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INVESTIGATING THE EFFECTS OF PURE *GALLIC ACID* IN WOUND HEALING

MUKHTAR DANJUMA IBRAHIM

MSc THESIS
BIOMEDICAL ENGINEERING PROGRAMME

THESIS ADVISOR

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ISTANBUL, MAY / 2016

T.C.

FATİH ÜNİVESİTES

BİYOMEDİKAL MÜHENDİSLİK ENSTİTÜSÜ

SAF GALLİK ASİDİN YARA İYİLEŞMESİ ÜZERİNE ETKİLERİNİN ARAŞTIRILMASI

MUKHTAR DANJUMA IBRAHIM

YÜKSEK LİSANS

BİYOMEDİKAL MÜHENDİSLİĞİ PROGRAMI

DANIŞMAN

YRD. DOÇ. DR. AYDIN ALBAYRAK

İSTANBUL, MAYIS / 2016

T.C.

FATIH UNIVERSITY

INSTITUTE OF BIOMEDICAL ENGINEERING

Mukhtar Danjuma Ibrahim, a **MSc**. Student, of Fatih University **Institute of Biomedical Engineering** student ID 520114016 successfully defended the **thesis/dissertation** entitled **"INVESTIGATING THE EFFECTS OF PURE GALLIC ACID IN WOUND HEALING",** which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family: Alhaji Danjuma Ibrahim (father) and Aisha Abdullahi (mother)

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Finally, I would like to thank all my friends for being a very important part of my life, making it more fun every day.

May, 2016 Mukhtar Danjuma Ibrahim

TABLE OF CONTENTS

ABBREVIATIONS

ROS	: Reactive oxygen species
PDGF	: Platelet-derived growth factors
VEGF	: Vascular endothelial growth factor
MMPs	: Metalloproteinases
ATCC	: American type cell collection
CFU	: Colony forming unit
FVII	: Plasma factor VII
IL – 6	: Interleukin-6
DMEM	: Dulbecco's Modified Eagle Medium
PBS	: Phosphate Buffer Saline
FBS	: Fetal Bovine Serum
NIH3T3	: Mouse Embryonic Fibroblast cell line
LB	: Luria Broth
ECM	: Extracellular Matrix
WST-1	: Water Soluble Tetrazolium-1
MIC	: Minimum Inhibitory Concentration

LIST OF FIGURES

Page

LIST OF TABLES

Page

SUMMARY

INVESTIGATING THE EFFECTS OF PURE *GALLIC ACID* IN WOUND HEALING

MUKHATR DANJUMA IBRAHIM

Biomedical Engineering Programme

MSc Thesis

Adviser: Asst. Prof. Dr. Aydin ALBAYRAK

Wound healing is a complex process that involves the activation of biological pathways to generate an effective tissue environment containing cells, cytokine, enzymes, growth factors, and extracellular matrix components. The phases of wound healing describe the organism reaction to injury. Tissue injury can be cured in various ways using traditional and conventional methods of wound treatment. *Staphylococcus epidermidis,* gram positive bacteria, is one of the most commonly known bacteria to colonize wounds, cause infection, and delay the wound healing process. In this study, gallic acid which is an organic acid naturally found in *Acacia nilotica* plant was investigated in its pure powder form for its cytotoxic and cell migratory activities on NIH3T3 fibroblast cells by using colorimetric assay. Antibacterial activity of gallic acid against *Staphylococcus epidermidis* was evaluated using antibacterial susceptibility assay. Our results show that gallic acid is a biocompatible therapeutic with antimicrobial properties that promotes wound healing in *in-vitro* wound models.

Keywords: Acacia nilotica, wound healing, gallic acid, cytotoxicity, antibacterial activity, minimum inhibitory concentration.

ÖZET

SAF GALLİK ASİDİN YARA İYİLEŞMESİ ÜZERİNE ETKİLERİNİN ARAŞTIRILMASI

MUKHATR DANJUMA IBRAHIM

Biyomedikal Mühendisliği Programi

Yüksek Lisans Tezi

Danişman: Yrd. Doç. Dr. Aydin ALBAYRAK

Yara iyileşmesi, etkili bir doku ortamının oluşturulması için bir çok biyolojik yapının (hücreler, sitokin, enzim, büyüme faktörleri ve hücre dışı matris bileşenleri) aktivasyonunu içeren, karmaşık bir süreçtir. Doku yaralanmaları tedavisinde geleneksel ve geleneksel olmayan bir çok yöntemler kullanılmaktadır. Bu çalışmada, *Acacia nilotica* bitkisinde bulunan bir organik asit olan *gallik as*it molekülünün, NIH3T3 fare fibroblast hücreleri üzerindeki sitotoksisitesi ve hücre göçüne olan etkileri kolorimetrik yöntemler ile araştırılmıştır. Ayrıca, *gallik asidin Staphylococcus epidermidis'*e karşı antibakteriyel aktivitesi, antibakteriyel duyarlılık testi kullanılarak değerlendirilmiştir. *Staphylococcus epidermidis*, yarada koloni oluşturarak enfeksiyona neden olan ve yara iyileşme sürecini geciktiren en yaygın bakterilerden biridir. Sonuçlarımız, *galik asidin in vitro* yara modellerinde yara iyileşmesini teşvik eden ve antibiyotiklerle kıyaslanabilecek derecede antimikrobiyal özelliğe sahip bir molekül olduğunu göstermiştir.

Anahtar kelimeler: Akasya nilotica, Yara İyileşmesi, Gallik asit, Sitotoksisite, Antibacteriyel aktivite, Minimum inhibitör konsantrasyonu.

CHAPTER 1

INTRODUCTION

1.1 Background

The wound is defined as the break of cells and anatomical function of living tissue. The injury may be developed by chemical, thermal, physical, immunological or bacterial abuse to the tissue [1]. Many bacterial organisms take advantage of the injury to enter into the tissue. To prevent bacteria from entering the tissue via lesion surfaces, wound site needs to be repaired quickly. Despite the fact that various kinds of materials such as hydrogels and creams are used to treat wounds and accelerate the healing process, the number of people with chronic lesions is on the rise. The existence of secondary a disease in a person such as diabetes usually can complicate the wound healing process further and led to delayed healing times. It has been projected that about 3% of the population of the USA will grow some type of leg ulcer in their lifetime as a result of aging and a higher frequency of metabolism related disorders [2, 3 and 4]. This means that 3 – 6 million people will be affected with chronic lesions that would cost circa $5 – $10 billion to treat these lesions each year [3].

1.2 Aim of the study

The purpose of this research is to investigate the effects of pure garlic acid on wound healing and biofilm formation.

1.3 Wound complications

Wound healing is a process that begins immediately after an injury**.** The process of wound healing comprises of continuous and overlapping phases: Homeostasis, Inflammation, Proliferation, and Remodeling. The injury's response to remediation is the basic host protective function, aimed to restore the tissue to its normal structure. The infection is regarded as the most significant factor that hampers with the healing phases. Invasions of microorganisms such as bacteria, fungi and virus prolong the inflammatory

stage. In wound care, microbial invasions can lead to infections and hence need to be prevented completely or at least reduced substantially for the normal healing process to take place. Health professionals and collaborators regularly care for a variety of skin wounds such as surgical incisions, ulceration, and turf burns which are regarded as the hardest to treat. In clinics, from acute wound management to increased scar tissue remodeling are some of the challenges that wait to be addressed to provide better better wound healing capabilities.

