ANTIFUNGAL AND INJURY REGENERATION POTENTIAL OF GREEN TEA

BSc Zoology

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RESEARCH COMPLETION CERTIFICATE

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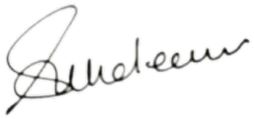

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ABSTRACT

Green tea has beneficial effects on human health including anticarcinogenic, antiviral, antibacterial and antifungal activity. The purpose of this study is to evaluate the wound healing and antifungal ability of green tea plants in acid burn rats and *Candida albican*. The antifungal activities were done by ditch plate method. The antifungal potential of green tea extract on Candida albican established remarkably positive results by demonstrating the inhibitory zone at a level of 40 ul. Biochemical assays such as antioxidant tests were conducted. ELISA protocol was used to evaluate the angiogenesis and the antibodies used were Annexin-V and VEGF. *Camellia Sinensis* plant extract showed remarkable wound healing capacity with elevated VEGF levels at a dosage of 100 mg/ml and decreases apoptosis level by using the same concentration. The ability of green tea extract to treat wounds was also proven by the histopathological examination. The wound index was measured to study the wound healing power of green tea extract and the results were significant. The study depicts that wound healing was observed after green tea plant treatment.

TABLE OF CONTENTS

Chapter Titles

Research Completion Certificate i
Anti-Plagiarism Declaration… ii
Acknowledgments… iii
Abstract iv
Table of Contents… v
List of Figures… vi
List of Tables… vii
List of Abbreviations… viii
1 Introduction 1
Rationale 4
Aims and Objectives 5
2 Literature Review 6
2.1 Plant Overview 6
2.1.1 Chemical Composition 7
2.2 Camellia Sinensis Properties: 8
2.2.1 Antimicrobial Activity 8
2.2.2 Antifungal Activity 8
2.2.3 Antioxidant Property 9
2.2.4 Antiviral Property 9
2.2.5 Antaging Property 10
2.2.6 Antiparkinson Effect: 10
2.2.7 Cardiovascular Effect: 10
2.3 Burns Wound 10
2.4 Wound Healing and Angiogenesis 11

2.5 Wound healing & VEGF 13

3 Methodology 16

3.1 Plant Powder Preparation: 16

3.2 Aqueous Extract Preparation: 16

3.3 Assessment of antifungal activity 16

3.3.1 Preparation of culture media for the study 16

3.3.2 Ditch plate method 16

3.4 Animal Model: 17

3.5 Ethical Clearance 17

3.6 Grouping of Rats with *Camellia Sinensis* 17

3.7 Acid Burn Injury in Rats: 18

3.8 Sampling after seven days: 20

3.9 Treatment of Rats with *Camellia Sinensis* 21

3.10 Wound Index Measurement Protocol 21

3.11 Enzyme-Linked Immunosorbent assay (ELISA) 21

3.12 Estimation of antioxidants analysis 22

3.1.1 Catalase measurement: 22

3.1.2 Glutathione measurement: 22

3.1.3 SOD measurement: 22

3.1.4 Apox measurement: 22

3.13 Histopathology Assay 23

3.14 Statistical analysis 24

4 Results 23

4.1 Plant powder: 24

4.2 Aqueous extract: 24

4.3 Antifungal Assay.....25
4.4 Wound index:.....26
4.5 ELISA VEGF.....29
4.6 ELISA Annexin V:.....30
4.7 Antioxidant Analysis.....31
4.7.1 Estimation of APOX.....31
4.7.2 Estimation of SOD:.....32
4.7.3 Estimation of GSH:.....33
4.7.4 Estimation of CAT:.....34
4.8 Histopathology.....35
4.8.1 Group 1: Normal group.....35
4.8.2 Group 2: Injury group.....35
4.8.3 Group 3: Placebo 1 group.....36
4.8.4 Group 4: Treatment 1 group.....36
4.8.5 Group 5: Placebo 2 group.....36
4.8.6 Group 6: Treatment 2 group.....37
5 Discussion.....38
6 Conclusion.....40
7 References.....41

LIST OF FIGURES

Figure no.	Title	Page no.
2.1	Green tea plant	**07**
2.2	Green tea extract contains major catechins with chemical structures	**09**
2.3	The effect of green tea on second-level burns on rats	**12**
2.4	Different stages of the wound healing process	**13**
3.1	Anesthesia Injected to Rats before Acid Burn Injury	**20**
3.2	Rat's Hair Removed	**20**
3.3	Acid Soaked Filter Paper were placed to the shaved region of Rats	**21**
3.4	Acid-burned Area of Rat, Highlighted by Marker	**21**
3.5	Blood sampling of rats	**22**
4.1	Green tea powder	**25**
4.2	Filtered green tea powder mixture	**25**
4.3	Candida Albican petri dish with less concentration of treated and control group	**26**
4.4	Candida Albican petri dish with more concentration of treated and experimental group	**27**
4.5	After receiving *Camellia Sinensis* extract treatment, a rat with acid burns showed wound contraction	**28**

4.6	Graphical representation of Wound Index	**29**
4.7	Graphical representation of ELISA-VEGF	**30**
4.8	Graphical representation of ELISA-Annexin V	**31**
4.9	Graphical representation of APOX	**32**
4.10	Graphical representation of SOD	**33**
4.11	Graphical representation of GSH	**34**
4.12	Graphical representation of CAT	**35**
4.13	Histological diagram of normal skin obtained from Albino rats	**36**
4.14	Histological diagram of acid burn injured skin obtained from Albino rats	**36**
4.15	Histological diagram of skin obtained from Placebo group 1 of Albino rats	**37**
4.16	Histological diagram of skin obtained from Treated group 1 of Albino rats	**37**
4.17	Histological diagram of skin obtained from Placebo group 2 of Albino rats	**38**
4.18	Histological diagram of skin obtained from Treated group 2 of Albino rats	**38**

LIST OF TABLES

Table no.	Title	Page no.
3.1	Samples and control groups incorporated in the 8 compartments of petri dish	**18**
3.2	Categorization of rat	**19**
3.3	Dose, Route and Duration of Anesthesia given to Rats before Acid Burn Injury	**19**
4.1	Observed antifungal activity	**26**
4.2	Summary of Graphical results of Wound index	**29**
4.3	Summary of Graphical results of ELISA VEGF	**30**
4.4	Summary of Graphical results of ELIS Annexin	**31**
4.5	Summary of Graphical results of APOX assay	**32**
4.6	Summary of Graphical results of SOD assay	**33**
4.7	Summary of Graphical results of GSH assay	**34**
4.8	Summary of Graphical results of CAT assay	**35**

LIST OF ABBREVIATIONS

No.	Abbreviations	Full forms
1	VEGF	Vascular endothelial growth factor
2	TMB	Tetramethylbenzidine
3	ELISA	Enzyme-immunosorbent assay
5	GTC	Green tea catechins
6	GC	Gallocatechin
7	CHD	Coronary heart disease
8	PF-4	Platelet factor-4
9	ROS	Reactive oxygen species
10	H&E	Hematoxyline and eosin stain
11	GSH	Glutathione
12	SOD	Superoxide dismutase
13	HRP	Horseradish peroxidase
14	BFGF	Basic fibroblast growth factor
15	PBS	Phosphate buffer saline
16	SEM	Standard Error of the Mean
17	ANOVA	Analysis of Variance
18	ECM	Extracellular matrix
17	HCL	Hydrochloric Acid

CHAPTER 1
INTRODUCTION

One of the most popular beverages worldwide is tea. Tea is enjoyed in around 30 nations all over the globe. It is best filled in moist, subtropical circumstances with a lot of downpour, great waste, and somewhat acidic soil (1). Green tea is grown in Pakistan's Hazara, Swat, Azad Kashmir, and Abbottabad areas (2). Green tea has a higher number of catechins than dark or oolong teas. In vitro and in vivo, catechins are strong cancer prevention agents. The cancer prevention agent capability of this kind of tea is also helped by its mineral and nutrient content.

Researchers have discovered a number of health benefits of tea, including anticancer, anti-carcinogenic, and anti-arteriosclerotic properties. Tea is consumed in powdered form, soft extracts, and strong infusions to obtain these health advantages. Green tea catechins (GTC) are an important component of tea that has attracted a lot of attention as a potential anti-cancer and cardiovascular disease agent (3). Green tea has been demonstrated in numerous logical and clinical examinations to have antiproliferative, antimutagenic, cell reinforcement, antibacterial, antiviral, and chemoproperties (4).

Hemostasis, inflammation, enlargement, and regeneration are four specific and highly regulated phases of wound healing as a natural biological development in the human body. For the wound to heal accurately, all four stages must be completed in a timely manner. Many factors can interfere with one or more of these processes, leading to infiltration or delayed healing of the wound. Wounds that do not heal properly, such as severe back pain and incurable wounds have not progressed to the normal stages of healing. As a result of the delayed, partial, or irregular healing process, such lesions often develop pathologic inflammation. Most chronic ulcers are caused by ischemia, diabetes, venous stasis syndrome, or depression (5).

Angiogenesis is the course of fresh blood vessels' framing. Angiogenesis, or the arrangement of fresh blood vessels from previous veins, considerably affects an assortment of sicknesses, including malignant growth, ischemic coronary illness, wound healing, and inflammation (6). The production of new blood vessels, also known as neovascularization, is particularly evident during tumour development, when the growth of solid tumours and their developed metastases is dependent on the induction

of a sufficient blood supply. Angiogenesis is linked to a variety of immunological activities, including autoimmune reactions and inflammation. Controlling angiogenesis has aroused much interest in the last 40 years as it has been recognised as a potential target for potential therapies. The study of how biological functions are regulated by interacting cells is known as angiogenesis processes. Macrophage tissue interaction regulates angiogenesis. During angiogenesis, endothelial cells multiply, migrate, and merge into new blood vessels (7).

Angiogenesis is important for wound healing because it invades the wound clot and organizes into a micro vascular network throughout the granulation tissue, creating new blood vessels from old capillaries. Reactivating blood circulation at the site of harm to tissues is critical for constructing a powerful restoration response, and wound angiogenesis is a paradigmatic version of this question regarding the improvement and reconstruction of blood vessel designs (8, 9). Due to the ease with which this process may be controlled and manipulated, skin defect repair is a great model for studying angiogenesis. During skin restoration and wound healing, the majority of the progress elements, matrix extracellular components, and cellular kinds that have recently been discovered and assumed to be significant in blood vessel development have been identified and examined. The relevance of local oxygen concentrations in wounds for the re-establishment of a blood vessel network, as well as how this influences healing outcomes from normal repair to regeneration (10-12).

Angiogenesis is a physiological cycle that is expected for typical injury recuperating. Wound angiogenesis is impacted by hypoxia, aggravation, and development factors. The molecular and cellular phases of angiogenesis have been discovered, and chronic wounds indicate flaws in this process (13). Angiogenesis is an intricate framework that is broadly managed by both serum and basic extracellular lattice (ECM) signals because of tissue injury. The most significant angiogenic cytokines in injury angiogenesis are vascular endothelial development factor, angiopoietins, fibroblastic development factor, and changing development factor. New vessels are framed because of an arrangement of organic occasions. Angiogenesis, angiogenesis-related growth factors, angiogenesis stages in wound healing, and angiogenesis-promoting nanocarriers are all discussed.

