COMPARATIVE EVALUATION OF SOYBEAN (*Glycine max L*) EXTRACTS AND OESTROGEN CREAM ON EXPERIMENTAL CUTANEOUS WOUND HEALING IN OVARIECTOMIZED ALBINO RATS

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BY

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DEPARTMENT OF VETERINARY SURGERY AND RADIOLOGY,

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ZARIA

NOVEMBER, 2018

Declaration

I declare that the work in this dissertation entitled **"Comparative evaluation of Soybean (*Glycine max L*) extracts and oestrogen cream on experimental cutaneous wound healing in ovariectomized albino rats"** had been carried out by me in the Department of Veterinary Surgery and Radiology, Ahmadu Bello University, Zaria, under the supervision of Dr. E. O. Abidoye and Dr. A. Y. Simon. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

Iskiil Oladehinde OYENEKAN

Name of student	Signature	date

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This dissertation entitled **"COMPARATIVE EVALUATION OF SOYBEAN (*GLYCINE MAX L*) EXTRACTS AND OESTROGEN CREAM ON EXPERIMENTAL CUTANEOUS WOUND HEALING IN OVARIECTOMIZED ALBINO RATS"** by Iskiil Oladehinde OYENEKAN, meets the regulations governing the award the degree of Master of Science of Ahmadu Bello University Zaria and is approved for its contribution to knowledge and literary presentation.

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Dedication

This dissertation is dedicated to my wife (Haneefat Olubukola Ajanaku) and my Children (Thaabit Oluwagbenga and Hafsah Omotayo) for their unending love, prayers and patience, throughout the course of this work.

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Abstract

This study was carried out to evaluate the comparative effects of topical soybeans extracts and oestrogen cream on cutaneous wound healing using excision wound model in ovariectomized Wistar albino rats. Twenty four (24) female Wistar albino rats of age 20 weeks weighing 200-250g were ovariectomised two weeks post acclimatisation and monitored for four weeks post-surgery for proper recovery. They were randomly divided into four groups (A, B, C, D) with 6 rats in each group. Two full-thickness skin excision measuring 1.5 x 1.5cm was surgically created at the dorsum of the thoraco-abdominal region of each rat. Wounds in all groups (A, B, C, D) were monitored and treated topically with normal saline, oestrogen cream, soybean crude-ethanolic extract and Soybean-chloroform extract respectively, for 20 days. Wound contraction was evaluated on day 4, 8 and 12 by wound tracing method after removing the scab while granulation tissue was harvested for histopathology, antioxidant assay, Marker of Oxidative Stress (Malondialdehyde (MDA)) and B-cell lymphoma 2 (Bcl-2) antiapoptotic protein assay on day 4, 8, 12 and 16. There was no significant ($p>0.05$) difference in the rate of wound contraction in the oestrogen cream (90.29 ± 0.75%) and soybean ethanolic extract (89.28 ± 0.95%) treated groups but both had a significant ($p < 0.05$) rate of wound contraction compared to normal saline treated group (64.56 ± 0.70%). Histopathology revealed that oestrogen cream and soybean ethanolic extract promoted formation of healthy granulation tissue, collagen deposition, fewer and regulated inflammatory cells infiltration, fibroblast proliferation and formation of new blood vessels. The wounds in normal saline, oestrogen cream, soybean crude-ethanolic extract and Soybean-chloroform extract treated groups healed on day 18.25 ± 0.25, 14.25 ± 0.25, 14.00 ± 0.00 and 16.25 ± 0.25 respectively.

Oestrogen cream and soybean ethanolic extract treated group had a significant ($p < 0.001$) increase in Reduced Glutathione (GSH) level (84.67 ± 2.34µg/ml, 82.27 ± 1.63 µg/ml respectively) on day 8 post wounding compared to normal saline (56.9 ± 8.09 µg/ml) treated group. Malondialdehyde (MDA) level reduced significantly ($p < 0.001$) on day 4 post wounding in oestrogen cream (77.37 ± 7.88 µmols/mgproteins), soybean ethanolic extract (90.13 ± 3.32 µmols/mgproteins), and soybean chloroform extract (86.60 ± 6.99 µmols/mgproteins) treated group compared to normal saline (180.97 ± 10.02 µmols/mgproteins). Also, there was significant ($p < 0.05$) increased expression of Bcl-2 antiapoptotic protein on day 4 and 8 of wound healing in the oestrogen cream with quick score (10.00 ± 1.00, 11.00 ± 1.00 respectively) and soybean ethanolic extract with quick score (8.00 ± 1.00, 10.00 ± 1.00 respectively) compared to normal saline control group with quick score (5.33 ± 0.67, 6.33 ± 1.45 respectively) indicating reduced cellular apoptosis during the proliferative phase of wound healing. In conclusion, this study demonstrated that topical treatment of cutaneous wound with soybean ethanolic extract had comparable efficacy to oestrogen in the management of wound in ovariectomized wistar albino rats.

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List of Abbreviations

DNA	Deoxyribonucleic Acid
FGF	Fibroblast Growth Factor
KGF	Keratinocyte Growth Factor
HRT	Hormone Replacement Therapy
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
EGF	Epidermal Growth Factor
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein-1
NOX	NADPH oxidase
OVX	Ovariectomised
PDGF	Platelet Derived Growth Factor
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
TGFβ	Transforming Growth Factor β
TNF-α	Tumour Necrosis Factor-α
VEGF	Vascular Endothelial Growth Factor
FRSE	Free Radical Scavanging Enzyme
SOD	Superoxide Dismutase

CAT	Catalase
GSH	Reduced Glutathione
Gpx	Glutathione Peroxidase
GST	Glutathione-S-Transferase
MIP	Macrophage inflammatory protein
Bcl-2	B cell Lymphoma-2
Bax	Bcl-2-like protein
Bad	Bcl-2 associated death receptor
Bak	Bcl-2 homologous antagonist/killer
Bcl-xL	B-cell-Lymphoma extra large
Bcl-w	Bcl-2-like-protein-2
ECM	Extracellular Matrix
NO	Nitric Oxide
PGE2	Prostaglandin E2
ERα	Oestrogen receptor ERα
ERβ	Oestrogen receptor ERβ
SERM	Selective Oestrogen Receptor Modulator
CEE	Conjugated Equine Oestrogen
ERT	Oestrogen Replacement Therapy
MMP-2	Matrix Metaloproteinase-2
SMA	Smooth Muscle Actin
RNS	Reactive Nitrogen Species
VSMC	Vascular endothelia smooth muscle

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

A wound involves the disruption (sequel to damage) of the normal continuity to an epithelial surface and its underlying connective tissues (Dunnill *et al*., 2017) and it is one of the most frequent reasons for seeking medical attention (Kokane *et al.*, 2009). An impaired wound healing is a major area of unmet clinical need that constitutes a substantial drain on global health services and leads to significant patient morbidity (Campbell *et al.*, 2010).Wound healing is an important process in replacement of tissue structure and function (Rubin and Farber, 1994; Reddy *et al*, 2008), restoration of dermal barrier and preventing infection following injury (Gilliver *et al.*, 2006). The healing is a sign of growth and an important biological phenomenon (Rahman *et al*., 2017). Wound healing phases involved activities of many cell types including epidermal keratinocytes, fibroblasts and so on (Rieger *et al*., 2015) which function in a number of overlapping events that include leukocyte recruitment, matrix deposition, epithelialization, and ultimately resolution of inflammation with scar tissue formation (Ashcroft and Mills, 2002; Gunay *et al.*, 2005; Sagliyan *et al.*, 2010; Wu and Chen, 2014). Healing is accompanied by the release of substances like eicosanoids, cytokines, prostaglandins, leukotrienes and reactive oxygen species (ROS) (Dwivedi *et al.*, 2017).

Among these substances, ROS play vital role in healing and serve as cellular messengers that drive numerous aspects of molecular and cell biology (Dwivedi *et al.*, 2017). Reactive oxygen species include hydroxyl radical (OH^-), superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) (Mohammad *et al.*, 2008). The OH^-, interacts with lipids, proteins and

nucleic acids resulting in loss of cell membrane integrity, structural or functional changes in protein and genetic mutations, respectively (Martin and Leibovich, 2005).

The classical view about the role of ROS in wound healing is to protect the host against invading bacteria and other micro-organisms (Sen and Roy, 2008; Sarsour *et al.*, 2009; Lande'n *et al.*, 2016). However, studies have suggested that cellular ROS are tightly regulated to prevent tissue damage and that they play a critical role as second messengers in regulation of cellular activities and tissue repair (Le Belle *et al.*, 2011; Morimoto *et al.*, 2013) such as wound healing process (Sen and Roy, 2008). They are used by number of cells responsible for wound healing, including platelets, macrophages, fibroblasts, endothelial cells and keratinocytes (Soneja *et al.*, 2005) where they modulate wound healing processes such as migration, adhesion, proliferation, neovascularization, remodeling, and apoptosis (Mohammad *et al.*, 2008).

Due to the underlying signaling and damage pathways, oxidative stress due to elevated and sustained ROS could result in disturbed wound healing (Schafer and Werner 2008; Suzuki *et al.*, 2012). Free-radical- scavenging enzymes (FRSE) are a cytoprotective enzymal group that has an essential role in the reduction, deactivation and removal of excessive ROS as well as in the regulation of the wound healing process (Kandemir *et al.*, 2013). These include both enzymatic such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, GSH-Px and glutathione-S-transferase (GST) and nonenzymatic such as glutathione, vitamin E components (Young and Woodside, 2001; Memisogullari *et al.*, 2003). Thus, without proper antioxidant activities, wound healing might be delayed through severe tissue damage or cellular apoptosis (Ponrasu *et al.*, 2013).

The presence of these free radicals can cause damage to the surrounding cells/ tissues around wound thereby worsen the healing process and induce apoptosis and necrosis (Houghton *et al.*, 2005; Hameedaldeen *et al.*, 2014). Apoptosis, a physiological cell death process, possesses very distinct features, and is almost different from necrosis which is characterised by morphological changes, including DNA fragmentation, membrane alteration, and formation of apoptotic bodies (Desmoulière, 1995). There is enormous evidence that the sequence of cellular events that characterize healing of cutaneous wound and other tissue repair processes is tightly regulated and controlled by a distinct temporal pattern of cellular apoptosis (Greenhalgh 1998). This pattern is responsible for the removal of inflammatory cells and the evolution of granulation tissue into scar tissue (Desmoulière, 1995). When the cell commits to apoptosis, the signal to activate death machinery is detected and transduced to downstream effectors (Mishra *et al*., 2008). Various regulators then carry out the apoptotic response (Bellamy *et al.*, 1995; Salvesen and Dexit, 1997) and finally the cell is removed by phagocytosis (Mishra *et al*., 2008). Thus, aberrant apoptosis can lead to abnormal wound healing by removing granulation tissue (Walker *et al.*, 1988). Research has showed that the rate of wound closure is associated with the extent of apoptosis in the wound: an increased rate of wound closure is associated with a reduced extent of apoptosis (Farahani and Kloth, 2008).

Several factors contribute to apoptosis, but the key elements are categorized in two main families of proteins including caspase enzymes and Bcl-2 family (Hornberry and Lazebink, 1998). As effectors of the apoptosis pathway, the Bcl-2 family of proteins either promotes or prohibits apoptosis. Proapoptotic proteins include Bax, Bad, and Bak whereas Bcl-2 and Bcl-xL, Bclw are antiapoptotic proteins or protooncogen (Levine, 1997; Skommer *et al.*,

2007). Overexpression of Bcl-2 leads to the inhibition of apoptosis which then contributes to neoplastic transformations (Hockenberry *et al.*, 1990; Reed, 1999). Moreover, expression of apoptosis-related marker such as Bcl-2 gives the new means of regulating the apoptosis pattern in wound healing (Bhan *et al.*, 2013).

Natural products have been proven to enclose wound healing property and their primary or secondary metabolites have been shown to contribute to the potential repair mechanisms including activation of immune epithelial cells, cytokines, extracellular matrix (ECM), reactive oxygen species (ROS), growth factors, and diferent inflammatory mediators (Tsala *et al.*, 2013).

Due to the lack of much side effects of plant (whole or extract) compared to synthetic drugs, approximately 60 % of the world's population relies almost entirely on plants for medication (Kandemir *et al.*, 2013). Medicinal plants continue to be an important therapeutic aid for alleviating the ailments of human kind (Sheela *et al.*, 2012). The use of plant-based strategies is opening a new perspective for the treatment of cutaneous wounds, mainly in developing countries, once it represents a simple, low cost, and affordable therapy (Wu *et al.*, 2012; Bahramsoltani *et al.*, 2014; Tan *et al.*, 2014; Gonc *et al.*, 2014; Sarandy *et al.*, 2015). Indeed, many plants have been shown to possess therapeutic potential as promoters of wound healing for example, *Jatropha curcas, Aloe barbadensis, Centella asiatica, Ficus bengalensis, Curcuma longa, Cynodon dactylon , Rubis cordifolia , Ficus recemosa , Glycyrrhiza glabra, Berberis aristata*, *Thevetia peruviana*, *Pongamia pinnata* (Villegas *et al.*, 1997; Shukla *et al.*, 1999; Esimone *et al.*, 2008; Gupta and Jain, 2010; Oryan *et al.*, 2010; Abdulla *et al.*, 2011; Al-Bayaty *et al.*, 2012; Cheng *et al.*, 2013; Hajiaghaalipour *et al.*, 2013: Rahman *et al.*, 2017; Dwivedi *et al.*, 2017).

Some of these plants have been screened scientifically for the evaluation of their wound healing activity in different pharmacological models and human subjects, but the potential of most of the plants remain unexplored (Ahmed *et al.*, 1995).

The soybean (*Glycine max (L.*) *Merr. Leguminosae*) is a species of legume that is native to East Asia and is considered a very important plant because of its phytochemical content (Uyar *et al.*, 2014). It is widely used as supplement in food industries and it is included in the formulations of many drugs (Uyar *et al.*, 2014). Soybean contain high amount of isoflavones, a class of organic compounds that are produced almost exclusively by members of the Fabaceae/Leguminosae (bean) family (Messina, 2010). Isoflavones are phytooestrogenic compounds that are structurally similar to oestrogenic steroids (Uyar *et al.*, 2014). The most important soy isoflavones are genistein, daidzein and glycitein (Uyar *et al.*, 2014).

It has been reported that isoflavone compounds possess antihaemolytic, antioxidative, antifungal, oestrogenic and antitumoral activities (Diplock, 1994; Rice-Evans *et al.*, 1996; Adlercreutz and Mazur, 1997; Onozawa *et al.*, 1999). Soy isoflavones have been demonstrated to possess many biological functions such as antioxidant (Kao and Chen, 2006), inhibition of cancer cell proliferation (Kao *et al.*, 2007a), anti-inflammatory activities (Kao *et al.*, 2007b), prevention of coronary heart diseases (Dalais *et al.*, 2003), as well as osteoporosis (Migliaccio and Anderson, 2003). In anti-inflammatory effects, soy isoflavones could decrease the secretions of interleukin-1 (IL-1), IL-6, nitric oxide (NO) and prostaglandin E2 (PGE2) in the cell supernatant and fluid of mouse peritoneal exudate (Kao *et al.*, 2007a). Soy isoflavones are considered to have beneficial effects on the skin via mechanisms such as the prevention of lipid oxidation, stimulation of fibroblast

proliferation, reduction of collagen degradation and inhibition of 5α -reductase and they have been widely used as ingredients in cosmetics (Uyar *et al.*, 2014). Soy isoflavones alone or in combination with other agents have many beneficial effects on the appearance and structure of skin (Skovgaard *et al*., 2006; Draelos *et al*., 2007; Izumi *et al*., 2007; Accorsi-Neto *et al*., 2009).

A significant characteristic of isoflavones is their capacity to bind both to oestrogen receptors (ERs) ERα and ERβ (Kuiper *et al.*, 1998) through their phenolic ring. Specific binding affinity to ERs enables isoflavones to elicit both oestrogenic and antioestrogenic effects depending on the tissue, as well as on isoflavone and endogenous estradiol levels (Makela *et al.*, 1995). Thus, isoflavones, as stress-induced phytochemicals, may provide health benefits through their selective oestrogen receptor modulator (SERM) activities (Cederroth and Nef, 2009).

Oestrogen, a class of steroidal hormones are produced in mammals majorly by the ovaries and responsible for many physiological activities that are important in homeostatic regulation of many cellular and biochemical events (Jyoti *et al.*, 2015). Based on *in vivo* (systemic and topical oestrogen administration) and *in vitro* studies, ooestrogen treatment accelerates wound healing through a number of mechanisms; re-epithelialisation, inflammatory response modulation, modulating cytokine expression, angiogenesis stimulation, enhancing matrix deposition and proteolysis regulation (Ashcroft *et al*., 1999a; Thornton, 2002; Ashcroft and Ashworth, 2003; Gilliver and Ashcroft, 2007; Pincus *et al* 2010; Al-Kadhimy and Ghani, 2015; Peržeľová *et al*., 2016; Zhou *et al*., 2016), while ooestrogen deprivation especially in the geriatrics results in delayed healing in geriatric patients (Tuttle *et al*., 2004; Engeland *et al*., 2006; Pincus *et al*., 2010). Also

studies have shown that conjugated equine oestrogen (CEE) administration resulted in 30 % increase in dermal thickness on skin biopsies as compared to placebo (Brincat *et al.*, 2005).

