EVALUATION OF WOUND HEALING EFFECTS OF LEAF EXTRACTS OF *Simarouba glauca* ON WISTAR RATS

Dissertation Submitted to

The TamilNadu Dr. M.G.R. Medical University, Chennai

In partial fulfillment for the award of the Degree of

MASTER OF PHARMACY

(Pharmacology)

OCTOBER-2016

DEPARTMENT OF PHARMACOLOGY

KMCH COLLEGE OF PHARMACY

KOVAI ESTATE, KALAPPATTI ROAD,

COIMBATORE-641048

EVALUATION OF WOUND HEALING EFFECTS OF LEAF EXTRACTS OF *Simarouba glauca* ON WISTAR RATS

Dissertation Submitted to

The TamilNadu Dr. M.G.R. Medical University, Chennai

In partial fulfillment for the award of the Degree of

MASTER OF PHARMACY

(Pharmacology)

OCTOBER-2016

Submitted by
Reg No:261425821

K	M
C	H

DEPARTMENT OF PHARMACOLOGY

KMCH COLLEGE OF PHARMACY

KOVAI ESTATE, KALAPPATTI ROAD,

COIMBATORE-641048

Dr. A. RAJASEKARAN, M. Pharm., Ph.D.,

Principal,
KMCH College of Pharmacy,
Kovai Estate, Kalapatti Road,
Coimbatore - 641 048,
Tamil Nadu.

CERTIFICATE

This is to certify that the dissertation work entitled "**Evaluation of wound healing effects of leaf extracts of *Simarouba glauca* on wistar rats"** is a bonafide research work carried out by the candidate **Reg No: 261425821** and submitted to The Tamil Nadu Dr. M.G.R Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology** at the Department of Pharmacology, KMCH College of Pharmacy, Coimbatore, Tamil Nadu during the academic year 2015-2016.

Date: **Dr. A. Rajasekaran,M.Pharm., Ph.D.,**

Principal

GUIDE,
KMCH College of Pharmacy,
Kovai Estate, Kalapatti Road,
Coimbatore -641 048,
Tamil Nadu.

CERTIFICATE

This is to certify that the dissertation work entitled "**Evaluation of wound healing effects of leaf extracts of *Simarouba glauca* on wistar rats"** is a bonafide research work carried out by the candidate **Reg No: 261425821** and submitted to The Tamil Nadu Dr. M.G.R Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology** at the Department of Pharmacology, KMCH College of Pharmacy, Coimbatore, Tamil Nadu during the academic year 2015-2016.

Date: **Guide**

Department of pharmacology

DECLARATION

I do hereby declare that the dissertation work entitled **"Evaluation of wound healing effects of leaf extracts of *Simarouba glauca* on wistar rats"** submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology**, was done by me at the Department of Pharmacology, KMCH College of Pharmacy, Coimbatore, Tamil Nadu during the academic year 2015-2016.

Date: **Reg No: 261425821**

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"evaluation of wound healing effects of leaf extracts of *Simarouba glauca* on wistar rats"** submitted by the candidate **Reg no: 261425821** to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology** is a bonafide work carried out by the candidate at the Department of Pharmacology, KMCH College of Pharmacy, Coimbatore, Tamil Nadu and was evaluated by us during the academic year 2015-2016.

Date:

Internal Examiner **External Examiner**

Convener of Examinations

Examination Centre: KMCH College of Pharmacy, Coimbatore.

For the ancestors who paved the path before me upon whose shoulders I stand. Also to my parents friends and my dear sister….

ACKNOWLEDGEMENT

On this fruitful occasion of the successful completion of this dissertation, I bow my head to the **God almighty** who is always showering blessings upon me and without whose blessing, I would not have been able to attain this stage in my life.

It is my first and foremost duty to express my sincere thanks and deep sense of indebtedness to my guide **Mr.G.Ariharasivakumar**, **M.Pharm.,** Assistant Professor, Department of Pharmacology, who has guided me and taken interest in my project. His scholarly guidance and inspiring suggestions have helped me in carrying out the present work. Words are inadequate to express my deep sense of gratitude to him for his invaluable guidance.

With great pleasure I wish to place my indebtedness to **Dr. K.T. Manisenthil Kumar M. Pharm., Ph.D.,** Professor and head, Department of Pharmacology for his support, guidance and all the timely help to do my project work.

It is my previlage to thank **Dr. A. Rajasekaran, Principal,** KMCH College of Pharmacy, Coimbatore who has provided excellent facilities to do research in this institution.

I will always remain indebted to **Dr. Nalla G. Palanisamy**, Chairman, and **Dr. Thavamani D. Palanisamy**, Managing Trustee, KMCH College of Pharmacy, Coimbatore for all the facilities, which have been provided to us at the institution, enabling me to do work.

I owe my heartfelt thank to me esteemed and beloved staffs to **Dr. N. Adhirajan, M.pharm, Phd., Dr. C. Sundaramoorthy M.pharm, PhD, Mr.Saravanan j, M.pharm, Mrs. A. Abarnadevika, M.pharm, Mr. M. Senthil Kumar, M.Pharm** for their sensible help and suggestions.

I am greatful to labtechnicians **Ms. Vanathi and Mr.Tamilarasan**, (Department of pharmacology) **Mrs.Anandhi, Mrs.Sudha and Mrs.Thiruveni,** Librarian and chemical store keeper **Mr.Viji** for their valuable support and timely help during the course of the entire work.

Specilal thanks are extended to **Pink panthers, Jopson, Kanchana, Manimaran, Aaron.**

I am thankful to all my seniors and juniors for their help and support.

My sincere thanks to **Dr.Pasupathy, Yarn Department, South India Textile research Association**, Coimbatore who extended his co-operation in my studies.

A word of thanks to **Mrs. Dhanalakshmi** for helping in animal maintenance in my animal studies.

I cherish the friendship I had and take this opportunity to thank each one of them. My friends have been an encouragement every time and their motivation my confidence that had helped me reach here. To my friends **Aswathi, Deepthi, Divya, Nisha, Revathy and Swathi** for their constant support and whole hearted solidarity. My special thanks to them for the great time we had during the hostel fest shall always be cherished.

With immense pleasure I express my deep sense thanks of gratitude to **Rida** for the valuable support throughout the entire course of my project.

Above all I would like to thank my loving **parents** for everything you have done for me over the past 24 years. You guys have always believed in me and have been so supportive.

Reg No: 261425821

ABBREVIATIONS

ABBREVIATIONS	FULL FORM
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AQSG	Aqueous extract of *Simarouba glauca*
CAT	Catalase
CFU	colony forming units
DMSO	Dimethyl Sulfoxide
DPPH	1, 1-diphenyl-2-picrylhydrazyl
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
EASG	Ethyl acetate extract of *Simarouba glauca*
EDTA	Ethylene Diamine Tetra Acetic acid
FBS	Fetal Bovine serum
GAE	Gallic acid Equivalent
GSH	Reduced glutathione
HAI	Hospital acquired infection (HAI)
HPTLC	High Performance liquid Chromatography
LPO	Lipid peroxidation
MEM	Modified Eagle's Medium
MIC	Minimum inhibitory Concentration
MTCC	Microbial Type Culture Collection
MTT	3-(4,5–dimethyl thiazol–2–yl)–5–diphenyltetrazolium bromide
NCCS	National Centre for Cell Sciences
NCIM	National Collection of Industrial Micro organisms
NINSS	Nosocomial Infection National Surveillance Service
PBS	Phosphate Buffered Saline
QE	Quercetin Equivalent
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TLC	Thin Layer Chromatography
WHO	World Health Organisation

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

Acknowledgment

Certificates

Introduction

Plan of work

Review of literature

Plant profile

Materials and Methods

Results

Discussion

Conclusion

Bibliography

ABSTRACT

The present investigation has been undertaken to study the wound healing properties of ethyl acetate and aqueous extracts of *Simarouba glauca*. The plant *Simarouba glauca* has a long history in herbal medicine in many countries. Experiments were conducted following standard procedures. Both extracts were evaluated for their *in vitro* anti oxidant, antimicrobial and cytotoxic properties. The EASG and AQSG ointments were administered topically, for evaluating the wound healing potential in excision wound model for fourteen days, in burn wound model for twenty one days, and in infected excision wound model for twenty one days. Povidone iodine was the standard for excision wound and silver sulfadiazine was used as the standard for both burn and infected excision wound models. Extracts treated groups showed higher *in vitro* antioxidant, anti microbial and cytoprotective activities. EASG exhibited similar wound healing activity that of the standard but with lesser magnitude.The result may be attributed to the phytoconstituents such as flavonoids and phenolics present in it which may be due to their individual or cumulative effect that enhanced wound healing and provided scientific evidence to the ethnomedicinal futures of *Simarouba glauca*. These findings could justify the inclusion of this plant in the management of wound healing.

Keywords: EASG, AQSG, Wound healing potential, Phytoconstituents.

1. INTRODUCTION

1.1 INTRODUCTION

This chapter presents; the background of the study, statement of the problem, definition of terms, purpose of the study, theoretical basis and organization of the remainder of the study.

1.1.1 Background of the Study

Wound types are diverse, from acute to chronic and traumatic to surgical to disease-driven. But the range of products and under development to address them is even more varied. Current estimates indicate the worldwide nearly 6 million people suffer from chronic wounds.[1] In developed countries, it is estimated that 1 to 2% of the population will experience a chronic wound during their lifetime.[2] Data shows a predictable global increase in surgical wounds, lacerations and traumatic wounds from 2012 to 2017, with a small projected increase in the prevalence of burns in this period. Other types of wound, such as amputations and carcinomas are also predicted to rise slightly. Even if it is small in number when compared to surgical wounds, the report shows that chronic wounds are the most challenging and high-cost wounds to manage, making them a target for development of technologies to manage them. It is also reported that, chronic wounds, such as pressure ulcers, venous ulcers and diabetic ulcers, are set to increase from a little over 40 million to over 60 million between 2012 and 2017.[3] An estimated 265,000 deaths every year are caused by burns – the vast majority occur in low and middle-income countries. In 2004, nearly 11 million people worldwide were burned severely enough to require medical attention.[4]

Thirty five million cutaneous wounds requiring major intervention occur yearly in the U.S.A. alone. And it is been estimated that the total number of chronic wounds exceeds 2 million and possibly up to 5 million annually in the U.S.A. alone (1998). The social and financial levy of chronic wounds is very high. The common cause of acute wounds is thermal injury, and in each year 2.5 million people are suffering from burn injury, especially in USA. Other major type of acute cutaneous

wounds are caused by excision of extensive skin cancer, trauma and medical conditions such as deep microbial infections, vasculitis, scleroderma, pemphigus, toxic epidermal necrolysis to name a few. Classes of chronic wounds include arterial ulcers, venous ulcers, diabetic ulcers and pressure ulcers. It is estimated that the prevalence of leg ulcers alone is between 0.5%± 1.5% with an annual cost of nearly $1 billion.[5] Chronic wounds represent a silent epidemic that affects a large fraction of the world population and poses major and gathering threat to the public health and economy of the United States.

In India, over 1,000,000 people are moderately or severely burnt every year. The prevalence of wounds in the population studied (n=6917) was 15.03 per 1000. The prevalences of acute and chronic wounds were 10.55 and 4.48 per 1000 of the population respectively. The most common site for both acute and chronic wounds was the lower extremity. In contrast to Western studies, the most common etiology for a chronic lower extremity wound was an untreated acute traumatic wound. This in turn highlights the need to establish community-based wound-care teams in India.[6]

Wound infection is not a modern phenomenon. As early as 14-37AD there is documentary evidence that Cornelius Celsus (a Roman physician) described the four principal signs of inflammation and used 'antiseptic' solutions. Another Roman physician, Claudius Galen (130-200 AD) had such an influence on the management of wounds that he is still thought of by many today as the 'father of surgery'. It should also be remembered that he and some of his followers instigated the 'laudable pus' theory, which incorrectly considered the development of pus in a wound as a positive part of the healing process

The 2002 survey report by the Nosocomial Infection National Surveillance Service (NINSS), which covers the period between October 1997 and September 2001, indicates that the incidence of hospital acquired infection (HAI) related to surgical wounds is as high as 10%. These infections complicate illness, cause anxiety, increase patient discomfort and can lead to death. The cost to the NHS is almost £1 billion pounds per annum.[7]

The incidence of burns in the US has dropped from 4.2 per 100,000 during the year 1961 through 1964 to 1.5 per 100,000 during the period 1993 through 1996. Improvement in burn assessment and delivery care however needs further improvement to reduce medical transport and treatment costs.[8]

1.1.2 Statement of the Problem

Wounds are normally resolved in a few days, but chronic wounds represent a major burden because of social and economic factors. Thereby, the search for new agents is ongoing and natural products become a great target. Individual factors such as stress or diabetes can cause delay in the healing process or increase the risk of infection in the wound. Due to unawareness of the society this can lead to chronic diseases which may damage the other organs. Impaired wound healing results in severe morbidity leading to long hospitalization of patients. States (US) population, which results in a significant economic burden estimated to be nearly 2–4% of the health budgets.[9]

Wound healing is the general repair response of the body immediately after the disruption of skin integrity. It is essentially composed of five interconnected and overlapping phases; haemostasis and inflammation, neovascularization, granulation, re-epithelization and remodeling. The treatment of wounds is sometimes challenging especially in chronic wounds with a prevalence of 4.5 per 1000 population. Although there has been vast development in pharmaceutical drug industry, wound healing drugs are not still satisfactory because of their low availability, high cost, and various detrimental side effects. Therefore, medicinal plant derived drugs is under great demand because of the common belief that they are safe, reliable, clinically effective, low cost, globally competitive and better tolerated by patients. Since ancient times, human beings have been using many plant resources based on empirical observations without any scientific knowledge for the treatment of wounds, cuts, and burns. Biologically active constituents of these plants such as tannins, triterpenoids, flavonoids and alkaloids have been found to affect one or more phases of wound healing process.

1.1.3. Definition of Terms

1.1.3.1 Wound: A wound may be defined as a break in the epithelial integrity of the skin or may also be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissue**.**[10]

1.1.3.2 Healing: Healing is a complex biological process including blood coagulation inflammation, fibroplasia, collagen deposition, and wound contraction.[11]

1.1.3.3 Epithelialization: The process of covering a denuded surface with epithelium or the process of assembling an epithelium from non-epithelial cells, such as in the process of mesenchymal to epithelial transition.[12]

1.1.3.4. Inflammation: A localized physical condition in which part of the body becomes reddened, swollen, hot, and often painful, especially as a reaction to injury or infection.[12]

1.1.3.5. Collagen: The main Structural protein in the extra cellular space in the various connective tissues.[14]

1.1.3.6. Angiogenesis: Physiological process through which new blood vessels from pre-existing vessels.[15]

1.1.3.7. Flavonoids: Flavonoids are ubiquitous in plants; almost all plant tissues are able to synthesize. There are also a wide variety of types at least 2000 naturally occurring flavonoids. They can be classified into seven groups: flavones, flavonones, flavonols, flavononols, isoflavones, flavonols (catechins) and anthocyanidins. In general, the leaves, flowers, fruits, or the plant itself, contain flavonoid glycosides, while the woody tissues contain aglycones, and the seeds may contain both.[16]

1.1.3.8. Phytochemicals: Biochemicals derived from naturally-occurring plant sources, and may be beneficial for health or treatment of disease**.**[17]

1.1.3.9. Antioxidant: A substance that inhibits oxidation or reactions promoted by oxygen or any substance that prevents or reduces damage caused by reactive oxygen species (ROS) or reactive nitrogen species (RNS). ROS and RNS are highly reactive chemicals that attack other molecules and modify their chemical structure synthetic or natural substances added to products to prevent or delay their deterioration by action of oxygen in air. In biochemistry and medicine, antioxidants are enzymes or other

organic substances, such as vitamin E or beta-carotene that are capable of counteracting the damaging effects of oxidation in animal tissue.[18]

1.1.4 Theoretical Framework

Wound is a type of injury which happens relatively quickly where skin is cut, torn or punctured or where blunt force trauma causes a contusion . In a pathological way it refers to a sharp injury which leads to the damage of dermis of the skin. Wound can be classified based on the contamination like clean wound contaminated wound, Infected wound and Colonized wound: or can be classified as acute and chronic wound based on the severity, or it may be open or closed. The cutaneous response to injury and stress comprises a temporary change in the balance between epidermal proliferation and differentiation as well as an activation of the immune system. Soluble factors play an important role in the regulation of these complex processes by coordinating the intercellular communication between keratinocytes, fibroblasts, and inflammatory cells. The process of wound healing may be presented as a series of separate events. In actuality, the entire process is much more complicated, as cellular events that lead to scar formation overlap. Many aspects of wound healing have yet to be elucidated.[19] Cutaneous wound healing is a complicated, multistep process with numerous mediators that act in a network of activation and inhibition processes. Current understanding of the complex process of wound repair is based on decades of study. Now that major wound processes are more fully understood, therapeutic strategies can be developed to manipulate wound repair.

Animal models always provide crucial insights into the mechanisms and pathophysiology of cutaneous wound repair. Various aspects of the healing process can be characterized and quantified in a controlled and reproducible environment. Various types of normal and pathological injury paradigms can be applied in both small and large animal species. To mimic clinical problems, rates of repair can be compromised by surgical impairment of blood supply or metabolic manipulations such as the diabetic state. Even though animal wound repair is an imperfect reflection of human wound healing and its clinical challenges, these models continue to be crucial tools for the development of new strategies and approaches to rational wound

therapy.[20] The number of studies that use animal models for wound healing has been increasing recently. However this is not surprising considering the number of new products that are introduced each year. There are numerous types of *in-vivo* models available, and each has unique benefits and disadvantages.

Rodent and small mammal models for wound healing have emerged as the model of choice for many researchers all over the world. This type of animal study is valuable to wound research for multiple reasons. Small animals are inexpensive, easily obtainable, and require less space, food, and water. Moreover, these animals often have multiple offspring, which develop quickly allowing experiments to proceed through multiple generations. Small animals generally have accelerated modes of healing when compared to humans, thus experiment duration lasts for days, whereas it takes weeks or months in human experiments. Likewise some small animals can easily be altered genetically and provide a wound model capable of resembling defective human conditions such as diabetes, immunological deficiencies, and obesity. Another advantage of these models is their ability to serve in experiments where death is an endpoint, as in some cases of bacterial or viral infection.[21]

Animal models have been available for decades, yet historically in the wound healing field translation of candidates identified in animal models to human therapies have been problematic. Indeed, most will agree that while invaluable for uncovering important pathways and processes, current animal models are not well suited to preclinical evaluation of therapeutic candidates. With the caveat of known intrinsic differences to human repair, the mouse model system provides an abundant, manipulatable, accurate tool for wound repair study and is likely to remain the model of choice to mechanistically probe the process for the foreseeable future.[22] The various animal models that are used to interpret the wound healing potential are acute Wound Models, Impaired Healing Models, Immunologic, Chronic Wound Models. The experimental models used in this study are:

- Excision wound model
- Burn wound model
- Organism induced wound model

Excision wound model: Excision wound involves the removal of great amount of tissues and is useful in evaluating the wound closure, re-epithelialization grade, inflammation, wound maturation, neo-angiogenesis and remodeling process. This model is useful in determining the biochemical and histological parameters. It include tape stripping, blisters, split-thickness, full thickness, splinting.

Burn wound model: Chemical or radiation burns the skin or other tissues produce a remarkably different healing response due to their effect on the viability of cells and tissues. The depth of the burn may be designated as first, second, or third degree. Thermal burns in particular create an extensive zone of frank necrosis that includes dead cells and denatured or even charred connective tissue. This model is used to study the pathogenesis of skin damage and allowing determination of three basic phases which occur during wound healing

Infecetd excision wound model: Creating wound on the back of mice followed by the inoculation of micro-organism to the wound area. In this model bacterial colonization-wound biofilm helps to explain many of the complex challenges that are clinically observed. A stabilized mature biofilm will induce an effect on the wound that will delay healing and induce a local infection.**[23]**

These models replicates the events observed in patients with acute and chronic wound, and therefore serve as a useful and relevant model for studying the healing potential.

1.1.5 Purpose of the Study

Traditional medicine practitioners have described the therapeutic efficacies of many traditional and indigenous plants against diseases. A large number of plants are used by folklore traditions in India for treatment of cuts, wounds and burns. Various research data revealed that plants may worked in healing and regeneration of the tissue by multiple mechanisms. There are several reports stating that the extracts of several plants, used for wound healing properties. Natural products that are safe, and possess physiological properties are excellent sources of new therapeutics for the treatment of conditions like mechanical damage of the skin. Some researchers therefore have shifted their focus to the potential wound healing properties of plants.

Wounds are normally resolved in a few days, but chronic wounds represent a major burden because of economic and social factors. Thereby, the search for new agents is ongoing and natural products become a great target. Various literatures and studies show that the active principles triterpinoids, Quassanoids and flavonoids are having a crucial role in healing acute and chronic wounds. *Simarouba glauca* is rich in these types of triterpinoids, Quassanoids and Flavonoids. The purpose of this study is to investigate and evaluate wound healing activity on various wound model by using ethylacetate and aqueous extracts of *Simarouba glauca*.[24]

1.1.6 Hypothesis

I hypothesizethat the presence of active constituents like triterpenoids, quassinoids and flavonoids in this plant *Simarouba glauca* after isolation and extraction is having protective effect in healing wounds. To test whether the plant possess the same activity, different wound models are selected. The result of these studies will have a translational value to wound healing effect.

The following specific aims will be pursued to achieve my objectives

1.1.7 Specific Aims

1. To determine the in-vitro anti-oxidant capacity of leaves of *Simarouba glauca*
2. To determine the In-vitro cytotoxic effect of leaves of *Simarouba glauca*
3. To determine the in-vitro anti-microbial potential of leaves of *Simarouba glauca* on different micro-organisms.
4. To employ the leaves of *Simarouba glauca* in short term different wound models to determine wound healing effect by
    - Excision wound model
    - Burn wound model
    - Organism induced wound model

1.1.8 Organization of the Remainder of the Study

The remainder of the study will consist of the following .Chapter II will review the literature on plant of *Simarouba glauca.* Chapter III will detail the research design and methodology of this study. Chapter IV will present the data collected. Chapter V will present the findings, conclusions and recommendations of this study. The references list works cited in the study

2. PLAN OF WORK

1. Review of Literatures

2. Selection, Collection And Authentication of Plant Material

3. Extraction of plant materials with petroleum ether, ethyl acetate and water

4. Preliminary phytochemical analysis & chromatographic Screening

5. Quantification of total phenol and flavanoid content

6. *In vitro* antioxidant study

- DPPH radical scavenging assay
- ABTS radical cation assay

7. Invitro cytotoxicity study by MTT assay

8. Invitro anti-microbial studies

9. Pharmacological study

- ✓ Screening of wound healing activity using various models
  - Excision wound model
  - Burn wound model
  - Infected excision wound model
- ✓ Invivo activity
  - Evaluation of parameters

a. Percentage wound contraction
b. Epithelialization time
c. Tissue breaking strength
d. Total protein
e. Hydroxy proline
f. Hexosamine
g. Uronic acid

- In-vivoAntioxidants assays
  - Enzymatic antioxidants(SOD,CAT,)
  - Non-enzymatic antioxidant(GSH)
  - Estimation of Lipid peroxidation(LPO)

10. Histopathological study

11. Statistical analysis

3. REVIEW OF LITERATURE

3.1. WOUND

A wound may be defined as a rupture or break in the epithelial integrity of the skin or it can be defined as a loss or breaking of cellular, anatomic and functional continuity of living tissue as a result of trauma or violence followed by damage or disruption of underlying normal tissue.

Or it can also be defined as a type of injury which happens relatively quickly where skin is cut, torn or punctured or where blunt force trauma causes a contusion . In a pathological way it refers to a sharp injury which leads to the damage of dermis of the skin.

Every year, millions of people experience burns, suffer from chronic wounds, or have acute wounds that become complicated by infection, dehiscence or problematic scarring. Now a day it is considered as the major problem in the field of clinical practice[26]

3.2 CLASSIFICATION[26]

Based on the level of contamination

- Clean wound
- Contaminated wound
- Infected wound
- Colonized wound

Clean wound : These type of wounds are made under sterile conditions where they are devoid of micro-organisms and they likely to heal without any complications.

Contaminated wound: These kind of wounds shows the presence of pathogenic micro-organisms and foreign bodies as a result of accidental injury.

Infected wound: infected wound also shows the presence of pathogens, clinical signs of infection where it will be yellow in color, filled with pus along with pain and redness.

Colonized wound: Chronic wound where the healing is very difficult and also filled with pathogens.

Based on the duration of healing it can be classified as:

- Acute wounds
- Chronic wounds

3.2.1 ACUTE WOUNDS[27]

Acute wounds heal without any complications in the predicted amount of time.

Causes of Acute Wounds

- Sharp pointed objects like nail, jagging or poking into the body
- Rough surfaces rubbing and scraping against the skin
- Sharp blades or edges like knife, cutting the skin cleanly
- Hard blows by objects, tearing the tissue roughly by sheer force

Types of acute wounds

1. Surgical wounds: These wounds are made purposefully, it may be closed or left open for healing. Surgical wound will be always clean, but sometimes it may be contaminated due to infection of micro-organism.
2. Traumatic wounds: Caused by force or impact of some nature. Eg: incision, abrasion.

Signs and symptoms:

The major signs and symptoms include pain, bleeding at the site of injury. The skin that surrounds the wound may be open and appeared to be torn. Infected wound may have foul smell, pus, and the area surrounds the infected wound may be red, tender and swollen.

3.2.2 CHRONIC WOUNDS[28]

These kinds of wound usually take longer time for healing and lead to complications.

Causes of chronic wounds:

Failure or delay in wound healing may due to lack of one or more of the main factors of healing including good supply of oxygen, blood, nutrients and clean and infection free environment.

Types:

1. Infectious wounds: If the infection is not treated properly, it will not heal in the expected time.
2. Ischemic wounds: The wound area will be ischemic that there will be insufficient blood supply. So the supply of oxygen and nutrients will be limited leading to delay in wound healing.
3. Radiation wound: Excessive exposure to ionizing or radiating materials weakens the immune system, causes damage to the exposed tissues and delay the healing time of all wounds.
4. Surgical wounds: This type of wounds can progress in to chronic wounds if the blood supply to the surgery area is accidentally damaged or the wound care is inadequate. It may eventually leads to delay in wound healing.
5. Ulcers (the most common type of chronic wounds):- Arterial ulcers, Venous ulcers, Diabetic ulcers, Pressure ulcer

Signs and Symptoms

The signs and symptoms may differ based on the type of chronic wounds.

Infectious wounds: these are typically associated with pus drainage, bad odor, debris (yellowish to greenish) or dead tissue, and ongoing symptoms of inflammation like fever, pain, redness, hotness and swelling.

Ischemic wounds: Typically the affected area will be pale and cold. Hair growth will be diminished and there might be a weak pulse sensation in the area.

Radiation poisoning wounds: Usually associated with redness, itching, blistering, inflammation and also other unspecified symptoms that include vomiting, nausea, fever and abdominal pain.

Surgical wounds: Generally occur at the site of surgical incision, but instead of a clean appearance, the tissue around the incision appears red, hot, and swollen, which can be inflamed or infected.

Ulcers: It may vary based on the type of ulcers, These ulcers are superficial, shallow, and irregularly shaped with pain and edema Diabetic ulcers: These can be either of neuropathic (secondary to nerve damage) origin, where the lack of sweat makes the skin dry and easy to crack and scale, forming callus (accumulation of dead skin layers). Pressure Ulcers includes itching, blistering, hotness, swelling and discoloration of the area.

3.2.3 OPEN WOUNDS[29]

In open wounds the underlying tissues will be exposed or the organs are open to the outside environment as seen in the case of penetrating wounds.

Causes, Types and Symptoms

1.**Abrasion**: Shallow irregular wounds appear on the upper layers of skin usually with minor to no bleeding. Abrasion occurs due to skin brushing with a smooth surface at a high speed or with rough surface. These kinds of wound have mild pain that subsides shortly after the initial injury.

2.**Lacerations**: Tear like wounds usually deeper than abrasions with irregularly torn edges and cause more pain and bleeding. Lacerations commonly caused by contact with objects like hard blows, accidents or collusions or trauma.

3.**Incisions**: incision type wound usually results from surgical procedure or skin cut with sharp objects such as; scalpels, knives and scissors. These are linear in shape with smooth, sharp edges. Depending on the depth and site of the wound it can be life threatening if it involves vital organs, nerves or major blood vessels.

4.**Punctures:** Punctures are small rounded wounds results from objects with very thin pointed tips like nails, needles, or other tapered objects and with teeth in cases of

animal or human bites. The wound depth, size, pain and bleeding are directly related to the force and size of causative object.

5. **Penetrating:** these types of wounds are caused by force or any objects that breaks through the skin to the underlying tissues or organs. And have variable shapes, size and presentations; based on the cause. These can be life threatening, causing severe damage especially to nerves, blood vessels and even to vital organs.

3.2.3.1 COMPLICATIONS[30]

1. Infection: Most open wounds are caused by contaminated objects that carry different variety of micro-organisms. These kinds of wound present a foul odor, pus, fever and pain.
2. Inflammation: Results from the body's immune response to foreign materials that caused a wound. This will make the wound area red, swollen, hot and even painful.
3. Scarring: These kinds of wounds will leave a scar after healing, sometimes lead to the deformity of the affected area; particularly with gunshot, penetrating or deep laceration wounds.
4. Loss of function: Depending on the severity of the wound and the damage to the affected area the loss of function can be temporary or permanent.

3.2.4 CLOSED WOUNDS[31]

Closed wound usually refers to internal injury where there is no break in the continuity of the skin and underlying tissues.

Causes, Types and Symptoms

Closed wounds are generally caused by forceful impact of a blunt object to the skin, where the skin is not broken but the tissues underneath it may be extensively crushed or the damage can reach down to the underlying tissue, muscle, internal organs and bones.

1. Contusions: These are mostly known as the bruises accompanied by bleeding for a few hours followed by swelling within 24-48 hours or immediately after the injury. Swelling occurs as a result of collection of blood beneath the skin of damaged tissues. Contusions are painful bruise with reddish to bluish discoloration that spread over the injured area.
2. Hematomas: Hematomas are the type of injuries that usually damages the capillaries and small blood vessels leading to pooling of blood in a limited area. These are normally painful, rubbery spongy lump like lesions. Depending on the severity and type of trauma it can be small or large, under the skin or deep inside.
3. Crush injuries: Crush injuries are most commonly caused by an external high pressure force that squeezes part of the body between two surfaces. Depending on the size, power, duration and site of the trauma the degree of pain and injury can range from a minor bruise to a complete destruction of the damaged area.

3.2.4.1 COMPLICATIONS

1. Severe bleeding, nerve damage, large bruises, and internal organ damage.
2. Compartment syndrome: increased pressure and swelling in the fascia that surrounds the nerves, muscles and blood vessels in that area. The blood supply to the affected limbs will be blocked due to increased pressure, causing damage to nerves and muscles. Permanent damage leads to loss of function and may necessitate amputation.

3.3 HEALING[32]

Tissue injury may result in tissue destruction and cell death. On the other hand it is the response of the body specifically to injury as an attempt to restore normal structure and function. Healing involves two distinct processes: At times these two processes take place simultaneously.

- Regeneration- where the healing takes place by proliferation of parenchymal cells and results in the complete restoration of the original tissues.

- Repair- Here instead of the parenchymal cells, proliferation of the connective tissue elements will takes place resulting in fibrosis and scarring.

Table 1: Phases of healing process

Phase	Cellular and bio-physiologic events
Hemostasis	1. Vascular constriction 2. Platelet aggregation, degranulation, and fibrin formation(thrombus)
Inflammation	1. Neutrophil infiltration 2. Monocyte infiltration and differentiation to macrophage 3. Lymphocyte infiltration
Proliferation	1. re-epithelialization 2. angiogenesis 3. collagen synthesis 4. ECM formation
Remodeling	1. collagen remodeling 2. vascular maturation and regression

3.3.1 REGENERATION

In order to maintain the proper structure and function of the tissues, the cells are under constant regulatory control of their cell cycle. It includes growth factors such as Fibroblast growth factor, epidermal growth factor, endothelial growth factor, platelet-derived growth factor, transforming growth factor-β.

Cell cycle: It is the phase between two successive cell divisions and is divided into 4 unequal phases.

M PHASE: Phase of mitosis

G_1 Phase: Gap 1 phase- After mitosis daughter cells enters G_1 phase.

S Phase: Synthesis Phase- Synthesis of nuclear DNA takes place at this phase

G_2 Phase: Gap 2 Phase- The cells enters G_2 phase after the completion of the nuclear DNA.

G_0 Phase: Gap 0 phase- this is the resting or inactive phase of the cell after an M phase.

Depending on the dividing capacity, the cells of the body can be classified into 3

1. Labile cells: Under normal physiologic conditions these types of cells multiply throughout the life. Eg: Surface epithelial cells of epidermis, respiratory tract, alimentary tract, vagina cervix, urinary tract, uterine endometrium, cells of lymph nodes, bone marrow and spleen.
2. Stable cells: The ability of the cells to proliferate will decrease or lose after reaching adolescence, but retains the capacity to divide in response to stimuli throughout their life. Eg: Parenchymal cells of various organs like liver, kidneys, pancreas, thyroid and adrenal; mesenchymal cells like fibroblasts, smooth muscle cells, vascular endothelium, cartilage and bone cells.
3. Permanent cells: These types of cells loss their ability at the time of birth. These include: cardiac and skeletal muscle cells, neurons.

