EVALUATION OF SKIN REGENERATION & ANTIBACTERIAL PROPERTIES OF LEMON PEEL

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SEHAR MUNIR

ABSTRACT

Medicinal plants are believed to have wound healing abilities and are found to be very effective in antibacterial, antiviral, and anticancer activities. One of these plants with curative properties is the lemon. This study aims to evaluate the antibacterial and skin regeneration potential of lemon peel using two *E. coli* strains and albino rats. The extract in different concentrations of 20 µl and 40 µl was tested against DH5 alpha and BL21 strains, and clear inhibitory zones were observed in both groups. For *in vivo* investigations, the wound index measurements, sandwich ELISA, antioxidant assays, and histopathological tests of rats were conducted. The two groups of rats were given extracts at doses of 50 mg/ml and 100 mg/ml, and the results were compared to those of injured or untreated rats, which showed wound healing with high levels of VEGF in the treated groups, especially in the group with dose 100 mg/ml. Antioxidant values were found to be highly significant. And the histopathology of skin samples revealed that the skin of treated rats is healing effectively and is almost back to normal. These findings indicate that lemon peel could be very efficient for the treatment of burn wounds and bacterial infections.

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LIST OF ABBREVIATIONS

No.	Abbreviation	Full Form
1	VEGF	Vascular endothelial growth factor
2	VEGF-A	Vascular endothelial growth factor-A
3	ECM	Extracellular matrix
4	VBM	Vascular basement membrane
5	IMG	Intussusceptive microvascular growth
6	ELISA	Enzyme-linked immunosorbent assay
7	E. coli	Escherichia coli
8	LB	Luria Bertani
9	Plac	Placebo
10	PBS	Phosphate buffered saline
11	BSA	Bovine serum albumin
12	OD	Optical density
13	CAT	Catalase
14	GSH	Glutathione
15	SOD	Superoxide dismutase
16	PMS	Phenazine methosulphate
17	NBT	Nitro blue tetrazolium

18	NADH	Nicotinamide adenine dinucleotide
19	DTNB	5,50-dithiobis-(2-nitrobenzoic acid)
20	HRP	Horse reddish peroxidase
21	TMB	Tetramethylbenzidine
22	SEM	Standard Error of the Mean
23	ANOVA	Analysis of variance

CHAPTER 1

INTRODUCTION

Infectious bacterial disorders kill the majority of people across the world (1). The major source of serious infections in people is bacterial organisms of various types, including gram-negative and gram-positive bacteria. Because of their diverse natural niches, these microorganisms can live under extreme conditions (2). Synthetic antibiotics have several drawbacks, including the fact that they are expensive and out of reach for most people. And antibiotic resistance develops over time as microorganisms evolve. As a result, many antibiotics lose their efficacy against bacteria over time (3, 4). Additionally, antibiotics may cause undesirable effects on the body, such as hypersensitivity, immunological suppression, and allergic responses. Natural products, on the other side, have accomplished much in serving as a guide for the development of novel antibacterial drugs. Furthermore, the antibiotics obtained in this manner are biocompatible (5, 6). Bioactive plant extracts, as they are widely known, are a prospective source for the majority of medications (7). Berberine and quinine, for example, are plant-derived antibiotics that are particularly powerful against microorganisms (8).

Plants and their extracts with pro- or antiangiogenic activity can be used as possible medicinal agents for wound healing (9). Several herbal medicines have been demonstrated to be helpful in wound therapy, particularly burn wounds. The best burn wound healing activity was found in garlic, aloe vera, gotu kola, and sea buckthorn (10). The active ingredients in many plants that helped to close wounds were flavonoids, alkaloids, saponins, and phenol compounds. The main active components were glycosides, including madecassoside and asiaticoside, as well as proteolytic enzymes (11).

Lemon is a valuable plant that has therapeutic properties and belongs to the Rutaceae family. It is grown primarily for its anticancer, antifungal, and antibacterial alkaloids, which are efficient against clinically significant strains (12). Urinary tract problems, constipation treatment, loss of weight, healthy digestion, pile worms, stomach ulcer, breathing difficulties, and wound healing are also some of the health advantages of lemon (13). Lemon has antioxidant properties that have been proven in several

investigations to aid wound healing. Vitamin C (ascorbic acid) is also abundant in lemon, which immensely promotes wound healing. Through a good drug delivery route, it speeds up the healing of wounds through its anti-apoptotic and anti-oxidative properties (14).

A wound is caused by an external chemical or physical agent, injuring the cellular and anatomic continuity of live tissue. Closed and open wounds are the two types of wounds. Closed wounds do not harm the protective bodily surface, also the wounded tissue is not bared to the external world. However, the protective skin layer is breached by open wounds, allowing outside elements to enter the tissue and causing complications throughout the healing process (15). Non-healing cutaneous wounds have become a serious medical and societal burden in modern times. Wound healing is a challenging process that is heavily influenced by the severity as well as method of injury (16).

The wound healing process, in general, may be categorized into various phases, including maturation, proliferation, acute inflammation, and immediate hemostasis. Angiogenesis is a significant concomitant activity in the proliferation stage (17). By producing capillary sprouts, which invade the extracellular matrix (ECM) stroma by breaking down the endothelial cells after it has breached the underlying vascular basement membrane (VBM), it performs a key role in wound healing. These tube-like structures spread, split out, and form networks. The advancement of angiogenesis capillaries in the ECM is driven by endothelial cell proliferation, and chemotaxis from the target site directs growth in the desired way. Growth factors, inflammation, and Hypoxia are all involved in wound angiogenesis regulation (18).

The development of new vessels of blood from preexisting ones is called angiogenesis. This occurs in both disease and health or wound healing throughout one's life, starting from the womb and continuing until expiring (19). Any tissue in the body that is active metabolically is only some hundred micrometers far from a blood capillary, which is produced via the angiogenesis process. To exchange nutrients and metabolites by diffusion in all tissues, capillaries are necessary. Sprouting and intussusceptive angiogenesis can both produce these capillaries. Intussusceptive angiogenesis was found around two decades ago (20) and sprouting angiogenesis was found approximately 200 years ago, so it is better understood. Sprouting angiogenesis occurs

when endothelial cell sprouts grow in the direction of a stimulus (21), resulting in parts of the body that lacked blood vessels being able to develop them because of sprouting angiogenesis. In comparison, intussusceptive angiogenesis refers to the split that encourages the growth of blood vessels when interstitial tissue components enter already available capillaries to produce transvascular tissue structures that enlarge. It is believed that both types occur in practically all tissues and organs (22).

The management of angiogenesis' distinct steps is referred to as molecular regulation of angiogenesis. An angiogenic chemical increases endothelial cell migration, proliferation, and tube formation, whereas an inhibitor prevents the processes from occurring (23). The ability to stretch and thin endothelial cells, as well as movement of the endothelium layer, is crucial in intussusceptive microvascular growth (IMG). The activation of endothelial cell membrane fusion to create transcellular holes, as well as matrix proteolysis, collagen fibril formation, and peri-endothelial cell recruitment, are additional requirements (24).

Angiogenesis tissue activity is determined by the balance of several stimulating and inhibitory stimuli (24, 25). Various factor for growth, including VEGF (Vascular endothelial growth factor), have been discovered (26-28). VEGF stimulates angiogenesis and vascular permeability in endothelial cells. In a healthy adult, VEGF is also involved in the menstrual cycle, wound healing, angiogenesis, and embryonic development (29). It causes endothelial cells to produce and release hydrogen sulfide, which leads to endothelial cell proliferation, migration, and permeability, micro vessel development, and wound healing (30, 31).

Activated platelets produce many cytokines, including VEGF, during wound healing. For the natural inflammation response, VEGF draws circulating monocytes and neutrophils to the site of damage. Monocytes, keratinocytes, and endothelial cells also release it near wound, there it might effect capillaries (32). Additionally, VEGF increases infiltration by changing the proteins at the junctions between endothelial cells, which may help the formation of granulation tissue. Endothelial cell migration and proliferation are brought on by VEGF's activation of receptors. Besides encouraging angiogenesis, VEGF also contributes to the development of blood vessels by luring endothelial progenitor cells from the bone marrow (29). VEGF also encourages the formation of pericytes, which cover and maintain the blood vessels.

Furthermore, it stimulates epithelialization and collagen deposition in wounds (33). The inhibition of VEGF by neutralizing antibodies causes a reduction in fibroblast migration and a delay in wound healing (34). Uncontrolled angiogenesis and many heterogeneous abnormalities are caused by high levels of VEGF (35). The basic purpose of this study is to determine the potential of lemon peel for skin regeneration on acid burn rats, as well as its antibacterial capabilities by testing against *E. coli* bacterial strains.

RATIONALE

The purpose of this study was to identify the potential of lemon peel in repairing the skin and its antibacterial properties. Medicinal characteristics can be found in a variety of plants, including citrus. Citrus plants are highly nutritive having flavonoids, fiber, vitamin C, etc. All of these serve crucial roles in maintaining overall health, protecting against various diseases, and promoting the healing of wounds. Every part of these citrus plants needs to be thoroughly studied as they offer significant advantages and cause little to no harm.

AIM

"To study the potency of lemon peel for the regeneration of skin along with its antibacterial properties."

OBJECTIVES

- To learn the preparation of aqueous extract of lemon peel
- Evaluation of regeneration of skin in acid burn rats
- To learn the antibacterial properties of lemon peel

CHAPTER 2

LITERATURE REVIEW

2.1 Plant Overview:

The lemon, or *Citrus limon*, is an edible fruit that belongs to the citrus family. Citrus trees yield fruits in a variety of forms and sizes, and they are evergreen trees that are rich in fragrance, flavor, and juice (36). Lemons are typically oval in form and have a smooth, spongy surface. Some are smaller than grapefruits, while others are larger. Lemons are typically greenish yellow in colour when they are young, but as they grow larger, they become bright yellow. The floppies fruit contains tiny seeds (37). Lemon leaves are dark green in colour and range in size from 0.65 to 1 cm, placed alternately on the stem. The lemon has five fragrant white petals. The flower of the lemon comes from the cultivar 'Pink Lemonade.' This cultivar's leaves are variegated, and the fruit is striped (38).

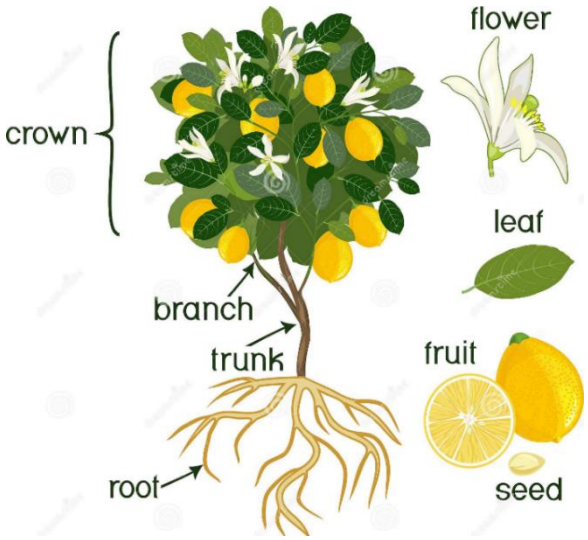


Figure 2.1: Lemon plant morphology (39)

Lemon plants were initially brought to southern Italy and then to Egypt and Iraq around several hundred years ago, lemons had spread throughout the Mediterranean region, and by 760–1297 A.D., they had been planted in China. In 1493, lemons were transported into island of Hispaniola and Spain. It was first produced in California in 17th century (40). Now, Argentina, Brazil, China, Italy, Iran, India, Mexico, Pakistan, Turkey, US and Spain are some of the countries where it is grown (13).