Many herbs and herbal extracts are used for wound healing and angiogenesis. For example, one of these medicinal plants is green tea (Camellia sinensis), which fights oxidants and inflammation and can help with wound healing. Green tea has been used as a drink and herb in many Asian countries to help you with everything from controlling bleeding and wound healing to having the ability to control your body temperature and blood sugar and improving digestion (14). The most common location for inexperienced tea polyphenols is flavonoids, along with catechins, catechingallates, and proanthocyanidins. The antifungal activity of green tea extract was time-dependent, and its inhibitory effect did not change significantly over time. Tissue solubility, impact on dentin structure, and function as an antioxidant are other features of green tea that should be investigated (15). Its ability to stop the growth of fungal strains is a sign of the tea plant extract's antifungal qualities, which can be employed to treat fungal infections in the future (16). Green tea, with its concentrate and the separated parts, has additionally been demonstrated to assist with diminishing oxidative pressure (17), and neural issues (18). The production of granulation tissue, which is dependent on epithelialization and angiogenesis, is required for healing. Currently, no growth factor that induces both epithelialization and angiogenesis is available to treat individuals with poor healing. Angiogenesis is a vital stage in injury healing. Compound middle people, the extracellular grid, metabolic inclinations, and actual powers all work locally to administer vessel development. In experimental wounds, manipulating some of these elements can promote healing (19).

The angiogenic factor, vascular endothelial development factor (VEGF), was first distinguished as a fundamental development factor for vascular endothelial cells. Many cancers up regulate VEGF, and its role in tumour angiogenesis is well understood (20). Vascular endothelial growth factor is the key regulator of both healthy and pathological angiogenesis (VEGF, VEGF-A). The setup of the vascular stockpile is significant for organ advancement and variety all through embryogenesis, as well as wound recuperating and concept exercise in grown-ups. This comprehensive overview provides a historical perspective on the challenges of VEGF availability as well as the first steps in assessing protein performance (21). The scope of our study was to evaluate the antifungal activity and wound healing capacity of green tea extract.

RATIONALE

To check the antifungal properties of green tea and to identify whether it is beneficial in the development of angiogenesis or wound healing in acid burn rats. Animal models were used for finding therapies instead of drugs for different diseases in preclinical studies. The world prefers neutraceutical therapies instead of drugs, which cause great damage to animals instead of treating them. Therefore, it is a need for the betterment of the world to use neutraceutical plants which are more effective, have fewer side effects, and are relatively low cost.

OBJECTIVES

The objectives of the research were as following:

- Preparation of aqueous extract of green tea.
- Evaluation of skin regeneration in acid burn rats.
- Evaluation of antifungal properties green tea.

CHAPTER 2

LITERATURE REVIEW

2.1 Plant Overview

One of the most popular drinks on the world is green tea. In many regions of the world, tea is brewed as green, dark, or oolong tea using the Camellia sinensis plant. Drinking green tea affects the human wellbeing of every one of them. Tea is prepared from the evergreen Camellia sinensis plant, which is commonly found in tropical and subtropical regions around the world. There are around 325 different species in the genus. Green tea is grown in Pakistan's Hazara, Swat, Azad Kashmir, and Abbottabad areas. Many tea types differ in terms of processing, as well as the levels of oxidation and fermentation associated with them, which affect flavour and aroma characteristics. Green tea is a non-fermented, minimally oxidised tea (22).

Figure 2.1: Green Tea Plant (23)

The antioxidant and anti-inflammatory properties of green tea are due to catechin polyphenols, which are important chemicals responsible for its health benefits. Green tea's stimulating qualities are enhanced by caffeine, while its calming properties are enhanced by the amino acid theanine. Environmental conditions and processing lead to significant variations in catechin and caffeine levels (24).

Tea plants might be developed in regions with in excess of 120 cm of yearly precipitation, temperatures somewhere in the range of 12 and 30 degrees Celsius, and heights ranging from ocean equal to 2,200 meters (25). The vast majority of green tea varietals are prepared from tender, immature leaves. There is three to four tea harvest seasons each year, depending on the width and length. The process of making green tea involves steps such as picking, post-harvest spreading, preparation, rolling, molding, and drying. When green tea is prepared and stored, it undergoes a number of metabolic changes. As the leaves of oxidation, hydrolysis, polymerization, and conversion are processed, the levels of polyphenolic catechin compounds decrease, while the levels of caffeine remain unchanged (26).

At six months of production, there is a major decrease in polyphenolic catechin levels, with some components decreasing by 50%. The important notes of green tea are bitter and sweet (27). A lower incidence of major illnesses of the lungs, colon, oesophagus, mouth, stomach, small intestine, kidneys, pancreas, and glands has also been associated with green tea use. Numerous epidemiological research studies and clinical trials have shown that drinking green tea (and, to a lesser extent, black tea and oolong tea) lowers the risk of developing chronic diseases. (28).

2.1.1 Chemical Composition

Green tea and its ingredients contain high levels of antioxidants called polyphenols. Catechins, which account for 10 to 20% of dried tea leaves, are thought to be responsible for the bitter taste of green tea (29). The chemical composition of green tea also includes: proteins (15-20% dry weight), of which compounds play an important role; amino acids (1-4%), valine, leucine, threonine, glutamic corrosive, tryptophan, aspartic corrosive, serine, tyrosine, lysine, and arginine; and starches (1-4%). minerals and minor components (5% dry weight) like sodium, phosphorus, cobalt, strontium, nickel, copper, zinc, calcium, magnesium, chromium, iron, potassium, aluminum, and fluorine; and follow measures of lipids (linoleic and-linolenic acids), sterols (stigmasterol), nutrients (B, C, E), colours (chl (esters, alcohols, aldehydes, lactones, hydrocarbons). Caffeine, theobromine, and theophylline are among the methylxanthines found in 3–4 percent of the fresh leaves (30).

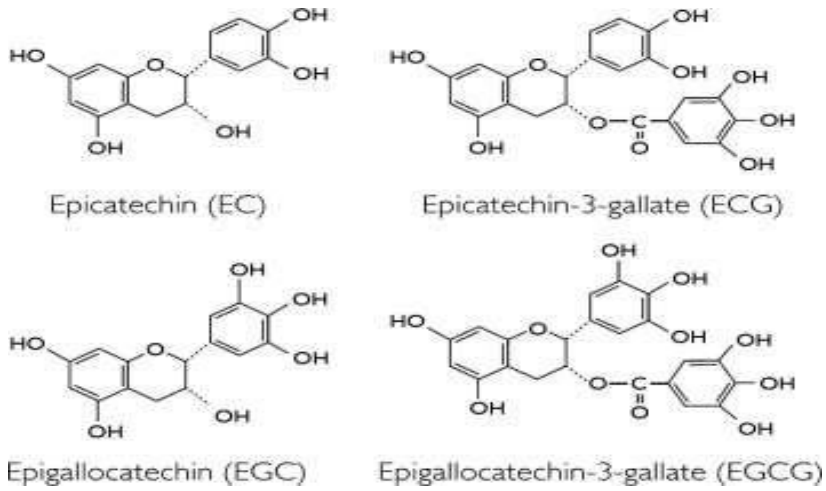


Figure 2.2: Green tea extract contains major catechins with chemical structures (31)

2.2 Camellia Sinensis Properties:

2.2.1 Antimicrobial Activity

Green tea leaves contain polyphenol, a bioactive compound with an antimicrobial effect against a wide range of bacteria. Several studies on the antimicrobial effects of green tea polyphenols have been conducted over the past two decades (32). According to several studies, drinking green tea lowers the risk of cardiovascular disease, stroke, and obesity (33). Using the well diffusion method and gravimetric analysis, green tea polyphenols were examined for antibacterial, antifungal, and anticorrosion activities (34).

2.2.2 Antifungal Activity

According to Koech and Wachira, the study also showed the antifungal effects of green, black, and white tea products based on germ plasma levels, which may lead to therapeutic use in the future (35). The antifungal properties of the green tea leaves are well known. Because traditional and universal antifungal drugs have side effects, and drug resistance is increasing, it is believed that the use of herbal medicines in the treatment of fungal infections of the disease and the occurrence of adverse effects antifungal it will be a good substitute (36). Citrus flavonoids and polyphenols contained in green tea have been the subject of a lot of recent research. These chemicals have been shown to have antiviral, antitoxin, antiviral, and antifungal properties (37).

2.2.3 Antioxidant Property

Antioxidants are of importance to biologists and clinicians because they continue to protect the body against free radical damage caused by atherosclerosis, ischemic heart disease, stroke, Alzheimer's disease, Parkinson's disease, and ageing. Natural substances and products have key antioxidant properties and are also linked to cancer, anti-aging, and inflammation, according to a growing body of studies.

Several studies have found that flavanols extracted from green, black, and red tea leaves have extremely significant antioxidant properties. Vastag discovered that a cup of tea leaf infusion is "an injection of antioxidants," capable of scavenging free radicals more effectively than vitamins C and E. Early Japanese studies on the antioxidant action of catechins in oil systems produced a lot of conflicting results (38). According to Roedig-Penman and Gordon, the extraction of green tea water was very effective as a natural antioxidant for storing an oil emulsion in water (pH 5.5, 40 days at 30 °C) (39).

In a recent study, food ingredients have been demonstrated to have antioxidant properties. Numerous epidemiological studies have found that polyphenol-rich foods and beverages help to prevent diseases, reduce mortality from cardiovascular and cancer-related disorders, and slow down the ageing process (40).

Natural antioxidants and extracts from the plant have been the subject of much attention in recent decades due to concerns about the possible health effects of antioxidants being produced. Green tea extract is especially well-suited to items that are prone to lipid oxidation, such as trans-free and low-fat diets, as well as those high in polyunsaturated fatty acids (41). There is little research in the literature that compares the effectiveness of green tea extract in a diet against lipid oxidation. Peroxide levels, formed dienes, thiobarbituric corrosive receptive substances (Ski lifts), Oil Dependability File (OSI), Oxipres enlistment term, and tangible assessment are some of the fundamental estimations used to decide the viability of green tea separate in food (42).

2.2.4 Antiviral Property

According to Kawai et al.'s investigation into the mechanism underlying the anti-HIV properties of green tea polyphenols, EGCg (but not ECG) binds specifically to CD4

molecules on the surface of cells. 51. Further study is required to determine whether these effects are discernible in people.

2.2.5 Antaging Property

Green tea protects serum lipids and proteins from oxidative damage that promotes aging. In addition, green tea also reduces the marking damage of DNA oxidative damage, 8-oxo-deoxyguanosine (8-oxodG), in the kidney liver, and brain. Polyphenols from green tea are therefore beneficial against damage from aging.

2.2.6 Antiparkinson Effect:

Green tea has been shown to have neuroprotective properties, which means it can play a role in preventing Parkinson's disease. Dopamine neurons are protected by green tea polyphenols, and drinking more green tea increases this protection, claim the authors. They further suggest that this protective effect is due to the regulation of the ROS-NO pathway, which may be responsible for the death of Parkinson's disease cells (43). Green tea includes antioxidants that protect brain cells from injury, which can contribute to Parkinson's disease.

2.2.7 Cardiovascular Effect:

The use of the components in green tea has been found to have hypolipidemic action in a variety of animal experiments. The leaves of 2.5 percent green tea were consumed by rats for several weeks, resulting in a drop in serum cholesterol and triglycerides as well as the absence of toxins in the kidneys and liver.

Plants are crucial to millions of people in our daily lives, and they serve as a source of new pharmaceuticals and the creation of prospective therapies for human health. Ethnomedicine's therapeutic systems reveal a distinction between civilizations. In Africa, the majority of citizens (70 to 80 percent) inquire about traditional physicians' medical services. Herbal medicines are widely used in the treatment of a wide range of disorders in various parts of the world, and they help to improve and produce experimental drug research.