3.3.2 REPAIR[33]

Repair is defined as the replacement of injured tissue by fibrous tissue.

Repair involves two processes:

1. Granulation tissue formation
2. Wound contraction

Granulation Tissue Formation

The name granulation tissue derived from the slight granular and pink appearance of the tissue. The granulation tissue consist of granules, histologically corresponds to proliferation of new small blood vessels which are slightly lifted from the surface by thin covering of fibroblasts and young collagen.

The phases of granulation tissue formation includes

- Phase of inflammation: The blood clots at the site of injury after trauma, followed by acute inflammatory response along with exudation of plasma, some monocytes and neutrophils within 24 hours.
- Phase of clearance**:** Here all the necrotic tissues, red blood cells and debris will be cleared off by the combination of liberated proteolytic enzymes from neutrophils, autolytic enzymes from dead tissues, and phagocytic activity of macrophages.
- Phase of ingrowths of granulation tissue: it includes two main steps or processes.

  Angiogenesis- Formation of new blood vessels at the injured site.

  Fibrogenesis- Formation or development of fibroblast from the fibrocytes and mitotic division of fibroblast.

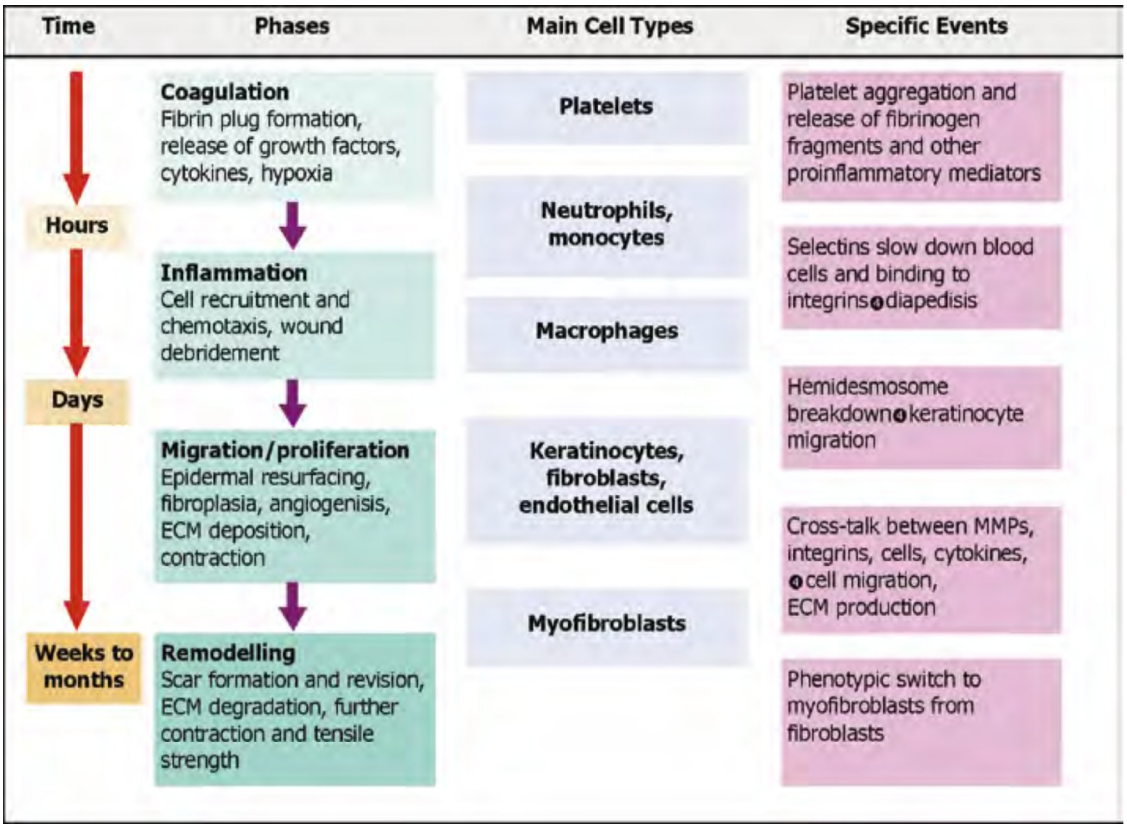


Figure 1. Phases of wound healing, major types of cells involved in each phase, and selected specific event

3.4 CONTRACTION OF WOUNDS[34]

The wound starts to contract after 2-3 days and the process will be completed in 14 days. During this time period the wound size is reduced by approximately 80% of its original size. This contraction of wound results in rapid healing since lesser surface area of the injured tissue has to be replaced.

The proposed mechanism of wound contraction can be explained by using various factors, These include:

1. Dehydration: Removal of fluid from drying wound leads to dehydration. This was the first suggested mechanism but without being substantiated.
2. Contraction of collagen: This was thought to be responsible for wound contraction but contraction proceeds even when the availability of collagen content in the granulation tissue is very less.
3. Discovery of myofibroblasts: The presence of myofibroblast in the active granulation tissue put to an end to the controversies surrounding the wound contraction and its mechanisms. The migration of these cells to the wound area and their active contraction is said to be responsible for the reduction of the wound area. The evidences that support this concept are morphological and functional characteristics of this modified fibroblasts or myofibroblasts, like
    a. Fibrils present in these cells resemble those seen in smooth muscle cells
    b. The actin-myosin found in these cells are similar to that found in the non-striated muscle cells.
    c. The nuclear membrane shows infoldings like in smoothmuscle cells
    d. They differ from the ordinary fibroblast cells as these cell have basement membrane and desmosomes.
    e. The drug responseof granulation tissue and smooth muscle are similar.

3.5 WOUND HEALING

Healing may be defined as a complex biological process including blood coagulation inflammation, fibroplasia, collagen deposition, and wound contraction.

3.5.1 CUTANEOUS WOUND HEALING[35]

Cutaneous wound healing is a typical example of combination of regeneration and repair. Although most of the skin lesions heal efficiently, the end product may not be functionally perfect. Epidermal appendages do not regenerate, and leads to the formation connective tissue scar in place of the mechanically efficient meshwork of collagen in the unwounded dermis. In case of superficial wounds the epithelium will be reconstituted and there may be little scar formation. Cutaneous wound healing is divided in to three phases:

1. Inflammation (early and late)
2. Granulation tissue formation
3. Re-epithelialization
4. Wound contraction
5. ECM deposition
6. Remodeling.

Wound healing is a fibroproliferative response which is mediated through growth factorsand cytokines.

Table 2: Growth Factors and Cytokines Affecting various Steps in Wound Healing

Monocyte chemotaxis	**PDGF, FGF, TGF-β**
Fibroblast formation	**PDGF, FGF, TGF-β, TNF, IL-1**
Fibroblast Proliferation	**PDGF, FGF, TGF-β**
Angiogenesis	**VEGF, Ang,FGF**
Collagen synthesis	**TGF- β, PDGF**
Collagenase secretion	**PDGF, FGF, EGF, TNF, TNF- β**

3.5.1.1 Healing by first intention OR Primary union (Wounds with opposed edges)

The least complicated examples of a repair is the healing of a wound with the following characteristics like

i. Clean and uninfected
ii. Surgically incised,
iii. without much loss of cells and tissues and
iv. the edges are approximated by surgical sutures

Healing of these kind of wounds are referred to as Primary union or healing by first intension.

The incision or the surgical incision causes death of a limited number epithelial cells and connective tissue cells, also it disrupts the continuity of the epithelial basement membrane. The surgical wound results in narrow incisional space which will be immediately filled by clotted blood containing blood cells and fibrin, And the clotted blood undergo dehydration and forms a well-known scab that covers the injured area(wound).

The primary union follows a series of sequential events:

i. Initial haemorrhage: It will start immediately after the injury, where the space between the approximated surface of the incised wound is filled with blood, which then clots and seals the wound from infection and dehydration.
ii. Acute inflammatory response: This stage occurs within 24 hours with the appearance of neutrophils which gradually moves towards the fibrin clot.
iii. Epithelial changes: The next 24 hour will witness for the movement of spurs of the epithelial cells from the wound edges(with little cell proliferation) along the cut margins of the dermis, where they deposit the basement membrane components as they move. These will fuse in the midline underneath the surface scab, producing a thin continuous epithelial layer that closes the wound. A well approximated wound will be roofed by

a layer of epithelium in 48 hours. The underlying viable dermis will be separated from the overlying necrotic material and clot by these migrated cells, leading to the formation of scab, which is cast off.

By third day these polymorphs will be largely replaced by the macrophages, and the granulation tissue invades the incision space progressively, and the collagen fibers will be present at the incision margins.

iv. Organisation: By the third day fibroblast invade the wound area and by the fifth day collagen fibrils starts forming, which dominates till healing is completed. The incisional space will be filled with the granulation tissue and neovascularization is maximal. The abundant collagen fibrils begin to bridge the incision. The epidermis will recover its normal thickness, and differentiation of the surface cells leads to the formation of a mature epidermal architecture with surface keratinization.

During the second week there will be continued proliferation of fibroblasts and accumulation of collagen. The edema, Leukocyte infiltrate and increased vascularity have mostly disappeared by this time. In four weeks, the scar tissue with scanty cellular and vascular elements, a few inflammatory cells and epithelialized surface is formed.

v. Suture tracks: Each suture track can be considered as a separate wound and comprehence the same phenomena as in the healing by primary union. And include the events like haemorrhage, inflammatory response, epithelial cell proliferation from both margins along the suture track, formation of young collagen and fibroblastic proliferation. The sutures can be removed on the seventh day and by that time most of th epithelialized suture tr acks will be avulsed and the epithelial tissue will be absorbrd. In some cases the suture track gets infected and it leads to stich abscess, or the epithelial cells may remain in the track owing to implantation or epidermal cysts.

In all the way the scar formed in the sutured wound will be neat because of the close apposition of the margins of the wound; on the other hand use of adhesive tapes avoids removal of stitches and its complications.

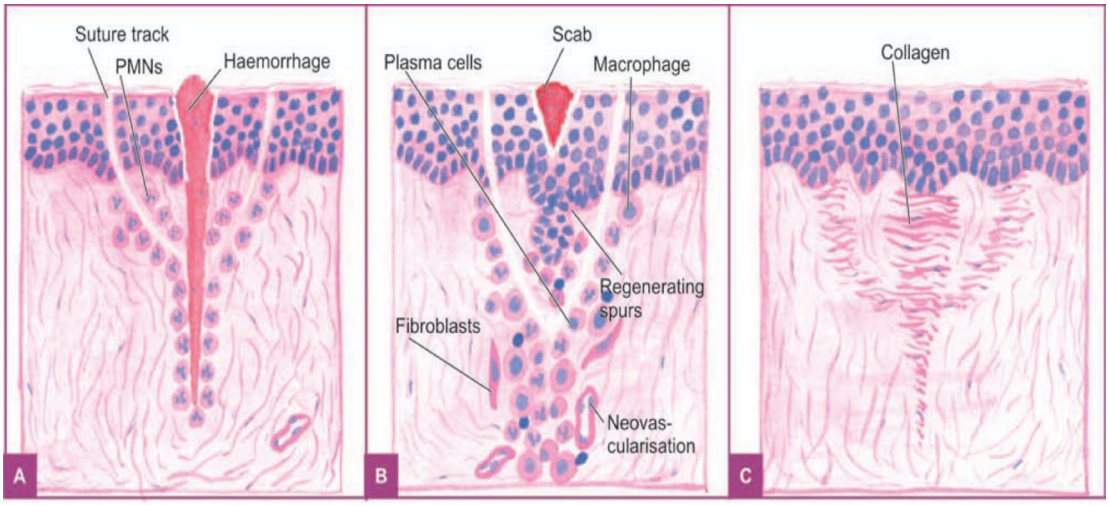


Figure 2**: Primary union of skin wounds**: **A,** The incised wound as well as suture track on either side are filled with blood clot and there is inflammatory response from the margins. **B**, Spurs of epidermal cells migrate along the incised margin on either side as well as around the suture track. Formation of granulation tissue also begins from below. **C**, Removal of suture at around 7th day results in scar tissue at the sites of incision and suture track.

3.5.1.2 Healing by second intention (secondary union) or wound with separated edges:

It is defined as the healing of a wound with the following characteristics like

1. Open with a large tissue defect and may be infected
2. Having extensive loss of cells and tissues and
3. The wound is not approximated by surgical sutures but left open

When there is extensive loss of cells and tissue, as in surface wounds which create large defects and in such cases the repair and healing process will be complicated. Regeneration of the epithelial cells fails to bring out the original architecture, and as a result there will be massive growth of the granulation tissue

from the margin to complete the process of repair. This particular type of healing is referred to as healing by second intention.

The healing events in both union are same, except in having large tissue defect which has not to be bridged. For this reason healing takes place from the margins inwards and from the base upwards. Hence when compared with primary union the healing by secondary union is a slow process leads to the formation of large, sometimes ugly scar.

The sequential events are

1. Initial haemorrhage: The wound area is filled with blood fibrin clot.
2. Inflammatory phase: Starts with initial acute inflammatory response followed by the appearance of macrophages.
3. Epithelial changes: The epidermal cells proliferate along the margins of the wound and migrate in to the wound as epithelial spurs and these spurs completely reepithelialize the gap. On the other hand the proliferating epithelial cells do not cover the surface fully till the granulation tissue from base has started filling the wound space. Thus the pre existing viable connective tissue will be separated from the necrotic material and clot on the wound surface, forming scab which is cast off. Eventually the regenerated epidermis becomes stratified and keratinized.
4. The main event in secondary healing is the granulation tissue formation, which is formed by proliferation of fibroblasts and angiogenesis from the adjoining viable elements. The newly formed such type of tissues will be deep red in colour, granular and very fragile. Gradually the matured scar became whilte and pale in color due to decreased vascularity and increased collagen deposition. The specialized structures of the skin like sweat glands and hair follicle are not replaced due to the failure of the structures to regenerate.
5. Wound contraction: The important feature of secondary healing is the wound contraction. The myofibroblasts of the granulation tissue aids in faster healing and it contracts the wound to one third to one fourth of the original size.
6. Presence of infection: infection or bacterial contamination delays the healing process as these organism release bacterial toxins that provoke necrosis,

thrombosis and suppuration. This bacterial contamination can be prevented by the surgical removal of the dead and necrosed tissue.

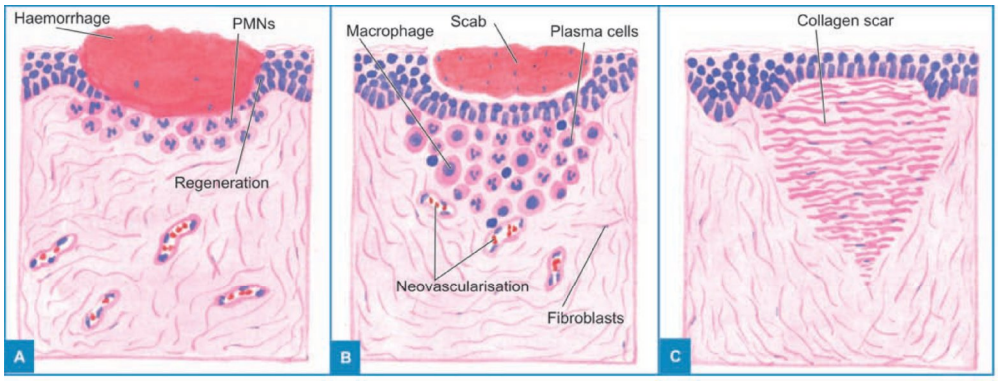


Figure 3: **Secondary union of skin wounds**. **A**, The open wound is filled with blood clot and there is inflammatory response at the junction of viable tissue. **B**, Epithelial spurs from the margins of wound meet in the middle to cover the gap and separate the underlying viable tissue from necrotic tissue at the surface forming scab. **C**, After contraction of the wound, a scar smaller than the original wound is left.

3.6 LOCAL AND SYSTEMIC FACTORS THAT INFLUENCE WOUND HEALING.[36]

There are number of known and unknown factors that modify the healing process, which frequently impairs the adequacy and quality of both inflammation and repair. And these influencing factors are

1. Local factors
2. Systemic factors

Local factors include:

3. Infection- leads to persistent injury and inflammation
4. Mechanical factors- like early motion of wounds delay the healing by compressing blood vessels and separating the edged of the wound.
5. Foreign bodies- fragments of steel, glass, or even bone and sutures delays the healing.

6. Size, location and type of wound also influence the healing rate. Wounds present in the highly vacsularized area like face heals faster than those of the poorly vascularized area such as foot

Table 3: Factors that retard healing

Factors that retard wound healing	
Local factors	
Blood supply	Mechanical stress
Denervation	Necrotic tissue
Local infection	Protection(dressings)
Foreign body	Surgical techniques
Hematoma	Type of tissue
Systemic factors	
Age	Anemia
Malnutrition	Obesity
Drugs(steroids, cytotoxic mediations,	Systemic infection
Intensive antibiotic therapy)	Trauma, hypovolemia and hypoxia
Genetic disorders(osteogenesis,	Temperature
imperfect, Ehlers-Danlos syndromes,	Uremia
Marfan syndrome)	Vitamin deficiency (vitamin C)
Hormones	Trace metal deficiency (zinc, copper)
Diabetes	Malignant disease

Systemic factors include:

A. Nutrition has profound effect on wound healing. Protein and vitamin deficiency leads to delay in wound healing especially in cases of Vitamin c deficiency.
B. Metabolic status- Metabolic status highly influences the repair process particularly in Diabetes mellitus. Delayed wound healing is one of the major complications faced by the diabetic patients, where it occur as a consequence of the microangiopathy.

C. Circulatory status: Inadequate blood supply caused by venous abnormalities like varicose veins or arteriosclerosis retards venous drainage and leads to impaired or delayed wound healing.
D. Hormones: Hormones like glucocorticoids have significant anti-inflammatory effect, and that influences various factors of inflammation. And these hormones having profound role in inhibiting collagen synthesis.

3.7 COMPLICATIONS OF WOUND HEALING [37][38]

The abnormalities in any of the basic components of healing process eventually lead to complications. These aberrations are grouped in to three main categories.

1. Deficient scar formation
2. Excessive formation of the repair components
3. Formation of contractures.

During the process of healing the following, the following complications may occur:

1. Infection: Infection of wound delays the healing
2. Implantation cyst : The persistence of epithelial cells in the wound after the healing process is known as implantation or epidermal cyst formation
3. Pigmentation: Healed wound sometimes have rust like color. This coloration is due to the haemosiderin staining. And sometimes these colored particulate materials may persist and impart specific color to the healed wound area.
4. Deficient scar formation: At times the formation of the granulation tissue will be inadequate and ends in deficient scar formation.
5. Incisional hernia: Formation of weak scar especially after laparotomy, ends in the formation of incisional hernia or wound dehiscence.
6. Excessive contraction: Excessive contraction of wound may result in formation of cicatrisation or contractures. Eg: Plantar contracture, Palmar contracture, and Peyronie's disease.
7. Hypertrophied scars and keloid formation: Sometimes there will be exaggerated scar formation and which will be painful. Excessive collagen formation result in

keloid (claw like) formation and it is more common in blacks. Hypertrophied wounds are confined to the borders of the initial wound whereas keloids consist of tumor like projection of connective tissue.

8. Neoplasia: The scars may act as the site for the development of the carcinoma like the squamous cell carcinoma in Marjolin's ulcer that is the scar following burns on the skin.

3.8 WOUND INFECTION[39]

The development of a wound infection depends on the complex interplay of many factors. If the integrity and protective function of the skin is breached, large quantities of different cell types will enter the wound and initiate an inflammatory response. This may be characterised by the classic signs of redness, pain, swelling, raised temperature and fever. This process ultimately aims to restore homeostasis

3.9 Potential wound pathogens

The most of the micro-organisms are less than 0.1mm in diameter and can therefore only be seen under a microscope.

3.9.1 Bacteria

These are relatively simple cells that can be further categorised according to differences in their shape and cell wall. Cocci (spherical shaped cells), bacilli (rods) and sprirochaetes (spirals) can be arranged singly; however cocci and bacilli can also be found in pairs, chains and irregular clusters. They can be visualised using a bacteriological staining process called Gram staining; after Gram staining, Gram-positive bacteria are purple and Gram-negative bacteria are red. Species that fail to stain with the Gram reaction, such as Clostridia, require specialised stains.

3.9.2 Fungi

These are composed of larger more complex cells than bacteria. They are either single-celled yeasts or multi-cellular organisms with a nuclei contained within a

cell membrane. Fungi can be responsible for superficial infections of the skin, nails and hair and, although they have been isolated from wounds, they are rarely pathogenic in this setting.

3.9.3 Protozoa

These are single celled organisms within a fragile membrane and without a cell wall. They are most significantly associated with infected skin ulcers.

3.9.4 Viruses

These are composed of genetic material (nucleic acid) enclosed within a protein coat or a membranous envelope. Although viruses do not generally cause wound infections, bacteria can infect skin lesions formed during the course of certain viral diseases.

It is important to remember that different micro-organisms can exist in polymicrobial communities and this is often the case within the margins of a wound

3.9 BURN WOUND[40]

Burns or burn wounds are so much different from other wounds that a separate medical superspeciality has been designated to manage them. Even though extensive burns primarily involve a single organ; however, almost all systems of the body are affected in this disease making it a generalized disorder. Unlike any other traumatic wound, involvement of intensivist and physician is more in burns.

3.9.1 ETIOLOGY

The cause of burn injury may be thermal or non thermal. Thermal include thermal contact, flame, or scald. Non thermal include such as chemical, electrical or radioactive. Chemical injuries are a result of contact with substance that are toxic to skin or lining of respiratory and alimentary tract loke acid,alkali etc. Electric burns results from conduction of electric current through body.

3.10 Treatment

Once a diagnosis of wound has been confirmed and antibiotic sensitivities identified, appropriate management regimens should be considered, with a high priority given to reducing the risk of cross infection. It is important to treat the patient as a whole and not the infection alone, so management strategies must be based on data derived from a holistic assessment of the needs of the individual. The main treatment objective will be to reduce rather than eradicate the bacterial burden within the wound margins. In addition to antibiotic therapy, there are two main generic groups of wound management products that have the potential to reduce the bacterial burden in the wound, these are compounds containing silver or iodine.

3.10.1 Antibiotic therapy

Antibiotics are chemical substances produced by a micro-organism that have the capacity, in dilute solutions, to selectively inhibit the growth of or to kill other micro-organisms. Whereas it is now generally accepted that systemic antibiotics are essential for the management of clinically infected wounds, the choice of antibiotic to be used is not always apparent. Only after a comprehensive assessment process including consideration of patient characteristics, the results of microbiological investigations and the identification of both the nature and location of the wound, can the most appropriate antibiotic be identified.

The routine use of topical antibiotics is not justified for colonised or infected wounds. In addition, a recent systematic review of antimicrobial agents has concluded that systemic or topical antimicrobials are not generally indicated for the management of chronic wound infections. However, there may be some value in the prophylactic use of topical antimicrobials for the initial management of acute cellulitus, whilst awaiting clarification of antibiotic sensitivity and the establishment of a therapeutic regimen.

Resistance to antibiotics has become a serious problem in recent years particularly with the rise of epidemic strains of MRSA. The overuse of broad-

spectrum antibiotics will only serve to exacerbate the situation. It could therefore be argued that all antibiotic use should be based on known sensitivities.

3.10.2 Iodine

Iodine is an element that has antiseptic properties. It is active against a number of pathogens. In the past its use has been limited by the fact that elemental iodine can be absorbed systemically, is almost insoluble and can be an irritant to the skin.

In wound management iodine is used in two forms:

- Cadexomer iodine - a polysaccharide starch lattice containing 0.9% elemental iodine that is released on exposure to wound exudate.
- PVP-1 (Povidone iodine) - an iodophor composed of elemental iodine and a synthetic polymer

Both have different physical characteristics that relate to the component parts and the iodine concentration of available iodine that is released when in use. Clinically iodine is indicated for wound cleansing, wound bed preparation (the stimulation and influence of specific cells involved with the immune system) and the prevention and management of wound infection.

3.10.3 Silver

Recently a number of dressings containing silver have become available, although silver and silver compounds have been routinely used in clinical practice as bactericidals for over a century. Silver interferes with the bacterial electron transport system and inhibits the multiplication of the bacteria. However, to achieve this, silver ions have to be able to enter a cell. The chemical bonding of silver with a sulphonamide antimicrobial - sulphadiazine - has resulted in the development of a safe broad-spectrum agent for topical use (eg Flamazine). In this formulation silver is released slowly from the transport medium in concentrations that are selectively toxic to micro-organisms such as bacteria and fungi. This type of silver product has been used successfully in the management of acute and chronic wounds.

Products that can sustain the interaction of silver with micro-organisms in the exuding wound are likely to be more effective in preventing/controlling local infection as potentially more silver ions will be available to enter bacterial cells. This assumes that the concentration of silver in the solution is both correct and maintained.

3.11 *Simarouba glauca*

Patil Manasi S. (2011) conducted a Critical review on medicinally important oil yielding plant Laxmitaru (*Simarouba glauca* DC.). This study reveals the traditional uses of this particular medicinal plant as it is well known for the gastro intestinal diseases especially for the dysentery. It also gives a detailed information about the major chemically active constituents of the plant. The study summarizes Phytochemical, Ethnobotanical, pharmacological, aspects and nursery practices of this medicinal plant.

Mishra S.R. (2012) established a method for the production of Bio-diesel (Methyl Ester) from Simarouba Glauca Oil. By using methanol they trans-esterified the Simarouba glauca oil in the presence of KOH. After verifying all the parameters it was found to be as a good and viable alternative for the diesel fuel

***Iasmine A.B.S. Alves* (2014)** have conducted a detailed study on Simaroubaceae family its botany, chemical composition and biological activities. The study reveals the charecterisics and properties of almost all the plants of this particular family. Quassanoids are considered as the major chemically active constituents of this particular family, and the study also reveals the major uses of this particular family.

K. Santhana Lakshmi(2013) studied the *In vitro* antibacterial, antioxidant, thrombolytic, haemolytic, activities and phytochemical analysis of *simarouba glauca* leaves extracts . The study was conducted by using various extracts. The methanolic extract showed maximum anti microbial activity, where as the heamolytic activity was found to be more in ethyl acetate extract. When compared to other extracts the chloroform exhibited maximum thrombolytic activity.

T. G. Umesh (2014) evaluated the *In vitro* antioxidant potential, free radical scavenging and cytotoxic activity of *simarouba gluaca* leaves. The study reveals the

percentage of phenolic and flavanoid content in the plant. Among the various extracts the aqueous extract gives the maximum anti-oxidant activity. Cytotoxic effect (SCC9) was found to be more in the methanolic extract.

Hélida M. L. Maranhao (2014) investigated Hepatoprotective effect of the aqueous extract of *Simarouba amara* Aublet (Simaroubaceae) stem and bark. Carbon tetrachloride (CCl4) was used to induce Hepatic hamage in Rats. The treated groups decreased levels of liver markers and lipid peroxidation in all given doses and showed increased levels of catalase at doses 250 and and 500mg/kg. Immuno histochemical tests shows positive signs of hepatocyte proliferation in all doses. It is suggested that the presence of catechins is responsible for these protective properties. Hence the study revealed that the plant can prevent the oxidative damage and it also increases the regenerative and reparative capacities of the liver.

Mail V.V (2014) reports the influence of various concentrations of fluoride on plant growth and metabolism. It was noticed that, the seedlings of *Simarouba glauca* shows tolerance to various concentrations of fluoride (5ppm, 10ppm, 25ppm, 50ppm, 100ppm.). The stable growth of Simarouba glauca has been reflected changes in secondary metabolites. It was reported that the total polyphenols, anthocyanins and flavonoid contents were increased in response to fluoride stress. This might be due to induction of secondary metabolites under stress condition which will be beneficial for induction of fluoride stress tolerance. Thus, the induction of secondary metabolites due to stress will improve the bioactive potential of this medicinally important plant.

Rajamane, N.N.(2014) evaluated the effect of sodium chloride stress on polyphenol, flavonoid, anthocyanins contents and Lipid peroxidation of leaflets of *Simarouba glauca.* The antioxidant capability of the plants was determined by measuring non-enzymatic antioxidant activities such as total polyphenols and total flavonoids. Anthocyanins and malondialdehyde content was also measured. Data indicate that *Simarouba glauca* reacted to salt-induced oxidative stress by increasing non-enzymatic antioxidant, defenses proportionally to the increasing of the stress imposed, and the total polyphenols, total flavonoids, anthocyanins and malondialdehyde contentwas found to be increased.

4. PLANT PROFILE [41][49][50][51]

Figure 4: *Simarouba glauca.*

Plant name : ***Simarouba glauca*** also known as Paradise tree

Family : Siamroubaceae

Synonyms : *Simarouba amara, Quassia simarouba, Zwingera amara, Picraena officinalis,*

Simarouba medicinalis

4.1 Vernacular names:

English : bitter ash, bitter damson princess tree, Simarouba, Paradise tree.

Spanish : acajou blanc, daguilla, daguillo gavilan, juan, primero, laguilla, olivio, palo

Creole : bwa blan, bwa fwenn, doliv fwenn.

French : bois amer, bois blance, bois frene, bois negresse, quinquina d Europe.

Trade name: *Simarouba*, Dysentry bark, Mountain Damson, Acituno.

Tamil : Laxmi taru

4.2 Natural habitat and distribution

S.glauca DC. is indigenous to Southern Florida, the West Indies and Brazil. It is native to Bahamas, Costa Rica, Cuba, EI-Salvador, Guatemala, Haiti, Honduras, Jamaica, Mexico, Puertorico, united states of America. While exotic to India, Srilanka, Phillippines and Myanmar. It grows under tropical conditions in Central America spreading from Mexico to Panama Southern Florida as well as

Caribbean Islands. *S. glauca* was introduced in Kenya and Burundi in Africa in 1957. It grows best in areas where the annual temperature ranges between 22-29^{0}c., but can tolerate a range of 18-34^{0}C. It prefers a mean annual rainfall in the range 2,000-3,000mm, but tolerates 1,200-4,000mm, growing in areas with a distinct dry season and where there is no dry season. It prefers sandy soils in the wild where it is found on rocky, shallow calcareous soils of mountain slopes and ridges, as well as on the deeper soils of the ravines and alluvial plains

4.3 Reproductive biology:

The tree starts flowering and fruiting at about three years of age. Flowering is annual beginning in December and continuing up to February. The tree starts bearing when they are 4-6 years old and reach stability in production of another 4-5 years. The droplets (blackish purple in pink genotypes and brownish yellow in green genotypes) are ready for harvest by March/April. Season and duration of reproductive phenoperiods vary according to location and climate. Individuals fruits have a development and ripening period of 1-2 months. Fruits is ellipsoid drupe,2-2.5cm long, with thin hard cuticle and juicy fruit pulp.

4.4 Botanical description:

It is a large, evergreen dioecious tree with a broad crown that can reach a height of 42 metres tall, though is usually smaller. The straight, cylindrical, unbuttressed bole is up to 50 - 60cm in diameter, strongly tapered and frequently unbranched for 20 - 27metre. Leaves are pinnately compound, entire, petiolate, alternately arranged with 9-16 leaflets per leaf. New flush of leaves produced every year. Flowers are pale yellow in colour. Male and female flowers are produced on separate trees. So are unisexuals. Fruits are Drupes, oval, elongated, 1-5 inch in length, brightly coloured, green to purplish-black containing large seeds which are dispersed by vertebrates with fleshy fruit covering.

4.5 Part used:

Bark, flower, stems, seeds, leaves, roots.

4.6 Chemical Constitutes:

QUASSINOIDS namely, Ailanthinone, Glaucarubinone, Holocanthone, Dehydroglaucarubinone, Benzoquinone, Canthin, Glaucarubine, Glaucarubolone, Simarubin, Melianone, Simaroubidin, Simarolide, Sitosterol, Tirucalla and Flavonoids.

4.7 Therapeutic Uses:

An infusion of the leaves or bark is considered to be astringent, a digestion and menstrual stimulant and an antiparasitic remedy. It is taken internally for Cancer, diarrhoea, dysentery, malaria, colitis, Hemorrhage, Anemia, Rheumatoid arthritis, Hepatitis, Hyperacidity, Dyspepsia, Fever, Ulcers and bleeding in alimentary system,; it is used externally for wounds and sores.

5. MATERIALS AND METHODS

5.1 MATERIALS USED FOR THE STUDY

Table 4: List of Instruments

Sl.No	Insruments	Manufacturer
1	Analytical weighing balance	Shimadzu
2	Cooling centrifuge	Remi
3	Deep freezer (-80^{0}C)	Remi
4	Electric water bath	Technico
5	Homogenizer	Remi
6	Hot air oven	Narang scientific works
7	HPTLC	Camag
8	P^H meter	Eutech
9	Rotary evaporator	IKA RV10
11	Ultra sonicator	Soltec
12	UV spectrometer	Pharmaspec UV-1700, Shimadzu

5.2 CHEMICALS USED FOR THE STUDY

Table 5: List of Chemicals.