CHAPTER 2

2.1 Skin physiology

The skin is regarded as the biggest organ of the human body which performs a vital function in sustaining life [5]. The skin serves as a barrier between the body and the external world protecting the body from the excessive temperature, radiation, and infection. The human skin is composed of three main layers, namely: epidermis, dermis and hypodermic layer (Figure 2.1).

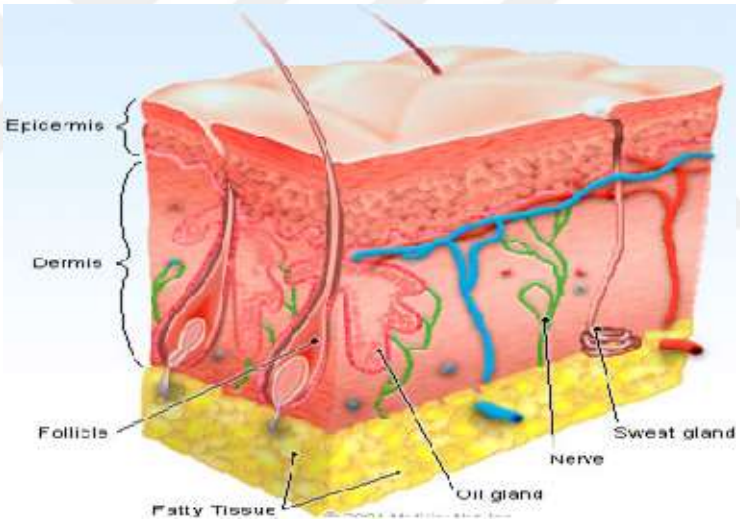

Figure 2.1 Structure of human skin [5]

The skin's outmost layer is the **epidermis** where special skin cells called *keratinocytes* reside and provide constant supply of new skin cells. These cells mature and rise to the skin surface and are discarded to be substituted by the new ones [5]. The underlying dermis supplies the epidermis with the nutrition because the epidermis lacks blood vessels. *Melanocytes* contains pigments that give the skin its color and are also responsible for engrossing free energy and protecting skin against injury triggered by electromagnetic radiation. Another important epidermal cell is *Langerhan cells* that are produced by the bone marrow. Langerhan cells migrate to the skin surface and assist to combat infection. The last type of epidermal cells is *Markel* specialize *cells*, which are

specialize in sensing the light touch. These cell types are found on the toes and fingertips or even on other specialized parts [5].

The **dermis** layer is found beneath the epidermis layer and consists of 2 layers that support epidermis: 1. Papillary dermis which is found just underneath the epidermis is characterized with a tinny layer of tissue containing a little elastic, collagen fibers, and blood vessel capillaries [5]. 2. Reticular dermis is deeper and comprises large bunches of elastic and collagen fiber running similar on the surface of skin. The function of these fibers is to assist the skin to resist damage from clipping or other kinds of traumatic injury. These layers also aid the skin when it is compressed or stretched to regain its normal state [5]. The sebaceous glands, sweat glands, and hair follicles are located in this layer.

Subcutaneous tissue is the deepest skin layer that contains *lipocytes cells* which store fats and act as an insulation layer [6].

2.2 Wounds

The wound is the break of cells and anatomical function of living tissue. The injury may be developed by chemical, thermal, physical, immunological or bacterial abuse to the tissue [1]. Wounds can be classified as closed and open wounds. In the case of open wounds, the skin is torn or ruptured and the underlying tissues are exposed to the exterior surrounding. The deepness of tissue injury in open wounds may be generally separated into: superficial (involving epidermis and upper dermis only), incomplete thickness (skin loses up to the lower dermis) or full thickness (loss of skin and hypodermic tissue). While in closed wounds the skin surface is undamaged (examples include: hematomas, contusions and stage 1 pressure ulcers) [7]. Therefore, wounds are divided into acute and chronic wound depending on the mechanism of the damage, and their relation to the healing time [8].

2.2.1 Acute wounds

Acute wounds typically heal at a foreseeable and expected rate based on the normal wound healing process. They progress through the normal wound healing phase that include: homeostasis, inflammation, proliferation and remodeling. Acute wound is of

two types, namely: (a) Traumatic wound, like minor cut and (b) Surgical wound, which is incised and suture performed by surgeon [8].

2.2.2 Chronic wounds

The chronic wound appears to be detained in one of the phases of wound healing. It is a kind of wound that does not heal within 3 months or at the expected rate of healing as most of the acute wounds do [9]. Examples of chronic wounds include: diabetic ulcer, arterial leg ulcer, and venous leg ulcer [9].

2.3 Wound healing physiology

Wound healing is an interconnected and a continuous process that begins immediately after an injury**.** The process of wound healing comprises of overlapping phases (Figure 2.7): Homeostasis, Inflammation (0 – 4 days), Proliferation (day 3 – 3 weeks), and Remodeling phase 4 (week 3 – 2 years) [10]. These stages are controlled by a complex linkage of interrelating cytokines, growth factors as well as their cellular receptors [11].

2.3.1 Phases of wound healing

Homeostasis: Homeostasis involves a sequential procedure that acts together to halt the bleeding from a lesion. The thrombomodulin which is the coagulation inhibitor is secreted by the endothelial cells of the undamaged blood vessels. The nitric oxide and prostacyclin were also produced to prevent accumulation of platelets [12]. Vasoconstriction, the biological process which determines the quantity of blood going out of the damaged vessel is accompanied by the establishment of a platelet plug that closes up the disrupted vessels. The final step in homeostasis is the establishment of a fibrin clot (Figure 2.2) through the production of plasma factor VII (FVII) [13, 14] and prothrombin. Fibrin is a type of collagen fiber and is created around the platelet plug attaching it in place [15].

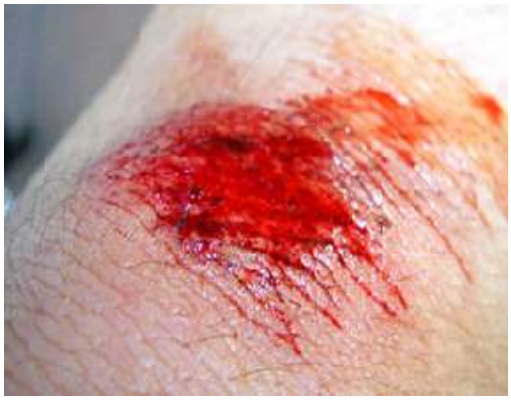

Figure 2.2 Homeostasis phase

Inflammation: In this phase, interleukins which are a type of cytokines are triggered. The activation of chemotaxis of monocytes and macrophage is stimulated by interleukin 6(IL – 6) [16]. Also interleukin 8(IL – 8) promotes the proliferation of neutrophils and neovascularization [17]. The platelet-derived growth factors (PDGF) are responsible for removing the injured tissue, producing fresh healthy tissue, and killing pathogens (Figure 2.3). The cytokines released by macrophage and other inflammatory cells generate reactive oxygen species (ROS) [18].

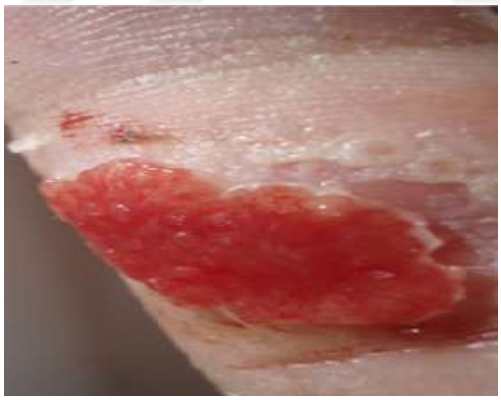

Figure 2.3 Formation of new healthy tissue

Proliferation: This stage is characterized by angiogenesis and epithelialization (Figure 2.4). The neutrophil macrophages allow the synthesis and restoration of extracellular matrix (ECM). Fibroblasts are drawn to the wound site by chemo-attractants secreted by the neutrophils [19]. Production of hyaluronic acid (HA) assists cell migration and gives tissue the ability to eliminate deformation [20]. The additional nutrients and oxygen needed for wound to heal are supplied by the vascular endothelial growth factor (VEGF).