1.2 Statement of Research Problems

Reactive oxygen species (ROS) that arise after cutaneous injury may worsen the healing process and induce apoptosis (Hameedaldeen *et al.*, 2014; Şener *et al.*, 2015). Investigation of an appropriate therapy to cure wounds, giving consideration to the evaluation of various factors including oxidative stress and cellular apoptosis during the process of wound healing and their interactions with each other is necessary(Şener *et al.*, 2015).

Studies have shown that reduced systemic oestrogen levels in ovariectomized mice or menopausal women results in a markedly impaired rate of wound healing associated with excessive inflammation and scarring (Margolis *et al.*, 2002; Ashcroft *et al.*, 2003). Meanwhile, it has been reported that oestrogen replacement substantially accelerates healing in both aged humans and oestrogen-depleted animal models (Ashcroft *et al.*, 1997; Margolis *et al.*, 2002).

In both mice and humans, oestrogen signals through two related but distinct nuclear hormone receptors, oestrogen receptor (ER) α and β, which function as either homo- or hetero-dimers (Campbell *et al.*, 2010). The receptors, which share >95% homology in their DNA- binding domain, are differentially expressed throughout mammalian tissues. Agonists selective for each oestrogen receptor are currently being used *in vivo* to elucidate the specific roles of ER isoforms in a range of tissues (Campbell *et al.*, 2010). In light of the detrimental side-effects of long term oestrogen treatment in post-menopausal women,

specifically increased cancer incidence, there is currently much interest in the development of oestrogenic alternatives (Campbell *et al.*, 2010). Selective oestrogen receptor modulators (SERMs) have been, and are currently being, developed that differentially bind ERs with tissue-specific agonist/antagonist profiles (Campbell *et al.*, 2010) and one of such is soy isoflavones. To our knowledge, the comparative effects of topical soybean extract and conjugated oestrogen on apoptosis marker (Bcl-2) and parameters of oxidative stress have not been investigated in the cutaneous excision wound healing process.

1.3 Justification

Our ageing population combined with an increase in cardiovascular and neurological diseases and diabetes, implies that there is an increasing number of patients presenting with these chronic and often complicated wounds (Gosain and Dipietro, 2004; Brem and Tomic-Canic, 2007; Guo and Dipietro, 2010). Consequently, the manipulation of obtunded healing processes due to severe complication in these individuals is required to reduce the morbidity, mortality, hospital resource and economic cost currently associated with this increasing health burden (Posnett and Franks, 2008; Sen *et al.*, 2009).

Many of the synthetic drugs currently used for the treatment of wounds are not only expensive but also pose problems such as allergy, drug resistance and so on, and this situation has forced the scientists to seek alternative drugs (Sai and Babu, 1998). Efforts are being made all over the world to discover agents that can promote healing and thereby reduce the cost of hospitalization and save the patient from amputation or other severe complications (Omale and Isaac, 2010). More than 80% of the world population still depends upon traditional medicines for their ailments (Kumara *et al.*, 2001), especially for wound management (Purna and Babu, 2000), as they provide a moist environment to

encourage the establishment of the suitable environment (Omale and Isaac, 2010). Many medicinal plants are claimed to be useful for wound healing in the traditional system of medicine though their mechanism of action and efficacy have not been evaluated scientifically (Omale and Isaac, 2010).

Beneficial effects of oestrogen on wound healing have been established. However due to its long-term effects, phytooestrogens are being explored as an alternative to the oestrogen replacement therapy (ERT) because of the similarity in chemical structures with estradiol (Vaya and Tamir, 2004). Meanwhile, Soybeans are the most common source of isoflavones and represent the most common group of phytooestrogens found in human foods which are structurally similar to estradiol-17, the most potent mammalian oestrogen (Setchell, 1998; Teekachunhatean *et al.*, 2013; Şener *et al.*, 2015). Thus, comparing the effects of soybean extract and oestrogen in regulating oxidative stress and apoptotic parameters will enhance our knowledge on the importance of oestrogen as well as benefits of soy-phytooestrogen as SERM during wound healing.

1.4 Aim and Objective of the Study

1.4.1 Aim of the study

The broad aim of this study was to evaluate the comparative effects of topical soybean extracts and oestrogen cream on experimental cutaneous wound healing in ovariectomized Wistar albino rats

1.4.2 Objectives of the study

The objectives of these studies were:

1. To evaluate grossly the comparative effects of topical Soybean extract and ooestrogen cream on cutaneous wound healing in Wistar albino rats
2. To evaluate the comparative effects of topical Soybean extract and ooestrogen cream on granulation tissue histopathology during wound healing in Wistar albino rats
3. To evaluate the comparative effects of topical Soybean extract and ooestrogen cream on oxidative stress during wound healing in Wistar albino rats.
4. To evaluate comparative effects of topical Soybean extract and ooestrogen cream on expression of anti-apoptotic (Bcl2) protein during wound healing in Wistar albino rats.

1.5 Research questions

1. Does topical application of ooestrogen and Soybean extract have any effect on gross appearance of wound healing process in in Wistar albino rats?
2. Is there any comparative effect of topical Soybean extract and ooestrogen cream in wound healing in Wistar albino rats?
3. Are there comparative effects of topical Soybean extract and ooestrogen cream on oxidative stress during wound healing in Wistar albino rats?
4. Do topical Soybean extract and ooestrogen cream affect expression of pro-apoptotic (Bcl2) protein during wound healing in Wistar albino rats?

CHAPTER TWO

2.0 INTRODUCTION

2.1 The Skin Structure

The skin is the outermost organ of the mammalian body which serves as defense system against the external environment (Lee *et al.*, 2006; Emmerson, 2010). Skin is the largest multi-functional organ of the body which plays important roles in sensation, thermoregulation, biochemical, metabolic, and immune functions (Wysocki, 1999; Zhao *et al.*, 2016). The skin in animals consists of four distinctly recognisable layers: the epidermis, the basement membrane, the dermis and the subcutaneous layer. It houses nerve endings, vasculature, hair follicles, sweat glands, sebaceous glands and nails (Gould, 1996). Murine (mice and rats) skin is composed of a far thinner epidermis (Sougrat *et al.*, 2002) and a dermis populated with hair follicles (Trainer and Alexander, 1997). In human and mouse skin the subcutaneous layer lies directly above a striated muscle layer, the *panniculus carnosus* (Emmerson, 2010). A crucial species difference is that rodents have loose skin, as do many other mammals, whereas humans, most primates and pigs do not (Dorsett-Martin, 2004).

2.1.1 The epidermis

The epidermis, the most superficial layer of skin is composed of four histologically distinct layers; the *stratum basale*, the *stratum spinosum*, the *stratum granulosum*, *stratum lucidum* and the *stratum corneum* (Madison, 2003; Elias, 2005). The lowest layer, the *stratum basale*, is attached to the basement membrane, the junction between the epidermis and the dermis, by hemidesmosomes, and comprises a single layer of columnar cells with elongated nuclei (Emmerson, 2010). *The stratum spinosum* lies immediately over the

stratum basale and consists of a number of rows of spinous cells, named so because of their histological spiny appearance under a light microscope, due to desmosomes between the cells. Above the *stratum spinosum* lies the *stratum granulosum*, which consists of one to three layers of angular shaped and flattened keratinocytes, with darkly stained nuclei and containing basophilic granules called keratohyaline granules, which contain filaggrin (Emmerson, 2010). The *stratum corneum* makes up the outermost layer of the epidermis and is made up of annucleate, cornified dead keratinocytes, rich in keratin (Clark, 1996). The *stratum corneum* acts as the primary barrier to stop percutaneous water loss and entry of micro-organisms (Elias, 2005). There are four different cell types that comprise the majority of the epidermis; stratified keratinocytes, the primary cell of the epidermis; melanocytes, which are the pigment producing cells and contribute to UV protection (Wickett and Visscher, 2006); Langerhans cells which have a role in immune surveillance and; Merkel cells which are involved in sensation (Clark, 1996).

2.1.2 The basement membrane

The basement membrane is the separating structure between the epidermis and the dermis. It contains proliferative granular cells and mediates the passage of chemical signals between the dermis and the epidermis (Clark, 1996) while acting as a mechanical barrier (Liotta *et al.*, 1980). It tightly anchors the epidermis and dermis together by interactions between hemidesmosomes and anchoring filaments and fibrils, while also providing support for the epidermis, resistance to shearing forces and determines the polarity of skin growth (Bruckner-Tuderman, 1999). The basement membrane is mainly composed of α6β4 integrin, laminin5, collagens IV and VII and elastic fibres that stretch across the basement

membrane, directly linking the epidermis to the dermis (Weber *et al.*, 1982; Leigh *et al.*, 1988).

2.1.3 The dermis

The dermis which forms the majority of the skin contains a network of mechanically strong fibres, comprised of collagens I and II (Epstein and Munderloh, 1978), elastin, polysaccharides and very few cells (Emmerson, 2010). Of these cells, most are fibroblasts, but mast cells, macrophages, lymphocytes, and melanocytes are also present (Emmerson, 2010). Fibroblasts are the primary cellular source of collagens and elastins (Wood, 1985). The dermis extends from the *lamina densa* of the basement membrane to the subcutaneous layer and contains various skin appendages including sweat glands, apocrine glands, sebaceous glands, hair follicles and nails and supports the epidermis (Marieb and Hoehn, 2007).

The dermis is composed of two layers: papillary and reticular (James *et al.*, 2005). The outer papillary layer is thin and contains loosely woven elastin and collagen fibres and a large number of blood vessels. The collagen fibers (mainly types I and III) are produced by fibroblasts, are arranged parallel to the skin surface, and are responsible for the main mass and tensile strength of skin (Brincat *et al.*, 2005). In contrast, elastin fibers are arranged as a thinly distributed sub-epidermal network and provide the skin with elasticity and resilience (Brincat *et al.*, 2005). The reticular layer comprises the lower 80% of the dermis and is composed of dense, disorganised connective tissue, mainly collagen fibres, primarily arranged in planes parallel to the skin surface (Emmerson, 2010).

2.1.4. The hypodermis

The hypodermis or subcutaneous layer is the deepest layer of the skin, composed mostly of mesenchymally derived adipose tissue (Emmerson, 2010). The hypodermis comprises two layers: the panniculus adiposus and the panniculus carnosus (Rook and Burns, 2004). The *panniculus adiposus* is comprised of adipose tissue while the *panniculus carnosus* is composed of striated muscle fibres (Spence and Mason, 1992).

2.2 Cutaneous Wound Repair

The epidermis and dermis of normal skin maintain a steady-state equilibrium to ensure a protective barrier against the external environment (Su *et al.*, 2010; Wang *et al.*, 2014; Ogawa, 2017). When the protective barrier is broken by trauma, radiation, chemical injury, and/or burns, the wound healing process is immediately set in motion and complex biochemical events take place to repair the damage (Profyris *et al.*, 2012; Butzelaar *et al.*, 2016; Zhang *et al.*, 2016; Janis and Harrison, 2016; Hofer *et al.*, 2017). The wound healing process is usually characterized as four sequential but overlapping phases: haemostasis (0–several hours after injury), inflammation (1–3days), proliferation (4-21days) and remodelling (21days–1year) (Reinke and Sorg, 2012; Walmsley *et al.*, 2015; Berman *et al.*, 2017; Hrosley and Watt, 2017; Plikus *et al.*, 2017; Seo *et al.*, 2017; Yannas *et al.*, 2017;). Deregulation of any of these steps results in impaired healing, e.g., chronic hard-to-heal ulcers or excessive scarring, which presents a major and increasing health and economic burden to our society (Mustoe *et al.*, 2006; Sen *et al.*, 2009).

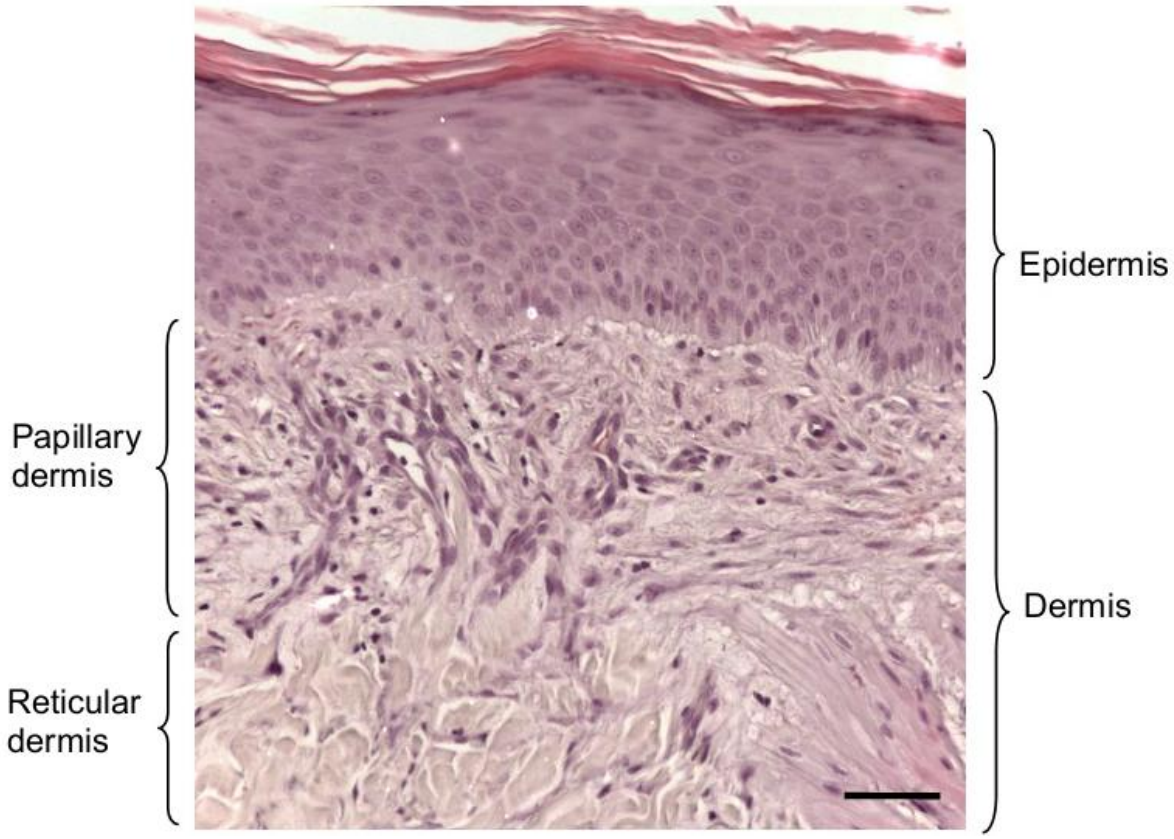


Plate 2.1 Structure of the rat skin (Emmersion, 2010)

2.3. Phases of Wound Healing

2.3.1 Haemostasis phase

Haemostasis is activated immediately after injury to the skin in a normal individual. The exposed sub-endothelium, collagen, and tissue factor will activate platelet aggregation, which leads to degranulation and releasing chemotactic and growth factors (GF), such as proteases, platelet-derived GF, and vasoactive agents (histamine and serotonin) (Martin; 1997; Woo *et al.*, 2004; Landen *et al.*, 2016; Brockmann *et al.*, 2017). Clot formation is occurred, thus achieving successful hemostasis (Horng *et al.*, 2017). The clot seals the injury site and prevents further bleeding and infection (Cohen, 1992). Apart of being the main player in blood clotting, hemostasis also provides a matrix scaffold for cells recruited to the site of injury, specifically fibrin in connection with fibronectin, where it acts as a scaffold for monocytes, fibroblasts and other different cell players (Tonnesen *et al.*, 2000; Landen *et al.*, 2016; Zhao *et al.*, 2016). A variety of cytokines and growth factors are released into the wound during this stage, mediating the communication and synergizing the activity of different cell players to accomplish the task of healing (Landen *et al.*, 2016)

2.3.2 Inflammatory phase

Inflammation is an essential, nonspecific, innate immune response involving the breakdown of tissue and clean-up of cellular, extra-cellular and pathogen debris (Zhao *et al.*, 2016). The phase which is actually run concurrently with hemostasis, proceeds for 48 to 72h and is thus described as the early stage of wound healing (Eming *et al.*, 2014; Horng *et al.*, 2017). The innate inflammatory cells involved in this phase of healing response include neutrophils, macrophages, mast cells, dendritic cells, eosinophils and basophils