2.3 Burns Wound

Burns are one of the most serious injuries that can occur. For burn research, a consistent burn wound is required. The rat is one of the most widely used animal species in the study of burns, and it has many similarities to the rat burn model. In terms of housing, storage, and manufacturing, both are much cheaper. The rat has a larger body size than the mouse, which makes it easier to handle and easily pressurized by human touch. Despite their popularity, methods of wound healing in rats are not compatible with the human wound healing process. Rats (mice and rats) have a subcutaneous panniculus carnosus muscle, which aids in skin healing through wound penetration and collagen synthesis (44, 45), this limitation exists. On the other hand, this rapid wound reduction allows researchers to quickly investigate the full range of wound healing equipment. The method of cremation and its preservation in animal images is important because it influences the effect of burns and the way wounds are treated. The structure and anatomy of the skin vary per species, as do their advantages and disadvantages as an experimental burn injury model.

Figure 2.3: The effect of green tea on second-level burns on rats (46)

2.4 Wound Healing and Angiogenesis

Although the terms "tissue repair," "wound repair," and "wound healing" are often interchangeable, "healing" and "repair" refer to two different types of procedures and outcomes. First and foremost, before performing any separation, it is important to note

that "healing" and "repair" are not limited to the skin, but can apply to any organ system. Wound healing is a technical term that should be used only in the context of actual regeneration, which occurs when the formation and formation of the original organ or anatomic part are completely restored to their original state of injury (47).

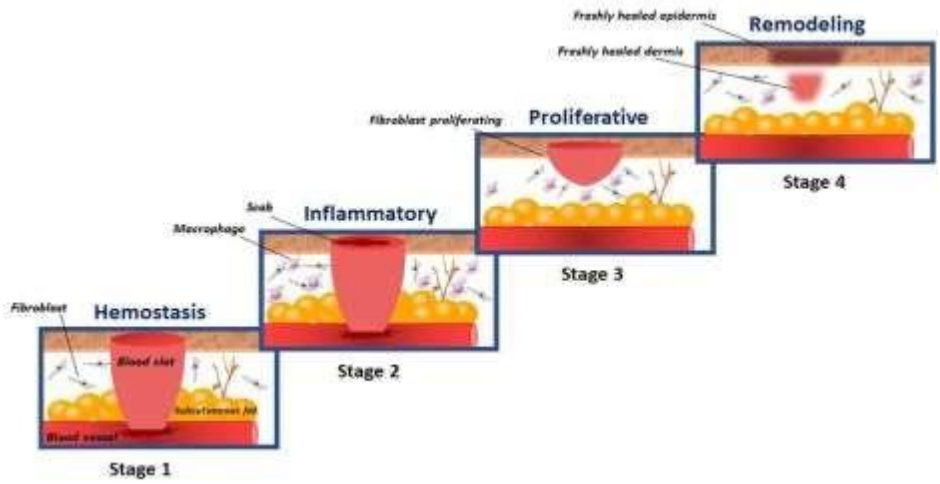


Figure 2.4: Several phases of the healing process for wounds (48)

Angiogenesis is the formation of new arteries from existing ones during normal development and homeostasis and certain pathological conditions. In 1787, the British surgeon John Hunter came up with the word of "angiogenesis". However, for almost a hundred years later, there were only a few reports of tumour angiogenesis, and these were the anatomical studies. The field of angiogenesis was started in the early to mid-1970s when Judah Folkman said that the growth rate of the tumour should be resolved if the tumour was deprived of blood sources (49). At the age of 10, after Folkman first proposed the concept of malignancy angiogenesis, and only in 65 editions of the publication of angiogenesis in the title, compared to more than 9,000 journals from 2000 to 2010 (50). The scientific achievements on the subject of angiogenesis were initially documented by John Hunter, a Scottish physician, anatomist, and surgeon. In both health and illness, his observations demonstrated that vascularity and metabolic requirements are proportionate. Hunter was the first to notice that angiogenesis is governed by a natural law established by Aristotle, which states that "shape follows function."(51). Controlling angiogenesis could lead to cancer therapies, which generated a frenzy of research in the field. For example, just two manuscripts dealing with angiogenesis were published in 1970; by 2009, over 5200 articles had been published (52).

Angiogenesis is significant for cancer development and metastasis. Numerous solid plants require angiogenesis, or the advancement of fresh blood vessels, to make due and flourish. As a result, a number of drugs have been developed that address important aspects of angiogenesis, including cell adhesion, cellular matrix degeneration, and endothelial cell activation by angiogenic cytokines or growth factors. The fact that tumour cells do not thrive in the absence of blood vessels and the fact that anti-cancer drugs have anti-cancer properties in animals have made it interesting to point to angiogenesis induced by tumours as a mechanism to stop tumour progression (53). Furthermore, unlike cytotoxic drugs (antimitotic, antimetabolites, and alkylating agents), endothelial cell-specific inhibitors should not produce cancer resistance as endothelial cells are genetically stable (54).

The biological and molecular processes behind angiogenesis have been extensively studied, and essential events related to tumor-induced neovascularization have been identified. (1) The stimulation of endothelial cells by angiogenic cytokines found in a plant, such as VEGF, leads to increased endothelial cell proliferation and migration; (2) the release of enzyme-reducing enzymes such as matrix metalloproteinase and plasminogen activators, leading to the digestion of ECM; and (3) the construction of a three-dimensional capillary network near the plant. All of these physiological and molecular phases have been recognised as potential antiangiogenic targets, resulting in the discovery and development of a number of medicines that target vessel formation, endothelial cell proliferation, and migration (55).

Angiogenesis, or the development of a new capillary network, is one of the most obvious aspects of normal healing. A new study casts doubt on the long-held idea that optimal healing necessitates a high level of capillary growth (56). According to new research, usually healed wounds have an abnormally vigorous and mostly ineffective angiogenic response, which could have a negative impact on repair outcomes. Healing wounds are good experimental models for learning about the nature of angiogenesis that would otherwise be unavailable.

Wound hypoxia is caused by vasculature disruption in the wound area (57). Despite the fact that hypoxia is widely accepted as the physiological basis for angiogenesis, unadorned hypoxia cannot support formation of active blood vessels (58, 59). Hyperoxia, like hypoxia, promotes wound angiogenesis and healing by inducing the

production of angiogenic factors. In wound clinics, oxygen therapy is routinely used to treat wound hypoxia (58) (60).

According to a recent study, the neuronal protein 3.1 (P311) is important in the treatment of skin lesions, suggesting that modifying the P311 gene may improve the healing function of MSC. This study found that increased vi3 expression of P311 improved the ability of MSCs to decrease the number of inflammatory cells, increase IL10 expression, decrease TNF and IFN levels, increase collagen uptake, promote angiogenesis, and ultimately accelerate the closure of the skin wound and promote wound healing. At the cellular level, the signalling pathway was found to be highly correlated with P311 control of VEGF synthesis in MSCs (61). Our findings reveal that altering the P311 gene in MSCs improves their ability to promote skin wound repair, paving the way for future therapeutic applications (62).

The onset of angiogenesis is influenced by a few soluble factors, the most important of which is VEGF-A. In several studies, VEGF-A, which is produced in response to hypoxia, has been shown to be a very important proangiogenic component in wound treatment. VEGF-A is a potent proangiogenic agent that also promotes wound edoema by increasing vascular decay (63). VEGF-A, fibroblast growth factor-2, platelet-derived growth factor, TGF-family members, and cardiac ankyrin repeat protein are among the other factors that promote wound angiogenesis (64). Many techniques have been employed to inhibit wound angiogenesis in these investigations, including the use of anti-VEGF antibodies, antiangiogenic drugs, and integrin signalling blockage (65). The findings support the view that the amount of angiogenesis seen in the skin lesion is excessive and possibly unnecessary. Evidence for this theory is strengthened by the study of well-healing wounds.

Wound healing necessitates angiogenesis. The vasculature makes up to 60% of the repair tissue, and the term "granulation tissue" originates from the predominance of the capillaries in the temporary organ of repair. The massive metabolic needs of debridement and fibroplasia necessitate a plentiful blood supply; the basal vasculature is rarely, if ever, adequate support. The endothelial cell is a healer's organiser and regulator, and angiogenesis is more than just a nutritive process (66).

2.5 Wound healing & VEGF

Vascular endothelial growth factor (VEGF) is a mitogen and angiogenesis factor found only in endothelial cells. The recent discovery that deletion of a single VEGF allele causes Oxford University Press embryonic mortality emphasises the importance of VEGF in vascular system development. According to previous studies, VEGF is also an important mediator of neovascularization in malignancies and retinopathies. In addition, in coronary arteries or organ ischemia, VEGF promotes coarse vessel development (67). High proximity According to studies of lipid autoradiography, VEGF binding sites are located in the vascular endothelium of vessels large and small but not in other cell types, indicating that VEGF is a specific endothelial cell-specific component.

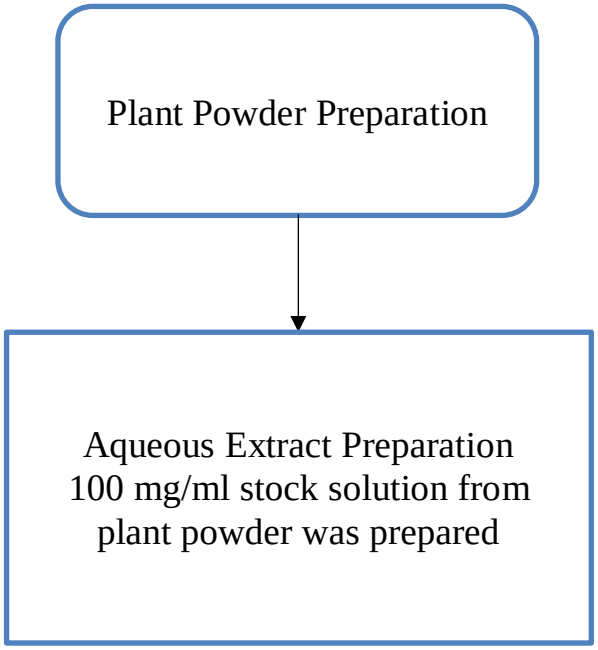
Vascular endothelial growth factor (VEGF) is one of the most significant survival and growth factors for the endothelium. Vasculogenesis is regulated by VEGF, which also promotes angiogenesis and the growth of endothelial cells. Stone and colleagues believe that hypoxia-induced VEGF mRNA up-regulation in neuroglial cells is critical for the growth of retinal vasculature (68, 69).

VEGF controls wound closure and epidermal repair, the creation of granulation tissue, the quality of wound healing capacity, and the quantity of scar tissue associated with enhanced angiogenesis. Despite the fact that VEGF has been found to alter multiple elements of wound healing in several studies, its proangiogenic activity has been linked to the majority of VEGF's effects during wound repair. During normal wound healing, high quantities of VEGF are produced, resulting in a robust angiogenic response(70).

