EFFECTS OF THE METHANOLIC LEAF EXTRACT OF *PALISOTA HIRSUTA* IN POST-SURGICAL WOUND MANAGEMENT

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JULY, 2013
EFFECTS OF THE METHANOLIC LEAF EXTRACT OF *PALISOTA HIRSUTA* 
IN POST-SURGICAL WOUND MANAGEMENT

A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE 
STUDIES OF THE UNIVERSITY OF NIGERIA IN PARTIAL FULFILLMENT 
FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE 
(VETERINARY SURGERY)

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DECLARATION

The studies presented in this dissertation are original and were carried out by me under the supervision of Dr. C. A. Eze and Prof. A. O. Anaga. References made to the works of other investigators are duly acknowledged. No part of this dissertation has been submitted previously or elsewhere for a degree or diploma.

___________________________ 26th July, 2013

UNAMBA-OPARAH, CHIOMA
Certification page
DEDICATION

TO

Ihemdirim and Chijioke.
ACKNOWLEDGEMENTS

My first gratitude goes to the Almighty God, who saw me through the ups and downs of this study.

I wish to thank my supervisors; Dr. C. A. Eze and Prof. A.O. Anaga for their understanding, guidance and support in making this work a reality.

This acknowledgement will not be complete if I fail to express my gratitude to Prof. S.V.O. Shoyinka and Prof. K.F. Chah, both of whom made invaluable inputs into the work.

I also thank the laboratory technicians of the Step-B Project lab, Department of Physiology and Pharmacology for their assistance in providing me with all the equipments needed to run some of the lab work. My thanks goes to Mrs Martina, of the Veterinary Biochemistry lab, and Mr Ngene, of the Veterinary Medicine lab.

And most importantly, I wish to appreciate my husband, Dr. Unamba-Oparah for his undivided attention, understanding and immense support all through this work; you are one of a kind.
TABLE OF CONTENT

Title page - - - - - - - - - - - - - i

Declaration - - - - - - - - - - - iii

Certification - - - - - - - - - - - iv

Dedication - - - - - - - - - - - v

Acknowledgements - - - - - - - - - - - vi

Table of content - - - - - - - - - - - vii

Abstract - - - - - - - - - - - xv

CHAPTER ONE

1.0 Introduction - - - - - - - - - - - 1

1.1 The Objectives of the Study - - - - - - - - - 2

1.2 Significance of the Study - - - - - - - - - 3

CHAPTER TWO

2.0 LITERATURE REVIEW - - - - - - - - - 4

2.1 Definition - - - - - - - - - - - 4

2.2 Causes of wound - - - - - - - - - 4
<table>
<thead>
<tr>
<th>Section</th>
<th>Topic</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3</td>
<td>Classification of wounds</td>
<td>4</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Classification of wounds based on aetiology</td>
<td>4</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Classification of wounds based on bacterial contamination</td>
<td>6</td>
</tr>
<tr>
<td>2.3.3</td>
<td>Open or closed wounds</td>
<td>6</td>
</tr>
<tr>
<td>2.3.4</td>
<td>Acute or chronic wounds</td>
<td>7</td>
</tr>
<tr>
<td>2.4</td>
<td>Wound healing</td>
<td>7</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Inflammatory phase</td>
<td>7</td>
</tr>
<tr>
<td>2.4.2</td>
<td>Debridement phase</td>
<td>8</td>
</tr>
<tr>
<td>2.4.3</td>
<td>Repair phase</td>
<td>9</td>
</tr>
<tr>
<td>2.4.4</td>
<td>Maturation phase</td>
<td>11</td>
</tr>
<tr>
<td>2.5</td>
<td>Types of wound healing</td>
<td>12</td>
</tr>
<tr>
<td>2.5.1</td>
<td>Factors affecting wound healing</td>
<td>13</td>
</tr>
<tr>
<td>2.6</td>
<td>Wound assessment and management</td>
<td>19</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Clinical actions to be taken in wound management</td>
<td>19</td>
</tr>
<tr>
<td>2.6.2</td>
<td>Agents used in wound management</td>
<td>20</td>
</tr>
<tr>
<td>2.6.2.1</td>
<td>Antibiotics</td>
<td>20</td>
</tr>
<tr>
<td>2.6.2.2</td>
<td>Enzymatic debriding agents</td>
<td>20</td>
</tr>
<tr>
<td>2.6.2.3</td>
<td>Wound healing promoters</td>
<td>21</td>
</tr>
<tr>
<td>2.6.2.4</td>
<td>Pulsed electromagnetic field treatment of open wound</td>
<td>21</td>
</tr>
<tr>
<td>2.6.2.5</td>
<td>Ultrasonography and Phototherapy</td>
<td>21</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Pages</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>2.7</td>
<td>Wound models and parameters measured</td>
<td>21</td>
</tr>
<tr>
<td>2.7.1</td>
<td>Excision wound model</td>
<td>22</td>
</tr>
<tr>
<td>2.7.2</td>
<td>Incision wound model</td>
<td>22</td>
</tr>
<tr>
<td>2.7.3</td>
<td>Dead space wound model</td>
<td>23</td>
</tr>
<tr>
<td>2.7.4</td>
<td>Burn wound model</td>
<td>23</td>
</tr>
<tr>
<td>2.8</td>
<td>Wound healing indices</td>
<td>23</td>
</tr>
<tr>
<td>2.8.1</td>
<td>Wound contraction</td>
<td>23</td>
</tr>
<tr>
<td>2.8.2</td>
<td>Epithelialization period</td>
<td>24</td>
</tr>
<tr>
<td>2.8.3</td>
<td>Wound breaking/Tensile strength</td>
<td>24</td>
</tr>
<tr>
<td>2.8.4</td>
<td>Granulation tissue formation</td>
<td>24</td>
</tr>
<tr>
<td>2.8.5</td>
<td>Hydroxyproline content</td>
<td>25</td>
</tr>
<tr>
<td>2.9</td>
<td>The role of plant extracts in wound management</td>
<td>25</td>
</tr>
<tr>
<td>2.10</td>
<td>Antibacterial activity of plants in wound healing</td>
<td>25</td>
</tr>
<tr>
<td>2.11</td>
<td>Commonly used wound healing plants</td>
<td>27</td>
</tr>
<tr>
<td>2.12</td>
<td>Active principles in plants involved in wound healing</td>
<td>28</td>
</tr>
<tr>
<td>2.12.1</td>
<td>Mechanism of action of these active principles</td>
<td>28</td>
</tr>
<tr>
<td>2.13</td>
<td>Herbalism and toxicity</td>
<td>29</td>
</tr>
<tr>
<td>2.13.1</td>
<td>Toxicity studies</td>
<td>29</td>
</tr>
<tr>
<td>2.13.2</td>
<td>Importance of liver and kidney enzymes in toxicity studies</td>
<td>30</td>
</tr>
<tr>
<td>2.13.3</td>
<td>The hemogram in toxicity studies</td>
<td>31</td>
</tr>
<tr>
<td>2.14</td>
<td><em>Palisota hirsuta</em></td>
<td>32</td>
</tr>
</tbody>
</table>
2.14.1 Classification - - - - - - -- - 32
2.14.2 Plant description - - - - - - - - - 32
2.14.3 Traditional uses - - - - - - - - - 32
2.14.4 Medicinal uses - - - - - - - - - 33
2.14.5 Relevant research findings of *Palisota hirsuta* - - - - 34
  2.14.5.1 Phytochemical constituents and lethal dose - - - - - 34
  2.14.5.2 Liver protective activity - - - - - - - 34
  2.14.5.3 Antinociceptive activity - - - - - - - 34
  2.14.5.4 Local anaesthetic activities - - - - - - - 34
  2.14.5.5 Antibacterial activity - - - - - - - 35
  2.14.5.6 Anti-arthritic activity - - - - - - - 35
  2.14.5.7 Anti-inflammatory and antipyretic activities - - - - 35
  2.14.5.8 Antiviral activity - - - - - - - 35

**CHAPTER THREE**

3.0 **MATERIALS AND METHODS** - - - - - - - 36
3.1 Materials - - - - - - - - - 36
3.1.1 Plant Materials - - - - - - - - - 36
3.1.2 Experimental animals - - - - - - - - - 36
3.1.3 Drugs - - - - - - - - 36
3.1.4 Equipments - - - - - - - - 37
3.1.5 Diagnostic test kits - - - - - - - - 37
3.1.6 Chemicals and Reagents - - - - - - - - 37
3.1.7 Surgical materials - - - - - - - - 38
3.2 Methods - - - - - - - - 38
3.2.1 Plant Preparation - - - - - - - - 38
3.2.2 Evaluation of the wound healing activity of PHLE - - - 39
3.2.2.1 Protocol 1: Excision wound model - - - - - - 39
3.2.2.2 Protocol 2: Incision wound model - - - - - - 41
3.2.2.3 Protocol 3: Dead space wound model - - - - - - 42
3.2.3 Protocol 4: Evaluation of the antibacterial activity of PHLE - - 45
3.2.4 Protocol 5: Sub-acute toxicity test - - - - - - 47
3.2.4.1 Determination of relative organ weight (ROW) - - - - 48
3.2.4.2 Hematology - - - - - - - - 48
3.2.4.2.1 Packed cell volume (PCV) - - - - - - - - 48
3.2.4.2.2 Hemoglobin concentration (Hb) - - - - - - - - 48
3.2.4.2.3 Total red blood cell (RBC) - - - - - 49
3.2.4.2.4 Total white blood cell (WBC) - - - - - 49
3.2.4.2.5 Differential WBC counts - - - - - 49
3.2.4.3 Biochemical Assay- - - - - 50
3.2.4.3.1 Alanine aminotransaminase (ALT) - - - - 50
3.2.4.3.2 Aspartate aminotransferase (AST) - - - - 50
3.2.4.3.3 Alkaline phosphatase (ALP) - - - - - 51
3.2.4.3.4 Total protein (TP) assay - - - - - 51
3.2.4.3.5 Total bilirubin assay - - - - - - 52
3.2.4.3.6 Creatinine assay - - - - - - 52
3.2.5 Data presentation and analysis - - - - - 54

CHAPTER FOUR

4.0 RESULTS - - - - - - - 55
4.1 Plant extraction - - - - - - - 55
4.2 Effect of Palisota hirsuta leaf extract (PHLE) on percentage wound contraction - - - - - - - 55
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3</td>
<td>Effect of PHLE on epithelialization period</td>
<td>56</td>
</tr>
<tr>
<td>4.4</td>
<td>Effect of PHLE on wound scoring (Excision wound model)</td>
<td>56</td>
</tr>
<tr>
<td>4.5</td>
<td>Effect of PHLE on wound breaking strength (Incision wound model)</td>
<td>56</td>
</tr>
<tr>
<td>4.6</td>
<td>Histopathology of the effect of PHLE on incision healing wound</td>
<td>57</td>
</tr>
<tr>
<td>4.7</td>
<td>Effect of PHLE on level of granulation tissue (Dead space wound model)</td>
<td>57</td>
</tr>
<tr>
<td>4.8</td>
<td>Effect of PHLE on hydroxyproline level (Dead space wound model)</td>
<td>57</td>
</tr>
<tr>
<td>4.9</td>
<td>Antibacterial activity of PHLE</td>
<td>57</td>
</tr>
<tr>
<td>4.10</td>
<td>Effect of PHLE on ROW of the heart</td>
<td>58</td>
</tr>
<tr>
<td>4.11</td>
<td>Effect of PHLE on ROW of the spleen</td>
<td>58</td>
</tr>
<tr>
<td>4.12</td>
<td>Effect of PHLE on ROW of the liver</td>
<td>58</td>
</tr>
<tr>
<td>4.13</td>
<td>Effect of PHLE on ROW of the kidney</td>
<td>58</td>
</tr>
<tr>
<td>4.14</td>
<td>Effect of PHLE on the packed cell volume</td>
<td>58</td>
</tr>
<tr>
<td>4.15</td>
<td>Effect of PHLE on the hemoglobin concentration</td>
<td>59</td>
</tr>
<tr>
<td>4.16</td>
<td>Effect of PHLE on the total red blood cell count</td>
<td>59</td>
</tr>
<tr>
<td>4.17</td>
<td>Effect of PHLE on the total white blood cell count</td>
<td>59</td>
</tr>
<tr>
<td>4.19</td>
<td>Effect of PHLE on neutrophil count</td>
<td>59</td>
</tr>
</tbody>
</table>
4.20 Effect of PHLE on lymphocyte count - - - 60
4.21 Effect of PHLE on serum AST level - - - 60
4.22 Effect of PHLE on serum ALT level - - - 60
4.23 Effect of PHLE on serum ALP level - - - 60
4.24 Effect of PHLE on serum total protein level - - - 61
4.25 Effect of PHLE on serum bilirubin level - - - 61
4.26 Effect of PHLE on serum creatinine level - - - 61

CHAPTER FIVE

5.0 DISCUSSIONS - - - - - - - 94
5.1 Conclusions - - - - - - - 97
5.2 Recommendations - - - - - - - 98
References - - - - - - - 99
Appendices - - - - - - - 110
Abstract

This study evaluated the use of the methanolic leaf extract of *Palisota hirsuta* in postsurgical wound management using different wound models in rats. It also assessed the antibacterial activity of the plant extract as well as the possible toxic effects on the liver, kidney and blood.

The yield of the methanolic leaf extract of *Palisota hirsuta* (PHLE) was 5.98 % w/w and it was dark green, pasty in consistency with a pungent smell. In the excision wound model, PHLE at 1% and 4 % significantly (p<0.05) increased the percentage wound contraction from days 6 - 21 post-surgery when compared with Petroleum Jelly and Cicatrin®. The PHLE –treated rats in group V (4 % PHLE) showed best activity in wound contraction when compared with rats in groups III (1 % PHLE) and IV (2 % PHLE) throughout the duration of the experiment. The epithelialization period of the PHLE-treated rats in group V (4 %) was significantly (p<0.05) lower than those of the control groups I and II. In the incision wound model, the wound breaking strength was significantly (p<0.05) higher in the PHLE and Cicatrin® treated groups compared to PJ-treated group. The rats that received 4 % PHLE also showed the highest wound breaking strength. In the deadspace wound model, the level of hydroxyproline production was significantly (p<0.05) increased by PHLE at 25, 50 and 100 mg/kg. There was also a significant (p<0.05) increase in the dry weight of the granulation tissue by PHLE (50 and 100 mg/kg) compared to the control. In the agar diffusion assay, PHLE induced no inhibition zones against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. In the sub-acute toxicity study, there were no significant (p>0.05) variations in the ROW of the kidney, liver and spleen of the rat groups on day 14. The PHLE at 40 mg/kg led to significantly (p<0.05) higher ROW of the heart on day 14 when compared with control but, by day 28, no significant (p>0.05) alteration was observed. The PHLE at 20 mg/kg significantly (p<0.05) reduced the size of the
spleen on day 28. Also on day 28, PHLE at 20, 40, and 160 mg/kg significantly (p<0.05) reduced the ROW of the liver. The PHLE significantly (p<0.05) increased the RBC count at 20, 40, and 60 mg/kg and Hb concentration (20 and 40 mg/kg). There was no significant (p>0.05) alteration of the PCV, total white blood cell (WBC) and differential WBC counts by PHLE. The serum biochemistry assay revealed no alteration in the serum AST, ALT, ALP, total protein by day 14 excepting total bilirubin. But by day 28, bilirubin level was not significantly (p>0.05) different from the control. Serum ALP, AST level was significantly (p<0.05) reduced while ALT level was significantly increased by PHLE though within the normal range. Creatinine level was not affected by PHLE. In conclusion, the methanolic leaf extract of *P. hirsuta* was well tolerated by the animals as no sign of toxicity was observed throughout the period of study. The extract showed wound healing activity by improving wound contraction, epithelialization period, hydroxyproline content, wound breaking strength and granulation tissue formation.
CHAPTER ONE

1.0 INTRODUCTION

The restoration of function is of fundamental importance in surgery. This is based on cells and tissues regaining biologic integrity through healing from wounds created naturally or iatrogenically (Harari, 2004). Wounds represent a significant burden on the patients and health care professionals worldwide in that not only do they affect the physical and mental health of millions of people; they also impose significant cost on them (Badri and Renu, 2011). Presently, estimates show that nearly 6 million people worldwide suffer from chronic wounds (Kumar et al., 2007), which have failed to progress through the normal stages of healing thereby entering a state of pathologic inflammation (Menke et al., 2007). These wounds may even lead to multiple organ failure or death of the patient (Roberts et al., 1998).