(Noble and Jiang, 2006). Both platelets and leukocytes release inflammatory cytokines that provide a chemotactic gradient for additional leukocytes to enhance the inflammatory process (Zhao *et al.*, 2016). These include interleukin (IL)-1, IL-6, IL-8, tumour necrosis factor (TNF)-, platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-. Notably, PDGF plays an important role in initiating the chemotaxis of neutrophils, monocytes, smooth muscle cells and fibroblasts, whereas TGF-stimulates cytokine secretion from macrophages and enhances fibroblast and smooth muscle cell chemotaxis (Diegelmann and Evans, 2004). In uninfected wound, initial leukocyte response is dominated by neutrophils for the first two to five days, with macrophages taking over from approximately day three in response to chemokines, complement and by-products of bacterial degradation (Reinke and Sorg, 2012; Sinno and Prakash, 2013; Brockmann *et al.*, 2017; Berman *et al.*, 2017; Horng *et al.*, 2017 Plikus *et al.*, 2017). Neutrophils have three main functions. Firstly, they generate free radicals via the myeloperoxidase pathway to kill bacteria (Goldman, 2004). They also debride the wound through secretion of proteolytic enzymes that break down nonviable tissue (Zhao *et al.*, 2016). These include proteases with broad substrate specificity such as serine proteases or with narrow specificity such as matrix metalloproteinase (MMP)-2 and -9 that degrade collagen and collagen fragments (Xue and Jackson, 2013). Lastly, neutrophils phagocytose the dead bacteria and matrix debris (Zhao *et al.*, 2016). Neutrophils also produce cytokines, e.g., tumor necrosis factor (TNF)-a, interleukin (IL)-1b and IL-6, to amplify the inflammatory response (Eming *et al.*, 2014). They usually undergo apoptosis once their tasks are completed and are mopped up by macrophages (Zhao *et al.*, 2016). Approximately 3 days after injury, monocytes are recruited to the injury site, where they differentiate into macrophages and support healing

(Landen *et al.*, 2016). Monocytes continue to enter the wound site in response to chemo-attractants [for example, Macrophage Chemoattractant Protein (MCP) -1, Macrophage Inflammatory Protein (MIP) -1 and Growth-Related Oncogene α (MIP-2 in mouse) (Werner and Grose, 2003)]. Fragments of ECM, including collagen, elastin, fibronectin, thrombin, and Transforming Growth Factor β (TGF-β) stimulate circulating monocytes to attach to the endothelium of blood vessels and migrate through the blood vessel wall into the tissue (Werner and Grose, 2003). As monocytes immigrate into the wound and transform into macrophages, they become the most important regulatory cell in the inflammatory reaction and facilitate inflammatory–proliferative phase transition during wound healing (Li *et al.*, 2007; Emmerson, 2010; Landen *et al.*, 2016). The highly phagocytic macrophages remove any non-functional host cells, bacterial-filled neutrophils, damaged matrix, foreign debris, and remaining bacteria (Diegelmann and Evans, 2004; Horng *et al.*, 2017). Activated macrophages secrete further growth factors, chemokines and cytokines such as TGF-, TGF-, basic fibroblast growth factor (bFGF), PDGF, and vascular endothelial growth factor (VEGF) to amplify and eventually resolve inflammation—recruiting endothelial cells and fibroblasts to initiate the next phase of healing (Zhao *et al.*, 2016). While inflammation is vital in clearing infection and debris, prolonged inflammation may results in increased scarring or chronic wounds (Zhao *et al.*, 2016).

2.3.3 The proliferative phase

Proliferative/Granulation tissue formation phase starts about 4 days post-injury (Halloran and Slavin, 2002). In the proliferation phase, the provisional wound matrix formed during haemostasis is replaced by granulation tissue, consisting of a large amount of fibroblasts,

granulocytes, macrophages, blood vessels, in complex with collagen bundles, which partially recovers the structure and function of the wounded skin (Schultz and Wysocki, 2009). Fibroblasts play a central role in the formation of the granulation tissue, which migrate mainly from the nearby dermis to the wound in response to cytokines and growth factors, e.g., PDGF, transforming growth factor (TGF)-b and bFGF, produced by platelets and macrophages in the wounds (Schultz and Wysocki, 2009; Hinz *et al.*, 2007). After migrating into the provisional wound matrix, fibroblasts proliferate and produce proteinases, e.g., matrix metalloproteinases (MMPs), to degrade provisional matrix (Gill and Parks, 2008; Nissinen and Kahari, 2015); while depositing collagen and other extracellular matrix (ECM) components, e.g., proteoglycans , hyaluronic acid, glycosaminoglycans, and fibronectin, to form granulation tissue (Xue and Jackson, 2015), which fill up the wound gap and provide a scaffold for cell adhes ion, migration, growth and differentiation during wound repair (Eckes *et al.*, 2010; Barker, 2011). Additionally, the recruited fibroblasts differentiate into myofibroblasts to achieve wound contraction by cytokines and/or molecules and GFs, including platelet-derivedGF, fibroblastGF, nerveGF, transforming GF-(TGF-, including TGF-1,TGF-2,andTGF-3), connective tissue GF (CTGF), cysteine-rich 61 (Cyr61), interleukins (IL, including IL-6, IL-8, and IL-10), homeobox 13, osteopontin, and early growth response protein 1 (Syeda *et al.*, 2012; Profyris *et al.*, 2012; Driskell *et al.*, 2014; Ojeh *et al.*, 2015; Zhang *et al.*, 2016; Janis and Harrison, 2016; Mateu *et al.*, 2016; Lindley *et al.*, 2016; Cohen et al, 2017; Gerarduzzi and di Battista, 2017; Darby and Weller, 2017).

2.3.3.1 Re-epithelialisation: As the inflammation subsides, proliferation becomes a major theme with the focus on bridging the wound edges (i.e., re-epithelialization), restoring the

vascular network and forming granulation tissue (Landen *et al.*, 2016). Re-epithelialisation commences as keratinocytes and epithelial stem cells proliferate and migrate over a fibrin/fibronectin-rich provisional ECM (Reinke and Sorg, 2012; Martin, 1997). It is initiated by cell-to-cell contact signalling that indicates the absence of neighbouring cells at the wound edge, termed "free edge effect" (Matsuzaki *et al.*, 2004). To generate more cells to cover the wound, keratinocytes at the basal layer of the wound edge and epithelia stem cells from nearby hair follicles or sweat glands start proliferating approximately 2–3 days after injury (Lau *et al.*, 2009). Epidermal growth factor (EGF), Keratinocyte growth factor (KGF) and Transforming growth factor beta 1 and 2 (TGFβ-1 and -2) secreted from multiple cell types in the wounds (Barrientos *et al.*, 2008) stimulate keratinocyte proliferation and migration (Matsuzaki *et al.*, 2004) while Matrixmetalloproteinases (MMPs) produced by the damaged keratinocytes at the wound edge cleave collagen in the basal lamina, allowing cell migration (Salo *et al.*, 1994). Migration is triggered by loss of contact inhibition and physical tension at cell adhesion structures (Landen *et al.*, 2016). Migration stops when the cells get in contact and new adhesion structures are formed. Keratinocytes secrete proteins to rebuild the basement membrane (Jacinto *et al.*, 2001). Re-epithelialization is essential for the re-establishment of tissue integrity (Horng *et al.*, 2017). After the completion of re-epithelialization, represented by keratinocytes from wound edge and neighboring adnexal structures migrating across the wound surface, apoptosis of myofibroblasts then occurs, and the skin defect is closed (Horng *et al.*, 2017).

2.3.3.2 Angiogenesis: This is the formation of new blood vessels in tissue (neovascularization), during development or in response to injury or tumour angiogenic factors. Within a couple of days of wounding, angiogenic capillary sprouts

invade the wound clot and the granulation tissue. Angiogenesis and fibroplasia occur simultaneously, providing oxygen, nutrients, and a much hydrated matrix to sustain the high levels of cellular activity (Zhao *et al.*, 2016). The process is initiated by growth factors, e.g., VEGF, PDGF, basic fibroblast growth factor (bFGF), and the serine protease thrombin in the wounds, which activate the endothelial cells of existing vessels (Li *et al.*, 2003). The upregulation of vascular endothelial growth factors (GFs) all provide a structural framework for endothelial cells to proliferate and lay down new vessels by the process of angiogenesis during this phase (Syeda *et al.*, 2012; Berman *et al.*, 2017; Cohen *et al.*, 2017).

2.3.3.3 Wound contraction: This is the centripetal movement of the wound edges, resulting in a reduction in wound size. Dermal fibroblasts at the wound edges start to proliferate after injury and in 3-4 days post-wounding, they begin to migrate into the wound provisional matrix (Martin, 1997). During intermediate stages of healing, 7-14 days post-wounding, a proportion of the recently invaded fibroblasts take on a myofibroblast phenotype, characterised by large bundles of microfilaments, which contain F-actin, along the plasma membrane and at cell-cell and cell-matrix linkages (Welch *et al.*, 1990).

Wound contraction involves two major mechanism: (1) organization and stiffening of the ECM (Ehrlich and Hunt, 2012) and (2) cellular contraction of myofibroblasts (Gabbiani *et al.*, 1971; Tomasek *et al.*, 2002). Myofibroblasts are specialized contractile fibroblasts largely responsible for extracellular matrix (ECM) remodeling and contraction in mature granulation tissue (Klingberg *et al.*, 2013). Myofibroblast differentiation occurs in response to growth factors and mechanical stress and is characterized by the formation of

large stress fibers and focal adhesions (Gabbiani *et al.*, 1971; Desmouliere *et al.*, 1993; Tomasek *et al.*, 2002; Hinz, 2007). One prominent feature of myofibroblast differentiation is the expression of -Smooth Muscle Actin (-SMA) in stress fibers, and this is considered to be the basis for their high contractility (Gabbiani *et al.*, 1971; Tomasek *et al.*, 2002).

2.3.4 Remodelling and maturation phase

Contraction and remodelling constitute the last phase of wound healing (Zhao, 2016). Tissue remodelling involves the transition from the provisional matrix, including the fibrin clot, to a collagenous scar. Alteration of the provisional matrix of fibrin and collagen involves lysis of the clot, deposition of fibronectin and proteoglycans and collagen remodelling, and can take up to six to nine months or up to a year to complete (Profyris *et al.*, 2012; Walmsley *et al.*, 2015; Janis and Harrison, 2016; Berman *et al.*, 2017). Mechanical tension and cytokines, e.g., TGF-b, drive fibroblasts to differentiate into myofibroblasts, which express a -smooth muscle actin (SMA) and contract the wound (Hinz, 2007) via their integrin receptor contacts to ECM components such as fibronectin and collagen (Darby *et al.*, 1990; Werner and Grose, 2003). The following are critical for normal wound healing: the orientation of type I collagen, the regression of the immature vessels, and the activation of myofibroblasts (Ashcroft *et al.*, 2013; Cheng *et al.*, 2013; Huang *et al.*, 2014; Berman *et al.*, 2017). At this phase, the quickly produced collagen III in the ECM is replaced by the collagen I, which has a higher tensile strength but takes longer time to deposit (Zhao *et al.*, 2016).

The end of healing is heralded by apoptosis of vascular cells and myofibroblasts as their work is completed, converting the cell rich granulation tissue into a collagen filled, hypocellular scar (Desmouliere *et al.*, 1995; Xue and Jackson, 2015). The final actin-rich

cells and reorganization of mature blood contribute to normal wound healing (Horng *et al.*, 2017). Once the wound is closed there is very little risk of infection, but the scar is cellular and highly vascularised, appears pigmented and is elevated (Clark, 1996). Some skin components, e.g., hair follicles and sweat glands, cannot be recovered after serious injury; and the healed skin can only achieve maximum 80% of the original tensile strength (Schilling, 1976). A keloid or hypertrophic scar may be formed when the phases of wound healing are disrupted, particularly granulation tissue formation and resolution (Ogawa, 2010). The distinguishing histological feature between keloid scars and hypertrophic scars is the presence of thick collagen bundles in keloid scars that are absent in hypertrophic scars (Ogawa *et al.*, 2009). The balance between production of TGFβ-1, -2 and -3 has a major impact on fibrosis and scarring (Yu *et al.*, 2005).

2.4 Oxidative Stress and Wound Healing

Oxidative stress and nitrosative stress is the harmful effect of free reactive oxygen species (ROS) and reactive nitrogen species (RNS) radicals respectively, leading to potential biological damage (Kovacic and Jacintho, 2001; Valko *et al.*, 2001; Ridnour *et al.*, 2005). Partially reduced and highly reactive O_2 species (ROS) (including superoxide anion $\cdot O_2^-$, peroxide $\cdot O_2^{2-}$, hydrogen peroxide H_2O_2, hydroxyl radicals $\cdot OH$ and hydroxyl OH^- ions) are often generated during mitochondrial oxidative phosphorylation and other electron transfer reactions (Rahal *et al.*, 2014). Reports have shown that in cellular homeostasis ROS plays vital roles (Dunnil *et al.*, 2017). This is because berrantly low levels of ROS induce cell cycle arrest (i.e. are cytostatic), basal ROS levels maintain normal cell function and homeostasis; increased amounts induce a number of transcription factors to drive a cell-mediated defence response while excessive ROS induction activates pro-apoptotic

proteins for subsequent induction of cell death, and in extreme cases, cellular necrosis (Liu *et al.*, 2002; Inoue *et al.,* 2003; Trachootham *et al.*, 2008; Shen and Pervaiz, 2009; Rahal *et al.*, 2014;). Thus, increased and/or sustained levels of ROS and other mediators of oxidative stress play a significant role in pathogenesis of disease conditions including; atherosclerosis, diabetes, cardiovascular diseases, cancer, neuro-degenerative diseases as well as in chronic inflammatory and fibrogenic diseases involving chronic activation of wound healing (Novo and Parola, 2012; Rahal *et al.*, 2014).

2.4.1 Mechanism of oxidative stress in wound healing

Oxygen is needed in virtually every step of wound repair as the energy needed during biosynthesis, intracellular transportation and cell movement relies on adenosine triphosphate (ATP), which is most efficiently synthesized in an oxygen-dependent manner (André-Lévigne *et al.*, 2017). Oxygen also plays pivotal roles in the wound healing process, such as oxidative bacterial killing, collagen synthesis, angiogenesis, and epithelialization (Sen, 2009; Schreml *et al.*, 2010). During wound repair, high proliferation rates and the production of extracellular matrix (ECM) components (most notably collagen) increase the demand for energy and thus for oxygen (Hunt *et al.*, 1969). The intense uptake and use of oxygen (O_2) during healing leads to tissue hypoxia and the levels of available O_2 influence the tissue repair response (Dunnil *et al.*, 2017). As the vascular supply is constricted in the early stages of the wound-healing response (mediated by ROS release), there is further reduction in O_2 bioavailability and with resultant exacerbated hypoxic state (Dunnil *et al.*, 2017). While oxygen is utilized to produce energy via oxidative phosphorylation, ROS are produced and cause oxidative injury (Kurahashi and Fujii, 2015). Low partial pressures of blood O_2 reduce the rate of the mitochondria energy

production, and many endogenous enzymes that utilise O_2 deplete ROS from the area (Dunnil *et al.*, 2017). This may deplete O_2 levels to an extent that ROS mediate responses are affected as its generation relies on O_2 presence (Sen, 2009). Several cells utilize these radicals, during the wound-healing response including platelets, macrophages, fibroblasts, endothelial cells and keratinocytes (Soneja *et al.*, 2005). However, elevated and sustained ROS have been detected in vivo and have been associated with impaired wound repair in chronic, non-healing wounds (Schafer and Werner, 2008). In fact, it is clear that the precise balance between low versus high levels of ROS is critical in determining functional outcome: low levels of ROS are essential in stimulating effective wound healing (Rodriguez *et al.*, 2008), whereas excessive ROS release results in cellular damage and impaired wound repair (Ponugoti *et al.*, 2013; Kurahashi and Fujii, 2015).

2.4.2 Roles of ROS during phases of wound healing

2.4.2.1. The role of ROS and redox signaling during the coagulation phase

Activation and recruitment of platelets following vessel-wall injury leading to blood coagulation usually occurs concomitantly with a sharp increase of ROS production at the wound site (Gregg *et al.*, 2004), suggesting that redox signaling regulates wound repair as early as during the coagulation phase (Roy *et al.*, 2006). Platelets have been shown to produce ROS (Salvemini and Botting, 1993), and the upregulation of tissue factor expression by activated platelets is mediated through ROS generation by NOX enzymes in vascular smooth muscle cells (VSMCs) (Gorlach *et al.*, 2000; Herkerl *et al.*, 2002). When platelets come into contact with collagen they commence production of ROS in a NOX-dependent manner, which results in increased platelet recruitment (Krotz *et al.*, 2002). Platelet-derived growth factor (PDGF), released by platelets upon activation, plays an

important role in the recruitment and proliferation of various cells during wound repair and is dependent on H_2O_2 (Sundaresan *et al.,* 1995).