2.1.1. Chemical composition:

Lemons are thought to have certain health benefits and contain a variety of compounds. Lemons have abundant vitamin C and are incredibly beneficial to one's health. A hundred milliliters of citrus juice contain roughly 50 milligrams of vitamin C and five milligrams of citric acid. P, Zn, Mg, Fe, Cu, Ca, K, and Na are also included in it (41). Iron, copper, zinc, and manganese are essential nutrients that are widely employed in health, environmental research, and medicine. Potassium has long been recognised as a necessary ingredient in animal feed. When potassium's diet is deficient, young animals will not properly grow and can die in a matter of days. Calcium is in charge of bone production. Ca regulates a variety of cell processes and serves an important structural role in living organisms (42). Phosphorus is an essential nutrient for all cattle. Mg has been linked to the maintenance of nerve electrical potential and the activation of certain enzyme systems.

Table 2.1: Citrus fruits chemical composition (per 1 cup of edible portion) (43).

Component	*C. Sinensis*	*C. paradisi*	*C. reticulate*	*C. aurantifolia*	*C. aurantium*	*C. Limon*
Moisture (g)	88.4	88.5	87.8	84.6	87.6	85
Protein (g)	0.8	1	0.9	1.5	0.7	1
Fat (g)	0.3	0.1	0.3	1	0.2	0.9
Fibre, g	0.5	-	-	1.3	0.3	1.7
Carbohydrates (g)	9.3	10	10.6	10.9	10.9	11.1
Minerals (g)	0.7	0.4	0.4	0.7	0.3	0.3
Calcium (mg)	40	30	50	90	26	70
Phosphorous	30	30	20	20	20	10
Iron (mg)	0.7	0.2	0.1	0.3	0.3	2.3
Thiamine (mg)	-	0.12	40	0.02	-	0.02 (in juice)
Riboflavin (mg)	-	0.02	-	0.03	-	0.01 (in juice)
Niacin (mg)	-	0.3	-	0.1	-	0.01(in juice)
Vitamin C (mg)	50	-	68	63	30	39 (in juice)
Carotene, µg	-	-	350	15	1104	-
Energy, K cal	43	45	-	59	48	57

Bioactive nutrients such as phytonutrients are derived for human use from the plants. Lemon is one of the several citrus fruits that are high in phytoconstituents. Flavonoids, glycosides, coumarins, and sitosterol are all abundant in citrus fruit (44). Citrus fruits account for very minimum percent of daily carbs and calories. Polyethoxylated flavones are extremely important, yet they are uncommon in other herbs. Citrus fruits' fibre includes polyphenols, with vitamin C as the most essential one (45). Lemons also include a variety of phytochemicals, such as polyphenols and terpenes. Citric acid is extremely concentrated in them, as it is in other citrus fruits. Lemons include a variety of phytochemicals, including polyphenols and terpenes. Fellander, camhenium,

terpineol, citrain, citric, calcium oxalates, mucilages, carotenoids, vitamin C, flavonoids, and limonene are all components of this complex essential oil. Sugar, pectin, citric acid, malic acid, and flavonoids are plentiful (46).

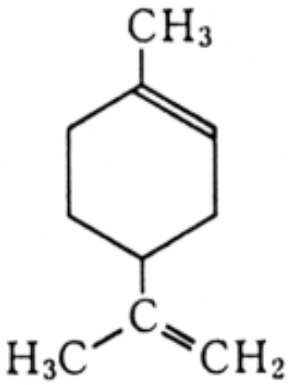


Figure 2.2: Chemical structure of Limonene (47)

2.2 Citrus limon properties:

Many species of citrus are known for their antibacterial, anticancer, hypoglycemic, anti-inflammatory, antioxidant properties because of their physiological, therapeutic, and pharmacological properties. The primary ingredient of lemon essential oil, citrus fruit peel's liquids, has been demonstrated in the studies to have a considerable relaxing effect via activating dopamine and serotonin receptors when supplied to mice by inhalation in concentrations of 0.5 percent and 1.0 percent. Furthermore, D-limonene, like indomethacin and hyoscine, has an inhibitory action on pain receptors (48).

2.2.1 Anticancer activities:

Inhibition of angiogenesis is a revolutionary cancer treatment method. Some plants might be employed as anti-angiogenic treatments and for cancer prevention (48). Flavonoids, carotenoids, dietary fibre, folate, and Vitamin C are found in citrus limonene and are thought to help prevent degenerative diseases and cancer. Citrus limonoids have antineoplastic properties in its extract since they include chemicals that protect cells from the damage that causes cancer (49).

2.2.2 Antioxidant activity:

Citrus juices and fruits are good sources of antioxidants, which include ascorbic acid, flavonoids, and phenolic compounds (50). Citrus peels are a good source of glycosides

and phenolic chemicals. The bioactive molecules include phenolic acids, which have antioxidant properties as well as a variety of other biological functions (51). Citrus fruits, such as lemon, have the highest antioxidant activity. Ascorbic acid, which is found in large amounts in various plants, can operate as an antioxidant. Other phytochemicals, such as glutathione, flavonoids, carotenoids, and different enzyme systems, might potentially play a role in antioxidant action (52).

2.2.3 Anti-ulcer activity:

A balance between offensive, e.g., *H. pylori*, pepsin, acid, and unsure features is involved in the pathogenesis of peptic ulcer. Many Indian medicinal plants, such as lemon and its derivatives, have been shown to be effective therapeutic agents for a variety of ailments, including treatment of ulcers. Aqueous extracts of fruits also has ulcer treating properties against stomach ulcers (53).

2.2.4 Anti-diabetic activity:

Polyphenols were found to significantly reduce weight, development of hyperlipidemia, insulin resistance, high blood glucose levels, and fat formation, all of these are hallmarks of obesity. Lemon and its peel contain a lot of polyphenols (54). Citrus peel has antioxidant and diabetes-lowering properties due to its high total polyphenol content (48).

2.2.5 Antimicrobial activity:

Lemon is an astringent as well as an antibacterial agent. This is a noteworthy observation since sebum may induce skin infections in specific types of skins, such as micrococcus, and Pseudomonas when released in excess. It may operate as a predisposing factor in other skin illnesses, such as acne, for a period of time. Citrus juice, when applied directly to the skin, can help to prevent certain disorders and maintain the skin healthy (12).

Lemon also prevents several harmful disorders, such as cancer, cardiovascular diseases, and stroke by deactivating free radicals. Its antibacterial and astringent characteristics contribute to its therapeutic abilities. Lignan et al. observed that when applied to the location of some insect stings and bites, lemon juice helps to alleviate the poison and discomfort (55). Oguwike et al applied lemon juice to rat wounds, they discovered that the test rats' bleeding and clotting times were reduced when corelated to the other rats

from control group (56). Ahmad et al. found that ministration through mouth with lemon juice extract enhanced healing of wounds and tissue growth in injured diabetic rats, and that total protein and hydroxyl proline levels indicated that collagen synthesis was significantly greater (57). In addition to wound healing/regeneration properties, lemon has antibacterial, antioxidant, and anticancer properties as well. By obtaining swabs from persons with pimples and using lemon juice on them, Shinkafi et al. discovered that lemon juice has a stronger antibacterial impact than the conventional cleansers used for the treatment of acne vulgaris (58). Canon et al. used an essential oil extracted from lemon to combat bacteria found in fish. The crude extract of naturally occurring lemon strongly reduced the growth of many fish pathogens (*C. freundii, L. anguillarum, A. hydrophila, and Y. ruckeri),* indicating that it has a stronger antibacterial effect (59).

2.3 Burn Wounds:

Burn injuries are completely different from other wounds, they have their own medical subspecialty. Despite the fact that significant burns predominantly affect a particular organ, they affect practically all bodily systems, making them a widespread illness. Burns require the intervention of an intensivist and a physician in a way that no other severe wound does (60). Burns are categorised based on their causal agent: Thermal burns, Electric burns, Radiation caused burns, Lasers caused burns, Chemical or Acid Burns. Each form of burn wound in the above categorization is distinct from the others, as well as from other types of injuries. In addition to that all of these burns cause skin damage, the local and systemic care for each of the aforementioned burns differs (61).

By absorption or inhalation, a large variety of acids can induce ocular and cutaneous burns as well as systemic consequences, necessitating medical and/or surgical treatment in most cases (62). Almost 24,000 chemicals are frequently used in household cleaning, agriculture, industry, and other areas, and a lot of them are recognised as potentially harmful to human health. Chemical burns are therefore a significant hazard in both the home and the workplace. In the household setting, awareness of the potential impact of these agents is minimal, whereas it is frequently underestimated in the industry (63). In last few years, there was an upsurge in the usage of chemical agents in domestic violence aggressions, primarily against women, spraying them on their faces and bodies, resulting in significant and disfiguring burn sequelae, but the illegal use of

chemicals to assault others is not unusual. On the other hand, the use and fear of chemical weapons has risen because of international instability in various regions of the globe, with numerous armed conflicts. In recent years, studies have revealed a preponderance of white phosphorous-related injuries (64). Dermal burns from chemicals make up a moderate but noteworthy fraction of wounds, with documented occurrences of about 11% and have been linked to about 31% of all deaths from the burns (65). Cutaneous chemical burns can cause plenty of problems for the physician dealing with them. It's not always easy to judge burn depth and deciding whether or not to excise the wound early isn't always easy either (66).

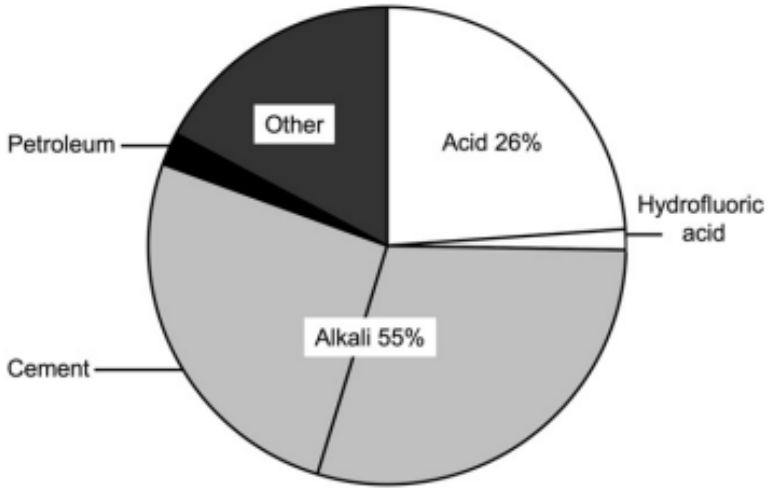


Figure 2.3: Different chemicals which cause cutaneous burn (67)

Protein denaturation is a typical side effect of all types of burns. Chemical injuries, on the other hand, differ significantly from thermal burns Thermal injuries are typically brought on by very brief exposure to extremely hot temperatures that are abruptly stopped, while acid injuries are more likely to be brought on by longer contact with chemicals (66). In addition, there are some significant biochemical distinctions between them. Biological proteins have a specific structure that is made by van der Waal's forces and hydrogen bonding, as well as a specific amino acid sequence. External forces may readily disturb these three-dimensional structures, which are critical for the biological function of proteins. Heat or chemicals, particularly pH changes, can cause the structures to disintegrate (68).

Protein coagulation is quick in thermal injuries due to irreversible cross-linking events, but protein degradation is continued in chemical burns by other mechanisms, mostly

hydrolysis. As long as remnants of the offending substance are present, especially in deeper layers, these processes may persist. Chemical agents can also have a systemic effect if their components are distributed throughout a person, potentially causing toxicity. The amount of burning agent, penetration, and time of skin contact, all are responsible for a chemical burn injury severity (65).

