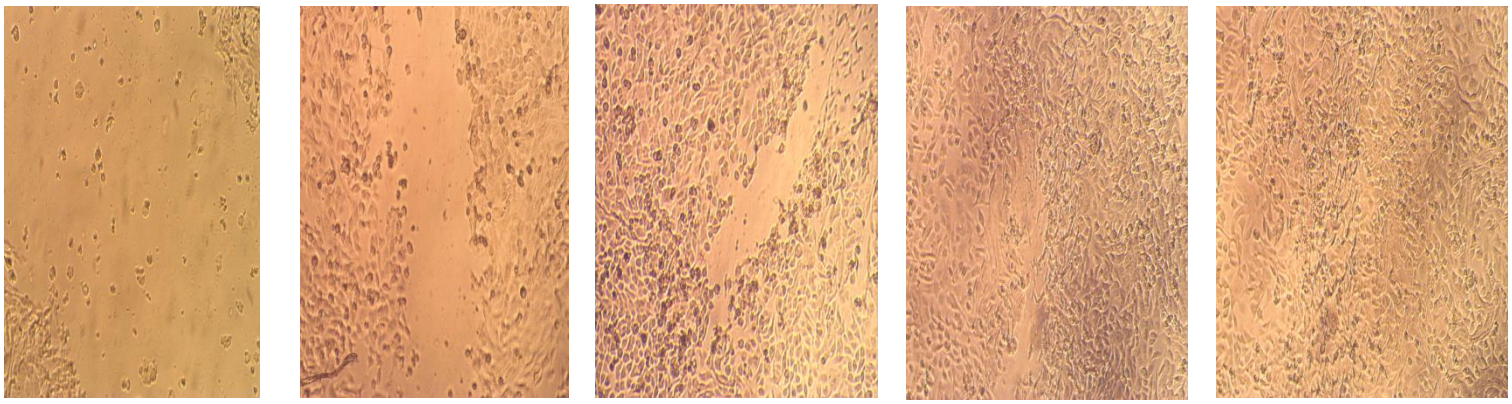

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Figure 3.49: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin(15.6 µg/ml) at different period of incubation.

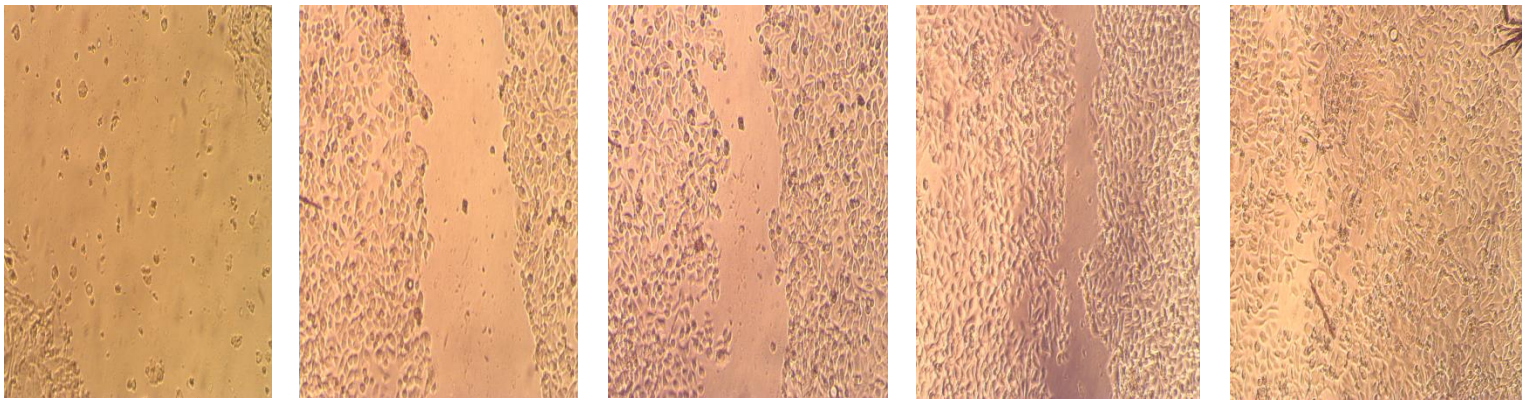

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Figure 3.50: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin(31.25µg/ml) at different period of incubation.

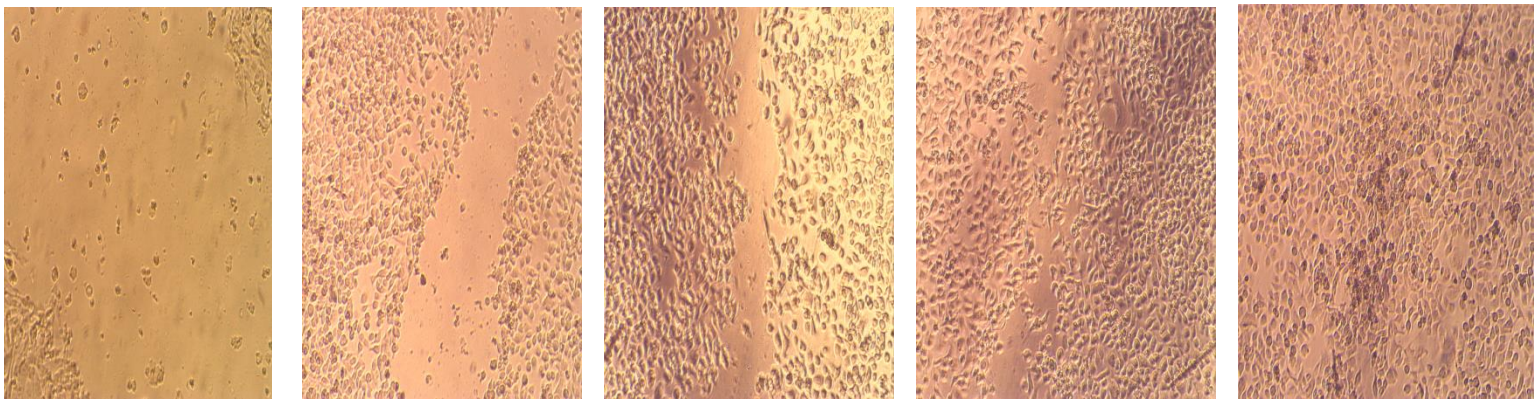

Figure 3.51: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin(62.5µg/ml) at different period of incubation.