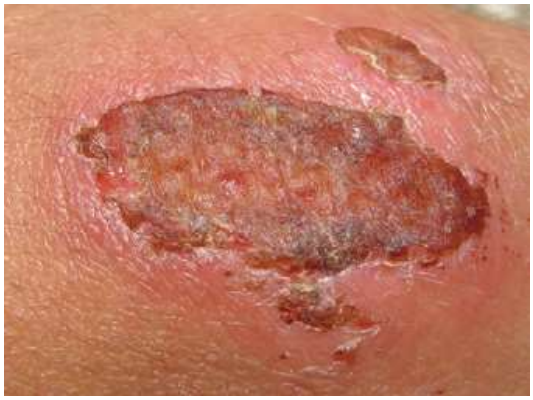

Figure 2.4 Epithelialization

Remodeling: Tissue recovers 80 – 90% of its original strength (Figure 2.5). In the human ECM, collagen is the highest structural protein available. The ECM remodeling is alleviated by the proteases like tissue-derived inhibitors (TIMPs) and matrix metalloproteinases (MMPs) [19].

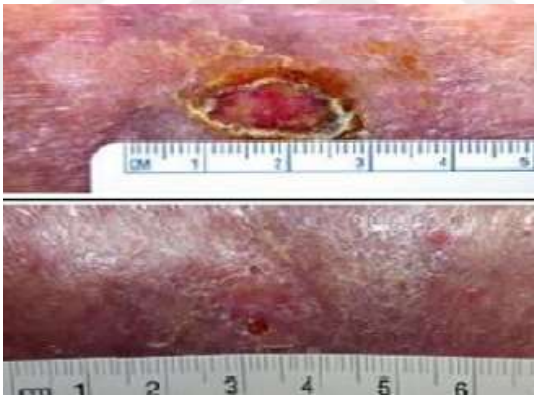

Figure 2.5 Wound closure

2.4 Biomaterials in wound healing

Biomaterials are pure bulk or composite materials that have natural or synthetic origin and can be used to improve or replace the function of any tissue or organ of the body partly or completely [21]. Biomaterials functionalization could bring possible ways to invent novel materials for wound healing with anti-inflammatory, adhesive, and anti-bactericidal properties [22]. Biomaterials are usually categorized into five broad categories: metals, polymers, ceramics, naturals and composites. Biomaterials should

not damage the tissue which they come in contact with. Also, biomaterials should be non-toxic, biodegradable, non-inflammatory, non-carcinogenic, and biocompatible [23].

2.4.1 Acacia nilotica

Acacia nilotica which belongs to the family of Fabaceae (Table 1) has a length of 14-18m with thick spherical treetops, branches, and stem. Its color varies amongst dark-black, cracked bark, pinkish – grayish slice, oozing out reddish modest gum [24]. There are nearly (1350 species) of *A. nilotica* scattered around warm climates and tropical regions. In Asia, there are 89 species whereas in Australia there are 957 species [29].

The plant also has been utilized effectively in folk medicine for different health conditions such as opthalmia, cough, tuberculosis, smallpox, antispasmodic, dysentery, aphrodisiac, cold, fever, sclerosis, skin-cancer, hypertension, bleeding piles, hemorrhoid and menstrual problems [25, 26, 27]. The seed, the bark, and the leaves of *A. nilotica* have also shown various activities such as antioxidant, antibacterial, cytotoxic, styptic, wound ulcers, skin diseases, leukoderma, burning sensation, dysentery, bleeding piles, and hemorrhage [28].

Table 2.1 Acacia nilotica taxonomic classification

Kingdom	**Plantae**
Subkingdom	**Tracheobionta**
Super division	**Spermatophyta**
Division	**Magnoliophyta**
Class	**Magnoliopsida**
Subclass	**Rosidae**
Order	**Fabales**
Family	**Fabaceae**
Genus	**Acacia**
Species	**Nilotica**

2.4.2 Phytochemical constituents

The phytochemical studies of Seigler et al., have shown that the seed of *A. nilotica* contains a high percentage of phenolic components (PC) (Figure 2.6). The PC consists of gallic acid, ellagic acid, protocatechuic and m-digallic acid, flavan 3,4-diol, oligomer 3,4,7-trihydroxy flavan – 3-ol, and 3,4,5,7-tetrahydroxy flavan (-) epicatechol [30]. There was a report from Katsina State of Nigeria on the traditional use of *A. nilotica* pod used for postnatal wound healing [31]. Gilani et al., reported that the leaves were used for the treatment of wounds, diarrhea, Alzheimer disease and bronchitis while the seedless pod for the treatment of ulcer [32].