Heat not only harms skin on a local level, but it also has several systemic impacts. These alterations are unique to burn wounds and are rarely seen in wounds generated by other types of traumas. The heat effect and damage cause a generalized increase in capillary permeability. Plasma leaks from capillaries into the interstitial spaces because of this. High capillary permeability and the resulting leak of plasma last for up to 48 hours, with the first 8 hours being the most intense (69). By 2 days, capillary permeability has been thrombosed and are no longer in circulation or either returned to normal. Because of the loss of plasma in burn patients, the body has low levels of extracellular fluid.

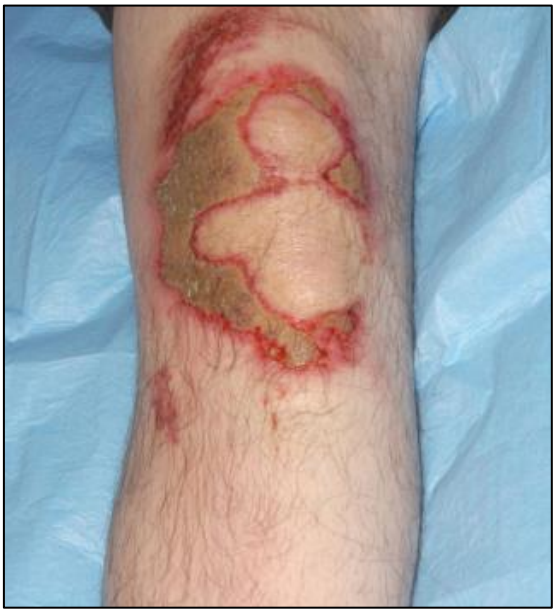
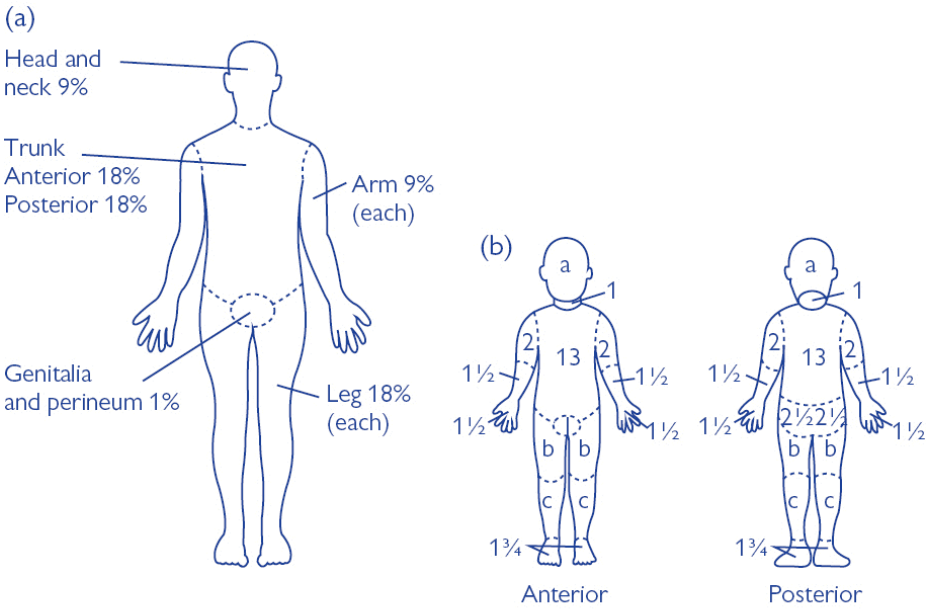


Figure 2.4: Burn injury caused by cement, 48 hours after injury (67)

The fluid lost amount will be determined by severity of the burns (70). In grown-ups, Wallace's rule of '9' determines the burns surface area, whereas in adolescents and children, Browder's chart is used (71). If not properly resuscitated, high burns can result in low level of extracellular fluids. There is a maximum conceivable fluid loss in burns involving half of the body, and this remains the same even if more than half of the body is burnt. This widespread rise in capillary infiltration has never been observed in any other wound (72). Due to inflammation, there is only a limited response at the wound

site, resulting in chronically increasing vasodilation and oedema. Blood loss causes hypovolemic shock in other large traumatic wounds that need full blood replacement right away. In the case of severe burns, 100% of the blood is replaced within 2 days (60).



Relative percentage of body surface area (% BSA) affected by growth

	Age				
Body part	0y	1y	5y	10y	15y
a = ½ of head	9½	8½	6½	5½	4½
b = ½ of one thigh	2¾	3¼	4	4¼	2½
c = ½ of one lower leg	2½	2½	2¾	3	3¼

Figure 2.5: (a) Wallace rule (b) The Browder and Lund's chart (71)

The following are the different types of burn wounds based on how much skin and deeper tissues are involved:

- Skin is inflamed without blistering in a **first-degree burn**.
- **In Second-degree burns** the upper layer and varying thicknesses of the skin is involved.
    i. Second-degree superficial—vesication and inflammation are visible in the dermis because of the papillary dermis.
    ii. Second-degree deep—eschar development is visible due to the involvement of the deep bottom layer of the dermis.
- **The Third-degree burns**, also known as full-density burns, are characterized by development of crust (73, 74).

Blisters form in second-degree superficial burns as plasma collects between the skin layers. This fluid contains high fluid protein and is an excellent growth medium for bacteria and other organisms, but it should not be kept for lengthy periods of time. Blisters can also form in wounds caused by repetitive contact, such as from shoe bites, which can be thought of as a broader spectrum of friction burns (75). Closed wounds may also include fluid-filled blebs, which might indicate an underlying fracture or hematoma. When skin dries, it becomes nonelastic, leathery and forms the crust. Because the blood vessels proximally are compressed by circumferential eschar across the extremities, distal vascularity may be compromised (76).

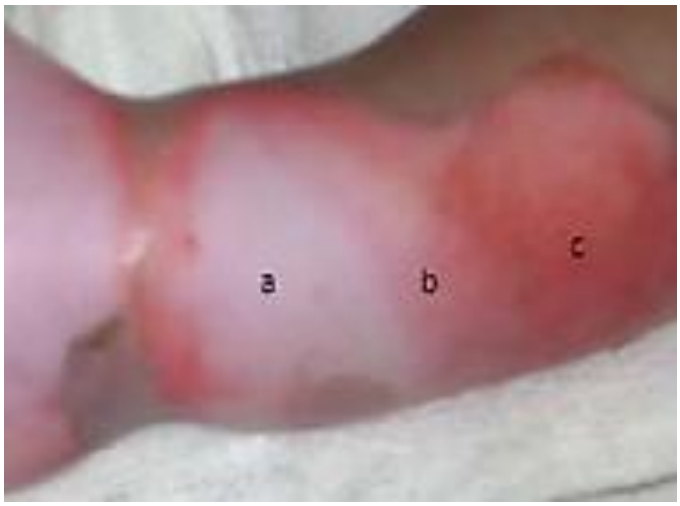


Figure 2.6: Jackson's three zones on a scald burn i.e., (a) coagulation zone, (b) stasis zone, (c) hyperaemia zone (60)

In injured charred tissue, Jackson (1959) identified three zones.

- Coagulation zone central: the middle part of the burns with complete clabber mortification.
- Stasis zone: This is located on the outskirts of the clabber zone. This zone's movement is slow, but it can be restored with prompt and appropriate resuscitation and careful wound care.
- The hyperaemia zone is on the outskirts of the stasis zone. It's the outcome of severe vasodilation, which can be noticed during the inflammatory phase following trauma. This returns to normal after a while (77).Infections, particularly drug-resistant infections, are common in burn wounds (78), resulting in higher mortality, longer greater hospital expenditures, and delayed wound healing (79). Strong immune reactions to infections can result in blood

poisonings by bacteria, which slows the healing of wounds by lowering blood pressure and decreasing blood flow to end organs such as the skin. Furthermore, because multiorgan failure and sepsis are the major reasons of mortality in injured patients, infection prevention and control are a top priority in their care (80-82). It is difficult to diagnose an infection early and accurately. Because the diagnostic value of procalcitonin in burns is uncertain, the most commonly employed tests are white blood cell count and C-reactive protein (83).

Systemic infections and mortality have steadily declined with the development of antibiotics such as silver sulfadiazine and mafenide, as well as early grafting and excision (81, 84, 85). However, bacterial infections, both Gram -ve and Gram +ve, are one of the leading reasons of death after an injury. Cultures of bacteria can help with antibiotic resistance, especially in situations of bacterial drug resistance. However, burns have different pharmacokinetic properties, so the dose should be adjusted properly to enhance antibiotic efficacy (86). Invasive fungal infections do not have effective topical antimicrobials, and fungal wound infections are linked to higher fatality rates in major burns (87). Because of its high mortality, a forwarding wound inflammation necessitates a quick treatment, frequently with histopathology, and wound excision (88).

2.4 Wound Healing:

First- and second-degree superficial burns can be healed by focusing on this intention. Second-degree superficial burns can be healed by the mucosa, which is plentiful in the inner skin layer. Within 5-7 days, the wound heals completely, and there are practically no scars. Healing occurs by secondary intention in third and second-degree wounds, which involves contractions and epithelization (89). At the area of injury, treatment using therapeutical plants, extensive study has been conducted. According to Fabricant and Farnsworth, since at least 60,000 years, medicinal plants have been widely recognised in medicine (90). Plants were employed to stimulate wound healing in many ancient civilizations. Many plants including Cinnamon, Neem, Aloe vera, *Anethum graveolens* (91), Eucalyptus, *Securigera securidaca*, Fenugreek, Lotus, Chamomile, Bael (92), *Astragalus membranaceous*, Indian mulberry, Lucidone, Genistein, Asiaticoside, Curcumin was studied for their anti-inflammatory, antibacterial, antimicrobial, and regeneration potential, as reviewed by the Reza Farahpour (93).

The wound healing phases are maturation (remodeling), proliferation (reparative), inflammatory (reactive), hemostasis (blood clot). The only variation is the time of each stage, which is the same for all sorts of wounds (94). The early steps following damage are intended to achieve blood clotting sometime after injury through a series of extracellular proteolytic activities aimed at limiting blood loss. (95). In this cascade, a series of catalytically inactive zymogens are transformed into fully active, catalytically active serine proteases, leading to fibrin clot formation and platelet activation. Platelet activation forms the growth factors, including immunological mediators and platelet-derived growth factors, which activate the immune response and initiate the inflammation phase of injury healing (96).

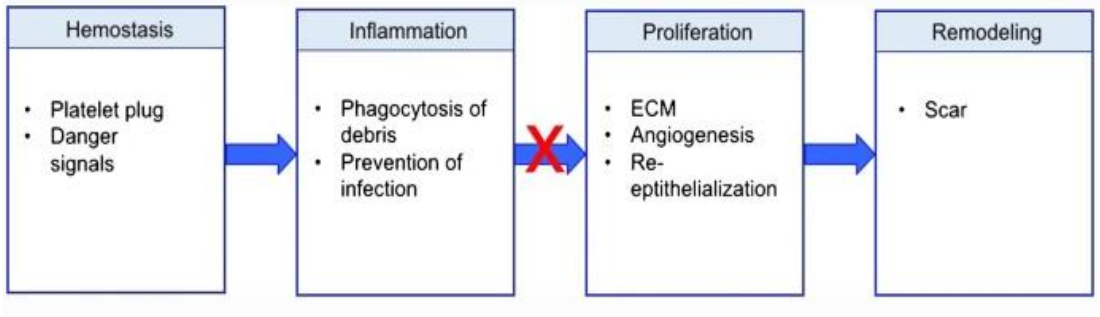


Figure 2.7: Four Phases of wound healing (96)

All burn wounds go through the same inflammatory phase. The body's inflammatory response, which includes vascular and cellular components, begins very immediately after the damage. Local vasodilation occurs shortly after burns, with fluid extravasation into the third space. Increased capillary permeability may be widespread in severe burns, resulting in significant plasma extravasation that needs fluid replenishment (97). The initial cells to travel to the place of injury are monocytes and neutrophils. Later, neutrophils begin to decline, and macrophages take their place. Chemotactic factors such as kallikreins and fibrin peptides generated during the coagulation process, as well as chemicals secreted by mast cells such as cytokines, leukotrienes, proteases, histamine, and tumour necrosis factor, cause these cells to migrate. The poisons generated by the burnt cells, cleaning of dead tissue and phagocytosis are aided by the cellular response (98).