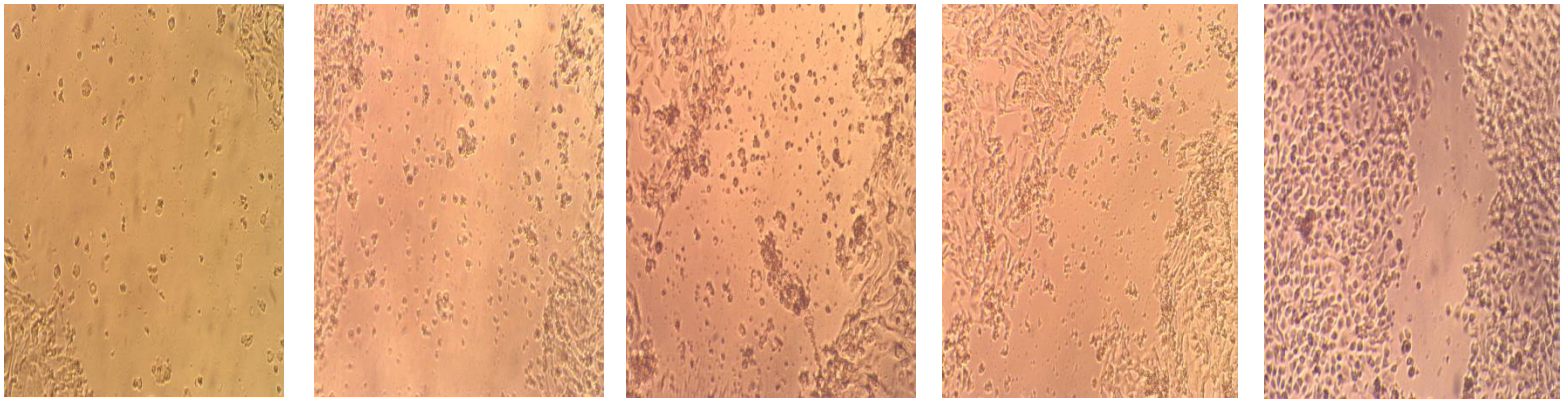

Figure 3.52: Photomicrograph (10XLence) of MDCK cell line without treatment of phenytoin plus 250 or 500 µg/ml of *Ricinus communis* leaf ethanolic extract (control group) at different period of incubation.

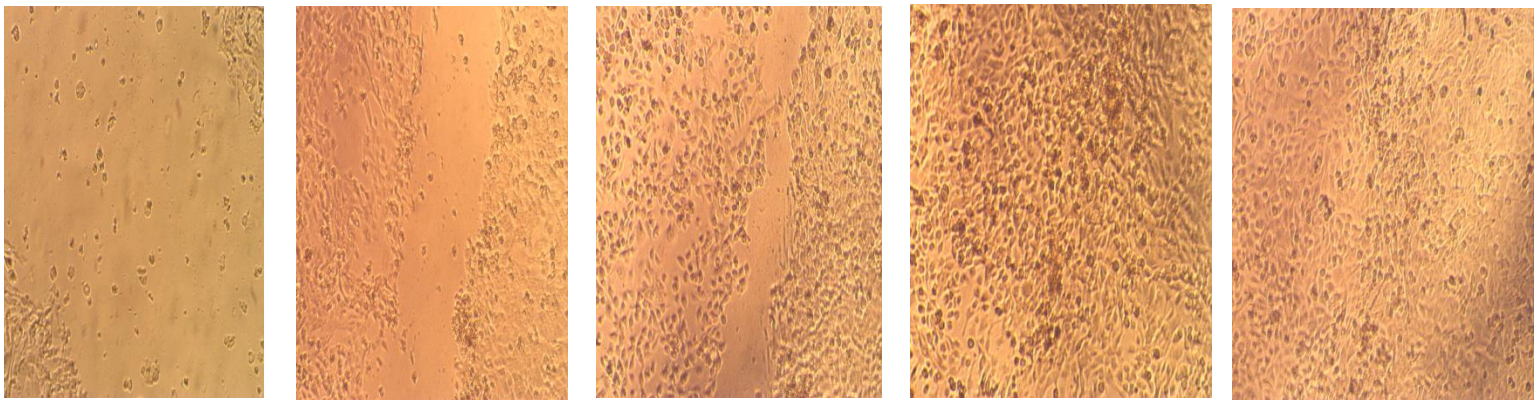

Figure 3.53: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin 1.9µg/ml plus 250µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

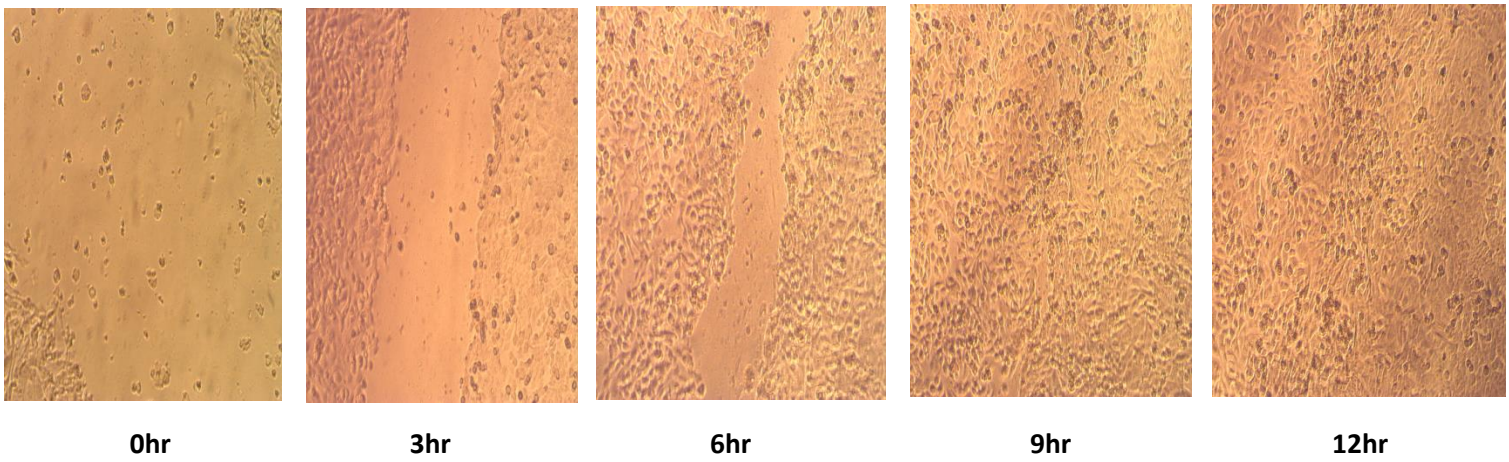

Figure 3.54: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin 3.9µg/ml plus 250µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