2.4.2.2. The role of ROS and redox signaling during the inflammation phase

Inflammatory phase of wound-healing involves invasion of wound site by neutrophils and macrophages. Upon arrival of neutrophils and macrophages at the wound site, they start to secrete large amounts of ROS along with pro-inflammatory cytokines (Goldman, 2004) and proteolytic enzymes such as matrix metalloproteinase (MMP) (Gill and Parks, 2008). Nicotinamide Adenine dinucleotide Phosphate (NADPH) oxidase (NOX2) is expressed at high levels in plasma membranes of inflammatory cells and activated during phagocytosis, which results in the production of large amounts of superoxide radical anions (Darr and Fridovich, 1994; Bedard and Kraue, 2007). The isoform NOX2 plays a predominant role in microbial destruction as it is responsible for producing large amounts of ROS from neutrophils and macrophages in what is called the respiratory burst (Inoguchi *et al.*, 2003; Schafer and Werner, 2008; Kanta, 2011). The increase in the concentration of superoxide and hydrogen peroxide is transient as the neutrophil and macrophage migration diminishes and as a result of the action of antioxidative defense systems (auf dem Keller *et al.*, 2006). High levels of SOD gene expression are found at early stage of wound repair (auf dem Keller *et al.,* 2006). Fibroblasts secrete ROS when they are stimulated with inflammatory cytokines in-terleukin-1 or TNF-alpha or with growth factors EGF and PDGF (Meier *et al.*, 1991).

The generated ROS by neutrophils and macrophages directly attack invading pathogens, and finally destroy them to aid phagocytosis (Kurahashi and Fujii, 2015). Inside

phagosomes, high concentrations of ROS create a toxic oxidative stress environment for phagocytozed microbes leading to DNA damage, lipid peroxidation and oxidation of amino acids (Brooker, 2012). As phagocytic macrophages and neutrophils destroy invading microbes in their phagosomes, they release high concentrations of H_2O_2, and this stunts growth of adjacent, contaminating bacteria (Nathan and Root, 1977; Fang, 2011) and removal of infectious agents which is essential in preventing complicated, chronic wounds a role played by ROS (Dunnil *et al.*, 2017). If the inflammatory phase does not resolve in time and the concentration of ROS exceeds the antioxidant capacity of the cell, the condition called oxidative stress results (Martindale and Holbrook, 2002). Oxidative stress mediated by radical ROS (superoxide anion, hydroxyl radical) and nonradical ROS (hydrogen peroxide, singlet oxygen) may inhibit cell migration and proliferation and cause tissue damage and perpetuation of inflammation (Steiling *et al.*, 1999; Wlaschek and Scharffetter-Kochanek, 2005).

2.4.2.3 The role of ROS and redox signaling during the proliferation phase

It is increasingly accepted that redox-signaling plays a central role during angiogenesis (Sen and Roy, 2008; Bretón-Romero and Lamas, 2014). Moderate levels of H_2O_2 upregulate the production of the vascular endothe lial growth factor (VEGF), the key angiogenic growth factor, in keratinocytes, and result in accelerating angiogenesis (Sen *et al.*, 2002; Roy *et al.*, 2006). The expression of VEGF increases within hours after keratinocytes, fibroblasts, and endothelial cells at the wound site are exposed to hypoxia (Berse *et al.*, 1992; Brown *et al.*, 1992). It seems that NOX enzymes are the major source of ROS during wound angiogenesis and that they are produced by a wide range of cells, including inflammatory cells, vascular endothelial cells, fibroblasts and epithelial cells

(Roy *et al.*, 2006; Ushio-Fukai, 2006). It is thus conceivable that NOX enzymes are critical for angiogenesis (André-Lévigne *et al.*, 2017). The notion that redox signaling plays an important role in angiogenesis is supported by evidence suggesting that a large number of antioxidants limit angiogenesis (Sen and Roy, 2008). This includes Vitamin C (Ashino *et al.*, 2003), quercetin (Jackson and Venema, 2006), resveratrol (Lin *et al.*, 2003), and several others.

Re-epithelialization is an important process in wound healing for effective wound closure (André-Lévigne *et al.*, 2017). H_2O_2 triggers the activation of receptors for epidermal growth factor (EGF) and the keratinocyte growth factor (KGF) (Gooldkorn *et al.*, 1998; Marchese *et al.*, 2003) and induces the production of TGF α (a member of EGF) in fibroblasts (Vivekananda *et al.*, 1994). Migrating keratinocytes dissect the wound, separating eschar from viable tissue, producing MMPs and activating plasmin by plasminogen activators (Bugge *et al.*, 1996). These MMPs (MMP-1, MMP-2 MMP-9 etc) are important constituent of the matrix-degrading apparatus of keratinocytes and are activated by either NOX4 or H_2O_2 through different pathways (Schaffer *et al.*, 1997; Yoon *et al.*, 2002; Ridgway *et al.*, 2005; Grange *et al.*, 2006; Lund *et al.*, 2006).

In order to guarantee rapid wound closure, wound contraction is of utmost importance. Wound contraction involves two major mechanisms: (1) organization and stiffening of the ECM (Ehrlich and Hunt, 2012) and (2) cellular contraction of myofibroblasts (Tomasek *et al.*, 2002; Gabbiani *et al.*, 1971). NOX2 might play a role in myofibroblast differentiation (André-Lévigne *et al.*, 2017), as a recent study found that oxidants produced by NOX2 mediate a-SMA and ECM upregulation in human dermal fibroblasts in response to TGF1 (Zhang *et al.*, 2014). It has been reported that TGF1 induces H_2O_2 production in human

fibroblasts (Thannickal and Fanburg, 1995). Also ROS produced by NOX4 were shown to be a prerequisite for TGF1-induced myofibroblast differentiation, ECM production and contractility in lung-derived fibroblasts (Hecker *et al.*, 2009).

2.4.2.4 The role of ROS and redox signaling during the maturation phase

After wound closure, controlled cell removal and ECM remodeling are crucial to terminate repair (André-Lévigne *et al.*, 2017). Myofibroblasts, as well as vascular and inflammatory cells, physiologically disappear by apoptosis and activities of proteolytic enzymes (mainly MMPs) secreted by macrophages, epidermal cells, endothelial cells, and fibroblasts degrade ECM components (Desmouliere *et al.*, 1995; Fluck *et al.*, 1998; Niland *et al.*, 2001). Cell persistence can lead to hypertrophic scarring, associated with increased levels of TGF1, -SMA expression, ECM deposition, and vascularization (Wang *et al.*, 2007; Sarrazy *et al.*, 2011). The role of NOX enzymes in apoptosis has been intensively investigated, especially in the fields of cancer and cardiovascular research, where strong implications of NOX enzymes in the regulation of apoptosis have been reported (Meitzler *et al.*, 2014). NOX-mediated ROS production has been repeatedly associated with different fibroblast growth factor signaling pathways and is likely to play a direct role in fibroblast apoptosis during scar maturation (Zhao *et al.*, 2009).

2.4.3 Roles of ROS in impaired/chronic wound healing

Chronic wounds are in the great majority of cases associated with a chronic state of inflammation (Rosner *et al.*, 1995). It has been shown that the proliferation phase can only effectively be initiated if the number of macrophages diminishes (André-Lévigne *et al.*, 2017). If macrophages persist, the inflammatory phase is prolonged and the proliferation

phase delayed (Newton *et al.*, 2004). ROS play an important role in wound repair via redox signaling (André-Lévigne *et al.*, 2017). The majority of ROS during wound repair are most likely being produced by neutrophils and macrophages during the inflammatory phase (Wlaschek and Scharffetter-Kochanek, 2005). As chronic wounds enter a state of chronic inflammation, the amount of ROS remains high over a prolonged period of time, with subsequent cell damage further accelerating inflammation (Wlaschek and Scharffetter-Kochanek, 2005). In fact, oxidative stress in chronic wounds seems to be dramatically increased when compared to acute wounds (James *et al.*, 2003). The senescence of fibroblasts is increasingly discussed as a hallmark in the development of chronic ulcers (André-Lévigne *et al.*, 2017). Oxidative stress has been suggested to be one possible explanation of how fibroblasts could enter a senescent state in chronic wounds (Clark, 2008), as high levels of oxidative stress lead to premature senescence in fibroblasts in culture (Von Zglinicki, 2002; Song *et al.*, 2005).

2.4.4 Antioxidant-mediated regulation of cellular ROS level

Low and normal levels of ROS play important roles in wound repair and signal transduction for reepithelialization and proliferation of cells, such as the collagenase activity and the epidermal growth factor signaling (Şener *et al.*, 2015). However, higher levels of ROS can cause oxidative stress and may damage intracellular macromolecules such as DNA, lipids, and proteins (Şener *et al.*, 2015). Therefore, regulating oxidative stress and the inflammatory response is important during the cutaneous wound healing process (Schäfer and Werner, 2008; Hameedaldeen *et al.*, 2014). In order to survive, aerobic organisms have developed evolutionary conserved mechanisms and strategies to carefully control generation of ROS and other oxidative stress-related mediators to

maintain intracellular redox homeostasis and to use these reactive intermediates to modulate signal transduction, gene expression and cellular responses (Cadenas and Davies, 2000; Thannickal and Fanburg, 2000; Dröge, 2002; D'Autrèaux and Toledano, 2007; Novo and Parola, 2008). The homeostatic control of cellular ROS levels (redox state) is the role of a specialist group of proteins known as antioxidants (Dunnil *et al.*, 2017). The utilization of antioxidants has been considered as an effective therapeutic approach in wound healing (James *et al.*, 2001; Sharifi *et al.*, 2012). The antioxidants are a system of proteins designed to remove the deleterious effects of ROS, and they do so by donating their own electrons, thus preventing them from capturing electrons from other important molecules, such as DNA, proteins and lipids (Dunnil *et al.*, 2017). Known members of the antioxidant system are Thioredoxin-1 (Trx-1) and −2 (Trx-2), glutathione (GSH)-related glutathione s-transferases (GSTs), superoxide dismutase (SOD), glutathione peroxidases (GPx), NADP(H) quinone oxidoreductase (NQO1), catalase, epoxide hydrolase, heme oxygenase-1 (HO-1), UDP-glucuronosyl transferases (UGTs) and glutamylcysteine synthetase (Dunnil *et al.*, 2017). In addition to proteinaceous ROS control, cells also utilise non-enzymatic metabolites that are small antioxidant molecules, such as vitamin C, vitamin E, β -carotene, glutathione, coenzyme Q, bilirubin, α-tocopherol, nicotinamide adenine dinucleotide phosphate (NADPH) and urate (Dunnil *et al.*, 2017). Moieties with a metal ion capable of oxidation/reduction reactions, such as transferrin and ferritin, possess an increased ROS-scavenging capability (Bryan *et al.*, 2012; Novo and Parola, 2012; Kurahashi and Fujii, 2015).

Expression of GPx increased after wounding with maximal mRNA levels being found between days 3 and 5 after injury (auf dem Keller *et al.*, 2006). At 14 days after the injury,

when the wound has completely healed, expression of this enzyme would have almost declined to the basal level (auf dem Keller *et al.,* 2006). By contrast, expression of catalase was not altered by skin injury (Steiling *et al.*, 1999). The increased mRNA expression of several ROS-scavenging enzymes observed after skin injury seems to be in contrast to findings of other investigators (Shukla *et al.*, 1997), who found reduced SOD and GPx activities in wound tissue between days 2 and 7 after cutaneous injury in rats (auf dem Keller *et al.,* 2006). The discrepancy between these results may be due to inhibition of the enzymatic activities of these enzymes at the wound site by the high levels of ROS (Pigeolet *et al.,* 1990; Vessey and Lee, 1993). Given the obvious importance of ROS detoxification in healing skin wounds, it seems likely that reduced levels of ROS-detoxifying enzymes result in healing impairments, a hypothesis, which is supported by the observation of reduced activities of SOD, catalase, and GPx in wounds of aged rats compared to young rats (Anamika and Shukla, 2000); by the beneficial effect of antioxidants on wound healing in ischemic rat skin (Senel *et al.,* 1997) and by the restoration of the delayed wound healing seen in diabetic mice by adenoviral delivery of Mn-SOD to the wound site (Luo *et al.,* 2004).

2.5 Apoptosis in Wound Healing

2.5.1 Apoptosis and BCL2 family protein

Apoptosis is an active process of programmed cellular destruction with distinctive morphological and biochemical features (Ding and Yin, 2009). Apoptosis was formally described and named in 1972 as a unique morphological response to many different kinds of cell stress that was distinct from necrosis (Shamas-Din *et al.,* 2013). Two major apoptotic pathways have been defined in the mammalian cells: the extrinsic or death

receptor pathway and the intrinsic pathway-also called the Bcl-2-regulated or mitochondrial pathway pathway (Ding and Yin, 2009; Johnson *et al.,* 2012). Several factors contribute to apoptosis, but the key elements are categorised into two main families of proteins including caspase enzymes and Bcl-2 family (Hornberry and lazebnik, 1998). The Bcl-2 is a protoonco-gene which is capable of inhibiting apoptosis or programmed cell death, and it encodes a 25 kDa protein, which can be found on inner mitochondrial membrane, nuclear envelope, and endoplasmic reticulum (Tsujimoto *et al.*, 1984; Hockenbery *et al.*, 1990).

The Bcl-2 family proteins are the most important regulators of the mitochondrial pathway (Youle and Strasser, 2008). This pathway predominantly leads to the activation of caspase-9 (Hakem, 1998) but, at least in certain cell types, the intrinsic pathway can proceed in the absence of caspase-9 or its activator, apoptotic protease-activating factor-1 (APAF1) (Marsden, 2002). They work primarily by binding to other related proteins that regulate permeabilization of the mitochondrial outer membrane (MOM) (Shamas-Din *et al.*, 2013). Signals from the death receptor pathway could also be bridged to the mitochondrial pathway via the Bcl-2 family proteins. The BCL-2 (B-cell lymphoma-2) gene was discovered at the t (14;18) chromosome translocation breakpoint in B-cell follicular lymphomas, where its transcription becomes excessively driven by the immunoglobulin heavy chain gene promoter and enhancer on chromosome 14 (Tsujimoto *et al.*, 1985; Cleary *et al.*, 1986). In mammals, there are at least 12 core BCL-2 family proteins, including BCL-2 itself and proteins that have either three-dimensional (3D) structural similarity or a predicted secondary structure that is similar to BCL-2 (Youle and Strasser, 2008). This family of proteins consists of both antiapoptosis and proapoptosis members

(Chipuk *et al.*, 2010). While the proapoptosis members serve as sensors to death signals and executors of the death program, the antiapoptosis members inhibit the initiation of the death program (Ding and Yin, 2009).

BCL-2 family members have classically been grouped into three classes (Youle and Strasser, 2008). One class inhibits apoptosis (BCL -2, BCL-XL, BCL-W, MCL1 , BCL-B (also known as BCL -2L10) and A1 (also known as BCL-2A1), whereas a second class promotes apoptosis (BAX , BAK and BOK (also known as MTD)) (Youle and Strasser, 2008). A third divergent class of BH3 - only proteins (BAD, BI K (also known as BLK or NBK), BID, H r K (also known as death protein-5 (DP5)), BI M (also known as BOD), BMF, NOXA and P uM A (also known as BBC3)) have a conserved BH3 domain that can bind and regulate the anti-apoptotic BCL-2 proteins to promote apoptosis (Youle and Strasser, 2008).

Bcl-2, the prototype of the Bcl-2 family proteins, was the first defined molecule involved in apoptosis (Ding and Yin, 2009). In the mitochondrial apoptotic pathway, the release of the mitochondrial apoptotic proteins results from an increase in the outer membrane's permeability, which may be due to opening of the pore formed by Bax or Bak, the mitochondrial permeability transition pore, or a pore made from components of the two (Ding and Yin, 2009). The mitochondrial dysfunction is often characterized by the mitochondrial depolarization and ROS generation, which are in part contributed by the loss of cytochrome c. Bcl-2 family protein could regulate cell cycle progression from G0/G1 to the S phase, which can be inhibited by the antideath molecules Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 and the adenoviral Bcl-2 homologue, E1B19K, but promoted by the prodeath molecules Bax (Knudson et al, 2001), Bad (Chattopadhyay *et al.*, 2001), and Bid (Bai *et*

al., 2005). This has been demonstrated in different cell types, including lymphocytes, hepatocytes, and fibroblasts (Ding and Yin, 2009). Immunohistochemical expression of Bcl-2 has been reported in several lymphatic, neurogenic, and epithelial neoplasms (van Golen *et al.*, 2000; Monni *et al.*, 1997; Munakata *et al.*, 2000). Alterations of apoptosis and cells proliferation have been implicated in wound healing process (Appleton *et al.*, 1996). Expression of apoptosis-related marker such as Bcl-2 gives the new mean of regulating the apoptosis pattern in diabetic and nondiabetic wound healing of rat model of diabetes (Bhan *et al.*, 2013)

2.5.2 Roles of apoptosis in wound healing

Tissue repair involves a series of events that involve rapid increases in specific cell populations to perform a specific task such as inflammation or collagen deposition (Greenhalgh, 1998). When each event is completed the effector cells must leave the wound to allow for the start of the next event in wound healing. Each subsequent phase involves increases in other cell populations that by necessity have to gradually decrease in number. Eventually, a mature wound ends up quite acellular and avascular. The cells that disappear from a wound can leave by one of three mechanisms: necrosis, emigration or apoptosis (Greenhalgh, 1998). Apoptosis is the most logical mechanism of cell elimination since it is energy efficient (cells do not have to migrate) and it does not lead to inflammation (Greenhalgh, 1998). Apoptosis or programmed cell death is the universal pathway for the elimination of unneeded cells and tissues in a phagocytotic process that does not elicit an inflammatory response. After eliminating invading organisms and nonviable tissue, inflammatory cells would be expected to undergo apoptosis and be removed without inciting more in inflammation. Likewise, when enough collagen has been deposited (at the

end of the proliferative phase) fibroblasts would start to undergo apoptosis. Finally, upon the completion of wound maturation, endothelial cells and remaining fibroblasts would silently disappear (Greenhalgh, 1998).