Restoration of epithelium begins in the form of keratinocyte movement from the skin, sometime after injury in partial thickness burns and normally fills the lesion within a week (99). The lower membrane arises in the middle of the epidermis and dermis during

re-epithelialization. Dermal restoration is aided by angiogenesis and fibrogenesis. After primary excision and grafting, delayed primary intention heals severe burns. The recovering phase of injury healing involves the placement of the skin grafts following first excision (100).

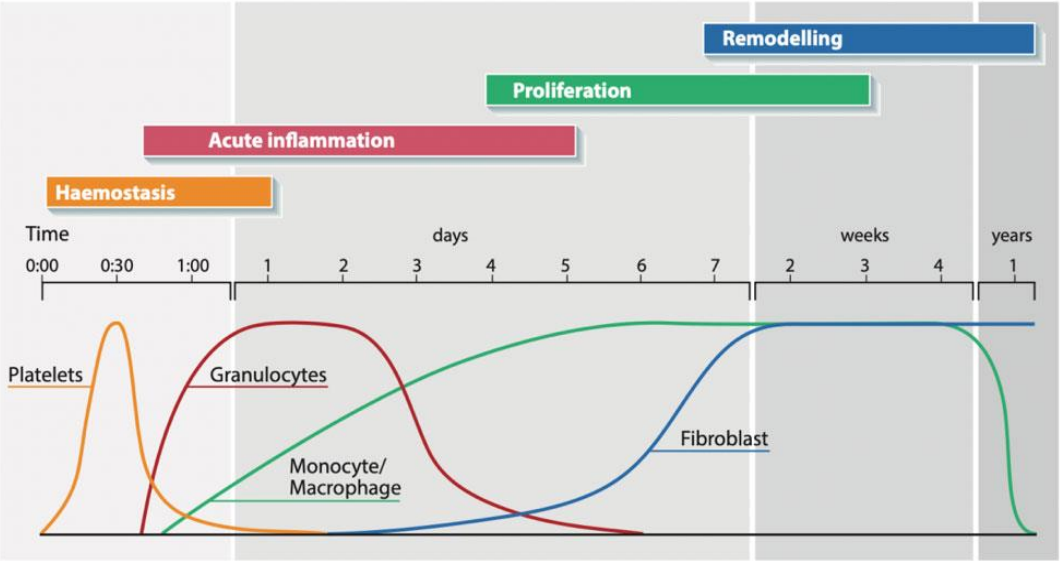


Figure 2.8: Time duration of different phases of healing wounds (109)

The remodeling is the last stage of recovery, during which the scar or graft matures. Initially, threadlike proteins such as elastin and collagen are used as extracellular matrix around the smooth muscle, endothelium and epithelium during the last phase of wound healing (101). This matrix rebuilt into blister tissues later in the resolution phase, and fibroblasts gets the newly formed fibroblast appearance, which causes the blister contraction. This stage is causes contractures and hypertrophic scarring in second-degree full thickness and deep dermal burns that are permitted to recover by themselves (102). It can take years. The hyperactive reaction of melanocytes to burn damage causes hyperpigmentation in superficial burns, whereas the loss of skin cells in the appendages causes hypopigmentation in deep burns. Once innervation begins in skin-grafted regions, the developing nerves disrupt melanocyte regulation, resulting in hypopigmentation of the skin in the white-skinned individuals and hyperpigmentation of the graft in dark-skinned (60).

Although angiogenesis has a crucial function in the healing of wounds, it has received little attention. However, according to Ribatti et al., in the late 1900s, significant progress was achieved in our knowledge of angiogenesis, with Judah Folkman's work

being particularly essential (103). Several cells of inflammation that colonised the injury area were also highlighted to be an angiogenic factor source by Sidkey et al. and Polverini et al (104, 105). Sidky & Auerbach, for example, found capillary proliferation in the skin of mice during a local graft-vs.-host reaction, implying that immunocompetent donor lymphocytes were responsible for this angiogenic response (105). By using the clear cornea of a rabbit, Fromer et al. discovered that ocular neovascularization caused by silver nitrate injuries was consistently coupled to a neutrophil-rich infiltration, implicating macrophages in angiogenesis (106-108).

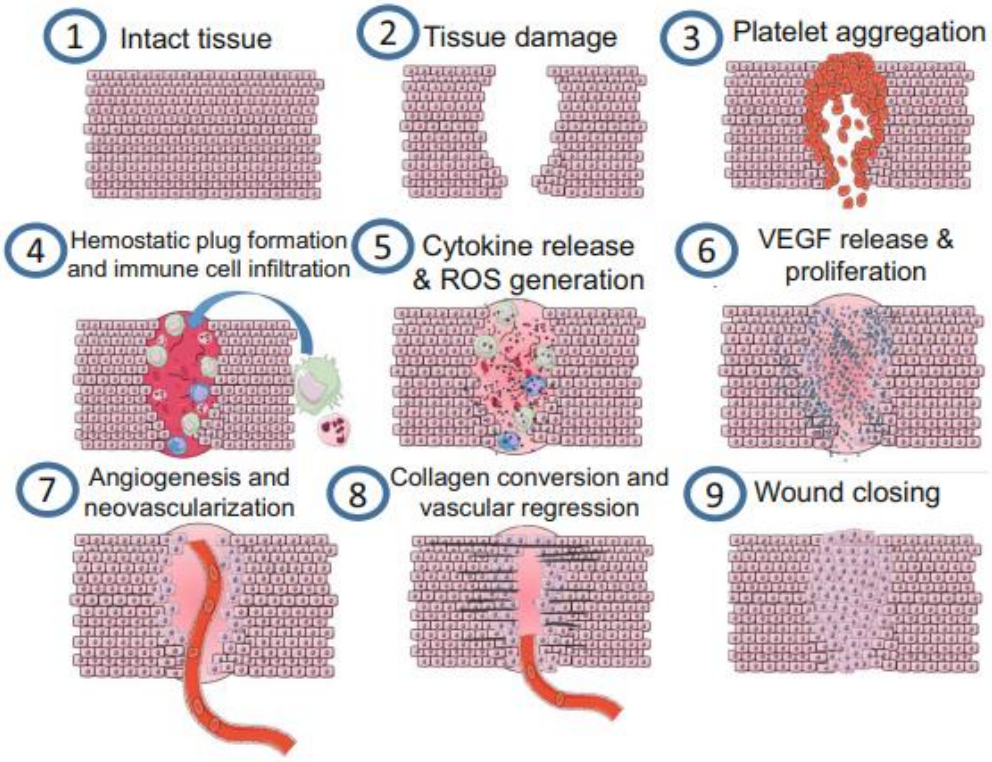


Figure 2.9: Angiogenesis and VEGF role in wound healing (116)

According to Pollard, angiogenesis is influenced by macrophages in a variety of ways. They promote sprouting angiogenesis and impact new vessel branching. They also shape and remodel them (110). Angiogenesis was first studied experimentally in the middle of the last century by Algire and Ide et al. (111-113). In the last few years, angiogenesis studies have flourished a lot. Because, according to Adair, Montani et al., angiogenic treatments have the potential to heal wounds, treat cancer, heart diseases, and many other life-threatening disorders (22).

Folkman and Haudenschild were the first to identify angiogenesis in vitro, when they noticed spontaneous organising of capillary endothelial cells into capillary-like

structures after their prolonged cell cultures (114). The cheek pouch of the hamster, ear chamber of the rabbit, chorioallantoic membrane of chick embryo, and eye of the rodent have all been used to examine angiogenesis in vivo and have yielded significant results as described by Ribatti et al. (115).

Ferrara and Henzel published a paper in June 1989 describing the extraction of VEGF from bovine pituitary folliculostellate cells, which proved to play an crucial part in angiogenesis (117). Two investigations into the embryonic development of the mouse's vasculature in 1996 established importance of the VEGF. Between days 11 and 12, only one allele of VEGF was inactivated, causing mortality of the embryo (118, 119). Nissen et. al detected rise in the amount of VEGF when a healing wound enters the phase of proliferation (30).

CHAPTER 3

METHODOLOGY

3.1 Plant powder preparation:

Lemon grows in Pakistan's four provinces, but Punjab produces most of the crop. It is easily available almost all year and has antibacterial, antifungal, anticancer, and many other benefits as well (13). Fresh lemons were taken from the market and washed with tap water. Their juice was extracted, and the peels were shade dried before being ground into a fine powder and stained with 80-mesh sieves.

Figure 3.1: Shade dried lemon peels

3.2 Aqueous extract preparation:

An aqueous extract was prepared by dissolving 10g of lemon peel powder in 100ml of water and allowing it to sit for 48 hours before filtering through Wittman filter paper (120). All the water-soluble fractions present in the solution were evaporated. The residual content was weighed and stored in falcon tubes at low temperatures for future experiments.

Figure 3.2: Solution of lemon peel powder and water

3.3 Bacterial culture:

Bacterial strains of *Escherichia coli* DH5 Alpha, SCC 2197 and BL21 (DE3) were utilized. These bacteria are available in the Zoology Laboratory, Kinnaird College for Women.

3.3.1 Preparation of inoculum:

For the inoculum of E. coli, six test tubes were used, and each was filled with 5 mL of LB (Luria-Bertani) broth. Bacteria were allowed to grow overnight in this LB broth (121).

3.3.2 Petri dish preparation:

Each petri dish was divided into 8 different compartments from the outside with the help of a marker. After bacterial culture development, these compartments were filled with varying amounts of antibiotic samples and control groups.

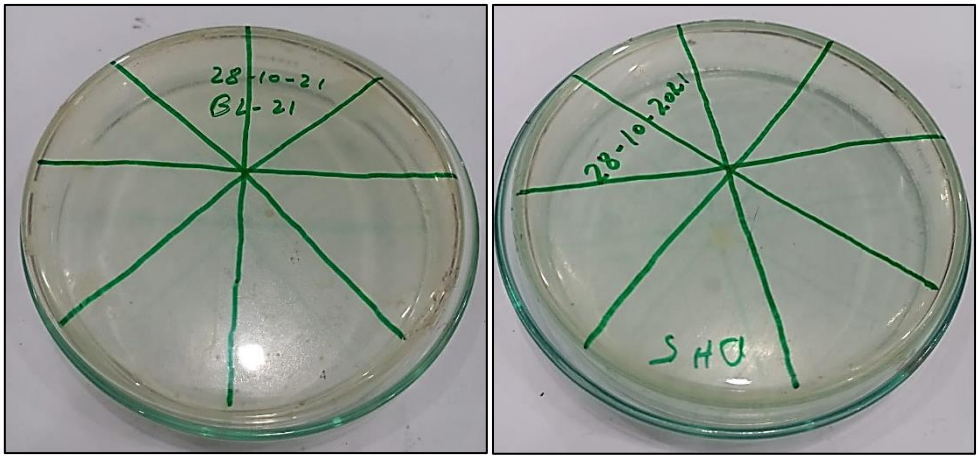


Figure 3.3: BL21 and DH5 petri dishes divided into eight compartments

3.3.3 Disc diffusion method:

LB agar was sterilized in the autoclave for 15 mins at 121 °C, cooled at room temperature, and then poured into sterilized Petri plates for microorganism testing. The medium was swabbed with the DH5 alpha and BL21 inoculum. The discs, which had a diameter of 6 mm, were placed on the surface of the medium using sterile forceps.