There are various surgical procedures that create wounds and these are; investigative or corrective surgery, minor or major surgery, open (traditional) or minimal access surgery, elective or emergency surgery, and incisions (simple cuts) or excision (removal of tissue). Under ideal conditions, a surgical wound, whether an incision or excision, will follow the normal wound healing pathway associated with acute wounds. Unfortunately, not all conditions are ideal for wounds as complications may set in and impede the healing process (Smith and Nephew, 2012). Thus the use of orthodox medications to assist wound healing.

The use of conventional medicine in wound healing has its challenges and these are cost (affordability), availability, and microbial resistance to mention but a few. This has necessitated the search for cheaper, more effective, quicker and longer acting alternatives to conventional
medications (Dunlap, 2010). The widespread availability of plant formulations with wound healing potentials, has led to the development and use of herbal remedies in wound healing, and thus its application in post surgery wound dressing.

Some plant extracts and their formulations had been reported to promote certain stages of the wound healing process. These are: *Centella asiatica* (Shukla et al., 1999), *Mimosa pudica* (Dnyaneshwar, et al., 2009), *Cassia fistula* (Muthusamy et al., 2005), *Aloe vera* (Krishnan, 2006; Choi et al., 2001), *Anthocephalus cadamba* (Prahalad et al., 2007) and others.

Research on plants as wound healing agents is one of the developing areas in modern biomedical sciences. Traditional forms of medicine, practiced for centuries are being scientifically investigated for their potentials in the treatment of wounds related disorders (Krishnan, 2006). Although *Palisota hirsuta* is used traditionally for wound healing (Burkill, 1985), there has been no scientific basis for its use hence this study.

1.1 The Objectives of the Study

The aims of this study are:

i. To evaluate the wound healing activity of the methanolic leaf extract of *P. hirsuta* using wound healing parameters such as percentage wound contraction, epithelialization period, wound breaking strength, granulation tissue formation and hydroxyproline content of the healing tissue.

ii. To investigate the antibacterial activity of the plant extract.

iii. To evaluate the toxicity of the extract on the liver, kidney and blood.
1.2 Significance of the Study

The results of this study will provide the scientific basis for:

1. The folkloric use of *Palisota hirsuta* as a traditional wound healing agent.
2. The use of the extract in post-operative wound management.
3. Its use in wound dressing.
4. The safety of the use of the extract as wound healing agent.
5. Its use as alternative source of wound healing agent since it is readily available.
CHAPTER TWO

LITERATURE REVIEW

2.1 Definition

Wound is a disruption of the anatomic continuity and metabolic functions of body structures which include organs, tissues, and cells (Harari, 2004). The cause of the wound is often the determinant of the extent of damage (Bright and Probst, 1985). A laceration for instance is an example of wound without tissue loss whereas; an avulsion injury can result in significant loss of tissue (Slatter, 2003).

2.2 Causes of wound

The causes of wound are surgery, trauma (vehicular, fire arms, fights), neoplasia, topical medications, infections, chemicals, excessive temperatures, and irradiations (Harari, 2004).

2.3 Classification of wounds

O’Dwyer (2007) classified wound based on the following parameters.

- Aetiology
- Nature and extent of the skin deficit
- Degree of bacterial contamination
- Extent of the trauma to the surrounding tissues.

2.3.1 Classification of wounds based on aetiology

Based on aetiology, wound is classified as follows (Johnston, 1990; Hedlund, 1997; Swaim and Henderson, 1997; O’Dwyer, 2007):
i. Abrasions: These are wounds that result from friction applied approximately parallel to the external surface of the skin. This friction usually results in the removal of variable amount of the epidermis, dermis and hypodermis.

ii. Degloving wounds: These result when the skin is torn from the underlying tissues, usually from a limb or occasionally the mandible.

iii. Avulsion injuries: These occur following forcible separation of tissues from their underlying attachments.

iv. Shearing: These represent a combination of degloving and abrasion injuries and are frequently seen in road traffic accidents.

v. Incisional wounds: These are wounds commonly seen as surgical wounds but can be caused by trauma.

vi. Puncture wounds: Puncture wounds are caused by sharp objects moving in a plane perpendicular to the skin surface. It can either be a penetrating wound, when there is an entrance only or perforating wound, when there is both an entrance and an exit. Animal bite wounds such as Snake bites are the most common cause of a puncture wound.

vii. Burns: Burn wounds are classified according to the method of infliction, the depth of the injury, or the surface area involved. They can also be further classified as partial-thickness or full-thickness injuries. First degree burns (superficial burns) involve only the epidermis. Second degree (partial-thickness) burns involves both the epidermis and varying amounts of the dermis. Third degree (full-thickness) burns results in the destruction of the entire skin thickness.
2.3.2 Classification of wounds based on bacterial contamination

Wound classification based on their level of bacterial contamination includes the following:

i. Clean wounds: Clean wounds are created under aseptic conditions and do not enter into the gastrointestinal, respiratory, genitourinary or oropharyngeal cavities. Therefore, they are limited to the wounds created during surgical procedures.

ii. Clean-contaminated wounds have minimal contamination. Surgical wounds involving the gastrointestinal, respiratory, genitourinary or oropharyngeal cavities are considered clean-contaminated wounds.

iii. Contaminated wounds are heavily contaminated and include wounds with foreign matter within it.

iv. Dirty wounds have active infections such as old traumatic wound with purulent exudates.

Clean, clean-contaminated, and contaminated wounds contain less than $10^5$ bacteria per gram of tissue (Pavleric, 1999). Infected wound contains more than $10^5$ per gram of tissue.

2.3.3 Open or closed wounds

Wound can also be classified as either open or closed wound (Jull, 2000).

a) Open Wounds: These are wounds in which the skin has been compromised and the underlying tissues exposed. Examples are lacerations and puncture wounds.

b) Closed Wounds: Here, the skin is not compromised but there is trauma to underlying tissues. Examples are cerebral contusions, hematomas etc (Jull, 2000).
2.3.4 **Acute or chronic wounds**

The above classifications can also be broadly classified as either acute or chronic wounds depending on the duration of the wound (Jull, 2000).

I. **Acute wounds** are wounds that do not last more than 6 weeks, and proceed normally through the wound healing process (Cohen *et al.*, 1999).

II. **Chronic wounds** last beyond 6 weeks as seen in pressure ulcers common in persons with diabetes as a result of skin breakdown.

The nature of wound determines the wound management approach. Basically, the aim of wound management is to optimize the conditions for wound healing.

### 2.4 Wound healing

Wound healing is the physiologic restoration of organ structure and function characterized by cellular and biochemical processes. These processes occur in an organized and sequential pattern lasting from days to months or even years (Harari, 2004). Fossum (2007), described wound healing as having four phases, and these are; inflammatory phase, debridement phase, repair phase and maturation phase.

#### 2.4.1 Inflammatory phase

The inflammatory stage is characterized by increase in vascular permeability, chemotaxis of the circulatory cells, release of cytokines and growth factors, and activation of macrophages, neutrophils, lymphocytes, and fibroblasts. The hemorrhage cleans and fills the wound immediately after injury. There is vasoconstriction for 5-10 minutes to limit hemorrhage, followed by vasodilation to allow leakage of fibrinogen and platelets into the wound.
Coagulation is activated by thromboplastin released from the injured cells. Fibrin and plasma transudates fill the wound and plug the lymphatics, localizing inflammation and holding the wound edges together. The blood clot formed provides stability for the wound edges. It also provides limited wound tensile strength. Eschar forms when the clot dries and this (eschar) protects the wound, prevent further hemorrhage and allow healing to progress beneath the surface. The inflammatory cells like mast cells, platelets, and macrophages secrete growth factors or cytokines and this initiate and maintain the proliferative phase of wound healing. Histamine, proteolytic enzymes, thromboxane, prostaglandins, kinins, complements, growth factors, and lysosomal enzymes are inflammatory mediators that initiate inflammation immediately after injury. Inflammation last for about 5 days. Debridement is initiated by white blood cells leakage from the blood vessels into the wound (Fossum, 2007).

2.4.2 Debridement phase

The debridement phase is characterized by the formation of an exudate made up of the white blood cells, dead tissues and wound fluid on the wound. Neutrophils and monocytes migrate into the wound, phagocytose and enzymatically destroy foreign and host cellular debris. Monocytes become macrophages in wound at 24 to 48 hours. Macrophages secrete chemotactic and growth factors. The chemotactic factors which includes complements, collagen fragments, bacteria endotoxins and inflammatory cell products direct macrophages to tissues. The growth factors made up of platelet-derived growth factor (PDGF), transforming growth factor-α (TGF-α), transforming growth factor-β (TGF-β), fibroblast growth factor (FGF) and interleukin-1 (IL-1) initiate, maintain and coordinate the formation of granulation tissues. Lymphocytes appear later after neutrophils and macrophages in the debridement phase and secrete soluble factors that may induce or inhibit migration and protein synthesis by other cells. The chemotactic substances
released by the macrophages stimulate fibroplasia, angiogenesis and collagen synthesis. When macrophage function is suppressed, wound healing is severely impaired, but neutropenia or lymphopenia does not affect wound healing (Harari, 2004; Fossum, 2007).

### 2.4.3 Repair phase

The repair phase occurs between 3 and 21 days and is characterized by fibroblastic proliferation, capillary infiltration and re-epithelialization. The fibroblasts produce an amorphous ground substance and collagen which strengthen the wound. The vascular invasion supplies oxygen and nutrition while the epithelial proliferation provides the surface coverage of the wound. Deoxyribonucleic acid (DNA) and fibroblast proliferation and collagen synthesis are also stimulated by macrophages (Fossum, 2007). The process of epithelial resurfacing is critical in order for the wound to be considered ‘healed’. The initial event in epithelialization is the migration of undamaged epidermal cells from the wound margins and from the epithelium of hair follicles and other adnexal structures, if the defect is superficial enough. This process occurs within hours of wounding and is a directed event that does not require an initial increase in cellular proliferation. After migration has begun, an increase in epithelial proliferation at the wound margins occurs to provide the additional cells needed for wound cover. Proliferation is maximal at 48 to 72 hours after wounding and is reflected by a 17 fold increase in mitosis and epithelial hyperplasia at the wound edges (Winter, 1972).

Keratinocytes assist in the process of re-epithelialization by producing fibronectin, collagenases, plasminogen activator, neutral proteases and type V collagen. Fibronectin is an important matrix component (Yamada and Clark, 1996) that promotes adhesion of keratinocytes and assist in their guidance across the wound base. Collagenase and other proteases are important in debridement
of devitalized tissue. Fibroblast proliferation and collagen synthesis are also stimulated by tissue oxygen content (about 20 mmHg) and slight acidity. Fibroblasts originate from undifferentiated mesenchymal cells in surrounding connective tissue and migrate to the wound. They are stimulated by Transforming growth factor-β (TGF-β) to produce fibronectin (Nickoloff et al., 1988). The roles of fibronectin in wound healing are as follows (Ruoslahti, 1999):

I. To cross link with fibrin to provide matrix for cell adhesion and migration.
II. It functions as an early component of the extracellular matrix.
III. It binds to collagen and interacts with matrix glycosaminoglycans.
IV. It has chemotactic properties for macrophages, fibroblasts and endothelial and epidermal cells.
V. To promote opsonization and phagocytosis.
VI. It forms a component of the fibronexus.
VII. It forms scaffolding for collagen deposition.

The migration of fibroblasts into the wound occurs just ahead of new capillary buds as inflammatory phase subsides (2-5 days) and their population peaks at 1-2 weeks post-wounding (Torre and Sholar, 2006). The fibroblasts invade wounds to synthesize and deposit collagen, elastin, and proteoglycans that mature into fibrous tissue. As collagen is deposited, wound fibrin disappears. Collagen is a marker for wound tensile strength. Also, as collagen content of wound increases, the number of fibroblasts and the rate of collagen synthesis decreases indicating the end of repair phase. If macrophages are absent, fibroblast migration and proliferation, collagen production and capillary in-growth are delayed.
2.4.4 Maturation phase

The maturation phase begins following adequate collagen deposition in wounds. This occurs 17-20 days after injury (Harari, 2004). Maturation phase consists primarily of a balance of collagen deposition and collagenolysis, remodeling of the collagen molecules, fibres and bundles, regression of the vascular elements, and the final appearance of the healed scar. The maturation phase continues for months after re-epithelialization has occurred. Fibroblast is the most important cell behind the synthesis of collagen. Rough endoplasmic reticulum in the fibroblast is the site of collagen synthesis. Hydroxylation of proline and lysin is important in collagen synthesis, and it occurs with the help of various co-factors like iron, copper, vitamin C etc (John et al., 2003; Slatter, 2003). Procollagen is the collagen molecule that is secreted by the fibroblast after hydroxylation. Collagen molecule is produced by the removal of the amino and carboxy terminal peptides. The primary collagen molecules crosslink with each other to form the fibrils and fibers that provides the tensile strength to the wound. As collagen is laid down, fibronectin gradually disappears. The nonsulfated glycosaminoglycans, hyaluronic acid are replaced by more resilient proteoglycans such as chondroitin-4-sulfate. Also, water is gradually reabsorbed from the scar allowing collagen fibers and other matrix components to lie closer together. This facilitates cross linking of collagen fibers mediated by lysyl oxidase that provides the increase in scar tensile strength (David et al., 2003; Hunt, 2003). Collagen bundles grow in bulk and become progressively reoriented from a random pattern to run parallel to the skin surface. Type I collagen becomes the major collagen present in the remodelled scar, reversing the earlier type III collagen predominance. Normal tissue strength is never regained in wounds, only about 80 % of the original strength may be regained (Levenson et al., 1965).
2.5 Types of wound healing

Hunt, (2003) classified wound healing into three types depending on the nature of the wound.

a. First (Primary) intention wound healing

b. Second intention wound healing

c. Tertiary intention wound healing

a. Primary intention wound healing (Closure by primary intent) occurs when there is no loss of tissue. Here, the wound is closed immediately following the injury by direct approximation of the wound edges, leading to rapid epithelial cover, faster healing and minimal scar tissue formation. The wound is closed within the initial 6 h of injury (that is, the “golden period”) (Fossum, 2007). Primary intention healing is the real goal of surgery i.e, to achieve healing without discharge or infection, separation of wound edges and minimal scar tissue formation.

b. Second intention wound healing is the method used for infected or contaminated wound. There is loss of tissue therefore; the wound is left open intentionally for granulation and epithelialization. The wound edges approximate in the natural way- by granulation and contraction (Gottrup, 1999). This type of healing is characterized by much scar tissue formation.