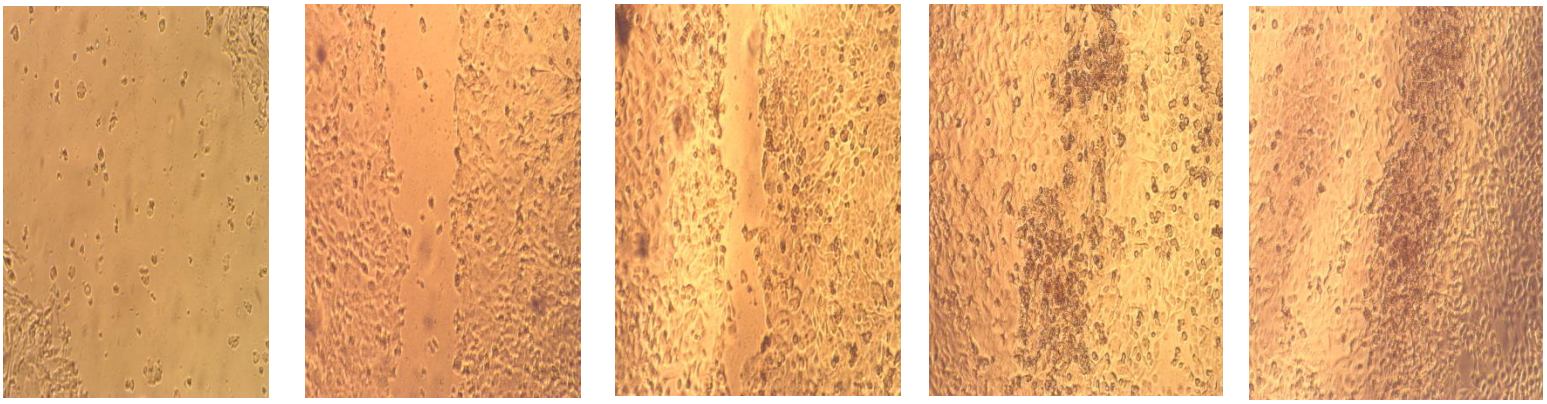

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Figure 3.55: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin 7.8µg/ml plus 250µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation .

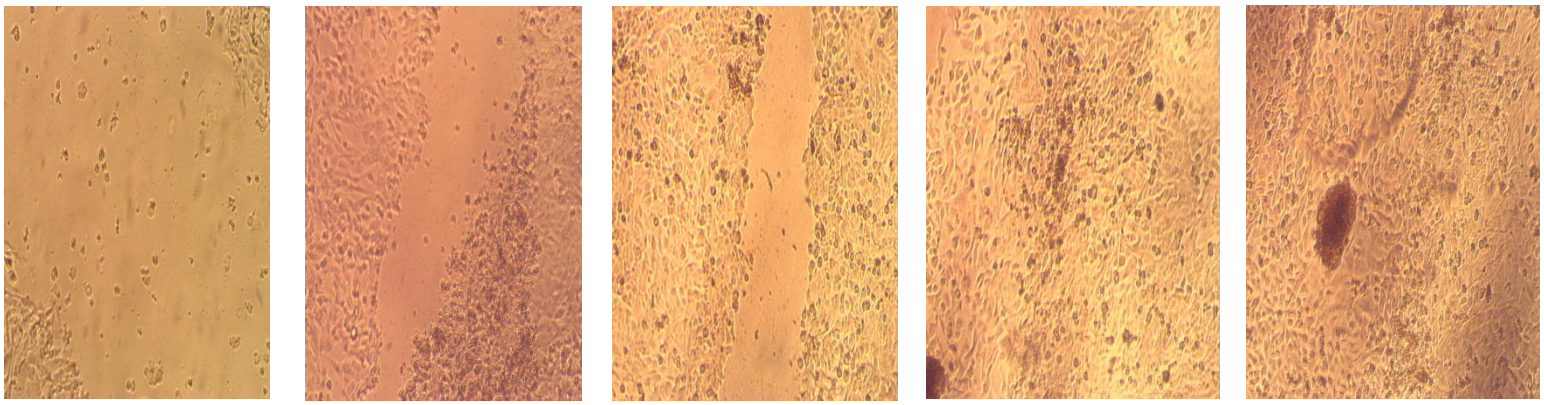

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Figure 3.56: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin 15.6µg/ml plus 250µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

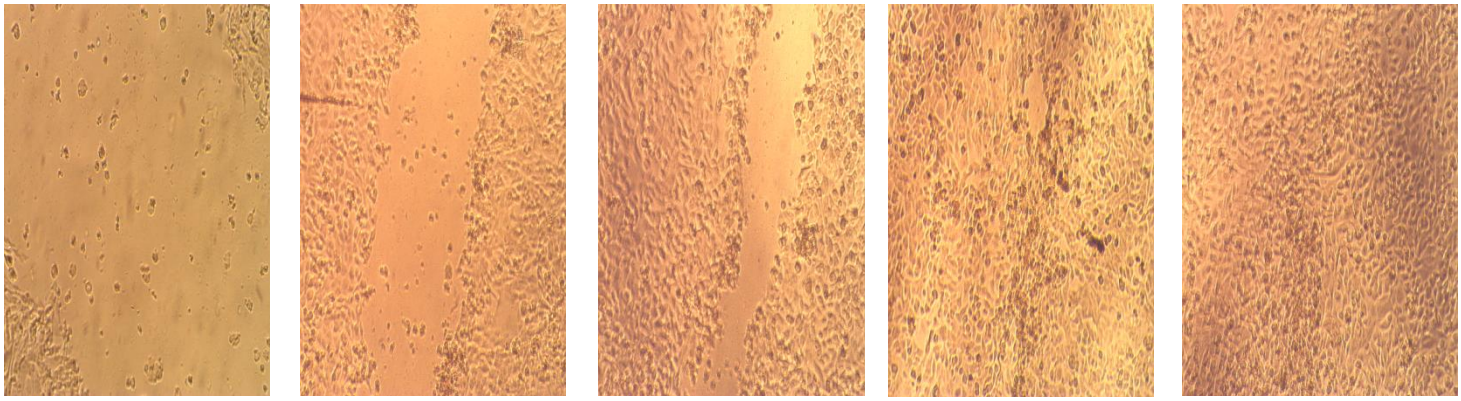

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Figure 3.57: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin 31.25µg/ml plus 250µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