Figure 3.4: Sterilizing LB agar and other equipments in the autoclave (left), placing filter papers on the petri dish (right)

3.3.4 Treatment of bacteria:

A 100µl micropipette was used to load the aqueous extract and control groups. The experiments were carried out at various extract and control group concentrations (20µl, 40µl). After inverting the petri dishes, they were incubated for one day. Then the zones were observed in millimeters (mm) (122).

Table 3.1: Samples and control groups incorporated in the 8 compartments of petri dish

Group 1	Normal
Group 2	Injection water 20 µl
Group 3	Ampicillin
Group 4	Plac-20 µl
Group 5	Treated-20µl
Group 6	Plac-40 µl
Group 7	Treated-40 µl
Group 8	Injection water 40µl

3.4 Animal Model:

The healthy albino rats weighing about 200 to 250 grams were raised at the “Molecular Medicine Research Centre (CRIMM) at the Molecular Biology and Biotechnology

Institute (IMBB)", University of Lahore. The usage of these animals in the research was approved by the Ethical review committee of Pakistan.

Figure 3.5: Albino rats

3.5 Grouping of rats:

Albino rats were brought and divided into 6 groups with n=3 in each group. One group of rats were not given any injury while the other 5 groups were given acid burn injury.

Table 3.2: Categorization of rats

No.	Group Name	Group detail
1	Normal rats	Rats given no injury
2	Untreated rats	Injured rats left untreated
3	Placebo (Plac) 1	Injured rats treated with 50 mg/ml placebo
4	Treated 1	Injured rats treated with 50 mg/ml aqueous extract
5	Placebo (Plac) 2	Injured rats treated with 100 mg/ml placebo
6	Treated 2	Injured rats treated with 100 mg/ml aqueous extract

3.6 Acid burn injury in rat:

Rats have also been used in experiments since 1960 because of their genetic, anatomical, and physiological similarities with humans (123). Rats were divided into

six groups. The normal rats with no burn injuries were dissected, and their skin was stored in formalin. The remaining 5 groups were shaved after they had been given ketasol mixed with precedex. The filter paper was soaked in acid, and second-degree burn injuries were given to two different areas of the rat's shaved body.

Figure 3.6: Injecting ketasol to rat (left), Giving burn injury to rats (right)

3.7 Treatment of rats:

One of these five rat groups was not given any treatment. Two groups were treated with the aqueous extract of lemon peel in different concentrations (50mg/ml and 100mg/ml), and normal saline in different concentrations (50mg/ml and 100mg/ml) was given to the remaining two groups, with the help of gavage tube. All the rats were closely monitored to ensure that their wounds did not become infected, and their injuries were examined (124, 125).

Figure 3.7: Gavage feeding rat with extract

3.8 Wound index Measurement:

In wound index measurement, all of the observations were recorded during the wound healing process to see if the lemon peel powder was acting effectively (126). At the first day of the experiment, the burn injury was measured and recorded. Its size, appearance, and other parameters were observed every day during the healing process. The wound and lemon powder's progress were tracked daily. Every day, photographs of the wounds were taken, and a sketch of the wounds was drawn by using a transparent sheet.

3.9 Collection of skin & serum samples:

After 7 days, all the rats were dissected and their skins were collected and stored for histopathology tests while serum was prepared from their blood, which was stored for sandwich Elisa (127).

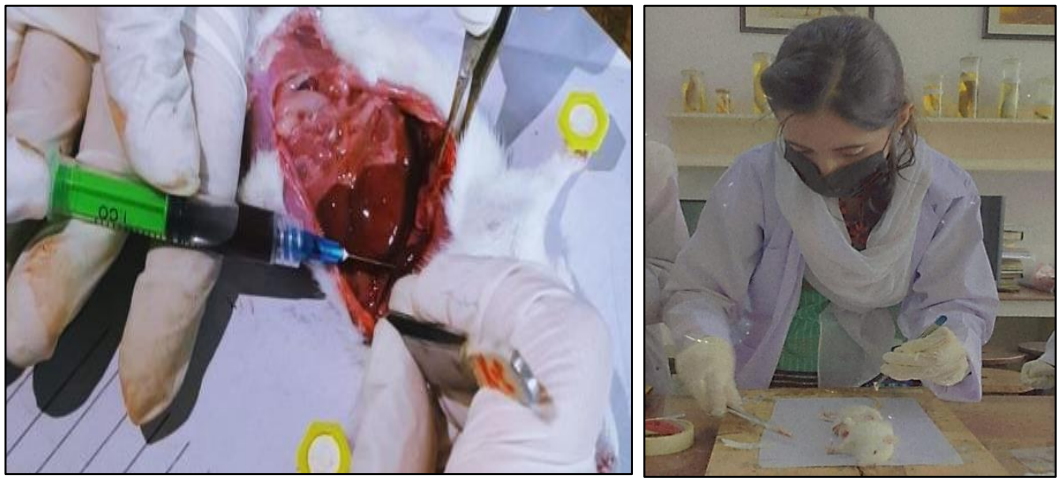


Figure 3.8: Blood withdrawal from the heart of rat (left), Injured skin sample collection after 7 days (right)

3.10 Sandwich ELISA:

In a microtiter plate, a sandwich ELISA (enzyme-linked immunosorbent assay) for annexin V and VEGF was conducted. Upon coating the plates with 100µl annexin V and 100µl VEGF antibodies, respectively, they were incubated for two hours. Then these wells were washed three times with phosphate buffered saline (PBS) with the help of a micropipette. After that, 100µl bovine serum albumin (BSA) was applied for half hour to prevent the antigens and antibodies from binding non-specifically to the wells. Then, each well was filled with a 100 µL sample (serum obtained from the blood of

treated rats) and incubated for one hour. The samples were separated, and wells were washed thrice with PBS before being incubated for 2 hours at 37°C with a horse reddish peroxidase (HRP) conjugated donkey anti-rabbit secondary antibody. Again, the walls were three time washed with PBS and, after 15 minutes of washing 100 μL of chromogenic solution 3, 3, 5, 5 TMB (tetramethylbenzidine) was added to it and later 0.18 M sulphuric acid was added to halt the reaction, and at 450nm, absorbance was taken (128, 129).

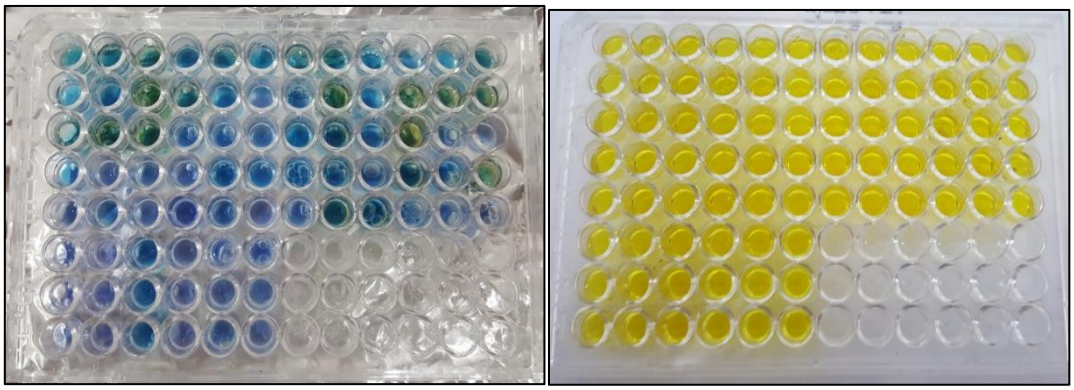


Figure 3.9: Addition of TMB to ELISA plate (left), After adding H_2SO_4 to stop the reaction (right)

3.11 Antioxidant analysis:

3.11.1 Estimation of catalase:

Catalase activity was monitored (130) by mixing 0.1 ml serum, 0.1 ml phosphate buffer (10mM, pH 7.0), and 0.4 ml H_2O_2 (0.2 M). The addition of 2.0mL dichromate acetic acid reagent halted the reaction. Samples were incubated for 10 minutes in a boiling water bath, cooled, and their absorbance was measured at 530nm (131).

3.11.2 Estimation of glutathione:

In a test tube, 0.5 ml serum, 2.0 ml disodium hydrogen phosphate buffer (0.3 M), and 0.25 ml (5,50-dithiobis-(2-nitrobenzoic acid) or DTNB) (0.001 M) were combined (132) to measure the amount of reduced glutathione. The absorbance was measured using a spectrophotometer at 412 nm after 15 minutes of incubation (131).

3.11.3 Estimation of superoxide dismutase:

The superoxide dismutase (SOD) activity was measured (133) by mixing 0.1 ml serum with 1.2 ml sodium pyrophosphate buffer (52 mM, pH 8.3), 0.1 ml PMS (phenazine

methosulphate) (186 lM), 0.3 ml NBT (Nitroblue tetrazolium) (300 lM) and initiating the reaction with 0.2 ml NADH (Nicotinamide adenine dinucleotide) (750 lM). By adding 0.1ml glacial acetic acid after 90 seconds of incubation at 30 C, the reaction was halted. 4.0 mL of n-Butanol was used to vigorously agitate the reaction mixture. The mixture was incubated for only 10 minutes before centrifuging for 5 minutes at 2,000 rpm. At 560 nm, absorption of the upper butanol layer was examined (131).

3.11.4 APOX assay:

One millilitre of the reaction mixture was comprised of 100 mM KH_2PO_4 buffer (pH 7.0), 0.5 mM ascorbate, and 0.3 mM H2O2, with the remainder being serum. After 3 minutes, OD290 was taken (as ascorbic acid oxidation was assessed as a reduction in absorbance at 290 nm for 3 minutes). The enzymatic activity was measured in enzyme units per gramme of fresh weight (U g-1 FW). One unit of enzyme is equal to the quantity required to degrade 1 μmol of H2O2 per minute at 25°C (134).

3.12 Histopathology test:

The stored skin samples from all six groups of rats were sent to histopathology labs for a thorough examination of the skins. Dehydrated skin samples were embedded in wax and sliced with a microtome (5um). To stain the skin segment and evaluate skin architecture, hematoxylin and eosin stain were used (135).

3.13 Statistical Analysis:

The statistical analysis was done through graph pad software by using all the quantitative data acquired from several experimental groups.

CHAPTER 4

RESULTS

4.1 Plant powder:

The shade-dried lemon peels were grounded and sieved to form yellow colour plant powder weighing 120 grams.

Figure 4.1: Grounded lemon peel powder

4.2 Aqueous extract:

The mixture of weighted lemon peel powder and water was filtered after 2 days and then evaporated to obtain the extract of lemon peel.

Figure 4.2: Filtered lemon peel solution

4.3 Antibacterial property of Lemon peel:

The purpose of this assay was to determine the antibacterial properties of the lemon peel powder. The lemon peel powder extract was tested against the *E. coli* strains DH5 alpha and BL21, and the obtained results showed that lemon peel has antibacterial properties.