Sl. No	Chemical	Manufacurer
1	2, 2'- azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)	Himedia
2	5,5-dithiobis (2-nitro benzoic acid) DTNB	Himedia
3	Ascorbic acid	SISCO
4	Bovine serum albumin (BSA)	SD-Fine chem
5	Chloramine T	Himedia
6	1,1- diphenyl-2-picrylhydrazyl (DPPH)	HIMEDIA
7	Folin-Ciocalteau reagent	HIMEDIA
8	Gallic acid	Zigma Aldrich
9	Glutathione reduced	Himedia
10	Quercetin	Himedia
11	Sodium azide	Himedia
12	Thiobarbituric acid	Himedia
13	Trichloro acetic acid	Himedia
14	P-dimethylaminobenzaldehyde	Himedia

5.3 METHODS

5.3.1 PLANT COLLECTION AND AUTHENTICATION

The aerial part of the plant *Simarouba glauca* was collected from Alapuzha District of kerala and authenticated from the Botanical survey of India (BSI), southern circle, Coimbatore, Tamil Nadu. The authentication certificate number is No. BSI/SRC/5/23/2016/Tech/605. Soon after collection, the leaves were cleaned and shade dried. After drying, these leaves were crushed to a coarse powder, stored in air tight plastic containers for further use.

5.3.2 EXTRACTION OF THE PLANT MATERIAL[44]

The extraction is done by using Sohxlet apparatus. The coarse powder of the leaves was first extracted with petroleum ether. Obtained defatted material is again extracted with ethyl acetate and aqueous. After extraction the extracts were evaporated or concentrated by using rotary evaporator and dried at room temperature to give a viscous mass. The obtained crude extracts were weighed and stored at 4^0C for the further analysis.

5.3.3 QUALITATIVE PHYTOCHEMICAL ANALYSIS OF EASG AND AQSG.[52][53][54][55]

5.3.3.1 Preparation of test sample

A small quantity of the extract was dissolved in 5ml of distilled water and filtered. The filtrate was tested to detect the presence of various phytochemical constituents in the sample.

5.3.3.2 TEST FOR CARBOHYDRATES

- **Molisch's test**

Few drops of Molisch's reagent was added to 2-3ml of filtrate, followed by addition of concentrated sulphuric acid along the sides of the test tube. Formation of violet colour ring at the junction of two liquids indicates the presence of carbohydrates.

- **Fehling's test**

1ml Fehling's-A (copper sulphate in distilled water) was added to 1ml of Fehling's-B (potassium tartarate and sodium hydroxide in distilled water) solution, boiled for one minute. To this added 1ml of filtrate and heated gently. Formation of brick red precipitate indicates the presence of reducing sugars.

- **Benedict's test**

Few ml of filtrate was mixed with equal volume of Benedict's reagent (alkaline solution containing cupric citrate complex) and heated in boiling water bath for 5min. Formation of reddish brown precipitate infers the presence of reducing sugars.

5.3.3.3 TEST FOR ALKALOIDS

Small amount of extract mixed with few ml of dilute hydrochloric acid. Shaken well and filtered. Following tests were performed with the obtained filtrate.

- **Dragendorff's test**

A few drops of Dragendorff's reagent (potassium bismuth iodide solution)was added to 2-3ml of filtrate. Orange red precipitate indicates the presence of alkaloids.

- **Mayer's test**

A few drops of Mayer's reagent (potassium mercuric iodide solution) was added to 2-3ml of filtrate. Cream (dull white) precipitate was formed.

- **Wagner's test**

A few drops of Wagner's reagent (solution of iodine in potassium iodide)was added to 2-3ml of filtrate,. Reddish brown precipitate was obtained.

- **Hager's test**

A few drops of Hager's reagent (Picric acid) was added to 2-3mlof filtrate. Yellow precipitate was obtained

5.3.3.4 TEST FOR TRITERPENOID

- **Libermann-Burchard test**

A small quantity of extract was treated with few drops of acetic anhydride, followed by a few drops of concentrated sulphuric acid. A brown ring was formed at the junction of two layers and the upper layer turns green colour, infers the presence of phytosterols and formation of deep red colour indicates the presence of triterpenoids.

- **Salkowski test**

A small quantity of the extract was treated with chloroform and few drops of concentrated sulphuric acid and allowed to stand for few minutes. Yellow colour at the lower layer indicates the presence of triterpenoids.

5.3.3.5 TEST FOR GLYCOSIDES

- **Legal's test**

1ml of pyridine and 1ml of sodium nitroprusside was added to 1ml of extract. Pink to red colour indicates the presence of glycosides.

- **Keller-Killiani test**

Glacial acetic acid was added to 2ml extract, followed by the addition of trace quantity of ferric chloride and 2 to 3drops of concentrated sulphuric acid. Reddish brown colour appears at the junction of two liquid indicates the presence of cardiac glycosides.

- **Baljet test:**

2ml of extract was addedto sodium picrate solution. Yellow to orange colour formation indicates the presence of glycosides.

5.3.3.6 TEST FOR STEROIDS AND STEROLS

- **Liebermann- Burchard reaction**

2ml of extract was mixed with chloroform. To that mixture added 1-2ml of acetic anhydride and 2drops of concentrated sulphuric acid along the sides of the test tube. The solution becomes red, then blue and finally bluish green colour.

- **Salkowski reaction**

2ml of extract was mixed with 2ml chloroform and 2ml concentrated sulphuric acid. Shaken well. Chloroform layer appears red and acid layer shows greenish yellow fluorescence.

5.3.3.7 TEST FOR PHENOLS

- **Ferric chloride test**

1ml of the alcoholic solution of the extract was added to 2ml of distilled water followed by few drops of 10% ferric chloride. Formation of blue or green colour indicates the presence of phenols.

- **Lead acetate test**

Diluted 1ml of alcoholic solution of extract with 5ml distilled water and to this added few drops of 1% aqueous solution of lead acetate. Formation of yellow colour precipitate indicate the presence of phenols.

5.3.3.8 TEST FOR TANNINS

- **Lead acetate test**

A few drop of lead acetate was added to 5ml of aqueous extract. Formation of yellow or red colour precipitate indicates the presence of tannins.

5.3.3.9 TEST FOR SAPONINS

- **Foam Test:**

1ml of test sample was diluted with 20ml of distilled water and shaken it in a graduated cylinder for 3minutes. Foam of 1cm after 10min indicates the presence of saponins.

- **Froth test:**

5ml of test sample was added to sodium bicarbonate solution. After vigorous shaking the mixture, kept it for 3minutes. A honey comb like froth formation indicates the presence of saponins.

5.3.3.10 TEST FOR FLAVONOIDS

- **Alkaline reagent test**

A few drop of sodium hydroxide solution was added to the extract. Formation of an intense yellow colour, which turns to colourless on addition of few drops of dilute hydrochloric acid, indicates the presence of flavonoids.

- **Shinodas test [Magnesium hydrochloride reduction test**]

Alcoholic solution of extract was treated with a small piece of magnesium ribbon and a few drops of concentrated HCl was added and heated. Appearance of crimson red or occasionally green to blue colour infers the presence of flavonoid.

5.3.3.11 TEST FOR PROTEINS AND AMINO ACIDS

- **Biuret test**

3ml of test solution was added to 4% sodium hydroxide and few drops of 1% copper sulphate solution. Formation of violet colour indicates the presence of proteins.

- **Ninhydrin test**

A mixture of 3ml test solution and 3drops of 5% Ninhydrin solution was heated in a boiling water bath for 10min. Formation of purple or bluish colour indicates the presence of free amino acids.

5.3.4 HIGH PERFOMANCE THIN-LAYER CHROMATOGRAHIC (HPTLC) METHOD FOR ESTIMATION OF FLAVONOIDS[56]

Standardization of plant materials and constituents is the need of the day. Also, the WHO has emphasized the need to ensure the quality of medicinal plant products using modern controlled techniques and applying suitable standards. Chromatographic fingerprinting techniques are the most significant methods which may be used for herbal drug analysis and for quality assurance.

High-performance thin layer chromatography (HPTLC) based methods could be considered as a good alternative, as they are being explored as an important tool in routine drug analysis. Major advantage of HPTLC is its ability to analyze several samples simultaneously using a small quantity of mobile phase. HPTLC also offers better resolution, accuracy, less time consuming and low cost of analysis. Additionally, it minimizes exposure risks and significantly reduces disposal problems of toxic organic effluents, thus reducing possibilities of environment pollution. HPTLC also facilitates repeated detection of chromatogram with same or different parameters.

The basic principle of HPTLC is adsorption. Where the mobile phase used is non-polar and the stationary phase is polar. Chemical or active constituents present in the plant extract will move through the plate according to the relative solubility of the constituents in the two phases and will be separated. The non- polar compound will be eluted first and the more polar later. The compounds can be identified based on the R_f value.

Experimental condition:

- Stationary phase : Aluminium plates precoated with Silica Gel $60F_{254}$ (10×10×0.2mm thickness)
- Mobile phase : Tolene:Ethyl acetate:Formic acid :Methanol(3:6:1.6:0.4)
- Sample for HPTLC : EASG, AQSG, and standard Quercetin, Rutin, Gallic acid, Apigenin And andrographolide solutions.
- Sample application : Camag Linomat 5
- Chamber type : Twin trough Chamber 10× 10cm
- Chamber saturation : 5min
- Development time : 30min
- Development distanc : 7cm
- Detection : Camag Scanner 3
- Data system : win CATS Planar Chromatography Manager.

Instrumental Parameters

- Number of track : 8
- Band length : 6.0mm
- Application position : 10mm
- Solvent front position : 80.0mm
- Solvent volume : 10ml
- Position of first track : 10mm
- Distance between tracks : 11.4mm
- Scan start position Y : 5.0mm
- Scan end position : 75.0mm
- Slit dimension : 6.00 × 0.45mm, Micro
- Optimized optical system : light
- Scanning speed : 20mm/sec
- Data resolution : 100µM/ step

Measurement table:

- Wavelength : 254nm
- Lamp : D_2 &W
- Measurement : Remission
- Measurement mode : Absorption
- Optical filter : Second order
- Detector mode : Automatic

Preparation of standard

Stock solutions of Standard compounds were prepared by dissolving accurately weighed 1mg of gallic acid, Rutin, Quercetin, Apigenin And andrographolide in 1ml of methanol (HPTLC grade), and stored at 4^0C. And 5µl of each standard were spotted on the HPTLC plate.

Preparation of Sample

Weighed accurately 1g of both ethyl acetate and aqueous extract and dissolved separately in 10ml of ethyl acetate and water. Each sample was then filtered by using Whatmann No.1 filter paper. 5µl of both extracts were spotted on the HPTLC plate.

Procedure:

The standards; gallic acid, Rutin, Quercetin, Apigenin And andrographolide (5µl), EASG and AQSG were spotted in form of bands with a Camag microlite syringe on pre-coated Silica Gel glass plate $60F_{254}$ (10×10cm with 0.2mm thickness) using a Camag Linomat 5 applicator. The plates were pre-washed with methanol and activated at 60^0c for 10min prior to chromatography. The sample loaded plate was kept in TLC twin trough developing chamber after chamber saturation with respective mobile phase. The optimized chamber saturation time for mobile phase was 5 min at room temperature. Linear ascending development was carried out and the plate was developed in the respective mobile phase up to 7cm. The developed plate was then dried by hot air to evaporate solvents from plate and also for the development of bands. The dried plate was observed under UV light at 254nm and 366nm and photo documentation was done. Densitometric scanning was performed on Camag TLC

scanner 3 in the absorbance mode at 280nm. The percentages of active constituents present in the both extracts were compared with that of standard.

5.3.5 QUANTIFICATION OF TOTAL PHENOLICS AND FLAVONOIDS[45][57]

5.3.5.1 ESTIMATION OF TOTAL PHENOLICS

Reagents

- Folin-Ciocalteu's reagent
- Gallic acid (1mg/ml)
- 20% sodium carbonate

Preparation of standard

Standard solution of was prepared by adding 10mg of accurately weighed Gallic acid in 10ml of distilled water.

Preparation of sample

10mg of the accurately weighed EASG and AQSG extracts were separately dissolved in 10ml ethanol and used for the estimation

.

Procedure

The total phenolic content of the EASG and AQSG were determined by Folin-Ciocalteau assay method. To an aliquot 100µl of both EASG and AQSG (1mg/ml) or standard solution of Gallic acid (10, 20, 40, 60, 80, 100µg/ml) added 50µl of Folin-ciocalteau reagent followed by 860µl of distilled water and the mixture is incubated for 5min at room temperature. 100µl of 20% sodium carbonate and 890µl of distilled water were added to make the final solution to 2ml. It was incubated for 30min in dark to complete the reaction. After that absorbance of the mixture was measured at 725nm against blank. Distilled water was used as reagent blank. The tests were performed in triplicate to get mean the values. The total phenolic content was found out from the calibration curve of Gallic acid. And it was expressed as milligrams of Gallic acid equivalents (GAE) per gram of extract.

5.3.5.2 ESTIMATION OF TOTAL FLAVONOIDS

Reagents

- Ethanol
- 10% aluminium chloride
- 1M Potassium acetate

Preparation of standard

Standard solution of was prepared by adding 10mg of accurately weighed Quercetin in 10ml of ethanol.

Preparion of sample

10mg of the accurately weighed EASG and AQSG extracts were separately dissolved in 10ml ethanol and used for the estimation.

Procedure

The total flavonoid content of the EASG ad AQSG was determined by using Aluminium chloride colorimetric method. To an aliquot of 1ml of extract (1mg/ml) or standard solutions of Quercetin (10, 20, 40, 60, 80, 100µg/ml) methanol was added separately to make up the solution upto 2ml. The resulting mixture was treated with 0.1ml of 10% aluminium chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water. Shaken well and incubated at room temperature for 30 minutes. The absorbance was measured at 415nm against blank, where a solution of 2ml ethanol, 0.1ml potassium acetate, 2.8ml distilled water and 0.1ml of aluminium chloride serve as blank solution. The total flavonoid content was determined from the standard Quercetin calibration curve. And it was expressed as milligrams of Quercetin equivalents (QE) per gram of extract.

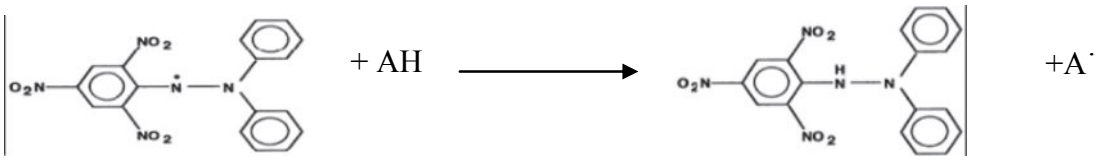
5.3.6 INVITRO-ANTIOXIDANT STUDY OF EASG AND AQSG

5.3.6.1 DPPH Free Radical Scavenging Assay[58]

Principle

The DPPH [1, 1-diphenyl-2-picrylhydrazyl (α, α-diphenyl-β-picrylhydrazyl)] assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517nm (purple colour). When Antioxidants react with DPPH, which is a stable free radical becomes

paired off in the presence of a hydrogen donor (e.g., a free radicalscavenging antioxidant) and is reduced to the DPPHH and as consequence the absorbance's decreased from the DPPH. DPPH radical is a stable radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet colour, characterized by an absorption band in ethanol/methanol solution centred at about 520nm Radical to the DPPH-H form, results in decolorization (yellow colour) with respect to the number of electrons captured. More the decolorization more is the reducing ability. This test has been the most accepted model for evaluating the free radical scavenging activity of any new drug. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (Diphenylpicrylhydrazine; non radical) with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present).



Procedure

The method adopted here is Blois method. Where by using the stable DPPH radical, the antioxidant capacity of the extract was measured in terms of hydrogen donating or radical scavenging ability. 1ml of 0.3mM solution of DPPH in ethanol was added to various concentrations of sample (10, 20, 40, 60,80, 100 µg/ml) and the reference compound (5, 10, 15, 20, 25 and 30 µg/ml), shaken vigorously, and left to stand in the dark at room temperature. After 30 min absorbance was measured at 517nm against a blank. Quercetin was used as Reference compound. A control reaction was also carried out without the test sample. All the tests were performed in triplicate in order to get the mean values. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples. Free radical scavenging activity was expressed as percentage inhibition (I%) and calculated using the following equation:

Percentage inhibition (I%) = (Abs $_{control}$- Abs $_{sample}$ /Abs $_{control}$) X 100

Different sample concentrations were used in order to obtain calibration curves and to calculate the IC50 values. (IC50 - concentration required to obtain a 50% radical scavenging activity).

5.3.6.2 ABTS Assay[59][60]

Principle

A method for the screening of antioxidant activity is reported as a decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants. The pre-formed radical monocation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS*+) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants present in the sample. The influences of both the concentration of antioxidant and duration of reaction on the inhibition of the radical cation absorption are taken into account when determining the antioxidant activity. Chemistry involves the direct generation of the ABTS radical monocation with no involvement of an intermediary radical. It is a decolorization assay; thus the radical cation is pre-formed prior to addition of antioxidant test systems, rather than the generation of the radical taking place continually in the presence of the antioxidant. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical and inhibits the absorption of the radical cation which has characteristic long-wavelength absorption spectrum showing maxima at 660,734, and 820nm.The relatively stable ABTS radical has a green colour and is quantified spectrometrically at 734nm. It is applicable to both aqueous and lipophilic systems.

Procedure

ABTS radical scavenging activity of the extract was measured by Rice-Evans method. ABTS was dissolved in water to a 7mM concentration. ABTS radical cation ($ABTS^{+}$) was produced by reacting ABTS stock solution with 2.45mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-

16h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the study, ABTS solution was diluted with phosphate buffer saline pH 7.4 (PBS) to an absorbance of 0.70 (± 0.02) at 734nm and equilibrated at 30^0C. After addition of 1ml of diluted ABTS solution to various concentrations of sample or reference compound (Quercetin), the reaction mixture was incubated for 6min and then absorbance was measured at 734 nm against a blank. A control reaction was carried out without the sample. All the tests were performed in triplicate in order to get the mean values. The percentage inhibition of $ABTS^+$ by the sample was calculated according to the formula:

Percentage inhibition (I %) = (Abs $_{control}$- Abs $_{sample}$ /Abs $_{control}$) X 100

Different sample concentrations were used in order to obtain calibration curves and to calculate the EC50 values. (EC50 - concentration required to obtain a 50% radical scavenging activity).

5.3.7ANTI-MICROBIAL ACTIVITY OF EASG AND AQSG[44]

5.3.7.1 Anti –bacterial study of EASG and AQSG

Preparation of inoculums

The inoculums for the experiment were prepared in fresh Nutrient broth from the preserved slant culture. The turbidity of the culture can be adjusted by the addition of broth or sterile saline (if it is excessive) or by further incubation to get the required turbidity, And the newly prepared inoculums were standardized by adjusting the turbidity of the culture to that of McFarland standards.

Preparation of sterile swabs

Cotton wool swab on wooden applicator or plastics were prepared and sterilized by autoclaving or by dry heat (only for the wooden swabs). It was sterilized by packing the swabs in culture tubes, papers or tins etc.

Sterilization of forceps

Forceps can be sterilized by dipping in alcohol and burning off the alcohol.

Experiment

The standardized inoculums is inoculated in the sterilized plates prepared earlier (aseptically) by dipping a sterile in the inoculums removing the excess of inoculums by passing and rotating the swab firmly against the side of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium 3 times rotating the plate through an angle of 60º after each application. Finally pass the swab round the edge of the agar surface. Leave the inoculums to dry at room temperature with the lid closed. The sterile discs are soaked overnight in sample solutions, EASG and AQSG. Each Petri dish is divided into 3 parts. First and second compartment were loaded with sample disc such as EASG and AQSG (100µg) and Std Ciprofloxacin disc (10µg), is placed on the fourth compartment of the plate with the help of sterile forceps. After that petri dishes are placed in the refrigerator at 4º C or at room temperature for 1 hour for diffusion. Incubate at 37 º C for 24 hours. Observe the zone of inhibition produced by different samples. Measure it using a scale and record the average of two diameters of each zone of inhibition.

Table 6: Bacterial strain used for the study with NCIM

Si no	organisom	strain	NCIM
1	Gram + ve bacteria	*Staphylococcus lentus*	*2169*
2		*Staphylococcus albus*	*2178*
3		*Staphylococcus aureus*	*2079*
4		*Bacillus subtilis*	*2063*
5		*Bacillus lentus*	*2018*
6	Gram –ve bacteria	*Vibrio cholerae*	*1738*
7		*Corynebacterium*	*2640*
8		*E-coli*	*2065*
9		*klebsilla*	*2707*
10		*pseudomonasaeurogenosa*	*2200*

5.3.7.2 Anti fungal activity of EASG and AQSG

Procedure

Preparation of inoculums

The inoculums for this particular experiment were prepared in fresh sabouraud Dextrose broth from preserved slant culture. The turbidity of the culture can be adjusted by the addition of broth or sterile saline (if it is excessive) or by further incubation to get the required turbidity, And the newly prepared inoculums were standardized by adjusting the turbidity of the culture to that of McFarland standards.

Preparation of sterile swabs

Cotton wool swab on wooden applicator or plastics were prepared and sterilized by autoclaving or by dry heat (only for the wooden swabs). It was sterilized by packing the swabs in culture tubes, papers or tins etc.

Sterilization of forceps

Forceps can be sterilized by dipping in alcohol and burning off the alcohol.

Experiment

The standardized inoculums is inoculated in the sterilized plates prepared earlier (aseptically) by dipping a sterile in the inoculums removing the excess of inoculums by passing and rotating the swab firmly against the side of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium 3 times rotating the plate through an angle of 60º after each application. Finally pass the swab round the edge of the agar surface. Leave the inoculums to dry at room temperature with the lid closed. The sterile discs are soaked overnight in sample solutions, EASG and AQSG. Each Petri dish is divided into 3 parts. First and second compartment were loaded with sample disc such as EASG and AQSG (100µg) and Std Clotrimazole disc (10µg), is placed on the fourth compartment of the plate with the help of sterile forceps. After that petri dishes are placed in the refrigerator at 4ºC or at room temperature for 1 hour for diffusion. Incubate at 37 ºC for 24 hours. Observe the zone of inhibition produced by different samples. Measure it using a scale and record the average of two diameters of each zone of inhibition.

Table 7; Fungal strain used for the study with MTCC

Si no	Fungi strains used	MTCC
1	*A.fumigatus*	1811
2	*M.purpureas*	1090
3	*A. paraticus*	2796
4	*Candida albicans*	3100
5	A.niger	1344

5.3.7.3 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION OF EASG and AQSG[61][62]

The minimum inhibitory concentration (MIC) is the lowest concentration of a chemical that prevents the visible growth of the organism.ie, the lowest concentration at which it has bacteriostatic activity.

5.3.7.3.1 Preparation of test drug:

The selected test drugs were prepared in DMSO at a concentration 1000µg/ml

5.3.7.3.2 Preparation of inoculum:

Staphylococcus albus , *Coryne bacterium* and *candida albicans* were the strains of organisms selected for the study. Overnight culture are grown at 37^0C Kirby- Bauer procedure and diluted to Muller Hinton Broth and Sabouraud Dextrose Broth for bacterial and fungal strains respectively. This overnight culture was diluted to 10^{-2}.

Inoculation

1. The sterile tubes were labeled 1-8 and 8^{th} tube was taken as control.
2. 1ml of Muller Hinton Broth was transferred to all tube.
3. 1ml of drug solution was added to 1^{st} tube and mixed well.
4. From the 1^{st} tube transfer 1ml of solution to the 2^{nd} tube and was repeated up to 7^{th} tube.

5. From the 7^{th} tube 1ml of solution was pipetted out and discarded.
6. 0.01ml of culture was added to all the test tubes.
7. All the tubes were incubated at 37^0C for 18-24hrs.
8. After incubation observe the turbidity by visually
9. The highest dilution without growth is the minimal inhibitory concentration.

5.3.8 DETERMINATION OF *IN VITRO* CYTOTOXICITY PROPERTIESOF EASG AND AQSG ON CULTURED FIBROBLAST CELLS BY MTT ASSAY[63][64]

5.3.8.1 Chemicals

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyltetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Modified Eagle's Medium (MEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India.

5.3.8.2 Cell lines and Culture medium

Fibroblast cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in MEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with Trypsin solution (0.2% trypsin, 0.02% EDTA in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

5.3.8.3 Preparation of Test Solutions

For Cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with MEM supplemented with 2% inactivated FBS to obtain a stock solution of 1mg/ml concentration and sterilized by

filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

5.3.8.4 Determination of cell viability by MTT Assay

Principle*:*

The ability of the cells to survive a toxic insult has been the basis of most Cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used.

Procedure*:*

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using MEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^o C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

5.3.9. PHARMACOLOGICAL EVALUATION OF EASG AND AQSG

5.3.9.1 ACUTE TOXICITY STUDY:[65]

Based on previously conducted study of *Simarouba glauca*, the dose was selected.

5.3.9.2 ANIMALS AND MANAGEMENT

Female wistar rats of 6-8 weeks old and 160-180g body weight were offered by KMCH College of pharmacy, Coimbatore. All rats were kept at room temperature and allowed to accommodate in standard conditions at 12-hr light and 12-hr dark cycle in the animal house. Animals were fed with commercial pellet diet and water *ad libitum* freely throughout the study. The experimental procedure was approved by IAEC (Institutional animal ethical committee of KMCH, governed by CPCSEA, Government of India. Proposal number: KMCRET/ M.PHARM/04/2015-16

5.3.9.3 DRUG:

Povidone Iodine and silver sulfadiazine Ointments were used as standard drug. The ointments were applied topically over the wound area.

5.3.9.4 PREPARATION OF EXTRAT

Both EASG and AQSG were formulated to ointment (1%). And it is applied topically over the wound area.

5.3.9.4.1 PREPARATION OF SIMPLE OINTMENT BASE[66]

Ingrediants:

Wool fat 50g

Hard paraffin 50g

Cetostearyl alcohol 50g

White soft paraffin 850g

Type of preparation: Absorption ointment base

Procedure:

Hard paraffin and cetostearyl alcohol taken in a china dish kept on water-bath at 70^0C. Wool fat and white soft paraffin are added to this mixture and stirred until all

the ingredients are melted. If required decanted or strained and stirred until cold and packed in suitable container.

1% of EASG AND AQSG were separately mixed with the above prepared simple ointment base.

5.3.8.5 EXPERIMENTAL MODELS

5.3.8.5.1 EVALUATION OF WOUND HEALING EFFECT OF EASG AND AQSG IN EXCISION WOUND MODEL

Rats were divided into 5 groups each containing 6 animals as follows.

Table 8: Experimental design for excision wound model

GROUP	GROUP SPECIFICATION	INTERVENTION
Group I	Control	Untreated
Group II	Simple ointment base	Only with simple ointment base
Group III	Standard	Povidone iodine 5%
Group IV	Test 1	EASG ointment 1%
Group V	Test 2	AQSG ointment 1%

INDUCTION OF WOUND[67]

On wounding day the rats were anaesthetized prior to creation of the wounds, by ether anaesthesia. The dorsal fur of the animal was shaved with an electric clipper and the area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of 1.5cm in width (circular area 2.25cm^2) created along the markings using toothed forceps, a surgical blade and pointed scissors. The entire wound left open. All the surgical interventions were carried out under sterile condition. After 24h of wound creation, the ointments was applied gently to cover the wounded area once daily until

complete healing Wound area and wound contraction, epithelializtion period and hydroxyproline content were monitored.

5.3.8.5.2 EVALUATION OF WOUND HEALING EFFECT OF EASG AND AQSG IN BURN WOUND MODEL

Rats were divided into 5 groups each containing 6 animals as follows.

Table 9: Experimental design for burn wound model

GROUP	GROUP SPECIFICATION	INTERVENTION
Group I	Control	Untreated
Group II	Simple ointment base	Only with simple ointment base
Group III	Standard	Silver sulfadiazine 5%
Group IV	Test 1	EASG ointment 1%
Group V	Test 2	AQSG ointment 1%

INDUCTION OF BURN WOUND[68]

On wounding day the rats were anaesthetized prior to creation of the wounds, by ethr anaesthesia. The dorsal fur of the animal was shaved with an electric clipper and the area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A partial thickness burn was made by putting a hot plate at a temperature of 84^0c on the prepared area for 30 sec under sterile condition. The burnt area was measured immediately after the burn and on 3^{rd}, 5^{th} , 7^{th} , 10^{th} , 14^{th} and 21^{st} day after burn injury. After 24h of wound creation, the ointment was applied gently to cover the wounded area once daily until complete healing achieved. Wound area and wound contraction, epithelializtion period and hydroxyproline content were monitored.

5.3.8.5.3 EVALUATION OF WOUND HEALING EFFECT OF EASG AND AQSG IN INFECTED EXCISSION WOUND MODEL

Rats were divided into 5 groups each containing 6 animals as follows.

Table 10: Experimental design for infected excision wound model

GROUP	GROUP SPECIFICATION	INTERVENTION
Group I	Control	Untreated
Group II	Simple ointment base	Only with simple ointment base
Group III	Standard	Silver sulfadiazine 5%
Group IV	Test 1	EASG ointment 1%
Group V	Test 2	AQSG ointment 1%

Selection of micro-organism:

The organism selected for inducing the infected model is based on the MIC repots. Where the Staphylococcus albus showed good MIC value.

INDUCTION OF INFECTED EXCISION WOUND MODEL[69]

On wounding day the rats were anaesthetized by ether anesthesia prior to creation of the wounds. The dorsal fur of the animal was shaved with an electric clipper and the area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of 1.5cm in width (circular area 2.25cm^2) created along the markings using toothed forceps, a surgical blade and pointed scissors (Figure 1). Infected model created by using 0.1mL of saline containing 10^6 colony forming units (CFU)/mL of Staphylococcus albus suspension, where the solution intradermally injected on the wounded area. The entire wound left. All the surgical interventions were carried out under sterile condition. After 72h of wound creation, the ointments

were applied gently to cover the wounded area once daily until complete healing achieved. Wound area and wound contraction, epithelializtion period and hydroxyproline content were monitored.

5.3.8.6 ESTIMATION OF PARAMETERS

5.3.8.6.1 Measurement of wound contraction[70]

The progression of wound healing was judged by the periodic assessment of the contraction of excision wounds. Wound contraction was monitored by tracing the outline of the wound on tracing sheet and then using graph sheet to calculate the area of the wound size. All animals in each group were monitored until complete healing of wounds occurred and the day at which each
wound healed was recorded. Mean of all healed wounds was determined.

$$\text{Percent wound contraction} = \frac{\text{healed area}}{\text{total area}} \times 100$$

5.3.8.6.1 Estimation of hydroxyproline [71][72]

Principle

The procedure is based on alkaline hydrolysis of the tissue homogenate and subsequent determination of the free hydroxyproline for the production of a pyrole. The addition of Ehrlich's reagent resulted in the formation of a chromophore that can be measured at 557nm. Optimal assay conditions were determined using tissue homogenate and purified acid soluble collagen along with standard hydroxyproline. Critical parameters such as the amount of chloramine-T, sodium hydroxide, p-dimethylaminobenzaldehyde, pH of the reaction buffer, and length of oxidation time were observed to obtain satisfactory results.

Procedure

Known amount of tissue (50mg) was taken in glass tubes and 4ml of 6N HCl was added to each tube to hydrolyse the tissue sample. The glass tubes were sealed and were incubated for 22 hours. The tubes are then opened and the contents are decanted into a china dish. HCl was then removed by evaporation and the residue was dissolved in water and made up to known volume (10ml) using a standard flask. A

series of standards were prepared containing 20-200µg of hydroxyproline with afinal volume of 2ml. One millilitre of the hydrolysed tissue samples was used to estimate the contents of hydroxyproline. Hydroxyproline oxidation was initiated by adding 1ml of chloramine-T to each tube in a predetermined sequence. The tube content were mixed by shaking a few minutes and allowed to stand for 20min at room temperature. Chloramine-T was then destroyed by adding 1ml of perchloric acid to each tube in the same order as before. The contents were mixed and allowed to stand for 5min. Finally 1ml of p-Dimethyl aminobenzaldehyde solution was added and the mixture was shaken well. Tubes were

placed in a 60°C water bath for 20min and then cooled in tap water for 5min. The colour developed was read spectrophotometrically at 557nm. Hydroxyproline value was determined from the standard curve.

5.3.8.6.3 Estimation of hexosamine [71] [73]

Principle

The method was based on the observation that in alkaline solution at 100°C, the amino sugars react with acetyl acetone to form chromogenic material which gives a chromophore or chromophores on treatment in acid solution with ethanolic p-dimethyl amino benzaldehyde. The method described is suitable for the estimation only for free amino sugars where a determination is carried to ascertain the amino sugar content of a polysaccharide or other material of high molecular weight. Any amino sugar units remaining as oligosaccharide or substituted amino sugar gives less color per unit weight of amino sugar than that found for free amino sugar. Therefore it is essential that the hydrolysis of granulation tissue is done without destruction of the amino sugar.

Procedure

Tissue samples (50mg) were hydrolysed with 2N HCL (5ml) at 100°C for 6 hrs. Hydrochloric acid was then removed by evaporation, then the residue was dissolved in water and made up to a known volume (10ml) using a standard flask. Aliquots containing 10-50mg hexosamine were treated with 1ml of freshly prepared 2% acetyl acetone in 0.5M sodium carbonate in capped tubes and kept in boiling

water bath for 15min. After cooling in tap water, 5ml of 95% ethanol and 1ml of Ehrlich's reagent (1.33% Dimethyl amino benzaldehyde in 1:1 ethanol: concentrated hydrochloric acid mixture) were added and mixed thoroughly. The purple red colour developed was read after 30 min at 530 nm. Water blank and standard glucosamine solution of various concentrations were also treated similarly to get a standard curve.

5.3.8.6.4 Estimation of total protein[74]

Total protein content of the granular tissue was determined by following Bradford (1976) method.

