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INVESTIGATION OF THE THERAPEUTIC EFFECTS OF *L-DOPA* AND *LAWSONE* IN WOUND HEALING

ELİF KESKİN

MSc THESIS

BIOMEDICAL ENGINEERING PROGRAMME

İSTANBUL, JANUARY / 2016 (DEFENSE)

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T.C.
FATİH ÜNİVESİTESİ
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L-DOPA VE *LAWSONE* BİYOMALZEMELERİNİN YARA İYİLEŞMESİ ÜZERİNE TEDAVİ EDİCİ ETKİSİNİN ARAŞTIRILMASI

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İSTANBUL, OCAK / 2016 (SAVUNMA)

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INSTITUTE OF BIOMEDICAL ENGINEERING

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ABREVIATIONS

L-dopa : L-3, 4-Dihydroxyphenylalanine

NIH3T3 : Mouse Embryonic Fibroblast Cell Line

MAP : Mussel Adhesive Protein

LB : Luria Broth (Miller)

WST-1 : Cell Proliferation Reagent

DMEM : Dulbecco's Modified Eagle Medium

CFU : Colony Forming Unit

FBS : Fetal Bovine Serum

ECM : Extracellular Matrix

FGF : Fibroblast Growth Factor

EGF : Epidermal Groth Factor

TFG-b : Transforming Growth Factor Beta

DMSO : Dimethyl Sulphate

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SUMMARY

INVESTIGATION OF THE THERAPEUTIC EFFECTS OF *L-DOPA* AND *LAWSONE* IN WOUND HEALING

ELIF KESKIN

Biomedical Engineering Programme

MSc Thesis

Advisor: Asst. Prof. Dr. Aydin ALBAYRAK

Wounds are known as injury or distruption of any type of tissue in the body and can be treated in many different ways. Currently, gauze, adhesive and non-adhesive fixation materials, and traditional wound dressings are the most commonly used methods for wound treatment. In recent years, biological wound fillers and dressings have also emerged as alternatives to traditional wound treatment materials by providing accelerated wound healing and reduced scar formation. However, an effective cure for most chronic wounds remains to be found. Especially, the chronic wounds caused by systemic diseases such as diabetes, cardiovascular diseases, and obesity are not only painful for patients but also financially and psychologically troublesome for caregivers. In addition, a significant portion of the budget that is allocated to health care goes to the

wound treatment. In this thesis, therapeutic effects of two different biomaterials namely *L-dopa* and *Lawsone* were investigated for their effects to accelerate the wound healing process and find a solution for non-healing or slowly-healed wounds by providing protection against infection. *L-dopa* is a catecholic amino acid that is part of the Mussel Adhesive Protein from marine mussel which can adhere strongly to non-specific surfaces in aqueous medium. *L-dopa* was used with polymeric materials in many surgeries such as tendon repair surgery, and placental laceration. *Lawsone* is a naphtaquinone derivative extracted from *Lawsonia Inermis* plant. Antimicrobial susceptibility testing, cell proliferation & cytotoxicity assay (WST-1) and migration assay or wound healing assay have been carried out as methods. Our results indicate that when L-dopa and Lawsone are used together as wound dressings on *in vitro* wound models, the duo show considerable therapeutic potential by accelerating the wound healing process and providing antimicrobial properties simultaneously.

Keywords: Wound Healing, Wound Dressing, Antimicrobial, Bioadhesive, Lawsone, L-Dopa.

ÖZET

TEZ BAŞLIĞI
L-DOPA VE *LAWSONE* BİYOMALZEMELERİNİN YARA İYİLEŞMESI ÜZERİNE TEDAVİ EDİCI ETKİSİNİN ARAŞTIRILMASI

ELİF KESKİN

Biyomedikal Mühendisliği Programı

Yüksek Lisans Tezi

Danışman: Yrd. Doc. Dr. Aydın ALBAYRAK

Doku hasarlanması yada tahribi olarak bilinen yara oluşumu, çok farklı şekillerde tedavi edilmeye çalışılmıştır. Günümüzde yara tedavisinde, gazlı bez, yapışkanlı-yapışkansız fiksasyon malzemeleri ve diğer geleneksel yara pansumanları halen en çok kullanılan yöntemlerdendir. Geleneksel yara örtülerine alternatif olarak gelişen teknoloji ile birlikte yara iyileşmesini hızlandırıcı biyolojik yara dolgu ve pansumalar geliştirilmiştir. Fakat halen kronik yaralar için daha etkili yara tedavi şekli ortaya konulamamıştır. Özellikle sistemik rahatsızlıkları (diyabet, obesite, kardiovaskular hastalıklar) olan hastaların yaraları kronikleşerek, hem hastaya acı hem yara bakımı yapan görevlilere

zahmet vermektedir. Ayrıca sağlık hizmetleri için ayrılan bütçenin önemli bir kısmı bu hastaların bakımı için ayrılmakta ve ülke ekonomisine giderek artan bir yük oluşturmaktadır. Bu tezde yara iyileşmesini hızlandırabilmek ve enfeksiyonlara karşı koruma sağlamak amacıyla, iki farklı biyomalzemenin *L-dopa ve Lawsone* tedavi edici özellikleri araştırıldı. L-dopa, midyenin salgısında bulunan, sulu ortamda güçlü bir şekilde biyolojik ve biyolojik olmayan birçok yüzeye yapışabilen bir tür katokolik amino asittir. Polimerik malzemeler ile birlikte, çeşitli cerrahi operasyonlarda örneğin; tendon onarımı, dikişsiz yara kapanması ve plasenta yırtılmaları tedavisinde kullanılmıştır. Lawsone ise *Lawsonia İnermis* bitkisinden çıkartılan, antimikrobiyal etkisi olan bir çeşit naftakuinondur. Metot olarak antimikrobiyal duyarlılık testleri, hücre çoğalma, sitotoksisite (WST-1) ve hücre göçü (*in vitro* yara modellerinde) tahlili kullanılmıştır. Bu çalışmada L-dopa ve Lawsone *in vitro* yara modelleri üzerinde beraber kullanıldığında, ortaya çıkan ikilinin yara iyileşmesini hızlandırıcı ve antimikrobiyal özelliğe sahip yeni nesil bir yara örtüsü olarak kullanılabileceği gösterilmiştir.

Anahtar kelimeler: Yara İyileşmesi, Yara Örtüsü, Biyoyapıştırıcı, Antimikrobiyal, Lawsone , L-Dopa.

CHAPTER 1

INTRODUCTION

1.1 Background

For many years, treatment ways of wound depending on natural sources have been getting interest due to the properties of the natural materials. Although currently, traditional wound dressings such as guaze and cotton packing materials are mostly used in clinical settings, they are not able to meet the needs for ideal wound healing. Alternatively, polymer wound dressings such as transparent film dressings, hydrocolloids, hydrogels, calsium alginates, and foams can be utilized to cure the wounds. There are also other types of gels and creams applied on wound surface in order to speed up healing. Wound treatment is also big concern for wound care sectors. Market size of wound dressings are tought to be around $18 millons up to 2020. Estimated total expenses for curing of wounds about 2-3 % total budget of health care system in European Community and USA[1]Especially chronic wounds increases the health care cost due to the slowly healing or failed heal. Seeking of solutions for these kind of wounds will improve mankind health and significantly decrease wound care cost.

1.2 Problem formulation

Wound healing is a natural biological process which has four main programmed phases: hemostasis, inflammation, proliferation, and remodeling. These phases must occur in appropriate order and time for a wound to heal successfully. Many factors can interfere one or more phases of this biological process resulting in impaired wound healing[11].

Infection is the most important factor that interferes with the healing steps. Bacterial, viral, fungal, and other microorganismal invasions prolong the inflammation phase. Hence, prevention of the microorganismal infections is crucial during wound treatment. Another significant problem is the insufficient and slow cell migration from the surrounding tissue toward the wound closure in the course of healing.

In this thesis, we investigated the potential effects of applying two novel biomaterials, *L-dopa* and *Lawsone* in preventing infection, promoting wound healing and increasing the rate of cell migration towards wound closure. *Lawsone*, a component of *Lawsonia Inermis* plant extract, has antimicrobial activity on numerous bacteria such as *Enterococcus faecium* and *Klebsiella pneumoniae* and fungi [2] *L-dopa* is an catechol amino acid of Marine Mussel Foot Protein that provide adhesion on various surfaces [29].

1.3 Aim of study

In this study, therapeutic effect of novel biomaterials namely Lawsone (obtained from Lawsonia Inermis Plant) and L-dopa Mussel Adhesive Protein Mimetic) have been investigated in order to estimate their relevance as wound care devices such as dressing and surgical bioadhesive for wound therapy. Future target is to develop a bioadhesive enriched with L-dopa and Lawsone which will have antimicrobial, biodegradable, biocompatible, waterproof and tissue constrictive properties due to the presence of these two molecules.

In this thesis, biological properties of L-dopa and Lawsone were evaluated on mouse fibroblasts as *in-vitro* wound models by using migration, cytotoxicity, and proliferation assays. Additionally, antimicrobial activity of Lawsone and L-dopa were also tested on *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeroginosa*.

CHAPTER 2

2.1 The skin structure

The skin is an exceptional organ that makes life possible by maintaining heat and water balance and providing a physical barrier to hazardous chemicals and microorganisms. It is also the largest organ of human body accounting for 15% of body weight of average person [4] Human integumentary system consists of the skin and its derivattives. Skin structure is formed by three layers from top to bottom: epidermis, dermis, and subcutaneous tissue (Figure 2.1).