c. Tertiary intention also known as delayed primary closure. Too heavily contaminated wound are best treated using this method. This is a procedure where the wound is left to heal by granulation for 4-6 days after which the wound edges are closed (Way et al., 2003). Considerable tissue loss is encountered in this method of wound treatment.
2.5.1 Factors affecting wound healing

The significance of wound healing phases may vary in different wounds and is dependent on the wound factors, host factors and other factors. These can also be broadly classified into;

a. Extrinsic factors
b. Intrinsic factors.

a. The Extrinsic Factors are;

i. Ischaemia and hypoxia: Oxygen is essential for normal wound healing. It is needed for fibroblast proliferation. The area surrounding the wound is normally found to have low oxygen tension and there exist an oxygen gradient between the nearest functional capillary and the wound edge. This oxygen tension is between 60 and 90 mmHg. Closer to the advancing edge of the granulation tissue, the oxygen tension approaches zero and this is due to diffusion gradient and the use of oxygen by cells in the wound margin (Niinikoski, 1977). Hyperbaric oxygen has been used successfully in the enhancement of wound healing in both humans and animals. Affected patients are exposed to 100% oxygen at a pressure above atmospheric pressure. The essence of this is to provide oxygen to hypoxic or poorly vascularized wounds (Hosgood, 1992; Werner, 1998). Hyperbaric oxygen not only saturate hemoglobin to 100%, but also increases plasma oxygen level (Thorn, 1989; Hosgood, 1992). It is this plasma oxygen that makes oxygen readily available to the wound. Hunt and Pai (1972); Hunt et al., (1969) and Knighton et al., (1981) showed that increase in oxygen gradient by hyperbaric oxygen treatment increases collagen synthesis and angiogenesis. Hyperbaric oxygen treatment was also shown by Grim et al., (1990); Kindwall and Goldmann, (1995) to improve wound healing.
ii. Malnutrition and hypoproteinemia: Malnutrition leading to hypoproteinemia can delay wound healing. Serum protein must be <2 g/dL for wound healing to be hindered. This is because serum protein level of <2 g/dL decreases fibrous tissue deposition. Incised wounds in animals fed diet without protein for a long time develop wound strength slowly. Cysteine is the critical amino acid for wound healing in animals (Madden, 1972; Peacock, 1984). Cysteine functions by activating Tumor Growth Factor (TGF) critical for fibroplasias.

iii. Deficiencies in Vitamins E, A, C, and trace elements like zinc, copper, iron and manganese: Vitamins deficiencies are associated with slowed epithelialization, decreased collagen synthesis and stability and increased susceptibility to infection.

Vitamin E, like cortisone stabilizes cell membranes but high dose reduces wound healing and collagen production.

Excess vitamin A increases inflammatory reaction by acting on lysosomal membranes, making them labile. High doses of vitamin A can reverse the inhibitory action of cortisone on wound healing, suggesting that the effect of cortisone is partly mediated by lysosomes. Vitamin A increases collagen deposition by stimulating fibroblast. Ehrlich and Hunt, (1968); Peacock, (1984) showed that administration of vitamin A does not alter the rate of wound healing in the absence of glucocorticoids or vitamin E.

Vitamin C deficiency delays wound healing. Vitamin C is required for the hydroxylation of proline and lysine in the synthesis of collagen thus, collagen molecules remain incomplete without vitamin C and may not be secreted by fibroblast (Slatter, 2003).
Vitamin K deficiency leads to a shortage in the production of vitamin-K dependent clotting factors (factors II, VII, IX and X) which result in susceptibility to bleeding, hematoma formation and hence, harmful effects on wound healing.

Trace elements like zinc, copper, iron and manganese are required as cofactors for various enzymes during wound healing. Clinically, zinc deficiency is more profound in that, zinc-dependent enzymes, DNA polymerase and reverse transcriptase are essential for normal epithelial and fibroblast proliferation. Inadequate level of zinc does not affect epithelial cells and fibroblast migration but their multiplication. This implies that epithelialization cannot occur and collagen synthesis will be inadequate to hold the wound edges together (Tengrup et al., 1981; Peacock, 1984). Zinc stabilizes the membranes of lysosomes and cells but at high concentration can inhibit macrophages thus decreasing phagocytosis. It may also interfere with collagen cross-linking (Peacock, 1984). The level of zinc can be restored by the administration of zinc to patient thereby enhancing normal wound healing. The administration of zinc to patients with normal zinc level does not increase the rate of wound healing (Tengrup et al., 1981; Peacock, 1984).

iv. Prolonged use of certain drugs like non-steroidal anti-inflammatory drugs (NSAID): The use of NSAIDs impedes wound healing especially during the first 5 days when inflammation is at its peak though, the total effect does not affect the course or quality of wound repair when they are administered at their pharmacological doses.

v. Immunosuppression induced by systemic steroid and chemotherapy impairs wound healing by reducing the mobilization of inflammatory cells.

vi. Chronic diseases like diabetes, hepatic disease, and chronic renal factors impede wound healing. Diabetes affect wound healing through its peripheral angiopathic and
neuropathic effect on wound healing including defect in leucocyte function, cell adherence, chemotaxis and collagen synthesis. These effects can be reversed by improvement in metabolic control (Silhi, 1998). Hepatic disease impair wound healing through its effect on protein and clotting factor while chronic renal factors has to do with uremia which alters enzyme system, biochemical pathways, and cellular metabolism. Uremia also reduces the rate of granulation and epithelial cell division. Acute uremia decreases wound strength by the synthesis of poor quality collagen or increased collagen degradation (Colin et al., 1979; Bucknall, 1984).

vii. Smoking: The association between cigarette smoking and delayed wound healing is well recognized in clinical practice (Silverstein, 1992). Apart from carcinogenic tars, hydrogen cyanide and carbon II oxide, nicotine is the only pharmacologically active substance in tobacco smoke. Smoking generally affects wound healing via tissue perfusion and oxygenation, cell function and reduction, epithelialization and collagen production (Cassidy, 2005). As stated by Cassidy (2005), smoking decreases tissue oxygen thereby retarding cell replication, collagen deposition and angiogenesis (all are oxygen-dependent); decreases keratinocyte, leucocyte and fibroblast migration, and induces decreased rate of type I and III collagen synthesis.

viii. Ageing: Physiologic ageing diminishes virtually all phases of wound healing. In fetal wounds, the mechanisms behind lack of scarring are unknown, but probably relate to the control of collagen fibrillogenesis. The role of collagen in the fetal wound matrix is controversial. Longaker et al., (1990) found that collagen was deposited in fetal wounds much more rapidly than in adults. Collagen deposition occurred in a normal dermal and mesenchymal pattern in second and early third trimester in fetal lambs. These findings
are consistent with the observation that fetal wounds heal faster and without scar formation.

ix. Foreign bodies in the wound: A foreign body in the wound serves as an appropriate surface for the activation of the alternate complement pathway and the generation of a prolonged inflammatory response, which interferes with the subsequent stages of wound repairs. Wounds containing foreign materials are characterized by low pH and low PO$_2$. These factors significantly slow down wound repair.

x. Topical antiseptics: Topical antiseptics like alcohol, povidone-iodine 1%, hydrogen peroxide 3%, chlorhexidine 0.5% can impair wound healing if the appropriate concentration is not used. Effective bacteriocidal concentration may also be cytotoxic to cells needed for wound repair. Certain concentrations are lethal to fibroblasts, polymorphonuclear neutrophils, induce capillary shut down, inhibit epithelialization and granulation tissue formation, increase wound infection through interference with host resistance and decrease in wound strength. Isotonic solution is the recommended wound lavage. Preferable to isotonic solution is a balanced electrolyte solution (Buffa et al., 1986).

xi. Topical medication and dressing: Faster epithelialization is enhanced with occlusive or semi-occlusive dressings (Bowersox and Sorgente, 1982). They provide the moist environment required for optimal wound repair and may also aid in preventing invasion and wound infection (Mertz et al., 1981).

xii. Surgical and destructive techniques like cryosurgery, local radiotherapy, electrosurgery and laser surgery impair wound healing (Slatter, 2003).
iii. Temperature: Warmth allow wound to heal faster and with greater tensile strength than if at room temperature.

b. The intrinsic factors

The intrinsic factors affecting wound healing include abnormalities with the wound that affect wound healing (Doherty et al., 2002; Graber, 2005). They include:

i. Extent of local tissue trauma: The extent of local tissue trauma affects the wound healing in that the more devitalized the tissues are, the slower the healing.

ii. Insufficient blood supply: The rate of healing depend on the blood supply which delivers oxygen and nutrients to cells. Any impairment of blood supply by trauma, tight bandages or wound movement slow wound healing.

iii. Wound infection: It is the most common local cause for prolonged healing. All wounds are contaminated postoperatively by resident bacterial flora. However, clinical infection occurs when a critical number of pathogenic organisms are present. Bacteria prolong healing by activating the alternate complement pathway and detrimentally exaggerating and prolonging the inflammatory phase of wound healing. They also elaborate toxins and proteases that can be damaging to cells. Finally, they compete for oxygen and nutrients in the wound milieu. Lactic acid, produced in this hypoxic state further stimulates the release of damaging proteolytic enzyme (Eaglestein, 1985). Excessive devitalised tissue, increased tension in the wound, hematoma and seromas, foreign bodies in the wound, are factors that predispose to bacterial secondary infection. These can be avoided by proper surgical techniques.
2.6 Wound assessment and management

Wound assessment is very important in wound management. It helps the clinician to classify the wound i.e if infected, contaminated e.t.c., and to know the nature of the wound (laceration, abrasion, puncture and so on). This knowledge determines the method of wound management to be employed. The initial wound management involves the removal of gross contaminants and the use of lavage (warm, sterile saline or tap water). Generally, wounds less than 6-8 h are treated by lavage, debridement and primary closure. Puncture wound should be surgically explored before closure. Wounds older than 6-8 h (or infected wounds) should be treated as open wounds. The objective of open wound management is to convert the wound to a surgically clean wound that can be closed (Fossum, 2007). Severely contaminated wound should be cultured.

2.6.1 Clinical actions to be taken in wound management

i. To evaluate the traumatized animal and stabilize its condition
ii. To prevent further contamination and trauma by temporarily covering of the wound
iii. To aseptically prepare the area around the wound
iv. To confirm the contaminating microorganism by carrying out culture and sensitivity tests
v. Debridement of the dead tissue and foreign debris
vi. Provision of wound drainage if need be
vii. To lavage the wound thoroughly
viii. Stabilization and protection of the wound in order to promote healing
ix. To perform appropriate wound closure
2.6.2 Agents used in wound management

Medical treatment of wound includes administration of drugs either locally (topical) or systemically (oral or parenteral) in an attempt to aid wound repair (Savanth & Shah, 1998). The topical agents used include antibiotics and antiseptics (Chulani, 1996), desloughing/debridement agents (Savanth & Mehta, 1996), wound healing promoters (e.g. Tretinoin, *aloe vera* extract, honey) and the experimental cytokines (Rajinder *et al.*, 2008). Other methods of treatment include pulsed electromagnetic field treatment, ultrasonography and phototherapy.

2.6.2.1 Antibiotics

This can be systemic or topical or a combination of the two. Systemic and topical antibiotics are essential in the treatment of wound especially very contaminated wound. Topical antibiotics are preferred over systemic antibiotics in the treatment of open wound. Antibiotics applied within 1-3 h of contamination are effective in preventing infection. The antibiotics used effectively as topical ointment or added to lavage are penicillin, ampicillin, bacitracin, carbanecillin, tetracycline, kanamycin, cephalosporin, neomycin and polymyxin (Slatter, 2003; Fossum, 2007). But, once infections set in, both systemic and topical antibiotics are of no use in preventing pus formation in a closed wound. Wound coagulum prevents antibiotics from getting into the wound and to prevent this, wound must be debrided in order to allow antimicrobials gain access to the bacteria in the wound.

2.6.2.2 Enzymatic debriding agents

These are employed as adjuncts to wound lavage and surgical debridement. They breakdown necrotic tissue and liquefy coagulum to allow for a better antibiotic contact with wound thus, enhancing exposure for the development of cellular and humoral immunity. If properly
administered, they do not damage living tissues. To produce the desired effect, enzymes must
remain in contact with the wound for adequate time.

2.6.2.3 Wound healing promoters

Aloe vera is used mainly for its activity against *Pseudomonas aeruginosa*, together with its
antiprostaglandin and antithromboxane properties. The last two properties enables it to maintain
vascular potency thus prevent ischemia of the wound.

2.6.2.4 Pulsed electromagnetic field treatment of open wound

This is still experimental and has been shown to enhance wound epithelialization and possibly
ey early wound contraction (Scardino *et al*., 1998).

2.6.2.5 Ultrasonography and Phototherapy

Ultrasonography and Phototherapy delivered by low-intensity lasers shortens inflammatory
phase of healing and enhances the release of factors that stimulate the proliferative phase of
wound healing (Dyson, 1997).

2.7 Wound models and parameters measured

There are different wound models and parameters, each evaluating different wound indices. It is
important to note here that the use of a single model is inadequate and there is no reference
standard that can collectively represent the various phases of wound healing hence, the different
models. These include:

1. Excision Wound Model
2. Incision Wound Model
3. Dead Space Wound Model
4. Burn wound model
In each model, the surgical procedure is carried out under aseptic condition with general anaesthesia. The predetermined area for wound infliction at the back of the animal is prepared for surgery by removing hairs either with depilatory cream, shaving machine or razor. The animal can be anaesthetized with ether or chloroform by open mask method or parenterally (intraperitoneally or intramuscularly) with anaesthetic drugs. The induction of local anesthesia can be done by subcutaneous injection of a lidocaine solution (2 ml, 2%) or lignocaine HCl (1 ml, 2%) at and around the area under investigation to render the area painless. The animals are allowed to recover and housed individually in their cages. They are fed standard feed and water *ad-libitum* throughout the course of the experiment.

### 2.7.1 Excision wound model

This is used to study wound indices like Collagen estimation, percentage wound contraction, and period of epithelialization. According to Nayak *et al.*, (2007) and Shenoy *et al.*, (2009), the wound is excised at the surgically prepared dorsum of the rats, 1-1.5 cm from the vertebral column and 5 cm from the ear. The full thickness wound of about 500 mm$^2$ is left open and treated topically with the drug or the extract in question for a specified period of time.

### 2.7.2 Incision wound model

Incision wound model studies the wound breaking/tensile strength. It is carried out by making a paravertebral incision 1.5 cm on either side of about 4-6 cm length through the skin and subcutis. The incision is sutured with interrupted suture pattern, 0.5-1 cm apart. The wound is treated with the drug or the extract topically or orally for ten days. On the 7th-8th day, the stitches are removed and on the 10th day, the breaking strength is evaluated (Perez *et al.*, 2005; Krishnaveni *et al.*, 2009) with a tensiometer.
2.7.3 Dead space wound model

Dead space wound model is used to evaluate the changes in the granuloma including its hydroxyproline content. The surgery is carried out by making a nick in the skin at the groin, axilla, or dorsal paravertebral skin, creating a pouch. This is followed by the implantation of a sterile grass piths (2.5 X 0.3 cm) or cotton pellets (5 -10 mg each) or metallic rings or cylindrical piths (2.5 X 0.3 cm) or polypropylene tubes (2.5 X 0.5 cm). The wound is sutured. The test material is applied topically or orally for 10 consecutive days.