Table 4.1: Observed antibacterial activity

No.	Groups	Antibacterial activity
1	Normal	-ve
2	Injection water 20 µl	-ve
3	Ampicillin	+ve
4	Plac-20 µl	-ve
5	Treated-20µl	+ve
6	Plac-40 µl	-ve
1	Treated-40 µl	+ve
2	Injection water 40µl	-ve

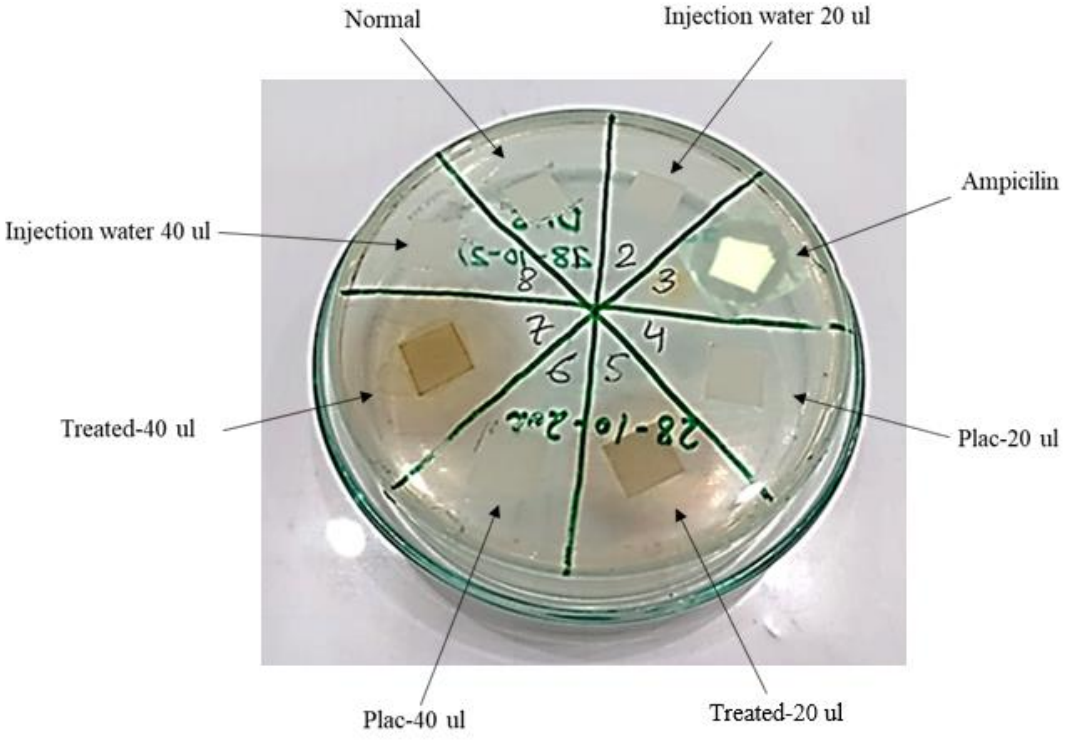


Figure 4.3: DH5 alpha petri dish showing inhibition zones in treated and ampicillin group

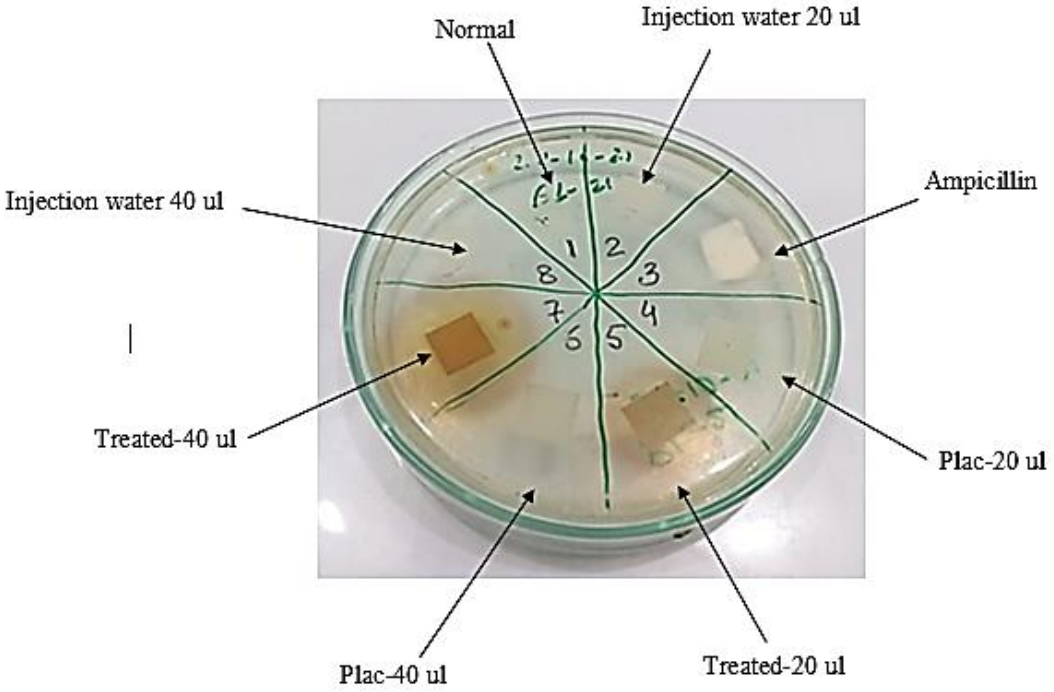


Figure 4.4: BL21 petri dish showing inhibition zones in treated and ampicillin group

4.4 Wound Index Measurement:

Wound shrinkage was observed in the treated groups of rats; the 100 mg/ml-dosed rats performed significantly better than the 50 mg/ml-dosed rats. while the wound index increased in the untreated and placebo groups of rats.

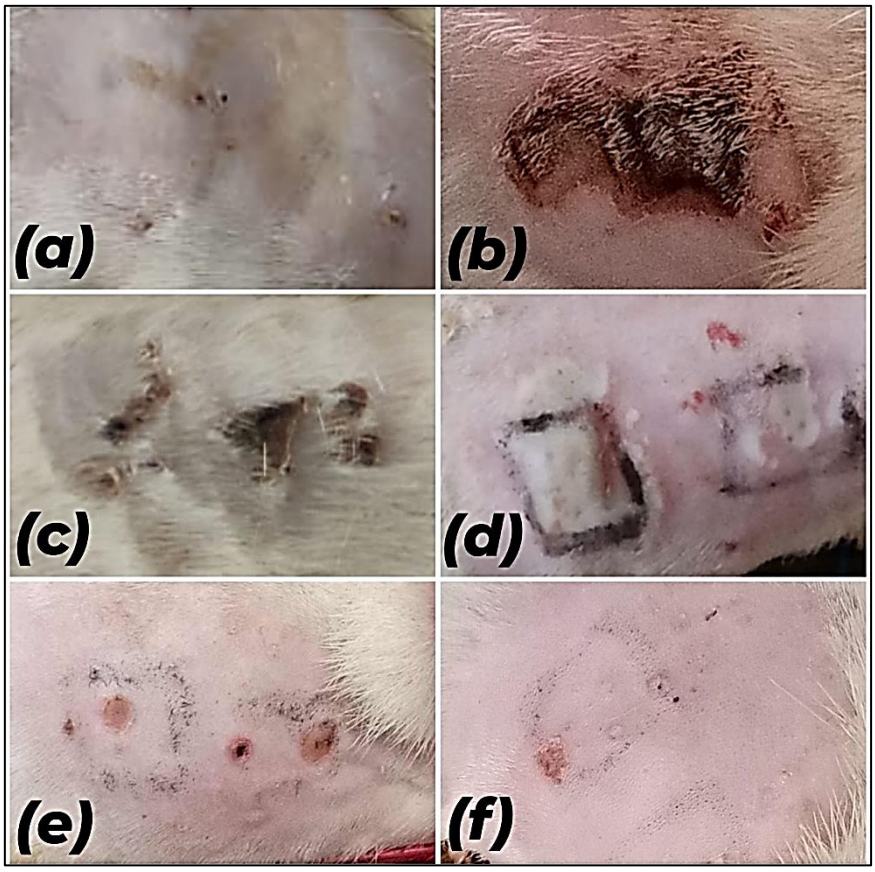


Figure 4.5: Wound measurement of all groups; (a) Normal skin of rat, (b) Injured and untreated rat skin, (c) Placebo (50 mg/ml) given rat skin, (d) Placebo (100 mg/ml) given rat skin, (e) Treated rat skin with dose 50 mg/ml, (f) Treated rat skin with dose 100 mg/ml

The graph data result indicates that the rats treated with lemon peel extract exhibit significant increase in wound contraction when compared to injured rats, and the results were estimated by applying one-way ANOVA using graph pad.

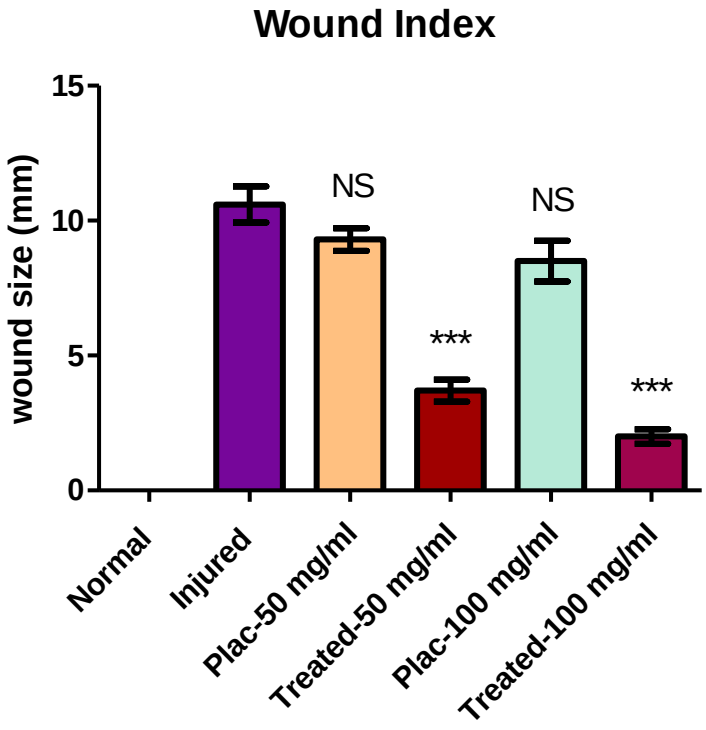


Figure 4.6: Wound index levels in treated groups of rats with selected doses of Lemon peel extract decreases as compared to injured groups of rats. Asterisk symbol *** indicates high statistical significance in results ($P < 0.001$). The mean ± SEM is used to express the values.

Table 4.2: Graphical results values of Wound index expressed in mean ± SEM

WOUND INDEX	**Normal**	**Injured**	**Placebo 50 mg/ml**	**Treated 50 mg/ml**	**Placebo 100 mg/ml**	**Treated 100 mg/ml**
	0.00±0.00	11±0.67	9.3±0.42	3.7±0.40	8.5±0.76	2.0±0.26

4.5 ELISA VEGF:

The graph data result indicates that the rats treated with lemon peel extract exhibit significant value of VEGF when compared to injured rats, and the results were estimated by applying one-way ANOVA using graph pad.