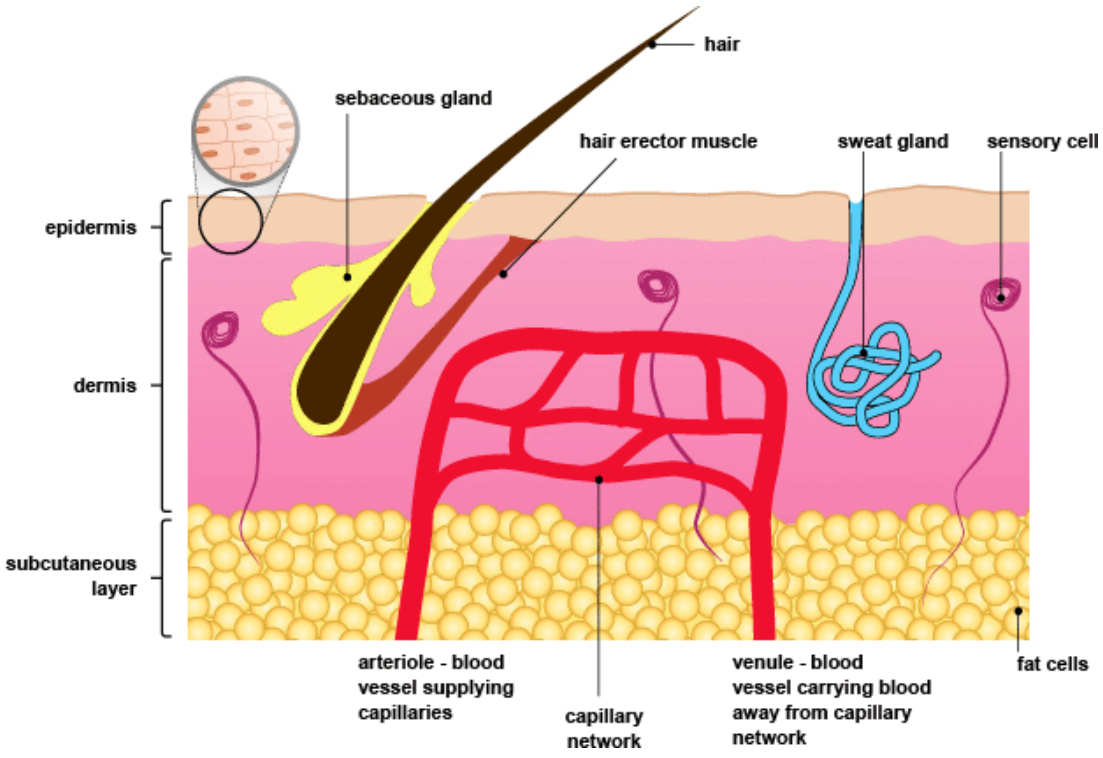


Figure 2. Human skin structure[30]

Epidermis is on the outer stratum. It has basal layer that always generate new cells by cell division. Epidermis consists of cell layers. Newly formed cells go through the surface in 1-2 months. After they move up, they gradually die and result in flattened keratinized cells. Therefore the outermost layer of epidermis is made up of dead cell that consistently come off by friction. Epidermis has also melanocytes, langerhans cells,

and merkel cells [4] Epidermis is renewed layer and forms derivatives structures such as nail and sweat glands.

The middle layer of the skin is dermis which includes supportive, elastic connective tissue protecting epidermis. It consists of fibroblasts cells, collagen, elastic fibers, capillaries, hair erector muscles, sensory cells, nerve fibers, pigment cells, sweat glands, hair follicles. The connective tissue holds the other structure in the skin. Fibroblasts are the main cells which produce extra cellular matrix (ECM) and collagen structure. Also fibroblasts take on critical roles during tissue repair by changing their morphology from normal quiescent state to proliferative and contractile myofibroblasts. Elastic fibers and collagens give elasticity and stretchy properties to skin. Sensory neurons are involved in sensation such as touch, pressure, heat, cold, and pain. Dermis components are continously recycled through degradation and synthesis [4]

The innermost part of the skin is the subcutenous tissue or panniculus which contains fat cells known as lipocytes. Thickness of this layer varies according to person and place in the body. Excess fat is stored in this layer which provides insulation to the body. The thickness of these three layers varies depending of location on the body. For instance, the thickness of epidermis ranges from 0.1 mm in eyelids to 1.5 mm in palms and soles and the dermis is thickest in the back [4].

2.2 Wounds

Physical disruption or injury in any kind of body tissue caused external damages is defined wounds. The restoration of injured tissue is called healing [5].Wound healing is a naturally process that occurs to repair the damaged tissue. When the tissue is injured seriously, body is not able to recover the wounds. Death cells and foreign materials should be removed and infection must be cured. Also injured tissues should be held together until it reaches the enough strength to withstand stress without mechanical support [5].To identify correct treatment in order for carefully assess the wounds is crucial issue. For this reason etiology of wounds are needed to be considered [25]. Wounds are classified as chronic and acute depending on the its healing time. Whereas acute wounds heal smoothly in expected time frame. Chronic wounds do not heal

properly in standard time interval and they may get longer for a week or month sometimes a year [25].

2.2.1 Acute wounds

Acute wounds usually heal in an expected time frame. The healing process includes homeostasis, infilammation, proliferation-reepithelization and remodeling. Traumatic and surgical wounds could be given as examples [25].

2.2.2 Chronic wounds

Chronic wounds are caused by disruption of normal healing process [7].They do not proceed in wound healing as acute wounds do. Pathophysiology of chronic wounds is not fully understood but there are significant changes in the amount of key molecules during healing. It has been indicated that the level of pro-inflammatory cytokines decreases in chronic wounds [7].Moreover, proliferation of cells which promote formation of new matrix in acute wounds is inhibited in chronic wounds [7].Venous leg ulcer, arterial leg ulcer, and neuropathic or diabetic ulcer are examples of chronic wounds [25].Chronic wounds can be caused by bacterial biofilms. Biofilmespeciallyoccurs in individual with underlying of pathology.

2.3 Wound healing

Wound healing is a dynamic biological process which is carried out through several sophisticated interactions at the molecular level [7].The physiology of biological healing process goes through the following phases ; hemostasis, inflammatory phase, proliferative phase, maturation phase(remodeling). This physiological framework provides knowledge about the attempts required for wound care especially complex task of tissue repair [8].

2.3.1 Healing phases

Hemostasis: Hemostasis is the first step of healing process begins immediately upon wounding. It is kind of body's natural defense against bleeding which is controlled via sealing the local blood vessels with platelets [9]. Circulating platelets that act as a

physical plug also release vasoconstrictive substances to reduce bleeding. This temporary plug of platelets blocks the damaged vessels and is replaced later with more stable fibrin cloth [9]. If there is no underlying clotting problem, hemostasis takes place in minutes at the beginning of injury [8].

Inflammatory phase: Inflammation is commonly known as clean up phase (Figure 2.2). Neutrophils and macrophages move through the wound area. Dead cells, tissue debris and microorganisms are phagocytosed by these cells. Neutrophils release and include high level of disruptive proteases and oxygen free radicals in order to ingest engulfed materials [10].Neutrophils also secrete inflammatory factors like tumor necrosis alpha (TNF-a) and interleukin-1(IL-1) that activate fibroblasts and epithelial cells. After two or three days neutrophils are destroyed in wound area and are replaced by macrophages [10]. Macrophages clear the wound area by phagocytosing bacteria,, damaged tissue, and depleted neutrophils [10]. Macrophages also release the chemotactic and growth factors like fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factor beta (TFG-b) and interleukin-1 (IL-1) [8].

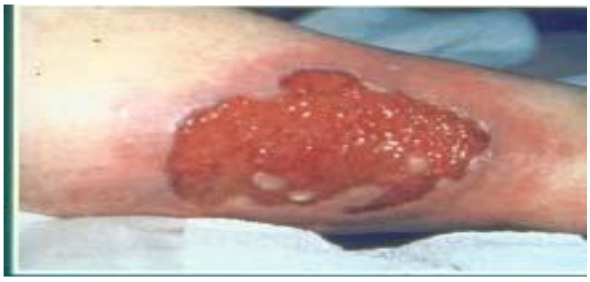


Figure 2.2 Inflammatory phase [9]

Proliferative phase: Proliferation is referred to new tissue growth depending on the depth of injury. It consists of granulation (Figure 2.3) and epithelization (Figure 2.4). As epithelial tissue is destroyed, dermal cells (fibroblasts) come up in response to cytokines and growth factor released from platelets at the beginning to carry on the healing. Fibroblasts form collagen matrix in wound area is known as granulation tissue. Collagens are proteins which contribute the connective tissue structure. Production of collagen fibers determine the tensile strength and elasticity of healing wound [5]. Newly formed granulation is filled with new blood vessels (angiogenesis) to carry nutrients and oxygen. This leads to a bright and reddish appearance on the wound site. Granulation

phase begins in 12-48 hours following tissue damage. Duration of granulations depends on the thickness of the wound. Granulation is known as scar tissue due to the vascularization, different appearance, and texture of newly formed tissue [11].

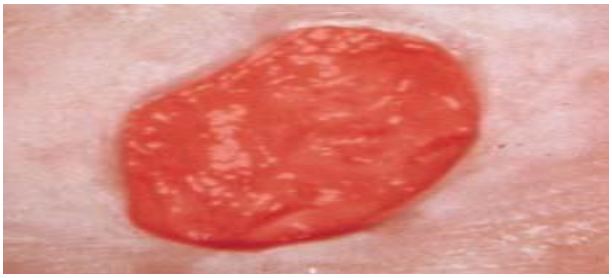


Figure 2.3. Granulation [9]

After granulation, *reepithelization* (regeneration of epithelial cells) occurs on the surface of the wound by growth and differentiation of epidermal cells, *keratinocytes* (Figure 2.4). The edges of wound undergo the contractions which are mediated by myofibroblasts (contractile fibroblast) [9].