2.7.4 Burn wound model

As the name implies, it evaluates wound indices including percentage wound contraction, hydroxyproline content and epithelialization time in burn wounds.

2.8 Wound healing indices

The wound healing indices are markers used to assess the rate and extent of wound healing. These include.

2.8.1 Wound contraction

Wound contraction is defined as the centripetal movement of the edges of a full thickness wound in order to facilitate closure of the defect (Grillo, 1964). Contraction is maximal between 5 and 15 days after wounding and is mediated to a great extent by the myofibroblast and its specialized connections with the surrounding extracellular matrix. The forces of contraction are mediated in a united fashion throughout the open wound (Peacock & When, 1990) by the fibronexus, as described by Singer (1979), a specialized network of structure. The contraction of the actin filaments within the myofibroblast is transmitted by the fibronexus to the surrounding matrix and
mediates the clinical phenomenon of wound contraction which draws the wound together, particularly skin lesions, thereby reducing the size of the final scar (Peacock, 1984; Hardy, 1989; Lorena et al., 2002). Enhancement of wound contraction measured as a percentage of the initial wound size is an indicator of wound healing.

### 2.8.2 Epithelialization period

This is the time it takes for the scab to fall off without any residual raw wound (Bhat et al., 2007). It is one of the wound healing indices. A significant reduction in the epithelialization period is an indication of enhanced wound healing.

### 2.8.3 Wound breaking/Tensile strength

Progressive increase in biochemical strength of healing tissue is important in dermal wound healing. This is a function of the collagen structure and elastic fibre networks. The breaking strength of the healed wound is measured as the minimum force required to pull the incision apart. Skin breaking strength gives an indication of the tensile strength of wound tissues and represents the degree of wound healing. Tensile strength is the resistance to breaking under tension and may indicate in part the quality of the repaired tissue (Shetty et al., 2008).

### 2.8.4 Granulation tissue formation

Granulation tissue formation begins within 3-5 days after wounding and the fibroblast is the critical cell in the formation of granulation tissue. An increase in granulation tissue dry weight implies enhanced wound healing.
2.8.5 Hydroxyproline content

Wound tissues or granulations are analyzed for hydroxyproline content which is the major component of extracellular tissue, collagen which gives strength and support to the healing wound. The breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of hydroxyproline can be used as a biochemical marker for tissue collagen and an index for collagen turnover (Nayak and Pinto, 2006). Significant increase in hydroxyproline content shows increased collagen synthesis which in turn shows enhanced wound healing (Shila and Natasa, 2008).

2.9 The role of plant extracts in wound management

Plants are of great importance in the management and treatment of wounds. Quite a large number of plants are used traditionally in many countries for the treatment of wounds and burns. They induce healing and regeneration of lost tissues by multiple mechanisms including inflammatory, antiseptic, and astringent mechanisms (Dunlap, 2010). The presence of life-preserving constituents in plants has made scientists to study these plants with a view to identify potential wound healing properties (Nayak and Pinto, 2006). Consequently, many pharmaceutical laboratories are now focused on identifying these active principles including the modes of action of these plants (Nayak and Pinto, 2006). This is because the medicinal values of these plants lie in their phytoconstituents (Hwang et al., 2000) and these include alkaloids, flavonoids, essential oils, tannins, terpenoids, saponins and phenolic compounds (Akinmoladun et al., 2007).

2.10 Antibacterial activity of plants in wound healing

The control of microbial infection is necessary for better wound healing and its management (Levine, 1970; Muhammad & Mohammad, 2005). Some plants have been shown to have wound
healing activity as a result of their antibacterial properties. The antibacterial activity of plants can be assessed using the disc diffusion assay or the agar well diffusion assay. The minimum inhibitory concentration (MIC) which is the lowest concentration of the plant extract in the nutrient agar that inhibited the visible growth of the organism is determined using the agar dilution technique (Odimegwu et al., 2008).
2.11 Commonly used wound healing plants

Table 2.1: Some commonly used wound healing plants as reported by Venkatanaraya et al., (2010).

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>FAMILY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allium cepa Linn</td>
<td>Liliaceae</td>
</tr>
<tr>
<td>Ocimum kilimandscharicum</td>
<td>Laminaceae</td>
</tr>
<tr>
<td>Kaempferia galanga</td>
<td>Zingiberaceae</td>
</tr>
<tr>
<td>Raxid paeomiae</td>
<td>Paeonaceae</td>
</tr>
<tr>
<td>Lantana camara</td>
<td>Verberaceae</td>
</tr>
<tr>
<td>Carica papaya</td>
<td>Caricaceae</td>
</tr>
<tr>
<td>Aloe vera</td>
<td>Liliaceae</td>
</tr>
<tr>
<td>Musa sapientum</td>
<td>Musaceae</td>
</tr>
<tr>
<td>Tragia plukenetii</td>
<td>Euphorbiaceae</td>
</tr>
<tr>
<td>Desmodium gangeticum</td>
<td>Fabaceae</td>
</tr>
<tr>
<td>Rubus sanctus</td>
<td>Rosaceae</td>
</tr>
<tr>
<td>Tephrosia purpurea</td>
<td>Leguminosae</td>
</tr>
<tr>
<td>Quercus infectoria</td>
<td>Fagaceae</td>
</tr>
<tr>
<td>Catharanthus roseus</td>
<td>Apocyanaceae</td>
</tr>
<tr>
<td>Sphaeranthus indicus Linn</td>
<td>Asteraceae</td>
</tr>
<tr>
<td>Hyptis suaveolens (L.) Poit</td>
<td>Lamiaceae</td>
</tr>
<tr>
<td>Tectona grandis</td>
<td>Verabinaceae</td>
</tr>
<tr>
<td>Morinda citrifolia Linn</td>
<td>Rubiaceae</td>
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<tr>
<td>Ageratum conyzoides Linn</td>
<td>Asteraceae</td>
</tr>
</tbody>
</table>
2.12 Active principles in plants involved in wound healing

Plants with wound healing activity do so with the aid of the active principle they contain. These are called the phytochemical constituents and include triterpenes, alkaloids, flavonoids and biomolecules (Sumitra et al., 2005). Others are asiaticoside isolated from Centella asiatica (Shukla et al., 1999), β-sitosterol (Krishnan, 2006) and glycoprotein (Choi et al., 2001) from Aloe vera gel, Oleanolic acid from Anredra diffusa (Letts et al., 2006), quercetin, isorhamnetin and kaempferol from Hippophae rhamnoides (Fu et al., 2005), curcumin from Curcuma longa (Jagetia and Rajanikant, 2004), proanthocyanidins and reseveratrol from Vitis vinifera (Khanna et al., 2002), acylated iridoid glycosides from Scrophularia nodosa (Stevenson et al., 2002), phenolic acids from Chromolaena odorata (Phan et al., 2001), (+)-epi-α-bisabolol from Peperomia galioides (Villegas et al., 2001), fukinolic acid and cimicifugic acids from Cimicifuga sps (Kusano et al., 2001), and Xyloglucan from Tamarindus indicus (Burgalassi et al., 2000). These are some of the reported important plant derived wound healing compounds that had been tested in animal models (Ayyanar and Ignacimuthu, 2009).

2.12.1 Mechanism of action of these active principles

The active principles in plants mediate their effects on the human body by binding to receptors present in the body and these processes are same with the already established and well understood mechanism for conventional drugs. Thus, herbal medicines do not differ greatly from conventional drugs in terms of their mechanism of action. This in principle, enables them to be as effective as conventional medicine and also, gives them the same potential to cause deleterious side effects.
2.13 Herbalism and toxicity

Major concerns in phytomedicines are toxicity and dosage. The dosage of herbal medicine has always been an issue especially when the plant in question has been recognized as toxic. Since all plants have some level of toxicity, it is of importance that all medicinal plants (fresh as well as dry) be subjected to toxicological studies (Schmelzer et al., 2010). Herbs and their formulations have continued to receive great attention because of the strong belief that they are safe (Farnsworth and Soejarto, 1985; Said et al., 2002). This belief to a large extent has influenced the indiscriminate use of these formulations by many. The incidence of adverse effects, sometimes, life-threatening conditions from these herbal remedies have been reported (Elvin-Lewis, 2001; Chan, 2003). As a result, it has become imperative to ascertain the toxicity profile of these medicinal herbs.

2.13.1 Toxicity studies

There are basically three (3) methods of assessing the toxicity of any test material/plant extract depending on the duration of exposure.

a) Acute toxicity test

b) Subacute/subchronic toxicity test

c) Chronic toxicity test

The evaluation of the toxicity is based on the observation of behaviour, growth, haematology and physiological tests – especially those relating to the excretory organs (Rock, 2007).
2.13.2 Importance of liver and kidney enzymes in toxicity studies

Tissue damage is usually associated with the release of enzymes specific to the affected tissue or organ in circulation. The consequence is an increase in the activity of such enzymes in body fluids (Aliyu et al., 2006).

The liver is the major site for drug metabolism (Rock, 2007). The frequency of its damage, an indication of toxicity, shows the active role it plays in the removal of drugs from the blood (Rock, 2007). In a toxic environment, blood level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are known to significantly increase (Adam, 1998; Crook, 2006). These two enzymes are reliable indices of hepatocellular toxicity (Hayes, 1989), though AST is less specific than ALT as an indicator of liver function (Aliyu et al., 2006). Alkaline phosphatase (ALP), gamma glutamyltransferase, 5’-nucleotidase, total bilirubin, or total bile acids are for hepatobiliary evaluation (Kurt et al., 1996). Serum protein is also a marker for assessing toxicity. An increase in tissue serum total protein is an indication of tissue damage while a significant decrease in total protein of the liver contents is a reflection of liver toxicity (Gatsing et al., 2005).

The kidneys have important physiological functions including maintenance of water and electrolyte balance, synthesis, metabolism and secretion of hormones, and excretion of the waste products from metabolism. In addition, the kidneys play a major role in the metabolism and excretion of drugs, hormones, and xenobiotics (Anders, 1980; Bock et al., 1990) hence, their exposure to toxicity. The enzymes that assess kidney function are creatinine and blood urea nitrogen, though serum creatinine is more reliable and predictive of renal failure. Creatinine is the major catabolic products of the muscle and is excreted in the kidneys (Aliyu et al., 2006).
Increase in blood urea is associated with increased tissue protein catabolism, excess breakdown of blood protein and diminished excretion of urea (Nduka, 1999).

2.13.3 The hemogram in toxicity studies

Blood is a very important tissue in the body, forming the main medium of transport for most substances including drugs and other xenobiotics. With almost all foreign compounds distributed through the bloodstream (Timbrel, 2000), the blood components are the cells initially exposed to significant concentrations of toxic compounds. According to Olson et al., (2000), the hematological system carries a higher predictive value (91 %) for human toxicity when the data are interpreted from assays of rodents and non-rodents thus, making the assessment of the hemogram very important in the toxicity studies. Apart from the reflection of the extent of deleterious effect of extracts on the blood of an animal, assessment of haematological parameters elucidate blood relating functions of a plant extract or its products (Yakubu et al., 2007). The main hematology tests recommended for animal toxicity and safety studies are; total leukocyte (white blood cell) count, absolute differential leukocyte (Zawidzka, 1990), erythrocyte (Red blood cell) count, evaluation of red blood cell morphology, platelet (Thrombocyte) count, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) (Kurt et al., 1996). The RBC count and PCV analysis will reflect the effect on the population of circulating red cells. While the Hb concentration tests will reveal if the general oxygen carrying capacity of the circulating red cells is affected, the MCHC, MCV and MCH are important in the diagnosis of anaemia in most animals (Coles, 1986).
2.14 **PALISOTA HIRSUTA**

2.14.1 **Classification**

Botanical name: *Palisota hirsuta* (Thunb.) K. Schum. (Sny. *P. thyrsiflora* Benth.)

Family: Commelinaceae.

Common names: Ikpere aturu (Igbo), Akerejupon (Yoruba) and Ighiguewe (Edo) (Burkill, 1985).

2.14.2 **Plant description**

The plant *Palisota hirsuta* is named after the French botanist and traveller Joseph Palisot de Beauvois (Boake-Gyasi, 2008). It is a tropical West African plant and a member of the spiderwort family (Akobundu *et al.*, 1987). *Palisota hirsuta* is a robust perennial herb of about 3m in height. It is found mainly in lowland rain-forest region extending over the Congo basin. The nodes are swollen with the internodes reaching up to 30 cm in length. This character (swollen nodes) is noted in the Liberia Bassa name meaning ‘swollen knee’, and Nigerian Igbo and Yoruba names meaning ‘Sheep’s knee’ and ‘Knee cap’ respectively. This species is the most commonly used of the Commelinaceae (Burkill, 1985).

2.14.3 **Traditional uses**

The plant is grown as ornamental and is sometimes a component of hedges (Burkill, 1985). The plant is also used as fish poison when combined with *Adenia lobata* (Jacq.) Engl. (Passifloraceae) in Senegal. In Gabon, the stem mixed with Tephrosia (Leguminosae: Papillionioideae) or the sap added to the pulverized seeds of Strophanthus (Apocynaceae) are used in making arrow poisons. The dried plant is used for soap-making in Sierra Leone (Burkill, 1985).
2.14.4 **Medicinal uses**

The preparation of the different plant parts are used in the treatment of cutaneous and subcutaneous skin infections, gastrointestinal disorders (diarrhea and dysentery), hemorrhoids, kidney problems (used here as diuretics), nasopharyngeal infections, venereal diseases, dropsy swellings, oedema and gout. It is also used for general healing, as pain-killers, ecbolics/abortifacient and as lactation stimulants (Burkill, 1985). The sap is hemostatic and is applied to yaws and guinea-worm sores, and also taken internally for difficult birth and female sterility. The crushed root is made into a suppository and used as an aphrodisiac (Burkill, 1985).

In Nigeria, the stem is chewed to serve as sedative for cough; the dried leaf smoked for toothache; the roots added to soup for pregnant women, and also added in water as enema for constipation. The Tiv use the leaves and roots to aid conception while the Igbo of Obonpa make an ointment of the plant for gun-shot wounds and swellings (Burkill, 1985).

In Sierra Leone, it is held that the washed and cut up root when boiled and taken immediately, cures gonorrhea in three (3) days. The roots are added into medicine for stomachache and also used to treat sore feet by fumigation (Burkill, 1985).

In Ghana, the plant is used for stomach pains and the stem is chewed as sedative for cough. The leaf infusion is taken for hemorrhoids and also given to babies for the healing of the navel (Burkill, 1985).

In Gabon, the stem shavings are used for healing of wounds especially the umbilicus; the heated leaves are placed over the lumber region for kidney pains. The leaves are also cooked with groundnut and taken by suckling mothers to cleanse their milk. For urethral discharge, draughts are made from pieces of stem exposed to the sun (Burkill, 1985).
In Liberia, it is believed that the plant treats deafness, and the sap from the roasted leaf is instilled into the ear for ear ache (Burkill, 1985).

2.14.5 Relevant Research Findings of *Palisota hirsuta*

2.14.5.1 Phytochemical constituents and lethal dose

Anaga *et al.* (2009) have shown the phytochemical constituent of the methanol leaf extract of *Palisota hirsuta* to include tannins, flavonoid, glycosides and proteins. The presence of alkaloid, flavonoids, tannins and terpenoids in ethanol leaf extract of *Palisota hirsuta* has been confirmed by Woode *et al.* (2009) with tannins and flavonoids being the dominant constituent. The lethal dose (LD$_{50}$) of *P. hirsuta* is 260.6 mg/kg body weight in fasted mice (Anaga *et al*., 2009).