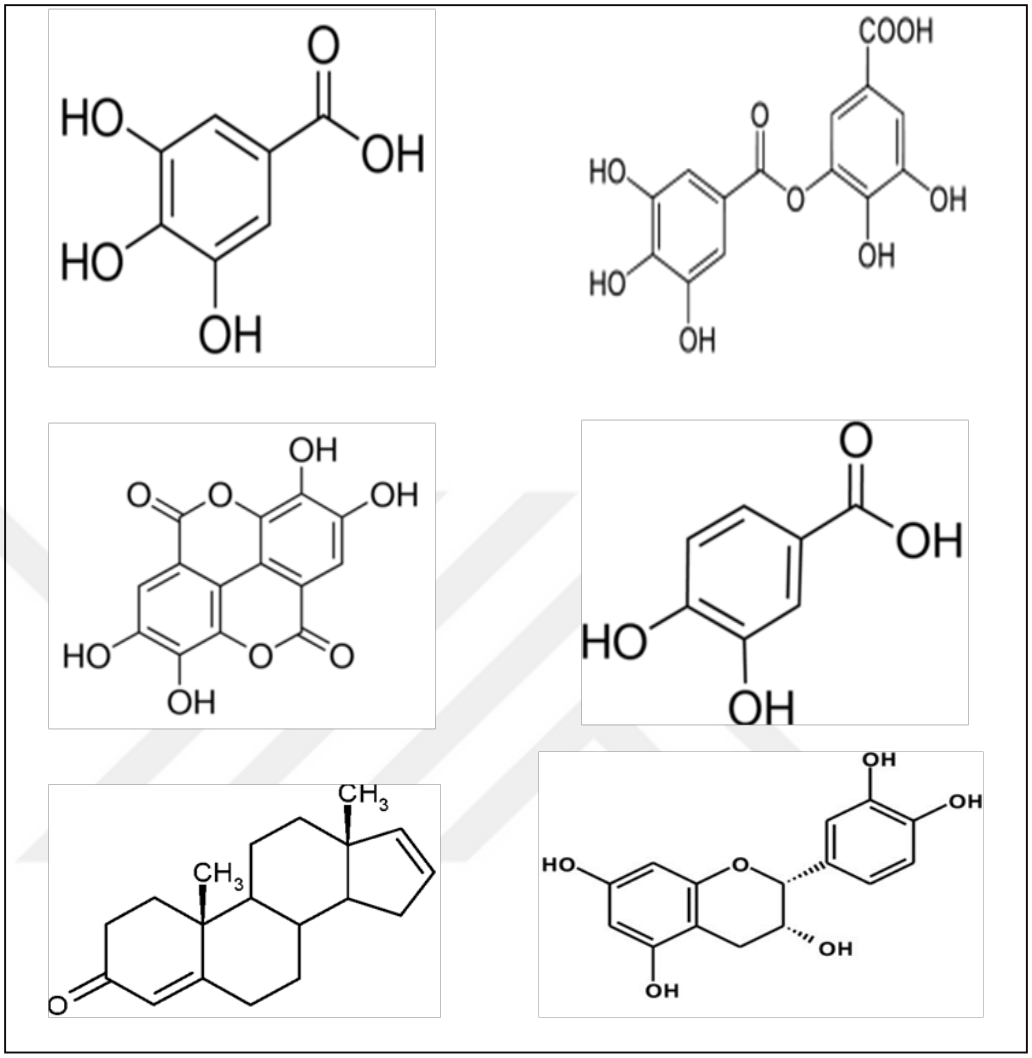

Figure 2.6 Phytochemical structures of phenolic components

a) Gallic acid b) Digallic acid c) Ellagic acid d) Protocatechuic e) Dimer f) Epicatechol

2.5 Gallic acid

Gallic acid is one of the phytochemicals that is found in *A. nilotica* plant. Gallic acid is an organic acid which is also called 3, 4, 5- trihydroxybenzoic acid with the chemical formula $C_7H_6O_5$. Pure gallic acid is a colorless crystalline organic powder [33].

2.5.1 Antibacterial and anti-fungal activity

While most wounds heal normally, problems do sometimes occur. In most cases, the problems are related to scar formation or delayed healing. Factors such as the number of microbial organisms, their pathogenicity, and the ability of a patient's immune system to fight infection, determine the consequences of wound bacterial contamination. The

infected wounds may show traditional symptoms of microbial infection (Figure 2.7) characterized by pain, swelling, redness, and heat.

The phytochemical studies on *A. nilotica* extracts have shown antimicrobial activity, against *S. aureus* and *E. coli*. [34, 35]. Bioactive components such as niloticane, cassane and diterpenes extracted from the stem bark revealed inhibitory activity against *E. coli, K. pneumonia, S. aureus* and *B. subtilis at* MIC values of 33µg/ml, 16µg/ml, 8µg/ml, and 4µg/ml, respectively [36].

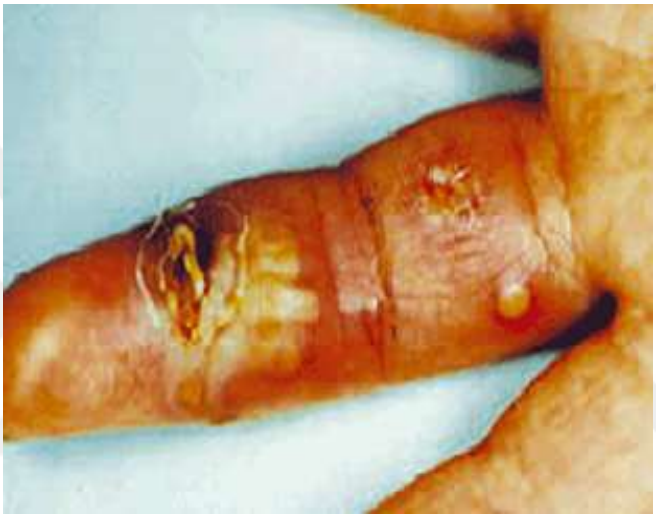

Figure 2.7 Bacterial infection

Banso et al., reported that bark extracts have antimicrobial activity against *B. subtilis, S. aureus, S. viridans, C. albicans, E. coli, S. sonnei,* and *X. malvacearum* using agar – well diffusion technique with MIC values between 35mg/ml and 50mg/ml, and high zone of inhibition [37, 38].

Dev et al., have shown that leaves, seed, and stem of *A. nilotica* have antimicrobial activity against *E. coli* at MIC value of 5µl and an inhibition zone of 12mm for leaf and seed, and 15.30mm for the stem extracts respectively [39]. Moreover, methanolic aqueous extracts show reasonable anti-microbial activity against multi-drugs tolerant *S. typhi* [40]. In another study, the anti-microbial potential of methanol extracts was examined against *E. coli, S. aureus,* and *P. aeruginosa* [41].

Recently, Pai et al., evaluated the bark extracts against *K. pneumonia* and showed that at the end of 48h, the inhibition zone is 7.62mm. The outcome is consistent with the reports of Runyaro et al., where the inhibition zone was < 10mm [42, 43]. Also the seed crude oil extract with MIC values of 0.158mg/ml displayed potent anti-fungal activity

against *A. niger,* and *C. albicans* holding inhibition zones of 37.5 and 36.1mm, respectively [44].

2.5.2 Anti-diabetic activity

Mamta et al. investigated methanol leaves extracts of *A. nilotica* in streptozotocin-induced diabetic's rats. Their results showed hypoglycemic activity on diabetic rates compared to glyburide [45]. The cook powder of *A. nilotica* that was absorbed by the diabetic animal models after 60min resulted in the lowering of blood glucose level starting from 132 to 106 [46].

Vijay et al. recently studied the hydroethanolic extract of a new seedless pod of *A. nilotica* in pylorus ligation-induced rat ulcer model. Their results revealed the extract to have antiulcer activity. Also, the extract with the more phenolic component showed higher antiulcer activity [47].

CHAPTER 3

MATERIALS AND METHODS

3.1 Reagents

All of the chemicals used were of research grade. The materials acquired from Sigma Aldrich include: Pure gallic acid powder, WST – 1 kit and Luria broth (LB). DMEM, PBS (1X), Streptomycin/Penicillin, and Trypsin (EDTA) were purchased from Lonza. The insert-plates for culture were purchased from Ibidi. The FBS was procured from Invitrogen.

3.2 Cells

In this research, the bacterial cultures used for antibacterial susceptibility testing were supplied by the American Type Cell Collection (ATCC, US). The gram positive bacteria *Staphylococcus epidermidis* that was used is of regularized strains (ATCC). The fibroblast cells (NIH3T3) (Figure 3.1) were obtained from the Biological sample preparation, and cultured in the Cell Culture Research Laboratory in the BioNano Technology Research and Development Center Fatih University.

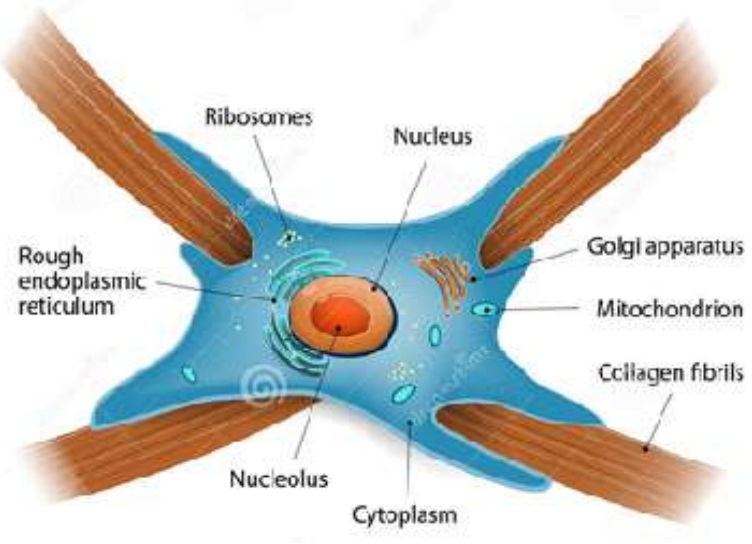

Figure 3.1 Structure of NIH3T3 Cell [50]

3.3 Preparation of gallic acid stock solution

The powder form of Gallic acid was purchased from Sigma Aldrich. DMEM was tested to dissolve the gallic acid powder. 1000 µg/ml concentration dissolved completely in DMEM but did not dissolve completely in water compared to the time it takes to dissolve in DMEM. 1000 µg/ml main stock of gallic acid was made ready. The ranges of gallic acid concentrations that were tested were from 50µg/ml to 650µg/ml.