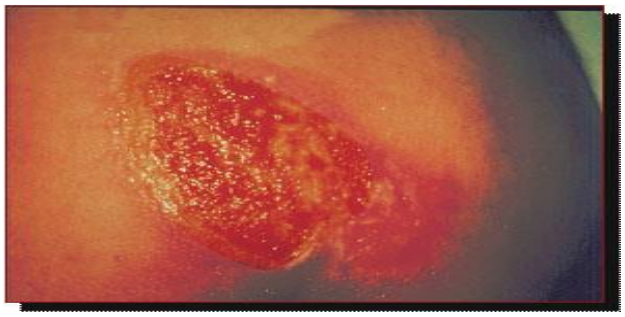


Figure 2.4 *Reepithelization* [9]

Maturation phase: Maturation phase is also known as remodeling or rebuilding stage. It may take up to 2 years to finish re-modelling. Newly formed or scar tissue is readjusted to be more long-lived according to its internal structure [9]. In this phase it is extensively brittle that can easily be damaged. Healed wound can restore upto 80% of the original strength of the skin [9]. During this stage, production and degradation of collagens lead to an equilibrium. Fibroblasts take vital role in organizing and crosslinking collagens in order to provide sufficient strength to wound. When durability of wound increase, fibroblast density decreases [8]

2.3.2 Factors affecting wound healing

Several factors impact the wound healing resulting in impaired wound. Factors that affect the healing are grouped into local and systemic (Table 2.1). Systemic factors influence the ability of individual to heal normally due to health or disease whereas local factors impair the characteristics of wound itself.

Table 2.1.Factors effecting Wound Healing [11]

Local Factors	**Systemic Factors**
Age and gender	Oxygenation
Sex hormones	Infection
Stress	Foreign body
Ischema	Venous sufficiency
Diseases: Diabetes, keloids, fibrosis, healing disorders, jaundice, uremia	
Medications: glucocorticoid steroids, non-steroidal, anti-inflammatory drugs, chemotherapy	
Obesity	
Alcoholism and smoking	
Cancer,radiation therapy, AIDS	
Nutrition	

2.4 Wound dressing materials

Wound dressings come into prominence taking into account of factors which affect the wound healing phases in wound care. Wound dressings cover the wound surface and accelerate wound healing. Wound dressings exert their wound healing effects by filling wound cavity, providing and maintaining a moist-micro environment around the wound surface and physically blocking the microbial organisms to infect the damaged tissue.

Wound dressings usually contain three layers: primary dressing which directly contacts the actual wound surface; secondary dressing that secures primary dressing in place;

and tertiary layer holds the dressing on skin surface with adhesive backing [12]. Commonly-used dressing types are polymer material dressings such as transparent film dressing, hydrocolloids, hydrogels, calcium alginates, and foams [12]. Transparent film dressings are mostly polyurethane membranes with semipermable adhesive backing that are suitable for dry skin [13]. Hydrocolloid dressings consist of pectin and carboxymethylcellulose with self-adhering properties [14]. They are useful for low to moderately leaking wounds but not for infected wounds [15]. Hydrogels dressings are natural humectants that cleanse the wound surface, accelerate the healing of the necrotic tissue and can be used to treat chronic wounds.

2.5 Biomaterials in wound therapy

Biomaterials are defined as substances which have been designed to perform a task alone or as part of the complex system in contact with living tissue for therapeutic or diagnostic purposes [16]. When biomaterials come in contact with living tissue, they should not harm it. They must be biocompatible, biodegradable, non-toxic, non-carcinogenic, non-inflammatory, inert as well as having proper mechanical features. There are four categories of biomaterials: metal, polymer, ceramic and natural. Polymeric biomaterials are usually used for drug delivery, tissue engineering and wound care. Biomaterials in wound dressings are generally hydrocolloids, alginates, collagens, chitosan, pectin and hydrogels. Polymeric wound dressings should exhibit biocompatibility, provide suitable moist environment and protect wound from microorganisms and dust [17].

2.6 Lawsone

Lawsone is obtained from dried and powdered leaves of *Lawsonia Inermis* plant. The extract of *Lawsonia Inermis* consists of tannic acid, resin composites, mannite acid derivatives, and lawsone (2-hydroxy-1, 4-naphtoquinone) [6] (Figure 2.5).

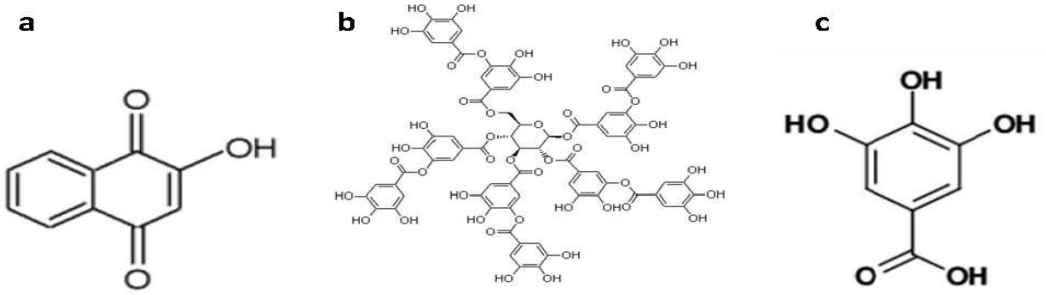


Figure 2.5. a) Lawsone b) Tannin c) Gallic acid

Lawsone is the main bioactive molecule of *Lawsonia Inermis* due to its high protein binding capacity [6]. It is also a member of naphtoquinones. Many naphtoquinones have pharmacological characteristics due to being functional components of diverse biochemical systems [18]. Naphtoquinones have wide-range of biological activities and effects such as antioxidant, antibacterial, antiinflammatory, antifungal, tissue-constricting, cooling, and photo sensitizing [23, 20, 21]. A list of earlier studies has revealed the following biological effects of *Lawsone* albeit at high concentrations.

Antibacterial: *Lawsone* is effective on various gram positive and gram negative bacteria such as *Bacillus subtilis, Proteus mirabilis* and *Escherichia coli* [23]

Antifungal: *Lawsone* has preventive effects on reproduction of almost all dermatophytes which can survive in human skin [22].

Wound constriction: *Lawsone* shrinks the tissues in necrotic and cut wounds and increase tissue granulation, collagen organization, and fibroblast cells [19].

Photosensitizer: *Lawsone* can be used as a photo sensitizer [24].

Cooling effect: *Lawsone* is used as a cooling agent [6].

2.7 Mussel adhesive protein (MAP)

Marine mussels have been frequently studied organisms in medicine and industry due to having natural bioadhesives obtained from their secretions. These bioadhesives are much stronger than chemically synthesized adhesives and can bind nonspecific surfaces in aqueous solutions. The adhesive and cohesive properties of mussel adhesive proteins (MAPs) are linked to the presence of L-3,4-dihydroxyphenylalanine (DOPA), a catecholic amino acid that is formed by modification of tyrosine (Figure 2.6). The catechol side chain of *L-dopa* is oxidized under basic conditions in the presence of oxygen. It also goes under oxidation reactions in the presence of enzymatic and chemical oxidants. Oxidation of *L-dopa* results in the formation of highly reactive quinone that can polymerize, an essential step for adhesive properties [28]. The quinone molecule also shows strong adhesion on biological surfaces by forming covalent bonds [25]. L-dopa has been currently used for coating biological scaffolds and meshes due to its bioadhesive properties. Polymeric bioadhesives enriched with *L-dopa* have been shown to treat damaged fetal membrane [21].

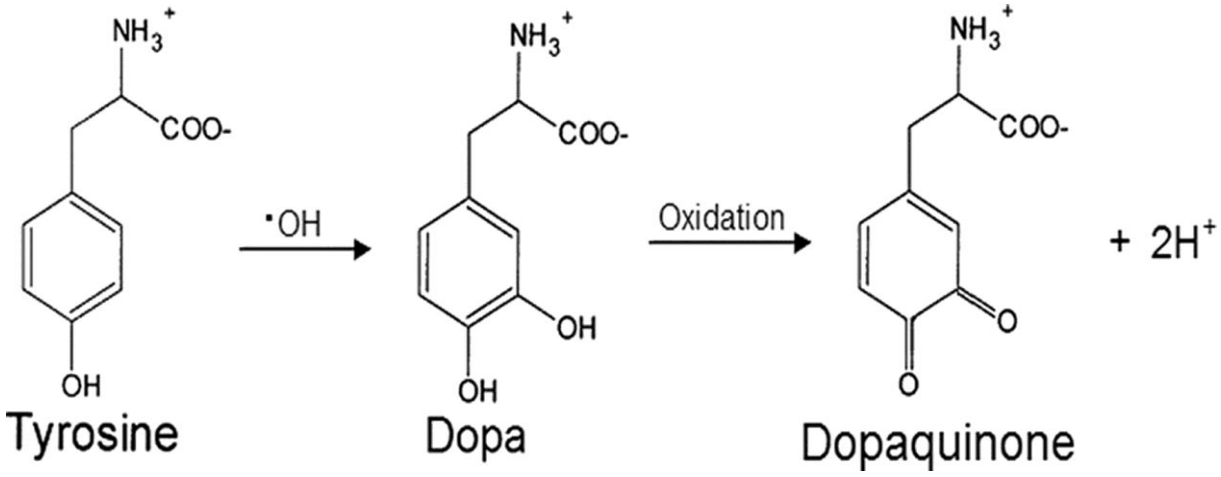


Figure 2.6 Biosynthesis of *L-dopa* from *L-tyrosine*

CHAPTER 3

Materials and method

Microorganisms

Bacteria were used for antimicrobial activity study. *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeroginosa* were obtained from American Type Cell Collection (ATCC). NIH3T3 fibroblast cells were provided by Fatih University Biology Department.