2.14.5.2 Liver protective activity

The methanol leaf extract of *Palisota hirsuta* has also been shown to have Liver protective activity (Anaga *et al*., 2009).

2.14.5.3 Antinociceptive activity

The antinociceptive property of the ethanol and methanol leaf extracts of *Palisota hirsuta* has been documented (Anaga *et al*., 2009; Woode *et al.*, 2009).

2.14.5.4 Local anaesthetic activities

The methanol leaf extract of *Palisota hirsuta* showed local anaesthetic activity (Anaga *et al*., 2009).
2.14.5.5 Antibacterial activity

The methanol leaf extract of *Palisota hirsuta* exhibited narrow spectrum antibacterial activity against *Staphylococcus aureus* and *Streptococcus pyogenes* (Anaga et al., 2009).

2.14.5.6 Anti-arthritic activity

The ethanol leaf extract of the plant *Palisota hirsuta* has anti-arthritic activity (Woode et al., 2009).

2.14.5.7 Anti-inflammatory and antipyretic activities

The ethanol root extract of *Palisota hirsuta* has been reported to have both anti-inflammatory and antipyretic properties (Boake-Gyasi et al., 2008).

2.14.5.8 Antiviral activity

The methanol extract of the leaves of the plant *Palisota hirsuta* has antiviral activity against herpes simplex, sindbis virus and poliovirus (Anani et al., 2000; Hudson et al., 2000).
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Material

The leaves of the plant, *Palisota hirsuta* used for the study were collected from Orba in Udenu Local Government Area of Enugu State, Nigeria. It was identified by a taxonomist as *P. hirsuta* at the Department of Botany, University of Nigeria, Nsukka.

3.1.2 Experimental animals

The albino rats used for the study were obtained from the Laboratory Animal Unit of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. The rats (105 males and 15 females) were between 3-4 months old, with mean weight of 175.40 ± 4.42 g at the commencement of the experiments. They were housed individually in stainless steel cages at room temperature, acclimatized for 2 weeks and fed commercial feed (Vital® Growers feed, GCOML, Jos, Nigeria) and water provided *ad-libitum* throughout the course of the study.

3.1.3 Drugs

Cicatrin® powder (Neomycin Sulphate BP and Bacitracin Zinc BP in a starch base, GlaxoSmithKline, Pakistan), Ketamine hydrochloride (Ketamine hydrochloride injection USP® Rotexmedica, Germany), Xylazine (Indian Immunologicals Ltd. Goliapadu, India), Distilled water, Sterile water for injection (Dana®, Nigeria), Chlorhexidine (Purit®, Nigeria),
3.1.4 Equipments

Hammer mill (Thomas-Wiley Laboratory Mill, USA), Hot air oven (Gallenkamp, England), Rotary evaporator (Buchi Labotechnik, Switzerland), Autoclave, Analytical Weighing balance (Mettler, Switzerland), Bench centrifuge (Hawksley™, England), Water bath (Tecam, England), UV-Visible spectrophotometer (CampSpec, U.K), Precision pipette: 50 µl (Red Leaf™, China), Eppendorf microlitre tubes (Red Leaf™, China), Test tubes (Pyrex®, England), Microscope slides/cover slips (Sail®, China), Petri dish (Alumco, China), Measuring cylinder (Pyrex®, England), Conical flasks (Pyrex®, England), Beakers (Pyrex®, England) and Disposable pipette tips (Red Leaf™, China).

3.1.5 Diagnostic test kits

Alanine aminotransferase (ALT) test Kit, Aspartate aminotransferase (AST)) test Kit, Protein test Kit, Creatinine test Kit and Bilirubin test Kit (All from Randox Laboratories Ltd., UK) and Alkaline phosphatase (ALP) test Kit (Quimica Clinica Aplicada S.A., Spain)

3.1.6 Chemicals and Reagents

Hexane (Sigma-Aldrich Laborchemikallen GMBH, Germany), Chloroform (Sigma-Aldrich Laborchemikallen GMBH, Germany), Methanol (Sigma-Aldrich Laborchemikallen GMBH, Germany) and Picric acid (Merck™, Germany).

Copper sulphate, Hydrochloric acid, Sodium hydroxide, Hydrogen peroxide, p-dimethylaminobenzaldehyde, n-propanol, Sulphuric acid and hydroxyproline (Sigma-Aldrich Laborchemikallen GMBH, Germany).
3.1.7 Surgical materials

Surgical blade (Helm®, Germany), Cotton wool (Lint®, Nigeria), Guaze, Tracing paper, Surgical gloves, Silk suture material (Anhuikanging, China), Thumb forceps (Sewards™, England), Scissors (Sewards™, England), Kidney-shaped dish (Sewards™, England) and Needle holder (Sewards™, England).

3.2 Methods

3.2.1 Plant Preparation

The fresh leaves of *Palisota hirsuta* (PH) were air-dried under shade, and pulverized into coarse powder using a hammer mill. The pulverized leaves (500 g) were defatted with 100% hexane for 24 h with intermittent shaking. This was followed by extraction with 100% methanol for 48 h, also with intermittent agitation. The extract was concentrated *in vacuo* using rotary evaporator at 40°C and designated as *Palisota hirsuta* leaf extract (PHLE). The percentage yield was calculated using the formula below and stored at 4 °C before use.

Percentage yield = \( \frac{\text{Weight of PHLE}}{\text{weight of pulverized leaves}} \times 100 = X \% \text{ w/w} \)

The doses of PHLE used in this study were based on previous work done on *Palisota hirsuta* by Anaga *et al.*, (2009).
3.2.2 Evaluation of the wound healing activity of PHLE

3.2.2.1 Protocol I: Excision wound model

A modified method of Morton and Malone, (1972) was adopted for this experiment. Thirty albino rats of either sexes (15 males and 15 females) were assigned randomly into 5 groups of 6 rats each. Different concentrations of the extract were suspended in 0.5 g Petroleum Jelly (PJ) and the rats were treated post-operatively as follows.

Group I served as the negative control and was treated topically with 0.5 g PJ once daily.

Group II served as the positive control and was treated with Cicatrin® powder (Neomycin Sulphate BP-1650 units and Bacitracin Zinc BP-125 units) once daily.

Groups III - V were treated with different concentrations (1, 2, and 4 %) of PHLE (all suspended in 0.5 g PJ) respectively.

Animal Preparation and Anaesthesia

The rats were sedated with Xylazine at the dose of 5 mg/kg bw intramuscularly, followed by ketamine hydrochloride injection (35 mg/kg bw intramuscularly) five minutes later to achieve dissociative anaesthesia.

The marked area on the dorsum of the rats; 1 cm from the vertebral column and 5 cm from the ear were shaved liberally and disinfected using chlorhexidine solution.

Surgical procedure

The rats were placed prone on the surgical table. A 3 cm diameter circular template was cut out from a tracing paper and placed on prepared dorsum of each rat. A marker was then used to
outline the circumference of the template on the dorsum of each rat. With a sterile scalpel blade and a thumb forceps, excision wounds 3cm diameter x 2 mm depth were aseptically created on the delineated areas. After recovery from anaesthesia, the animals were placed in their cages. Wounding day was considered as day 0 (Plate 1).

**Post-surgical wound management**

The wounds created on the rats were treated daily for 21 days post surgery (PSD).

**Measurement of wound contraction**

The wounds were monitored and wound areas measured on PSD 3, 6, 9, 12, 15, 18, and 21, using tracing papers and pencil. The tracings were read off on a graph sheet and recorded. The graph readings were used in calculating the wound contractions which were expressed as a percentage of the original size (Bairy and Rao, 2001) as follows:

\[
\% \text{ wound contraction} = \frac{\text{wound area on day 0} - \text{wound area on day n}}{\text{wound area on day 0}} \times 100
\]

where \( n \) = number of days i.e day 3, 6, 9, 12 as the case may be.

**Epithelialization period**

Epithelialization periods, which were the periods it took the scabs to fall off without raw wounds (Bhat et al., 2007) were recorded for all the groups.

**Wound Scoring**

The gross appearances of the wounds as healing progressed were scored as:

- Wet (W)------------------------------------1
Fairly wet (FW)---------------------------2
Dry (D)-------------------------------------3
Very Dry Crusty (VDC)------------------4
Very Dry with Scar (VDS)-------------- 5
Complete healing (CH)------------------6

3.2.2.2 Protocol 2: Incision wound model

The method described by Ehrlich and Hunt, (1969) was used in this experiment. Thirty male rats used for this study were randomly assigned into 5 groups of 6 rats each. Different concentrations of the extract were suspended in 0.5 g of Petroleum Jelly (PJ) and the rats were treated as follows:

Group I (Negative control) received 0.5 g Petroleum Jelly topically once daily.

Group II (Positive control) received Cicatrin® ((Neomycin Sulphate BP-1650 units and Bacitracin Zinc BP-125 units) powder topically once daily.

Group III-V received 1, 2 and 4 % of PHLE in PJ topically once daily, respectively.

Animal preparation and Anaesthesia

The rats were sedated with Xylazine (5 mg/kg bw intramuscularly) and after 5 minutes, received ketamine hydrochloride injection (35 mg/kg bw intramuscularly) to achieve dissociative anaesthesia.
The marked areas on the dorsums of the rats (1 cm left of the vertebral column and 5 cm from the ear) were shaved liberally and disinfected using chlorhexidine solution.

**Surgical procedure**

The rats after preparation and anaesthesia were positioned prone on the surgical table. Incision wounds 6 cm length x 2 mm depth were aseptically made through the skin and subcutis, 1 cm to the left of the vertebral ridge and 5 cm from the ear. The incision wounds were sutured with silk. Wounding day was considered as day 0 (Plate 2).

**Post-surgical wound management**

The incision wounds created on all the rats were treated once daily with the extract (at 1, 2, and 4 %) and standard for 10 days. The stitches were removed on PSD 8.

**Measurement of wound breaking strength**

On PSD 10, the breaking strengths of the healed wounds were measured with a tensiometer using the continuous constant water technique (Lee, 1968).

**Histopathology**

Samples of the healing tissue were harvested on (PS) day 10 from all groups and fixed in 10 % phosphate buffered formal saline for at least 48 h. They were dehydrated in ascending concentrations of alcohol, cleared in xylene for 1 h 30 minutes, and embedded in paraffin wax. Sections 5 microns thick were made and mounted on slides. The slides were stained with
hematoxylin, counterstained with eosin (H &E stains) and viewed under a light microscope.

3.2.2.3 Protocol 3: Dead space wound model

The modified method of Ilango and Chitra (2010) was adopted for this experiment. A total of 30 male rats were used and they were randomly assigned into 5 groups of 6 rats each and PHLE administered orally as follows:

- **Group I (Control)** - 10 ml/kg 5% dimethylsulfoxide (DMSO)
- **Group II** - 12.5 mg/kg PHLE
- **Group III** - 25 mg/kg PHLE
- **Group IV** - 50 mg/kg PHLE
- **Group V** - 100 mg/kg PHLE

The PHLE was dissolved in 5% DMSO in groups II - V.

**Animal preparation and Anaesthesia**

The left groin region of the rats were liberally shaved and disinfected with chlorhexidine solution.

The rats were sedated with Xylazine (5 mg/kg bw im), followed by induction of anaesthesia with Ketamine hydrochloride (35 mg/kg bw im).

**Surgical procedure**

The rats were placed on supine positions. Nicks were made on the shaved areas. Dead space wounds were created by implanting sterilized polypropylene tube (2.5 cm length by 0.25 cm
diameter) into nicks using a sterile forceps. The wounds were sutured by placing one or two silk interrupted sutures. The day of surgical implantation was considered as PSD 0.

**Post-surgical wound management**

The rats were orally administered with the extract and DMSO daily as stated above for 10 PSDs.

**Granulation tissue harvest**

On PSD 10, the rats were euthanized using chloroform in a gas chamber. An incision was made on the skin close to the implant. The implanted tubes were removed and granulation tissues formed on the tubes were harvested. The granulation tissues were weighed using a weighing balance to get the wet weight. They were dried in an oven at 42°C for 16 h and weighed to determine their dry weights.

**Hydroxyproline Assay**

A modified method of Neuman and Logan, (1950) was adopted for the hydroxyproline assay.

**Procedure**

Each dried granulation tissue (50 mg) was added to 1 ml 6 N HCl and autoclaved at 15 pound pressure for 3 hours. The acid hydrolysate obtained was neutralized with sodium hydroxide and made up to 10 ml with distilled water. One millilitre of each neutralized hydrolysate served as the test sample for the assay.

Nine test tubes were set up for each test sample and labelled accordingly and the followings were pipetted into each of the test tubes:
Test tube 1: 1 ml of distilled water.

Test tube 2 & 3: 1 ml standard containing 5 µg Hydroxyproline

Test tube 4 & 5: 1 ml standard containing 10 µg Hydroxyproline

Test tube 6 & 7: 1 ml standard containing 15 µg Hydroxyproline

Test tube 8 & 9: 1 ml test sample containing 5 µg Hydroxyproline

Pipetted in succession into each test tube were 1 ml 0.01 M Copper sulphate, 2.5 N Sodium hydroxide and 6 % Hydrogen peroxide. The solution in each tube was mixed and shaken occasionally for a period of 5 minutes, after which the tubes were placed in a water bath at 80°C for 5 minutes with frequent vigorous shaking. The tubes were chilled in ice and water bath. Into each tube, 4 ml of 3 N Sulphuric acid (H₂SO₄) was added with agitation. Two mililitres (2 ml) p-dimethylaminobenzaldehyde solution was added to each tube and the content of each tube was thoroughly mixed. The tubes were placed in a water bath at 70°C for 16 minutes, and in tap water to cool. The content of each tube was transferred into a cuvette and read at 540 nm wavelength. Test tube 1 was the blank. The amount of hydroxyproline, measured in microgram in 1 ml of the test sample was established by finding the point corresponding to its optical density on the standard curve.

### 3.2.3 Protocol 4: Evaluation of the antibacterial activity of PHLE

The agar diffusion method (Murray et al., 1995) was used in evaluating the antibacterial activity of PHLE. The petri dishes used were sterilized by autoclaving at 121°C for 15 minutes. The media was prepared by strict adherence to the manufacturer’s instructions.

**Media preparation and inoculation**
The dry media (5.6 g) was weighed and mixed with 200 ml of distilled water in a media bottle and homogenized by heating to boiling. The homogenized media was sterilized by autoclaving at 121°C for 15 minutes at 15 pound pressure and allowed to cool to 50°C. The media (25 ml) was dispensed aseptically into each sterile petri dish, left to cool and solidify.

Three of the nutrient agar plates (labelled plate 1, 2 and 3) were used for this experiment and into the plates, Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli were inoculated as follows:

Plate 1 was inoculated with Staphylococcus aureus

Plate 2 was inoculated with Pseudomonas aeruginosa

Plate 3 was inoculated with Escherichia coli

The preparations of the plant extract solution, dispensing and incubation of the plates

A solution of PHLE at the concentration of 20 mg/ml was prepared by dissolving 20 mg PHLE in 1 ml 100 % DMSO in a sterile vial labeled vial ‘a’. A two-fold dilution was made from vial ‘a’ to have:

vial ‘b’ (10 mg/ml)

vial ‘c’ (5 mg/ml)

vial ‘d’ (2.5 mg/ml)

Five (5) wells were punched into each nutrient agar plate using a sterile cork borer and labelled 1-5. The extract at the various concentrations was dispensed into the wells using a micropipette as follows:
Well 1 (50 µL 20 mg/ml PHLE)

Well 2 (50 µL 10 mg/ml PHLE)

Well 3 (50 µL 5 mg/ml PHLE)

Well 4 (50 µL 2.5 mg/ml PHLE)

Well 5 (50 µL 100 % DMSO)

The plates were incubated uninverted at 37°C for 24 h. After incubation, the plates were read and the diameter of each zone of inhibition recorded.