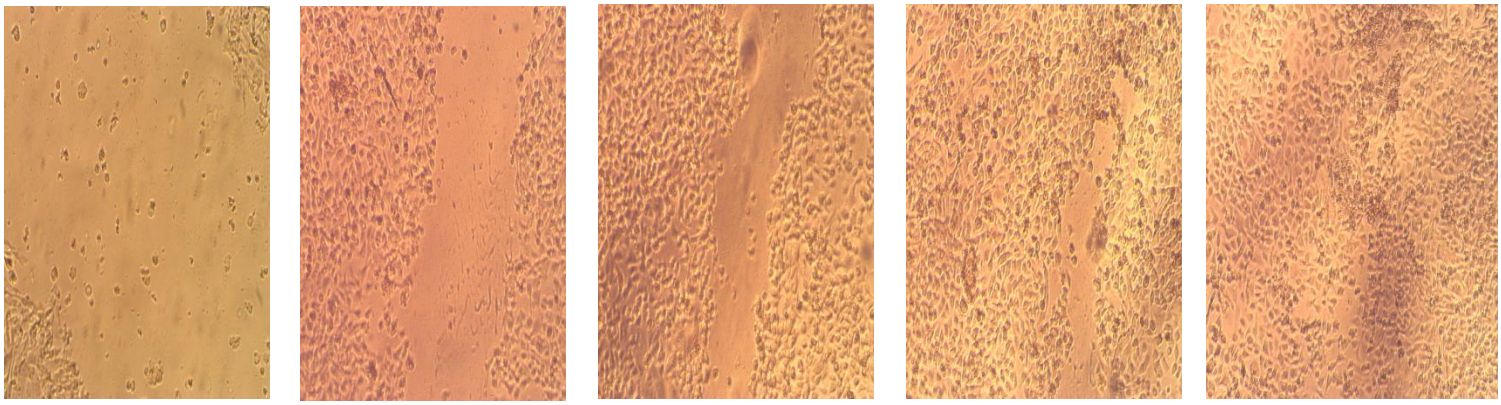

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Figure 3.58: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin 62.5µg/ml plus 250µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

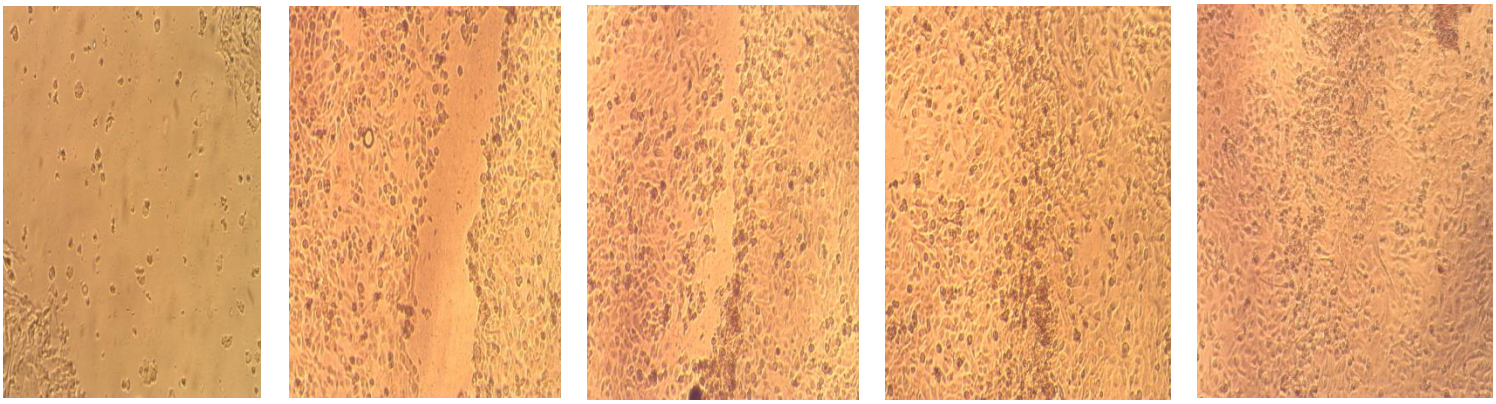

Figure 3.59: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin 1.9µg/ml plus 500µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

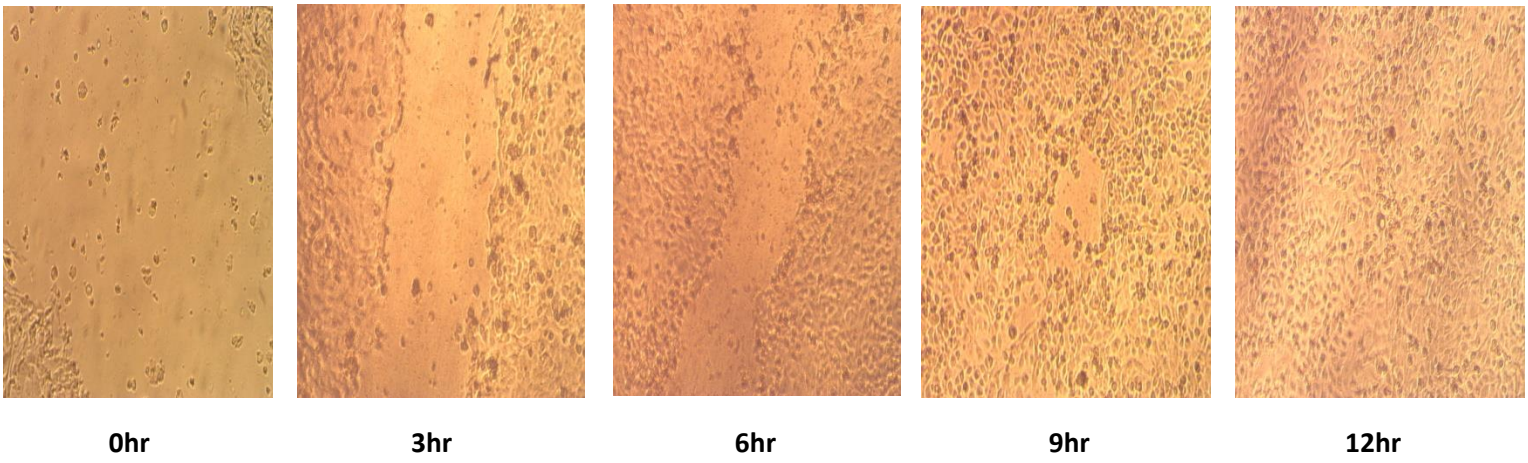

Figure 3.60: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin 3.9µg/ml plus 500µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