2.5.3 Roles of apoptosis in various phase of wound healing

Apoptosis is involved in the resolution of various phases of tissue repair. There is mounting evidence that the sequence of cellular events that characterizes healing of cutaneous wounds and other tissue repair processes is tightly regulated by a distinct temporal pattern of cellular apoptosis (Green-halgh, 1998). The early phase of inflammation is characterized by the invasion of neutrophils, macrophages, and lymphocytes to the wound area (Şener *et al.*, 2015). The fibroblasts then migrate and synthesize extracellular matrix components (Şener *et al.*, 2015). Inflammatory cells must be removed in order to begin this next phase of wound healing (Şener *et al.*, 2015). Remodeling of granulation tissue during the wound healing process is also accompanied by the apoptosis of fibroblasts (Rai *et al.*, 2005; Wu and Chen, 2014). It has been discussed that this rapid increase in cell proliferation is allowed by an initial decrease of apoptosis (Bhan *et al.*, 2013). Later, when the inflammatory process begins to shut down with wound closure and scar evolution, there is a dramatic decrease of cellularity, which has been clearly shown to be mediated by an increase of apoptotic cell death (Desmoulière *et al.*, 1995, 1997). Dysregulation in these apoptotic processes may result in abnormal wound healing, such as hypertrophic scars and keloid formation (Greenhalgh, 1998), or may delay wound healing (Deveci *et al.*, 2005; Blakytny and Jude, 2006).

2.5.3.1 Inflammatory phase

In the early phases of tissue repair, inflammatory cells underwent apoptosis starting as early as 12h after wounding and peak at the 4th day after wounding but slowly decreased afterwards (Brown *et al.*, 1997; Greenhalgh, 1998; Wu and Chen, 2014). Apoptotic inflammatory cell are usually localized beneath the leading edge of the migrating epithelium. Brown *et al.* (1997) reported that while the granulation tissue that was immediately next to the wound edge and covered with an epithelium had no labeling for apoptosis and tended to have less inflammatory cells and more fibroblasts. Most neutrophils entering a wound perform their task of eliminating microorganisms, undergo apoptosis and are rapidly and efficiently phagocytosed by macrophages in a process that does not elicit further inflammation (Riwaldt *et al.*, 2017). The mechanisms of neutrophil apoptosis have started to be elucidated by many researchers (Ohata *et al.*, 1994; Greenhalgh, 1998). In vitro studies suggest that TNF-α is involved in the signaling of neutrophil apoptosis (Ohata *et al.*, 1994). Macrophages also undergo apoptosis, although less has been published as to the mechanisms of the process (Greenhalgh, 1998).

2.5.3.2 Proliferation and maturation phase

Animal studies provide information on the role of apoptosis in eliminating fibroblasts in the later stages of tissue repair (Greenhalgh, 1998). Desmouliere *et al.* (1995) reported that apoptosis in myofibroblasts usually began on day 12, peak at 20days and resolved by 60days. These findings suggest that myofibroblast apoptosis begins about the same time as wound closure (Wu and Chen, 2014). Apoptosis patterns in the cells of the neovasculature similarly started at around day 12 and had maximal expression of apoptosis between 16

and 25days (Greenhalgh, 1998). The parallel apoptosis patterns of fibroblasts and endothelial cells suggests that there is a coordinated decrease in cellularity with wound maturation that may involve cell to cell communication (Greenhalgh, 1998). It appears that if the signal to down-regulate fibroblast and myofibroblast activity is delayed beyond a specific time point then apoptosis is permanently impaired. The extra fibroblast activity leads to an imbalance between collagen synthesis and degradation leading to excessive scar formation (Greenhalgh, 1998).

The regulation of keratinocyte apoptosis has received more attention in investigations of dermatologic diseases. Keratinocytes exposed to ultraviolet light (sunburn) rapidly underwent apoptosis (Raskin, 1997). Several signals have been found to be increased in response to ultraviolet light including p53 and fas (Raskin, 1997; Sung *et al.*, 1997). The loss of basement membrane attachment will also lead to apoptosis in a process associated with a decrease in bcl-xL expression (Gniadecki *et al.*, 1997; Rodeck *et al.*, 1997).

2.5.4: Bcl-2 protein and wound healing

Majority of the reports involving roles of Bcl-2 protein have been related to its role in cancer development with few report on tissue repair. However, there is recent paradigm shift as to its roles in wound healing especially during skin cancer management (Bhan *et al.*, 2013; Riwaldt *et al.*, 2017). In a report, Kane and Greenhalgh (2000) compared the expression of p53, Bcl-2, and apoptosis between normal and diabetic mice for 42 days and reported that during wound healing in normal mice, there is an inverse relationship between Bcl-2 and p53 over time (Kane and Greenhalgh, 2000). Upon injury, Bcl-2 expression was increased, while that of p53 decreased, in order to allow proliferation of cells which is an important step for wound repair (Kane and Greenhalgh, 2000; Bhan *et al.*,

2013). During the time course, BCL2 levels were down-regulated, while contrarily the p53 mRNAs were up-regulated to reduce the proliferative response (Riwaldt *et al.*, 2017). The levels of both p53 and Bcl-2 decreased over the days from 21 to 42, and the peak of apoptosis did not occur until day 14 (Bhan *et al.*, 2013).

2.6 Oestrogen Signaling and Skin Physiology

2.6.1 Oestrogen

Oestrogens, discovered by Allen and Doisy (1923) (Korach, 1994), are a group of steroid compounds which function primarily as female sex hormone. There exist three major forms of oestrogens in humans (Fig 2.1) including estradiol, estrone, and estriol, with estradiol being the predominant form (Tsai *et al.*, 2016; Tsui *et al.*, 2016; Leblanc *et al.*, 2017; Casarini *et al.*, 2017; Parodi *et al.*, 2017). Both estradiol and estrone are secreted by the granulosa cells of the ovaries (Lai *et al.*, 2014; Tsui *et al.*, 2017; Wang *et al.*, 2017; Worku *et al.*, 2017) during premenopausal periods, while small amounts of estrone, particularly in post-menopausal women, are produced by the conversion of androstenedione, secreted by the adrenal glands, in fatty tissue of the female body, the liver and the breasts (Emmerson, 2010). However, apart from ovarian oestrogen production, skin have been found to also involve in the synthesis of oestrogen (Mukai *et al.*, 2016), presenting a concept termed “intracrinology” (Wilkinson and Hardman, 2017).

2.6.2 Oestrogen receptors signaling

The first human ER, now called ERα, was cloned in 1985 (Green *et al.*, 1986). In 1996 a second human oestrogen receptor was discovered from a prostate cDNA library, now named ERβ (Kuiper *et al.*, 1996). Therefore, classical ER is referred to as ER α and the

new one has been named ERß. Major differences in the tissue and cell distribution of ER α and ERß have been well documented (Pelletier *et al.,* 2000). Oestrogen actions are regulated by binding to oestrogen receptor (ER)-α or ER-β receptors, which then subsequently interact with oestrogen-responsive elements (EREs) on target gene sequences for transcriptional induction or repression (Kuiper *et al.,* 1996; Enmark and Gustafsson, 1999; Rieger *et al.,* 2015).

Sex steroid hormones are involved in the regulation of skin development and function, with both ERs expressed in skin (Horng *et al.,* 2017). However, receptor distribution varies with body site: (Miller and Mac Neil, 1997; Wilkinson and Hardman, 2017). The recent ER localization studies have shown that ERß is widely expressed in human skin and its appendages, whereas ER α is only found in very few subsets of structures (Thornton *et al.,* 2003b). Some studies agree that ERβ is the predominant isoform in keratinocytes of human scalp skin (Mosselman *et al.,* 1996; Thornton *et al.,* 2003a, b), while in keratinocytes isolated from neonatal foreskin both ERα and ERβ protein are present, but in the case of ERα only with induction by estradiol (Verdier-Sevrain *et al.,* 2004). Both isoforms are expressed in hair follicles and sebaceous glands (Mosselman *et al.,* 1996; Thornton *et al.,* 2003a; Thornton *et al.,* 2006), while eccrine and apocrine glands express ERβ only (Mosselman *et al.,* 1996; Thornton *et al.,* 2003a; Beier *et al.,* 2005). Both ER isoforms are expressed in fibroblasts (Haczynski *et al.,* 2002), keratinocytes (Stevenson and Thornton, 2007), macrophages (Ashcroft *et al.,* 2003b) and neutrophils (Yu *et al.,* 2006) which act as a source of adult stem cells critical to the repair process (Ito *et al.,* 2005; Langton *et al.,* 2008). The differential expression of the two ER isoforms throughout tissues of the body is increasingly being exploited for therapeutic use: an

excellent example of which is the use of Selective Oestrogen Receptor Modulators (SERMs) and phytooestrogens, which rely on ligands binding to the ERs and exhibiting selective tissue specific activity (Klotz *et al.*, 2000; Lee *et al.*, 2013; Ahangarpour *et al.*, 2016; Chen *et al.*, 2016; Leblanc *et al.*, 2017).

2.6.3 Oestrogen signaling and wound healing

The recognition of the importance of oestrogen in skin physiology would suggest it that may have an important role in wound healing, and a number of studies are available to provide evidence that topical and systemic application of oestrogen or its precursor might have an important role in all phases of wound healing by modifying the inflammatory reaction, accelerating re-epithelialization, stimulating granulation formation, regulating proteolysis, and balancing collagen synthesis and degradation (Mills *et al.*, 2005; Stevenson and Thornton, 2007; Hardman and Ashcroft, 2008; Archer, 2012; Thornton, 2013; Midgley *et al.*, 2016; Mukai *et al.*, 2016; Brufani *et al.*, 2017; Chenu *et al.*, 2017; Pepe *et al.*, 2017; Leblanc *et al.*, 2017; Wilkinson and Hardman, 2017). It has been shown that primary oestrogen deficiency contributes to cutaneous aging and delayed wound repair or impaired wound healing (Margolis *et al.*, 2002; Mukai *et al.*, 2016; Horng *et al.*, 2017; Wilkinson and Hardman, 2017). Women who received hormone replacement therapy were less likely to develop a venous leg ulcer (age-adjusted relative risk (RR) 0.65, 95% confidence interval (CI) 0.61–0.69) or a pressure ulcer (RR 0.68, 95% CI 0.62–0.75) than those who did not use hormone replacement therapy, according to a case-cohort study in the UK General Practice Research Database (Margolis *et al.*, 2002).

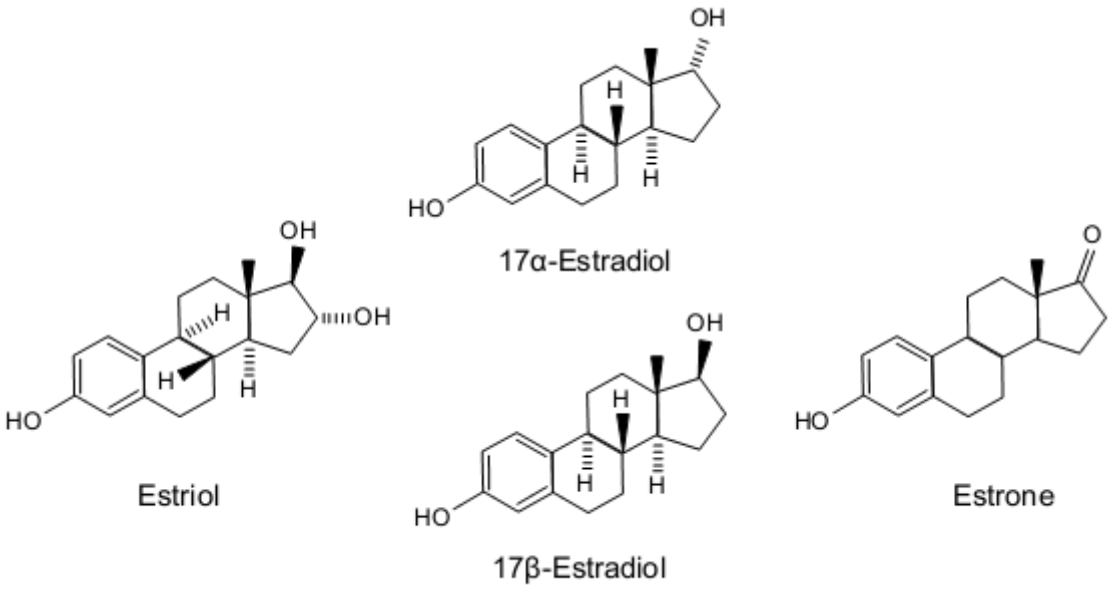


Figure 2.1: Major forms of oestrogen (Emmerson, 2010).

2.6.3.1 Oestrogen signaling on hemostasis/inflammation Process

The first step of wound healing is immediate hemostasis and a prompt initiation of the inflammatory process (Horng *et al.*, 2017). These conditions are mediated by local activation of the coagulation system, hematopoietic system, inflammatory cells, and immune system (Horng *et al.*, 2017). Coagulation systems, including coagulation factors, are significantly influenced by oestrogen signaling transduction (Koh *et al.*, 1997; Canonico, 2014; Chen *et al.*, 2016). Therefore, oestrogen deficiency might slow down the activation of coagulation and subsequently impair the immediate hemostasis, which initiates the wound healing process, suggesting the important role of oestrogen in hemostasis during wound healing (Horng *et al.*, 2017).

During the following inflammation process, the aggregation of megakaryocytes, leukocytes, monocytes, macrophages, lymphocytes, and mast cells is needed (Horng *et al.*, 2017). These immune cell populations as well as hematopoietic progenitors in bone marrow express ERs, suggesting that oestrogen directly affects the functions of these cells, including cytokines, in growth factor production (Murphy *et al.*, 2009; Cooper *et al.*, 2015; Gubbls Bupp, 2015; Kovats, 2015; Khan and Ansar, 2016; Laffont *et al.*, 2017;). Delay in wound healing has been associated with an excessive and prolonged inflammatory phase and increased pro-inflammatory cytokines (Ashcroft and Ashworth, 2003). Findings suggest that oestrogen attenuate the inflammatory phase of wound repair with adverse effects observed in hypooestrogenemia (Ashcroft *et al.*, 1999). Oestrogen signaling pro-inflammatory cytokine production is varied by the cell type and local oestrogen concentration (environmental factors) (Horng *et al.*, 2017). Oestrogen therapy can reduce the number of wound neutrophils, and reduce the neutrophil adhesion molecule L-selectin,

leading to diminished neutrophil localization at sites of inflammation (Ito *et al.*, 1995; Emmerson, 2010; Mukai *et al.*, 2016). Also oestrogen receptors are expressed by both neutrophils and macrophages, with many reports demonstrating that E2 can exert anti-inflammatory activities (Ashcroft *et al.*, 1997; Vegeto *et al.*, 1999; Vegeto *et al.*, 2001; Hardman *et al.*, 2008). Administration of oestrogen can increase wound fibronectin levels, as well as reduce elastase activity and lessen the degradation of fibronectin in wound tissue (Ashcroft *et al.*, 1999; Ashcroft *et al.*, 2000; Cooper *et al.*, 2015). In vivo oestrogen treatment in humans is associated with a reduction in inflammatory cell influx to the wound site while in vitro oestrogen treatment prevents the ability of excessive neutrophil accumulation at sites of inflammation (Ashcroft *et al.*, 1999). Oestrogen has long since been believed to have direct effects on monocytes and macrophages, due to the presence of oestrogen receptors (Gulshan *et al.*, 1990), a result that has more recently been supported (Harkonen and Vaananen, 2006; Murphy *et al.*, 2009). *In vivo* Ovx mice have increased macrophage infiltration to wounds, indicating a role for oestrogen in the control of excessive inflammation (Ashcroft *et al.*, 2003b). Oestrogen down-regulates the expression of pro-inflammatory cytokines, including TNF-α, Monocyte Chemotactic Factor (MCP-1), Interleukin (IL) -1β and -6, and, of particular importance, Macrophage Migration Inhibitory Factor (MIF), a cytokine that plays a major role in the beneficial effects of oestrogen on wound healing and repair. TNF-α and IL-1β expression is reduced after estradiol treatment in an animal model of stroke (Zhang *et al.*, 2010), while in post-menopausal women oestrogen deficiency increases T cell secretion of IL-1, IL-6 and TNF-α (Pfeilschifter *et al.*, 2002). E2 also enhances the production of basic fibroblast growth factor (bFGF) and transforming growth factor1 (TGF-1) in fibroblasts

(Ashcroft *et al.*, 1997; Fujimoto *et al.*, 1997). Although oestrogen is important for wound healing, the effects of oestrogen on wounds should be a balancing act, since both low and high levels of oestrogen will slow inflammation, and over-inhibition of inflammation is not good for wound healing (Horng *et al.*, 2017).