3.2.4 Protocol 5: Sub-acute toxicity test

A total of 30 male rats were used to evaluate the effects of PHLE on the liver, kidney and hemogram. The rats were randomly assigned into 5 groups of 6 rats each. Each group received oral medication daily for 28 days as follows:

Group I - Control (10 ml/kg 5% DMSO)

Group II - 20 mg/kg PHLE

Group III - 40 mg/kg PHLE

Group IV - 80 mg/kg PHLE

Group V - 160 mg/kg PHLE

The PHLE was dissolved in 5 % DMSO in groups II - V.
On days 14 and 28, blood samples were collected from 3 rats from each group using microcapillary tube on the retro-bulbar plexus of the median canthus of the eye (the orbital technique). The blood samples for hematology were collected in sample bottles containing ethylene-diamine-tetra-acetic acid (EDTA). The bottles were rocked to mix the blood properly with EDTA, to prevent clotting. The samples for biochemical assay were collected in test tubes and kept at room temperature for 30 minutes to clot. The tubes were thereafter, centrifuged at 3000 revolutions per minutes for 10 minutes to enhance total separation of the serum from the clotted blood. The clear supernatants (serum) were aspirated with syringe and needle and stored for use. The 3 rats from each group were sacrificed and the liver, heart, kidney and spleen were harvested. The surrounding fats were also trimmed off.

3.2.4.1 Determination of relative organ weight (ROW)

The weights of the liver, heart, kidney and spleen were recorded using a weighing balance. The ROW, an expression of the weight of an organ over the body weight, was calculated.

3.2.4.2 Hematology

3.2.4.2.1 Packed cell volume (PCV)

This was carried out using the microhematocrit method as described by Coles, (1986). The microcapillary tube used was nearly filled with blood sample and sealed at one end. It was centrifuged at 10,000 revolutions per minute (rpm) for 5 minutes with a microhaematocrit centrifuge. The PCV was read using a microhaematocrit reader.

3.2.4.2.2 Hemoglobin concentration (Hb)
The Cyanomethemoglobin method, as described by Kachmar, (1970) was adopted. To a test tube, 5 ml of Drabkin’s haemoglobin reagent was dispensed. This was followed by the addition of 0.02 ml of the blood sample with proper mixing. The mixture was allowed to react for 20 minutes. The absorbance was read at 540 nm wavelength against a reagent blank. The standards were prepared as above and read at 540 nm too. The haemoglobin concentration of the blood sample was obtained by the multiplication of the absorbance of the blood sample by a calibration factor obtained from the absorbance and concentration of the standard.

3.2.4.2.3 Total red blood cell (RBC)

The haemocytometer method (Schalm et al., 1975) was followed. To 4 ml of the red blood cell diluting fluid in a test tube, 0.02 ml of the blood sample was added making a 1:200 dilution of the blood sample. The diluted blood sample was loaded on to the Neubauer counting chamber. The red blood cells in the 5 groups of 16 small squares in the central region of the Neubauer chamber were counted using a light microscope at X40 objective. The number of cells counted for each sample was multiplied by 10,000 to arrive at the red blood cell count per µl of blood.

3.2.4.2.4 Total white blood cell (WBC)

The haemocytometer method (Schalm et al., 1975) was adopted. To 0.38 ml of white cell diluting fluid in a test tube, 0.02 ml of the blood sample was pipette to make 1:20 dilution of the blood. The diluted blood was loaded on to the Neubauer counting chamber. The cells on the 4 corners squares were counted using a light microscope at X10 objective. The number of cells counted for each blood sample was multiplied by 50 to arrive at the total white blood cell count per µl of blood.
3.2.4.2.5 Differential WBC counts

Differential WBC Counts were carried out using the Leishman technique (Schalm et al., 1975). The blood sample was gently shaken and a drop was placed on a clean grease-free slide. A thin smear was carefully made with the drop of blood using a cover slip. The smear was air-dried and stained with Leishman stain. The stained slides were viewed with an immersion objective using a light microscope. By the longitudinal counting method, 200 cells were counted with each cell type identified and scored using the differential cell counter. The result for each type of WBC was expressed as a percentage of the total count and converted to the absolute value per µl of blood by multiplying with 1000.

3.2.4.3 Biochemical Assay

3.2.4.3.1 Alanine aminotransaminase (ALT): The serum ALT was determined by the Colorimetric method (Reitman and Frankel, 1957; Schmidt and Schmidt, 1963). Three test tubes (for reagent blank, test sample and sample blank) were used and into the tubes were dispensed:

Reagent blank: Solution 1 (0.5 ml) and 0.1 ml of distilled water.

Sample blank: Solution 1 (0.5 ml).

Test sample: Serum (0.1 ml) and 0.5 ml of solution 1.

The solution in each test tube was mixed, and the tubes were placed in a water bath for 30 minutes at 37°C. Solution 2 (0.5 ml) was dispensed into each tube. The serum (0.1 ml) was added into the sample blank tube. The solution in each tube was mixed, allowed to stand for 20 minutes at room temperature. Sodium hydroxide (5 ml) was dispensed into each tube and mixed.
After 5 minutes, the absorbance was read against sample blank using a spectrophotometer at 540 nm wavelength.

**3.2.4.3.2 Aspartate aminotransferase (AST):** The Colorimetric method of Reitman and Frankel, (1957) and Schmidt and Schmidt, (1963) were adopted for this assay. Three test tubes were set up for this assay and into the tubes were dispensed:

Test tube 1 (Reagent blank): Solution 1 (0.5 ml) and 1 ml of distilled water.

Test tube 2 (Test sample): Serum (0.1 ml) and 0.5 ml of solution 1.

Test tube 3 (Sample blank): Solution 1 (0.5 ml) only

The content of each tube was mixed. The three tubes were placed in a water bath for 30 minutes at 37°C. This was followed by the addition of about 0.5 ml of the solution 2 into all test tubes which were allowed to stand for 20 minutes at room temperature. Sodium hydroxide (5 ml) was dispensed into each tube, mixed and left to stand for 5 minutes. The absorbance was read on a spectrophotometer at 546 nm wavelength in a cuvette.

**3.2.4.3.3 Alkaline phosphatase (ALP):** Phenolphthalein Monophosphate Method (Klein et al., 1960; Babson, 1966) was followed in running this assay. Two test tubes (Sample and Standard) were set up. Into each test tube, 1 ml of water and 1 drop of substrate was added. The solution in each tube was mixed and incubated at 37°C for 5 minutes. The Standard solution (0.1 ml) was added to the Standard test tube while 0.1 ml of the sample was added to the Sample tube. The content of each tube was mixed and incubated at 37°C for 20 minutes. The colour developer (5 ml) was added to each tube. The blank is water. The absorbance of the sample and standard was read against the blank at 550 nm wavelength.
The value of ALP was calculated with this formula:

\[
\text{Sample absorbance} \times 30 = \text{U/L of ALP}
\]

Standard absorbance

3.2.4.3.4 Total protein (TP) assay – The Biuret method described by Tietz, (1995) was used for this assay.

Standard: The standard solution (0.02 ml).

Test sample: The test serum (0.02 ml).

Blank: Distilled water (0.02 ml).

To all the test tubes, 1 ml each of Biuret reagent was dispensed. Each tube was thoroughly mixed and allowed to stand for 30 minutes at room temperature. The absorbance of the sample and standard were read against the blank at 540 nm wavelength. The total protein was calculated with the formula below.

\[
\text{Total proteins (g/dl)} = \frac{\text{Absorbance of sample} \times \text{Standard concentration}}{\text{Absorbance of standard}}
\]

3.2.4.3.5 Total bilirubin assay (Jendrassik and Grof, 1938; Sherlock, 1951).

Into the test tubes (Sample blank and Sample), 0.2 ml of Reagent A (Sulphanilic acid solution), was dispensed. One drop of Reagent D (Sodium nitrite) was added to the Sample tube. One millilitre (1 ml) of Reagent B (caffeine solution and 0.2 ml of the serum sample were dispensed in succession to the tube. The content of each tube was mixed and incubated for 10 minutes at 20-25°C. One millilitre of Reagent C (Tartarate solution) was added to each tube, mixed and
allowed to stand for 5 minutes at 25°C. The absorbance was read at 578 nm wavelength against
the blank. The total bilirubin was calculated with this formula

\[ \text{Mg total bilirubin/dl} = \text{Absorbance of sample} \times 10.8 \]

3.2.4.3.6 Creatinine assay: This assay was carried out using the method described by Henry,

The serum (1 ml) was deproteinized by adding 1 ml of 19.6 % Trichloroacetic acid (TCA) to it in
a test tube. This was mixed with a glass rod to evenly distribute the precipitate formed and
centrifuged at 2500 rpm for 10 minutes. The supernatant was aspirated into a test tube for use in
the assay. Three test tubes were set up for the blank, standard and test sample respectively.

Blank: Distilled water (0.5 ml), 0.5 ml TCA was added.

Standard: Solution 1 (0.5 ml), 0.5 ml TCA

Test sample: Supernatant (1 ml)

The 1 ml reagent mixture obtained in each tube was mixed.

The tubes were allowed to stand for 20 minutes at room temperature.

The absorbance of the sample and standard were read against the blank using at 520 nm
wavelength.
3.2.5 Data presentation and analysis

Statistical analysis was performed using SPSS software® (Version 13.0 for Windows, SPSS Inc., Chicago, USA). The result of the wound scoring was analyzed using Mann Whitney non-parametric test. The parametric data were presented as mean ± standard error of means (SEM) and analyzed using univariate and multivariate general linear model. The variant means were separated using least significant difference (LSD) post hoc test at p < 0.05.
CHAPTER FOUR

4.0 RESULTS

4.1 Plant extraction

The percentage yield of the methanolic leaf extraction of *Palisota hirsuta* was 5.98 % w/w. The extract was dark green, pasty in consistency with a pungent smell.

4.2 Effect of *Palisota hirsuta* Leaf Extract (PHLE) on percentage wound contraction

Figure 4.1 shows the percentage wound contraction effect of PHLE in the excision wound model. On PSD 3, no wound contraction was shown in all the groups. On PSD 6, the percentage wound contraction at 1 % (31.49 ± 6.74 %) and 4 % PHLE concentration (26.85 ± 3.81 %) significantly (p<0.05) increased when compared with cicatrin® (10.27 ± 1.20 %). However, there was no significant (p>0.05) variation observed as the PHLE-treated groups were compared with PJ-treated group. The increase in percentage wound contraction was sustained to the PSD 9 by the extract at both 1 % and 4 % concentration compared to PJ and Cicatrin® treatments. No significant (p>0.05) variation in percentage wound contraction was shown on PSD 12 in all the groups. However, on PSD 15 and 18, the extract-treated group V (4 % concentration) significantly (p<0.05) increased percentage wound contraction when compared with the PJ-treated group I. Wound contraction was significantly (p<0.05) increased by the extract at 4 % (100.00 ± 0.00 %) (Plate 6) and 1 % (96.77 ± 1.45 %) when compared with PJ (Plate 4.7). Similar increase was observed at 4 % (100.00 ± 0.00 %) when compared with Cicatrin® (90.34 ± 3.07 %) on PSD 21 (Plate 4.8). The extract at 2 % (group IV) showed no significant (p>0.05) variation in wound contraction when compared with PJ (group I) and Cicatrin® (group II) throughout the experimental period.
4.3 Effect of PHLE on epithelialization period

The epithelialization period of group V rats treated with 4% PHLE (21.33 ± 0.21 days) was significantly (p<0.05) lower than those of the control groups I (29.83 ± 4.29 days) and II (29.67 ± 2.56 days). The extract-treated groups III and IV showed no significant (P>0.05) variation in epithelialization period when compared with the control groups I and II (Fig. 4.2).

4.4 Effect of PHLE on wound scoring (Excision wound model)

On PSD 3, the gross appearance of the wounds in all the groups were all wet and inflamed (W, IN) Table 2. The wounds were fairly wet (FW) in PJ-treated group, very dry with crust (VDC) in cicatrin® treated group (Plate 4.3) and dry (D) in PHLE-treated groups (Plate 4.4) on PSD 6. On PSD 9, groups II-V appeared very dry with crust (VDC), while group I appeared dry (D). The wounds in all the groups were very dry with crust (VDC) by days 12 and 15 post surgery. However, by PSD 18, the wounds in group V were already very dry with scar (VDS) while those of groups I-IV remained very dry with crust (VDC). The wounds in group V rats were completely healed (CH) by PSD 21 (Plate 4.5), while those of groups I (Plate 4.6), II (Plate 4.7), III and IV were very dry with scar (VDS).

4.5 Effect of PHLE on wound breaking strength (Incision wound model)

The results as presented in figure 4.3 showed that the wound breaking strength of 427.01 ± 15.95, 451.49 ± 8.55, 550.58 ± 10.43 and 569.57 ± 4.44 g for groups II-V respectively were significantly (p<0.05) higher than that of group I (391.94 ± 16.16 g). PHLE-treated groups IV (550.58 ± 10.43 g) and V (569.57 ± 4.44 g) showed significantly (p<0.05) higher wound breaking strength than group II (427.01 ± 15.95 g) but PHLE-treated group III showed no significant (P>0.05) variation from group II.
4.6  Histopathology of the Effect of PHLE on incision healing wound

Healing skin sections from PJ-treated group I rats showed the absence of adnexa, presence of acanthosis of the epidermis and epidermal pegs (Plate 4.8). The healing skin sections of the cicatrin®-treated rat (group II) showed development of adnexa at the incision line, absence of acanthosis but the epidermis was not as near-normal as in PHLE- treated group V (Plate 4.9). PHLE- treated group V showed development of more adnexa, absence of acanthosis and a near-normal epidermis and dermis (Plate 4.10).

4.7  Effect of PHLE on level of granulation tissue (Dead space wound model)

The wet granulation tissue was significantly (p<0.05) higher in PHLE-treated groups than in the DMSO-treated (control) group. When dry, the granulation tissue in PHLE-treated groups IV (0.18 ± 0.01 g) and V (0.28 ± 0.04 g) were significantly (p<0.05) higher than the control (0.06±0.01 g). However, groups II and III showed no significant (p>0.05) variation from the control (Table 4.2).

4.8  Effect of PHLE on hydroxyproline level (Dead space wound model)

In dead space wound model, the level of hydroxyproline production was 433.32 ± 3.00, 469.28 ± 2.68, 460.00 ± 11.15 mg/100g dry tissue for groups III, IV and V, respectively; and was significantly (p<0.05) higher than that of group I (394.68 ± 6.47 mg/100g dry tissue). However, there was no significant (p>0.05) difference between PHLE-treated groups IV and V (Fig. 4.4).

4.9  Antibacterial activity of PHLE

The PHLE induced no inhibition zones against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* in the three incubated nutrient agar plates.
4.10 Effect of PHLE on ROW of the heart

The effect of PHLE on the ROW of the heart is presented in Table 4.3. On day 14, there was a significantly (p<0.05) higher ROW of the heart of the rats that received the extract at 40 mg/kg (4.55 ± 0.32 g) compared with the DMSO-treated (control) rats (3.59 ± 0.20 g). There was no significant (P>0.05) variation in the ROW of the heart of the rat groups (I-V) on day 28.