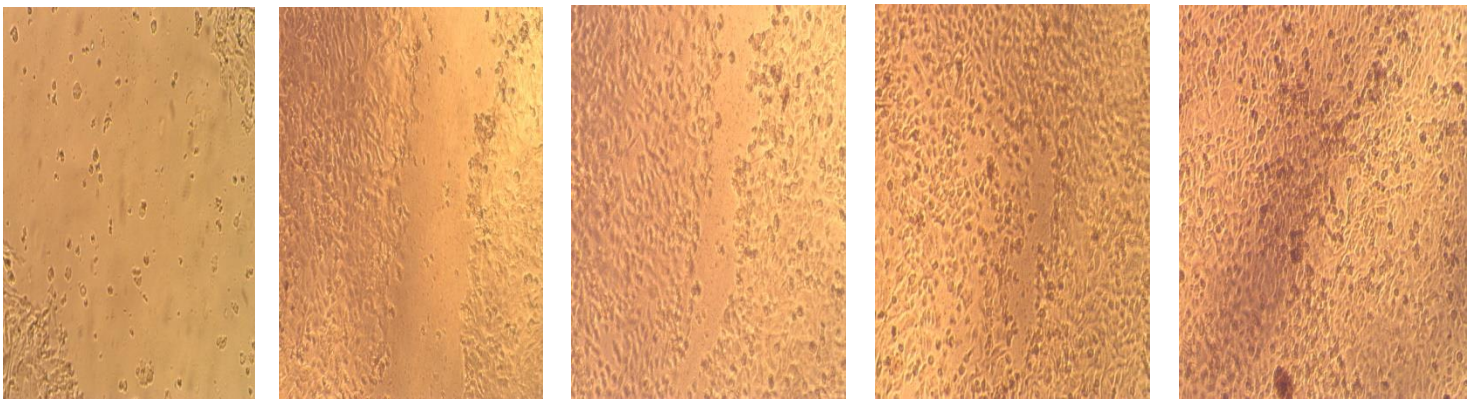

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Figure 3.61: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin 7.8µg/ml plus 500µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

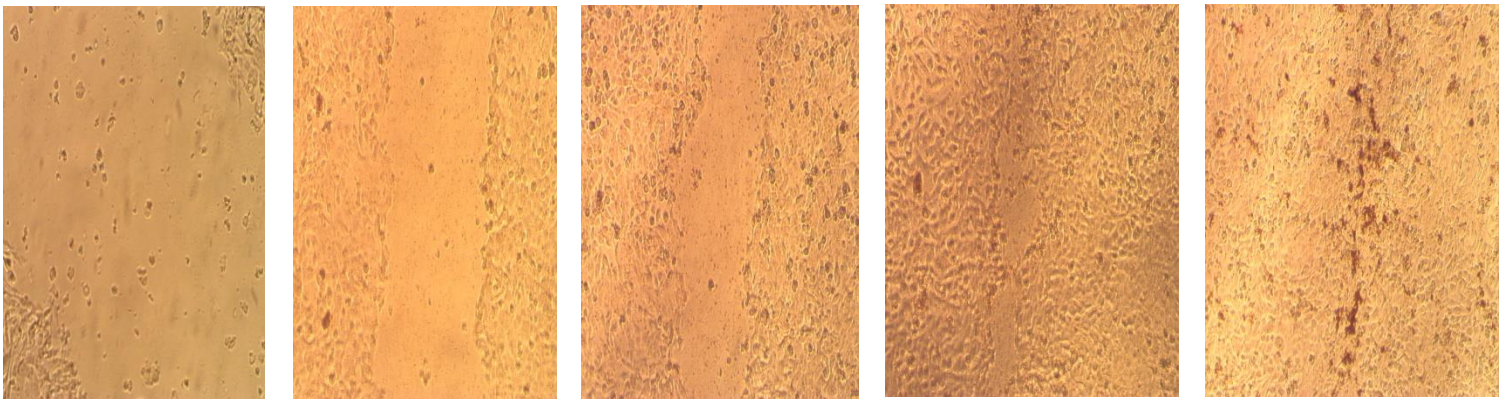

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Figure 3.62: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin 15.6µg/ml plus 500µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

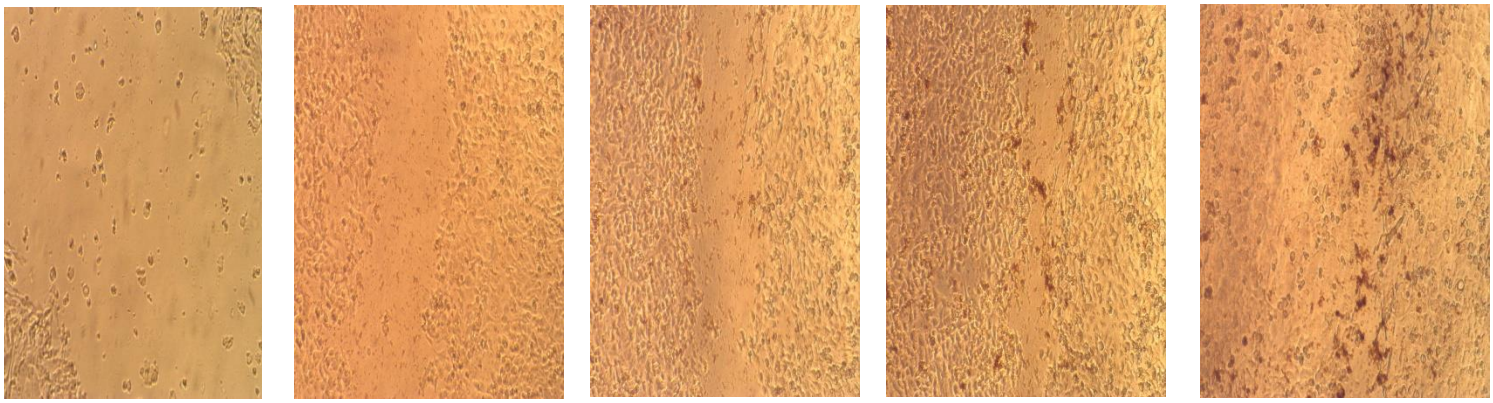

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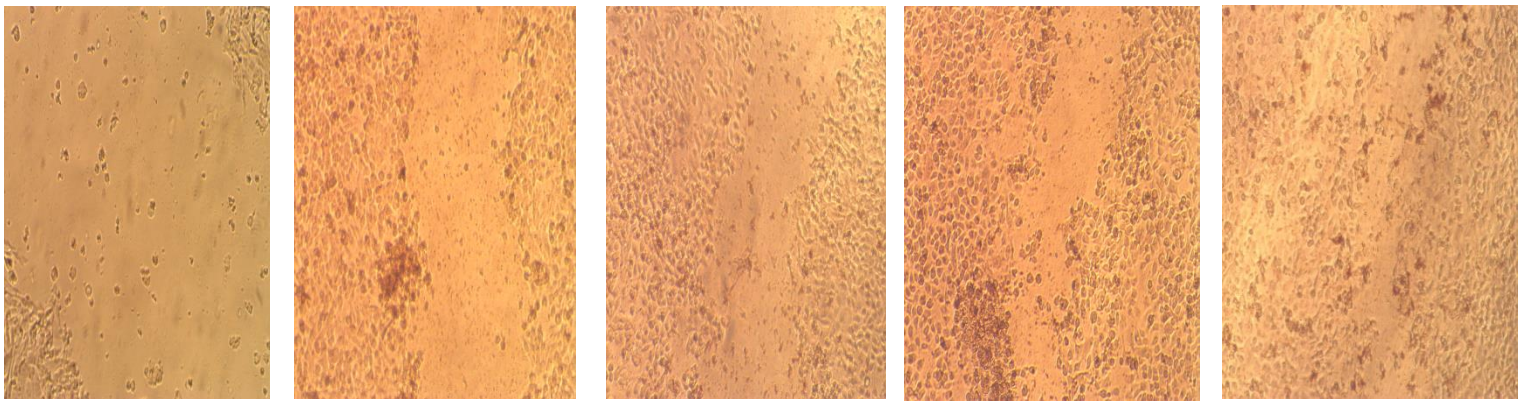
Figure 3.63: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin 31.25µg/ml plus 500µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.