3.4 Cell migration assay

The fibroblast cells used in this study were cultured in 75cm^2 flask containing DMEM supplemented with penicillin – streptomycin and 10% FBS. The cell culture was incubated at 37ºC in 5% CO_2 incubator, and monitored under microscope until 90 – 100% cell confluency was obtained. The cells were passaged and adequate number of cells were obtained. 35,000 NIH3T3 cells were seeded into each well with total volume of 70µl in culture – insert plates, containing cells, 10% FBS and DMEM as a cell suspension (Figure 3.2). The plates were incubated at 37ºC in 5% CO_2 incubator for 24h for the attachment of cell on the plates. Then, silicon wells were detached by holding the corner of silicon using tweezers. The 2ml total volume is put into the culture – insert plates comprising of testing agent 50µg/ml, 100µg/ml gallic acid concentrations, DMEM and FBS 10%. A control group was used which contains DMEM and 10% FBS that gives 2ml total volume. The images of cell migration were captured every 6h using the Leica inverted microscope system, and the area covered by migrating cells was also measured.

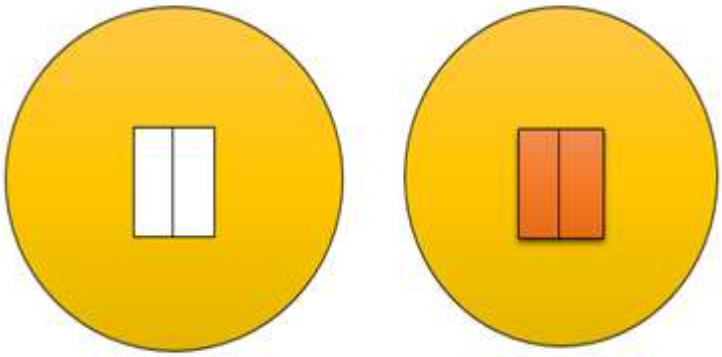

Figure 3.2 Insert plate loaded with NIH3T3 cells inside the wells

3.5 Cytotoxicity Assay

In order to determine the cytotoxic potency of gallic acid, 100 µl of 1×10^4/ml of the NIH3T3 cells were seeded into 96 – well plates (Figure 3.3) containing DMEM and FBS with penicillin and streptomycin. They were then incubated in a 5%CO_2 incubator at 37ºC for 24hrs and the media was discarded. Then replenished with DMEM and a different concentrations (50µg/ml, 100µg/ml, 200 µg/ml, 300µg/ml) of gallic acid. Also, 10% FBS was put into each well. The positive control group was treated with media added freshly. The plates were incubated for 24hrs, after that 10µl water soluble tetrazolium-1 (WST-1) was injected into all of the wells. The absorbance value was measured with an ELISA reader under 450nm. The rate of viable cell has been calculated with the following formula in accordance with absorbance value protocol;

$$\%\ \text{viable rate} = \frac{\text{Asample} - \text{Ablank}}{\text{Acontrol} - \textit{Ablank}}$$

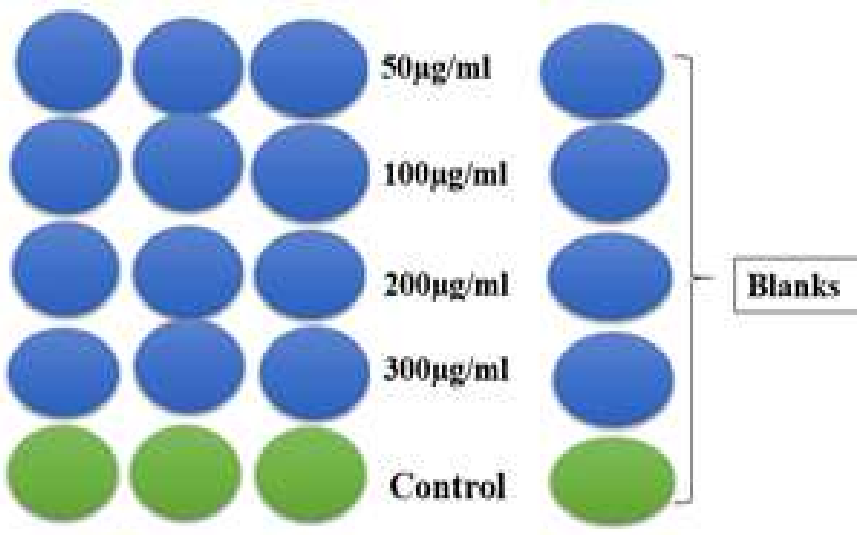

Figure 3.3 Wells loaded with different concentrations of gallic acid

3.6 Antibacterial Susceptibility Testing

The antibacterial effect of gallic acid was tested on gram positive bacteria *Staphylococcus epidermidis*. The strains of bacteria were inoculated into Luria Broth (LB). Its cell density was adjusted with UV – visible spectrophotometer, where around 10^8 bacterial CFU was obtained as the final concentration, and optical density (OD_{600} = 0.08 – 0.1) [48]. 500 µg/ml was prepared as the main stock of gallic acid dissolved in LB. The 50µg/ml, 100µg/ml, 200µg/ml concentrations were tested to get the MIC_{90} at least 90% of bacteria. Dissolved gallic acid and 100µl inoculum comprising 10^6 CFU bacteria were put into Erlenmeyer 50ml flasks for each concentration (50µg/ml, 100µg/ml, 200µg/ml), with fresh LB completing 5ml total volume in each 50ml flask for each concentration. The three different concentrations of gallic acid and two control groups (positive and negative groups) were studied to determine the antibacterial activity of gallic acid on one gram positive bacteria *S. epidermidis*. 200µl Penicillin – Streptomycin, pure LB media and 10^6 CFU bacteria were contained in positive control while negative control contains LB and bacteria. Then the flasks were incubated in a shaker incubator for 24hrs at 37ºC through 200rpm. After the incubation, 100µl of samples were taken (Figure 3.4) from each flask and put into 96-well plate with the blanks. Thus, samples were all screened in the ELISA microplate reader where the optical density of bacteria was measured at 600nm and inhibition % is obtained from the relation using the following formula [49].

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

where:

I = Inhibition

X = Optical density of bacteria in a negative control set

Y = Optical density of bacteria in a test set

Figure 3.4 Main gallic acid concentrations and positive and negative control (50µg/ml, 100µg/ml, 200µg/ml)

CHAPTER 4

RESULTS

4.1 Cell migration assay

In this research, we investigated the effects of gallic acid in its pure form on mouse fibroblast cells (NIH3T3) for their migratory activities. It is known that cell migration plays a vital function during the proliferation and the remodeling phases of wound healing. The outcome of this migration assay is represented in Figure 4.1 and Table 4.1 respectively where the speed at which the cells treated with gallic acid at 50µg/ml and 100µg/ml, with the control (cells without gallic acid) moves closing the gap area created after removing the silicon wells. Cell migration for the samples and control was measured after the images were captured in a time interval of 0h, 6h, 12h, and 24h (Figure 4.1) using Leica Inverted Microscope System. The images captured sequentially were significantly different for both treated cells and the control in terms of the rate at which they migrate to fill the gap area, showing significant reduction in the total gap area (Figure 4.1).