4.11 Effect of PHLE on ROW of the spleen

The effect of PHLE on the ROW of the spleen (Table 4.4) showed no significant (P>0.05) variation between the PHLE-treated groups and the DMSO-treated (control) group I on day 14. But on day 28, the ROW of the spleen was significantly lower in group II (1.98 ± 0.19 g) when compared with group I (5.06 ± 0.37 g).

4.12 Effect of PHLE on ROW of the liver

There was no significant (P>0.05) variation in ROW of the Liver among the groups on day 14. But on day 28, the PHLE at 20, 40 and 160 mg/kg significantly (P<0.05) lowered ROW of the liver (Table 4.5).

4.13 Effect of PHLE on ROW of the kidney

There was no significant (P>0.05) variation on the ROW of the kidney among the groups on days 14 and 28 (Table 4.6).

4.14 Effect of PHLE on the packed cell volume

It was observed that the PCV did not significantly (P>0.05) vary in all the groups on days 14 and 28 (Table 4.7).
4.15 Effect of PHLE on the hemoglobin concentration

There was no significant (P>0.05) variation in Hb concentration among the groups on day 14 (Table 4.8). The rats treated with 20, 40 and 160 mg/kg PHLE showed significantly (P<0.05) higher Hb concentration on day 28 compared with those treated with DMSO (control).

4.16 Effect of PHLE on the total red blood cell count

Total RBC count as presented in Table 4.9 showed that there was no significant (P>0.05) variation in RBC count on day 14 among the groups. However, on day 28, the rats that received the extract at 20 mg/kg and 40 mg/kg had significantly (P<0.05) increased RBC count compared to the rats that received DMSO.

4.17 Effect of PHLE on the total white blood cell count

The results of the effect of PHLE on the Total White Blood Cell Count is shown on Table 4.10. The total WBC count did not significantly (P>0.05) differ when the PHLE-treated groups were compared with control on days 14 and 28.

4.19 Effect of PHLE on neutrophils count

The table of values for the effect of PHLE on neutrophils count (Table 4.11) showed that there was no significant (P>0.05) difference in the neutrophils count among the groups on days 14 and 28.
4.20 Effect of PHLE on lymphocyte count

The results of the effect of PHLE on Lymphocyte Count presented in Table 4.12 showed no significant (P>0.05) variation in differential lymphocyte count when the PHLE-treated groups were compared with the DMSO-treated (control) group on days 14 and 28.

4.21 Effect of PHLE on serum AST level

The results of the effect of PHLE on serum AST is presented in Figure 4.5. Serum AST level in the PHLE-treated groups were not significantly (P>0.05) different from that of the DMSO-treated (control) rats on day 14. On day 28, the extract groups (20 and 80 mg/kg) had significantly (P<0.05) increased level of serum AST.

4.22 Effect of PHLE on serum ALT level

The results of the effect of PHLE on serum ALT is presented in Figure 4.6. There was no significant (P>0.05) difference on serum ALT level in both the PHLE-treated groups and the DMSO-treated (control) group on day 14. But on day 28, the serum ALT level of the PHLE (40 mg/kg) group was significantly (P<0.05) higher than the control.

4.23 Effect of PHLE on serum ALP level

The results of the effect of PHLE on Serum ALP level showed that there was no significant (P>0.05) difference in the serum ALP in all the rat groups on day 14. The serum ALP level was significantly (p<0.05) decreased in PHLE (80 mg/kg) treated group compared with the DMSO-treated (control) group on day 28(Figure 4.7).
4.24 Effect of PHLE on serum total protein level

The results of the effect of PHLE on Serum Total Protein level is presented in Fig. 4.8. PHLE-treated groups and DMSO-treated (control) group showed no significant (P>0.05) difference in the serum total protein level on days 14 and 28.

4.25 Effect of PHLE on serum bilirubin level

The bilirubin level was significantly (P<0.05) increased in the PHLE-treated group V (160 mg/kg) on day 14, though on day 28, there was no significant (P>0.05) variation in serum bilirubin level among the groups (Figure 4.9).

4.26 Effect of PHLE on serum creatinine level

There was no significant (P>0.05) variation (Figure 4.10) in the level of creatinine on day 14 and day 28 when the PHLE-treated groups were compared with the DMSO-treated (control) group.
Table 4.1: Effect of PHLE on Gross Appearance of the wound

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>DAY 3</th>
<th>DAY 6</th>
<th>DAY 9</th>
<th>DAY 12</th>
<th>DAY 15</th>
<th>DAY 18</th>
<th>DAY 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (PJ)</td>
<td>W, IN</td>
<td>FW</td>
<td>D</td>
<td>VDC</td>
<td>VDC</td>
<td>VDC</td>
<td>VDS</td>
</tr>
<tr>
<td>II (CICATRIN)</td>
<td>W, IN</td>
<td>VDC</td>
<td>VDC</td>
<td>VDC</td>
<td>VDC</td>
<td>VDC</td>
<td>VDS</td>
</tr>
<tr>
<td>III (1 % PHLE)</td>
<td>W, IN</td>
<td>D</td>
<td>VDC</td>
<td>VDC</td>
<td>VDC</td>
<td>VDC</td>
<td>VDS</td>
</tr>
<tr>
<td>IV (2 % PHLE)</td>
<td>W, IN</td>
<td>D</td>
<td>VDC</td>
<td>VDC</td>
<td>VDC</td>
<td>VDC</td>
<td>VDS</td>
</tr>
<tr>
<td>V (4 % PHLE)</td>
<td>W, IN</td>
<td>D</td>
<td>VDC</td>
<td>VDC</td>
<td>VDC</td>
<td>VDS</td>
<td>CH</td>
</tr>
</tbody>
</table>

W, IN- Wet, inflamed; FW-Fairly wet; D- Dry; VDC- Very dry with crust; VDS- Very dry with scar tissue; CH- Complete healing.


Table 4.2: Effect of PHLE on Level of Granulation Tissue (Dead space wound model)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Wet granulation (g)</th>
<th>Dry granulation (g)</th>
<th>Difference (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10 ml/kg DMSO</td>
<td>0.30 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>0.24</td>
</tr>
<tr>
<td>II</td>
<td>12.5 mg/kg PHLE</td>
<td>0.42 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.10 ± 0.00</td>
<td>0.32</td>
</tr>
<tr>
<td>III</td>
<td>25 mg/kg PHLE</td>
<td>0.47 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11 ± 0.01</td>
<td>0.36</td>
</tr>
<tr>
<td>IV</td>
<td>50 mg/kg PHLE</td>
<td>0.65 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.18 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.47</td>
</tr>
<tr>
<td>V</td>
<td>100 mg/kg PHLE</td>
<td>0.81 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.28 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM (n=6)

<sup>a</sup>p<0.05 when compared to control (group I)
Table 4.3: Effect of PHLE on ROW (g) of the Heart

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 mg/kg 5 % DMSO)</td>
<td>3.59 ± 0.20</td>
<td>4.08 ± 0.48</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>3.78 ± 0.17</td>
<td>3.84 ± 0.17</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>4.55 ± 0.32(^a)</td>
<td>4.51 ± 0.19</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>3.98 ± 0.51</td>
<td>4.19 ± 0.10</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>3.78 ± 0.10</td>
<td>4.13 ± 0.03</td>
</tr>
</tbody>
</table>

\(^a\)P<0.05 when compared with control (group I)
Table 4.4: Effect of PHLE on ROW (g) of the Spleen

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 mg/kg 5 % DMSO)</td>
<td>3.06 ± 0.14</td>
<td>5.06 ± 0.37</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>2.56 ± 0.75</td>
<td>1.98 ± 0.19\textsuperscript{a}</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>2.62 ± 0.34</td>
<td>4.71 ± 1.13</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>3.79 ± 0.53</td>
<td>3.88 ± 0.32</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>4.01 ± 1.06</td>
<td>3.34 ± 0.01</td>
</tr>
</tbody>
</table>

\textsuperscript{a}P<0.05 when compared with control (group 1)
**Table 4.5: Effect of PHLE on ROW (g) of the Liver**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 mg/kg 5 % DMSO)</td>
<td>4.03 ± 0.27</td>
<td>4.50 ± 0.12</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>4.38 ± 0.94</td>
<td>3.81 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>4.72 ± 0.12</td>
<td>3.97 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>4.69 ± 0.40</td>
<td>4.35 ± 0.05</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>4.25 ± 0.19</td>
<td>3.91 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>P<0.05 when compared with control (group I)
Table 4.6: Effect of PHLE on ROW (g) of the Kidney

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 mg/kg 5 % DMSO)</td>
<td>7.11 ± 0.41</td>
<td>6.40 ± 0.12</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>6.13 ± 1.62</td>
<td>6.44 ± 0.27</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>6.34 ± 0.21</td>
<td>6.81 ± 0.10</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>5.87 ± 0.16</td>
<td>6.51 ± 0.07</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>6.49 ± 0.17</td>
<td>6.23 ± 0.14</td>
</tr>
</tbody>
</table>
Table 4.7: Effect of PHLE on the Packed Cell Volume (%)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 mg/kg 5 % DMSO)</td>
<td>36.33 ± 2.03</td>
<td>30.00 ± 1.73</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>34.67 ± 1.20</td>
<td>32.33 ± 1.20</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>35.67 ± 2.73</td>
<td>34.00 ± 2.31</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>35.00 ± 1.73</td>
<td>31.67 ± 2.60</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>36.67 ± 0.33</td>
<td>31.67 ± 1.45</td>
</tr>
</tbody>
</table>
Table 4.8: Effect of PHLE on the Haemoglobin concentration (g/dl)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 mg/kg 5 % DMSO)</td>
<td>12.80 ± 0.26</td>
<td>9.93 ± 0.20</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>12.07 ± 0.07</td>
<td>12.97 ± 0.23^a</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>13.33 ± 0.70</td>
<td>13.27 ± 0.84^a</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>12.83 ± 0.88</td>
<td>10.00 ± 0.35</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>13.40 ± 0.12</td>
<td>12.70 ± 0.52^a</td>
</tr>
</tbody>
</table>

^aP<0.05 when compared with control (group I)
Table 4.9: Effect of PHLE on the Total RBC count (x 10^6/µL)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 mg/kg 5 % DMSO)</td>
<td>6.28 ± 0.17</td>
<td>4.97 ± 0.20</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>5.90 ± 0.12</td>
<td>5.93 ± 0.10^a</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>6.30 ± 0.39</td>
<td>6.13 ± 0.39^a</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>6.12 ± 0.36</td>
<td>4.67 ± 0.22</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>6.43 ± 0.08</td>
<td>5.48 ± 0.17</td>
</tr>
</tbody>
</table>

^aP<0.05 when compared with control (group I)
Table 4.10: Effect of PHLE on the Total WBC count (cells/mm$^3$)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 mg/kg 5 % DMSO)</td>
<td>$8.80 \times 10^3 \pm 1473.09$</td>
<td>$10.23 \times 10^3 \pm 216.51$</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>$8.17 \times 10^3 \pm 656.59$</td>
<td>$9.73 \times 10^3 \pm 1293.68$</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>$8.43 \times 10^3 \pm 1393.24$</td>
<td>$7.90 \times 10^3 \pm 1270.17$</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>$11.63 \times 10^3 \pm 1898.54$</td>
<td>$11.55 \times 10^3 \pm 866.03$</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>$8.60 \times 10^3 \pm 1270.17$</td>
<td>$11.50 \times 10^3 \pm 230.94$</td>
</tr>
</tbody>
</table>
Table 4.11: Effect of PHLE on Neutrophils count (%)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 mg/kg 5 % DMSO)</td>
<td>25.00 ± 3.21</td>
<td>17.67 ± 4.91</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>27.33 ± 2.33</td>
<td>22.67 ± 1.76</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>36.67 ± 9.39</td>
<td>20.67 ± 1.45</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>31.00 ± 3.79</td>
<td>19.67 ± 2.60</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>27.67 ± 5.49</td>
<td>23.00 ± 3.46</td>
</tr>
<tr>
<td>Groups</td>
<td>Day 14</td>
<td>Day 28</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td>I (10 mg/kg 5 % DMSO)</td>
<td>75.00 ± 3.21</td>
<td>82.33 ± 4.91</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>72.67 ± 2.33</td>
<td>77.33 ± 1.76</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>63.33 ± 9.39</td>
<td>79.33 ± 1.45</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>69.00 ± 3.79</td>
<td>80.33 ± 2.60</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>72.67 ± 5.49</td>
<td>77.00 ± 3.46</td>
</tr>
</tbody>
</table>
Fig. 4.1: Effect of PHLE on Percentage wound contraction
**Fig. 4.2**: Effect of PHLE on Epithelization period (Excision wound model)

* P< 0.05 when compared with Pet. Jelly and Cicatrin®
Fig. 4.3: Effect of PHLE on Wound breaking strength (Incision wound model)
* P< 0.05 when compared with control (group I)
Fig. 4.4: Effect of PHLE on Hydroxyproline level (Dead space wound model)

*P< 0.05 when compared to control (group I).
Fig. 4.5: Effect of PHLE on Serum aspartate aminotransferase (SAST) Activity

*P<0.05 when compared with control (group I)
Fig. 4.6: Effect of PHLE on Serum alanine aminotransaminase (SALT) Activity

*P<0.05 when compared with control (group I)
Fig. 4.7: Effect of PHLE on Serum Alkaline Phosphatase (SALP) Activity

*P<0.05 when compared with control (group I)
Fig. 4.8: Effect of PHLE on serum total protein
Fig. 4.9: Effect of PHLE on serum bilirubin activity

*P<0.05 when compared with control (group I)
Fig. 4.10: Effect of PHLE on serum creatinine activity
Plate 4.1: Excision wound (EW) on the dorsum of the experimental rats (Day 0).
Plate 4.2: Incision wound on the dorsum of the experimental rat (Day 0) showing the incision line (I) and the interrupted sutures (IS).
Plate 4.3: Gross appearance of the excision wound of rats treated with Cicatrin® (Group II) on PSD 6 showing the very dry crusted (VDC) wound.
Plate 4.4: Gross appearance of the excision wound of rats treated with 4 % PHLE (Group V) on PSD 6 showing the dry (D) wound.
Plate 4.5: Gross appearance of the excision wound of rats treated with 4 % PHLE (Group V) on PSD 21 showing complete healing (CH).
Plate 4.6: Gross appearance of the excision wound of rats treated with PJ (Group I) on PSD 21 showing the very dry wound with scar (VDS).
Plate 4.7: Gross appearance of the excision wound of rats treated with Cicatrin® (Group II) on PSD 21 showing the very dry wound with scar (VDS).
Plate 4.8: Histopathological section of the skin from rats treated with PJ (Group I) x 10. (H&E stain)
A, Acanthosis; I, the incision line showing no adnexia; EP, Epidermal peg; DN, Dermis of the normal skin; DH, Dermis of healing skin.
Plate 4.9: Histopathological section of the skin from rats treated with Cicatrin® (Group II) x 10. (H&E stain)

I, line of incision; AD, Adnexia; E, Epidermis; DN, Dermis of the normal skin; DH, Dermis of healing skin.
Plate 4.10: Histopathological section of the skin from rats treated with PHLE (Group V) x 10. (H&E stain)

I, line of incision; AD, Adnexia; DH, Dermis of healing skin; DN, Dermis of the normal skin; E, Epidermis
CHAPTER FIVE

5.0 DISCUSSIONS

The effects of the methanolic leaf extract of *Palisota hirsuta* on wound healing using different wound models were studied in rats. Sub-acute toxicity of PHLE was investigated in rats over 28 days of treatment. The antibacterial activity of PHLE was also studied using agar diffusion method. The results demonstrated the efficacy of PHLE in post-surgical wound management.