0hr 3hr 6hr 9hr 12hr

Figure 3.64: Photomicrograph (10XLence) of MDCK cell line treated with phenytoin 62.5µg/ml plus 500µg/ml of *Ricinus communis* leaf ethanolic extract at different period of incubation.

Chapter Four

Discussion

4.1-Effect of *Ricinus communis* leaves ethanolic and aqueous extracts on the viability of MDCK normal cell line

In the present study the insignificant difference in the viability of MDCK normal cell line after treated with different concentrations of *R.communis* ethanolic extract and the increase in the viability of MDCK normal cell line after treated with 125 µg/ml of *R.communis* aqueous extract agree with that reported by Gad-Elkareem *et al*., (2019) study which revealed that both two extracts (ethanol and aqueous-ethanol) of *R.communis* leaf extract given by oral route were safe up to a dose of 2,000 mg/kg/of body weight, and did not show any mortality and toxic effects in the behavior of the treated animals.

Another study by Masoko and Nemudzivhadi's , (2014) disagree with this results which revealed that the low concentrations of *R.communis* (hexane, dichloromethane, acetone, and methanol)leaf extracts had low toxic effect on Bud-8 cell line, while at high concentrations the extracts are highly toxic. Also the morphology of cells was altered from its normal shape of fibroblast to oval shape, because of the toxic effect of *R. communis* leaf extracts to the cells. Cell viability decreased with the increase in concentrations of *R.communis* (hexane, dichloromethane, acetone, and methanol)leaf extracts. Leaves of *R. communis* contain ricin but in low concentration. Ricin was reported to have high toxic effect on mammalian cells. It kills the cells by disrupting protein synthesis in the cells.

4.2-Effect of *Ricinus communis* leaves ethanolic and aqueous extracts on the healing of MDCK normal cell line- wound model

In the present study the decrease in the diameter and complete healing of the MDCK normal cell line-induced wound after the treatment with ethanolic leaf extract of *R.communis* at all concentrations except(250,500) µg/ml is in agreement with Mohammed and Albozachri ,(2020) who reported that the methanolic extract of castor leaf accelerate wound healing in rabbits through the acceleration of epithelial cell migration, angiogenic response and thus time of wound healing.

The enhancement of wound healing after treatment with ethanolic extract of *R.communis* leaf may be attributed to the presence of steroids, saponins, alkaloids, flavonoids, tannins, phenols, phytates, oxalates and glycosides in different parts of the plant including leaves as all these compounds have antimicrobial properties (Yadav *et al*., 2019).

Moreover it had been found that the antimicrobial properties of phytochemical compounds that are present in different parts of *R.communis*, promote the wound healing process, as they enhance wound contraction and increase rate of epithelialization (Bhakta and Kumar, 2015).

The higher effectiveness of aqueous *R.communis* leaf extract in comparison to that of ethanolic extract reported by the present study is disagree with that found by Naz and Bano, (2012) study which revealed that the aqueous extract of leaf had low activity against both fungal and bacterial strain as compared to methanol extracts and explaining this result by the

solubility of most phytochemical compounds which having antimicrobial potential in the methanolic more than aqueous solvents. According to the previous study the antibacterial and antifungal activity vary with the different solvents of plant leaf material used. *R. communis* is effective even at low concentration against bacterial and fungal pathogens.

The wound healing enhancement of the *R.communis* leaves extracts pointed by the present study could related to the presence of flavonoids: rutin, quercetin, epicatechin and polyphenols (Gallic and ellagic acid) and gentesic acid, these compounds which are potent anti-oxidant and anti-inflammatory agents (Saini *et al*., 2010). Inflammation is the major characteristics as a result of wound due to the release of eicosanoids, prostaglandins, leukotriene and reactive oxygen species, thus compounds such as flavonoids which is one of an important bioactive source from medicinal plants that possess different pharmacological activities such as antioxidant(free radical scavenger), antimicrobial, anti-inflammatory, anticancer activities, growth regulators could play an important role as wound healing enhancer Aslam *et al*.,(2018).

4.3-Effect of metformin on the viability of MDCK normal cell line

In the present study the increase in the viability of MDCK normal cell line after treated with 62.5 µg/ml of metformin is in agreement with Cozma *et al*.,(2022) who reported that metformin increases cell viability of hepatocyte cell line—HepaRG, without causing morphological changes to cells, mitochondria, or nuclei, as explained by the authar in his research.

The toxicity at high concentration of metformin 2000 µg/ml reported by the present study is disagree with that found by Gholamhosseyni *et al*., (2018) study which revealed that metformin had no significant cytotoxic effects on non-cancerous kidney cells (Hek293) after treated with different concentrations (0.01, 0.1, 1, and 10) mg/ml of metformin, may be due to using different cell line has effect on the result.

4.4-Effect of metformin on the healing of MDCK normal cell line- wound model

The enhancement of wound healing in the wounded MDCK normal cell line reported by the present study agree with Han *et al*.,(2017) study which found that metformin accelerated wound closure, stimulated angiogenesis and improved the function of impaired bone marrow - endothelial precursor cells (BM-EPC) in diabetic mice and increases the production of intracellular nitric oxide(NO) in BM-EPC, because loss of protection from NO due to reduced synthesis from endothelial nitric oxide synthase (eNOS) is the main cause of EPC dysfunction. Thus, as the EPCs are important for the promotion of angiogenesis and the maintenance of vascular homeostasis that can provides regenerating tissue with the required oxygen and nutrients.

Also result of the present study goes with that of Wen *et al*., (2016) in vitro study which showed that metformin improves the impaired function of BM-EPC (caused by high glucose) and increases the expression of the phosphorylated- eNOS and NO production in the cultured BM-EPCs, via AMPK dependent pathway. Metformin activated AMPK in cultured endothelial cells (ECs), AMPK could directly phosphorylate - (eNOS) in

ECs and promote endothelial function. The wound healing enhancement of metformin pointed by the present study could related to improve BM-EPC functions possibly with an AMPK/eNOS dependent pathway.