Similarly, the observed gap area covered by the migrated control cells at 0hour was 60%, after 6hours 63%, after 12hours 68%, and 87% after 24hours (Table 4.1) consecutively. Whereas the cells treated with 50µg/ml gallic acid, the observed gap area closure stood at 0hour was 63%, after 6hours 66%, after 12hours 73%, and 94% after 24hours. Also, cells treated with 100µg/ml gallic acid indicate faster closure of the gap area, the rate for 0hour was 64%, after 6hours 68%, after 12hours 73%, and 97% after 24hours (Table 4.1) respectively. It was observed that the velocity of the cells migrated and filled the gap area treated with the gallic acid at a concentration of 100µg/ml is slightly greater than the 50µg/ml, and then followed by the control (Figure 4.1). Considering control as a reference, we therefore conclude that 50 µg/ml and 100 µg/ml of gallic acid increased the speed of cell migration (Table 4.1).

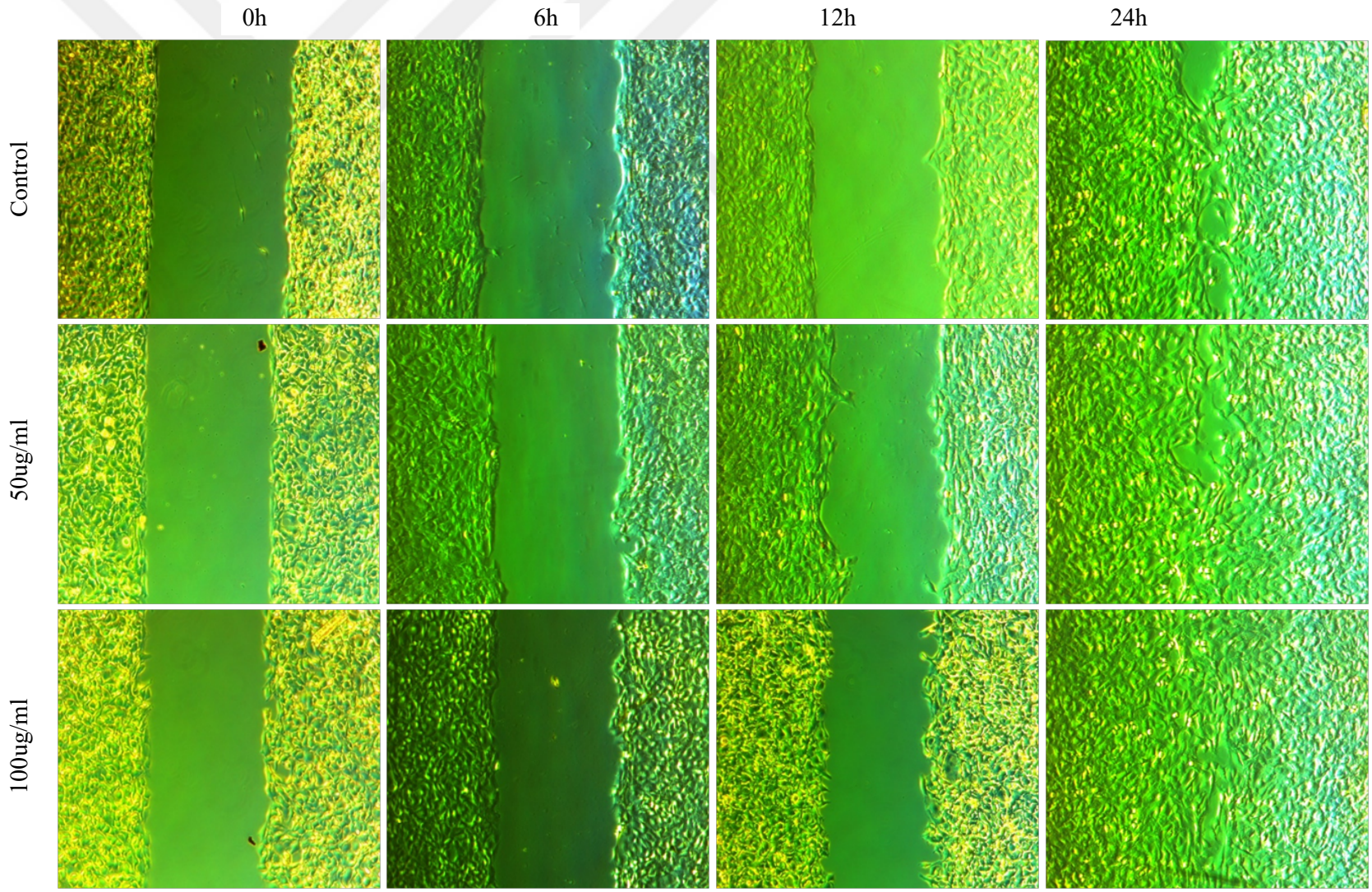

Figure 4.1 NIH3T3 cell migrations at different time intervals.

However, the two determined concentrations of gallic acid tested on the fibroblast cells revealed significance difference in the percentages for each concentration which is related due to the efficacy of the test material. Upon equated with the differences observed the control showed a little slower rate of cell migration. Hence, the percentages of the gap area filled by the cells that migrated at 50µg/ml and 100µg/ml concentrations of gallic acid under each time interval were computed with percentage of the area covered by the control as a reference (Table 4.1).

Moreover, the formula used as; (T1-Control)/(Control) and (T2-Control)/(Control). T1=50µg/ml which is the first concentration of gallic acid and T2=100µg/ml the second concentration utilized referenced against control (Table 4.1). There was a substantial difference of 0.08 for the T1 after 24hours and 0.11 for the T2 after 24hours (Table 4.1). This was as a result of cells response to the gallic acid being applied and proved its stimulating effects biomaterial agent that enhances the speed at which the cells migrate to close the gap area within a specific period of time under investigation.

Table 4.1 Percentage of area covered by cells at different time intervals

	0 Hour	6 Hours	12 Hours	24 Hours
Control	0.60	0.63	0.68	0.87
50µg/ml	0.63	0.66	0.73	0.94
100µg/ml	0.64	0.68	0.75	0.97
(T1-Control)/(Control)	0.05	0.04	0.07	0.08
(T2-Control)/(control)	0.06	0.07	0.10	0.11

T1=50µg/ml
T2=100µg/ml

4.2 Cytotoxicity Assay

The outcome of the cytotoxic activity of the three different gallic acid concentration evaluated on NIH3T3 cells is presented in Figure 4.2. In this work, the only non-cytotoxic concentration having cell survival rate of more than 50% are considered to be effective. The cytotoxic investigation indicate that gallic acid has no cytotoxic effect on NIH3T3 cells given that cell viability is around 52 – 93%, dependent upon IC_{50} for the tested concentrations (Figure 4.2). Hence, at 50µg/ml concentration of gallic acid the number of viable cells was 93%, for the 100 µg/ml concentration the cells survived was 80.10% and at 200µg/ml the cell survival was at 52% (Figure 4.2).