2.6.3.2 Oestrogen signaling on the proliferation and remodeling

The proliferative process of wound healing involves profuse fibroblasts, keratinocytes, and endothelial cells, as well as an accumulation of ECM (Berman *et al.*, 2017; Plikus *et al.*, 2017). All these cells contain ERs. Cells with low-level differentiation potential, for example mesenchymal cells, have the ability to stimulate tissue renewal (Horng *et al.*, 2017). The fibroblast is the key mesenchymal cell type in connective tissue and deposits the collagen and elastic fibers of the ECM, critical for wound healing (Horng *et al.*, 2017). Aging and oestrogen deficiency result in defects in fibroblast differentiation and functionality associated with impaired hyaluronan synthase 2 and epidermal growth factor receptor function, as a result of upregulated microRNA-7 expression, which mediates the over-activation of JAK/STAT1 (Brufani *et al.*, 2017). Oestrogen is important for the proliferation, migration, and differentiation of fibroblasts (Brufani *et al.*, 2017), which is primarily mediated by ER (Billon *et al.*, 2017). Similar to the impact of oestrogen on fibroblasts, oestrogen also imparts potent mitogenic effect on keratocytes, promoting in vitro and in vivo migration and improving re-epithelialisatiion of the wound (Verdier-Sevrain *et al.*, 2004; Son *et al.*, 2005; Zhou *et al.*, 2016; Mukai *et al.*, 2016). Stimulation of epidermal regeneration through keratinocyte activation may ensue after treating a wound with a targeting to ER (Peržel'ová *et al.*, 2016). Ashcroft *et al.* (1997) described that post-

menopausal women who have never taken HRT exhibited impaired re-epithelialisation of wounds compared to pre-menopausal women (Horng *et al.*, 2017).

Several reports are available on the relationship of oestrogen to endothelial cells (Tsui *et al.*, 2015; Yang *et al.*, 2017). Oestrogen signaling induces the mobilization of circulating endothelial progenitor cells from bone marrow, and these cells help to build and restore injured and/or damaged endothelium (Horng *et al.*, 2017). It has been suggested that oestrogen affects angiogenesis by influencing endothelial cells, by increasing in vitro attachment of endothelial cells to laminin, collagen I and IV and fibronectin (Horng *et al.*, 2017). Oestrogen also induces proliferation and migration, and inhibits the apoptosis of endothelial progeny cells (Horng *et al.*, 2017).

The last step of the wound healing is the remodeling phase, which relies on a controlled balance between the synthesis and degradation of the ECM, and oestrogen is thought to involve both (Billon *et al.*, 2017). Collagens are the most abundant proteins in the human body and the main components of the ECM (Blum, 2015). Type I collagen is dominant in normal wounds; by contrast, in hypertrophic scar or keloid scar, type III is dominant (Butzelaar *et al.*, 2016) and the formation and degradation of collagen is controlled by a myriad of factors (Amar *et al.*, 2017; Freitas-Rodriguez *et al.*, 2017; Jobin *et al.*, 2017; Van Doren *et al.*, 2017; Levin *et al.*, 2017; Figleton, 2017; Irrera *et al.*, 2017). Among these, matrix metalloproteinases (MMPs), a family of zinc dependent proteases, are crucial (Okonkwo and Dipietro, 2017). Estradiol (E2) reduces expression of metalloproteinase P-8 (MMP-8) and 13 (MMP-13) in ovariectomized rats, which inhibit collagenolysis and restores collagen content in the wound bed (Pirila *et al.*, 2001). Furthermore, E2 increases tissue inhibitor of metalloproteinase (TIMP) production in fibroblasts, (Sato *et al.*, 1991;

Pirila *et al.*, 2002) which maintains the collagen content as well. Exogenous oestrogen can reverse the impaired wound healing observed in menopausal women associated with improving matrix synthesis and deposition, leading to amelioration of scarring (Margolis *et al.*, 2002).

2.7 Soy Phytooestrogen and Wound Healing

Phytooestrogen are plant compounds with oestrogen-like biological activity. The first report on oestrogenic activity in plant extract was made in 1926 (Loewe *et al.*, 1927). By 1975, several hundred plants had been found to exhibit oestrogenic activity on bio-assay or to contain oestrogenically active compounds (Farnsworth *et al.*, 1975). Phytooestrogens assumed biological and economic importance in the 1940s, with the outbreak of infertility in sheep grazing on pastures rich in subterranean clover in Western Australia, later known as "Clover Disease" (Bennetts *et al.*, 1946). Phytooestrogens were identified in the urine of nonhuman primates in 1979, (Setchell and Adlercreutz, 1979) and in humans in 1982 (Axelson *et al.*, 1982; Setchell *et al.*, 1984).

The soybean *Glycine max (L.) Merr.* (Leguminosae) is a species of legume that is native to East Asia and is considered a very important plant because of its phytochemical content (Uyar *et al.*, 2014). It is widely used for centuries as food and it is included in the formulations of many drugs (Uyar *et al.*, 2014). Soybean include minerals, protein, vitamins and different kinds of carbohydrates. Sixty (60) % of total dry weight is fat and protein that means 40-67/36 % protein and 17-20 % fat (Modaresi *et al.*, 2011). Since soy protein is full of vitamins B1 and B2, Pantotenic acid, Colin, Niacin and all necessary amino acids for the body, it is considered an invaluable source of nutrient for human beings (Modaresi *et al.*, 2011). The protein content of soybean varies from 36% to 46%

depending on the variety (Garcia *et al.*, 1997; Grieshop and Fahey, 2001; Grieshop *et al.*, 2003). Soybean also contains micronutriments, which include isoflavones, phytate, soyaponins, phytosterol, vitamins and minerals (Modaresi *et al.*, 2011).

Isoflavones are natural occurring substances, present in some plants, which are structurally similar to oestrogens and can exibit weak oestrogen-like effects (Tepavcevic *et al.*, 2011). For this reason, they are classified as phytooestrogens: plant-derived compounds with activity similar to oestrogens (Tepavcevic *et al.*, 2011). Soy (*Glycine max*) contains the largest concentration of isoflavones (Anderson and Wolf, 1995; Philips *et al.*, 2002; Romani *et al.*, 2003; Tepavcevic *et al.*, 2011), a class of phytooestrogens (Tepavcevic *et al.*, 2011; Teekachunhatean *et al.*, 2013). The major dietary isoflavones, daidzein and genistein, have oestrogen-like activities (Hodgson *et al.*, 1998; Wong and Rabie, 2008; Teekachunhatean *et al.*, 2013) since they can bind to the oestrogen receptor in place of oestrogen due to their structural and functional similarities to oestrogen (Setchell, 1998; Tikka-nen and Adlercreutz, 2000; Dixon, 2004). Isoflavones have a non-steroidal structure but possess a phenolic ring that enables them to bind the oestrogen receptor (ER) and act either as oestrogen agonists or antagonists (Makela *et al.*, 1994, Makela *et al.*, 1995).

Phytooestrogens contain all the physiological and physiochemical characteristics of oestrogens (Modaresi *et al.*, 2011). Soy and phytooestrogens receive increasing attention due to the health benefits associated with their consumption in prevention and treatment of various chronic diseases (Adlercreutz *et al.*, 2004; Kondratyuk and Pezzuto, 2004; Manach *et al.*, 2004; Rostagno *et al.*, 2009). The conclusions that were reached by most of the above-mentioned studies indicated that the positive effects of soybean products can be attributed to its isoflavones content (Cederroth and Nef, 2009; Messina, 2010). The most

important soy isoflavones are genistein, daidzein and glycitein. Isoflavones are phytooestrogenic compounds that are structurally similar to oestrogenic steroids (Uyar *et al.*, 2014).

Studies have shown that there is a large variability in isoflavone content and composition in soybeans (Cederroth and Nef, 2009). This is function of the variety of soy grown, as well as environmental conditions (Wang and Murphy, 1994; Caldwell *et al.*, 2005). In soybean, isoflavones are tightly associated with proteins (Cederroth and Nef, 2009). Indeed, isoflavones can be dissociated from soy-proteins using alcohol extraction which significantly diminishes the amount of bound-isoflavones (Bhathena and Velasquez, 2002).

2.7.1 Synthesis, metabolism and mechanism of action of soy-phytooestrogen

In plants, the synthesis of phytooestrogens, such as soy isoflavones, generally coincides with environmental stresses such as pest infection, drought or lack of nutrients (Howitz and Sinclair, 2008). The amount of isoflavonoids present in soybean seed is a complex trait that is highly variable (Hoeck *et al.* 2000; Gutierrez-Gonzalez *et al.* 2010). It has been suggested that stress-induced plant compounds upregulate stress resistance pathways in animals (Cederroth and Nef, 2009). Soybean seeds contain nine different isoflavonoids with three core isoflavone aglycones (daidzein, genistein and glycitein) (Dhaubhadel, 2011) (Fig. 2.2). The aglycones are the most bioactive forms of isoflavonoids (Dhaubhadel, 2011). Each of these aglycones may also be present as their corresponding 7-O-glycosides (daidzin, genistin and glycitin) (Fig. 2.2) and malonyl glycosides (6”-O-malonyldaidzin, 6”-O-malonylgenistin and 6”-O-malonylglycitin) (Kudou *et al.* 1991)

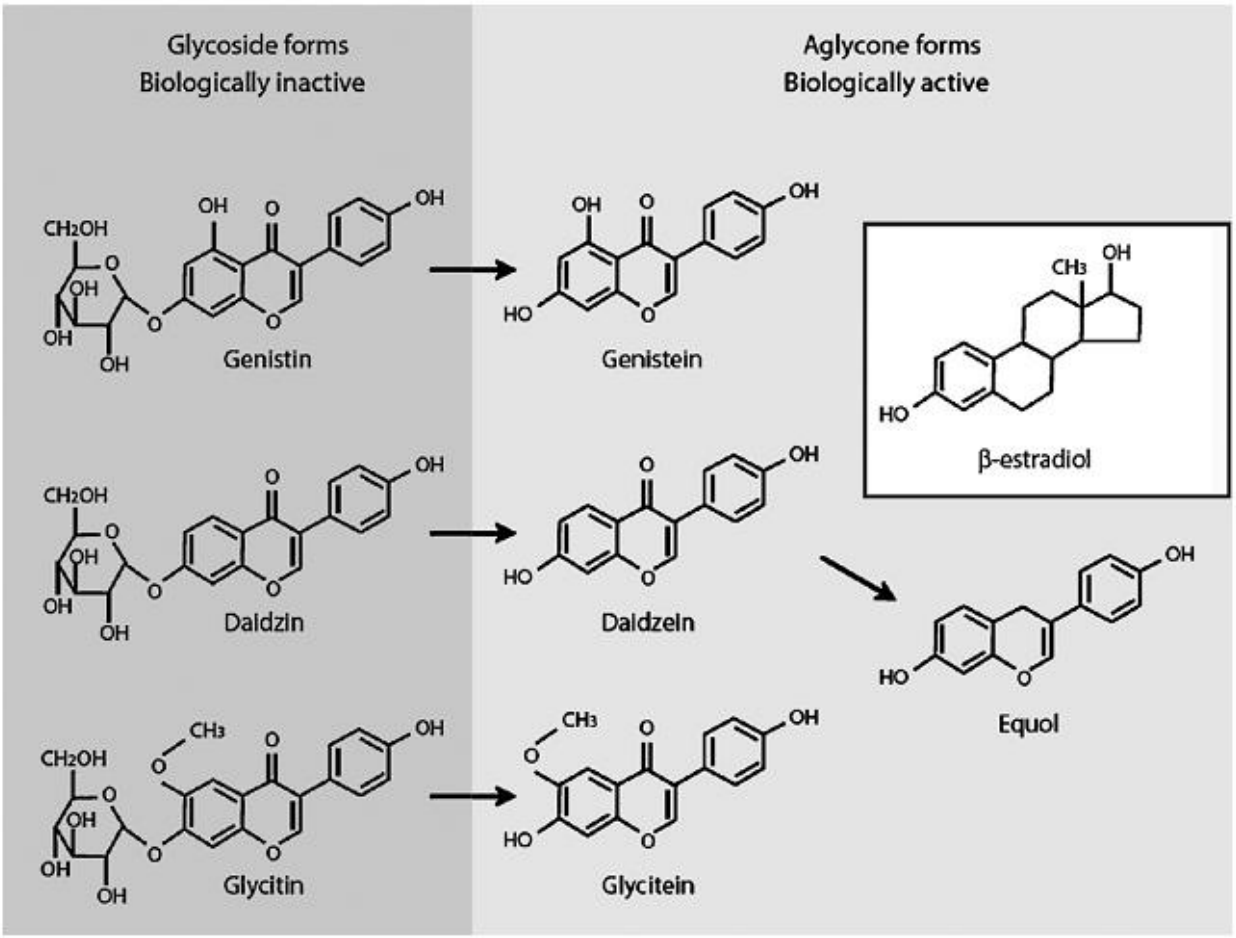


Figure 2.2: The molecular structure of isoflavones and 17β-estradiol (Cederroth and Nef, 2009)

The metabolism of isoflavones is rather complex. The two major isoflavones, genistein and daidzein, are present in soy as β-D-glycosides, namely genistin and daizin (Cederroth and Nef, 2009). These glycoside forms are biologically inactive (Setchell, 1998). Once ingested, isoflavone glycosides are hydrolyzed by bacterial β-glucosidases in the intestinal wall, resulting in the conversion to their corresponding bioactive aglycones (genistein and daidzein) (Cederroth and Nef, 2009). Only the aglycone forms are absorbed by the intestinal tract and are there-fore biologically active. Daidzein can be further metabolized to equol and O-demethyangolensin, and genistein to p-ethyl phenol (Cederroth and Nef, 2009). In fact, genistein, daidzein, equol and O-demethyangolensin are the major isoflavones detected in the blood and urine of humans and animals (Setchell, 1998). In rodents, equol is the major circulating metabolite among isoflavones representing up to 70–90% of all circulating isoflavones (Cederroth and Nef, 2009). While all rodents are equol producers, only 30% of humans are able to metabolize daidzein into equol (Atkinson *et al.*, 2005)

Isoflavones perform most of their biological effects through modulation of oestrogenic receptors (ER), as a result of their structural similarity with human oestrogens (Cederroth and Nef, 2009). The isoflavone affinity towards oestrogenic receptors results in numerous effects on oestrogen-regulated systems, including cardiovascular, metabolic, reproductive, skeleton, and central nervous system (Tepavcevic *et al.*, 2011). Isoflavone provide health benefits through their selective oestrogen receptor modulator (SERM) activities (Cederroth and Nef, 2009). A significant characteristic of isoflavones is their capacity to bond to both subtypes of oestrogenic receptors (ERα and ERβ) (Nachtigall, 2001; Montani *et al.*, 2008) but mainly to β receptors (Cederroth and Nef, 2009). Such specific affinity towards

oestrogenic receptors allows them to perform oestrogenic and anti-oestrogenic effects, depending on the type of the tissue and the endogenous oestrogen levels (Kupier *et al.*, 1997; Kupier *et al.*, 1998). Tissue-selective activity of isoflavones is important because anti-oestrogenic effects in reproductive tissue can decrease the risk of hormone-dependent cancers (breast, uterus, and prostate cancer), while oestrogenic effects in other tissues can impact preventively towards osteoporosis and hypercholesterolemia (Tepavcevic *et al.*, 2011). In comparison with physiological oestrogens, isoflavones are very weak oestrogens which, on the molar basis, have from 10^{-2} to 10^{-4} activities of 17β-estradiol (Biggers and Curnow, 1954; Bickoff, 1962). Genistein has ten times higher oestrogenic activity comparing to daidzein (Diel *et al.*, 2000; Branham *et al.*, 2002; Diel *et al.*, 2004), whereas glycitein has the highest oestrogenic potential in vivo (Song *et al.*, 1999). Genistein and glycitein can be biodegraded into metabolites with no oestrogenic activity (Cassidy *et al.*, 2000; Simons *et al.*, 2005), while daidzein can be metabolised into equol, which has higher oestrogenic potential than daidzein (Setchell *et al.*, 2002). In vitro and in vivo studies have shown that genistein demonstrates weak oestrogenic and antioestrogenic properties (Tiulea *et al.*, 2011).