While the wound healing improvement induced by metformin treatment reported by present study disagree with Gonzalez *et al*.,(2016) study which revealed through in vitro and in vivo experiments that metformin delays the process of wound healing which is independent of the blood glucose levels that can importantly effect proliferation of cells, metformin reduces keratinocytes proliferation, by interfering at least with AMPK and mTOR, altering the cell cycle of keratinocytes, without inducing apoptosis ,thus delayed wound healing as well as tissue remodeling contributing to increased wound size and increasing the time for complete healing of the lesion.

4.5-Effects of metformin plus 250 or 500 µg/ml *Ricinus communis* leaf ethanolic extract combination on the healing of MDCK normal cell line- wound model

The decrease in the diameter but incomplete healing of the MDCK normal cell line-induced wound after it is treatment with the combination of 250 or 500µg/ml of *R. communis* leaf ethanolic extract plus different concentrations of metformin may be due to antagonist effect between these two agents.

Up to knowledge there is no study that is dealing with the combined effect of *R.communis* leaf ethanolic extract plus metformin on the healing of wound to which result of the present study can be compared.

4.6-Effect of phenytoin on the viability of MDCK normal cell line

The increase in the viability of MDCK normal cell line after the treatment with lowest concentration of phenytoin reported by the present study agree with that found by Porojan , *et al* (2012) study which revealed that low concentrations of phenytoin stimulate growth and cellular proliferation in melanocyte culture.

4.7-Effect of phenytoin on the healing of MDCK normal cell line-wound model

The decrease in the diameter of MDCK normal cell line-induced wound found by the present study agree with Hasamnis *et al*., (2010) which revealed that topical phenytoin accelerate healing of excisional wound in albino rats. In a paper published by FDA office of orphan products development (1984), phenytoin was listed as an important agent for wound healing, subject to confirmation by fresh preclinical, and multi centric double blind clinical studies. Topical phenytoin therapy take a shorter time to complete healing and formation of granulation tissue when compared with triple antibiotic ointment application (Baharvand *et al*., 2014).

According to Sivarajah,(2017) study. The enhancement of wound healing by phenytoin may be attributed to its ability to inhibit collagenase which gives it a real role in facilitating the healing of ulcers. Phenytoin is used in hard-to-treat conditions that involve excessive or dysfunctional production of collagen, such as morphea and epidermolysis bullosa. Moreover the topical application of phenytoin decreases bacterial load,

forms healthy granulation tissue and helps in better graft take up then the conventional dressing.

Also this effect of phenytoin may be related to it is ability to stimulation of fibroblastic proliferation, enhancing the formation of granulation tissue, decreasing collagenase activity (by reducing its production or secretion or both), promoting deposition of collagen and other connective tissue components, decreasing bacterial contamination, and by decreasing the formation of wound exudate. As had been suggested by clinical study (Ashima and Surya, 2004).

4.8-Effects of phenytoin plus 250 or 500 µg/ml *Ricinus communis* leaf ethanolic extract combination on the healing of MDCK normal cell line- wound model

The decrease in the diameter and complete healing of the MDCK normal cell line-induced wound after it is treatment with the combination of 250 µg/ml of *R. communis* leaf ethanolic extract plus different concentrations of phenytoin may be due to synergistic effect between these two agents especially phenytoin which had been found to has a good wound healing potential when used even alone to treat excisional wound in albino rats by using 1% phenytoin cream (Hasamnis *et al*., 2010) . Up to knowledge there is no study that is dealing with the combined effect of *R.communis* leaf ethanolic extract plus phenytoin on the healing of wound to which result of the present study can be compared especially the effect high concentrations of phenytoin plus 500 µg/ml of *R.communis* leaf ethanolic extract which showed incomplete wound healing.

Conclusions and Recommendation

Conclusions

1- The wound healing effect of *Ricinus communis* aqueous leaf extract against MDCK cell line is more than that of ethanolic leaf extract of plant.

2- High concentrations of both metformin and phenytoin had a great cytotoxic effect on the MDCK normal cell line.

3-Both low concentrations of metformin and phenytoin have a good wound healing effect on MDCK cell line.

4- *Ricinis communis* leaf ethanolic extract plus metformin is not a good combination for wound healing on the MDCK cell line.

5- *Ricinis communis* leaf ethanolic extract plus phenytoin is a good combination for wound healing on the MDCK cell line.

Recommendation

1- Study the cytotoxic and wound healing activity of different parts of *Ricinus communis* on different types of normal cell lines.

2-Investigate the wound healing effects of aqueous and ethanolic extract of *Ricnus communis*, on animal models-induced wound.

3- Molecular study to the completely healed wound to understand the mechanisms by which the tested agents can accelerate the wound healing.

4-Study the effect of *Ricinus communis* on the inflammatory markers, growth factor, apoptotic and oxidative stress markers.

5- Gene expertness i.e certain genes involved in wound healing and could have correlation with *Ricinus communis* or used drugs.

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الخلاصة

الجرح : هو كل إصابة، مهما كانت بسيطة، تصيب الجسم أو تؤثر بصحته نتيجة عنف خارجي واقع عليه. ويعرف الجرح طبيا بأنه انقطاع استمرارية الجلد وغيره من انسجة الجسم نتيجة للتعرض لشدة خارجية.

التئام الجروح : هي عملية بيولوجية طبيعية في الجسم البشري. وتشمل العملية أربع مراحل مبرمجة بدقة عالية: تخثر الدم (الإرقاء)، والالتهاب، نمو الانسجة (الانتشار)، وإعادة البناء. يمكن أن تتداخل العديد من العوامل مع واحد او اكثر من مراحل هذه العملية. مما يتسبب في التئام الجروح بشكل غير لائق او ضعيف.

هدفت هذه الدراسة الى البحث في تأثير كل من مستخلص اوراق نبات الخروع(*Ricinus communis*) (الايثانولي و المائي) ,متفورمين و الفينيتوين لوحدها و مع بعضها في التئام جرح احدث على الخلايا الظهارية المأخوذة من النبيب الكلوي(MDCK).هذه الدراسة تتضمن جزأين:

الجزء الاول: من اجل تحديد سلامة العوامل المختارة(المستخلص الايثانولي و المائي للنبات, متفورمين والفينيتوين) تم اجراء دراسة استطلاعية للتحقيق في سميتها الخلوية على خط الخلايا الطبيعية (MDCK) وفقا لذلك مجموعة من التراكيز تم استخدامها لفحص التئام الجروح.