Chemicals

LB (Luria broth), Lawsone, L-dopa, Trypan blue and WST-1 kit were obtained from Sigma Aldrich. Cell culture media DMEM (4.5 g/glucose w/l-Gln), DPBS (1x w/ Ca++& Mg++), Trypsine-Versene (EDTA), Penicillin-streptomycine (5K/5K) were received from Lonza. FBS was bought from Invitrogen. Culture insert plates were obtained from İbidi

3.1 Optimization of Lawsone and L-dopa concentrations

Lawsone was obtained from Sigma Aldrich in powder form dissolved in three different solvents at a concentration of 1000 µg/ml: H_20, DMSO (5 %) and LB. Whereas it did not dissolve in H_20, it was soluble in DMSO but after a day in room temperatureit formed crystals. The best result has been achieved when LB was used as a solvent. Ranging from 25 µg/ml to 600 µg/ml of Lawsone was tested. Finally effective dosage of Lawsone has been accomplished in 300-400 µg/ml. Similarly, L-dopa was in powder form and dissolved in H_20 and cell culture media (DMEM). L-dopa was completely dissolved in DMEM but not in H_20. The main stock of L-dopa 1000 µg/ml was prepared. Relevant concentrations has been searched from the litreture and 1 Mm, 0.5 Mm and 0.25 Mm have been tested in cell culture. Main stocks of Lawsone and L-dopa dissolved in LB and DMEM respectively and results have been shown below;

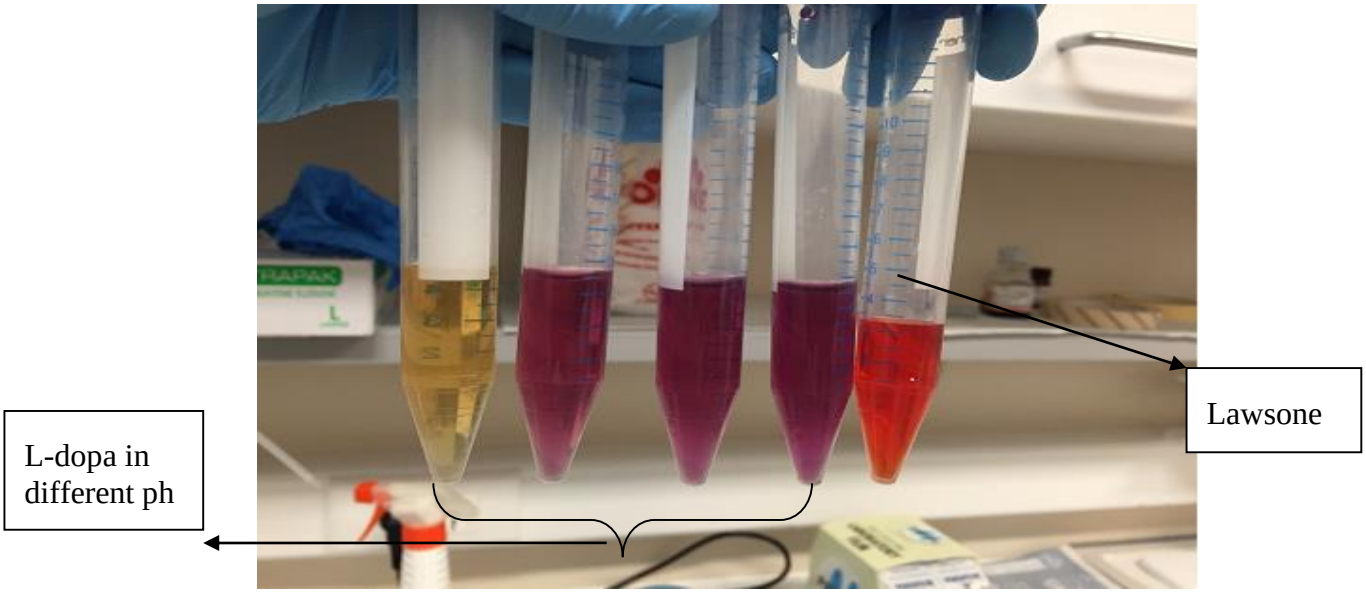


Figure 3.1 Main stocks of L-dopa and Lawsone

3.2 Antimicrobial susceptibility testing

Antimicrobial effects of Lawsone were tested on *Staphylococcus aureus* and *Staphylococcus epidermidis* (both gram positive) and *Pseudomonas aeroginosa* (a gram negative bacteria). The chosen bacteria are commonly known to colonize in many types of wounds. Bacterial strains were inoculated into LB adjusting their cells density with UV-visible spectrophotometer in order to get final concentration about 10^8 colony forming unit(CFU)(optical density OD_{600}=0.08-0.1) [23]. Main stock of Lawsone (1000 µg/ml) was prepared via dissolving powder Lawsone in LB in 70^{o}C for 15 min. Three different concentrations of Lawsone have been tried (200 µg/ml, 300 µg/ml and 400 µg/ml) to find the Minimal Inhibitory Concentration (MIC_{90}) at least 90% of bacteria. 100 µl of an inoculum containing 10^6CFU bacteria and dissolved Lawsone according to determined concentration (200µg/ml, 300µg/ml, 400µg/ml) were added into 50 ml erlenmeyer flask completing final volume of 5 ml with pure LB. Three concentrations of Lawsone and two control groups (negative and positive groups) were studied to observe the antimicrobial activity of Lawsone on three types of bacteria (Table 2.2). The negative control contains only bacteria and LB. The positive control has10^6 CFU of bacteria, LB pure media and 200 µl Penicilline- streptomycine (5000 U Penicilline and 5000 U streptomycine).

Table 3.1. Bacteria and Experimental samples

Staphylococcus aureus	*Staphylococcus epidermidis*	*Pseudomonas aeroginosa*
400 µg/ml	400 µg/ml	400 µg/ml
300 µg/ml	300 µg/ml	300 µg/ml
200 µg/ml	200 µg/ml	200 µg/ml
Negative contol	Negative contol	Negative contol
Positive control	Positive control	Positive control

After incubation of all flasks in shaker incubator for 21 hours in 37^{o} C via 200 rpm, 100 µl samples were taken from each flask into 96 well plates with blanks (Figure 3.2) All samples were screened in ELISA microplate reader. Optical density was measured at 600 nm for bacteria and % inhibition (I) is obtain from the relation [26].

X = Optical density of bacteria in negative control set

Y= Optical density of bacteria in test set I= Inhibition

$$I = \frac{(X - Y)}{X} \times 100$$

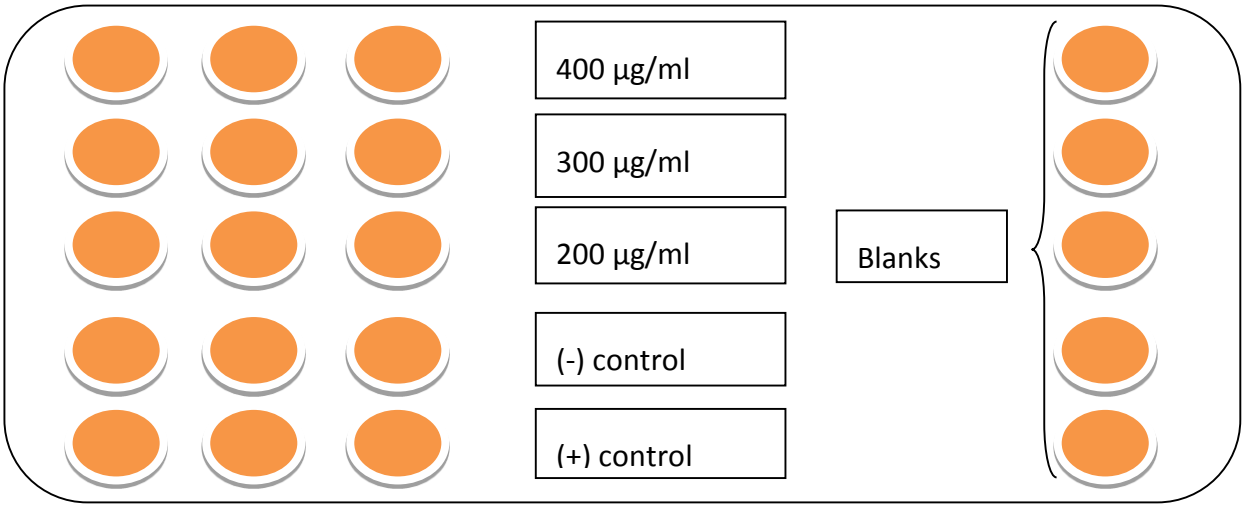


Figure 3.2 Samples arrangement in 96 well plate

3.3 Proliferation & Cytotoxicity Assay

The cell counting kit WST-1 is a colorimetric assay for determination of viable cells number. It is used for proliferation assay and as well as cytotoxicity assay. It is a spectrophotometric quantification of cell proliferation and viability in cell culture using 96-well plate format. 10.000 cells of NIH3T3 were seeded into 96-well plates which contained 100 µl total volumes in each well including DMEM with penicillin-streptomycine and FBS. They were incubated in 5% CO_2 incubator , 37^oC . After 24 hours, media was removed and pre-deteremined concentrations (400µg/ml, 350µg/ml, 300 µg/ml) of Lawsone, DMEM with 10% FBS were put into each well as triple for each concentration of Lawsone. As positive control group, fresh media was added. After 24 hours incubation, 10 µl WST-1 was inserted into each well and absorbance values were measured under 450 nm with ELISA reader. According to absorbance values, cell survival rate has been calculated with following formula (Protocol from Sigma A.);

$$Survival\ rate\ (\%) = \frac{Asample - Ablank}{Aconrol - A\ blank}$$

Same process has been done for L-dopa in different concentrations (50 µg/ml and 25 µg/ml) with different ph values; ph 5.5, ph 7.4, ph 8.0, ph 8.5. After seeding and incubating 10^4 cells of NIH3T3 into 96 well plates for 24 hours in 37^oC with 5% CO_2, media was discarded and 100 µl suspension was added containing DMEM(with 2% penicillin-streptomycin), 10% FBS, and L-DOPA with above ph values. For all concentrations and ph values, three wells were used (Figure 3.3). After cells were treated with L-dopa in distinct ph values for 24 hours, 10 µl WST-1 kit was put into each well. After one hour, absorbance values have been recorded and then applied on same formula as above to find the survival rate.