The surveys and nutritional intervention studies in humans and animals suggest that dietary phytooestrogens play an important role in controlling of menopausal symptoms and a variety of disorders (Tepavcevic *et al.*, 2011), including cardiovascular disease, cancer, hyperlipidemia, osteoporosis, and various forms of chronic renal disease (Barnes *et al*, 1994; Adlercreutz and Mazur 1997; Potter *et al.*, 1998; Scheiber *et al.*, 2001; Chiechi *et al.*, 2002; Sarkar and Li, 2003; Lee *et al.*, 2003b; Moriguchi *et al.*, 2004; Ikeda *et al.*, 2006; Wu *et al.*, 2008).

Soy isoflavones, found in soybean and soybean products, have been reported to possess many physiological activities and biological functions such as antioxidant (Kao *et al.*, 2006), inhibition of cancer cell proliferation (Kao *et al*, 2007a), anti-inflammatory activities (Kao *et al*, 2007b), prevention of coronary heart diseases (Dalais *et al.*, 2003), as well as osteoporosis (Migliaccio and Anderson, 2003). In anti-inflammatory effects, soy isoflavones could decrease the secretions of interleukin-1 (IL-1), IL-6 (Hassan *et al.*, 2010), nitric oxide (NO) and prostaglandin E2 (PGE2) in the cell supernatant and fluid of mouse peritoneal exudate (Kao *et al.*, 2007a). Thus, soy isoflavones may have potentially protective benefits at sites of inflammation (Kajiyama, 1993; Kamnaing, 1999). Chiang *et al* (2007) reported that the four groups of isoflavone extract from soybean cake significantly increase cell viability and inhibit intracellular H_2O_2 production. Given that ROS act as mediators to regulate MAPK pathways (ERK1/2, p38 and JNK), suppression of ROS production might lead to inhibition of MAPK pathways (Huang *et al.*, 2010). Genistein may increase production of super oxide dismutase (SOD)–a powerful antioxidant that quenches superoxide radicals (Wei *et al.*, 1995). This further explains why its regarded by many authors as a potent antioxidants (Wei *et al.*, 1993; Wiseman *et al.*, 2000; Djuric *et al.*, 2001; Malencic *et al.*, 2008).

2.7.2 Effect of soy-phytooestrogen on the skin

A number of studies have included soy isoflavones alone or in combination with other agents and showed positive effects on skin appearance and composition (Skovgaard *et al.*, 2006; Draelos *et al.*, 2007; Izumi *et al.*, 2007; Accorsi-Neto *et al.*, 2009). Genistein aglycone (hereinafter genistein), an isoflavone found in low concentrations in soybeans and in increased amounts in certain fermented soy foods, has been recently considered as

an ideal natural SERM and might play a preventive role in age-related skin changes accelerated by the menopause without the harmful ooestrogenic side effects on reproductive tissues (Cassidy, 2003).

Soy isoflavones are considered to have beneficial effects on the skin via mechanisms such as the prevention of lipid oxidation, stimulation of fibroblast proliferation, reduction of collagen degradation and inhibition of 5a -reductase and they have been widely used as ingredients in cosmetics (Uyar *et al.*, 2014). Moreover, cosmetics containing isoflavones are reported to retard skin aging (Izumi *et al.*, 2007). Accorsi-Neto *et al.* (2009) reported a thickened epidermis, increased collagen and elastin fiber content within the dermis, decreased thickness papillary dermis and a significantly increased amount of dermal blood vessels in 30 women with menopause who ate a soy isoflavone-rich diet for 6 months. Polito *et al.* (2012) revealed that both estradiol and genistein are more effective than raloxifene for improving skin damage related to decreased oestrogen conditions. In contrast, systemic treatment with genistein has been found to accelerate wound healing in ovariectomized rats (Marini *et al.*, 2010a). This finding was further supported by the finding that genistein can increase cell migration, inflammation, provisional matrix synthesis, collagen deposition, angiogenesis and re-epithelialization, all of which are crucial processes in skin repair (Uyar *et al.*, 2014). Interestingly, the experimental data obtained by Polito *et al.* (2012) also indicated that the systemic administration of genistein in the same dose range as that used in postmenopausal women had positive effects on ovariectomy-induced skin changes (Squadrito *et al.*, 2003; Atteritano *et al.*, 2007; Marini *et al.*, 2007; Marini *et al.*, 2008a Marini *et al.*, 2008b; Marini *et al.*, 2010a; D'anna *et al.*, 2009). Genistein has recently been accepted as the ideal natural SERM that might play a

preventative role in the age-related skin changes accelerated by menopause without causing harmful oestrogenic side effects in reproductive tissues (Cassidy, 2003). Currently, genistein-containing cosmetic creams are used to improve skin dryness and wrinkles related to oestrogen deprivation (Rona *et al.*, 2004). The efficacy and safety of genistein in a low-oestrogen environment were demonstrated in experimental and clinical studies (Squadrito *et al.*, 2003; Atteritano *et al.*, 2007; Marini *et al,* 2007; Bitto *et al.*, 2008; Bitto *et al.*, 2009a; Bitto *et al.*, 2009b; D'anna *et al.*, 2009; Marini *et al,* 2008a Marini *et al,* 2008b; Marini *et al,* 2010a;)

Biodegradable soy wound materials have been found to be very effective in wound management. The biomaterials uniquely combine adaptability to various clinical applications to clear tissue regeneration properties (especially burn injury) (Shevchenko and Santin, 2014; Santin and Ambrosio, 2008). Previous investigations about the use of soybean as a potential source for biomaterials have highlighted that these biomaterials in the form of films, granules, hydrogel or paste exert several specific bioactivities on various types of tissue cells and promote bone regeneration in vivo (Giavaresi *et al.*, 2009; Meroli *et al.*, 2010). Recent studies have also shown that these tissue regeneration properties can be ascribed to the unique combination of the scaffolding properties of these biomaterial and presence of isoflavones, plant oestrogens capable of selectively interacting with the oestrogen receptor (ER) β of the nuclear membrane rather than the ER α, thus activating cell differentiation rather than proliferation with a consequent induction of the production of extracellular matrix components, that is, collagen (Santin *et al.*, 2007; Meikle *et al.*, 2012).

Oestrogens exhibit their effect on dermal component of the skin by increasing fibroblasts proliferation. These observations prompted the pre-clinical validation of the soybean-based biomaterials when formulated as hydrogel or as paste (mix of thermoset granules and hydrogel) and to the purpose of using them either as wound dressing material or as dermal substitute to support split-thickness skin grafts in burns or other types of extended skin loss (Shevchenko and Santin, 2014). These hydrogel also promote an inflammatory response maintaining the neutrophil/macrophage balance in favor of the neutrophils, thus reducing the risks of infections and of a chronic inflammation leading to scarring (Santin *et al.*, 2007; Chien *et al.*, 2013).

The soy biomaterial for skin injury were found to accelerate epithelialization and neoangiogenesis, provide thicker dermis, fewer white blood cell count, more hair follicles, and greater collagen content (Shevchenko and Santin, 2014) . In vitro studies on fibroblasts, osteoblasts and macrophages as well as in vivo studies of bone regeneration have clearly demonstrated the healing properties of these biomaterials (Santin *et al.*, 2007; Giavaresi *et al.*, 2009; Meroli *et al.*, 2010; Meikle *et al.*, 2012). They possess a unique pattern of early inflammatory response, eliciting neutrophils and controlling macrophage infiltration, followed by tissue cell colonization of the wound bed with a significant deposition of collagen fibers (Shevchenko and Santin, 2014).

Topical application of soy has been used to reduce hyperpigmentation, enhance skin elasticity, control oil production, moisturize the skin, and delay hair regrowth (Waqas *et al.*, 2014). It shows highest efficiency in reducing UVB-induced oxidative stress and prevention of human keratinocyte death (Huang *et al.*, 2010). Soy isoflavone decrease

photoaging of the skin (Polito *et al.*, 2012) while the antioxidant effects of its metabolite and prevent skin cancers (Polito *et al.*, 2012; Waqas *et al.*, 2013; Waqas *et al.*, 2014).

Relatively long-term, systemic treatment with genistein aglycone shows comparable efficacy to ooestrogen in reversing some molecular, histological and functional changes of the skin associated with ovariectomy in aged rats (Polito *et al.*, 2012). It improves skin dryness and wrinkles (Rona *et al.*, 2004). Also systemic treatment with genistein has been found to accelerate wound healing in OVX rats (Marini *et al.*, 2010b). However, Emmerson *et al.* (2010) demonstrated that some of genistein's effects on the skin are mediated by non-classical ERs. Specifically, genistein accelerates keratinocyte migration and re-epithelialization via insulin growth factor-1 receptor and its anti-inflammatory activity is likely to be attributable to an effect mediated via the MAPK pathway (Polito *et al.*, 2012). Isoflavone genistein, thus have the ability ability to improve cell migration, inflammation, provisional matrix synthesis, collagen deposition, angiogenesis and re-epithelialization, all crucial processes in skin repair (Polito *et al.*, 2012).

The topical use of phytooestrogens-based cosmetics improves the overall condition of the skin. It became evident that the phytooestrogens complex favored an increase in IL-1 and reduced levels of anti-inflammatory cytokine IL-10 in the human keratinocytes cultures, this demonstrates the complex's potential immunomodulatory stimulus, which promotes skin homeostasis (Costa *et al.*, 2012). In addition to exerting the capacity to contract and organize connective tissue, fibronectin promotes cell adhesion inwound healing and re-epithelialization, and is also primarily responsible for the integrity of the DEJ (Rocquet and Bonte, 2002; Wierzbicka-Patynowski and Schwarzbauer, 2003). Soy isoflavone significantly increased the expression of mRNA for fibronectin in a study (Costa *et al.*,

2012). More recently, soy protein has received attention for a variety of its potential health benefits, including enhanced skin regeneration (Ahn *et al.* 2018) . In wound healing, oral and topical soy has been heralded as a safe and cost-effective alternative to animal protein and endogenous oestrogen (Ahn *et al.* 2018). Soy Protein Hydrosylate nanofibers promote fibroblast proliferation, migration, infiltration, and integrin beta1 expression while soy protein scaffolds accelerate re-epithelialization and epidermal thinning as well as reduce scar formation and collagen anisotropy (Ahn *et al.* 2018). Also nutritional supplements with isolated soy protein attenuates post-burn oxidative stress and inflammation, leading to improved wound healing in burn patients (Babajafari *et al.* 2017). Soy protein is among the newly developed bioderived materials employed for cutaneous wound healing and have proven to have great potential for skin tissue repair (Da *et al.* 2017). There is growing interest in the development of biodegradable materials from renewable biopolymers, such as soy protein, for biomedical applications especially in skin regeneration (Egozi *et al.* 2015). Glycitin, a major soy isoflavone, has been reported to stimulates dermal fibroblast proliferation, reduce scarring and is a promiing bioactive therapeutic for wound treatment (Seo *et al.* 2017). Genistein substantially accelerated wound repair, associated with a dampened inflammatory response (Emmerson *et al.* 2010).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. Ethical Approval

Ethical approval to use Albino rats in this study was sought from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC) Ahmadu Bello University, Zaria.

3.2. Experimental site

This experiment was carried out in the Department of Veterinary Surgery and Radiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria**,** North-Western Nigeria with Geographical coordinate of Zaria: $11^0$4'0'' North and $7^0$42'0'' East (Anon, 2016)

3.3 Materials

3.3.1 Materials and consumables

The following materials and consumables were used:

Liquid soap, Chlorhexidine gluconate B.P (0.3% w/v- Purit®), Surgical gloves, Sterile disposable syringe (2mls), Gauze bandage, Cotton wool, Adhesive plaster, Standard general surgical packs, Surgical gowns, Surgical drapes, Surgical blades, Vicryl® suture size 2/0, silk suture size 2/0, Surgical caps and masks, Ketamin HCl (Rotex Medica, Germany), Xylazine HCl (Xylase®, M.S. Global Mumbai) Bcl-2 antibody (Elabscience Inc USA), 10% Neutral buffered formalin, Phosphate buffered saline (PBS pH 7.0), Glass slide, Digital camera (Sony Corp UK), Ethanol, Paraffin wax, Xylene, Insulin syringe, Microtome(Medimas, Germany), Sample bottle, Centrifuge, Graph paper, Test tubes, Pipettes, Hematoxylene and Eosin dye, Colorimeter.

3.4 Collection and identification of soybean seed

Dried seed of Soybean (*Glycine max L*) was purchased from Samaru market, Zaria, Kaduna State and identified in the Herbarium of the Department of Botany, Ahmadu Bello University, Zaria and a voucher number 01293 was issued

3.5 Experimental Animals management

A total of twenty four (24) apparently healthy female Wistar albino rats were purchased from Nigerian Institute of Trypanosomiasis and Onchocerciais Research (NITOR) Kaduna State and used for the study. The rats were housed in the metal cages in the laboratory animal unit, Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria; acclimatized and conditioned for two weeks to ensure uniform weight of 250±50 grams in all the rats. Each rat was fed commercial poultry pelletized feed (Vital feed® Grower mash) daily with water given *ad libitum*. The animals were evaluated clinically through physical and laboratory evaluations (complete blood count, urinalysis, clinical chemistry, haemoparasitic and helminthic).

3.6 Ovariectomy procedure

Ovariectomy was carried out at small animal surgical theatre of the Department of Veterinary Surgery and Radiology, Ahmadu Bello University, Zaria two weeks after acclimatization (Plate 3.1a-f). Ovariectomy was done by single midline dorsal skin incision technique as described by Lasota *et al.*, (2004) and Khajuria *et al.*, (2012). The animals were anesthetized with a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg), intramuscularly and were aseptically prepared for invasive surgery by clipping the area of the skin incison and disinfecting with chlorhexidine gluconate and methanol. The

anaesthetised rats were place on the operating table on sternal recumbency position (plate 3.1a). Thereafter, a single midline dorsal skin incision, 3cm long, was made approximately half way between the middle of the back and the base of the tail (3.1b). The subcutaneous connective tissue was gently freed from the underlying muscle on each side using blunt forceps. Bilateral incisions were then made into the dorsolateral abdominal muscles to gain entry into the peritoneal cavity one after the other. After peritoneal cavity was accessed, the ovary was found, surrounded by a variable amount of fat (plate 3.1c). The edges of the incisions were held securely with tooth forceps and the ovarian fat pad surrounding ovaries was retracted with blunt forceps to expose oviduct. The ovarian vessel running longitudinal to the proximal oviduct was double ligated alongside the oviduct with 2/0 Vicryl® absorbable suture. The connection between the Fallopian tube and the uterine horn was cut and the ovary removed (plate 3.1d). The uterine horn and the remaining part of the oviduct were returned back into the abdominal cavity. The wound was closed in two layers (muscle and skin) using sterile sutures. The peritoneum and the muscle layers were sutured with one absorbable suture (Ethicon® chromic sutures-2/0, Johnson and Johnson Ltd., India) and the skin was sutured with one non-absorbable suture (silk sutures-2/0, Johnson and Johnson Ltd., India) (plate 3.1e). Povidone iodine was applied on the area to disinfect the skin after suturing (plate 3.1f). After surgery, the rats were housed individually in a metal cage with paper beddings for a period of two weeks to allow recovery. The skin sutures were removed by 12th day post surgery in all the rats. All the rats were randomly placed into groups in their cages.