لفحص السمية الخلوية خط الخلايا الطبيعية (MDCK) قد تم انباتها في طبق زرعي من 96 مكان زرعي وتمت معاملتها بكل نوع من المستخلص الايثانولي والمائي لأوراق نبات الخروع(*Ricinus communis*) مع مختلف التراكيز(1000 ,250,500 ,125 ,62.5 ,31.25) ميكرو غرام/مليلتر

تم اجراء نفس التجربة ولكن بتراكيز مختلفة من المتفورمين (2000، 1000 ،500 ،250 ،125 ،62.5,31.25,15.6,7.8,3.9) ميكرو غرام/مليلتر او الفينيتوين (2000 ،1000 ،500 ،250 ،125,62.5) ميكرو غرام/مليلتر بعد كل ذلك تم حضن جميع الخلايا المعالجة لمدة 24 ساعة عند 37 درجة مئوية. ثم تم اجراء الفحص باستخدام التحليل اللوني للفعالية الحيوية MTT.

الجزء الثاني: تم اجراء جرح عمودي باستخدام micropipette tip200µl بالضغط بقوة على الطبقة الاحادية للخلية في طبق الزرع النسيجي. وبعد ذلك تم تعريض الخلايا المجروحة لمختلف

التراكيز لكل عامل مفحوص من الجزء الاول لهذه الدراسة . ست تجارب تم اجراؤها على النحو التالي

التجربة الاولى: تم معاملة خط الخلايا الجريحة (MDCK) مع(500، 250، 125 ،62.5 ،31.25 ،15.6) ميكرو غرام/مليلتر للمستخلص الايثانولي لأوراق نبات الخروع.

التجربة الثانية: تم معاملة خط الخلايا الجريحة (MDCK) مع(500، 250 ،125 ،62.5 ،31.25 ،15.6) ميكرو غرام/مليلتر للمستخلص المائي لأوراق نبات الخروع.

التجربة الثالثة: تم معاملة خط الخلايا الجريحة (MDCK) مع(1000، 500 ،250 ،125 ،62.5 ،31.25,15.6,7.8,3.9) ميكرو غرام/مليلتر للمتفورمين

التجربة الرابعة: تم معاملة خط الخلايا الجريحة (MDCK) مع متفورمين بسلسلة تخافيف (1000,500,250,125) ميكرو غرام/مليلتر بالاشتراك مع 250 او 500 ميكرو غرام/مليلتر للمستخلص الايثانولي لأوراق نبات الخروع.

التجربة الخامسة: : تم معاملة خط الخلايا الجريحة (MDCK) مع(62.5، 31.25 ،15.6 ،7.8 ،3.9,1.9) ميكرو غرام/مليلتر للفينيتوين.

التجربة السادسة: : تم معاملة خط الخلايا الجريحة (MDCK) مع فينيتوين بسلسلة تخافيف (62.5، 31.25 ،15.6 ، 7.8، 3.9,1.9) ميكرو غرام/مليلتر بالاشتراك مع 250 او 500 ميكرو غرام/مليلتر للمستخلص الايثانولي لأوراق نبات الخروع.

بعد ذلك تم تصوير منطقة الجرح رقميا كل ثلاث ساعات بدأ من 9 صباحا(0hours)6,3,12 و9 مساءا على التوالي.

بالمقارنة مع الخلايا التي لم تتعرض للعلاج اظهرت نتائج السمية الخلوية للاستخلاص المائي لأوراق نبات الخروع (*Ricinus communis*) بتركيز 125ميكرو غرام/مليلتر زيادة معنويا($p\leq0.05$) في حيوية الخلايا الطبيعية (MDCK) بينما لا يوجد تغير ملحوظ ($p>0.05$) في حيوية الخلايا للمستخلص الكحولي للنبات.

اظهرت النتائج زيادة معنوية($p\leq0.05$) في حيوية الخلايا الطبيعية(MDCK) بعد معاملتها بتركيز 62.5 ميكرو غرام/مليلتر لكل من المتفورمين والفينيتوين بينما التركيز العالي للمتفورمين 2000 ميكرو غرام/مليلتر والفينيتوين (2000,1000,500,250) ميكرو غرام/مليلتر اظهرت حيوية الخلايا انخفاضا كبيرا($p<0.001$).

بالمقارنة مع الخلايا التي لم تتعرض للعلاج اظهرت النتائج ان كل تراكيز الاستخلاص الايثانولي لأوراق نبات الخروع(*Ricinus communis*) ماعدا 500 ميكرو غرام/مليلتر انخفاضا كبيرا ($p<0.001$) في قطر الجرح بينما كل تراكيز الاستخلاص المائي لأوراق نبات الخروع سببت انخفاضا كبيرا ($p<0.001$) في قطر الجرح مع الشفاء التام.

كما اظهرت النتائج أن جميع تراكيزا لمتفورمين ماعدا(15.6، 31.25 ،62.5) ميكرو غرام/مليلتر وجميع تراكيز الفينيتوين ماعدا أدنى واحد 1.9 ميكرو غرام/مليلتر على التوالي سببت انخفاضا كبيرا ($p<0.001$) في قطر الجرح مع الشفاء التام.

فيما يتعلق بالتأثير المشترك لمستخلص الايثانول لأوراق نبات الخروع (*Ricinus communis*) مع المتفورمين أو الفينيتوين اظهرت النتائج أن كلا المجموعتين سببتا انخفاضا كبيرا ($p\leq0.001$) في قطر الجرح. اما التئام الجروح اظهرت النتائج الشفاء الغير التام للجرح الذي تم معالجته مع كل تراكيز المتفورمين مع مستخلص الايثانول لأوراق نبات الخروع ماعدا تركيز 250 ميكرو غرام/مليلتر للنبات مع 125و 250 ميكرو غرام/مليلتر للمتفورمين اظهرت النتائج التئام الجرح كاملا. وكل من تراكيز الفينتوين مع مستخلص الايثانول لأوراق نبات الخروع باستثناء تلك التي تحتوي على 500 ميكرو غرام/مليلتر من المستخلص النباتي مع 62.5 و 31.25 ميكرو غرام/مليلتر للفينتوين سببت التئام الجرح كاملا.

في الختام: مستخلص أوراق نبات الخروع المائي والايثانولي ،المتفورمين والفينتوين تمتلك تاثير الشفاء على خط الخلايا الجريحة (MDCK) افضل من التاثير المشترك ماعدا تاثير فينتوين مع 250ميكروغرام/مليلتر للمستخلص النباتي مما يؤدي الى الشفاء في وقت اسرع.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل

كلية الطب

تأثير نبات الخروع, والفينيتوين والمتفورمين ومزيجهم على شفاء الجروح. دراسة مختبرية.

رسالة

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كجزء من متطلبات نيل درجة الماجستير في الادوية / الادوية والسموم

من قبل

دنيا عبد الحسين شاكر ناجي

(بكالوريوس صيدلة،2015)

اشراف

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