Reagents

- Alkaline copper reagent
- Solution A: 2% sodium carbonate in 0.1 N NaOH.
- Solution B: 0.5% copper sulphate in 1% sodium potassium tartarate
- Solution C: 50ml of solution A was mixed with 1 ml of solution B just before use.
- Folin's phenol reagent (commercial reagent, 1:2 dilutions) Bovine serum albumin (BSA).

Principle:

This method involves two steps;

Step: 1-protein binds with copper in alkaline medium and reduces it into cu++.

Step: 2- the cu++ formed catalyses the oxidation reaction of aromatic amino acids by reducing phosphomolybdotungstate to heteropolymolybdanum, which leads to the formation of blue colour and absorbance was measured at 640nm.

Procedure:

0.1 ml homogenate was made up to 1ml with distilled water and to this; 5ml of alkaline solution was added, mixed well and allowed to stand for 10min. Then a volume of 0.5ml Folin's reagent was added, mixed well and incubated at room temperature for another 10min. The blue colour developed was measured at 660nm against blank. Bovine serum albumin (1mg/ml) served as the standard and form the standard graph obtained; the amount of protein in the sample was calculated and expressed as mg/100mg tissue

5.3.8.6.5 Estimation of uronic acid[71][75]

Principle

The galacturonic acid content of the hydrolyzed sample is quantified colorimetrically using a modification of the Cu reduction procedure originally described by Avigad and Milner. This modification, substituting the commonly used Folin-Ciocalteau reagent for the arsenic containing Nelson reagent, gives a response that is linear, sensitive, and selective for uronic acids over neutral sugars. This method also avoids the use of concentrated acids needed for the commonly used m-phenylphenol method. This combined enzymatic and colorimetric procedure correctly determined the galacturonic acid and methanol content of purified sample. In both cases good agreement was obtained between this method and commonly used methods.

Procedure

A buffered copper solution was prepared by adding 23.2g NaCl, 3.2g sodium acetate, and 1.0mL glacial acetic acid to 80mL water. Once dissolved, 0.5 g CuSO4 is added, the pH adjusted to 4.8 with NaOH, and the final volume brought to 100mL. This solution is stable for weeks at room temperature. For the assay, equal volumes of this solution and the sample are mixed, giving final reagent concentrations in the assay of 2 M NaCl, 0.2M acetate, and 10mM CuSO4. In our standard assay, sample and assay solution volumes of 0.1mL each are mixed in test tubes, then the tubes are covered with glass marbles and placed in an aluminum heating block at 100 C. A diluted Folin–Ciocalteau reagent is then prepared by mixing 1mL of 2N Folin–Ciocalteau with 39mL of water. After 40min, the samples are removed from the heat and 8 volumes (0.8mL in our standard assay) of the 40-fold diluted Folin–Ciocalteau reagent is added. A colored product forms immediately; absorbance was measured at 750nm. Where the BCA reagent was used the procedure was the same, except instead of adding the diluted Folin– Ciocalteau reagent, 0.8mL of solution ‘‘A” from the procedure described in Waffenschmidt and Jaenicke, containing 5.0mM BCA in pH 10.1 carbonate buffer, was added. Absorbance was measured at 560nm.

5.3.8.6.6 Tensile strength[76][77]

Principle

Tensile strength is the resistance to break, under tension. It indicates how much the repaired tissue resists breakage under tension and may indicate in part the quality of repaired tissue. The healed rat skin was used for the test. The strength of the tissue was represented in gram.

Procedure

The tensile strength of the samples was tested using DAK SYSTEM BENCH. Speed was set at 100mm per minute and the load cell used was of 500kg. The jaws of the tensile tester were set 50mm, apart for the samples. The test specimen was clamped in the jaws and the machine was run at the rate of 100±2mm/min. until the specimens tore apart. The highest load reached was recorded when the sample was subjected to breaking. The distance between the jaws when rapture of the test specimen occurred was noted.

5.3.8.7 *IN VIVO* ANTIOXIDANT ACTIVITY[78][79]

Preparation of tissue homogenate

For the estimation of non-enzymatic and enzymatic antioxidants, tissue was minced and homogenized (10% w/v) in 0.1M saline phosphate buffer (pH 7.4) and centrifuged for 10min and the resulting supernatant was used for enzyme assays.

5.3.8.7.1ENZYMATIC ANYI-OXIDANT ACTIVITY

5.3.8.7.1.1 ESTIMATION OF CATALASE (CAT)[80]

Reagents

- Dichromate acetic acid reagent (5% potassium dichromate + glacial acetic acid were mixed at 1: 3 ratio (v/v).
- 0.01M Phosphate buffer (pH-7.0).
- 0.2M hydrogen peroxide.

Principle:

The normal antioxidant activity of the enzyme catalase is due to acceleration of decomposition of hydrogen peroxide to water and oxygen. This method is based on the principle that by measuring the rate of decomposition of hydrogen peroxide by the enzyme catalase spectrophotometrically at 570 nm, since hydrogen peroxide has the absorbance at this range.

$$2H_2O_2 \longleftrightarrow H_2O + O_2$$

Procedure:

To 1ml of tissue homogenate 4ml of hydrogen peroxide and 5ml of phosphate buffer was added and mixed well. From this, 1ml of solution was taken and mixed with dichromate acetic acid reagent and allowed to incubate for 30min at room temperature. The absorbance was measured at 570nm. The activity of catalase was expressed as μmole of H_2O_2 consumed /min/mg protein.

5.3.8.7.1.2 ESTIMATION OF SUPEROXIDE DISMUTASE (SOD)[81][82]

Principle:

Pyrogallol autoxidizes rapidly in aqueous solution, where the reaction will be faster at higher pH, and leads to the formation of several intermediate products. Thus the solution first becomes yellow-brown with a spectrum showing a shoulder between 400 and 425nm. Molecular oxygen, carrying two unpaired electrons with parallel spins, has a preference for univalent reduction because spin restrictions arise when reduction with electron pairs is attempted. The recently discovered enzyme superoxide dismutase rapidly dismutases univalently reduced oxygen $O_2.^-$ i.e., the superoxide anion radical ($2O_2.^- + 2\ H^+ \rightarrow O_2 + H_2O_2$). The enzyme has proven to be a useful probe for studying the participation of the radical in reactions involving oxygen such as autoxidations. Thus O_2^-. has been shown to be involved in the autoxidation of e.g. sulphite, adrenalin and 6-hydroxydopamine.

Procedure

This method might be used for determination of antioxidant activity of a sample, and it was described by McCord and Fridovich. 5% of tissue homoginate was added to 75mM, 30mM, and 2mM from Tris-HCL (pH 8.2), EDTA, and pyrogallol respectively. Then, the absorbance was measured at 420nm. The percentage of inhibition was calculated depending on that the ability of enzyme to inhibit of oxidation. So, any changes might be happened on the absorbance, it will give a clear picture on the ability of enzyme activity to prevent oxidation.

5.3.8.7.2 NON ENZYMATIC ANTIOXIDANT ACTIVITY

5.3.8.7.2.1 ESTIMATION OF REDUCED GLUTATHIONE (GSH)[83]

Reagents

- 5% TCA
- 0.6mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 0.2M sodium phosphate
- 0.2M Phosphate buffer, pH 8.0

Principle:

DTNB known as Ellman's reagent was developed for the detection of thiol compounds. DTNB and sulfhydryl groups present in glutathione (GSH) react to generate 2-nitro-5- thiobenzoic acid and glutathione disulfide (GSSG). Since 2-nitro-5-thiobenzoic acid is a yellow coloured product, GSH concentration in a sample solution can be determined by the measurement at 412nm [73]

Procedure

To 1ml of the homogenate, 1ml of the TCA solution was added and centrifuged. The supernatant was collected and the precipitate formed was removed. To 0.5ml of supernatant 2ml of DTNB was added, the volume was made up to 3ml with phosphate buffer. Then absorbance was read at 412nm. The amount of glutathione was expressed as µg/mg protein.

5.3.8.7.3 DETERMINATION OF LIPID PEROXIDATION[84]

Reagents

· Thiobarbituric acid 0.37%

· 0.25N HCL

· 15% TCA

Principle:

This assay is based on the reaction between Thiobarbituric acid with malonyldialdehyde which is formed as a result of polyunsaturated fatty acid oxidation. This reaction leads to the formation of pink coloured TBA-MDA complex which is measured at 532nm.

Procedure:

To 0.1ml of the sample, 2ml of TBA-TCA-HCL reagent (ratio of 1:1:1) was added mixed and kept in a water bath for 15minutes. Afterwards the solution was cooled and supernatant was removed and the absorbance was measured at 535nm against reference blank. The level of lipid peroxidase was given as n moles of MDA formed/mg protein.

5.3.8.8 HISTOLOGICAL ASSESSMENT[71]

Histological studies of wounded tissues provide accurate diagnosis of level of healing of the wound. Histopathology is the microscopical study of tissues for pathological alterations. This involves collection of morbid tissues from biopsy or necropsy, fixation, preparation of sections, staining and microscopical examination.

Collection of materials

Thin pieces of 3 to 5 mm, thickness were collected from tissues showing gross morbid changes along with normal tissue.

Fixation:

Kept the tissue in fixative for 24-48 hours at room temperature

The fixation was useful in the following ways:

a) Serves to harden the tissues by coagulating the cell protein,
b) Prevents autolysis,
c) Preserves the structure of the tissue, and
d) Prevents shrinkage

Common Fixatives: 10% Formalin

Haematoxylin and eosin method of staining:

Deparaffine the section by xylol 5 to 10 minutes and remove xylol by absolute alcohol. Then cleaned the section in tap water and stained with haematoxylin for 3-4 minutes and again cleaned under tap water. Allow the sections in tap water for few minutes and counter stained with 0.5% eosin until section appears light pink (15 to 30seconds), and then washed in tap water. Blotted and dehydrated in alcohol and cleared with xylol (15 to 30 seconds). Mounted on a Canada balsam or DPX Moutant and kept the slide dry and remove air bubbles.

5.3.8.9 STATISTICAL ANALYSIS

Datas of all the parameters were analyzed using the Graph pad 5.0 software. Analysis of Variance (ANOVA); one way ANOVA followed by Tukey's multiple comparison test was performed. The values were expressed as Mean ± SEM. P value <0.05 was considered as significant.

6. RESULTS

6.1 EXTRACTIVE YIELD OF EXTRACTS OF *Simarouba glauca*

Percentage Yield

Coarsely powdered *Simarouba glauca* leaves were extracted with ethyl acetate and aqueous using soxhlet apparatus after deffating with petroleum ether.

The percentage yield of each extract was found to be

- Ethyl acetate extract (EASG) : 2.85% w/w
- Aqueous extract (AQSG) : 23% w/w

6.2 PRELIMINARY PHYTOCHEMICAL ANALYSIS OF EASG AND AQSG

Table 11: Phytochemical Analysis of *Simarouba glauca*

Sl.No	Phytochemicel constituents	EASG	AQSG
1	Carbohydrates	Positive	Positive
2	Glycosides	Positive	Positive
3	Alkaloids	Positive	Positive
4	Phenolics	Positive	Positive
5	Flavonoids	Positive	Positive
6	Tannins	Positive	Positive
7	Triterpenoids	Positive	Positive
8	Saponons	Positive	Positive
9	Steroids	Positive	Positive
10	Proteins and amino acids	Positive	Positive

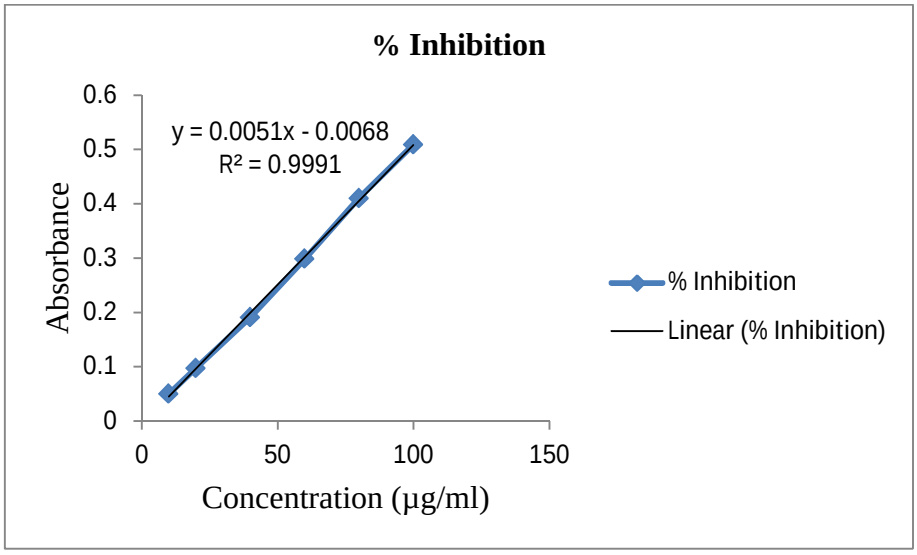
6.3 QUANTIFICATION OF TOTAL PHENOL AND FLAVONOIDS

6.3.1 ESTIMATION OF TOTAL PHENOL OF EASG AND AQSG

Table 12: Estimation of total phenolic content of EASG and AQSG

Sample	Concentration (µg/ml)	Absorbance
Standard 1mg/ml	10	0.0498
	20	0.0971
	40	0.1907
	60	0.2983
	80	0.410
	100	0.5086
EASG	1000	1.1589
AQSG	1000	0.4472

Figure 5: Total phenolic content of EASG and AQSG



The total phenolic content in EASG was found to be 198.42mg/g of extract calculated as Gallic acid equivalent.

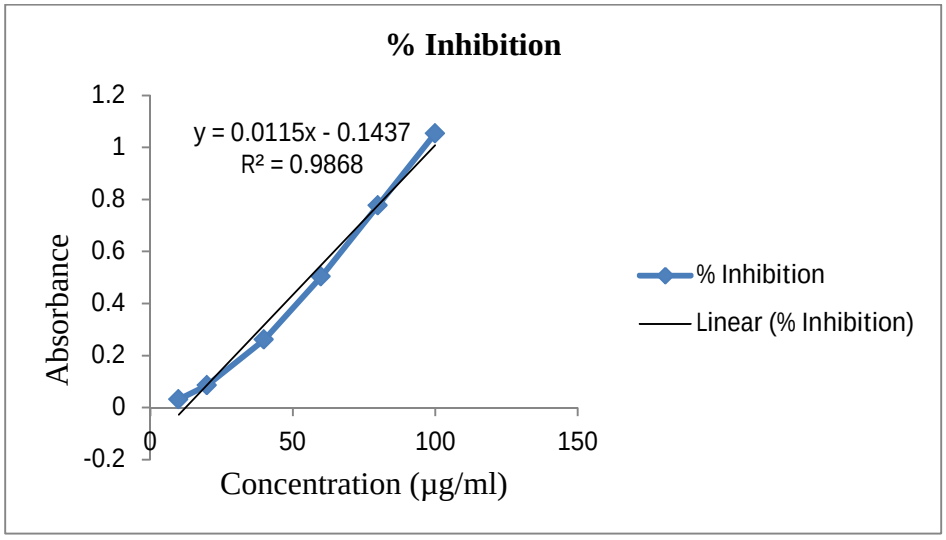
The total phenolic content in AQSG was found to be 90.64mg/g of extract calculated as Gallic acid equivalent.

6.3.2 ESTIMATION OF TOTAL FLAVONOID CONTENT OF EASG AND AQSG

Table 13 : Quantification of Total Flavonoid Content

Sample	Concentration (µg/ml)	Absorbance
Standard 1mg/ml	10	0.031
	20	0.085
	40	0.26
	60	0.5026
	80	0.776
	100	1.053
EASG	1000	0.7046
AQSG	1000	0.1513

Figure 6: Estimation of total flavonoid content of EASG and AQSG



The total flavanoid content in EASG was found to be 77.05mg/g of extract calculated as Quercetin equivalent.

The total flavanoid content in AQSG was found to be 26.75mg/g of extract calculated as Quercetin equivalent.

6.4 CHROMATOGRAPHIC SCREENING ANALYSIS

HPTLC study was carried out for the quantification of flavonoids in extract. Visualization was performed as done (figure). After development the plate was scanned in densitometer under 254nm and the chromatogram obtained is depicted in figure 7.

Figure 7: Detection of bands:

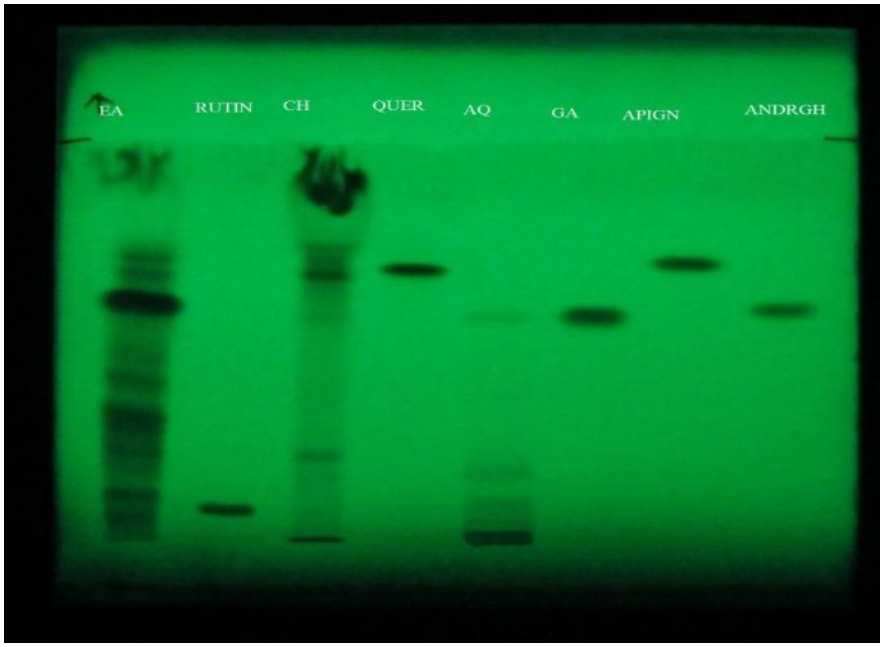
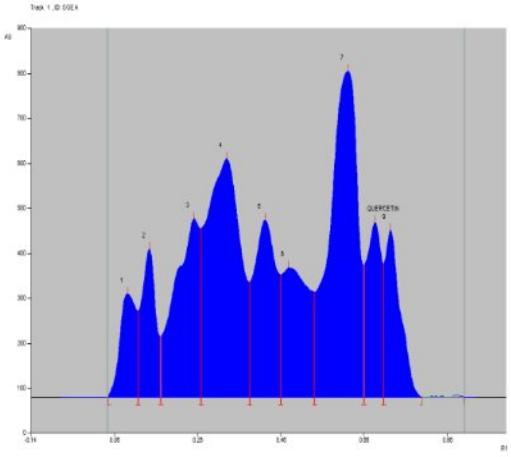


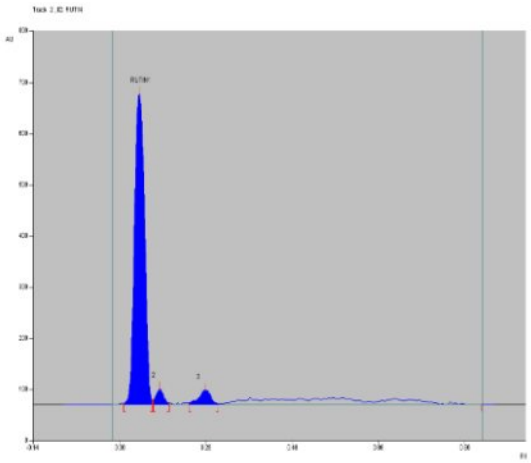
Table 14: Lists of spots applied on HPTLC plate:

Track number	Sample(5µL)
1	SGEA
2	RUTIN
3	QUERCETIN
4	SGAQ
5	GALLIC ACID
6	APIGENIN
7	ANDROGRAHOLIDE

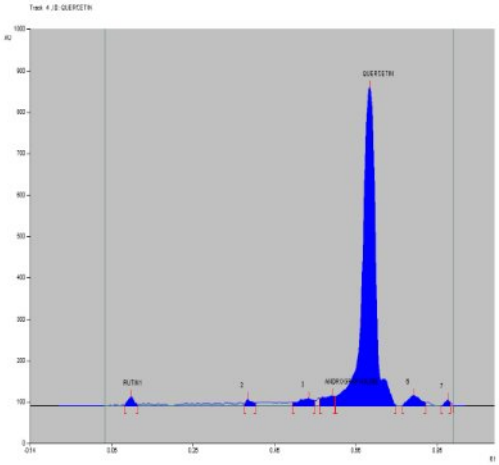
Figure 8: Chromatogram of Standards, EASG and AQSG



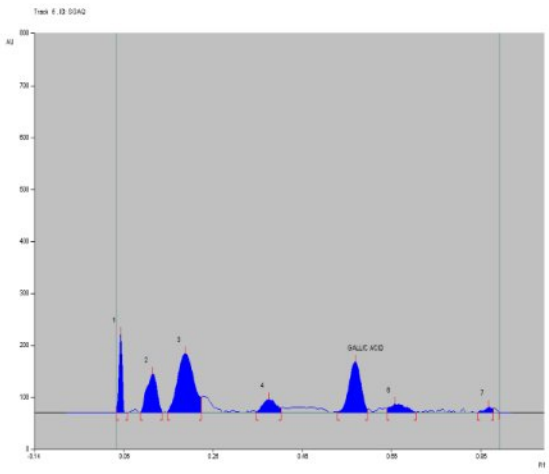
Chromatogram of EASG(5µl)



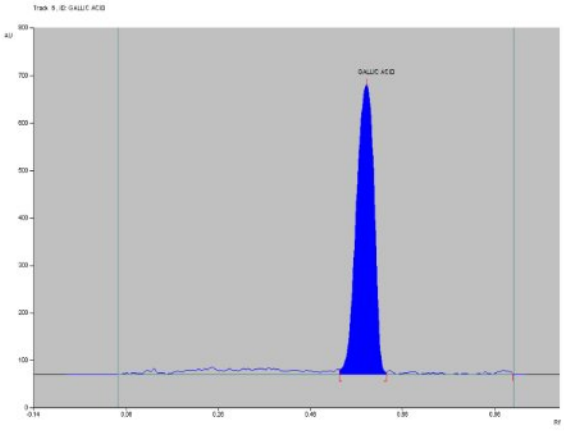
Chromatogram of RUTIN (5µl)



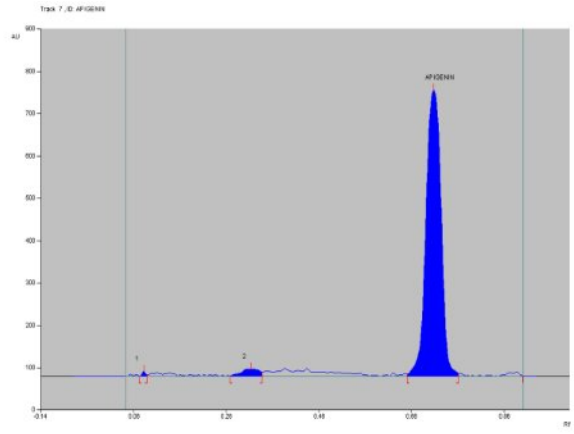
Chromatogram of QUERCETIN (5µl)



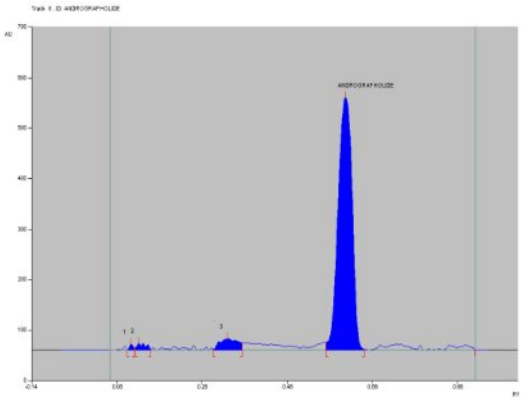
Chromatogram AQSG (5µl)



Chromatogram of GALLIC ACID (5µl)

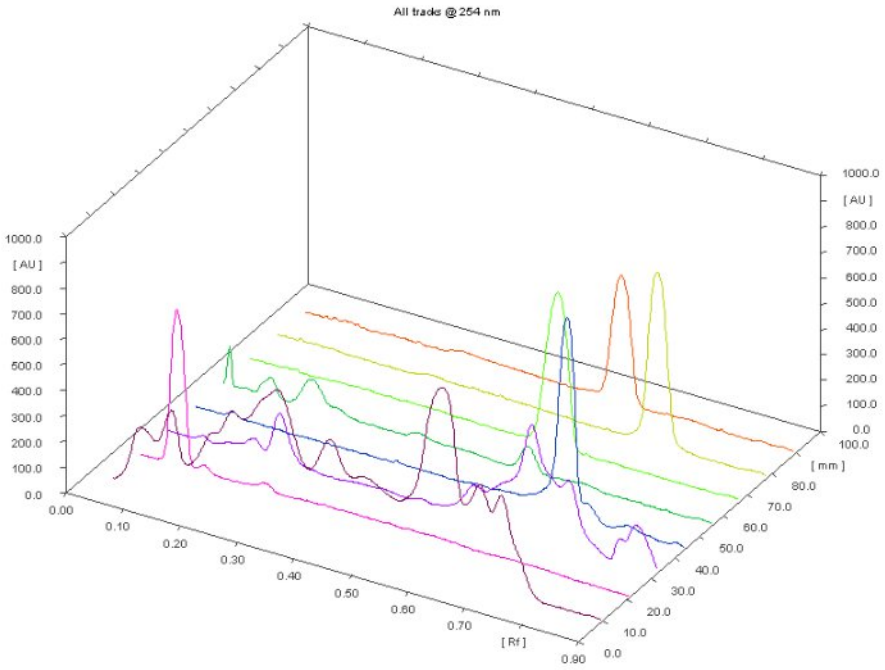


Chromatogram of APIGENIN (5µl)



Chromatogram of ANDROGRAPHOLIDE (5µl)

Figure 9: 3D Display of Standards, EASG and AQSG



6.4.1 Quantification of QUERCETIN and GALLIC ACID in EASG and AQSG by using HPTLC

Table 15:Amount of QUERCETIN in EASG

Volume applied (µl)	Concentration (µg/ml)	Area	Amount of Quercetin (µg)	% of Quercetin In 100mg of extract
5	500	11238	2.77	0.5

Table 16: Amount of GALLIC ACID in AQSG

Volume applied (µl)	Concentration (µg/ml)		Area	Amount of Gallic acid (µg)	% of gallic acid In 100mg of extract
5	500		18840.3	1.87	0.36

6.5 IN VITRO ANTIOXIDANT ACTIVITY OF EASG AND AQSG

6.5.1 DPPH RADICAL SCAVENGING ACTIVITY

Table 17: Percentage inhibition and IC50 values of DPPH radical by Quercetin

Sl.No	Concentration (µg/ml)	Percentage inhibition	IC 50 (µg/ml)
1	5	66.58	2.989
2	10	74.34	
3	15	80.58	
4	20	87.48	
5	25	94.56	
6	30	99.38	

Fig 10: DPPH radical scavenging activity of Quercetin

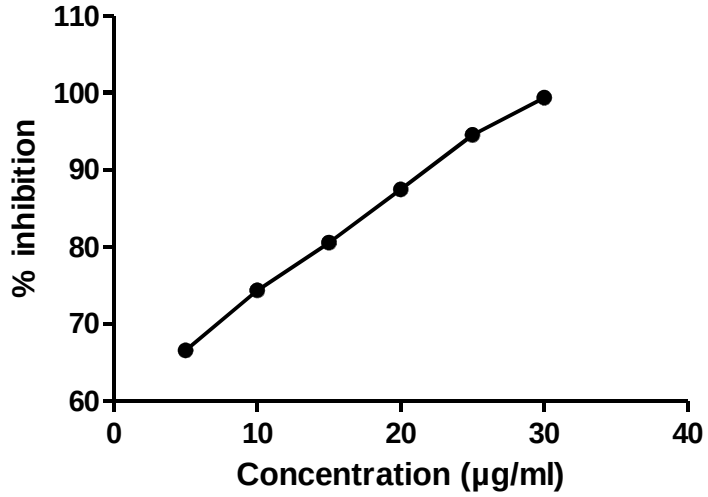


Table 18: Percentage inhibition and IC50 values of DPPH radical by EASG

Sl.No	Concentration (µg/ml)	Percentage inhibition	IC 50 (µg/ml)
1	10	42.67	13.22
2	15	46.55	
3	20	55.10	
4	25	60.6	
5	50	76.9	
6	100	91.40	

Fig 11: DPPH radical scavenging activity of EASG

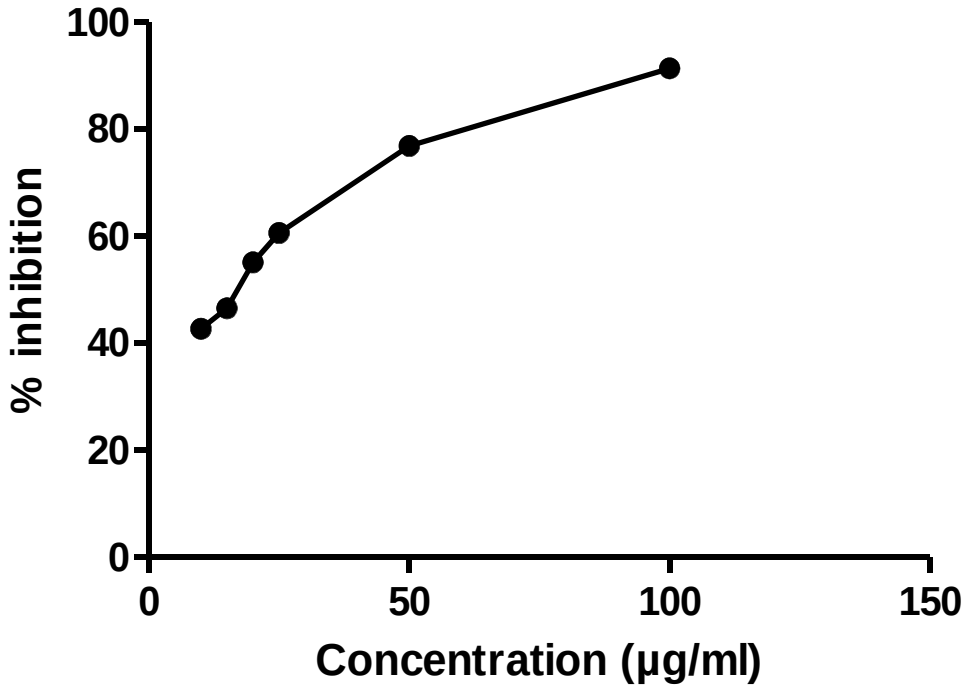
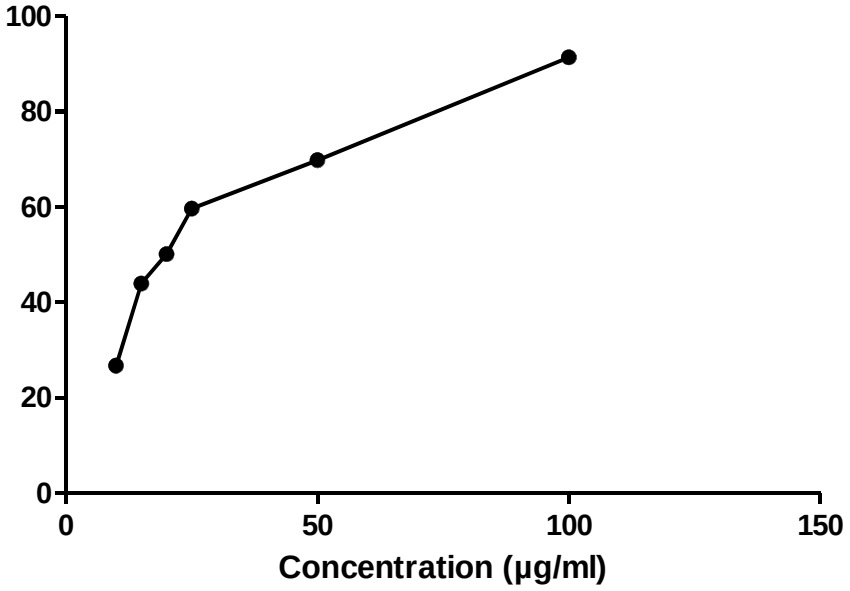


Table 19: Percentage inhibition and IC50 values of DPPH radical by AQSG

Sl.No	Concentration (µg/ml)	Percentage inhibition	IC 50 (µg/ml)
1	10	26.69	17.77
2	15	43.89	
3	20	50.12	
4	25	59.67	
5	50	69.77	
6	100	91.36	

Fig 12: DPPH radical scavenging activity of AQSG



6.5.2 TOTAL ANTIOXIDANT ACTIVITY BY ABTS RADICAL CATION ASSAY

Table 20 : Percentage inhibition of ABTS radical by Quercetin

Sl.No	Concentration (µg/ml)	Percentage inhibition	IC 50 (µg/ml)
1	0.25	70.08	0.1142
2	0.5	75.22	
3	0.75	79.62	
4	1.0	85.88	
5	1.25	91.9	
6	1.75	98.85	