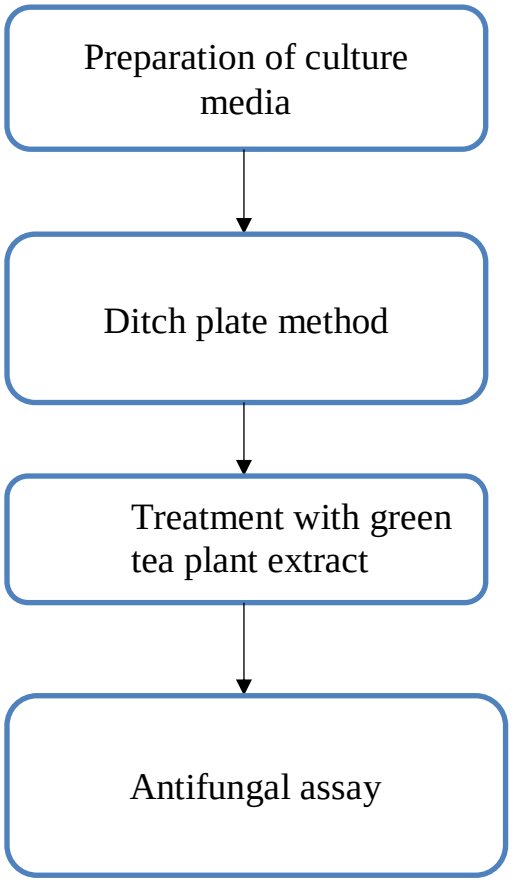
According to research on human and animal models, VEGF is produced by keratinocytes at the beginning of the wound healing process. However, new research suggests that keratinocytes release VEGF later in the healing process. VEGF is produced by regenerated fibroblasts, mast cells, and macrophages in damaged skin. After an injury, myeloid cells (monocytes and macrophages) have been demonstrated to be a significant source of VEGF. Both keratinocyte-derived and myeloid cell-derived VEGF have been proven to influence a number of aspects of the repair process.

PLAN OF WORK

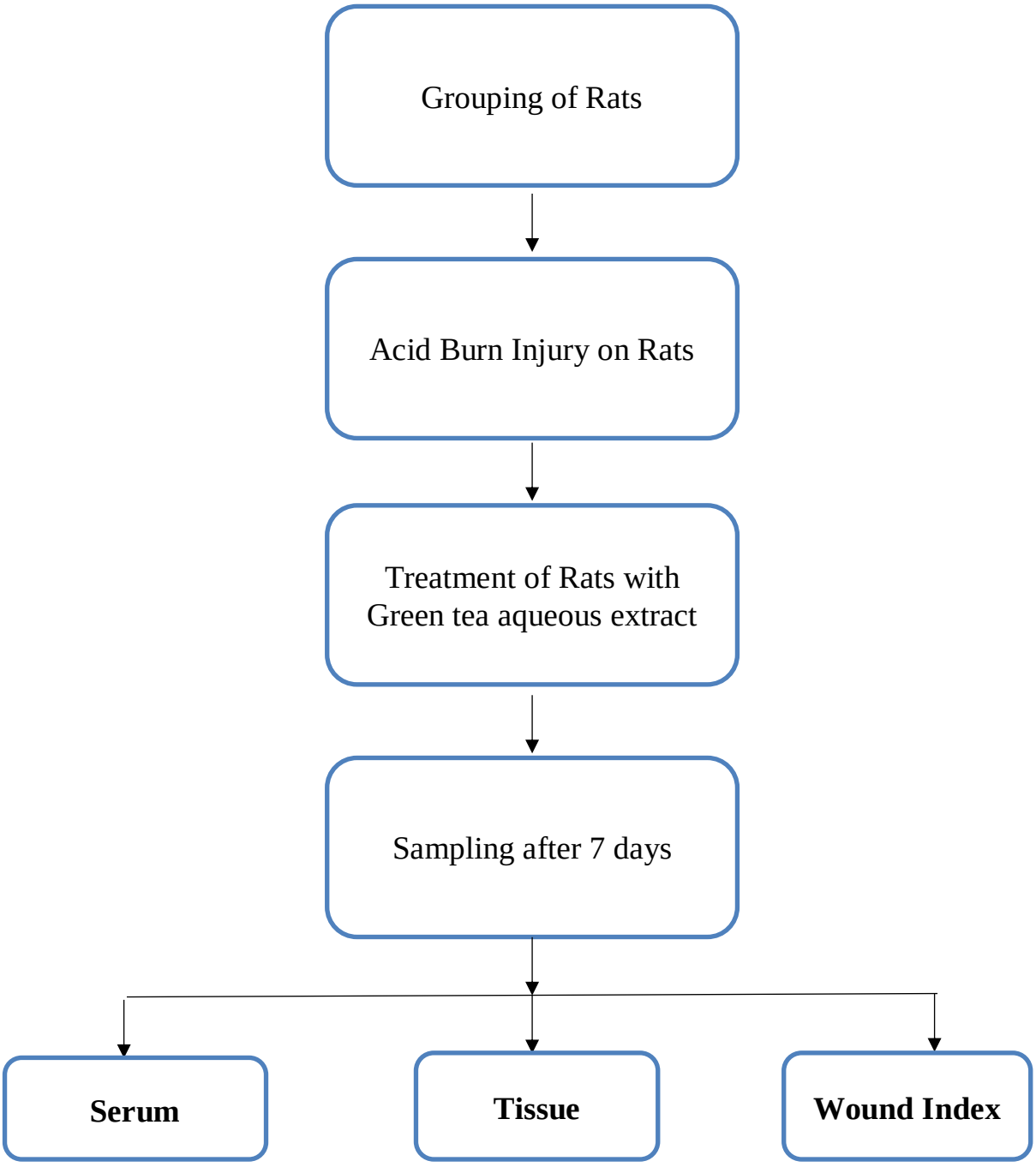
IN-VITRO ASSAYS

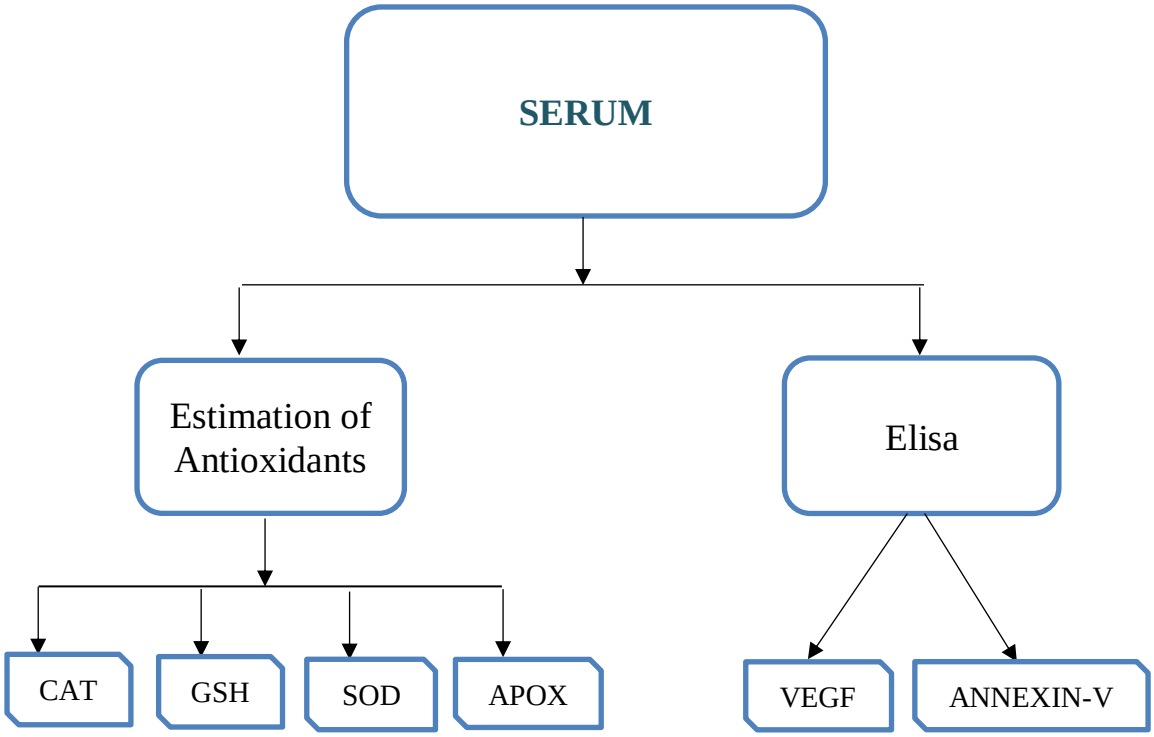


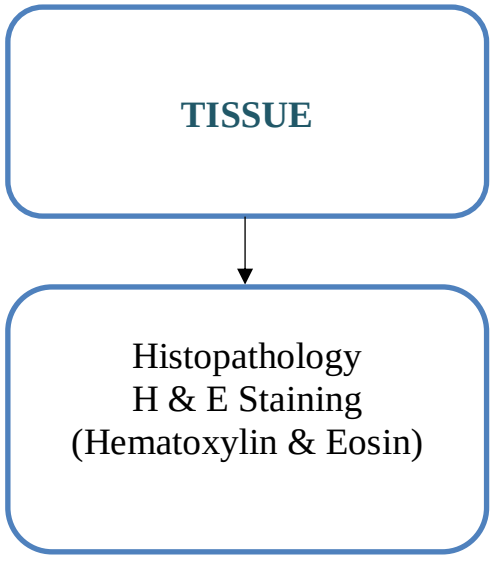
Antifungal Assay



IN-VIVO ASSAYS




SERUM
Estimation of Antioxidants
Elisa
CAT
GSH
SOD
APOX
VEGF
ANNEXIN-V


TISSUE
Histopathology
H & E Staining
(Hematoxylin & Eosin)

CHAPTER III

METHODOLOGY

3.1 Plant Powder Preparation:

The leaves of the Camellia sinensis plant, a member of Thiaceae family, are used to make the well-known beverage known as green tea. In Pakistan Green Tea grows in regions of Hazara, Swat, and Azad Kashmir and Abbottabad districts of Pakistan. Fresh green tea plants were prepared by taking dry green tea plants and grinding them into a fine powder.

3.2 Aqueous Extract Preparation:

Aqueous plant extract was made by dissolving 10g of plant powder in 100ml of water, then passing the solution through filter paper after two days. The water soluble fraction of the filtered extract was evaporated using petri dishes in the refrigerator.

3.3 Assessment of antifungal activity:

Antifungal activity of the given Candida albican strain was carried out by following the protocol described by:

3.3.1 Preparation of culture media for the study:

According to the manufacturer's instruction, Ampoule containing freeze dried form of the microorganism was opened and the contents were added to the Yeast Extract Peptone Dextrose broth which was incubated at 25 ± 2°C for 72 h. The 30 ml of molten sterile agar was poured aseptically in each four sterile petri plates and were allowed to solidify at room temperature. Hundred microliter of inoculum was spread with a sterile steel spreader to prepare a lawn of microorganism.

3.3.2 Ditch plate method:

Four wells on three plates were set up. The wells were assigned numbers between 1 and 12. Using a micropipette, a predetermined volume (250 ul) of the appropriate stock solution of green tea plant extract was added to each well. Then three wells were made

similarly on the fourth plate. Flucanozole served as the positive control, while the same plate also included 50% DMSO and sterile distilled water to serve as the negative controls. The plates were all then incubated for 24 hours at 25 °C while standing upright. The entire process was carried out twice. After 24 and 48 hours, the inhibition zones were measured on the underside of the plates using the Hi-Media zone scale.

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	Treated-20 µl
Group 4	Plac-20 µl
Group 5	Plac-20µl
Group 6	Amphotericin B
Group 7	Fluconazole
Group 8	Treated-40µl

3.4 Animal Model:

In the animal house of the: ―Molecular Medicine Research Centre (CRIMM) at the Molecular Biology and Biotechnology Institute (IMBB)‖, University of Lahore Pakistan albino rats were raised, average weight between 160 and 200 g. It was approved by ethical review committee of Pakistan for using this animal in research.