The excision wound model measured the wound contraction and epithelialization period. Wound contraction is the centripetal movement of wound margin to close up the open wound area. This movement is caused by myofibroblast activity (Gabbaiani, 1976). *Palisota hirsuta* at 1 % and 4 % concentration enhanced wound contraction from days 6-21 post-surgery when compared with cicatrin® and PJ- treated groups. No contraction was observed in all the groups on day 3 probably because they were all in the inflammatory phase of wound healing. This was also shown in the wound scoring data. PHLE-treated group V (4 %) showed best activity in wound contraction when compared with groups III (1 % PHLE) and IV (2 % PHLE) throughout the duration of the experiment. This also was evidenced in the wound scoring data that showed the PHLE group V reaching complete healing (CH) faster. The enhancement of wound contraction in group V is probably either due to the ability of the extract at this concentration (4 %) to enhance the contractile property of myofibroblasts or increase myofibroblasts population recruited into the wound area. The extract at 1 % concentration enhanced wound contraction higher than at 2 %. This most likely could be due to an experimental error. The period of epithelialization was significantly shortened by the extract at 4 % when compared with the control groups I and II, and also when compared with groups III and IV, an indication that
epithelialization was enhanced by the extract at this concentration. This showed that clinically, 4% PHLE is more effective in inducing wound healing.

The wound breaking strength is a measure of the rate of collagen synthesis and also the maturation process (Shanbhag, 2005). The increase in wound breaking strength by the extract and cicatrin® treated groups in incision wound model is an indication of enhanced collagen maturation. The rats that received the extract at 4% also showed the highest wound breaking strength. The histopathology of the healing skin sections in incision wound model further confirmed the above result in that the skin sections from PHLE-treated group V (4%) (the best of the PHLE-treated groups in wound healing activity) showed proliferation of adnexa, absence of acanthosis and a near-normal epidermis and dermis, a mark of advanced wound healing compared to the control (PJ) which showed evidence of delayed healing with absence of adnexa, presence of acanthosis of the epidermis and epidermal pegs. The cicatrin® treated group showed development of adnexa, absence of acanthosis but the epidermis and dermis were not as near-normal as observed in PHLE treated group V; a confirmation that the wound healing activity of cicatrin® treated group was not as good as that of PHLE-treated group V. The PHLE treated groups III and IV were comparable with group I (PJ).

Dead space wound model in this study showed significant increase in hydroxyproline concentration in P. hirsuta-treated groups III-V and increase in dry weight of the granulation tissue (PHLE-treated groups IV and V) which also indicated high collagen lay and maturation like was shown in incision wound model.
In the agar diffusion assay, PHLE induced no inhibition zones against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, an indication of no activity against these microorganisms. This was contrary to the work of Anaga *et al.*, (2009) where PHLE showed narrow spectrum antibacterial activity against *Staphylococcus aureus* and *Streptococcus pyogenes*. This could be due to differences in the age of the plant and season of the year when harvested (Lampe *et al.*, 1985). The reason been that these factors affect the concentration of active principles in plants thereby affecting their activity.

The subacute toxicity study evaluated the effects of the extract on the blood, liver and kidney function. It also measured the relative organ weights (ROW) of the heart, kidney, spleen and liver. The increase in the relative organ weight of the heart observed with the extract may be due to increase in the functional ability of the heart (Ashafa *et al.*, 2009). The absence of significant effect on the ROW of the kidneys is an indication that the PHLE has no harmful effect on the size of the kidney in relation to the weight of the animals. The extract at 20 mg/kg significantly reduced the size of the spleen on day 28. Also, on day 28 the extract at 20, 40, and 160 mg/kg significantly reduced the ROW of the liver. This probably may be due to cell constriction induced by the plant extract (Moore and Dalley, 1999). *P. hirsuta* significantly increased the RBC count at 20, 40, and 60 mg/kg and Hb concentration (20 and 40 mg/kg) though the values were within the normal range of values for RBC count and Hb concentration (Johnson-Delaney, 1996). This is an indication of erythropoiesis which is within the normal range. There was no significant alteration of the PCV by the extract. The total white blood cell (WBC) and differential WBC counts were also not altered by the extract showing that the extract has no immunomodulatory activity. From this study, serum AST, ALT, ALP, total protein were not
altered by day 14 except total bilirubin, an indication that there was no hepatocellular change in the PHLE-treated groups. Surprisingly, the bilirubin level that was significantly high on day 14 in PHLE-treated group (160 mg/kg) showed no significant alteration from the control and other groups on day 28. This, with the reduction in serum ALP level on day 28 is an indication that PHLE has no cholestatic effect since a rise in plasma alkaline phosphatase (ALP) level is usually a characteristic finding in cholestatic liver disease (Kaneko, 1989). On day 28, Serum AST level was significantly lower in the extract-treated groups (20 and 80 mg/kg), showing that there was no toxicity. On day 28 also, though the serum ALT level was significantly higher in the extract treated group (40 mg/kg), it was still within the normal range (Johnson-Delaney, 1996). This is an indication of the absence of hepatocellular damage. There was no significant change in serum total protein which is largely produced in the liver, this indicates absence of toxicity. The main source of creatinine in the body is Creatinine-phosphate. Baron, (1982) reported that creatinine is a marker of renal function and elevated concentration is often an indication of muscular dystrophy. The result of this study showed that creatinine level was not affected by the extract, an indication of no deleterious effect on the renal function.

5.1 Conclusions

In conclusion, the study justified the folkloric use of the plant as wound healing agent. The extract significantly decreased epithelialization period and increased the wound healing indices like: percentage wound contraction, wound breaking strength, granulation tissue formation and hydroxyproline concentration. This confirms its wound healing activity.

The best activity of the extract was seen at 4% concentration (for percentage wound contraction, epithelialization period and wound breaking strength) and 50 mg/kg for hydroxyproline
concentration. The study also showed no organ toxicity effect as evidenced in the sub-acute toxicity test.

5.2 Recommendations

i. From the study, the use of methanolic PHLE for post-surgical wound management and wound dressing is recommended.

ii. However, further studies to isolate/separate the active principle(s) in *P. hirsuta* responsible for its wound healing activity, and chronic toxicity test should be carried out.
REFERENCES


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107


Smith and Nephew (2012). Treatment options for Surgical Wounds. Htm


APPENDICES

Appendix 1: Table of values for graphs

Effect of PHLE on Percentage Wound Contraction

<table>
<thead>
<tr>
<th>Groups</th>
<th>I (Pet. Jelly)</th>
<th>II (Cicatrin® pwd)</th>
<th>III (1 % PHLE)</th>
<th>IV (2 % PHLE)</th>
<th>V (4 % PHLE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>day3</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>day6</td>
<td>18.39 ± 7.00</td>
<td>10.27 ± 1.20</td>
<td>31.49 ± 6.74(^b)</td>
<td>15.90 ± 5.47</td>
<td>26.85 ± 3.81(^b)</td>
</tr>
<tr>
<td>day9</td>
<td>54.36 ± 5.09</td>
<td>52.98 ± 5.35</td>
<td>72.97 ± 2.83(^{a,b})</td>
<td>52.56 ± 9.13</td>
<td>72.78 ± 2.83(^{a,b})</td>
</tr>
<tr>
<td>day12</td>
<td>69.64 ± 5.80</td>
<td>70.48 ± 6.64</td>
<td>82.33 ± 1.85</td>
<td>69.14 ± 9.54</td>
<td>83.43 ± 2.23</td>
</tr>
<tr>
<td>day15</td>
<td>74.34 ± 5.40</td>
<td>80.39 ± 4.06</td>
<td>87.43 ± 0.95</td>
<td>76.56 ± 8.00</td>
<td>90.73 ± 2.61(^a)</td>
</tr>
<tr>
<td>day18</td>
<td>82.67 ± 4.70</td>
<td>82.91 ± 3.85</td>
<td>90.17 ± 1.30</td>
<td>83.67 ± 7.01</td>
<td>95.18 ± 1.11(^a)</td>
</tr>
<tr>
<td>day21</td>
<td>87.00 ± 3.26</td>
<td>90.34 ± 3.07</td>
<td>96.77 ± 1.45(^a)</td>
<td>93.83 ± 2.85</td>
<td>100.00 ± 0.00(^{a,b})</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM (n=6)

\(^a\)p<0.05  when compared to control (group I), \(^b\)p<0.05  when compared to the standard drug (group II).
Appendix 2: Effect of PHLE on Epithelialization Period (days)

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Epithelization period (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Pet. Jelly)</td>
<td>29.83±4.29</td>
</tr>
<tr>
<td>2 (Cicatrin®)</td>
<td>29.67±2.56</td>
</tr>
<tr>
<td>3 (1 % PHLE)</td>
<td>24.00±1.10</td>
</tr>
<tr>
<td>4 (2 % PHLE)</td>
<td>25.83±2.18</td>
</tr>
<tr>
<td>5 (4 % PHLE)</td>
<td>21.33±0.21*</td>
</tr>
</tbody>
</table>

* P< 0.05 when compared with Pet. Jelly and Cicatrin®

Appendix 3: Effect of PHLE on Wound Breaking Strength

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Breaking strength (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Control (PJ)</td>
<td>391.94 ± 16.16</td>
</tr>
<tr>
<td>II</td>
<td>Cicatrin®</td>
<td>427.01 ± 15.95</td>
</tr>
<tr>
<td>III</td>
<td>1 % PHLE</td>
<td>451.49 ± 8.55^a</td>
</tr>
<tr>
<td>IV</td>
<td>2 % PHLE</td>
<td>550.58 ± 10.43^a</td>
</tr>
<tr>
<td>V</td>
<td>4 % PHLE</td>
<td>569.57 ± 4.44^a</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SEM (n=6)

^aP<0.05 when compared to control
### Appendix 4: Effect of PHLE on Level of Granulation Tissue

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Wet granulation (g)</th>
<th>Dry granulation (g)</th>
<th>Difference (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10 ml/kg 5 % DMSO</td>
<td>0.30 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>0.24</td>
</tr>
<tr>
<td>II</td>
<td>12.5 mg/kg PHLE</td>
<td>0.42 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.10 ± 0.00</td>
<td>0.32</td>
</tr>
<tr>
<td>III</td>
<td>25 mg/kg PHLE</td>
<td>0.47 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11 ± 0.01</td>
<td>0.36</td>
</tr>
<tr>
<td>IV</td>
<td>50 mg/kg PHLE</td>
<td>0.65 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.18 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.47</td>
</tr>
<tr>
<td>V</td>
<td>100 mg/kg PHLE</td>
<td>0.81 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.28 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.53</td>
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</tbody>
</table>

Values are expressed as Mean ± SEM (n=6)

<sup>a</sup>p<0.05 when compared to control

### Appendix 5: Effect of PHLE on Hydroxyproline Level

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>HDP content (mg/100 g dry tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10 ml/kg 5 % DMSO</td>
<td>394.68 ± 6.47</td>
</tr>
<tr>
<td>II</td>
<td>12.5 mg/kg PHLE</td>
<td>398.68 ± 5.34</td>
</tr>
<tr>
<td>III</td>
<td>25 mg/kg PHLE</td>
<td>433.32 ± 3.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IV</td>
<td>50 mg/kg PHLE</td>
<td>469.28 ± 2.68&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>V</td>
<td>100 mg/kg PHLE</td>
<td>460.00 ± 11.15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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</table>

Values are expressed as Mean ± SEM (n=6)

<sup>a</sup>p<0.05 when compared to control.
### Appendix 6: Effect of PHLE on Serum AST Activity

<table>
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<tr>
<th>Group</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 ml/kg 5% DMSO)</td>
<td>33.33 ± 17.25</td>
<td>38.00 ± 5.20</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>29.00 ± 9.87</td>
<td>23.00 ± 2.31*</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>31.67 ± 4.33</td>
<td>38.67 ± 1.45</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>50.33 ± 12.88</td>
<td>17.67 ± 4.63*</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>38.67 ± 16.46</td>
<td>31.67 ± 2.60</td>
</tr>
</tbody>
</table>

*p<0.05 when compared to control (group I).

### Appendix 7: Effect of PHLE on Serum ALT Activity

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 ml/kg 5% DMSO)</td>
<td>9.00 ± 1.15</td>
<td>6.67 ± 0.88</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>9.33 ± 3.93</td>
<td>5.33 ± 0.88</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>5.67 ± 1.20</td>
<td>10.00 ± 1.15*</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>8.33 ± 2.03</td>
<td>6.00 ± 0.58</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>9.00 ± 0.58</td>
<td>7.67 ± 0.33</td>
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</tbody>
</table>

*p<0.05 when compared to control (group I).

### Appendix 8: Effect of PHLE on Serum ALP Activity

<table>
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<tr>
<th>Group</th>
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<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 ml/kg 5% DMSO)</td>
<td>55.67 ± 4.41</td>
<td>48.00 ± 3.46</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>45.00 ± 5.51</td>
<td>37.33 ± 1.76</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>52.33 ± 6.12</td>
<td>43.00 ± 4.04</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>65.33 ± 12.77</td>
<td>26.00 ± 0.58*</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>64.00 ± 8.08</td>
<td>35.00 ± 7.51</td>
</tr>
</tbody>
</table>

*p<0.05 when compared to control (group I).
### Appendix 9: Effect of PHLE on Serum Total Protein

<table>
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<tr>
<th>Group</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 ml/kg 5 % DMSO)</td>
<td>5.55 ±0.17</td>
<td>6.40 ±0.12</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>6.74 ±0.56</td>
<td>6.13 ±0.35</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>6.48 ±0.05</td>
<td>6.57 ±0.03</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>6.64 ±0.71</td>
<td>6.30 ±0.29</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>5.67 ±0.08</td>
<td>6.10 ±0.17</td>
</tr>
</tbody>
</table>

*p<0.05 when compared to control (group I).

### Appendix 10: Effect of PHLE on Serum Creatinine Activity

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 ml/kg 5 % DMSO)</td>
<td>0.42 ±0.02</td>
<td>0.48 ±0.05</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>0.44 ±0.05</td>
<td>0.48 ±0.05</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>0.50 ±0.03</td>
<td>0.41 ±0.02</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>0.53 ±0.08</td>
<td>0.57 ±0.03</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>0.49 ±0.01</td>
<td>0.47 ±0.03</td>
</tr>
</tbody>
</table>

### Appendix 11: Effect of PHLE on Serum Total Bilirubin

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (10 ml/kg 5 % DMSO)</td>
<td>0.05±0.01</td>
<td>0.09±0.02</td>
</tr>
<tr>
<td>II (20 mg/kg PHLE)</td>
<td>0.06±0.01</td>
<td>0.07±0.02</td>
</tr>
<tr>
<td>III (40 mg/kg PHLE)</td>
<td>0.10±0.02</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>IV (80 mg/kg PHLE)</td>
<td>0.12±0.05</td>
<td>0.08±0.00</td>
</tr>
<tr>
<td>V (160 mg/kg PHLE)</td>
<td>0.16±0.04a</td>
<td>0.08±0.01</td>
</tr>
</tbody>
</table>

*ap<0.05 when compared to control (group I).*