Additionally, despite the fact that gallic acid proved non-toxic at 50 µg/ml, 100 µg/ml, and 200 µg/ml concentrations, but it showed toxic to NIH3T3 cells at 300 µg/ml concentration with 23% cells viability (Figure 4.2) which is less than IC_{50}. Therefore, the most effective dosage of gallic acid that proved no cytotoxicity on NIH3T3 cells was 50 µg/ml 93% cell survival, which is very close to the control. Then followed by 100µg/ml that gives 80.1% cell survival. The absorbance values observed from the samples screened by ELISA microplate reader were calculated and the average values were obtained (Figure 4.3). The averages of the values observed were used to find the amount of cell survived as a percentage (cell survival %) by using the formula below;

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

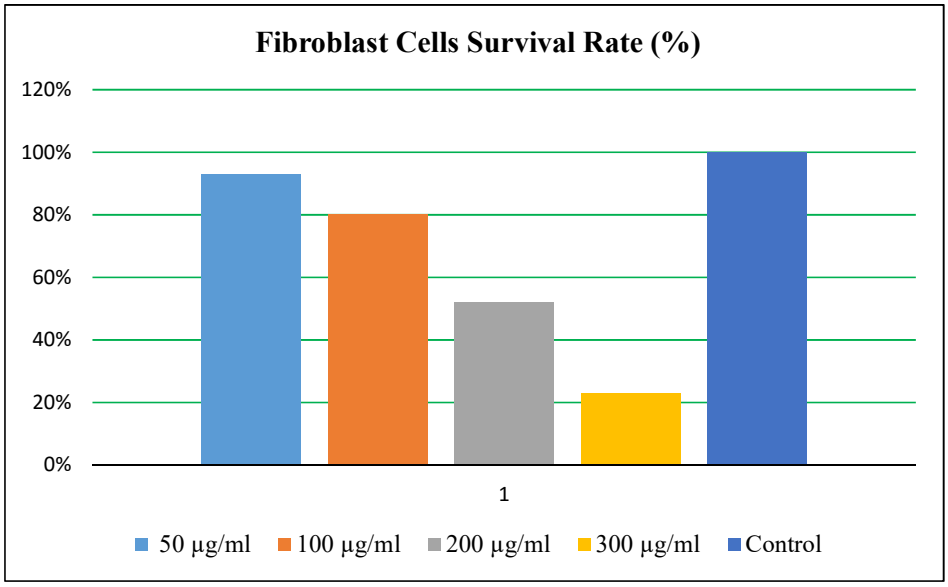

Figure 4.2 NIH3T3 Cell survival rate treated with gallic acid at four different concentrations.

The percentages of the cell survival rate were plotted in contrast to different gallic acid concentrations 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml (Figure 4.2). However, absorbance values mean were statistically analyzed to get their standard deviation (Figure 4.3). The ■ = average values, and the black bar on the top are the standard deviation. The statistical mean of the absorbance values for the percentage of 50 µg/ml, 100 µg/ml, and 200 µg/ml, concentrations of gallic acid (Figure 4.3) at p=0.01 they are significantly relevant compared to the control with 0.9859 average value.

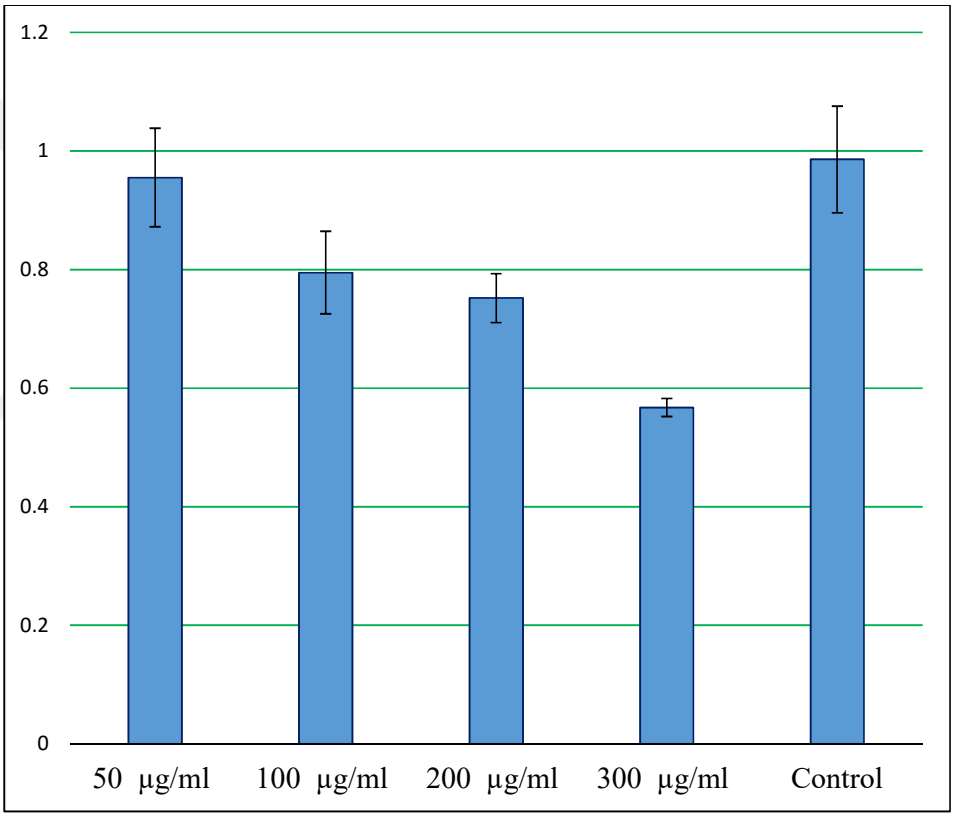

Figure 4.3 Absorbance values at different concentrations of gallic acid sample.

4.3 Antibacterial Susceptibility Testing

The three different gallic acid concentrations were examined for their antibacterial efficacies on *Staphylococcus epidermidis* growth inhibition. At 50µg/ml gallic acid inhibited 72.2%, and at 100µg/ml dosage it inhibited 90.4%, whereas 97.4% of inhibition was stated at a dose of 200µg/ml (Figure 4.3) gallic acid respectively. In contrast with the control sample where the percentage of inhibition was 98% for 200µl penicillin, gallic acid at 200µg/ml concentration inhibited 97.4% of bacterial growth. All three concentrations that were used in this work inhibited the growth of

Staphylococcus epidermidis. Similarly, the minimum inhibitory concentration (MIC) was achieved at 100µg/ml gallic acid concentration (Figure 4.3). Based on our findings the utmost effective dosage of gallic acid is 200µg/ml and then followed by 100µg/ml (Figure 4.3). Therefore, considering our results, gallic acid has antibacterial effect on *Staphylococcus epidermidis*. The average of absorbance values (Figure 4.4) of the three different concentrations of gallic acid tested on gram positive bacteria *Staphylococcus epidermidis* were utilized to compute the percentage of inhibition (Figure 4.3) .