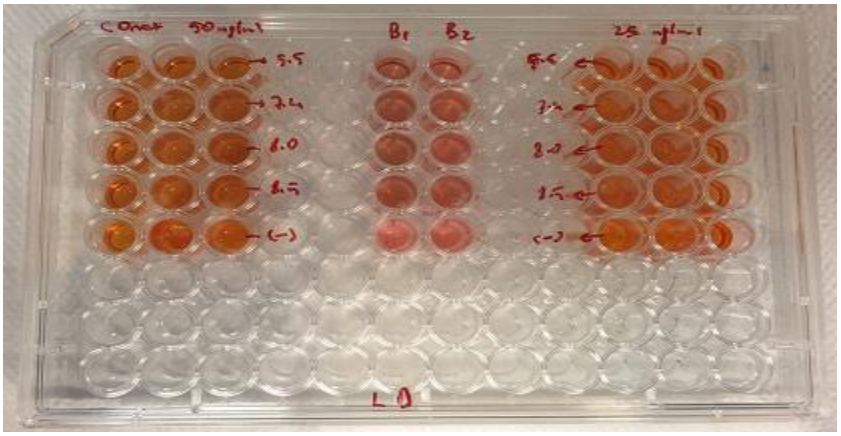


Figure 3.3 96 well plate design for L-dopa different ph and concentration.

3.4 Migration assay

NIH3T3 fibrobast cells was cultured in 25 cm^2 flasks with DMEM (with 2% penicillin-streptomycin) and 10% FBS in 5% CO_2 incubator in 37ºC in order to get enough number of cells. 35.10^3 cells of NIH3T3 (70 µl) were seeded into wells in culture-insert plates as cell suspension containing DMEM and 10% FBS. After incubation in 5% CO_2 incubator in 37ºC for 24 hours for attachment of cells on the plate, silicon wells were removed by the tweezer (Figure 3.4). 2 ml in total volume containing testing agents (400 µg/ ml Lawsone and 25µg/ml L-Dopa), DMEM and 10% FBS were added into culture-insert plates. One control group containing only DMEM and FBS was utilized. The images were captured and gap closures were measured by the Leica inverted microscope camera every 6 hours.

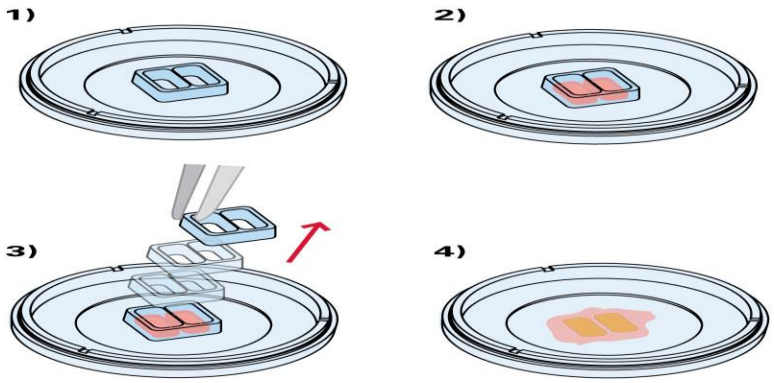


Figure 3.4 Culture-insert plates (ibidi)

1-Empty wells and plate, 2-Seeding cells into the wells, 3-After 24 hours, allowing cell attachment, wells are removed, 4- free media or agents are inserted.

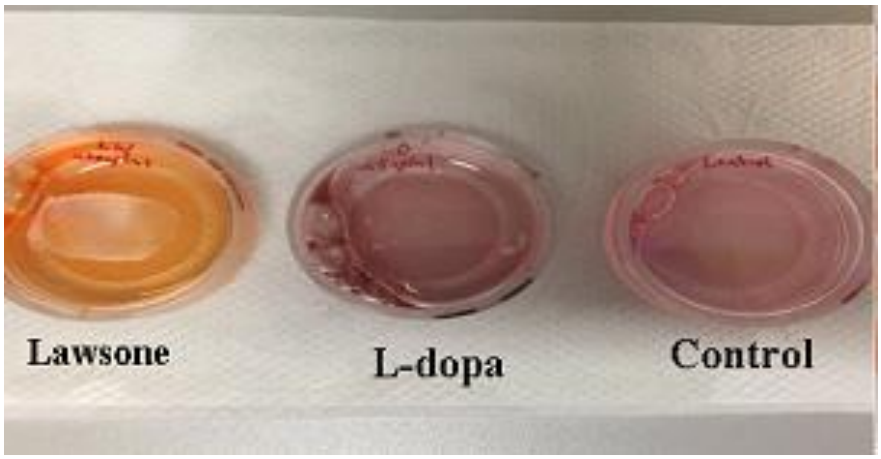


Figure 3.5 Treatment of L-dopa and Lawsone in wound healing assay.

CHAPTER 4

RESULTS

4.1 Antimicrobial Susceptibility Testing

The means of absorbance values of samples containing three different concentration of Lawsone and bacterias namely *Staphylococcus aureus*, *Staphlylococcus epidermidis*, *Pseudomonas auriginosa* were used to calculate the percentage of inhibition by the formula below.

$$I = \frac{(X - Y)}{X} \times 100$$

Antimicrobial effect of Lawsone on *Staphyloccocus aureus* has been shown as percentage (%) in the graph (Figure 4.1). The highest inhibition result was reached with 400µg/ml Lawsone for *Staphyloccocus aureus.*

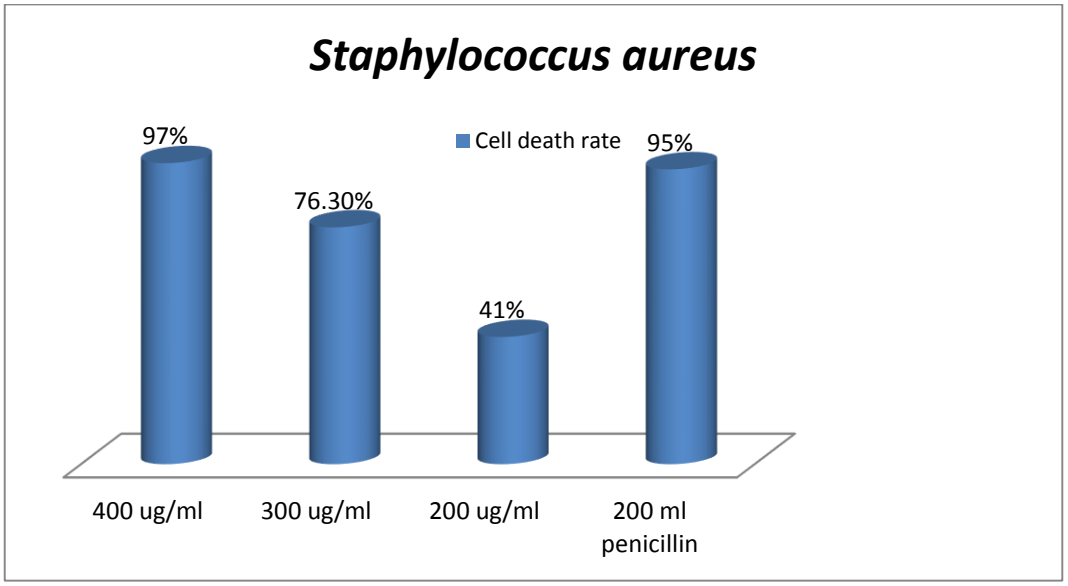


Figure 4.1 Cell death rate of *Staphylococcus aureus*

Antimicrobial activity of different concentration of Lawsone on *Staphylococcus epidermidis* has been indicated in (Figure 4.2) Lawsone has showed more inhibitory

effect on *Staphylococcus epidermidis* than others**.** The best inhibitive concentration of Lawsone was 400µg/ml on this bacteria.

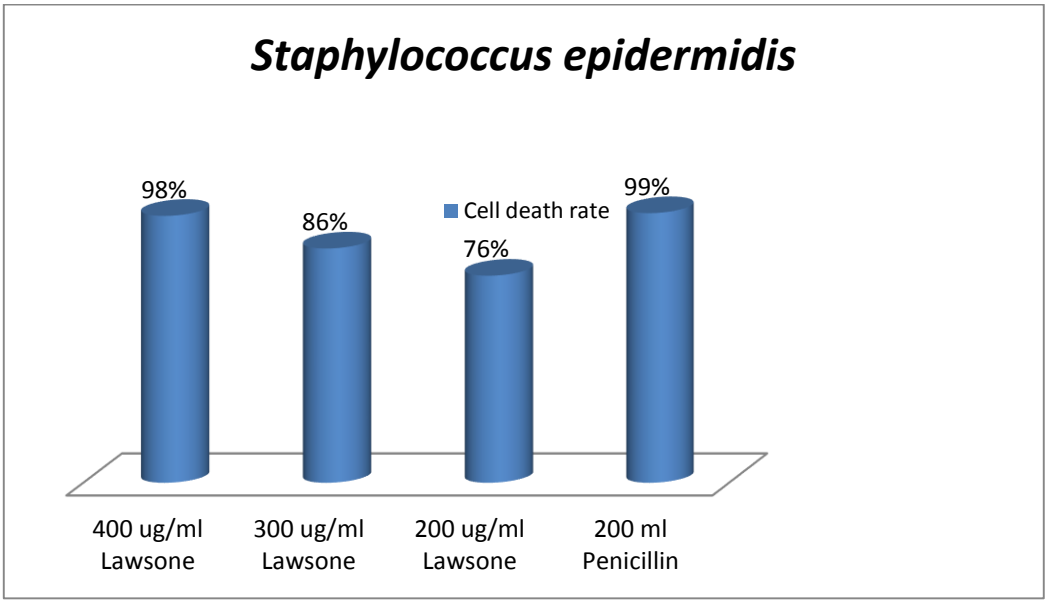


Figure 4.2 Cell death rate of *Staphylococcus epidermidis*

Antimicrobial activity of different concentrations of Lawsone on *Pseudomonas aeruginosa* was demonstrated in Figure 4.3.