Fig 13: ABTS radical scavenging activity of Quercetin

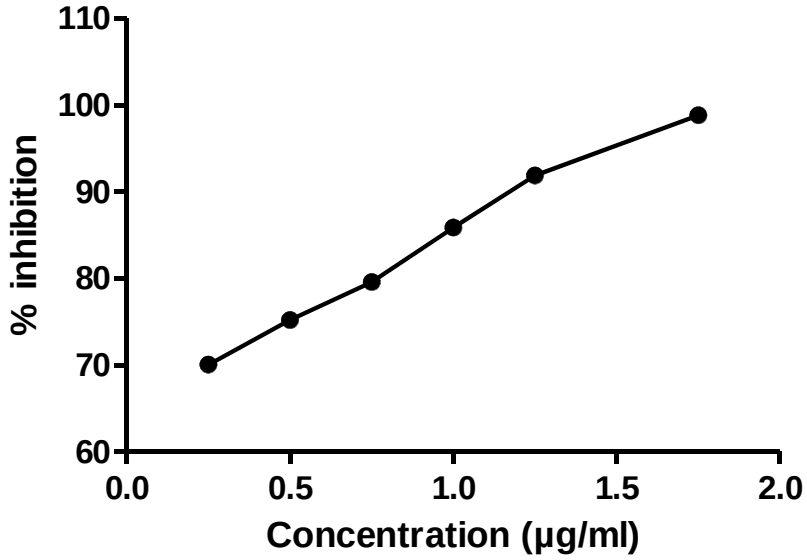


Table 21: inhibition and IC50 values of ABTS radical by EASG

Sl.No	Concentration (µg/ml)	Percentage inhibition	IC 50 (µg/ml)
1	1	53.61	1.160
2	2	57.41	
3	3	61.63	
4	4	71.18	
5	5	76.92	
6	10	97.37	

Fig 14: ABTS adical scavenging activity of EASG

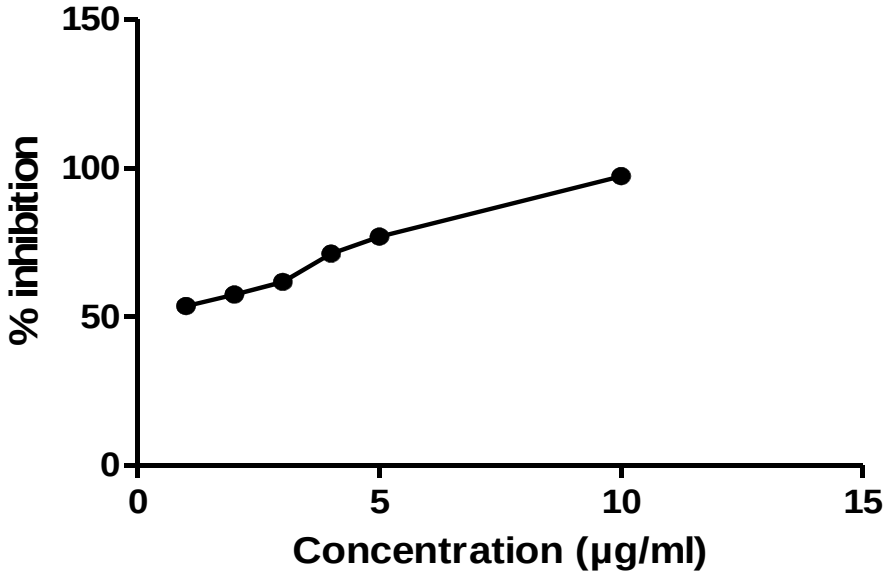
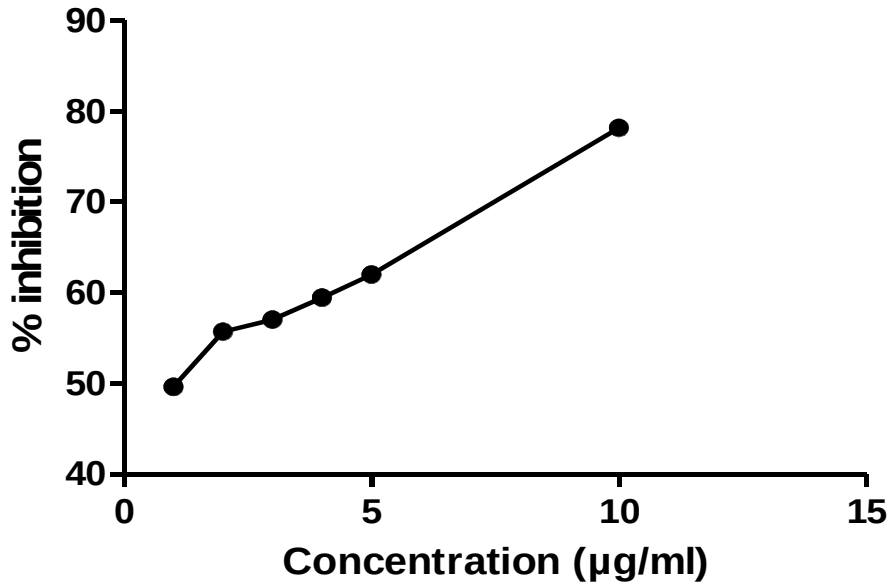


Table 22: inhibition and IC50 values of DPPH radical by AQSG

Sl.No	Concentration (µg/ml)	Percentage inhibition	IC 50 (µg/ml)
1	1	49.64	1.429
2	2	55.72	
3	3	57.06	
4	4	59.46	
5	5	62.00	
6	10	78.18	

Fig 15: ABTS radical scavenging activity of AQSG

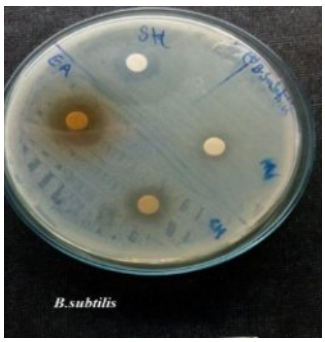


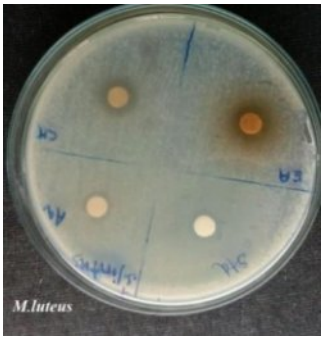
6.6 ANTI MICROBIAL ACTIVITY OF EASG and AQSG

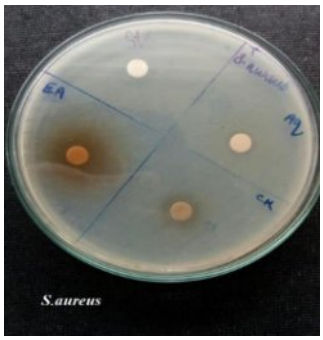
Table 23: Zone of inhibition for Gram +ve organisms

Organism	Standard (mm)	AQSG(mm)	EASG (mm)
Bacillus subtilis	12	8	15
Micrococcus luteus	10	8	13
Staphylococcus albus	11	11	15
Bacillus lentus	21	7	15
Staphylococcus aureus	12	11	14

Figure 16: Zone of inhibition for Gram +ve organisms







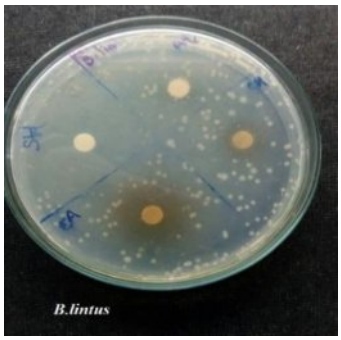
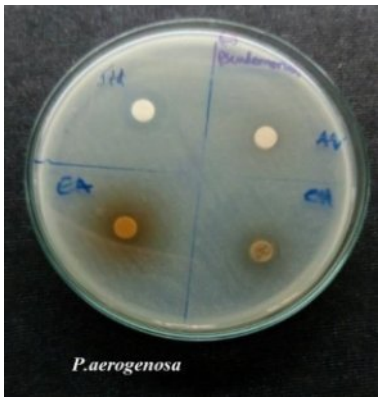
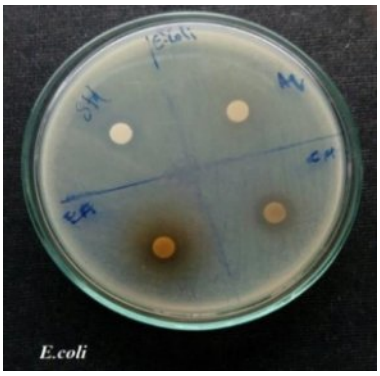


Table 24: Zone of inhibition for Gram –ve organisms

Organism	Standard (mm)	AQSG (mm)	EASG (mm)
Corynae bacterium	30	10	13
E.coli	12	8	12
Klebsella	12	8	14
Pseudomonas	13	10	12
V.cholera	34	8	14

Figure 17: Zone of inhibition for Gram –ve organisms



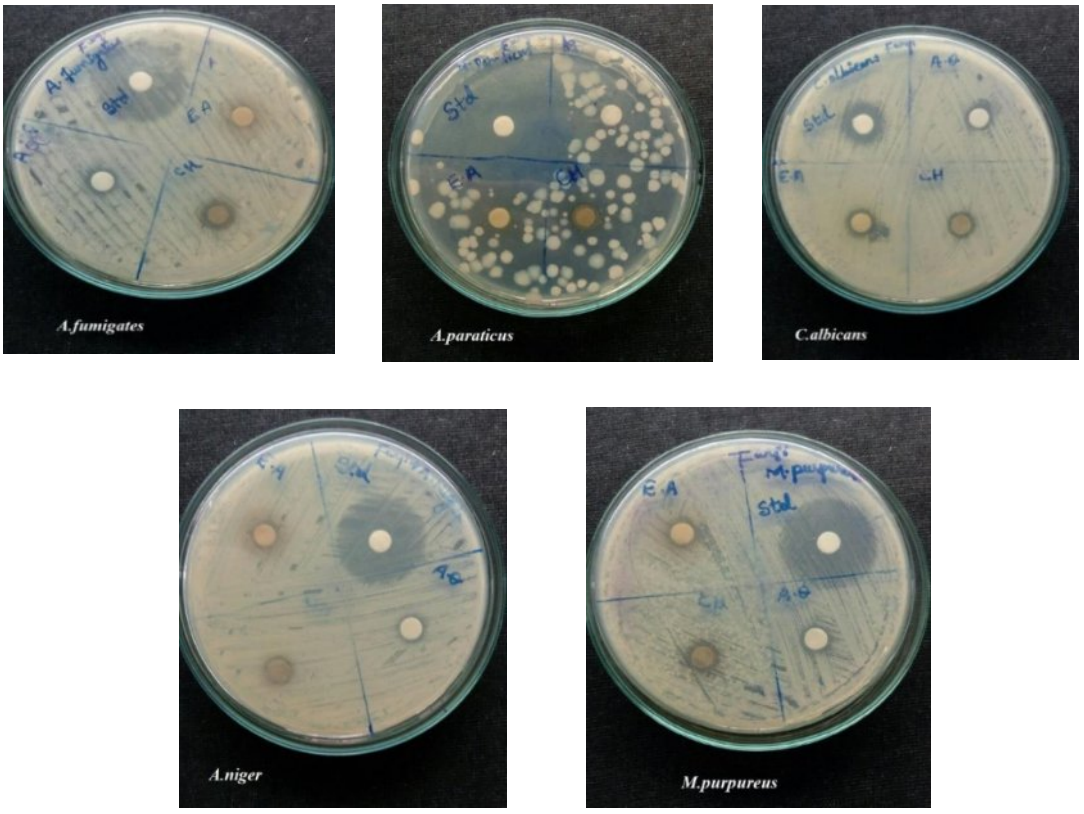


Antifungal activity of EASG and AQSG

Table 25: Antifungal activity of EASG and AQSG

organism	Standard (mm)	AQSG (mm)	EASG(mm)
Candida albicans	15	10	11
Aspergillus paraticus	15	6	10
Aspergillus fumigatus	25	7	10
Monascus purpureus	28	8	10
Aspergillus niger	27	6	9

Figure 18: Antifungal acitivity of EASG and AQSG



6.6.1 MINIMUM INHIBITORY CONCENTRATION OF EASG AND AQSG

Table 26: MIC values of EASG and AQSG

MIC value	*Staphylococcus albus* (µg/ml)	*Coryne bacterium* (µg/ml)	*Candida albicans* (µg/ml)
EASG	62.55	125	62.5
AQSG	125	125	250

Figure 19: MIC of EASG and AQSG

6.7 DETERMINATION OF *IN VITRO* CELL VIABILITY ASSAY ON CULTURED FIBROBLAST CELLS

Table 27: Percentage cell viability of EASG against MTT assay on Fibroblast

Sl. No	Name of Test sample	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	EASG	62.5 125 250 500 1000	1.52±0.7 8.27±4.6 11.57±4.2 19.99±1.3 24.27±1.9	>1000

Figure 20: Cytotoxic properties of EASG

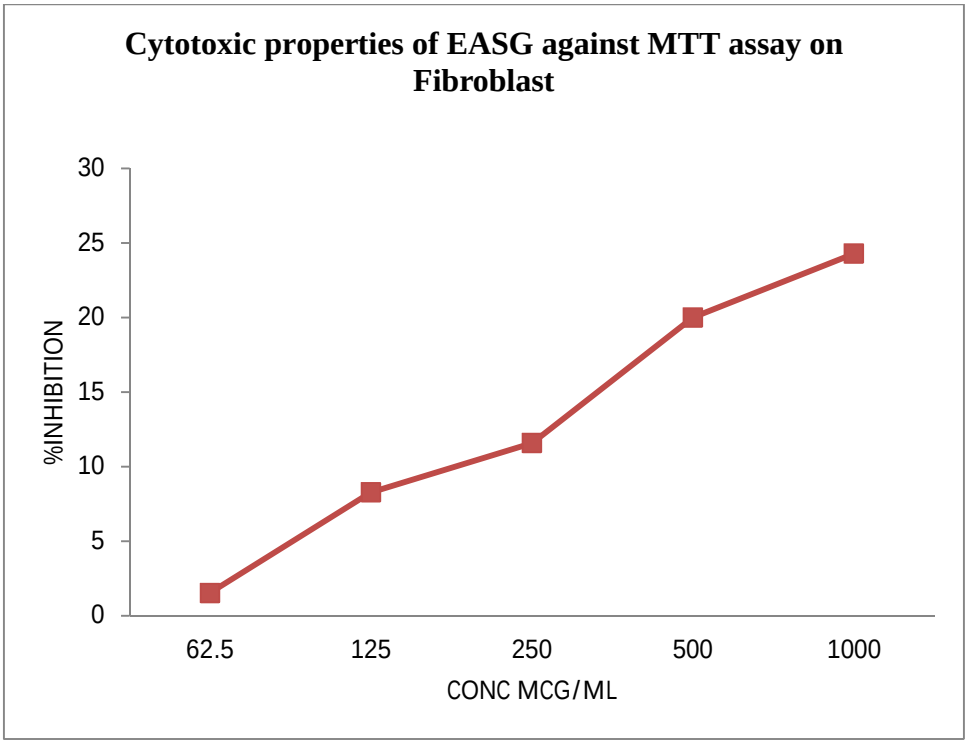


Table 28: Percentage cell viability of AQSG against MTT assay on Fibroblast

Sl. No	Name of Test sample	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	AQSG	62.5 125 250 500 1000	8.94±2.3 13.45±2.0 16.57±3.9 22.00±0.9 71.33±0.7	785.00±5.0

Figure 21: Percentage cell viability of AQSG

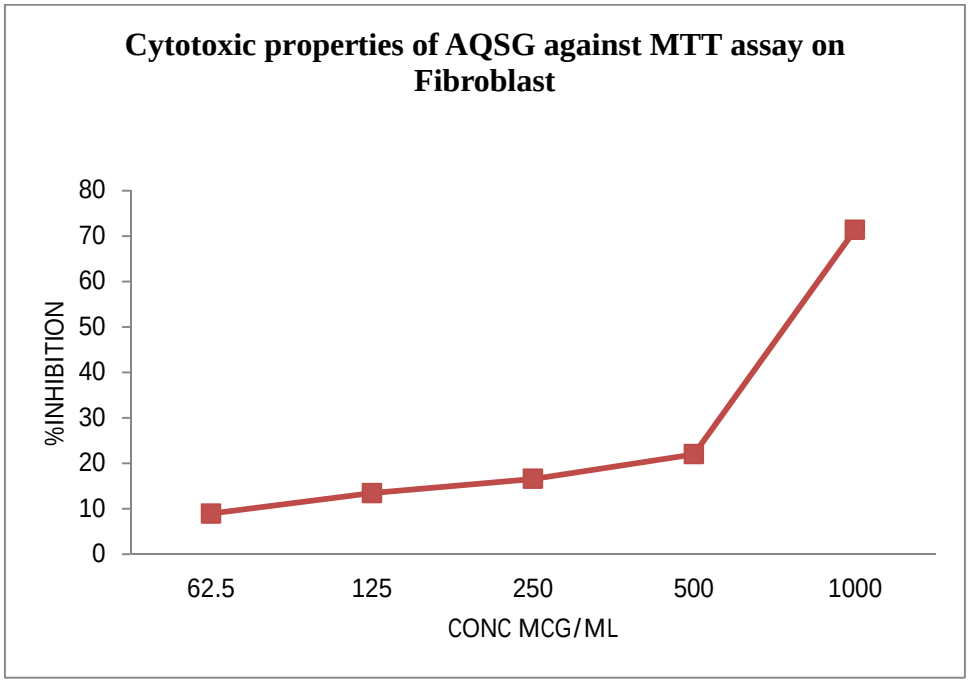
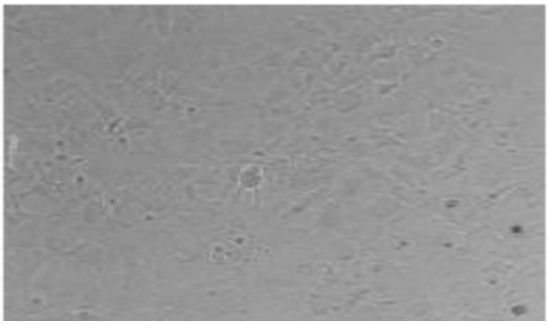
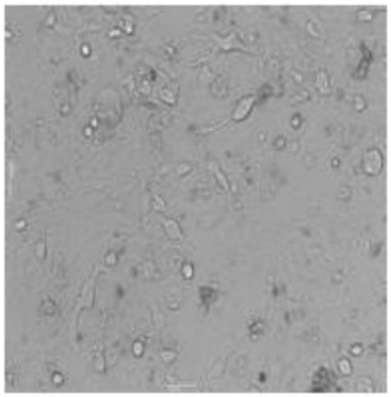


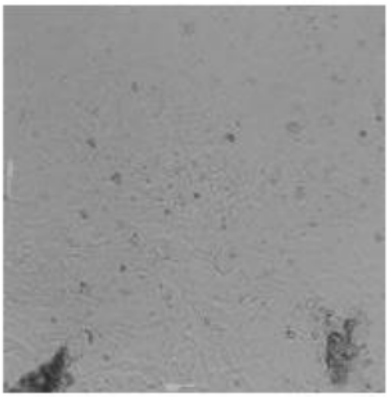
Figure 22: Cytotoxic properties of of EASG and AQSG



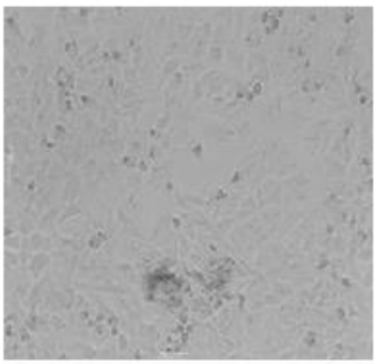
CONTROL Fibroblast



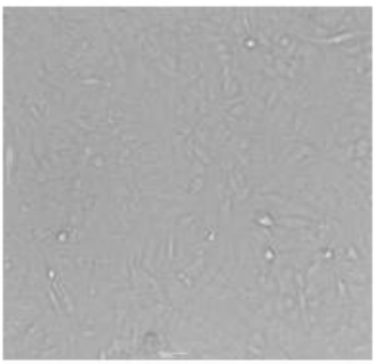
EASG 1000 (µg/ml)



EASG 500 (µg/ml)



AQSG 500 (µg/ml)



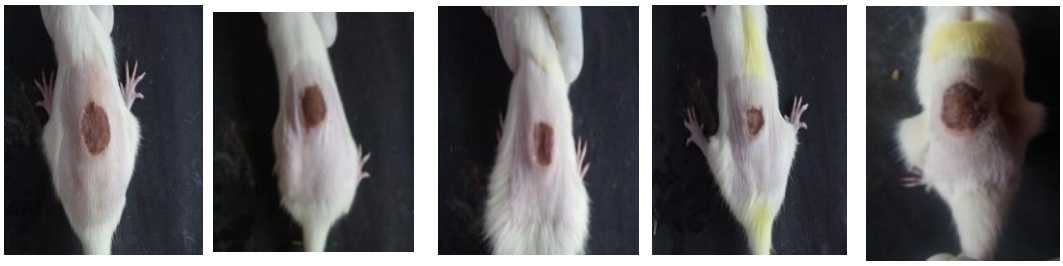
AQSG 1000 (µg/ml)

6.8 PHARMACOLOGICAL STUDIES

6.8.1 EVALUATION OF WOUND HEALING EFFECT OF EASG AND AQSG IN EXCISION WOUND MODEL

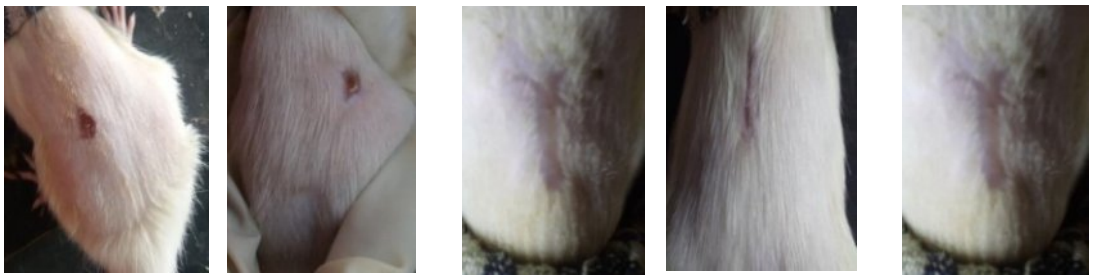
Figure 23: Percentage wound contraction on Excision wound model

On 1st day



Control Simple ointment base Standard EASG AQSG

After treatement-4th day



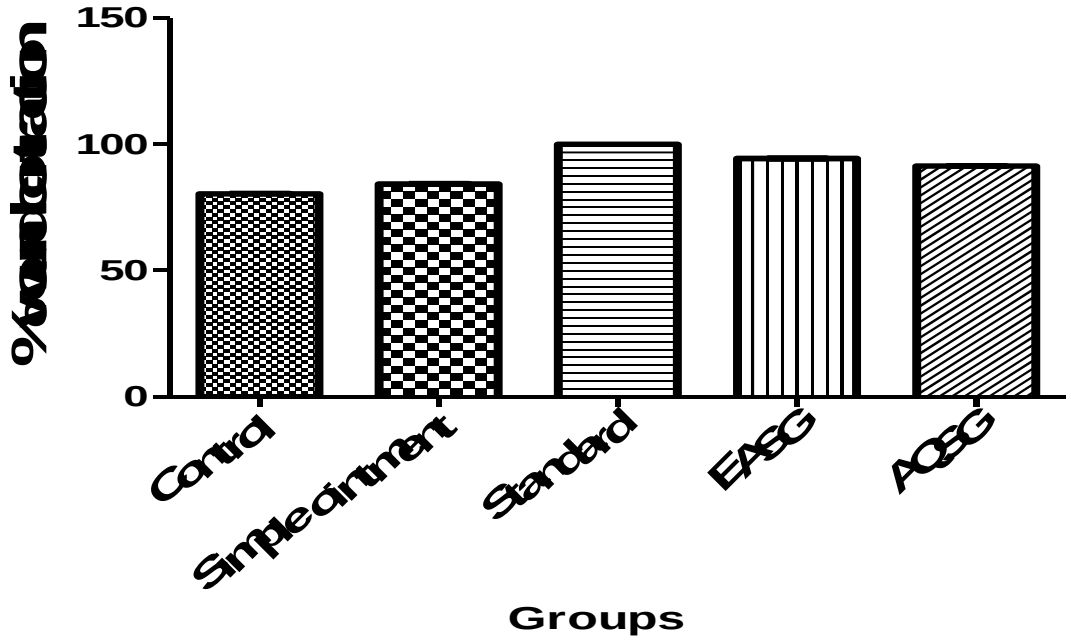
Control Simple ointment base Standard EASG AQSG

6.8.1.1 PERCENTAGE WOUND CONTRACTION

Table 29: Percentage wound contraction in excision wound model

	3rd day (%)	5th day (%)	7th day (%)	9th day (%)	11th day (%)	14th day (%)
Control	7.15±0.1784	20.93±0.0333	46.37±0.1406	65.36±0.1030	72.35±0.0482	80.37±0.0409
Simple ointment	7.933±0.0494	21.10±0.0365	49.12±0.3073	69.22±0.0860	75.12±0.3073	84.22±0.3742
Standard	13.33±0.0760	30.52±0.0307	60.08±0.0477	79.66±0.0400	95.20±0.0516	99.92±0.0374
EASG ointment	12.28±0.1014	28.18±0.0600	55.32±0.0600	73.46±0.0400	82.25±0.0341	94.26±0.0509
AQSG ointment	19.30±0.0966	26.17±0.0494	50.60±0.7746	70.96±0.0748	79.22±0.0477	91.28±0.0886

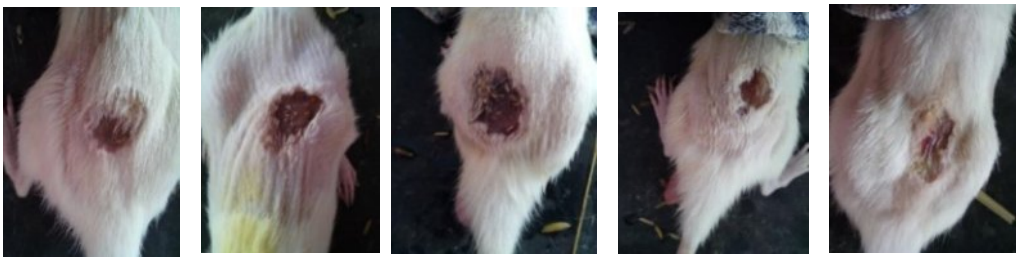
Figure 24: Effect of EASG and AQSG on % wound contraction in excission wound model



6.8.2 EVALUATION OF WOUND HEALING EFFECT OF EASG AND AQSG IN BURN WOUND MODEL

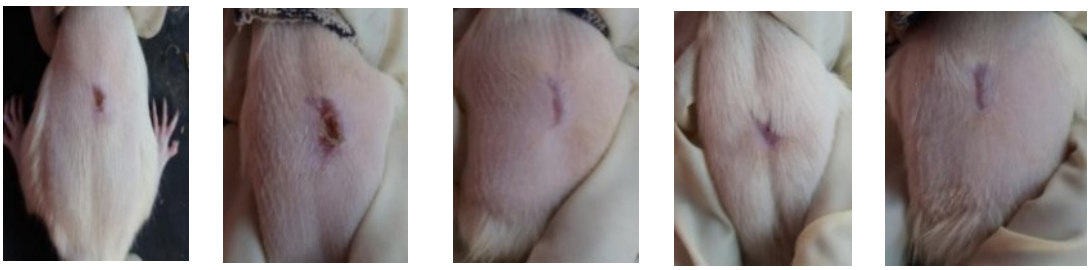
Figure 25: Percentage wound contraction in burn wound model

On 3rd day



Control Simple ointment base Standard EASG AQSG

After treatment 21st day



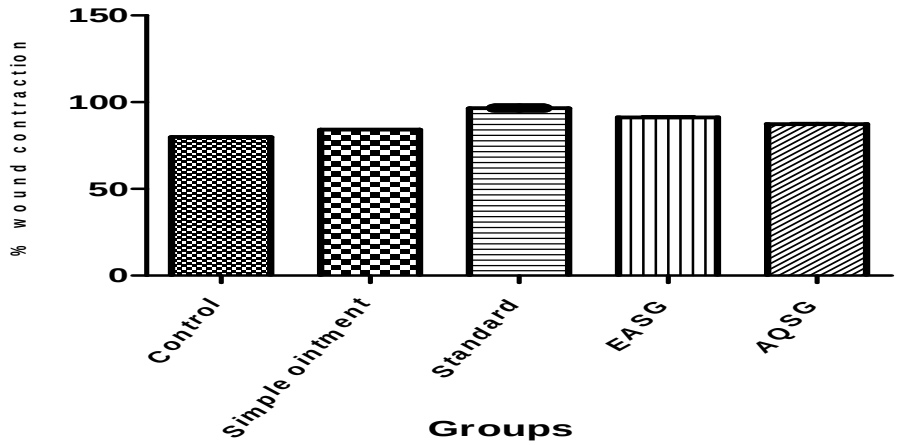
Control Simple ointment base Standard EASG AQSG

Table 30: Percentage wound contraction in burn wound model

	3rd	5th	7th	9th	11th	13th	15th	17th	19th	21st
Control	4.19±0.1934	9.12±0.0945	15.01±0.0786	22.13±0.0113	34.42±0.0678	42.12±0.0345	53.02±0.0224	62.02±0.058	76.98±0.0401	79.95±0.0763
Simple ointment	5.29±0.2457	10.65±0.1232	16.73±0.0763	24.43±0.0558	35.32±0.0213	44.67±0.0600	56.65±0.0432	68.22±0.0766	79.17±0.0494	84.18±0.0401
Standard	13.28±0.4567	19.67±0.0365	26.34±0.0201	34.87±0.0976	46.67±0.0374	58.89±0.0558	70.12±0.0881	79.45±0.0111	88.30±0.0966	96.62±0.0600
EASG ointment	11.07±0.9661	16.72±0.0601	21.57±0.0374	30.45±0.0453	41.24±0.0456	51.90±0.0112	62.32±0.0122	73.12±0.0866	80.18±0.0600	91.32±0.0600
AQSG ointment	10.34±0.1014	15.52±0.0491	20.12±0.0600	29.02±0.0432	39.78±0.0345	48.92±0.0866	60.34±0.0409	71.02±0.0374	79.46±0.0400	87.47±0.0881

All values are expressed as mean ± S.E.M; (n=6) [a]P<0.001, [b]P<0.01,[c]P<0.05, when all groups compared with control; [d]P<0.001, [e]P<0.01, [f]P<0.05 when all groups compared with Simple ointment; [g]P<0.001, [h]P<0.01, [i]P<0.05, when all groups compared with Standard.

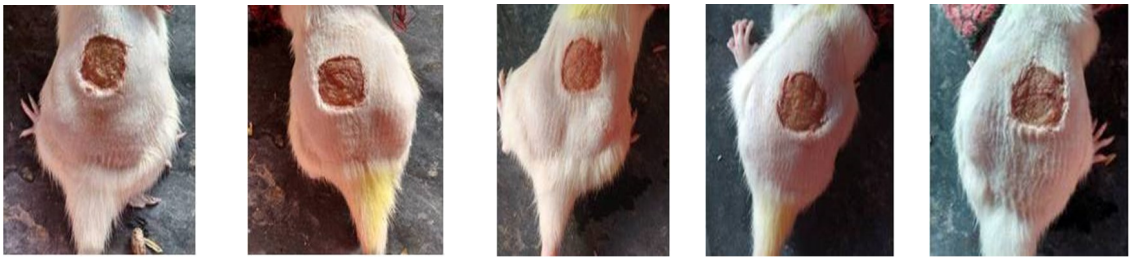
Fig 26: Effect of EASG and AQSG on % wound contraction in Burn wound model



6.8.3 EVALUATION OF WOUND HEALING EFFECT IN INFECTED EXCISSION WOUND MODEL

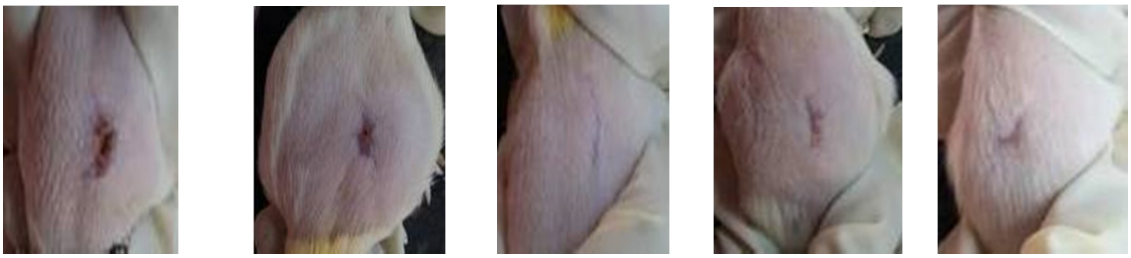
Figure 28: Percentage wound contraction iin infected excision wound model

On3rd day



Control Simple ointment base Standard EASG AQSG

After treatment -21st dat



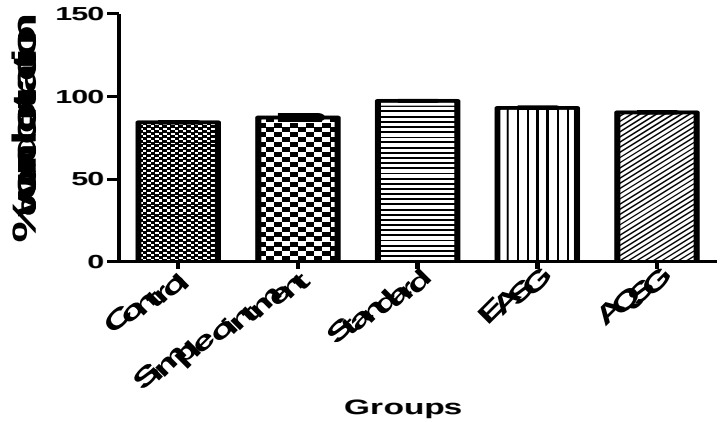
Control Simple ointment base Standard EASG AQSG

Table 31: Percentage wound contraction in infected excision wound model

	3rd	5th	7th	9th	11th	13th	15th	17th
Control	5.01±0.0333	11.24±0.4012	20.12±0.0900	29.03±0.0178	36.32±0.0881	56.34±0.0218	69.17±0.7438	72.65±0.09
Simple ointment	5.67±0.0760	12.28±0.1030	21.43±0.0600	29.95±0.0307	37.32±0.0561	59.41±0.1013	72.43±0.0345	76.28±0.06
Standard	13.33±0.0966	19.98±0.1406	29.93±0.0103	35.89±0.0775	44.76±0.0760	67.30±0.0966	79.65±0.0865	88.17±0.04
EASG ointment	11.933±0.172	17.15±0.0365	26.76±0.0860	33.14±0.0516	42.01±0.0365	60.67±0.0498	73.12±0.0600	84.18±0.06
AQSG Ointment	9.87±0.0400	16.32±0.0302	24.67±0.0482	30.22±0.0776	40.45±0.0365	59.87±0.0748	71.37±0.0881	81.46±0.04

All values are expressed as mean ± S.E.M; (n=6) [a]P<0.001, [b]P<0.01,[c]P<0.05, when all groups compared w
[e]P<0.01, [f]P<0.05 when all groups compared with Simple ointment; [g]P<0.001, [h]P<0.01, [i]P<0.05, when all g
Standard.