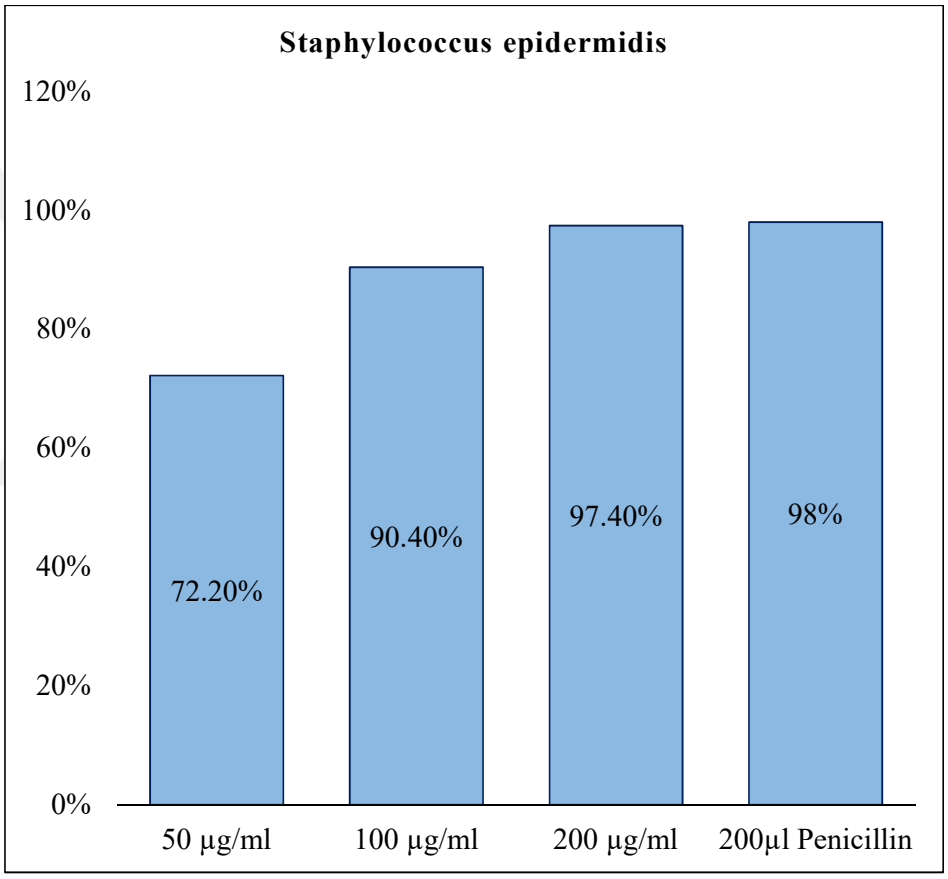

Figure 4.4 Growth inhibition rates of *Staphylococcus epidermidis*

Antibacterial effects of gallic acid on *Staphylococcus epidermidis* have been presented as percentage of inhibition (Figure 4.4). Gallic acid is an organic acid type biomaterial and bioactive element found in *A. nilotica* plant. The growth of bacteria was inhibited after being treated with gallic acid using three different concentrations. Penicillin as a positive control also inhibited the bacterial growth (see Figure 4.3) compared with negative control that contains bacteria only. The statistical analysis of averages of the absorbance values of the bacterial inhibition plotted in (Figure 4.5) for each

concentration of gallic acid including positive control at p=0.01 when compared with negative control all of them are significant (Figure 4.5).

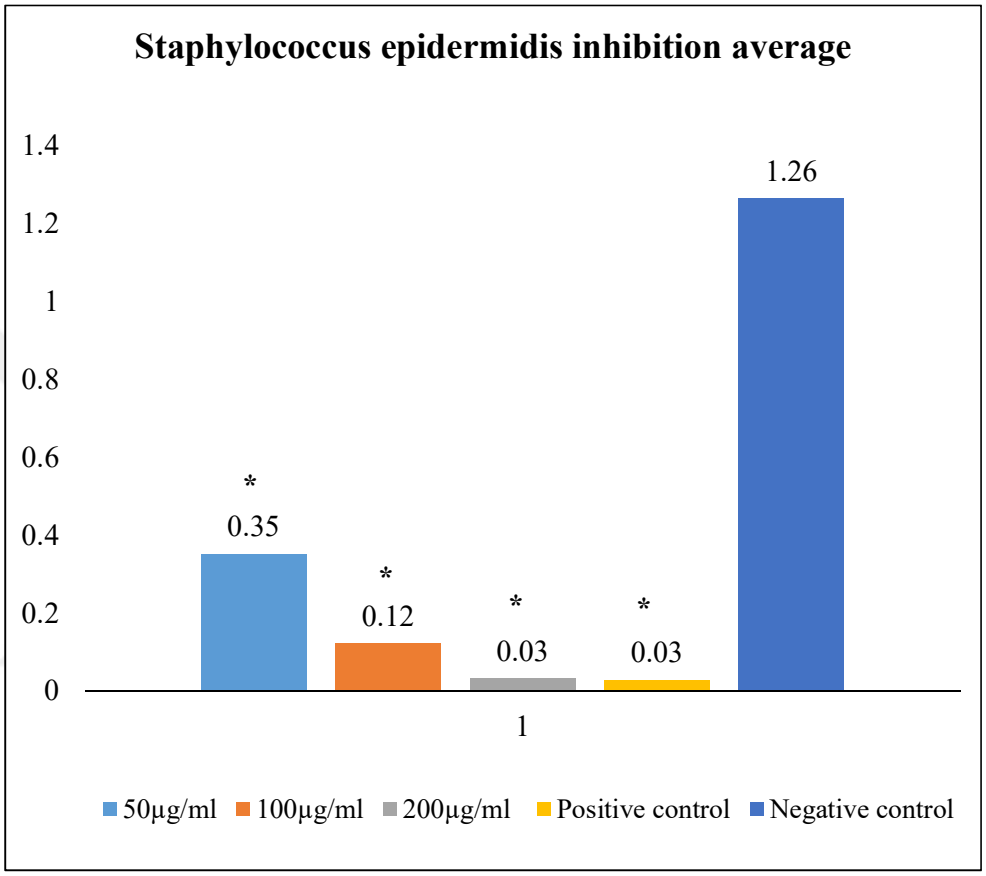

Figure 4.5 Inhibition averages of gallic acid concentrations, positive and negative controls

DISCUSSION

Even though plant bioactive components derived from medicinal plant, these compounds seem to contribute immensely to the deterrence of various kinds of infections. The most important aspect of consideration is the knowledge of each compound's safety and side effects to ensure their effectiveness in treating diseases. Gallic acid is an organic acid type and one of the bioactive elements found in *A. nilotica* plant.

The cell migration assay carried out in this study was done by means of culture – insert plate which is an *in vitro* cell migration model. It is known that cell migration plays a vital function during the proliferation and the remodeling phases of wound healing. The speed of fibroblast cell migration treated with gallic acid was significantly greater than that of untreated cells (control).

The result of cytotoxic test (Figure 4.2) has shown that gallic acid does not have any significant cytotoxic effect on NIH3T3 fibroblast cells. The most effective dosage of gallic acid that proved no cytotoxic effect on NIH3T3 cells was 50µg/ml and resulted in 93% cell survival rate which is very close to the control. The 100µg/ml concentration of gallic acid gave 80.1 % cell survival.

In the same way, the result of the antibacterial activity of gallic acid on *Staphylococcus epidermidis* demonstrated its effectiveness for inhibiting bacterial growth with high percentage compared to the positive control.

CONCLUSIONS

In this study, pure gallic acid has been investigated for its potential use as a wound dressing agent. Our results have shown that gallic acid at different concentrations exerted excellent *in vitro* non-cytotoxic activity on NIH3T3 cells indicating its biocompatibility. Also we have shown that gallic acid displays strong anti-bacterial activity (comparable to penicillin) against the gram positive *S. epidermidis* bacteria. Moreover, at certain concentrations such as 50µg/ml and 100µg/ml, gallic acid improves the speed of cell migration in NIH3T3 cells. The 100µg/ml has been found to be the optimum concentration of gallic acid which exerts high antimicrobial activity,

improves cell migration and shows almost no cytotoxic effect on in vitro wound models. Our results further indicates that gallic acid has immense potential as a novel wound dressing material alone or in conjunction with a other functional biomaterials and further investigations should be carried out to determine its other wound treatment related properties such as its effects on collagen production, genotoxicity and biodegradability.

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