3.5 Ethical Clearance:

The Ethical committees of University of Lahore and of Kinnaird College for Women in Lahore provided approval for the use of animals (albino rats).

3.6 Grouping of Rats with *Camellia Sinensis:*

Albino rats was taken and placed into 6 groups with n=3 in each group. Three rats remained normal while the other was subdivided into 5 groups which were given acid burn injury.

Table 3.2: Categorization of rats

No.	Group Name	Group Description
1	**Group 1 Normal (N)**	Normal rats with no injury
2	**Group 2 Injured (Inj)**	Acid-burned injured rats with no treatment
3	**Group 3 Placebo 1**	Injured rats treated with 50mg/ml placebo
4	**Group 4 Treatment 1**	Injured rats treated with 50mg/ml green tea aqueous extract
5	**Group 5 Placebo 2**	Injured rats treated with 100mg/ml placebo
6	**Group 6 Treatment 2**	Injured rats treated with 100mg/ml green tea aqueous extract

3.7 Acid Burn Injury:

First, injections of ketamine and xylazine were used to anaesthetize the rats.

Table 3.3: Dose, Route and Duration of Anesthesia given to Rats before Acid Burn Injury.

Drug	Dose	Route	Duration of Anesthesia
Ketamine + xylazine (Rompun®)	40-90 mg/kg ket + 5-10mg/kg xyl.	IP, SQ	45-90 minutes

Six rats were used in total, one was normal (uninjured), and the remaining five (n=5) were given acid burn injuries while under the influence of xylazene and ketamine solution (anaesthesia).

Figure 3.1: Anesthesia Injected to Rats before Acid Burn Injury

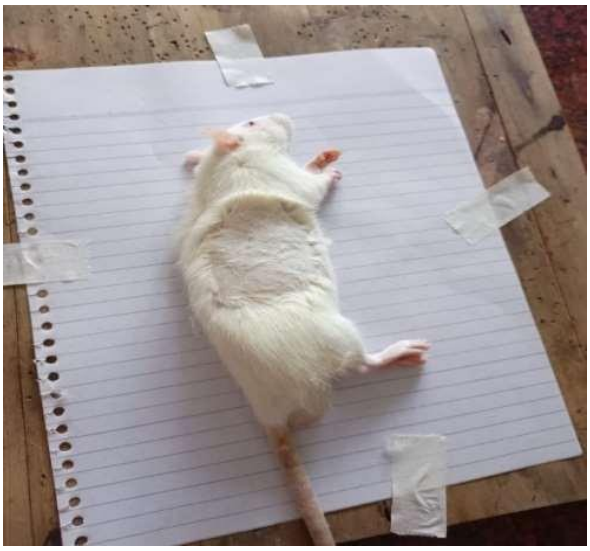


Figure 3.2: Rat's Hair Removed

Figure 3.3: Acid Soaked Filter Paper was placed to the shaved region of Rats

Figure 3.4: Acid-burned Area of Rat, Highlighted by Marker

3.8 Sampling after seven days:

After seven days, the rats were slaughtered and samples were gathered from all thesis groups of rats and the blood samples were centrifuged to get the serum on which ELISA was performed and antioxidant analysis was done. From all the groups tissue samples were collected for the histological analysis.

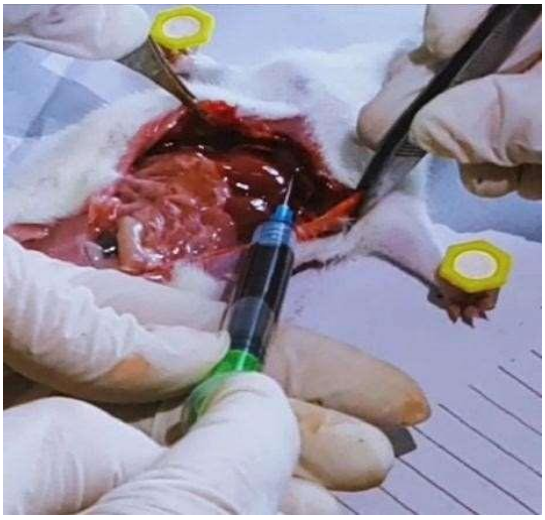


Figure 3.5: Blood sampling of rats

3.9 Treatment of Rats with *Camellia Sinensis:*

Rats were placed in cages and given access to clean water and food. A green tea aqueous extract was administered to two of the rat groups at concentrations of 50 mg/ml and 100 mg/ml, while the other two groups received placebos at the same concentrations.

3.10 Wound Index Measurement Protocol:

The reduction in the damaged area was measured using a wound-healing trial for 5, 10, and 15 days (71). A clear plastic sheet was used on the wounds of soft mice placed on their inner side. The cursor was used to draw a line in the damaged region on the sheet. Initial and current values were used to calculate the percentage reduction in wound size (72).

3.11 Enzyme-Linked Immunosorbent assays (ELISA):

This assay was used to determine the peptide ligands in large molecules in cells. In a 96-well plate, a solid phase sandwich ELISA for VEGF and annexin V will be performed. Add 100 ul of antibodies to 96 well plates in both the normal and treated groups. In a coating buffer, VEGF antibody was diluted to a concentration of 2-10 ug/ml. After that, a 96-well plate was coated with 100 ul of the diluted antibody and

left to incubate for 120 minutes. The antibody was extracted and saved in the tube after incubation. The solution was removed after three washes with PBS in each well. The blocking solution was then added, along with 200 ul of BSA, and the mixture was placed in an incubator for 30 minutes. BSA was then eliminated, and the wells were cleaned with PBS. After adding 200 ul of medium from each experimental group to each well and saving the medium, the wells were washed three times with PBS for five minutes. In every single well, add 100 ul of secondary antibody and rise for 1 hour. Take secondary antibodies after incubation and wash three times with PBS for five minutes each time. The chromogenic substrate TMB was added to all of the wells for detection, and inhibit the TMB reaction was done by using 100 ul of stop solution (0.18 M H2SO4). The absorbance of the spectrophotometer was measured at 450 and 620 nm.

3.12 Estimation of antioxidants analysis:

3.1.1 Catalase measurement:

The Sinha method was used to determine the catalytic activity (1972). A solution would be composed of 0.1 ml of conventional herbicide, 1.0 ml of phosphate buffer (pH 7.0,10 mM), and 0.4 ml of H2O2 (0.2 M). The reaction was stopped by adding 2.0 mL of dichromate acetic acid reagent. After 10 minutes in a boiling water bath, the samples was cooled and the absorbance was measured at 530 nanometers (73).

3.1.2 Glutathione measurement:

The amount of reduced glutathione (GSH) in the cell culture medium was calculated using the Beutler et al. method. A test tube was filled with 0.5 ml of cell culture media from both groups, 2.0 ml of disodium hydrogen phosphate buffer (0.3 M), and 0.25 ml of (5,50-dithiobis-2-nitrobenzoic acid) or DTNB (0.001 M). The absorbance was measured using a spectrophotometer at 412 nanometers after 15 minutes of incubation (74).

3.1.3 SOD measurement:

The activity of superoxide dismutase (SOD) was assessed by the method of Kakkar et al. (1984). Briefly, 0.1 ml of traditional media was mixed with 1.2 ml of sodium pyrophosphate buffer (pH 8.3, 52 mM), 0.1 ml of phenazine methosulfate (PMS) (186

lM), 0.3 ml of nitrobutytrazolium (NBT) (300 lM) and (750 lM). They was be added together. The reaction was fixed by addition of 0.1 ml of glacial acetic acid after 90 seconds of incubation at 30 C. With 4.0 mL of N-butanol, the reaction mixture was accelerating. This mixture was incubated for 10 min before being centrifuged at 2000 rpm for 5 min. The absorbance of the top layer of butanol was measured at 560 nanometers (75).

3.1.4 Apox measurement:

The procedures of Israr et al. and Nakano and Asada were used to test APOX. From 100 mM of KH2PO4 buffer (pH 7.0), 0.3 mM H2O2, 0.5 mM ascorbate, and post-treatment media, make one ml of the reaction mixture. After 3 min, the OD290 would be taken (since the oxidation of ascorbic acid would be measured as a 290 nanometer reduction in absorbance over 3 min). Enzyme activity was measured in units of the enzyme per gram of new weight (U g-1 FW). At 25 ° C, one unit of the enzyme is required to degrade 1 mol of H2O2 per minute mixture (76).

3.13 Histopathology Assay:

Skin tissues from all the rats were collected immediately after rats were slaughtered and submitted to histopathology lab for histological analysis. 0.5 centimetre square skin samples from the injured area were obtained and fixed in formaline for 7 days following the acid burn injury. Skin samples were dried up, embedded in wax, and sliced with 5 millimetre microtome. To dye the skin section and evaluate skin architecture, H&E (hematoxyline and eosin stain) was used.

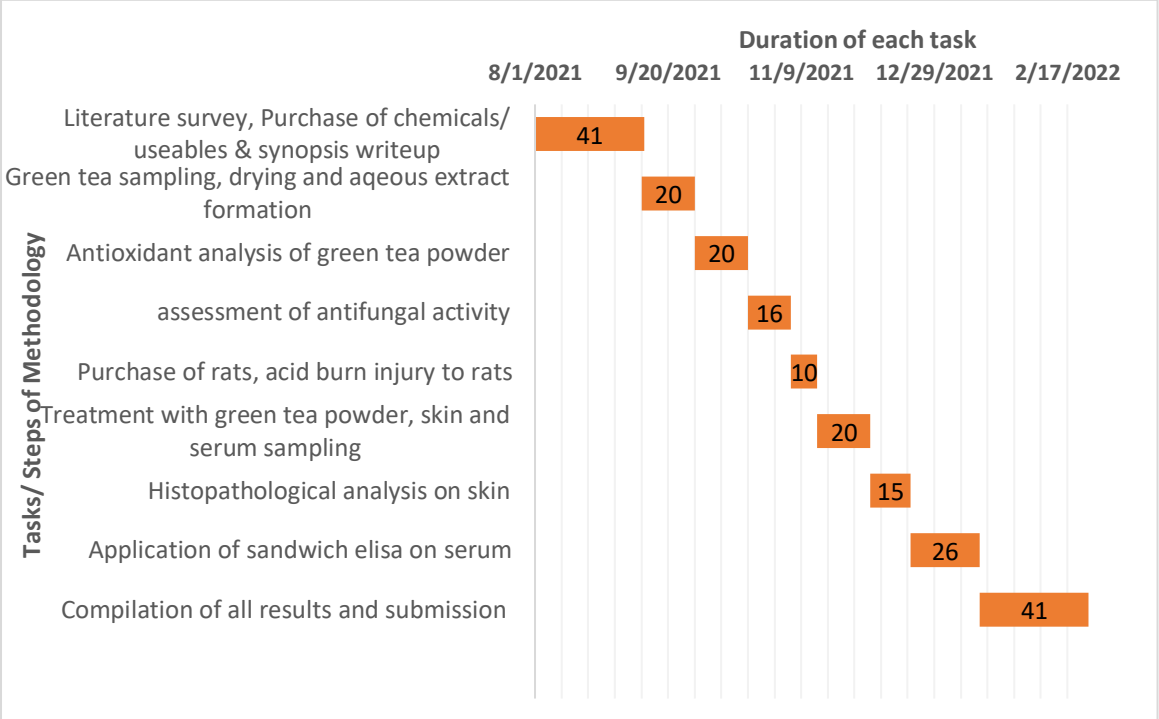
3.14 Statistical analysis:

All groups was statistically analysed using one-way ANOVA. Graphs and prisms were used to create all graphs. Image software was used to examine the images, and the references were added using ENDNOTE.