Figure 27: Effect of EASG and AQSG on % wound contraction in infected excision wound



6.9 EPITHELIALIZATION STAGES OF WOUND HEALING

Table 32: Epithelialization stages of excision, burn and infected excision wound models

Group	Excision wound	Burn wound	Infected burn wound
Control	16.67±0.3333	24.83±0.3073	25.00±0.6325
Simple ointment	15.17±0.3073^{c}	24.07±0.2108^{ns}	23.33±0.2108^{c}
Standard	11.00±0.2882^{ad}	19.83±0.3073^{ad}	19.83±0.3073^{ad}
EASG ointment	14.00±0.2582^{agns}	21.07±0.2108^{adg}	21.67±0.2167^{adg}
AQSG ointment	14.75±0.2882^{bgns}	21.50±0.2236^{adns}	22.83±0.3073^{afg}

All values are expressed as mean ± S.E.M; (n=6) $^{a}P<0.001$, $^{b}P<0.01$,$^{c}P<0.05$, when all groups compared with control; $^{d}P<0.001$, $^{e}P<0.01$, $^{f}P<0.05$ when all groups compared with Simple ointment; $^{g}P<0.001$, $^{h}P<0.01$, $^{i}P<0.05$, when all groups compared with Standard.

Figue 28: Effect of EASG and AQSG on epithelialization stages in excision wound model

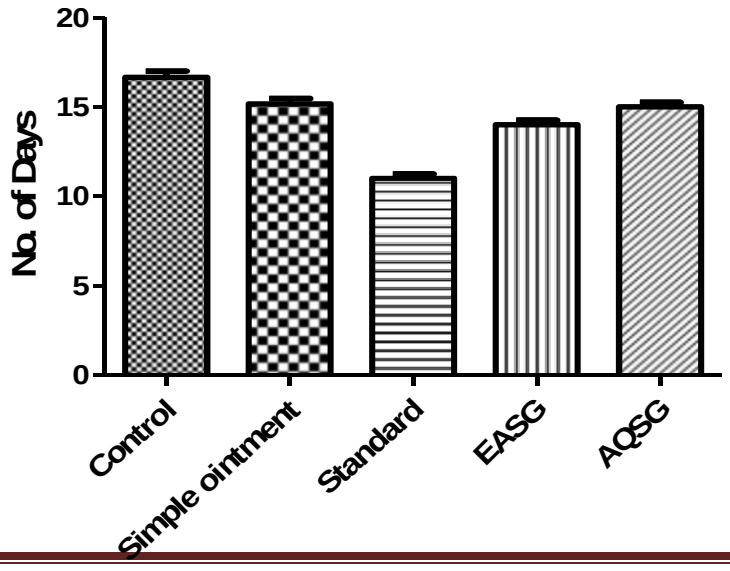
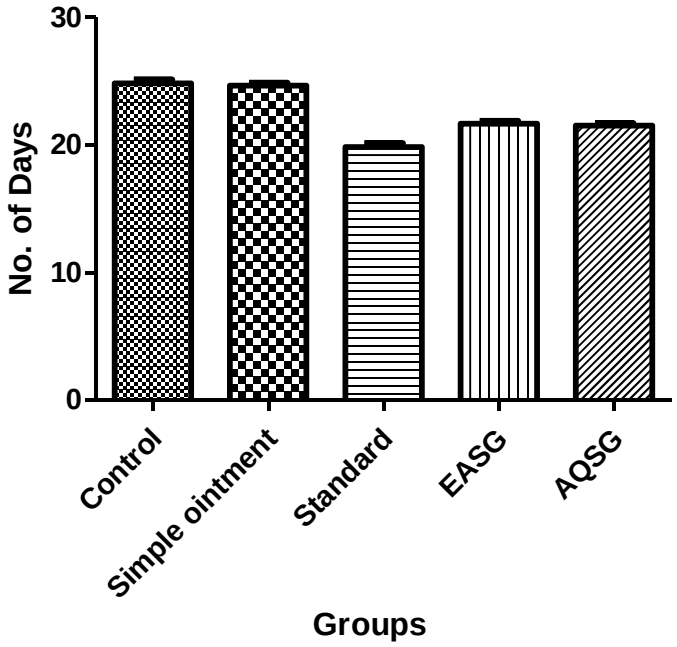
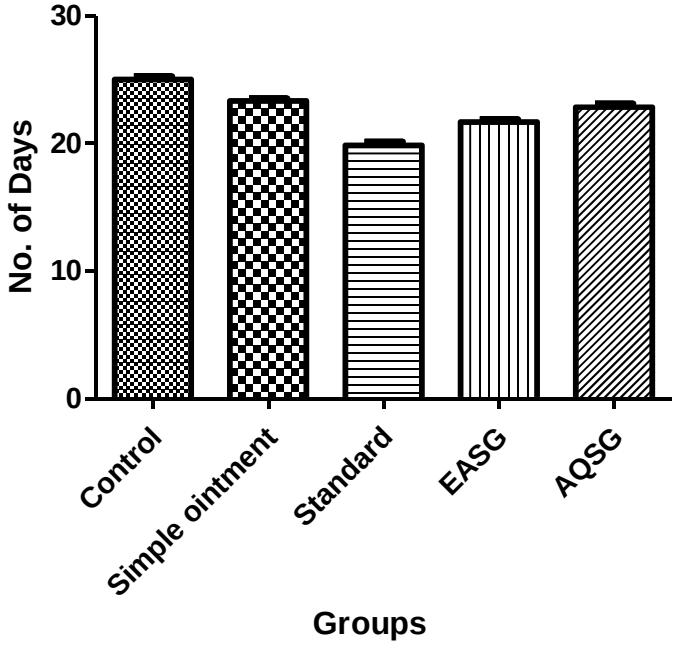


Figure 29: Effect of EASG and AQSG on epithelialization stages in burn wound model



Figue 30: Effect of EASG and AQSG on epithelialization stages in infected excission wound model



6.10 TENSILE STRENGTH

Table 33: Tensile strength of excision, burn and infected excision wound models

Group	Excision wound (g)	Burn wound (g)	Infected burn wound (g)
Control	336.4270	319.4820	321.4570
Simple ointment	412.1450	322.3680	347.6800
Standard	1019.4650	1133.3490	1025.3870
EASG ointment	917.0040	890.9870	901.1380
AQSG ointment	816.6840	785.1340	822.3870

6.11 ESTIMATION OF HYDROXYPROLINE

Table 34: Hydroxy proline levels in three wound models. Values are expressed as µg/g of tissue.

Group	Excision wound	Burn wound	Infected burn wound
Control	37.67±2.704	42.50±3.686	41.67±1.994
Simple ointment	40.00±3.152^{ns}	44.33±2.951^{ns}	43.17±2.315^{ns}
Standard	82.00±3.088^{ad}	81.00±4.041^{ad}	80.17±2.798^{ad}
EASG ointment	70.83±2.903^{adns}	71.83±2.120^{adns}	70.50±3.125^{adns}
AQSG ointment	64.33±2.431^{adh}	61.67±2.813^{beh}	70.33±2.152^{adns}

All values are expressed as mean± S.E.M; (n=6) $^{a}P<0.001$, $^{b}P<0.01$,$^{c}P<0.05$, when all groups compared with control; $^{d}P<0.001$, $^{e}P<0.01$, $^{f}P<0.05$ when all groups compared with Simple ointment; $^{g}P<0.001$, $^{h}P<0.01$, $^{i}P<0.05$, when all groups compared with Standard.

Fig 31: Effect of EASG and AQSG on Hydroxy proline level in excision wound model

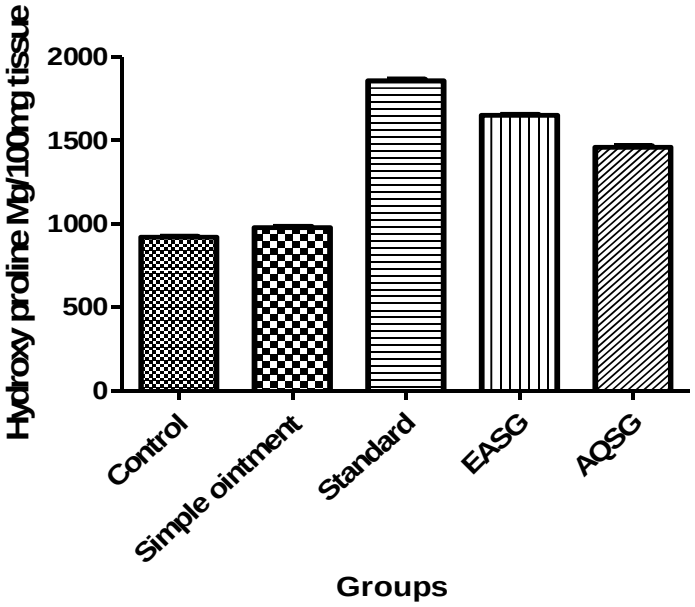


Fig 32: Effect of EASG and AQSG on Hydroxy proline level in Burn wound model

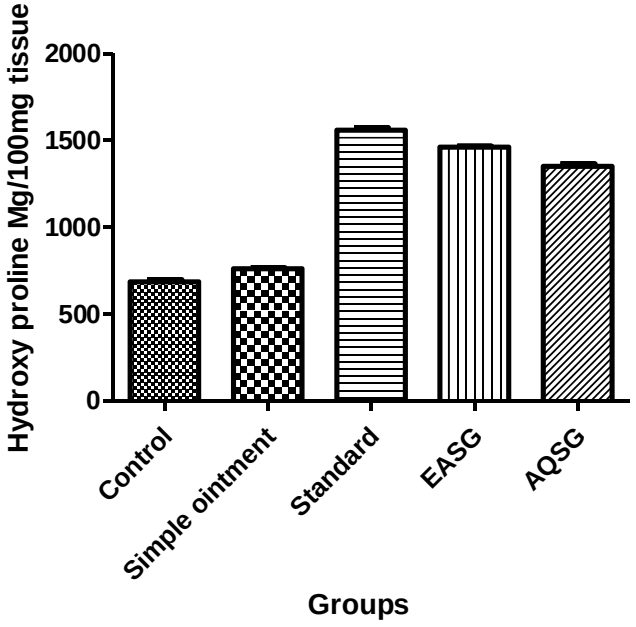
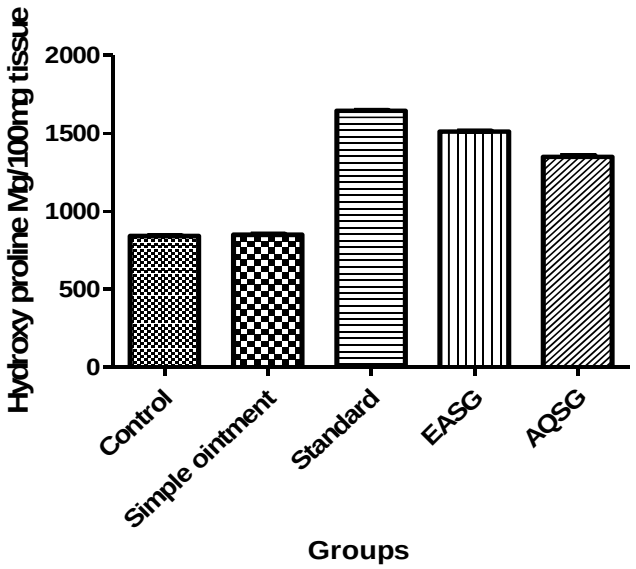


Fig 33: Effect of EASG and AQSG on Hydroxy proline level in infected excision wound model



6.12 ESTIMATION OF HEXOSAMINE

Table 35: Hexosamine levels in three wound models. Values are expressed as µg/mg of protein

Group	Excision wound	Burn wound	Infected burn wound
Control	78.83±1.778	64.00±1.317	81.67±1.145
Simple ointment	89.83±1.600^{c}	74.83±1.08^{ns}	91.67±0.8819^{c}
Standard	138.2±0,9458^{ad}	128.2±0.9458^{ad}	129.5±1.176^{ad}
EASG ointment	122.8±0.9458^{adg}	114.5±1.335^{adg}	123.7±1.145^{adh}
AQSG ointment	114.4±1.358^{adg}	106.2±1.014^{adg}	119.8±1.352^{adg}

All values are expressed as mean± S.E.M; (n=6) $^{a}P<0.001$, $^{b}P<0.01$,$^{c}P<0.05$, when all groups compared with control; $^{d}P<0.001$, $^{e}P<0.01$, $^{f}P<0.05$ when all groups compared with Simple ointment; $^{g}P<0.001$, $^{h}P<0.01$, $^{i}P<0.05$, when all groups compared with Standard.

Fig 34: Effect of EASG and AQSG on Hexosamine level in excision wound model

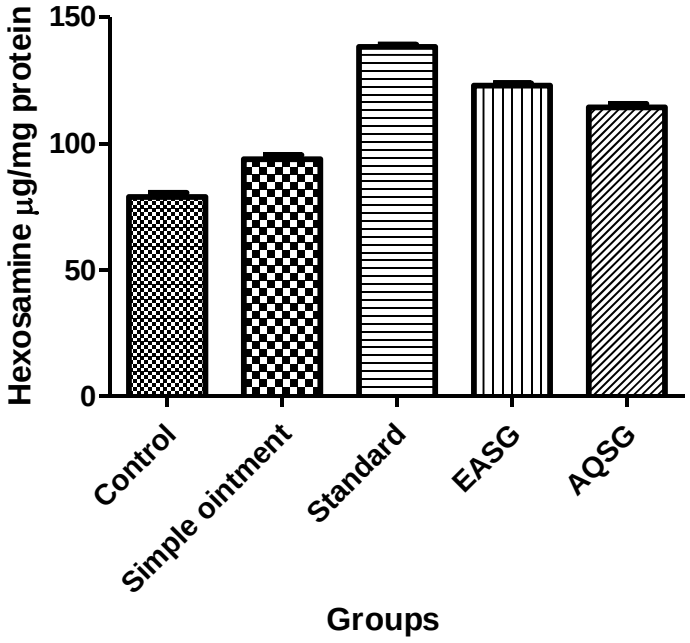


Fig 35: Effect of EASG and AQSG on Hexosamine level in Burn wound model

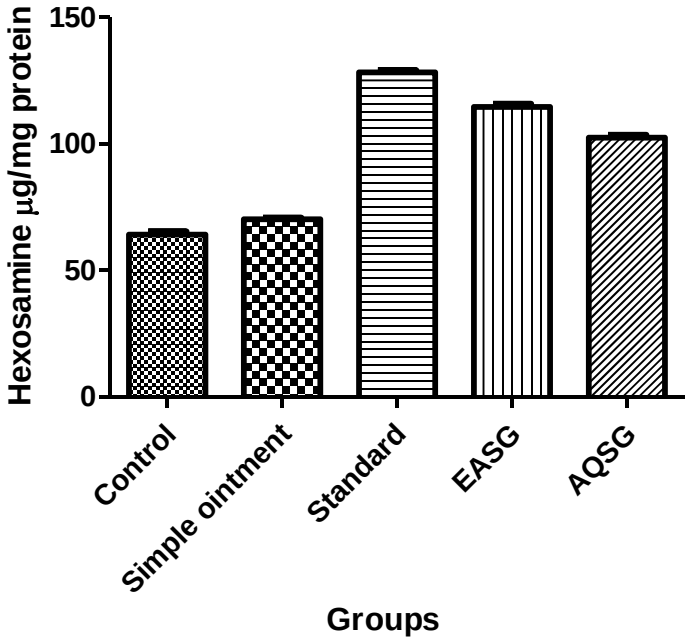
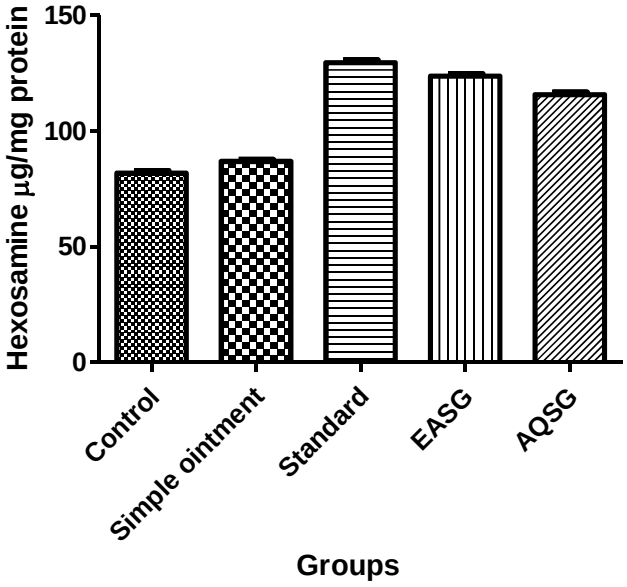


Fig 36: Effect of EASG and AQSG on Hexosamine level in infected excision wound model



6.13 ESTIMATION OF URONIC ACID

Table 36: Uronic acid levels in excision, burn and infected excision wound models. Values are expressed as µg/mg tissue

Group	Excision wound	Burn wound	Infected burn wound
Control	19.00±0.3651	15.83±0.6009^{ns}	17.50±0.7638^{ns}
Simple ointment	20.67±0.5774^{ns}	20.00±0.5774^{c}	23.33±1.054^{c}
Standard	52.50±0.7638^{ad}	51.50±0.7638^{ad}	55.50±0.4282^{ad}
EASG ointment	45.50±0.7638^{adg}	45.50±0.4285^{adg}	48.50±0.7638^{adg}
AQSG ointment	40.83±0.6009^{adg}	39.83±0.4773^{adg}	41.83±0.4773^{adg}

All values are expressed as mean± S.E.M; (n=6) $^{a}P<0.001$, $^{b}P<0.01$,$^{c}P<0.05$, when all groups compared with control; $^{d}P<0.001$, $^{e}P<0.01$, $^{f}P<0.05$ when all groups compared with Simple ointment; $^{g}P<0.001$, $^{h}P<0.01$, $^{i}P<0.05$, when all groups compared with Standard.

Fig 37: Effect of EASG and AQSG on Uronic acid level in excision wound model

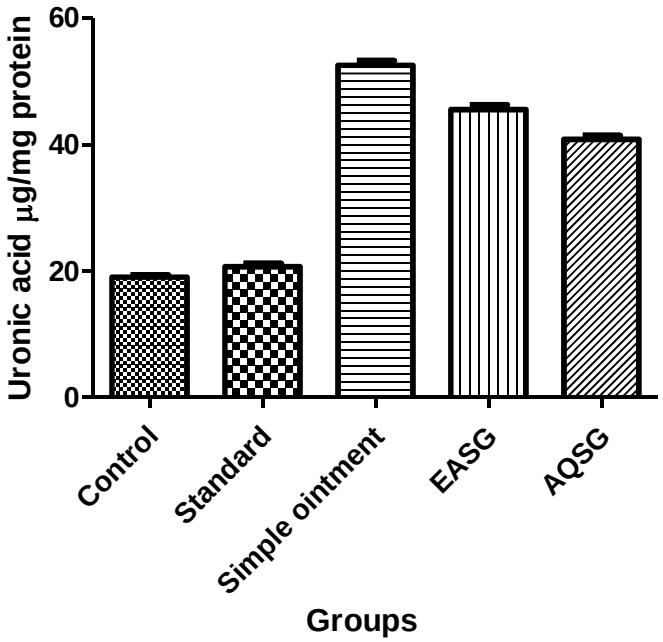


Fig 38: Effect of EASG and AQSG on Uronic acid level in infected Burn wound model

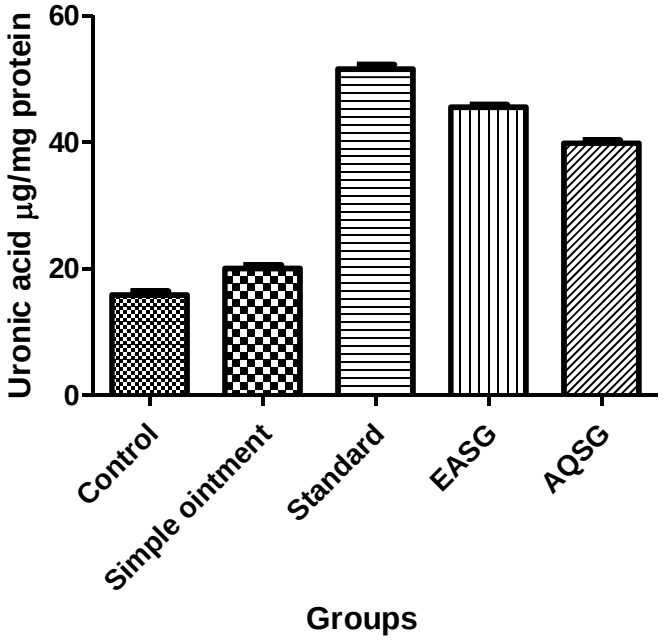
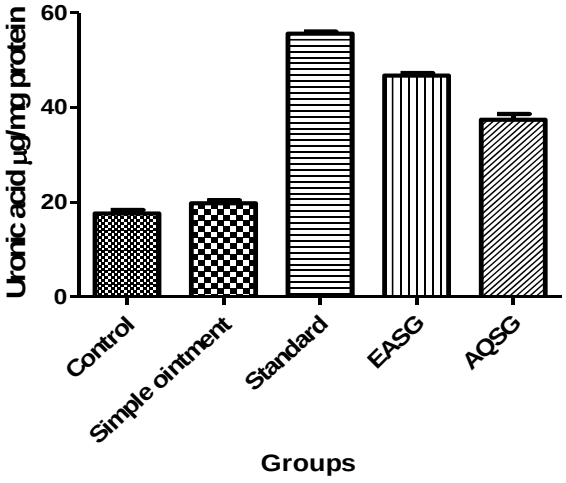


Fig 39: Effect of EASG and AQSG on Uronic acid level in infected excision wound model



6.14 *INVIVO* ANTIOXIDANT ACTIVITY

6.14.1 ESTIMATION OF TOTAL PROTEIN

Table 37: Effect of EASG and AQSG on total protein in skin tissue, values expressed in mg/g of tissue

Group	Excision wound	Burn wound	Infected burn wound
Control	46.00±0.9661	41.33±0.7149	45.00±0.5774
Simple ointment	50.83±1.014[b]	45.17±0.6009[c]	48.67±0.4216[c]
Standard	72.50±0.7688[ad]	68.50±0.7638[ad]	68.17±0.6009[ad]
EASG ointment	64.17±1.195[adg]	57.33±0.8433[adg]	58.67±0.8208[adg]
AQSG ointment	60.00±0.5774[adg]	51.50±0.9916[fdg]	53.83±0.4773[adg]

All values are expressed as mean ± S.E.M; (n=6) [a]P<0.001, [b]P<0.01,[c]P<0.05, when all groups compared with control; [d]P<0.001, [e]P<0.01, [f]P<0.05 when all groups compared with Simple ointment; [g]P<0.001, [h]P<0.01, [i]P<0.05, when all groups compared with Standard.

Fig 40: Effect of EASG and AQSG on on Total Protein level in excision wound model

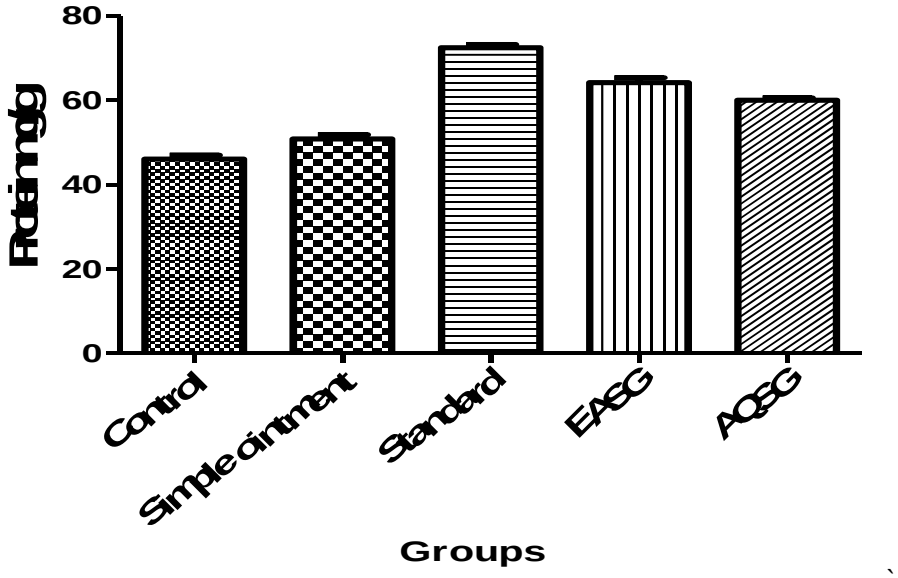


Fig 41: Effect of EASG and AQSG on Total Protein level in Burn wound model

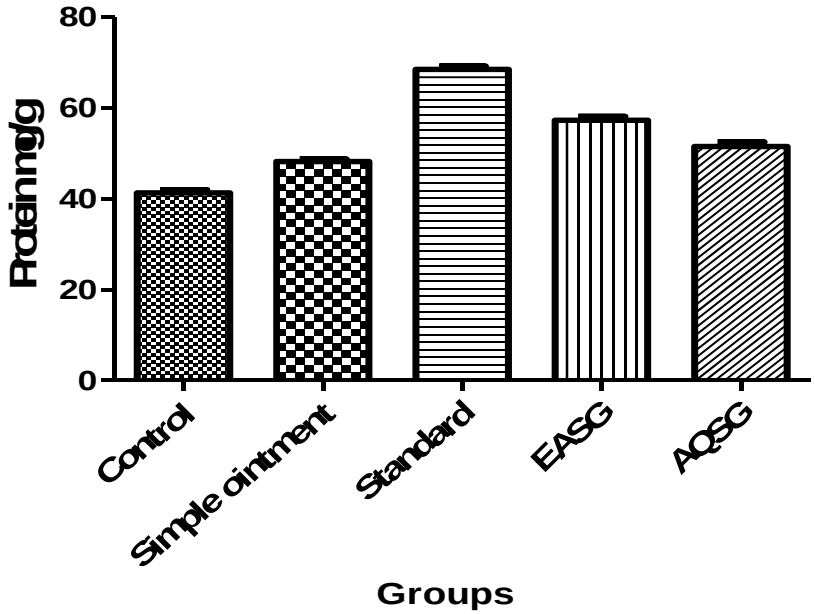
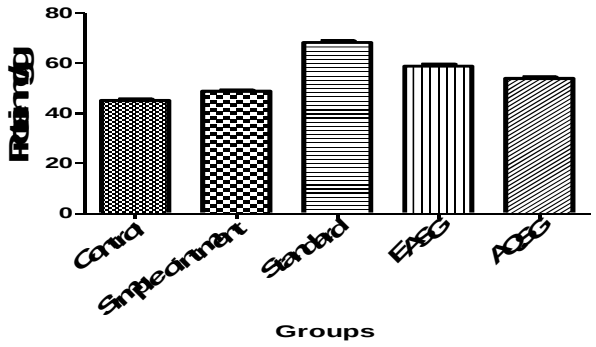


Fig 42: Effect of EASG and AQSG on Total Protein level in infected excision wound model



6.14.2 Effect of EASG AND AQSG on *In vivo* antioxidants of wound tissues of excision wound model.

Table 38: Effect of EASG AND AQSG on *In vivo* antioxidants of wound tissues of excision wound model

Groups	Antioxidant enzymes			
	SOD (unit/mg protein)	CAT (µmol of H2O2 consumed/min/ mg protein)	GSH (Glutathione µg/mg)	LPO (nmol of MDA/mgprotein
Control	0.48±0.0358	12.70±0.7080	7.16±0.4282	24.50±1.765
Only simple ointment	0.60 ± 0.0174^{ns}	14.65 ± 0.2592^{c}	8.16 ± 0.7032^{ns}	24.00 ± 1.265^{ns}
Standard	$2.26\pm0.1621^{a,d}$	$21.30\pm0.5228^{a,d}$	$13.17\pm1.352^{a,c}$	$9.167\pm1.302^{a,d}$
EASG ointment	$1.55\pm0.0428^{a,d,g}$	$19.05\pm0.3334^{a,d,i}$	$11.83\pm1.014^{c,ns}$	$13.83\pm1.721^{a,d,ns}$
AQSG ointment	$1.183\pm0.1046^{a,e,g}$	$17.17\pm0.2940^{a,e,g}$	$11.67\pm0.7601^{c,ns}$	$15.50\pm1.088^{a,d,ns}$

All values are expressed as mean± S.E.M; (n=6) $^{a}P<0.001$, $^{b}P<0.01$,$^{c}P<0.05$, when all groups compared with control; $^{d}P<0.001$, $^{e}P<0.01$, $^{f}P<0.05$ when all groups compared with Simple ointment; $^{g}P<0.001$, $^{h}P<0.01$, $^{i}P<0.05$, when all groups compared with Standard.