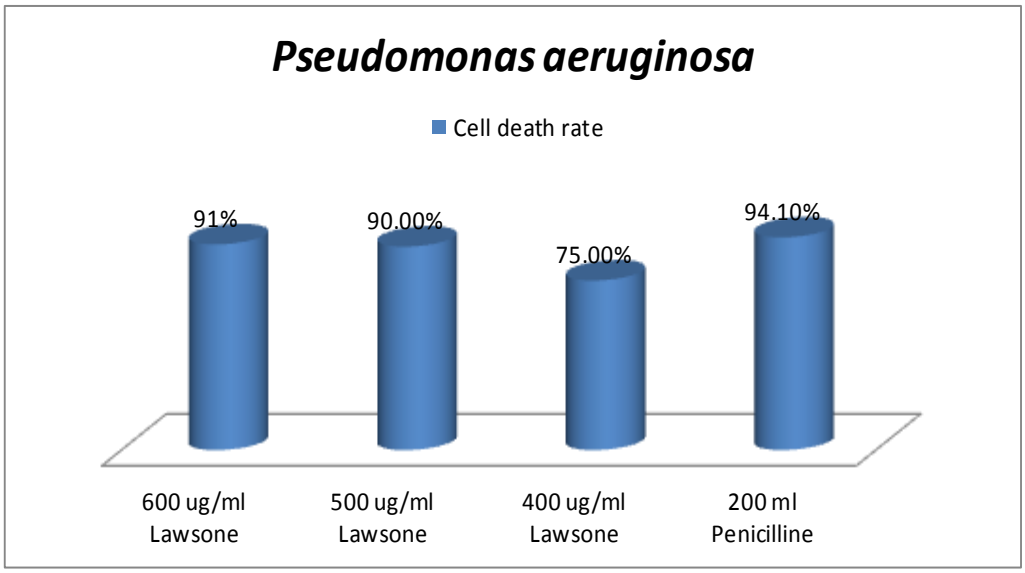


Figure 4.3 Cell death rate of *Pseudomonas aeruginosa*

Lawsone exhibited slightly less action on *Pseudomonas aeruginosa* than others. Less than 400µg/ml of Lawsone did not display desired influence. 400µg/ml of Lawsone hindered 75% bacterial growth. 500 and 600µg/ml of Lawsone have been tried to find effective dosage.

4.2 Proliferation & Cell Viability& Cytotoxicity Assay

Average of the absorbance values were utilized to find the cell survival rate as percentage by using following formula;

$$Survival\ rate\ (\%) = \frac{Asample\ - Ablank}{Aconrol - A\ blank}$$

Percentages of cell survival were plotted against respective ph of 50 ug /ml L-dopa (Figure 4.4), 25 ug /ml L-dopa (Figure 4.5) and Lawsone concentrations (Figure 4.6). Cytotoxicity of 25 and 50 µg/ml L-dopa and Lawsone have been indicated in the graphs (Figure 4.7) , (Figure 4.8) and (Figure 4.9) respectively.

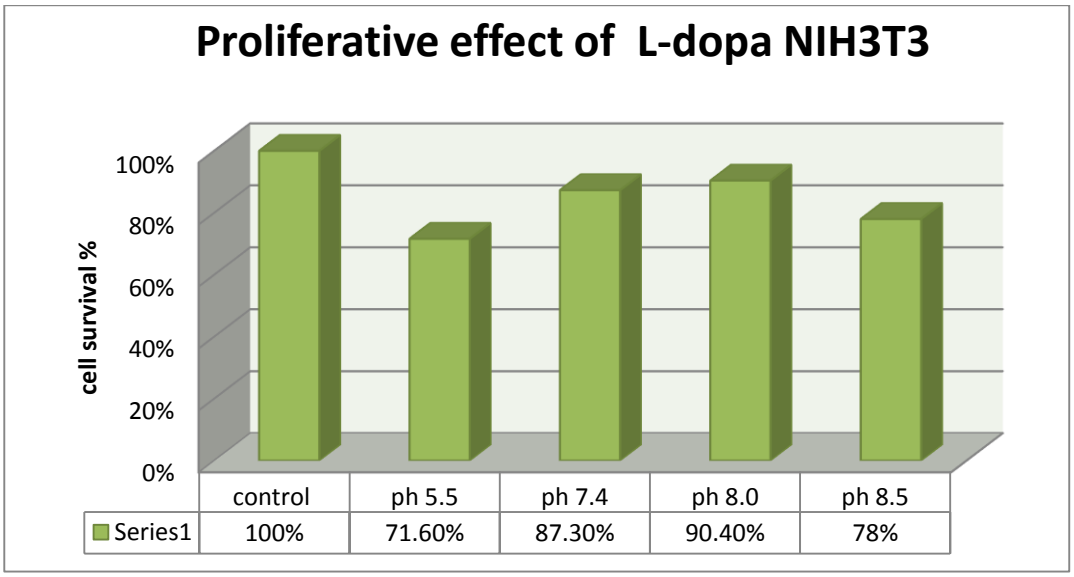


Figure 4.4 Cell survival rate of 50 µg/ml L-dopa-treated NIH3T3

The highest cell viability was observed at ph 8.0 of 50µg/ml L-dopa compared to the control.

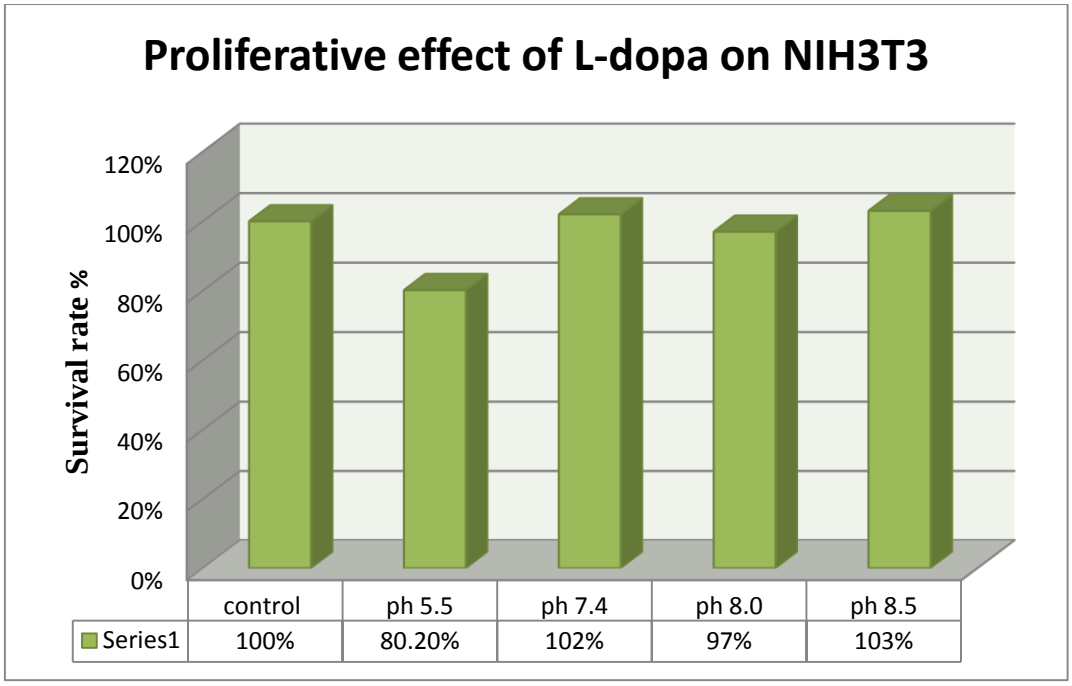


Figure 4.5 Cell survival rate of 25 µg/ml L-dopa-treated NIH3T3

25µg/ml L-dopa has indicated significantly proliferative effects except that at ph 5.5