GANTT CHART

Procedure/ Tasks	Start date (DMY)	End date (DMY)	Duration (days)
Literature survey, Purchase of chemicals/ useables & synopsis writeup	8/1/2021	9/10/2021	41
Green tea sampling, drying and aqueous extract formation	9/10/2021	9/30/2021	20
Antioxidant analysis of green tea powder	9/30/2021	10/20/2021	20
Assessment of antifungal activity	10/20/2021	11/5/2021	16
Purchase of rats, acid burn injury to rats	11/5/2021	11/15/2021	10
Treatment with green tea powder, skin and serum sampling	11/15/2021	12/5/2021	20
Histopathological analysis on skin	12/5/2021	12/20/2021	15
Application of sandwich Elisa on serum	12/20/2021	1/15/2022	26
Compilation of all results and submission	1/15/2022	2/25/2022	41

GANTT CHART


Duration of each task
8/1/2021
9/20/2021
11/9/2021
12/29/2021
2/17/2022
Tasks/ Steps of Methodology
Literature survey, Purchase of chemicals/ useables & synopsis writeup
Green tea sampling, drying and aqeous extract formation
Antioxidant analysis of green tea powder
assessment of antifungal activity
Purchase of rats, acid burn injury to rats
Treatment with green tea powder, skin and serum sampling
Histopathological analysis on skin
Application of sandwich elisa on serum
Compilation of all results and submission
41
20
20
16
10
20
15
26
41

CHAPTER 4

RESULTS

4.1 Plant powder:

Dry green tea leaves were ground into a fine powder that weighed 120 grams to create fresh green tea plants.

Figure 4.1: Green tea powder

4.2 Aqueous extract:

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

Figure 4.2: Filtered green tea powder mixture

4.3 Antifungal Assay:

The purpose of this assay was to determine the antifungal properties of the green tea powder. Green tea powder extract was tested against the *Candida Albican* strain and the results obtained were highly significant.

Table 4.1: Observed antifungal activity

No.	Groups	Antifungal activity
1	Normal	-ve
2	Injection water 20 µl	-ve
3	Treated-20µl	+ve
4	Plac-20 µl	-ve
5	Plac-20µl	-ve
6	Amphotericin B	++ve
1	Fluconazole	+ve
2	Treated-40µl	++ve

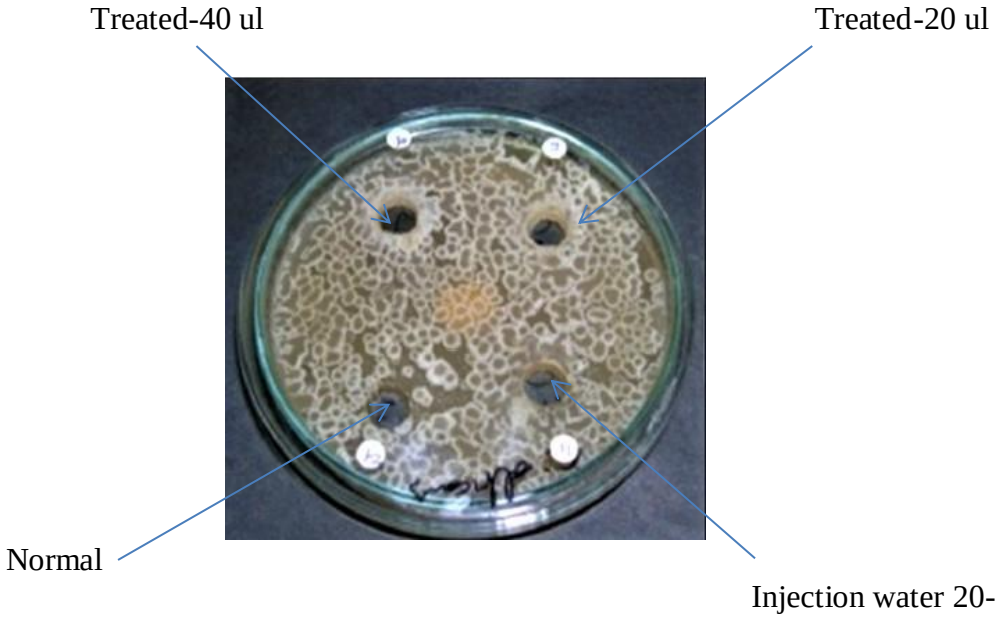


Figure 4.3: *Candida albican* petri dish showing inhibition zone in treated group (20 µl, 40 µl)

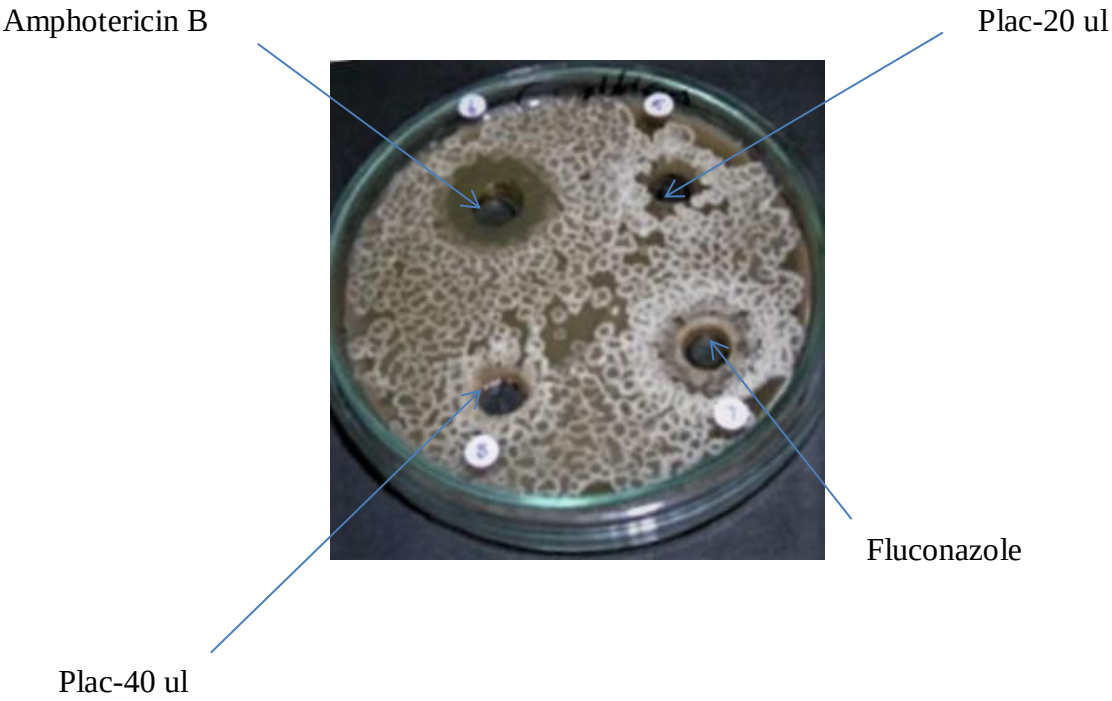


Figure 4.4: *Candida albican* petri dish showing inhibition zones in amphotericin B (more), Fluconazole, and treated (40 µl)

4.4 Wound index:

The graphical data shows that the group of rats treated with the *Camellia Sinensis* plant extract in comparison to injured groups shows a significant increase of wound contraction and results were estimated by applying one-way ANOVA using graph pad.

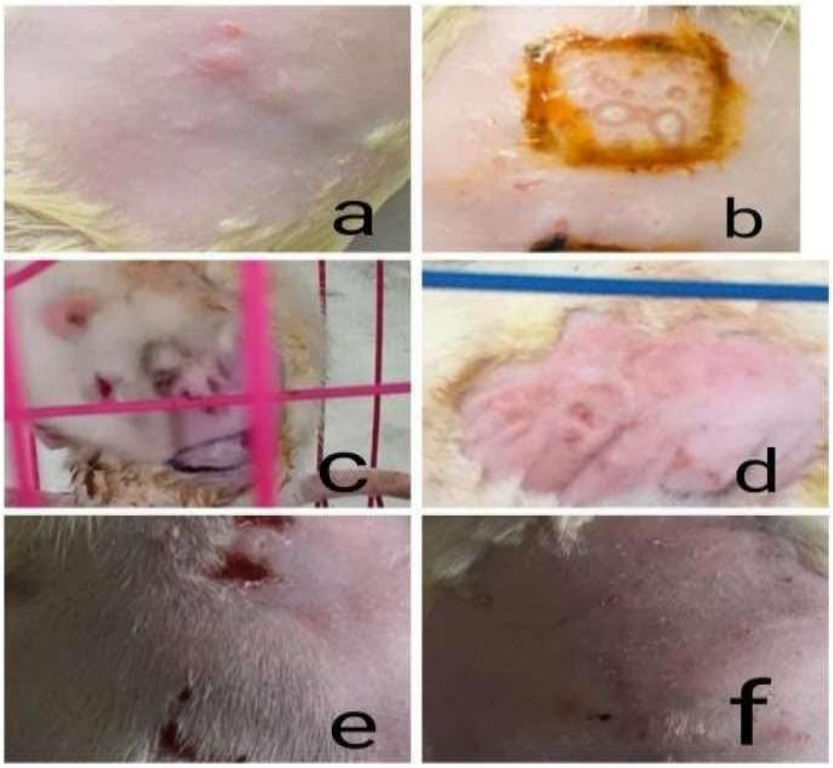


Figure 4.5: After receiving *Camellia Sinensis* extract treatment, a rat with acid burns showed wound contraction. **(a) Normal rat skin (b) Acid burned injury on rat (c) shows placebo 1 (d) shows treated rats 1 (e) shows placebo 2 (f) shows treated 2**

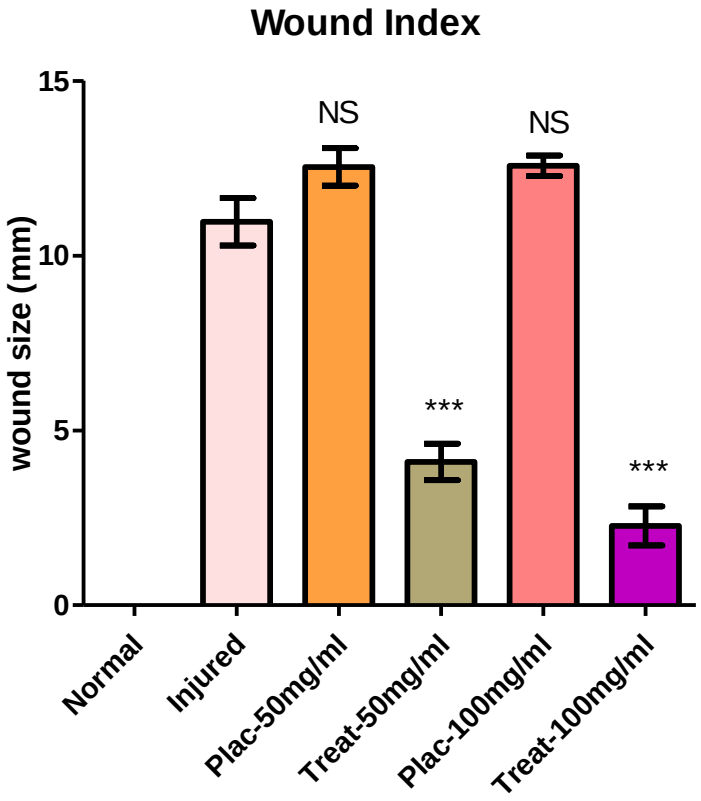


Figure 4.6: Wound index levels in treated group of rats with selected doses of *Camellia Sinensis* plant extract and in comparison to injured group of rats. The symbol *** denotes high statistical significance in results (P < 0.001). The mean ± SEM is used to expressthe values.