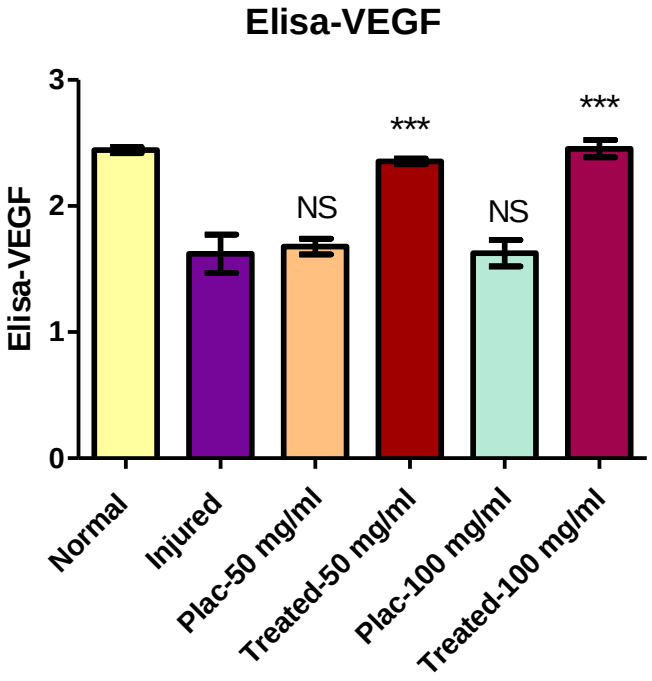


Figure 4.7: VEGF levels in treated groups of rats with selected doses of Lemon peel extracts increases as compared to injured groups of rats. Asterisk symbol *** indicates that the results are highly significant ($P < 0.001$). The mean ± SEM is used to express the values.

Table 4.3: Graphical results values of ELISA VEGF expressed in mean ± SEM

ELISA VEGF	Normal	Injured	Placebo 50 mg/ml	Treated 50 mg/ml	Placebo 100 mg/ml	Treated 100 mg/ml
	2.4±0.025	1.6 ±0.15	1.7±0.063	2.4±0.022	1.6±0.11	2.5±0.069

4.6 ELISA Annexin V:

The graph data result indicates that the rats treated with lemon peel extract exhibit decreased value of annexin V when compared to injured rats, and the results were estimated by applying one-way ANOVA using graph pad.

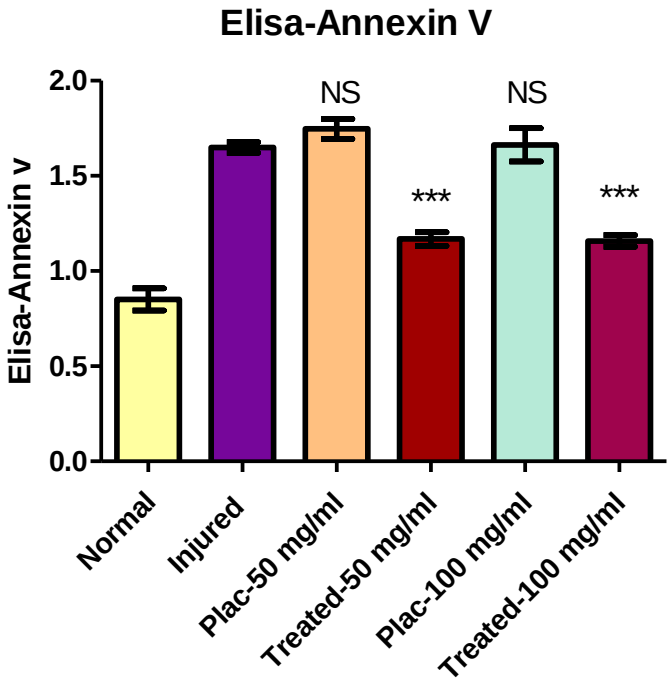


Figure 4.8: Annexin V levels in treated group of rats with selected doses of Lemon peel extract decreased as compared to injured group of rats. *** indicates that the results are highly significant ($P < 0.001$). The mean ± SEM is used to express the values.

Table 4.4: Graphical results values of Annexin V expressed in mean ± SEM

Elisa Annexin-V	Normal	Injured	Placebo 50 mg/ml	Treated 50 mg/ml	Placebo 100 mg/ml	Treated 100 mg/ml
	0.85±0.058	1.6±0.029	1.7±0.052	1.2±0.036	1.7±0.087	1.2±0.030

4.7 Antioxidant Analysis:

4.7.1 Estimation of Catalase:

A biochemical assay which is responsible for finding out the estimation of CAT levels in Lemon peel.

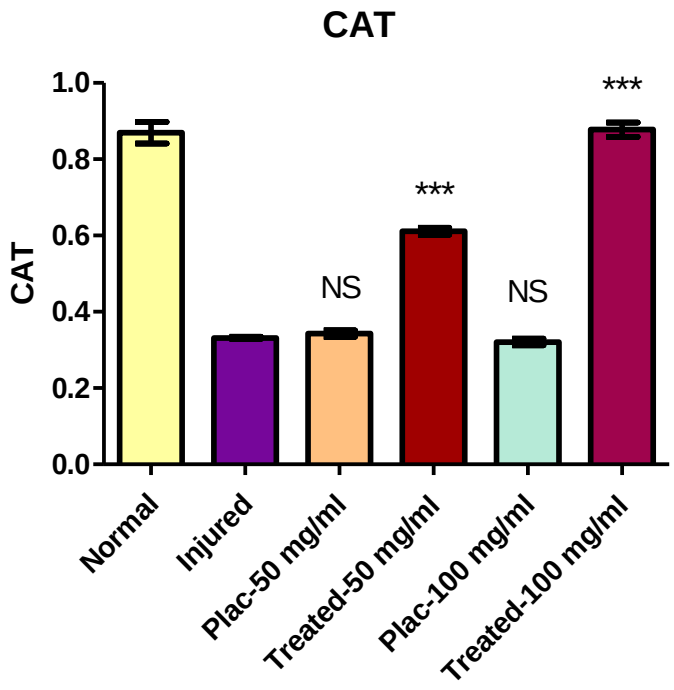


Figure 4.9: CAT levels in treated groups of rats with selected doses of Lemon peel extract in comparison to injured group of rats. *** symbols indicates that the results are highly significant ($P < 0.001$). The mean ± SEM is used to express the values.

Table 4.5: Graphical result values of CAT assay expressed in mean ± SEM

	Normal	Injured	Placebo 50 mg/ml	Treated 50 mg/ml	Placebo 100 mg/ml	Treated 100 mg/ml
CAT	0.87±0.028	0.33±0.028	0.34±0.082	0.61±0.091	0.32±0.088	0.88±0.019

4.7.2 Estimation of Glutathione:

A biochemical assay which is responsible for finding out the estimation of GSH levels in Lemon peel.

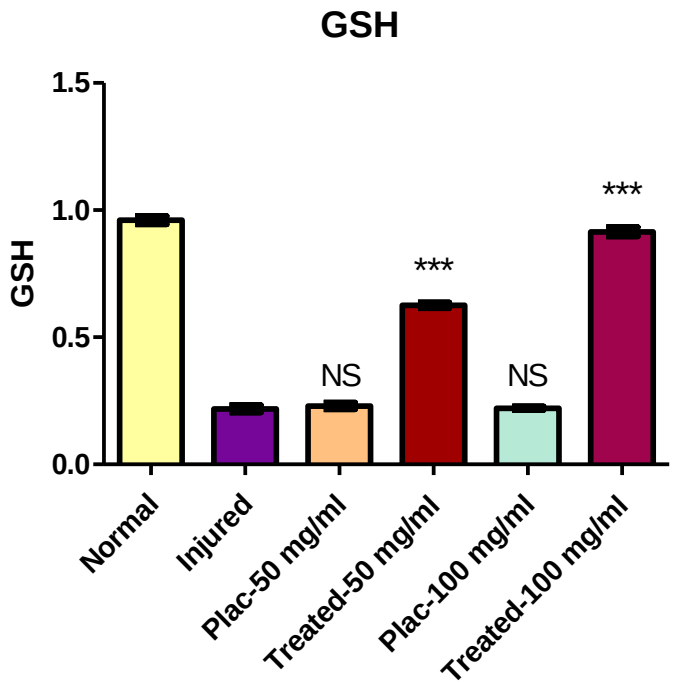


Figure 4.10: GSH levels in treated groups of rats with selected doses of Lemon peel extract in comparison to injured group of rats. *** symbols indicates that the results are highly significant ($P < 0.001$). The mean ± SEM is used to express the values.

Table 4.6: Graphical result values of GSH assay expressed in mean ± SEM

	Normal	Injured	Placebo 50 mg/ml	Treated 50 mg/ml	Placebo 100 mg/ml	Treated 100 mg/ml
GSH	0.96±0.016	0.22±0.014	0.23±0.013	0.63±0.011	0.22±0.0067	0.91±0.018

4.7.3 Estimation of Super oxide dismutase:

A biochemical assay which is responsible for finding out the estimation of SOD levels in lemon peel.

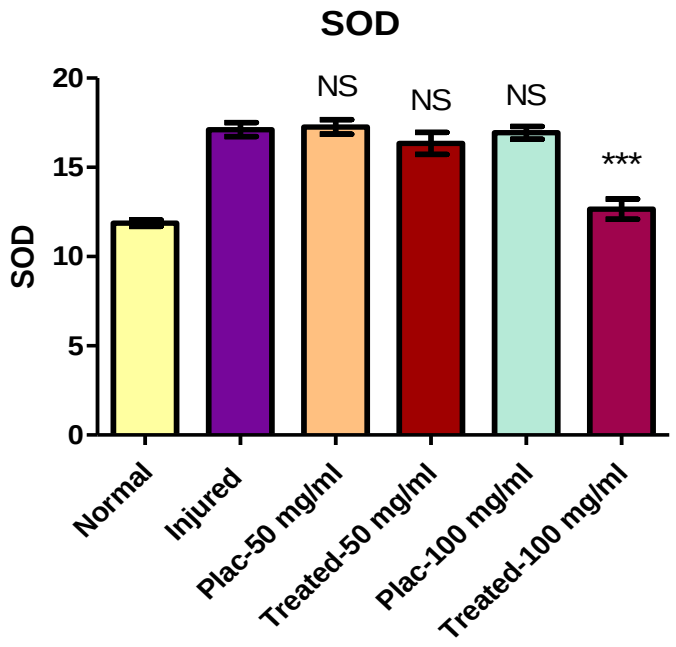


Figure 4.11: SOD levels in treated groups of rats with selected doses of Lemon peel extract in comparison to injured group of rats. *** symbols indicates that the results are highly significant ($P < 0.001$). The mean ± SEM is used to express the values.

Table 4.7: Graphical result values of SOD assay expressed in mean ± SEM

SOD	Normal	Injured	Placebo 50 mg/ml	Treated 50 mg/ml	Placebo 100 mg/ml	Treated 100 mg/ml
	12±0.18	17±0.39	17±0.41	16±0.62	17±0.36	13±0.57

4.7.4 Estimation of APOX:

A biochemical test which is responsible for finding out the estimation of APOX level in Lemon peel.