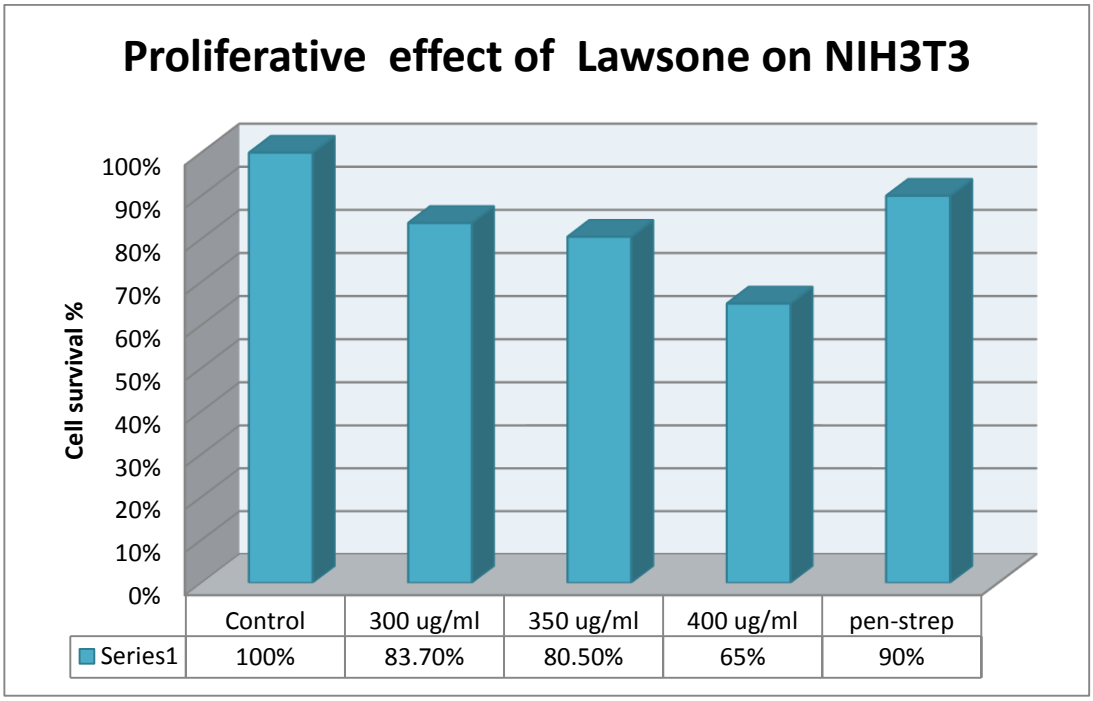


Figure 4.6 Cell survival rate of Lawsone –treated NIH3T3

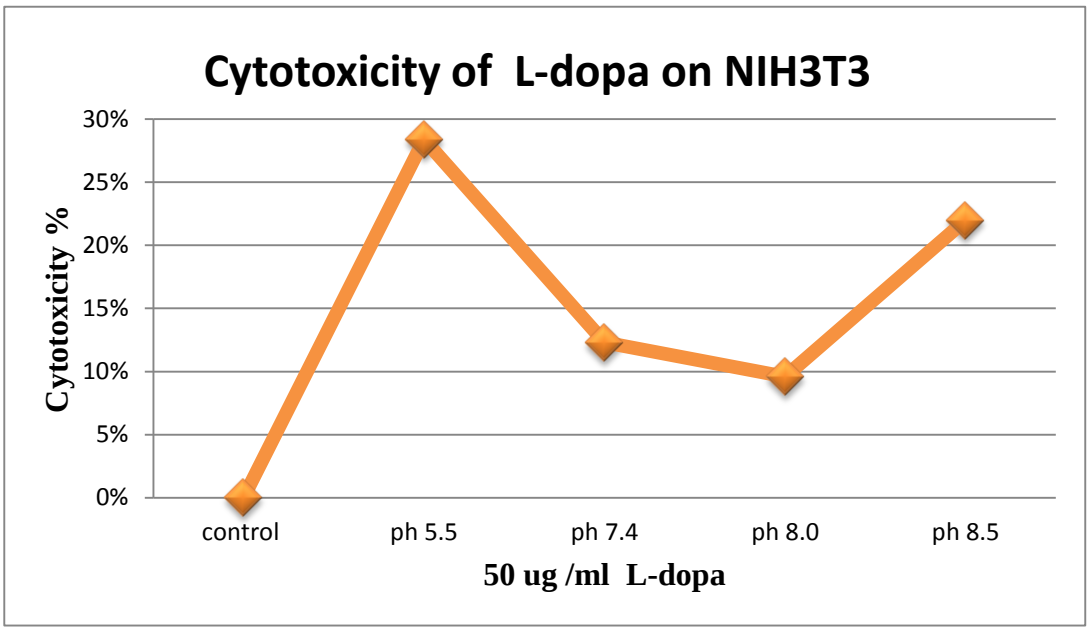


Figure 4.7 Cytotoxicity of 50 ug/ml L-dopa in ph 5.5, 7.4, 8.0, 8.5 on NIH3T3 cells L-dopa did not demonstrate cytotoxicity effect at concentration of 50µg/ml.

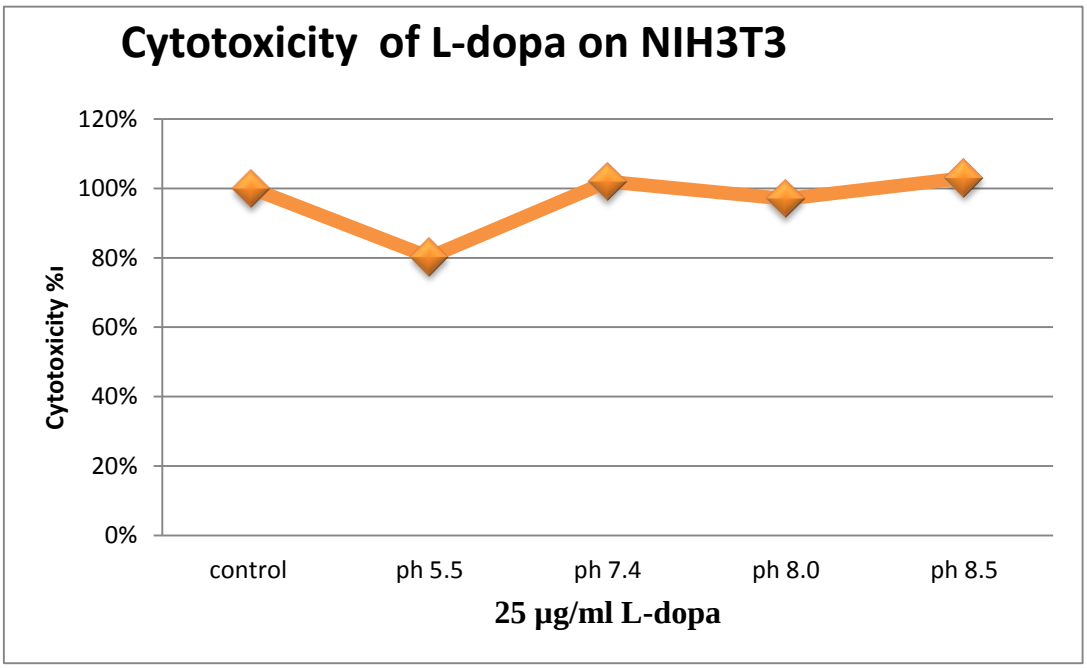


Figure 4.8 Cytotoxicity of 25 ug/ml L-dopa in ph 5.5, 7.4, 8.0, 8.5 on NIH3T3 cells 25µg/ml of L-dopa had no cytotoxicity on NIH3t3 cells.

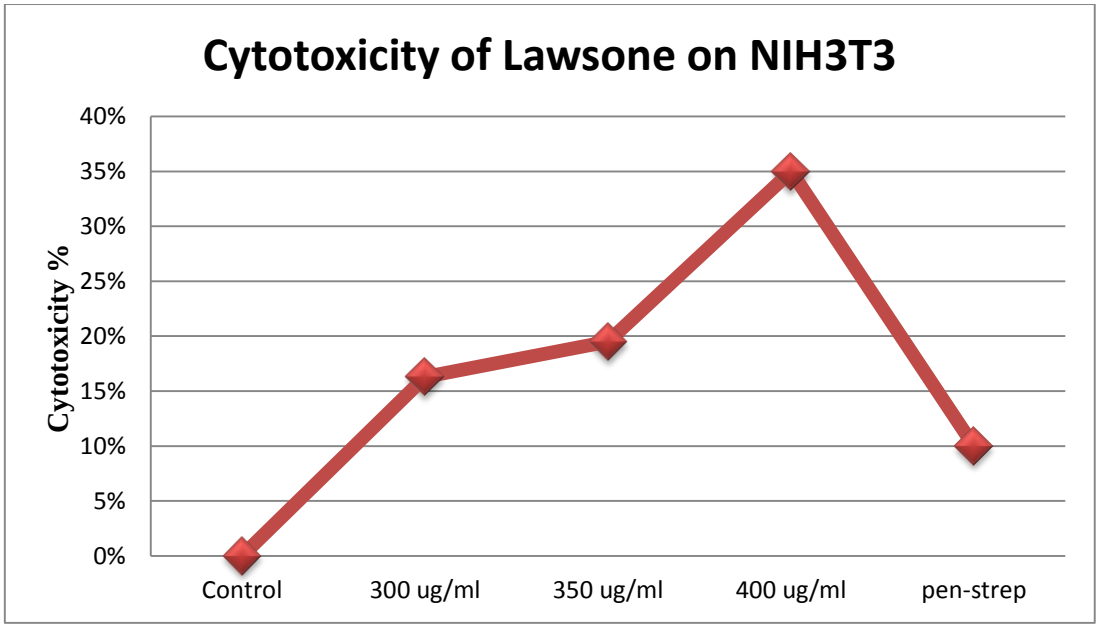


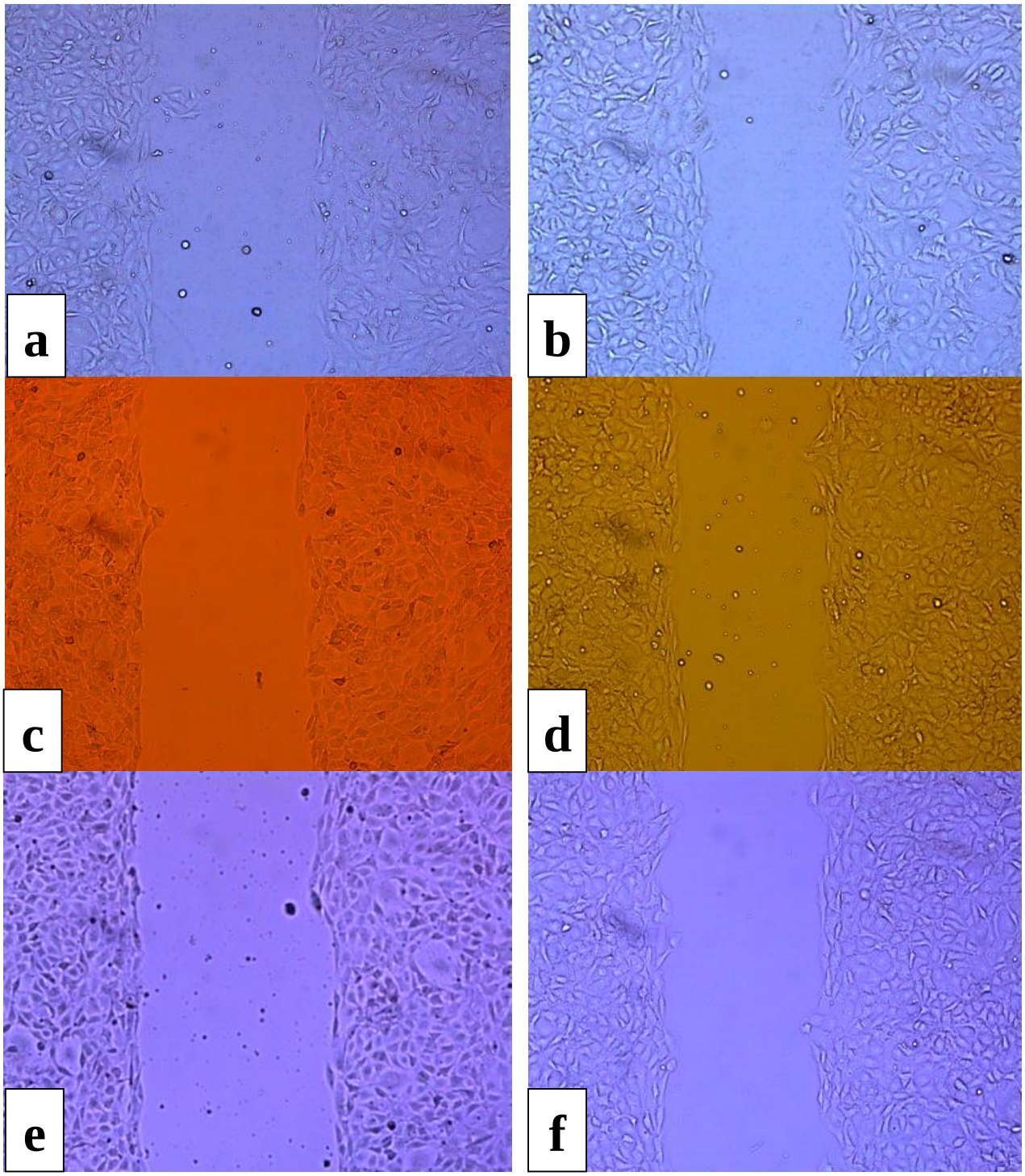
Figure 4.9 Cytotoxicity of Lawsone on NIH3T3 cells in different concentration 450 ug/ml, 350 ug/ml, 300 ug/ml.