Fig 43: Effect of EASG and AQSG on SOD level in excision wound model

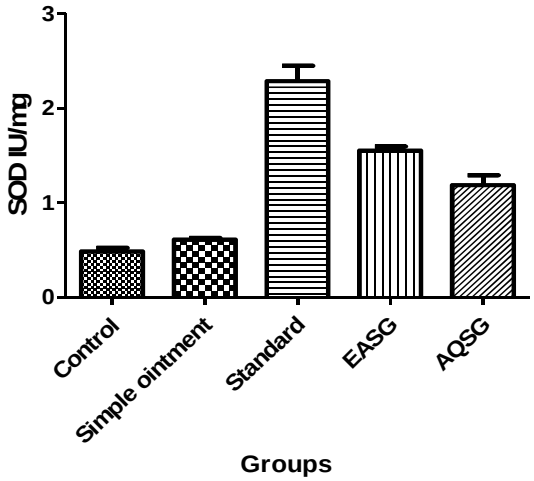


Fig 44: Effect of EASG and AQSG on CAT level in excision wound model

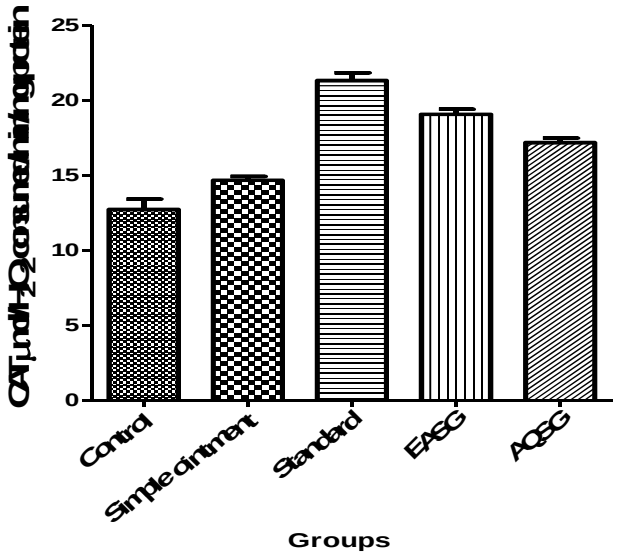


Fig 45: Effect of EASG and AQSG on GSH level in excision wound model

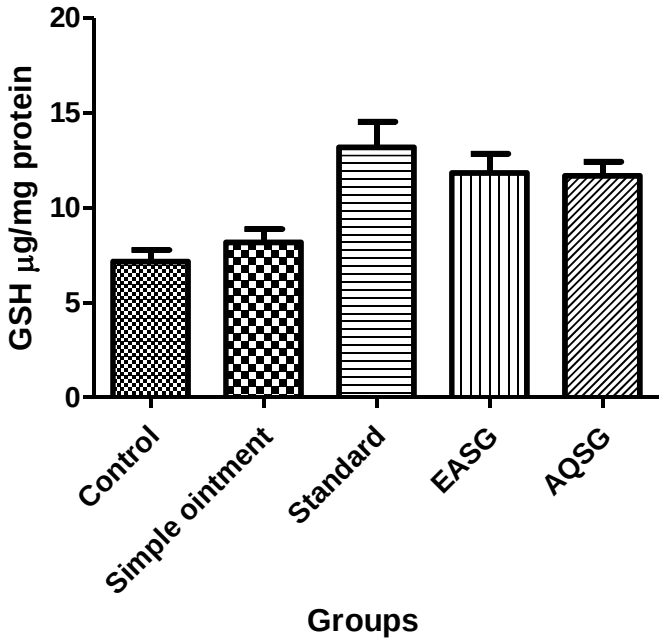
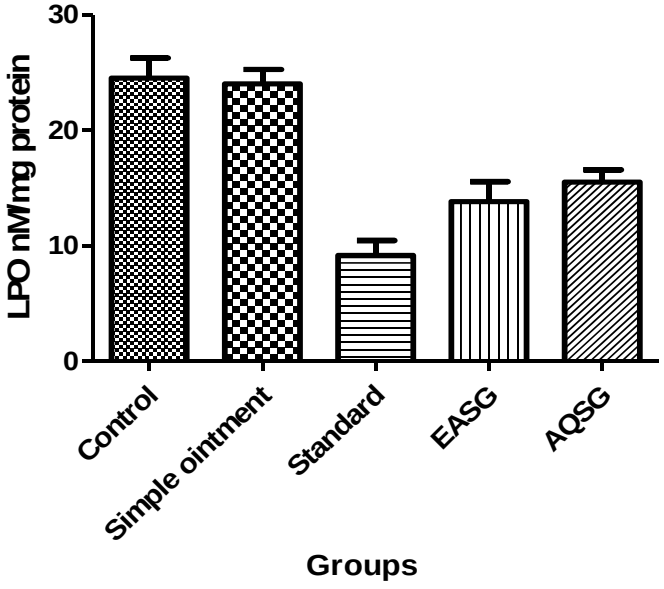


Fig 46: Effect of EASG and AQSG on LPO level in excision wound model



6.15.2 Effect of EASG and AQSG on *In vivo* antioxidants of wound tissues of Burn wound model

Table 39: Effect of EASG AND AQSG on *In vivo* antioxidants of wound tissues of Burn wound model

Groups	Antioxidant enzymes			
	SOD(unit/min/ mg protein)	CAT (µmol of H2O2 consumed/min/ mg protein)	GSH (Glutathione µg/mg)	LPO (nmol of MDA/mgprotein
Control	0.39±0.0281	12.42±0.7350	7.5±0.4282	25.67±1.626
Only simple ointment	0.54 ± 0.0199^{ns}	12.67 ± 0.8819^{ns}	8.66 ± 0.6146^{ns}	23.83 ± 0.8333^{ns}
Standard	$2.65\pm0.2125^{a,d}$	$20.50\pm1.176^{b,e}$	$13.50\pm0.8466^{a,c}$	$9.50\pm1.176^{a,d}$
EASG ointment	1.55 ± 0.078^{adg}	$19.67\pm1.476^{b,e,ns}$	$10.33\pm0.6667^{c,ns}$	$12.17\pm0.9804^{a,d,ns}$
AQSG ointment	1.13 ± 0.087^{adg}	$18.33\pm1.978^{c,f,ns}$	$10.63\pm0.5578^{c,ns}$	$13.50\pm0.7638^{a,d,ns}$

All values are expressed as mean± S.E.M; (n=6) $^{a}P<0.001$, $^{b}P<0.01$,$^{c}P<0.05$, when all groups compared with control; $^{d}P<0.001$, $^{e}P<0.01$, $^{f}P<0.05$ when all groups compared with Simple ointment; $^{g}P<0.001$, $^{h}P<0.01$, $^{i}P<0.05$, when all groups compared with Standard.

Fig 47: Effect of EASG and AQSG on SOD level in burn wound model

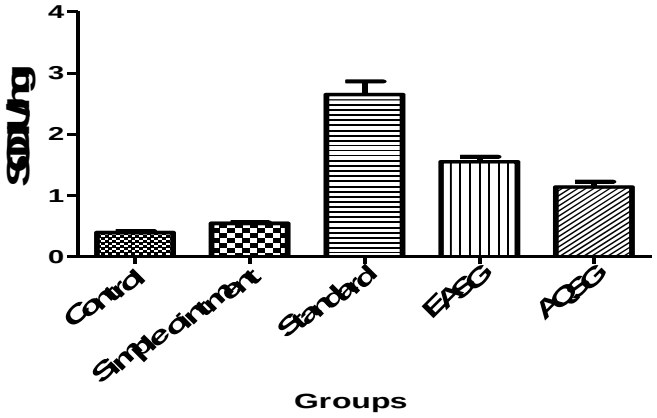


Fig 48: Effect of EASG and AQSG on CAT level in burn wound model

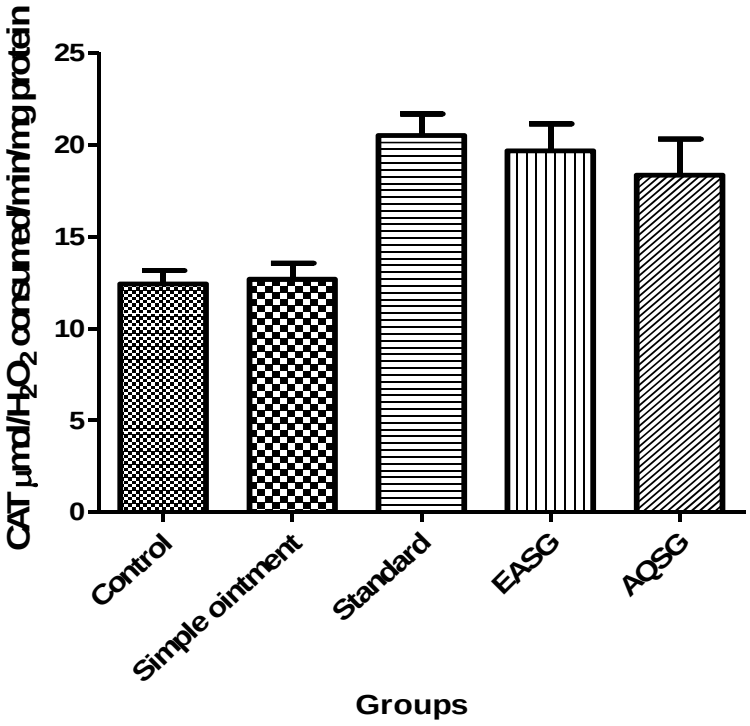


Fig 49: Effect of EASG and AQSG on GSH level in burn wound model

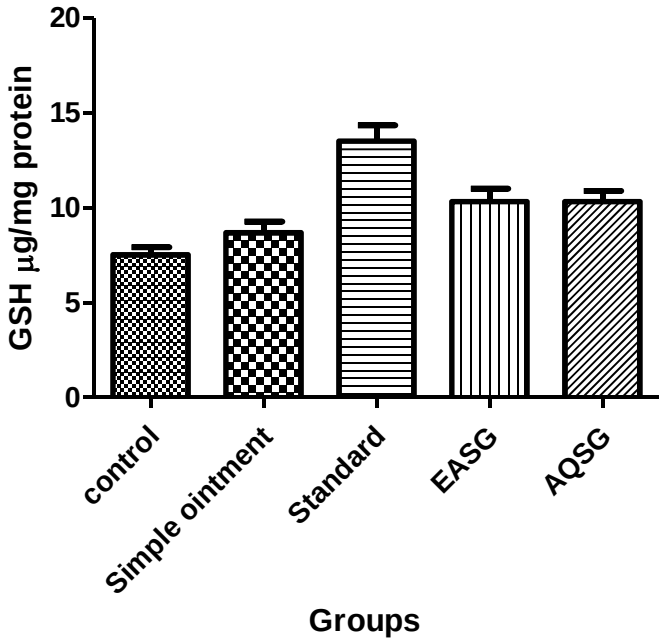
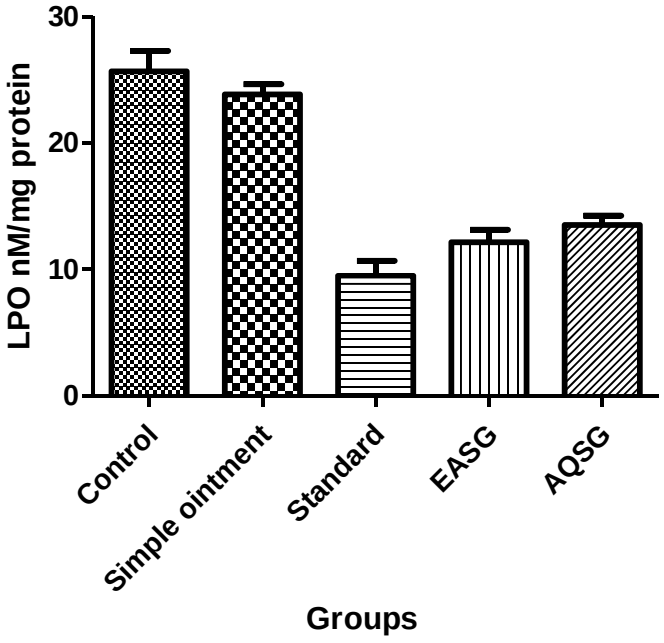


Fig 50: Effect of EASG and AQSG on LPO level in burn wound model



6.15.3 Effect of EASG AND AQSG on *In vivo* antioxidants of wound tissues of Infected excision wound model

Table 40: Effect of EASG and AQSG on *In vivo* antioxidants of wound tissues of Infected excision wound model

Groups	Antioxidant enzymes			
	SOD(unit/min/ mg protein)	CAT (µmol of H2O2 consumed/min/ mg protein)	GSH (Glutathione µg/mg)	LPO (nmol of MDA/mgprotein
Control	0.456±0.0395^{ns}	12.50±0.7638	7.83±0.6009	28.17±2.056
Only simple ointment	0.54±0.0423^{c}	13.17±0.9458^{ns}	8.667±0.6146^{ns}	22.33±0.8433^{c}
Standard	2.533±0.2231^{a,d}	21.50±1.057^{a,d}	11.83±0.7923^{b,f}	10.50±0.7638^{a,d}
EASG ointment	1.49±0.0521^{a,d,g}	19.50±1.0^{a,e,ns}	10.83±0.6009^{c,ns}	12.83±1.014^{a,d,ns}
AQSG ointment	1.25±0.0600 ^{a,e,g}	18.17±1.138^{b,f,ns}	10.67±0.5578^{c,ns}	14.00±1.00^{a,d,ns}

All values are expressed as mean± S.E.M; (n=6) ^{a}P<0.001, ^{b}P<0.01,^{c}P<0.05, when all groups compared with control; ^{d}P<0.001, ^{e}P<0.01, ^{f}P<0.05 when all groups compared with Simple ointment; ^{g}P<0.001, ^{h}P<0.01, ^{i}P<0.05, when all groups compared with Standard.

Fig 51: Effect of EASG and AQSG on SOD level in infected excission wound model

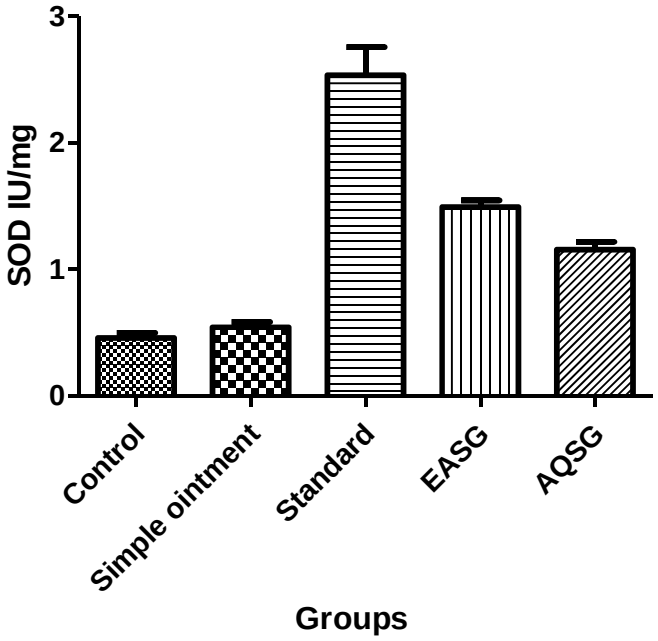


Fig 52: Effect of EASG and AQSG on CAT level in infected excision wound model

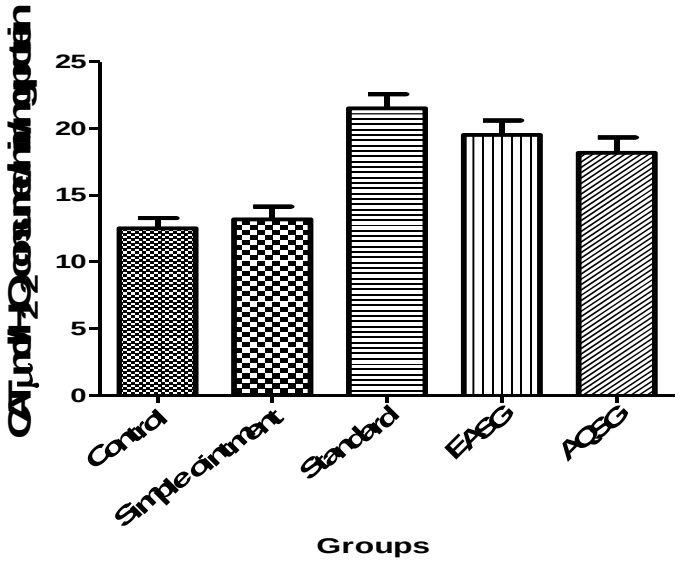


Fig 53: Effect of EASG and AQSG on GSH level in infected excision wound model

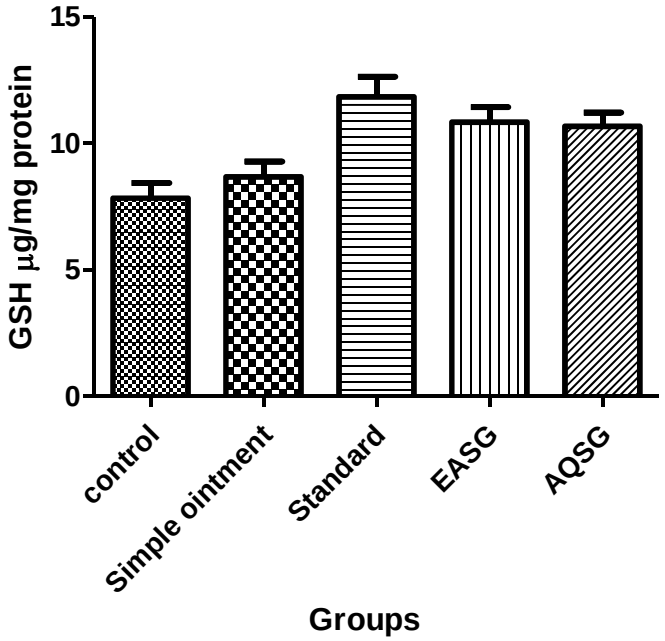
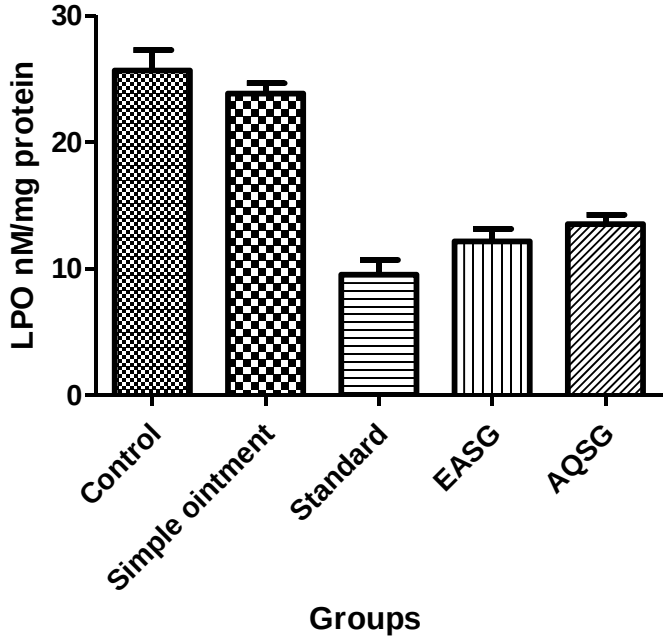


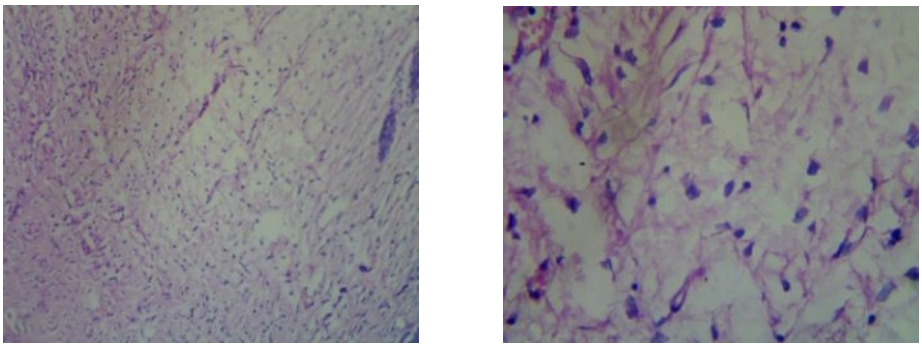
Fig 54: Effect of EASG and AQSG on LPO level in infected excision wound model



6.16 HISTOPATHOLOGICAL EVALUATION

6.16.1 Histopathological evaluation of excision wound model.

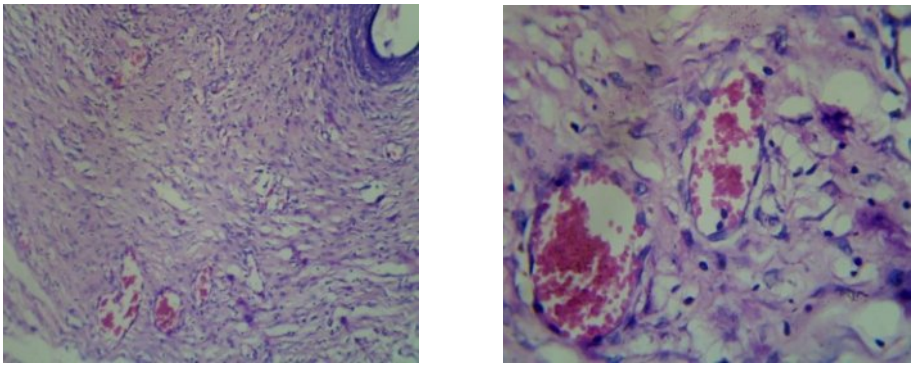
Figure 55: Group 1- Control



10X 40X

The section of the skin and epidermis shows incomplete healing with less epithelialization and lesser collagen formation indicated the incomplete wound healing. remodeling, granulation tissue and dispersed inflammation cells were observed in this group.

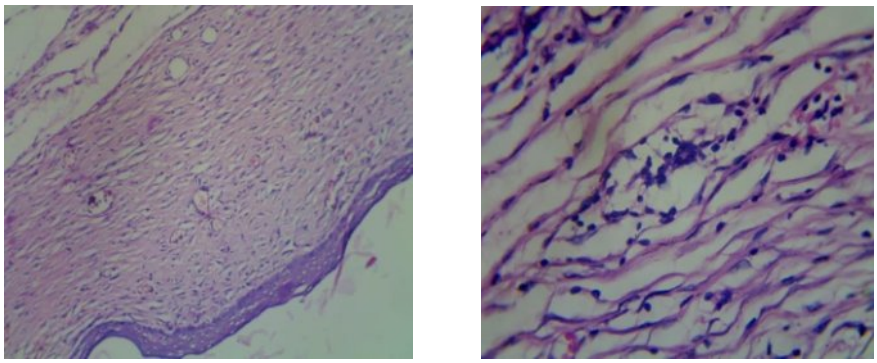
Figure 56: Group 2- Simple ointment base



10X 40X

Section of the skin and epidermis shows proliferation of fibroblasts and few macrophages and plasmacells with with infilteration of large number of lymphocytes, microphages and a few neotrophils. Dermis also shows mild edema.

Figure 57: Group 3- Standard

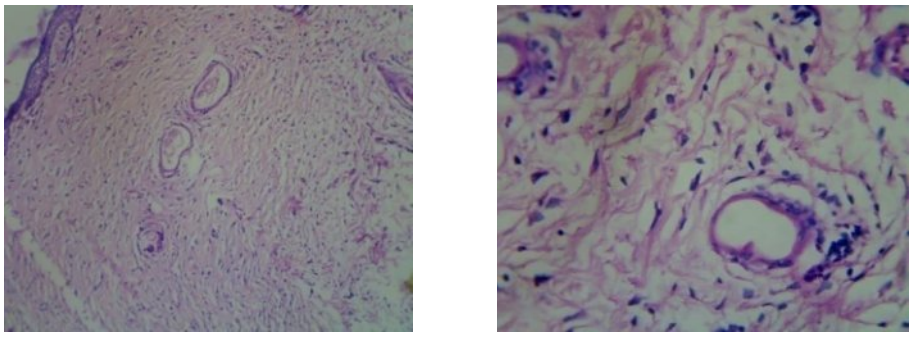


10X 40X

Section of the epidermis shows Re-epithelialisation with the proliferation of fibroblastgs and few lymphocytes in the sub epithelium with thin walled congested blood vessels. Mature connective

tissue and a few capillaries with a thick epidermal lining at the periphery of the wound were observed

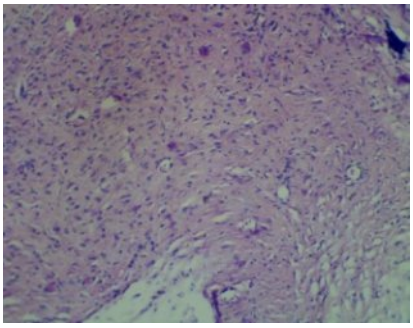
Figure 58: Group 4- EASG treated

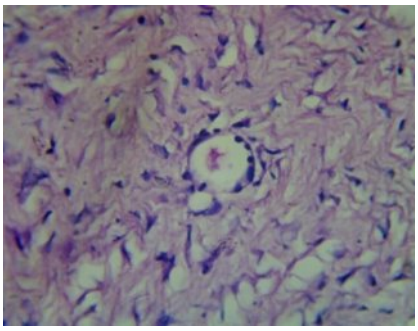


10X 40X

Section of the epidermis shows Re-epithelialization with the proliferation of fibroblasts, of dense fibrous tissue and blood capillaries was observed. Few macrophages were also present.

Figure 59: Group 5- AQSG treated



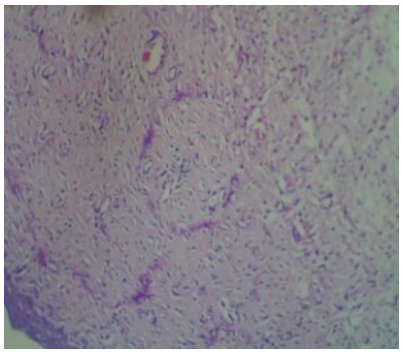


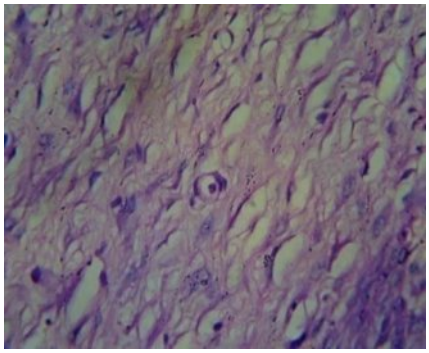
10X 40X

Section of the epidermis shows Re-epithelialization with the proliferation of fibroblasts, of dense fibrous tissue and blood capillaries was observed. Few macrophages were also present.

6.16.2Histopathological evaluation of Burn wound model

Figure 60: Group 1-Control

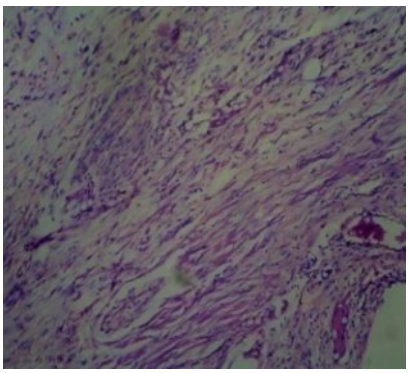




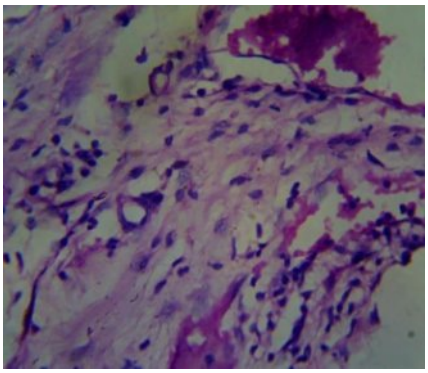
10X 40X

Section shows marked proliferation of angioblasts and fibroblast with infilteration of large number of lymphocytes, microphages and a few neotrophils

Figure 61: Group 2- Simple ointment base



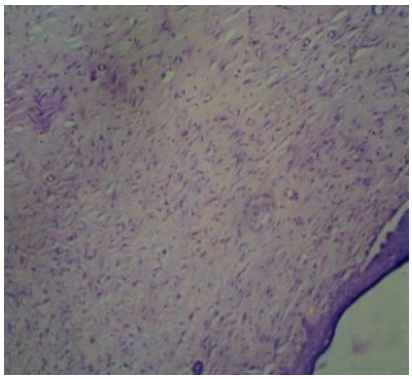
10X



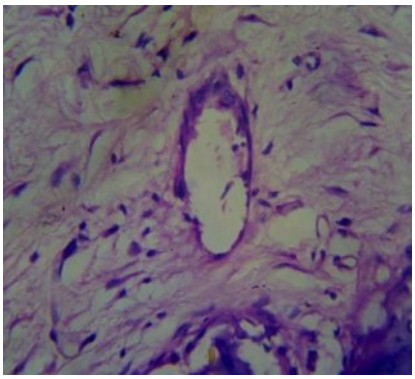
40X

Section shows skin and the epidermis shows **RE EPITHELIALIZATION.** Dermis shows proliferation of fibroblasts, scattered lymphoplasmacytic infiltrates and thin walled congested vessels (**Scar**)

Figure 62: Group 3- Standard



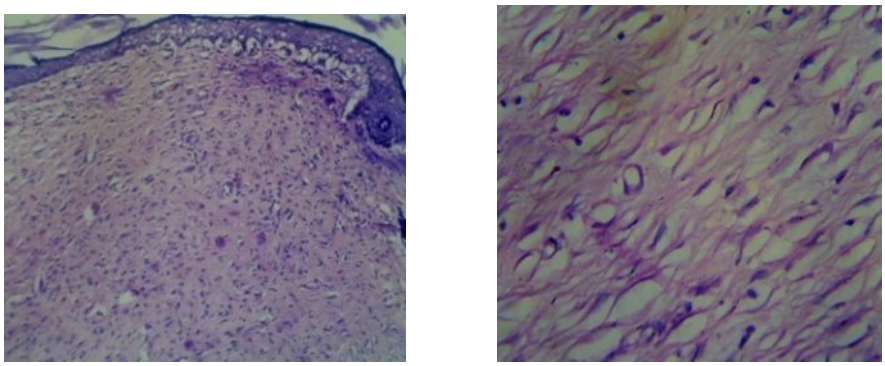
10X



40X

Section shows skin and the epidermis shows **RE EPITHELIALIZATION.** Dermis shows proliferation of fibroblasts and blood vessels (**Scar**).

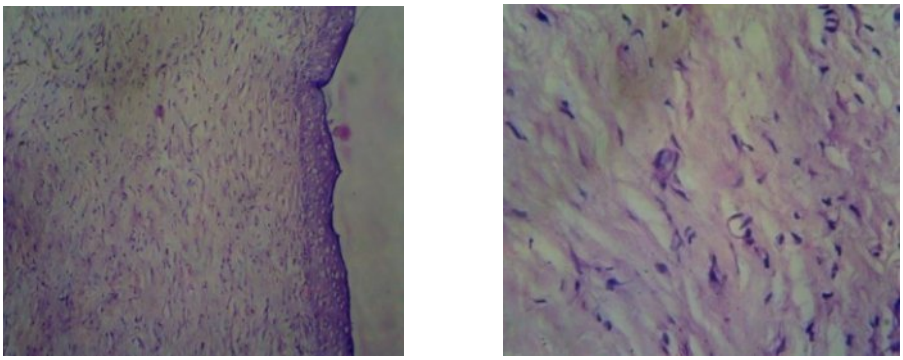
Figure 63: Group 4- EASG



10X 40X

Section shows skin and the epidermis shows **RE EPITHELIALIZATION.** Dermis shows proliferation of fibroblasts and thin walled vessels (**Scar**).

Figure 64: Group 5- AQSG

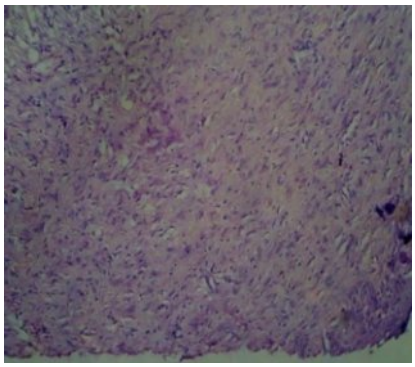


10X 40X

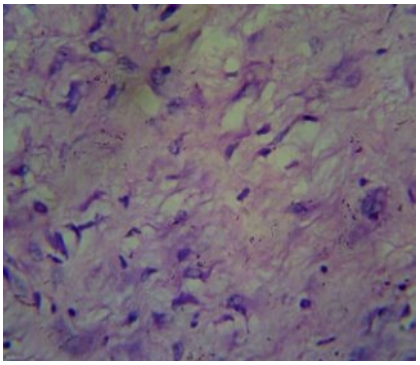
Section shows skin and the epidermis shows **RE EPITHELIALIZATION.** Dermis shows proliferation of fibroblasts and thin walled vessels (**Scar**).

6.16.3Hisopathological evaluation of Infected excision wound model

Figure 65: Group 1- Control



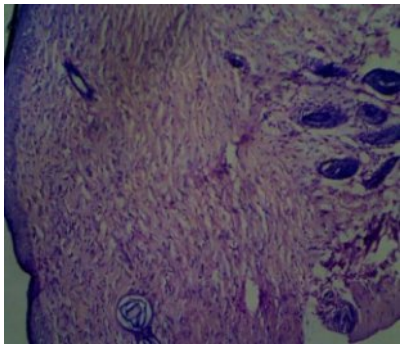
10X



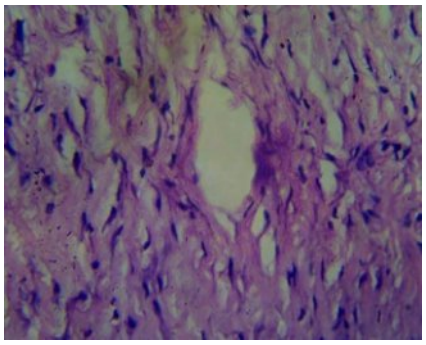
40X

Section shows skin and the epidermis shows **RE EPITHELIALIZATION,** one focal shows surface ulceration. Dermis shows proliferation of fibroblasts and thin walled vessels and scattered lymphocytes and few neutrophils seen in the section studied.

Figure 66: Group 2- Simple Ointment Base



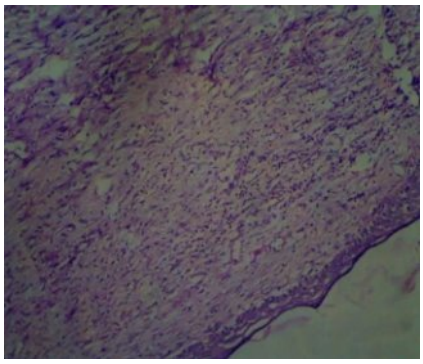
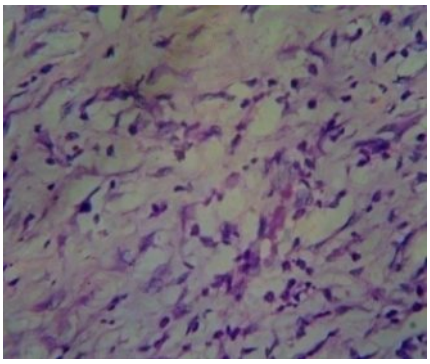
10X



40X

Section shows skin and the epidermis shows **RE EPITHELIALIZATION** with one area shows horn cyst**.** Dermis shows proliferation of fibroblasts, scattered lymphocytic infiltrates and thin walled vessels.

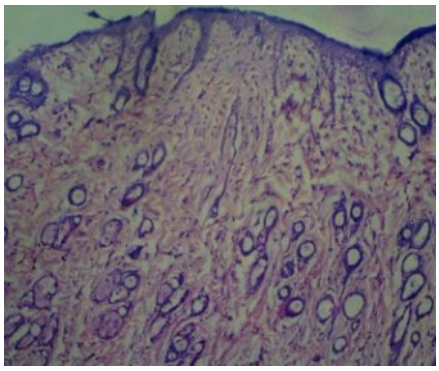
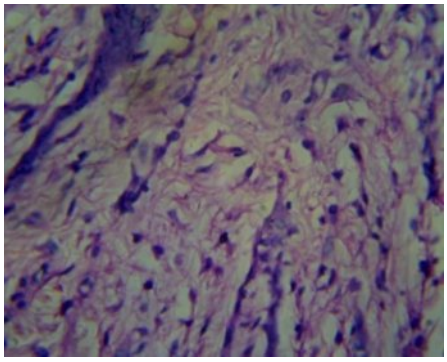
Figure 67: Group 3- Standard

10X 40X

Section shows skin and the epidermis shows **RE EPITHELIALIZATION.** Dermis shows proliferation of fibroblasts, scattered lymphoplasmacytic infiltrates, few neutrophils and thin walled vessels with extravasated rbc's.