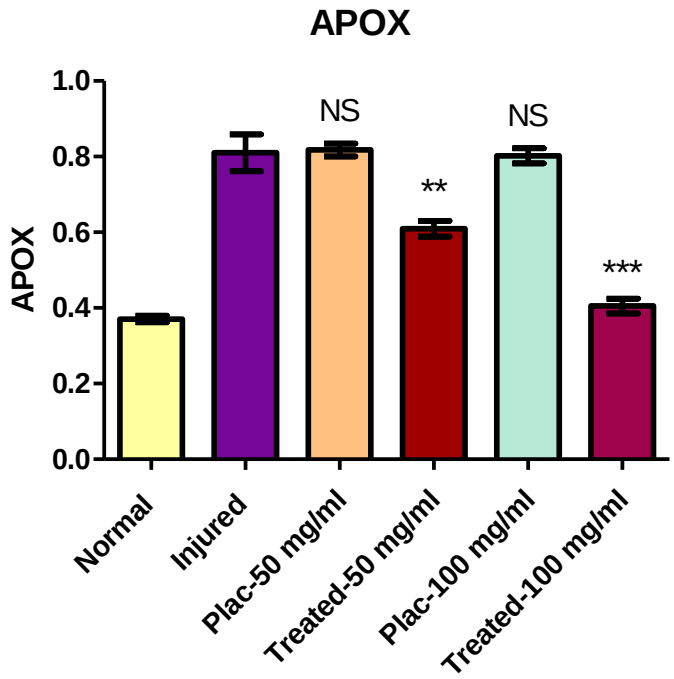


Figure 4.12: APOX levels in treated group of rats with selected doses of Lemon peel powder in comparison to injured group of rats. *** indicates that there is high statistical significance in the results ($P < 0.001$). The mean ± SEM is used to express the values.

Table 4.8: Graphical result values of APOX assay expressed in mean ± SEM

	Normal	Injured	Placebo 50 mg/ml	Treated 50 mg/ml	Placebo 100 mg/ml	Treated 100 mg/ml
APOX	0.37±0.0090	0.81±0.049	0.82±0.017	0.61±0.021	0.80±0.020	0.41±0.020

4.8 Histopathology:

4.8.1 Group 1: Normal group:

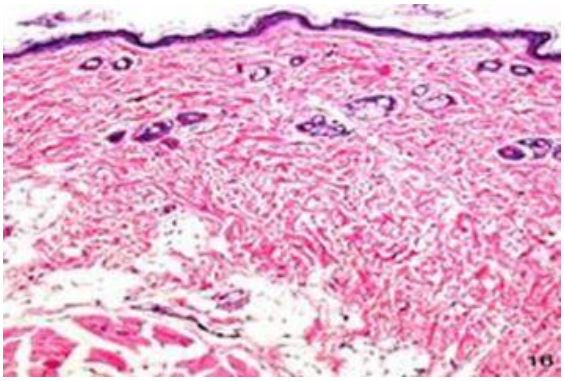


Figure 4.13: Histopathological diagram of normal skin obtained from rats

The two main layers of skin in the diagram are the epidermis and dermis. There is the hypodermis, a subcutaneous fascia located deep within the dermis. Skin thickness is normal. This demonstrates that skin architecture is normal in the group with normal skin.

4.8.2 Group 2: Injured group:

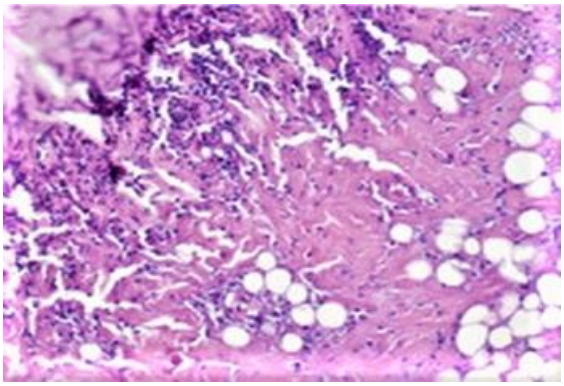


Figure 4.14: Histopathological diagram of injured skin obtained from rats

The diagram shows a burn wound, which exhibits an inflammatory reaction and fast oedema production. Skin thickness is impaired. Infiltration of inflammatory cells can be seen.

4.8.3 Group 3: Placebo group 50 mg/ml:

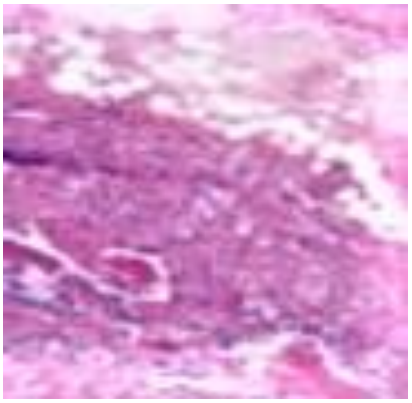


Figure 4.15: Histopathological diagram of skin obtained from placebo group 50 mg/ml of rats

The diagram shows a burn wound, which exhibits an inflammatory reaction and fast oedema production. Skin thickness is impaired. Infiltration of inflammatory cells can be seen.

4.8.4 Group 4: Treated group 50 mg/ml of Lemon Peel powder:

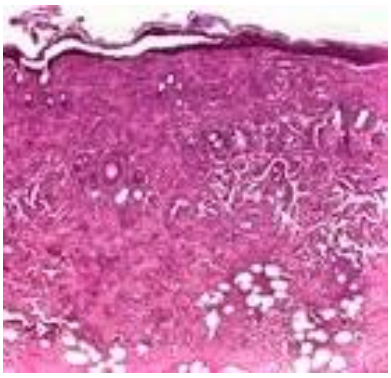


Figure 4.16: Histopathological diagram of skin obtained from treated group 50 mg/ml of rats

In the diagram above, using 50 mg of Lemon peel powder had a minor effect on wound healing. There is a little infiltration of inflammatory cells. The recovery process has begun.

4.8.5 Group 5: Placebo group 100 mg/ml:

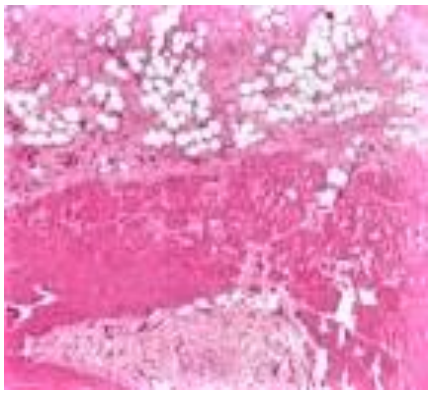


Figure 4.17: Histopathological diagram of skin obtained from placebo group 100 mg/ml of rats

The diagram shows a burn wound, which exhibits an inflammatory reaction and fast oedema production. Skin thickness is impaired. Infiltration of inflammatory cells can be seen.

4.8.6 Group 6: Treated group 100 mg/ml of Lemon peel powder:

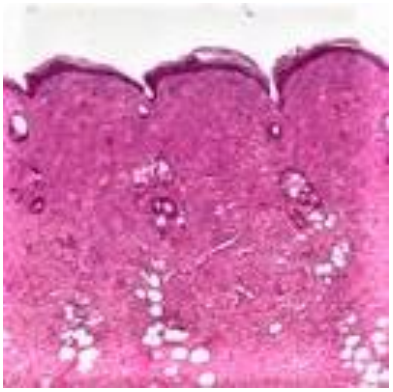


Figure 4.18: Histopathological diagram of skin obtained from treated group 100 mg/ml of rats

Lemon peel powder dosed at 100 mg was used as a cure for wounds. As observed in the above diagram, the morphology is really closed to being normal. The skin's structure is smooth. Rapid burn healing is ensured by the release of several substances by dendritic cells, which speed up early cell proliferation. Therefore, drugs that enhance dendritic cell activity are regarded as drugs for burn wound treatment.

CHAPTER 5

DISCUSSION

Antibacterial drug resistance in microbial pathogens is becoming a major concern globally. In hospitals, the number of infections caused by commercially available antibiotic-resistant isolates has risen dramatically in recent decades. It can often be difficult to control the spread of these bacteria in scientific settings since there are several acquired and inherent mechanisms of antibiotic resistance (136). A lot of focus has been placed on extracts and physiologically active substances that have been extracted from well-known plant species. In developing nations, the use of medicinal plants is essential for meeting basic health needs. And these plants may provide a new supply of antibacterial, antifungal, and antiviral compounds that have potent activity against infectious microbes (137, 138). Lemon is one such plant, it is an antioxidant that neutralizes free radicals, protecting against many harmful illnesses like cancer, stroke, and cardiovascular disease. Additionally, it combats pathogens. It aids in the generation of white blood cells and antibodies in the blood that fight off invasive microbes and stop infections. Its antibacterial and astringent qualities may be the cause of its healing abilities (139).

In this study, lemon peel extract exhibited strong antibacterial activity against two *E. coli* strains, DH5 alpha and BL21. When varied doses of extract (20 µl and 40 µl) and several other control groups were applied to the medium, distinct inhibitory zones were clearly observed in both the treated and ampicillin groups, demonstrating that natural products have the potential to replace the currently available drugs, which are causing antibacterial resistance and have many other drawbacks.

Medicinal plants also have wound healing abilities. The process by which wounded tissue regenerates or is repaired is referred to as wound recovery (140). A multi-step process known as wound healing involves antioxidant immunity and epithelialization, which recover damaged cell structures and tissue layers through overlapping hemostasis, inflammatory, proliferative, and maturation levels. Numerous herbal compounds can aid in the treatment of wounds and can help in their healing, according to the reported articles (141).

For *in vivo* studies on lemon peel extract, second-degree burn wounds were given to 5 groups of rats, and 1 group was kept normal. Among the five injured groups, group one was injured and left untreated, and the other two groups were given placebos in amounts of 50 mg/ml and 100 mg/ml, respectively, while the remaining two groups were given oral lemon peel extract in doses of 50 mg/ml and 100 mg/ml body weight. The burn wound reduction was observed in both the extract-treated groups, especially in the group with 100 mg/ml extract concentration. One way ANOVA was applied to their wound index measurements which showed highly significant values with p value < 0.001, confirming the wound-healing capabilities of lemon peel.

For further investigation, all 6 groups of rats were dissected, their skins were collected and stored for histopathological analysis, and the serum was obtained from their blood to perform sandwich ELISA for the evaluation of VEGF and Annexin V markers. The absorbance of plates was taken using an ELISA reader, and a one-way ANOVA was applied to the readings, which showed an increase in VEGF and a very clear decrease in annexin V in the treated groups, with high significance. VEGF plays an important role in angiogenesis and regeneration (142). Several antioxidant assays were also performed on the rats' serum, and one-way ANOVA was used to analyze the results. CAT, GSH, SOD and APOX levels in the serums of treated groups were compared to the injured group, the results obtained were highly significant with p value < 0.001. Antioxidants are believed to help regulate oxidative stress in wounds and hence promote wound healing (143).

The histopathology results for the normal group of rats displayed the normal structure of all layers of skin. The results for the rats in the injured group indicated inflammation, impaired skin thickness, and inflammatory cells that were integrated into the burned skin. The structure of the skin was completely damaged. Skins treated with 50 mg/ml and 100 mg/ml placebos demonstrated the same damaged skin structures. The histopathology results of the treated group of rats revealed that the skin thickness is rebuilding, and that the skin's overall structure is gradually returning to normal.

All these findings indicate that lemon peel has antibacterial properties and could be very effective for the regeneration of the skin.

CONCLUSION

With all the experiments and proven results, it is clear that lemon peel powder does have antibacterial and skin regeneration properties. The initial testing of alternative doses of lemon peel extract on DH5 alpha and BL21 strains confirmed the antibacterial properties of lemon peel by showing clear inhibition zones, particularly at the concentration of 40µl. For wound healing potential investigation, wound index measurements of burn injuries, sandwich enzyme-linked immunosorbent assays, antioxidant assays on serum, and histopathological tests on the skin of acid-burned and normal rats were conducted. With varying amounts of extract, it demonstrated skin regeneration by repairing skin layers and increasing VEGF levels, especially at 100 mg/ml dose. The confirmation of the antibacterial and skin regeneration properties of lemon peel extract opens new doors for further testing of the plant to treat burn wounds and bacterial disorders with natural products instead of synthetic ones.

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