4.3 Migration assay

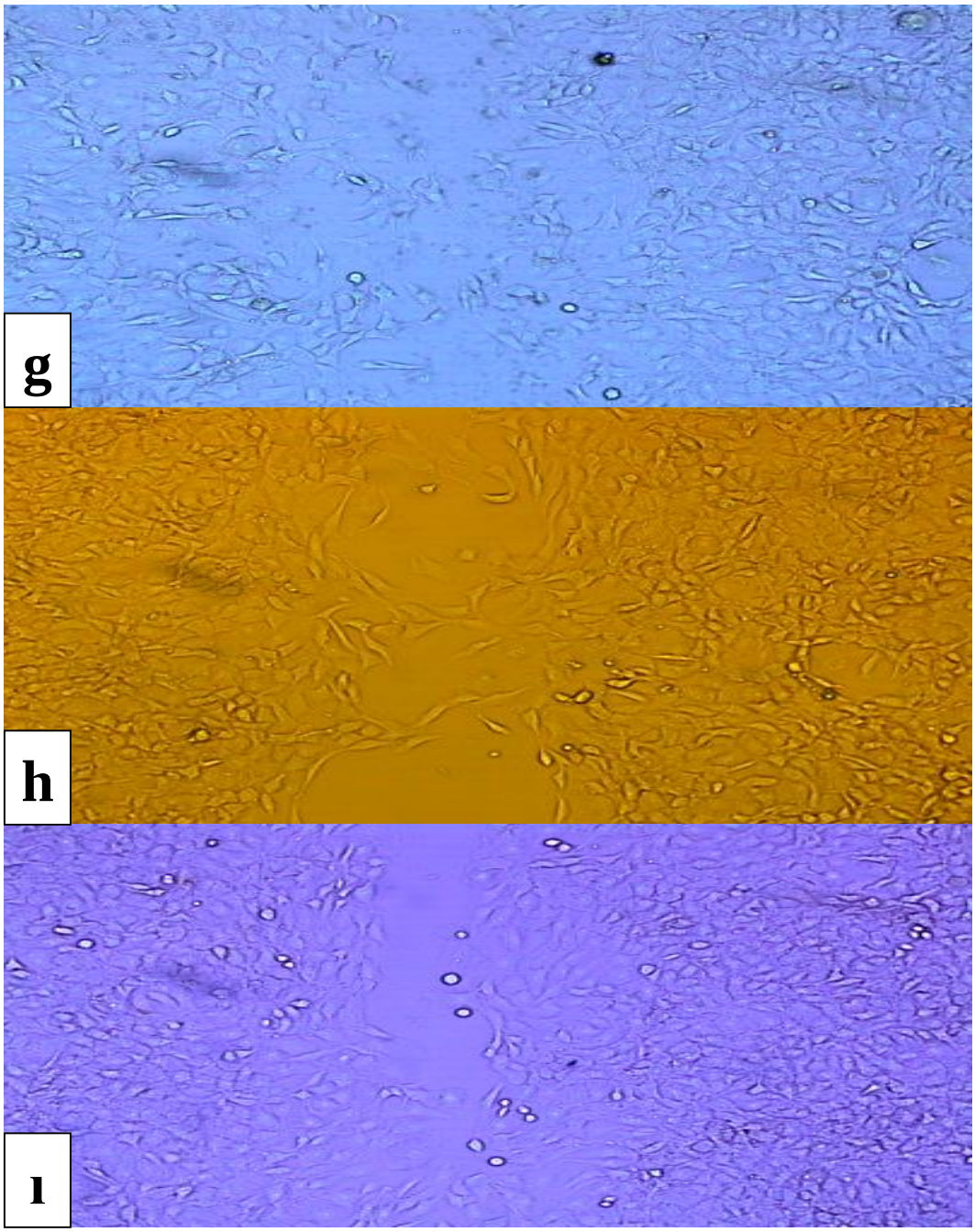
Testing agents were added into culture-insert plate after attachment of cells for 24 hours. And images were taken and closure of *in vitro* wound models were measured for each sample (Table 4.1) every 6 hours via Leica inverted microscope camera.

Table 4.1**.** Measurement of *in vitro* wound closure

	6 hours	12 hours
L-dopa treated closure	215.2 µm	176.2 µm
Lawsone treated closure	210.4 µm	170.4 µm
Untreated closure	242.1 µm	190.6 µm



6 hours **12 hours**



24 hours

Figure 4.10 Migration assay on NIH3T3 fibroblast cells in different time interval a),b) and g) migration of L-dopa treated cells c), d) and h) migration of Lawsone treated cells, e), f) and ı) untreated cells as control.

DISCUSSION

Antimicrobial susceptible testing

Lawsone is a type of naphtoquinone which is a naturally found biomaterial. It is a bioactive molecule of Lawsonia Inermis plant. Our results indicated that the effective dose of Lawsone is 400µg/ml. At this dose, it inhibited 97-98% of *Staphylococcus aureus* and *Staphylococcus epidermidis* compared to the 95% that was achieved by the positive control penicillin-streptomycin. Therefore, Minimum Inhibitory Concentration (MIC) is 400 µg/ml. According to our results, Lawsone has more antibacterial effect on *Staphylococcus epidermidis* than *Staphylococcus aureus*. On the other hand, Lawsone has less influence on *Pseudomonas aeruginosa* than others. It has prevented around 75% of *Pseudomonas aeruginosa* while penicillin-streptomycin showed 94.1% inhibition activity. We managed to dissolve Lawsone in LB instead of DMSO or other cytotoxic solvents chemicals which have cytotoxic effect on the bacteria. Therefore we could get direct effect of Lawsone on bacteria.

Proliferation & Cell Viability& Cytotoxicity Assay

Considering the results, Lawsone has no cytotoxicity effect providing around 63-69 % cell viability depending on IC_{50}. Respectively, at concentration of 400 µg/ml Lawsone cell survival rate was 63%, at concentration of 350 µg/ml it was 63.1% and at 300 µg/ml Lawsone it was 69%. Two different concentrations of L-dopa (25, 50 µg/ml) were utilized in different ph values (ph 5.5, 7.4, 8.0, 8.5). Cell viability was little close to what measured for control at concentration of 50 µg/ml L-dopa. The most effective ph was 8.0 providing 90.4 % cell survival, then ph 7.4 was secondly efficient resulting in 87.3% cell survival.The less influential ph was acidic 5.5 has around 71% cell viability. As a result, 50µg/ml L-dopa has no cytotoxicity effect on NIH3T3 fibroblast cells. The concentration of 25µg/ml L-dopa in distinct ph values has showed approximately similar cell viability as control. Cell survival rate was 80.2% at ph 5.5, 102% at ph 7.4, 97% at ph 8.0 and 103% at ph 8.5 respectively. 25µg/ml L-dopa slightly promoted proliferation of cells.

Migration assay

Cell migration takes very important roles during proliferation and remodeling phase of wound healing. Thus we investigated effect of Lawsone and L-dopa on migratory behavior of NIH3T3 fibroblast cells using *in vitro* wound healing model. The wounds started to be closed by removal of the square silicon-insert, leaving a cell free gap size (500µm). The migration speed of NIH3T3 cells treated with 25µg/ml L-dopa at ph 8.0 was greater than in the plate treated with 400 µg/ml Lawsone and control (Table 4.1). The closure was measured 215.2 µm in the insert plate treated with L-dopa, 210.4 µm in that with Lawsone and 242.1 µm in control for first 6 hours. After 24 hours the closure was 176.2 µm for L-dopa, 170.4 µm for Lawsone and 190.2 µm for control. Migration speed of NIH3T3 treated with L-dopa was significantly higher than that in Lawsone and control (Figure 4.10). We concluded that 25µg/ml L-dopa at ph 8.0 and 400 µg/ml Lawsone increased the speed of migration according to control.

CONCLUSIONS

In this study, therapeutic effects of Lawsone and L-dopa have been investigated to estimate their relevance as wound care devices such as dressing and surgical bioadhesives for wound treatment. Our results indicate that Lawsone has strong antibacterial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* milder antibacterial effect on *Pseudomonas aureginosa*. Furthermore, both L-dopa and Lawsone in certain concentrations and pH values promote the migration of NIH3T3 fibroblast cells. These novel biomaterials can be used as wound dressing materials either separately or together as part of a multifunctional bioadhesive formulation with antimicrobial properties.

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