Table 4.2: Summary of graphical results of Wound Index

	Normal	**Injured**	**Placebo 50 mg/ml**	**Treated 50 mg/ml**	**Placebo 100 mg/ml**	**Treated 100 mg/ml**
WOUND INDEX	0.00±0.00	11±0.67	13±0.54	4.1±0.52	13±0.29	2.3±0.56

4.5 ELISA VEGF:

The graph data shows that the group of rats treated with the *Camellia Sinensis* plant extract in comparison to injured groups shows a significant value of VEGF marker and results were estimated by applying one-way ANOVA.

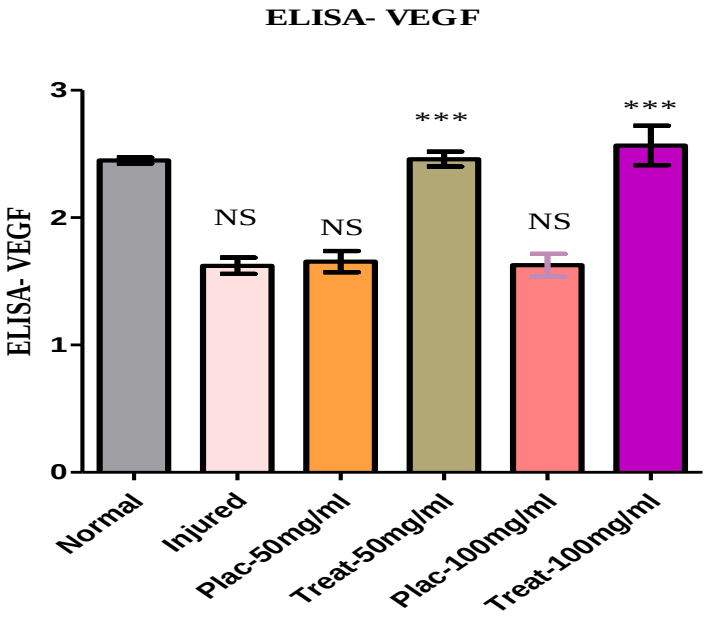


Figure 4.7: VEGF levels in treated group of rats with selected doses of *Camellia Sinensis* plant extract increase as compared to injured groups of rats. The symbol * * *indicates that the results are highly significant (P < 0.001). The mean ± SEM is used to expressthe values.

Table 4.3: Summary of graphical results of Elisa VEGF

	Normal	**Injured**	**Placebo 50 mg/ml**	**Treated 50 mg/ml**	**Placebo 100 mg/ml**	**Treated 100 mg/ml**
ELISA VEGEF	2.4±0.023	1.6±0.064	1.7±0.083	2.5±0.058	1.6±0.089	2.6±0.16

4.6 ELISA Annexin V:

The graph data shows that the group of rats treated with *Camellia Sinensis* plant extract in comparison to injured groups shows a significant value of Annexin-V marker and results were estimated by applying one-way ANOVA.

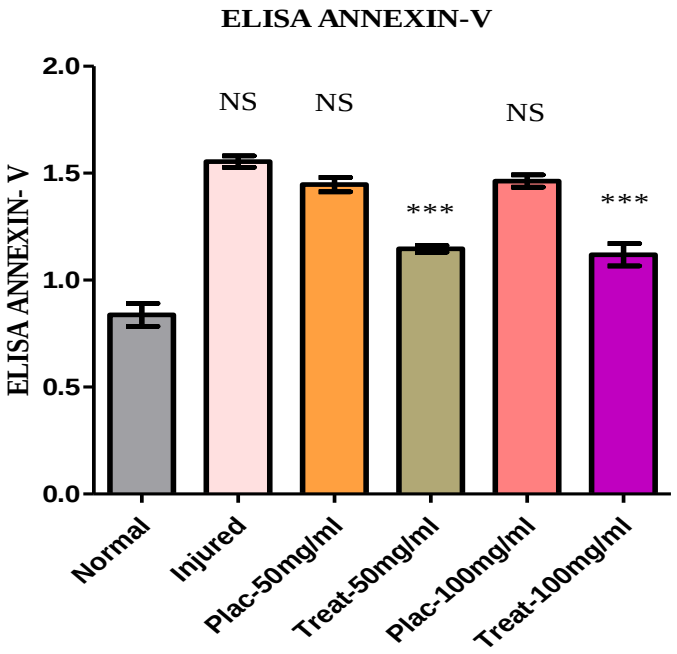


Figure 4.8: Annexin-V levels in treated group of rats with selected doses of *Camellia Sinensis* plant extract decreased as compared to injured groups of rats. The symbol* * * indicates that the results are highly significant (P < 0.001). The mean ± SEM is used to expressthe values.

Table 4.4: Summary of graphical results of Elisa Annexin-V

	Normal	**Injured**	**Placebo 50 mg/ml**	**Treated 50 mg/ml**	**Placebo 100 mg/ml**	**Treated 100 mg/ml**
ELISA ANNEX IN V	0.84±0.054	1.6±0.027	1.4±0.033	1.1±0.016	1.5±0.029	1.1±0.053

4.7 Antioxidant Analysis:

4.7.1 Estimation of APOX:

Camellia Sinensis is a biochemical test that determines the estimation of APOX levels.

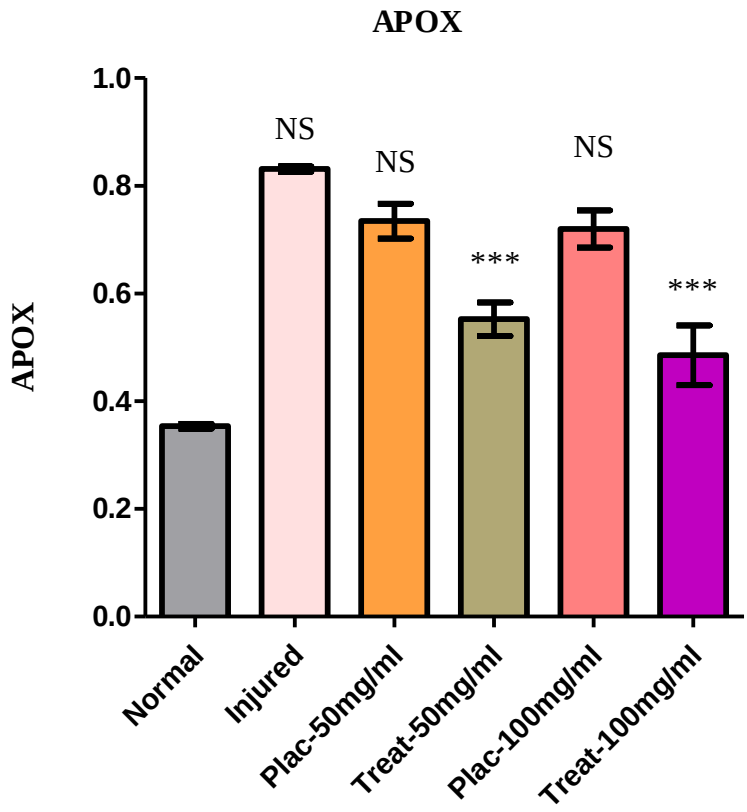


Figure 4.9: Apox levels in injury vs. treated groups of rats with selected doses of *Camellia Sinensis* plant extract. The symbol*** indicates that the results are highly significant ($P < 0.001$). The mean ± SEM is used to express the values.

Table 4.5: Summary of graphical results of APOX

	Normal	**Injured**	**Placebo 50 mg/ml**	**Treated 50 mg/ml**	**Placebo 100 mg/ml**	**Treated 100 mg/ml**
APOX	0.35±0.0045	0.83±0.049	0.73±0.032	0.55±0.031	0.72±0.034	0.45±0.055

4.7.2 Estimation of SOD:

Camellia Sinensis is a biochemical test that determines the estimation of SOD levels.

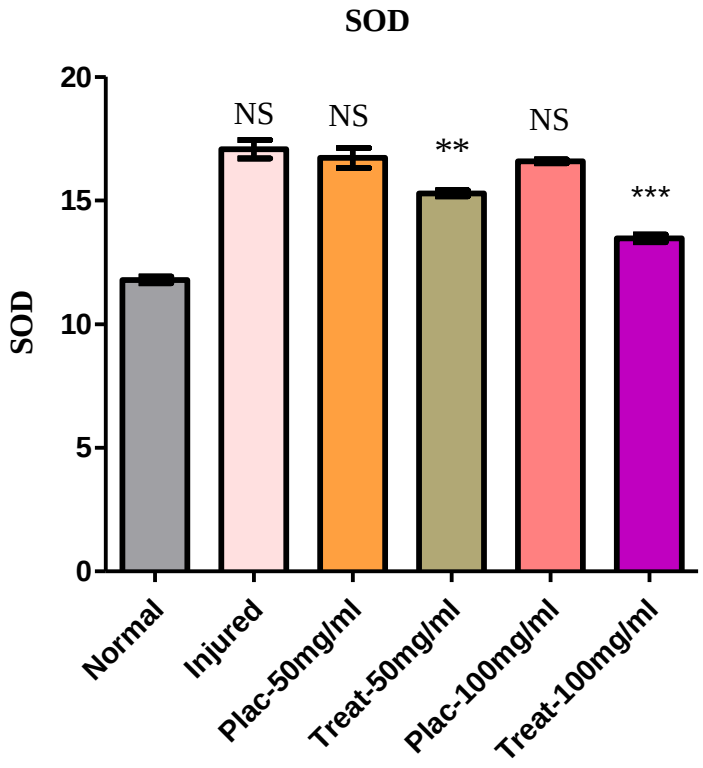


Figure 4.10: SOD levels in treated group of rats with selected doses of *Camellia Sinensis* plant extract and in comparison to injured group of rats. The symbol* * * indicates that the results are highly significant (P < 0.001). The mean ± SEM is used to expressthe values.

Table 4.6: Summary of graphical results of SOD

	Normal	**Injured**	**Placebo 50 mg/ml**	**Treated 50 mg/ml**	**Placebo 100 mg/ml**	**Treated 100 mg/ml**
SOD	12±0.14	17±0.38	17±0.40	15±0.12	17±0.079	13±0.15

4.7.3 Estimation of GSH:

Camellia Sinensis is a biochemical test that determines the estimation of GSH levels.