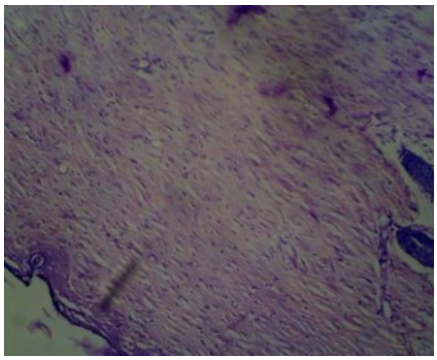
Figure 68: Group 4- EASG

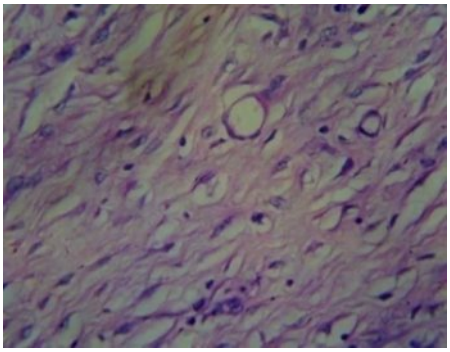
10X 40X

Section shows skin and the epidermis shows **RE EPITHELIALIZATION.** Dermis shows proliferation of fibroblasts and thin walled vessels.

Figure 69: Group 5- AQSG



10X



40X

Section shows skin and the epidermis shows **RE EPITHELIALIZATION.** Dermis shows proliferation of fibroblasts, thin walled vessels and few scattered inflammatory infiltrates.

7. DISCUSSION

Wound is a clinical entity and is as old as mankind, often considered as major problem in clinical practice. Each year, millions of people experience burns, suffer from chronic wounds, or have acute wounds that become complicated by infection, dehiscence or problematic scarring. Wounds are normally resolved in a few days, but chronic wounds represent a major burden because of economic and social factors. There by, the search for new agents is ongoing and natural products become a great target. Individual factors such as stress or diabetes can cause delays in the healing process or increase the risk of infection in the wound. Due to unawareness of the society this may lead to chronic diseases which may damage the other organs. Impaired wound healing can result into severe morbidity leading to long hospitalization of patients. States (US) population, which results in a significant economic burden estimated to be nearly 2–4% of the health budgets.[85]

Simarouba glauca or the paradise tree has a long history in herbal medicine in various countries. The leaves and bark of *Simarouba* have long been used as a natural medicine in tropics. The bark and leaf extract of Simarouba is well known for its different types of pharmacological properties such as haemostatic, antihelmenthic, antiparasitic, antidysentric, antipyretic and anticancerous. Externally it is used for wound and sores. The major active constituents are Quassinoids namely, Ailanthinone, Glaucarubinone, Holocanthone, Dehydroglaucarubinone, Benzoquinone, Canthin, Glaucarubine, Glaucarubolone, Simarubin, Melianone, Simaroubidin, Simarolide, Sitosterol, Tirucalla and Flavonoids.[41] Since Simarouba glauca have not been studied for its Wound healing activity, the present study was aimed to evaluate the wound healing potential of ethylacetate and aqueous extracts of SG in different wound models of rats.

Phytochemical screening of EASG and AQSG were performed and the results revealed the presence of carbohydrates, saponins, flavonoids, alkaloids, triterpenoids, glycosides, steroids, tannins, proteins and amino acids. The main attraction of the phytochemical was the presence of phenols and flavonoids which was concluded by the colorimetric estimation of these constituents in the extract.

Inorder to justify and quantify the presence of flavonoids, the extract was subjected to HPTLC screening against marker compounds such as, Gallic acid, Quercetin, Rutin and Apigenin.

Flavonoids are coming under the category of polyphenols, where its action is mainly attributed to anti-inflammatory action. And it provides a symptomatic relief in wound and sores. .From the results obtained from HPTLC study, it was found that EASG contains Quercetin & AQSG contains Gallic acid as flavonoids. The percentage of Quercetin present in 100mg of EASG was found to be 0.5% and 0.36% of Gallic acid was present in 100mg of AQSG.

Phytopharmaceuticals are gaining importance in modern medicine as well as in traditional system of medicine owing to their therapeutic effect due to the presence of phytochemicals such as polyphenols, flavonoids, terpenoids etc.

Flavonoids are widespread plant secondary metabolites that have shown free radical scavenging activity and protection against oxidative stress. Studies were revealed that flavonoids are also known to promote the wound healing process mainly due to their astringent and antimicrobial properties which appear to be responsible for the wound healing and increased rate of epithelialisation.[86] Polyphenols are the major plant compounds with antioxidant activity that is mainly due to their redox properties which enables them to act as reducing agents, hydrogen donors and singlet and triplet oxygen scavengers. Results obtained in the present study revealed that the levels of these phytoconstituents were considerable and the total phenol and flavonoid content was found to be EASG-198.42mg/g, AQSG-90.64mg/g and EASG extract-77.05mg/g , AQSG-26.75mg/g respectively.

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propogation of oxidizing chain reactions. They are radical scavengers which protect the human body against free radicals that may cause pathological conditions such as ageing, dementia, cancer etc.[87] Therefore in the present study, the potential of the EASG & AQSG to serve as antioxidants was assayed.

Free-radicals play an important role in the oxidative damage of biological systems. Several complementary methods have been adopted to trap free radicals through antioxidant activity, among which DPPH* assay is the most common. The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability. The free radical scavenging activity of the extract was estimated by comparing the %inhibition of EASG &AQSG with standard Quercetin. IC_{50} was also calculated to determine the amount of extract needed to quench 50% of radicals. Leaf extracts of *S. glauca* exhibited a dose dependent scavenging activity with IC50 values of 13.22 µg/ml, 17.77 µg/ml respectively for EASG and AQSG. Where the IC_{50} for standard Quercetin was found to be 2.99 µg/ml This low IC50 values implies the much stronger scavenging of free radicals than other simaroubaceae members like *Ailanthus altissima* and *Brucea amarissima*.[45]

The ABTS.+ scavenging assay, which employs a specific absorbance (734 nm) at a wavelength remote from the visible region and requires a short reaction time, can be used as an index that reflects the antioxidant activity of the test samples. The presence of specific chemical compounds in the extracts of *Simarouba glauca* may inhibit the potassium persulfate activity and hence reduced the production of ABTS•+.[88] *Simarouba glauca* extracts were found to be effective in scavenging radicals and the increase was concentration-dependent. IC_{50} value of Quercetin was 0.1149 µg/ml whereas 1.160 µg /ml and 1.429 µg/ml for EASG and AQSG respectively. This shows that *Simarouba glauca* extract presents a good ability to scavenge the ABTS radical. The antioxidant activities against ABTS or DPPH were correlated with the concentration, chemical structures, and polymerization degrees of organ antioxidants.

Thus from the result obtained it could be concluded that EASG & AQSG shows a good antioxidant activity which might be attributed to the presence of phytochemicals such as polyphenols and flavonoids.

The anti microbial study was carried out for ethyl acetate and aqoues extract of *Simarouba glauca* against different strain of bacteria (5 Gram + ve and 5 G – ve) and fungai (5 strains), that are known to cause infection in human and plants, by disc

diffusion method at 200µg/disc. The ciprofloxacin 10µg/disc and fluconazole 10µg/disc were used as standard for bacteria and fungi respectively.[89]

The standard ciprofloxacin and fluconazole was found to have significant antimicrobial activity against bacteria and fungi respectively. The various zones of inhibition were observed from all the extracts against various strains. Among the extracts the EASG of *Simarouba glauca* was observed to have significant antimicrobial activity than that of AQSG. The zone of inhibition was observed from both Gram +ve and Gram –ve bacteria and fungal strains .The maximum zone of inhibition (15mm) was found in EASG of *Simarouba glauca* against *B.lentus* and *Staphylococcus albus* for the Gram +ve organisms. And for Gram –ve organisms the maximum zone of inhibition (14mm) was found in EASG against *Klebsella* and *V.cholera.*

Both the extracts showed anti fungal activity. The anti fungal activity was observed maximum in EASG. Among all, the zone of inhibition was found to be maximum for *Candida albicans* (11mm).

Minimum Inhibitory Concentration (MIC) is defined as the highest dilution or least concentration of the extracts that inhibit growth of organisms. Determination of the MIC is important in diagnostic laboratories because it helps in confirming resistance of microorganism to an antimicrobial agent and it also monitors the activity of new antimicrobial agents.[90][91]

For bacterial MIC study, EASG showed least MIC value i.e. 62.55µg/ml against *S. albus* while MIC value of AQSG was 125µg/ml against *S. albus.* Whereas in case of *Coryne bacterium* the least MIC value for both the extracts EASG and AQSG was found to be 125µg/ml. In the MIC study of fungal strain EASG showed least MIC value i.e. 62.5µg/ml against *Candida albicans.* While in case of AQSG the observed MIC value was 250µg/ml.

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells

present and on the mitochondrial activity per cell.[64] The IC_{50} value after treatment with EASG and AQSG was found to be greater than 1000µg/ml, and785.00±5.0 respectively. Therefore both EASG and AQSG were found to be nontoxic to human dermal fibroblast cells since it did not affect the cellular activity of fibroblast cells even at high concentration.

Extracts of leaves of of *Simarouba glauca* demonstrated wound healing properties comparable with that of antibiotic standard. Animals in the untreated group showed some degree of healing. As earlier suggested, healing in this untreated group may be due to self-immunity. It is important to note that throughout the period of wound treatment, the extracts did not cause irritation or pain to the animals as the rats neither show any signs of restlessness nor scratching/biting of wound site when the extracts were applied.

In this investigation three models were used to assess the effect of the EASG and AQSG extracts as applied topically. The plant may have a beneficial influence on the various phases of wound healing like fibroplasias, collagen synthesis, and wound contraction, resulting in faster healing. The results of the present investigation showed that both plant extracts have definite wound healing action.

Extract treated excision wounds showed an increased rate of wound contraction, leading to faster healing as confirmed by the increased healed area when compared to the control untreated group. The EASG extract was recorded similar effectiveness when compared to the group treated with a commercial brand of povidone iodine ointment in but the magnitude was lesser than standard.

Burn wound model also showed marked increase in wound healing rate. Among the two extract treated groups the EASG group exhibited significant rate of wound contraction when compared to AQSG. The standard used here was the silver sulfadiazine.

Increased wound contraction rate was also observed in the extract treated infected excision wounds, when compared to control. Silver sulfadiazine was used as the standard. The rate of healing was more for the EASG treated group.[92]

Measurement of the hydroxyproline could be used as an index for collagen turnover. Collagen is a major protein of the extracellular matrix and is the major component that ultimately contributes to wound strength. Breakdown of collagen liberates free hydroxyproline and its peptides.[24] It was observed that *Simarouba glauca* increased the collagen content of the skin ultimately and contributed to wound strength. Previous studies stated that there is a strong correlation between the collagen fiber formation and acceleration of wound healing.

Tensile strength was measured to confirm the wound healing activity claimed for this plant. The increase in tensile strength of treated wounds may be due to increase in collagen concentration and stabilization of the fibers.[93] The extract showed significant tensile strength when compared to control animals. Our result conforms to the earlier studies that the increase in hydroxyl proline content of the granulation tissue in treated animal indicates the presence of higher collagen content and its turnover leads to rapid healing with concurrent increase in the tensile strength of the treated wounds. An increase in tensile strength of the treated wounds may be due to the increase in collagen concentration and stabilization of the fibers. The collagen molecules synthesized are laid down at the wound site and become cross linked to form fibers. Tensile strength is acquired from both remodeling of collagen and the formation of stable intra and intermolecular crosslink.

It was found that hexosamine content increased considerably when EASG and AQSG extracts applied topically on a wounded rat. Moreover, Hexosamine, which is a ground substratum for collagen synthesis, is known to increase during early stages of wound healing and decrease thereafter. A similar trend has been observed in the present study in hexosamine content of granular tissues. By correlating hexosamine content it can be judged how fast the wound heals. It is important to note that hexosamine content will increase during wound healing process and decreases when maturation and remodeling phase is attained.[93]

The glycosaminoglycans are known to stabilize the collagen fibres by enhancing electrostatic and ionic interactions with it and possibly control their ultimate alignment and characteristic size. Their ability to bind and alter protein-protein interactions has identified them as important determinants of cellular responsiveness in development, homeostasis, and disease. We observed an increase in uronic acid content in treated animals than in control.[71][93] An increase in uronic acid level in treated animals as compared to control animals represents an enhanced synthesis of glycosaminoglycans which is similar to earlier observations. Both extracts treated groups showed elevated levels of uronic acid in the three models, when compared to the controlled untreated group. In our study, hexuronic acid and hexosamine concentrations which are the components of glycosaminoglycans were significantly increased with EASG when compared with control indicating stabilization of collagen fibres. From all the parameters studied it was found that EASG showed significant wound healing activity when compared to AQSG.

An increase in the level of antioxidants was observed in granuloma tissues of wound. These antioxidants are known to quench the superoxide radical and thus prevent the damage of cells caused by free radicals. There is plenty of evidence to suggest that increased production of reactive oxygen species, lipid peroxidation and ineffective scavenging play a crucial role in various skin lesions and in modulation of fibroblast proliferation.[94] Cutaneous wounding causes a depression in the overall antioxidant status making it more vulnerable to oxygen radical attack. All these finding indicate that in wound healing antioxidants plays an important role. Experimental and clinical lines of evidence suggest that chronic wound undergoes substantial oxidative stress by neutrophils-derived oxidants which contribute markedly to tissue damage during chronic wound inflammation. Over production of reactive oxygen species (ROS) results in oxidative stress thereby causing cytotoxicity and delayed wound healing and elimination of ROS could be an important strategy in healing of chronic wounds.[95] Therefore, estimation of antioxidants like GSH, SOD, and CAT in granulation tissues is relevant because the antioxidants have been reported to hasten wound healing by decreasing the free radicals. Our studies on the in

vivo antioxidants revealed that EASG and AQSG increased the antioxidant activity by enhancing the levels of these antioxidant enzymes, thereby reducing free radicals stress, preventing inflammation and oxidative damage, ultimately promoting the healing process. During the treatment the anti oxidant levels (like SOD, CAT and GSH) of the treated groups was increased whereas, there was a significant reduction in the LPO levels. Among the two extract treated groups EASG revealed significant in vivo anti oxidant activity when compared to AQSG.

From histopathological studies, it was observed that the phases of wound healing occurred in a timely manner. The untreated (control) wound healed slowly when compared to the wounds treated with EASG and AQSG. Extracts treated groups shows large number of collagen tissue (fibrosis) and neovascularisation with minimal inflammatory cells. EASG treated showed near to normal features, collagen tissue (fibrosis), and neovascularisation when compared to control group.

8. CONCLUSION

Wound healing is a complex and continuous process that begins immediately after injury, followed by homeostasis, blood clotting, inflammation, proliferation and remodeling phases. All these phases can promote or prolong healing by influencing external or internal factors including infection sex hormones and nutrition. Delay in healing process increases the possibility of getting infected, improper recovery, and formation of unpleasant scar.

The study thus demonstrated the wound healing activity of ethyl acetate and aqueous extracts of the leaf of *Simarouba glauca* and found to be effective in the functional recovery of the wound. The extracts promote wound contraction; increases tensile strength, hydroxyproline, hexosamine and uronic acid of excision, infected excision and burn wounds as compared to control group. The result may be attributed to the phytoconstituents such as flavonoids and phenolics present in it which may be due to their individual or cumulative effect that enhanced wound healing and provided scientific evidence to the ethnomedicinal futures of *Simarouba glauca*. These findings could justify the inclusion of this plant in the management of wound healing.

9. BIBLIOGRAPHY

1. http://blog.mediligence.com/2009/12/13/incidence-and-prevalence-of-wounds-by etiology /
2. Sen CK, Gordillo GM, Roy S, Kirsner R, Lambert L, Hunt TK, Gottrup F, Gurtner GC, Longaker MT. Human skin wounds: a major and snowballing threat to public health and the economy. Wound Repair and Regeneration. 2009 Nov 1;17(6):763-71.
3. https://www.woundcare-today.co.uk/news/world-at-glance/projected-global-wound prevalence -by-wound-types
4. http://www.who.int/mediacentre/factsheets/fs365/en/
5. Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing. InJournal of Investigative Dermatology Symposium Proceedings 2000 Dec 1 (Vol. 5, No. 1, pp. 40-46). Nature Publishing Group.
6. Gupta N, Gupta SK, Shukla VK, Singh SP. An Indian community-based epidemiological study of wounds. Journal of wound care. 2004 Sep;13(8):323-5
7. Collier M. Recognition and management of wound infections. Worldwide wounds. 2004 Jan:1-0.
8. Church D, Elsayed S, Reid O, Winston B, Lindsay R. Burn wound infections. Clinical microbiology reviews. 2006 Apr 1;19(2):403-34.
9. Demirci S, Doğan A, Demirci Y, Şahin F. In vitro wound healing activity of methanol extract of Verbascum speciosum. International Journal of Applied Research in Natural Products. 2014 May 19;7(3):37-44.
10. Ramadevi D, Rao BG. In vivo wound healing activityon leaf extract of chloroxylon swietenia dc. group.;100:2-5.
11. Mercandetti M, Cohen AJ. Wound healing: healing and repair. Emedicine. com. Accessed January. 2005 Aug;20(2008):38.
12. medicine_en_ru.academic.ru/26135/epithelialization
13. http://www.healthyfunction.com/Inflammation _Health.html
14. https://en.wikipedia.org/wiki/Collagen
15. https://en.wikipedia.org/wiki/Angiogenesis

16. Edenharder R, Tang X, von Petersdorff I, Rauscher R. Structure-Activity Relations of Flavonoids as Antimutagens Against Cooked Food Mutagens and Nitroarenes in the Salmonella Reversion Assay. InFood Factors for Cancer Prevention 1997 (pp. 603-606). Springer Japan.
17. www.todaysdietitian.com/newarchives/090313p70.shtml
18. Awasthi S, Srivatava SK, Piper JT, Singhal SS, Chaubey M, Awasthi YC. Curcumin protects against 4-hydroxy-2-trans-nonenal-induced cataract formation in rat lenses. The American journal of clinical nutrition. 1996 Nov 1;64(5):761-6.
19. Florin L, Knebel J, Zigrino P, Vonderstrass B, Mauch C, Schorpp-Kistner M, Szabowski A, Angel P. Delayed wound healing and epidermal hyperproliferation in mice lacking JunB in the skin. Journal of investigative dermatology. 2006 Apr 1;126(4):902-11.
20. Davidson JM. Experimental animal wound models. Wounds-a compendium of clinical research and practice. 2001 Jan 1;13(1):9-23.
21. Perez R, Davis SC. Relevance of animal models for wound healing. Wounds: a compendium of clinical research and practice. 2008 Jan;20(1):3-8.
22. Fang RC, Mustoe TA. Animal models of wound healing: uility in transgenic mice. Journal of Biomaterials Science, Polymer Edition. 2008 Jan 1;19(8):989-1005.
23. Ansell DM, Holden KA, Hardman MJ. Animal models of wound repair: Are they cutting it?. Experimental dermatology. 2012 Aug 1;21(8):581-5.
24. Das K. Wound healing potential of aqueous crude extract of Stevia rebaudiana in mice. Revista Brasileira de Farmacognosia. 2013 Apr;23(2):351-7.
25. Topic: WOUNDS. Authors: prof.Mikhailychenko V.Yu, Starykh A.A. Department of General Surgery Medical Academy named after S.I. Georgievskiy, The Federal State Autonomous Educational Establishment of Higher Education “Crimean Federal University named after V.I. Vernadsky” Ministry of Education and Science of the Russian Federation 2016.
26. http://www.woundcarecenters.org/article/wound-basics/different-types-of-wounds
27. http://www.woundcarecenters.org/article/wound-types/acute-wounds
28. http://www.woundcarecenters.org/article/wound-types/chronic-wounds

29. Open WoundWritten by Julie Roddick and Valencia Higuera Medically Reviewed by Steven Kim, MD Healthline reference library on September 16, 2015
30. http://www.woundcarecenters.org/article/wound-basics/open-wound-basics
31. http://www.woundcarecenters.org/article/wound-basics/closed-wound-basics
32. Harsh Mohan,textbook of pathology,6th edition ,jaypee brothers medical publishers(P) LTD.2010:165-171
33. Robbins.Basic pathology.8th edition, published by Elsevier 2007:74-79
34. Robbins and Cotran. Pathologic basis of disease.7th edition. Published by Elsevier 87-118
35. Kathryn, L.Mc Cance sue.E Haether. Pathophysiology-The biologic Basis for disease in adults and children. 5th edition. Published by Elsvier Mosby 201-07, 1642-1676.
36. BN Datta.Textbook of pathology.second edition.Published by Jaypee brothers medical publishers(P limited Ltd) :207-214.
37. Russel J Greene and Norman D Harris. A basis for clinical pharmacy practice.published by pharmaceutical Press .2008; 55-56.
38. carol mattson porth, RN,MSN,PhD(physiology).Essentials of pathophysiology;concepts of altered Health states.Second edition,Published by Lippincott Williams and Wilkins.2007:280-284
39. Collier M. Recognition and management of wound infections. Worldwide wounds. 2004 Jan:1-0.
40. Tiwari VK. Burn wound: How it differs from other wounds?. Indian journal of plastic surgery. 2012 May 1;45(2):364.
41. Patil Manasi S, Gaikwad DK. A Critical Review on Medicinally Important Oil Yielding Plant Laxmitaru (Simarouba glauca DC.). Department of Botany, Shivaji University, Kolhapur416004 (MS) India. 2011;3(4):1195-213.
42. Mishra SR, Mohanty MK, Das SP, Pattanaik AK. Production of Bio-diesel (Methyl Ester) from Simarouba glauca oil. Research Journal of Chemical Sciences. 2012 May;2231:606X.

43. Alves IA, Miranda HM, Soares LA, Randau KP. Simaroubaceae family: botany, chemical composition and biological activities. Revista Brasileira de Farmacognosia. 2014 Aug;24(4):481-501.

44. Lakshmi KS, Sangeetha D, Sivamani S, Tamilarasan M, Rajesh TP, Anandraj B. In vitro antibacterial, antioxidant, haemolytic, thrombolytic activities and phytochemical analysis of Simarouba glauca leaves extracts. International Journal of Pharmaceutical Sciences and Research. 2014 Feb 1;5(2):432.

45. Umesh TG. in vitro antioxidant potential, free radical scavenging and cytotoxic activity of simarouba gluaca leaves. International Journal of Pharmacy and Pharmaceutical Sciences. 2014 Dec 29;7(2):411-6.

46. Maranhão HM, Vasconcelos CF, Rolim LA, Neto PJ, Neto JD, Fernandes MP, Costa-Silva JH, Araújo AV, Wanderley AG. Hepatoprotective effect of the aqueous extract of Simarouba amara Aublet (Simaroubaceae) stem bark against carbon tetrachloride (CCl4)-induced hepatic damage in rats. Molecules. 2014 Oct 31;19(11):17735-46.

47. Dc Mail.V V,Bhoite. A S, D K .Gaikwad Effect of Fluoride Stress on Phenolics of Medicinally Important Plant *Simarouba glauca*. Indian journal of applied researchVolume : 4 Issue : 11 November 2014

48. Rajamane NN, Gaikwad DK. Effect of sodium Chloride stress on polyphenol, flavonoid, anthocyanins contents and Lipid peroxidation of leaflets of Simarouba glauca.

49. Simarouba Monograph 3/1/04 *Simarouba amara, glauca*

50. http://tropical.theferns.info/viewtropical.php?id=Simarouba+amara

51. http://www.rain-tree.com/simaruba.htm#.V7QCj1t97IU

52. Kirtikar K.R, Basu, B.D. Indian medicinal plants. 2006; II: 936-938

53. Khandelwal K.R. Text book of practical pharmacognosy. 11th ed. Pune: Nirali prakashan. 2004; 149-155.

54. Trease G, Evans W. Textbook of Pharmacognosy, XIII Ed., BaillIere Tindal, London. 1989; 799 – 803.

55. Kokate C.K. Practical Pharmacognosy, IV edition. Nirali Prakashan. 1994; 108-109. (1994).

56. Tambe R, Kulkarni M, Bhise K. Preliminary phytochemical screening and HPTLC fingerprinting of bark extracts of Symplocos racemosa. Journal of Pharmacognosy and Phytochemistry. 2013 Sep 1;2(3).

57. Raghavendra H Lakshmanashetty, Vijayananda B Nagaraj, Madumathi G, Hiremuth and Vadalapudi Kumar. Invitro antioxidant activity of Vitex negundo L. Leaf extract. Chiang. Mai. J. Sci, 2010; 37(3): 489-497.

58. Shekhar TC, Anju G. Antioxidant activity by DPPH radical scavenging method of Ageratum conyzoides Linn. leaves. American Journal of Ethnomedicine. 2014 Aug 31;1(4):244-9.

59. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of agricultural and food chemistry. 2005 May 18;53(10):4290-302.

60. Paulsamy S, Karthika K. Screening of in vitro antioxidant activity of methanolic leaf and root extracts of Hypochaeris radicata L.(Asteraceae). Journal of Applied Pharmaceutical Science. 2012 Jul 1;2(7):149.

61. Ullah N, Parveen A, Bano R, Zulfiqar I, Maryam M, Jabeen S, Liaqat A, Ahmad S. In vitro and in vivo protocols of antimicrobial bioassay of medicinal herbal extracts: A review. Asian Pacific Journal of Tropical Disease. 2016 Aug 31;6(8):660-7.

62. Sen A, Batra A. Evaluation of antimicrobial activity of different solvent extracts of medicinal plant: Melia azedarach L. Int J Curr Pharm Res. 2012 Mar;4(2):67-73.

63. Muhammad AA, Pauzi NA, Arulselvan P, Abas F, Fakurazi S. In vitro wound healing potential and identification of bioactive compounds from Moringa oleifera Lam. BioMed research international. 2013 Dec 31;2013.

64. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nature protocols. 2007 Feb 1;2(2):329-33.

65. Dr. Shankara Sharma, N.Sriram. Anti-ulcer activity of Simarouba glauca against ethanol and indomethacin induced ulcer in rats. Int.J.of Res.in pharmacology and Pharmacotherapeutics Vol-3(2);2014:85-89

66. Azubuike chukwuemeka evaluation anti microbial properties of herbal ointments formulated with ethanolic extract of acalypha wilkesiana. Journal of biological and scientific opinion; vol1(2):2013

67. Das K. Wound healing potential of aqueous crude extract of Stevia rebaudiana in mice. Revista Brasileira de Farmacognosia. 2013 Apr;23(2):351-7

68. Somboonwong J, Kankaisre M, Tantisira B, Tantisira MH. Wound healing activities of different extracts of Centella asiatica in incision and burn wound models: an experimental animal study. BMC complementary and alternative medicine. 2012 Jul 20;12(1):1.

69. George BP, Parimelazhagan T, Kumar YT, Sajeesh T. Antitumor and Wound Healing Properties of Rubus ellipticus Smith. Journal of acupuncture and meridian studies. 2015 Jun 30;8(3):134-41.

70. Rashed AN, Afifi FU, Disi AM. Simple evaluation of the wound healing activity of a crude extract of Portulaca oleracea L.(growing in Jordan) in Mus musculus JVI-1. Journal of Ethnopharmacology. 2003 Oct 31;88(2):131-6.

71. Prashanthi R, Mohan N, Siva GV. Wound healing property of aqueous extract of seed and outer layer of Momordica charantia L. on albino rats. Indian Journal of Science and Technology. 2012 Jan 1;5(1):1936-40.

72. Edwards CA, O'Brien WD. Modified assay for determination of hydroxyproline in a tissue hydrolyzate. Clinica chimica acta. 1980 Jun 10;104(2):161-7.

73. Rondle CJ, Morgan WT. The determination of glucosamine and galactosamine. Biochemical Journal. 1955 Dec;61(4):586.

74. Lowry OH, Rosebrough NJ, Farr LA, Randall AJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; 193:265–75.

75. Anthon GE, Barrett DM. Combined enzymatic and colorimetric method for determining the uronic acid and methylester content of pectin: application to tomato products. Food chemistry. 2008 Sep 1;110(1):239-47.

76. Lodhi S, Pawar RS, Jain AP, Singhai AK. Wound healing potential of Tephrosia purpurea (Linn.) Pers. in rats. Journal of Ethnopharmacology. 2006 Nov 24;108(2):204-10.

77. Nayak BS, Pereira LM. Catharanthus roseus flower extract has wound-healing activity in Sprague Dawley rats. BMC Complementary and Alternative Medicine. 2006 Dec 21;6(1):1.

78. Nazima Ameen and Sabeeha Shafi. Biochemical and in-vivo antioxidant parameters for evaluation of memory enhancing activity. International Journal of Pharmaceutical, Chemical and Biological sciences. 2016, 6(3), 265-270

79. Alam MN, Bristi NJ, Rafiquzzaman M. Review on in vivo and in vitro methods evaluation of antioxidant activity. Saudi Pharmaceutical Journal. 2013 Apr 30;21(2):143-52.

80. Sinha A.K. Colorimetaaric assay of catalase. *Anal. Biochem*.1972; 47: 389-95.

81. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. European journal of biochemistry. 1974 Sep 1;47(3):469-74.

82. Al-Temimi A, Choudhary R. Determination of antioxidant activity in different kinds of plants in vivo and in vitro by using diverse technical methods. Journal of Nutrition & Food Sciences. 2013 Feb 14;2013.

83. Sedlak J, Lindsay RHC. Estimation of total, protein bound and nonprotein sulfhydryl groups in tissue with Ellmann's reagent. *Anal Biochem* 1968;25:192–205.

84. Okawa HN, Ohishi K, Yagi M. Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. *Anal Biochem*. 1979; 95(2): 351-58.

85. Sen CK, Gordillo GM, Roy S, Kirsner R, Lambert L, Hunt TK, Gottrup F, Gurtner GC, Longaker MT. Human skin wounds: a major and snowballing threat to public health and the economy. Wound Repair and Regeneration. 2009 Nov 1;17(6):763-71.

86. Nayak S, Nalabothu P, Sandiford S, Bhogadi V, Adogwa A. Evaluation of wound healing activity of Allamanda cathartica. L. and Laurus nobilis. L.

extracts on rats. BMC Complementary and Alternative Medicine. 2006 Apr 5;6(1):1.

87. Rasik AM, Shukla A. Antioxidant status in delayed healing type of wounds. International journal of experimental pathology. 2000 Aug 1;81(4):257-63.
88. Nishaa S, Vishnupriya M, Sasikumar JM, Hephzibah PC, Gopalakrishnan VK. Antioxidant activity of ethanolic extract of Maranta arundinacea L. tuberous rhizomes. Asian Journal of Pharmaceutical and Clinical Research. 2012;5(4):85-8.
89. Mikawlrawng K, Kaushik S, Pushker AK, Kumar S, Kameshwor Singh M, Sharma GS. Comparative in vitro antifungal activities of Simarouba glauca against Fusarium oxysporum and Aspergillus parasiticus. J Med Plant Stud. 2014;2(3):1-7.
90. Savitasagwan, D. V. Rao and R. A. Sharma *In vivo* and *in vitro* comparative study to determine the minimum inhibitory concentration (MIC) of *Maytenus emarginata* (Willd.) Ding Hou. J. Microbiol. Biotech. Res., 2012, 2 (6):845-851
91. Sen A, Batra A. Evaluation of antimicrobial activity of different solvent extracts of medicinal plant: Melia azedarach L. Int J Curr Pharm Res. 2012 Mar;4(2):67-73.
92. Chah KF, Eze CA, Emuelosi CE, Esimone CO. Antibacterial and wound healing properties of methanolic extracts of some Nigerian medicinal plants. Journal of Ethnopharmacology. 2006 Mar 8;104(1):164-7.
93. Rashed AN, Afifi FU, Disi AM. Simple evaluation of the wound healing activity of a crude extract of Portulaca oleracea L.(growing in Jordan) in Mus musculus JVI-1. Journal of Ethnopharmacology. 2003 Oct 31;88(2):131-6.
94. Lodhi S, Jain AP, Sharma VK, Singhai AK. Wound-healing effect of flavonoid-rich fraction from Tephrosia purpurea Linn. on streptozotocin-induced diabetic rats. Journal of herbs, spices & medicinal plants. 2013 Apr 3;19(2):191-205.
95. Shukla A, Rasik AM, Dhawan BN. Asiaticoside-induced elevation of antioxidant levels in healing wounds. Phytotherapy Research. 1999 Feb 1;13(1):50-4.

96. Abd-Elmaleck BS, Abed GH, Mandour AM. Histopathological and Hematological Changes in Rat Tissue after Injected with Babesia and Theileria parasites. Int. J. Curr. Microbiol. App. Sci. 2015;4(7):260-75.

97. Kesiova M, Alexandrova A, Yordanova N, Kirkova M, Todorov S. Effects of diphenhydramine and famotidine on lipid peroxidation and activities of antioxidant enzymes in different rat tissues. Pharmacological reports. 2006 Mar 1;58 (2):221.