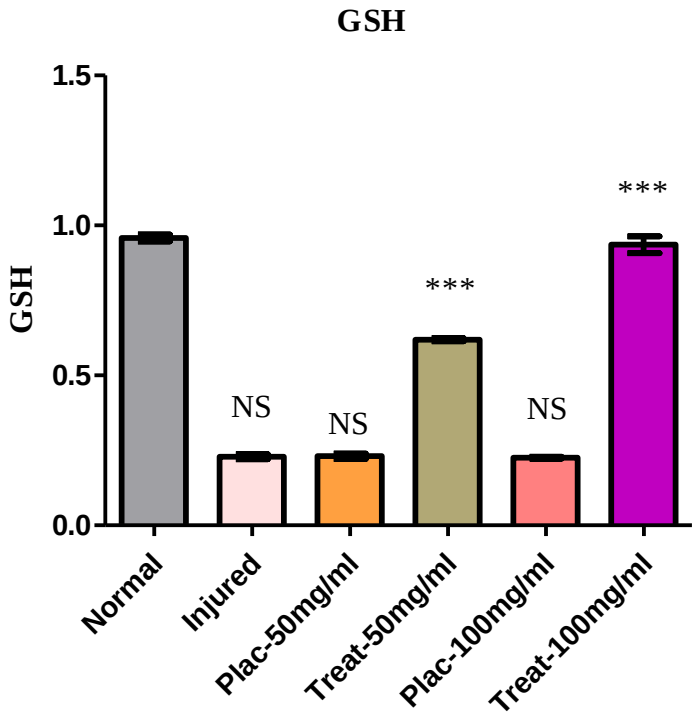


Figure 4.11: GSH levels in treated group of rats with selected doses of *Camellia Sinensis* plant extract and in comparison to injured group of rats. The symbol * * * indicates that the results are highly significant (P < 0.001). The mean ± SEM is used to expressthe values.

Table 4.7: Summary of graphical results of GSH

	Normal	**Injured**	**Placebo 50 mg/ml**	**Treated 50 mg/ml**	**Placebo 100 mg/ml**	**Treated 100 mg/ml**
GSH	0.96±0.012	0.23±0.087	0.23±0.091	0.62±0.056	0.23±0.033	0.94±0.028

4.7.4 Estimation of CAT:

Camellia Sinensis is a biochemical test that determines the estimation of GSH levels.

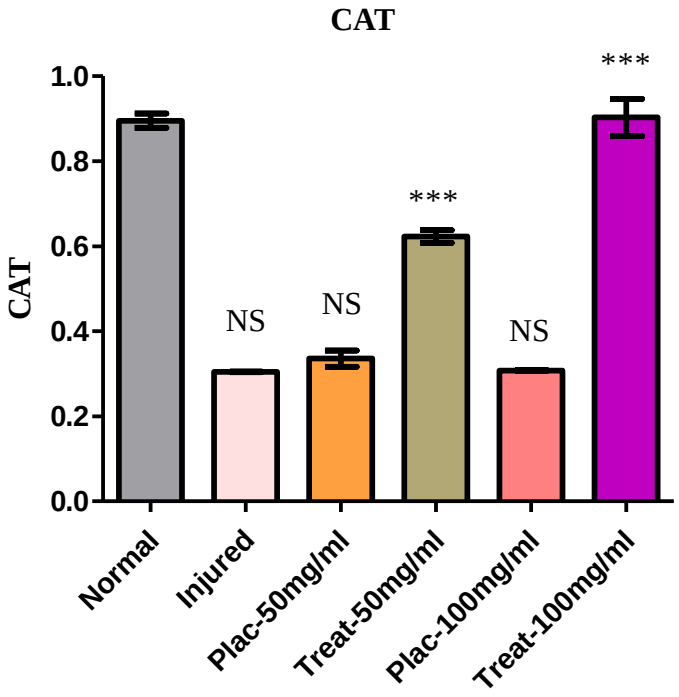


Figure 4.12: CAT levels in treated group of rats with selected doses of *Camellia Sinensis* plant extract and in comparison to injured group of rats. The symbol* * * indicates that the results are highly significant (P < 0.001). The mean ± SEM is used to expressthe values.

Table 4.8: Summary of graphical results of CAT

	Normal	**Injured**	**Placebo 50 mg/ml**	**Treated 50 mg/ml**	**Placebo 100 mg/ml**	**Treated 100 mg/ml**
CAT	0.90±0.017	0.30±0.00088	0.34±0.019	0.62±0.015	0.31±0.0012	0.90±0.044

4.8 Histopathology:

4.8.1 Group 1: Normal group

The epidermis and dermis are the two primary layers shown in the diagram. There are four to five cell layers in the epidermis. All glands appear to be normal. This demonstrates that the skin layers in this group have normal architecture.

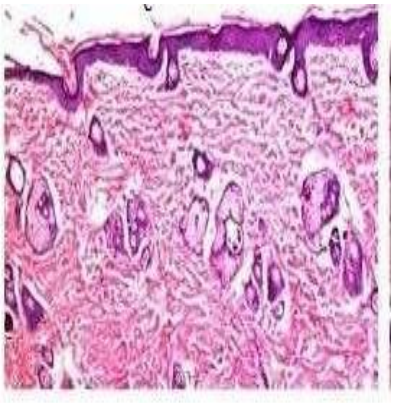


Figure 4.13: Histological diagram of Normal skin obtained from Albino rats

4.8.2 Group 2: Injury group

Acid burn wound injury shows that inflammation occurs. Skin layer thickness was compromised. In the diagram, dense infiltrations could be noticed. A change in skin architecture was observed.

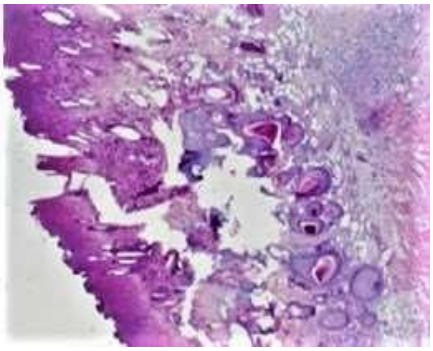


Figure 4.14: Histological diagram of Acid Burn injured skin obtained from Albino rats

4.8.3 Group 3: Placebo group (50mg/ml)

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

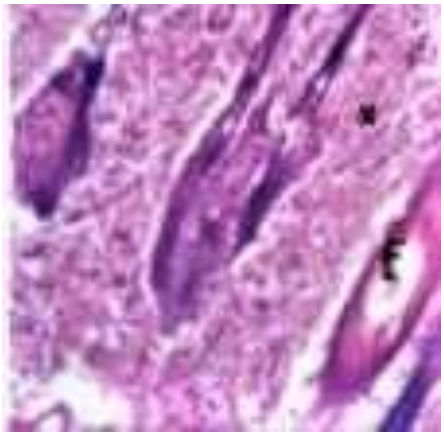


Figure 4.15: Histological diagram of skin obtained from Placebo group (50mg/ml) of Albino rats

4.8.4 Group 4: Treatment group (50mg/ml)

When using 50mg/kg of green tea extract, little effect on wound healing is seen in the diagram above. There is a little infiltration of inflammatory cells. The healing process has begun.

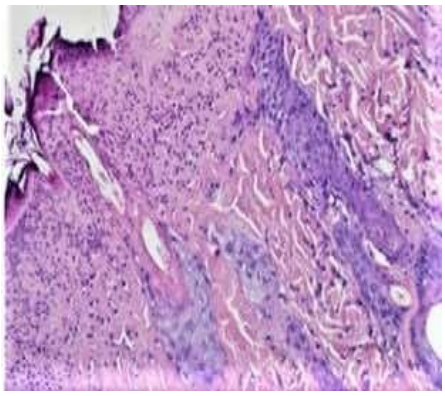


Figure 4.16: Histological diagram of skin obtained from Treated group (50mg/ml) of Albino rats

4.8.5 Group 5: Placebo group (100mg/ml)

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

Figure 4.17: Histological diagram of skin obtained from Placebo group (100mg/ml) of Albino rats

4.8.6 Group 6: Treatment group (100mg/ml)

Green tea extract at a level of 100mg/ml in this treated group demonstrated that skin morphology is similar to normal. This demonstrates that the wound's natural healing process took place and that the skin recovered.

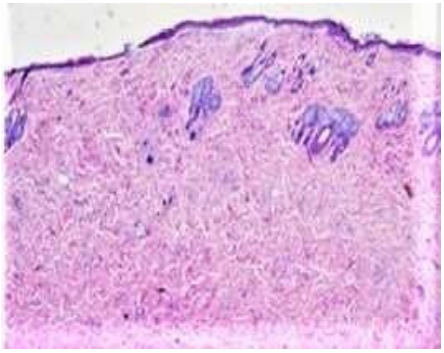


Figure 4.18: Histological diagram of skin obtained from Treated group (100mg/ml) of Albino rats

CHAPTER 5

DISCUSSION

An injury that breaks the skin or other body tissues is called a wound. Accidents and injuries are common causes of wounds. Healing from wounds takes time. The wound gets smaller as it heals more quickly. The longer it takes to heal, the larger or deeper the wound becomes. Herbal medicines have the ability to cure wounds. Plants with medical properties or those with favourable pharmacological effects on the human or animal body are considered medicinal plants. Herbal medicines are used to treat a wide range of illnesses. Minimizing tissue damage, ensuring appropriate tissue perfusion, oxygenation, and nourishment, and restoring the anatomical continuity and function of the afflicted tissue are the cornerstones of the most effective wound healing (77).

According to studies, drinking green tea regularly lowers the risk of developing cancer by preventing the onset and spread of the disease. The green tea plant possesses anti-aging properties, anti-fungal, anti-inflammatory, antioxidant, anti-microbial, antiviral, anti-parkinson, anticancer and wound healing activities have been demonstrated in vitro and in vivo using plant material. With its anti-oxidant, anti-cancer, and anti-aging, this plant may also have an impact on collagen formation and accumulation (78).

According to studies green tea extract has anti-inflammatory properties as well. The polyphenolic components in green tea are what give it its anti-inflammatory and anti-carcinogenic qualities (79). The primary and most chemopreventive component of green tea, (-)-epigallocatechin-3-gallate, is what gives the beverage its biochemical or pharmacological properties (EGCG).

A wound index level was measured in treated group of rats with selected doses of Green tea extract and in comparison to injured group of rats. The symbol *** denotes high statistical significance in results ($P < 0.001$). The ELISA assay was conducted, VEGF levels with doses of Green tea extract in treated group of rats compare to injured group shows, * ** indicates that the results are highly significant ($P < 0.001$). The symbol ** denotes statistical significant in results ($P < 0.05$). While the Annexin levels in treated group of rats with selected doses of Green tea extract and in comparison to injured

group of rats shows * * * which indicates that the results are highly significant ($P < 0.001$).

The antioxidant tests including APOX, GSH, CAT and SOD were estimated. APOX level, GSH level, CAT level, and SOD levels in injury vs. treated group of rats with selected dose of green tea extract indicates *** that results are highly significant ($P < 0.001$).

According to the results of the histopathology analysis, Camellia Sinensis treatment proves to be protective and advantageous in the treatment of wounds and demonstrates the medicinal role in the diseases as a cure.

CONCLUSION

The herbal medications have been utilized as healing properties. According to numerous studies, green tea possesses anti-inflammatory, antioxidant, antibacterial, and antifungal activities. Based on our treatment, present study concludes that green tea extract has potential for wound healing in albino rats and antifungal activities in Candida albican. The highest antifungal effects of green tea extract were seen at 40 ul as compared to 20 ul. At different doses of *Camellia Sinensis* extracts, the wound healing potential investigation was conducted using antioxidant estimation, wound index measurement, Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) Protocol, and Histopathological test. The wound index of albino rats was examined, and the results showed significant. The 100 mg/ml dosage of green tea extract is the cause of increased VEGF markers. In other cases, 100 mg/ml of green tea extract causes less apoptosis, which results in cell death. The results were highly significant. The histopathological results of green tea extract were also examined. The demonstration of green tea extract's antifungal and wound-healing abilities opens a novel possibility for further research to treat acid burn injuries.

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