Plate 3.1a: Draping of experimental animals before bilateral ovariectomy

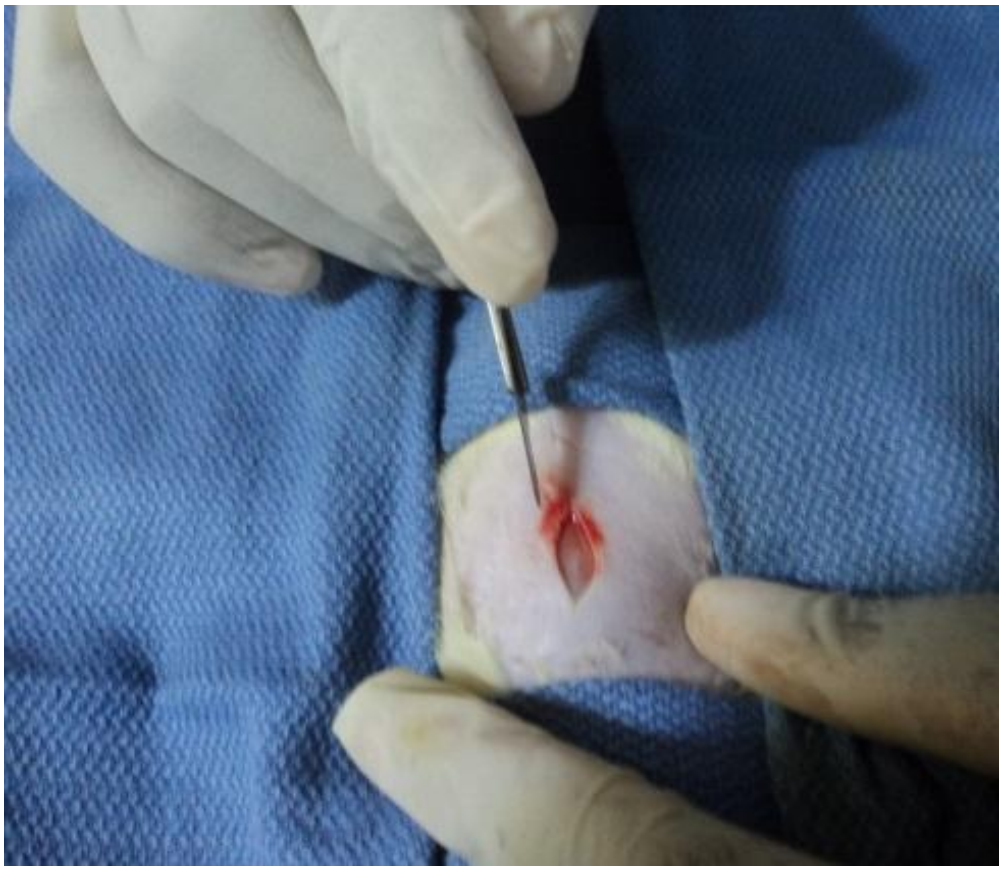


Plate 3.1b: Creation of single midline dorsal skin incision (3cm) half way between the middle of the back and the base of the tail to have access to the abdominal cavity on either side of the body**.**

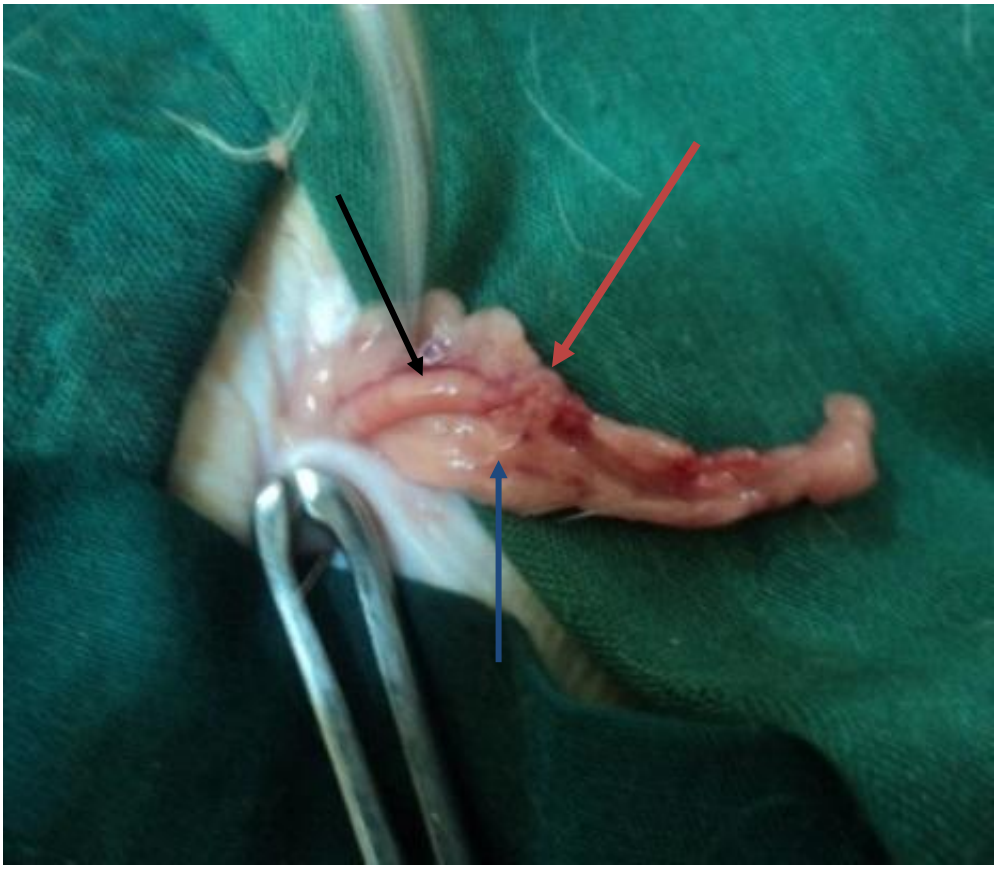


Plate 3.1c: Retraction of the ovarian fat pad (blue arrow) surrounding ovaries (Red arrow) long with the oviduct (black arrow) with blunt forceps



Plate 3.1d: Ligation of ovarian vessel running longitudinal to the proximal oviducted alongside the oviduct with 2/0 Vicryl® absorbable suture.

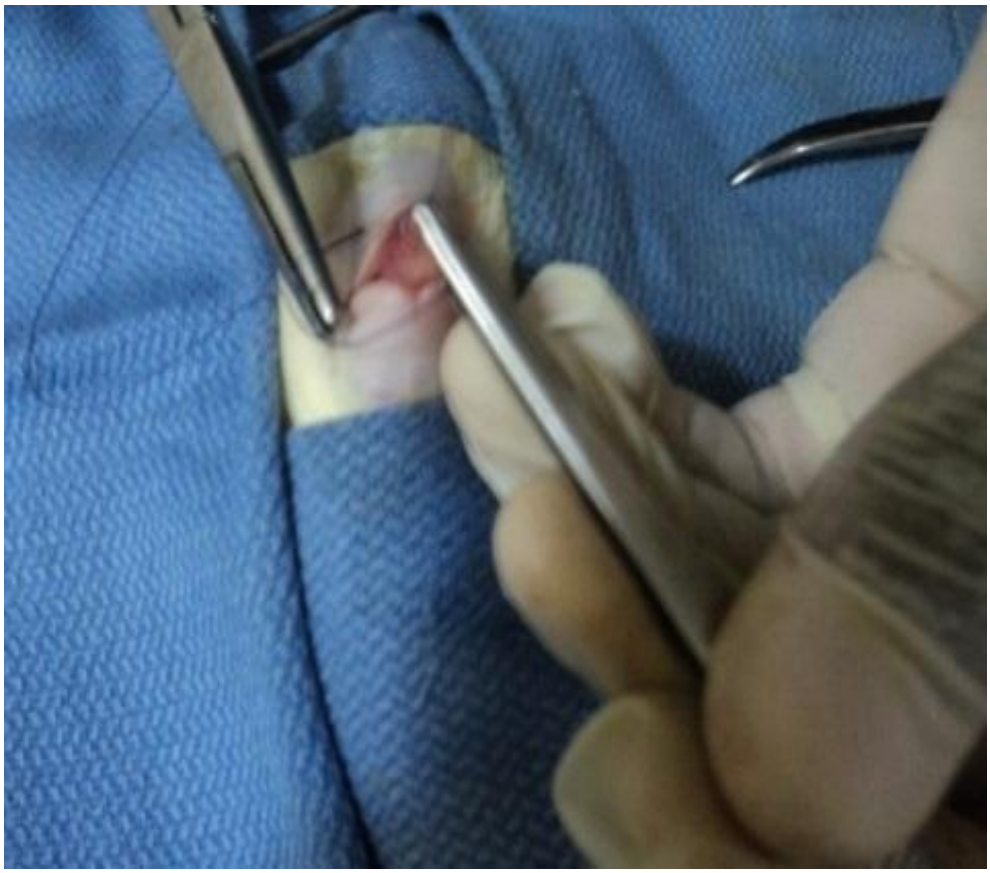


Plate 3.1e: Suturing and closure of muscle and skin



Plate 3.1f: Animal under recovery post-surgery

3.7 Extraction and Fractionation of Soybean extract

Soybean extraction amd fractionation was done as described by Prashanthi *et al.*, (2012). Dried soybean was pounded into a coarse powder using mortar and pestle. Dried powder was extracted with absolute ethanol in a 1 liter flat bottom flask by cold maceration for 48 hours and stirred occasionally within the period. The supernatant was collected and filtered through muslin cloth and then concentrated on a water bath for 24hours at 45°C. It was stored at 4° C in air tight bottles. Thereafter, 100g of the crude extract was dissolved in 50ml of distill water and then treated with 500ml of chloroform. Organic fraction and aqueous fraction was then concentrated on a water bath for 24hours. 80grams of oily crude ethanolic extract and 47grams of chloroform extract was used for the study.

3.8 Thin Layer Chromatography (TLC)

Thin layer chromatography technique was used in identifying the phytochemical constituents present in the ethanolic and chloroform fraction of the soybean extract. The TLC of the extract was carried out on pre-coated silica plates (TLC silica gel 60 F254, Merck KgaA, Darmstadt, Germany) using the ascending technique with suitable solvent system. TLC pre-coated plates were cut into suitable size. A line was drawn horizontally at 1cm from the bottom to serve as the origin. The soybean extract was dissolved in ethanol for spotting. Hexane and Ethanol (8:2) was used as solvent system and poured into the developing chamber and allowed to saturate. A capillary tube was dipped into the prepared extract sample and gently spotted at the marked region from the base of the plate (plate 3.2a). This was then transferred into the developing chamber and allowed to elute until it develops to the solvent front of about 1cm before the plate apex; at which it was removed (plate 3.2b). The plate was sprayed with P-anisaldehyde and heated in an oven at 105^{o}C for

5 minutes (plate 3.2c). The procedure was repeated but this time around the plate was sprayed separately with Dragedroff, Ferric Chloride, Borntrager's, Alluminium chloride and Liebermann-Bucchard stain for alkaloid, phenolic, anthraquinone, flavonoids and steroids/triterpens to detect the specific compounds (Hahn, 2007) (plate 3.2d).

3.9 Experimental Wound Creation and Management

Experimental wounds were created in all the rats 4 weeks post-ovariectomy and were randomly divided into four groups with each group containing 6 rats. The animals were anesthetized by intra-muscular administration of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (10 mg/kg). The dorsal region of each animal was surgically prepared for aseptic surgery and the sterile dorsal surface of skin marked with a sterile square shaped (15x15 millimeter) stencil (plate 3.3a). Two full-thickness skin wound were created at the dorsal surface of thoracoabdominal region (from the skin and panniculus carnosus muscle) of each rat having 10mm between adjacent wound and 40mm from the base of the corresponding ears using sterile scissors and forceps (plate 3.3b). Each wounded animal was housed in a separate metal cage with paper beddings. All rats in group A-D had their wound monitored and dressed daily with normal saline. Additionally, oestrogen cream, soybean crude-ethanolic extract and Soybean-chloroform extract was applied topically to the wounds of rats in group B, C and D respectively for 20 days. The wounds were treated daily for the first seven days and every other day afterwards till the end of the experiment (Day 20).

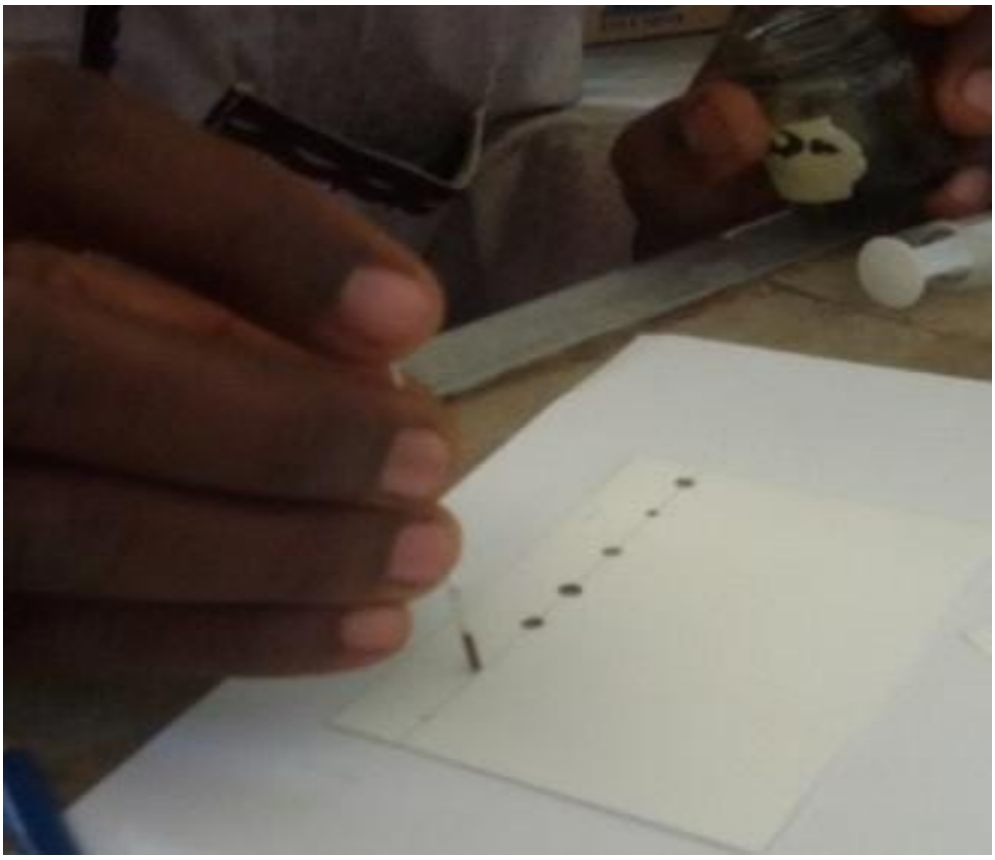


Plate 3.2a: Spotting of the extract on chromatographic plate

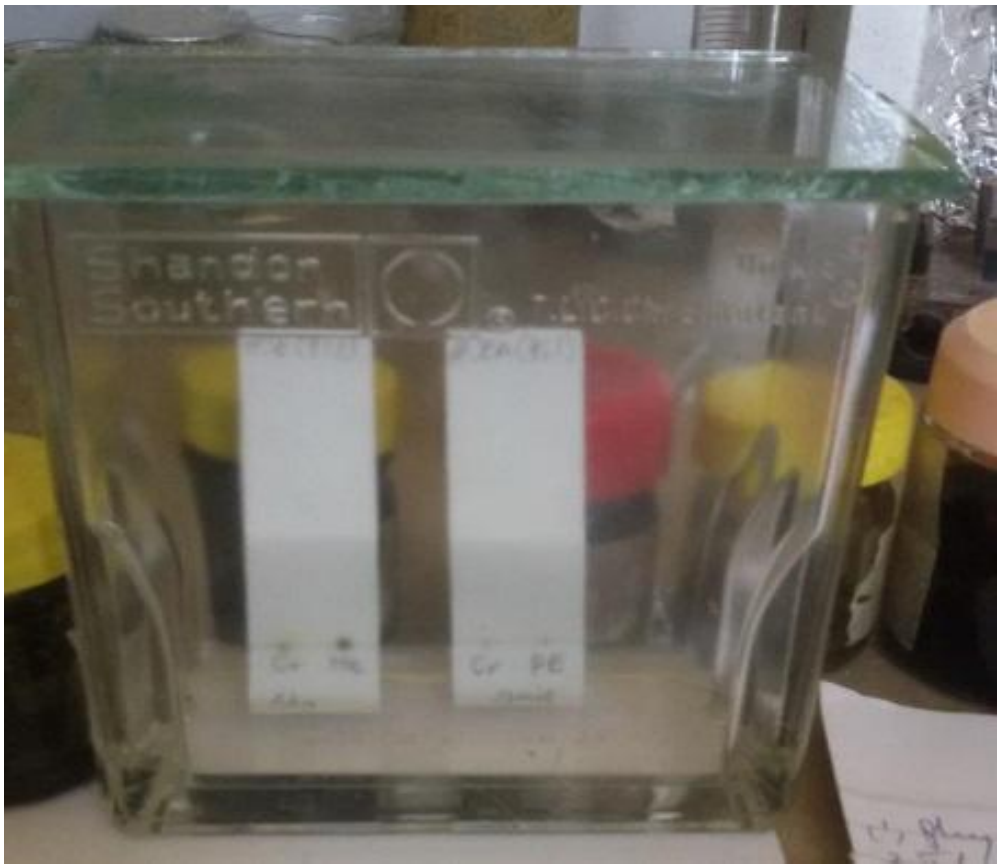


Plate 3.2b: Developing the spotted plate in glass chamber containing solvent system



Plate 3.2c: Spraying the plate with Dragedroff to detect presence of alkaloid

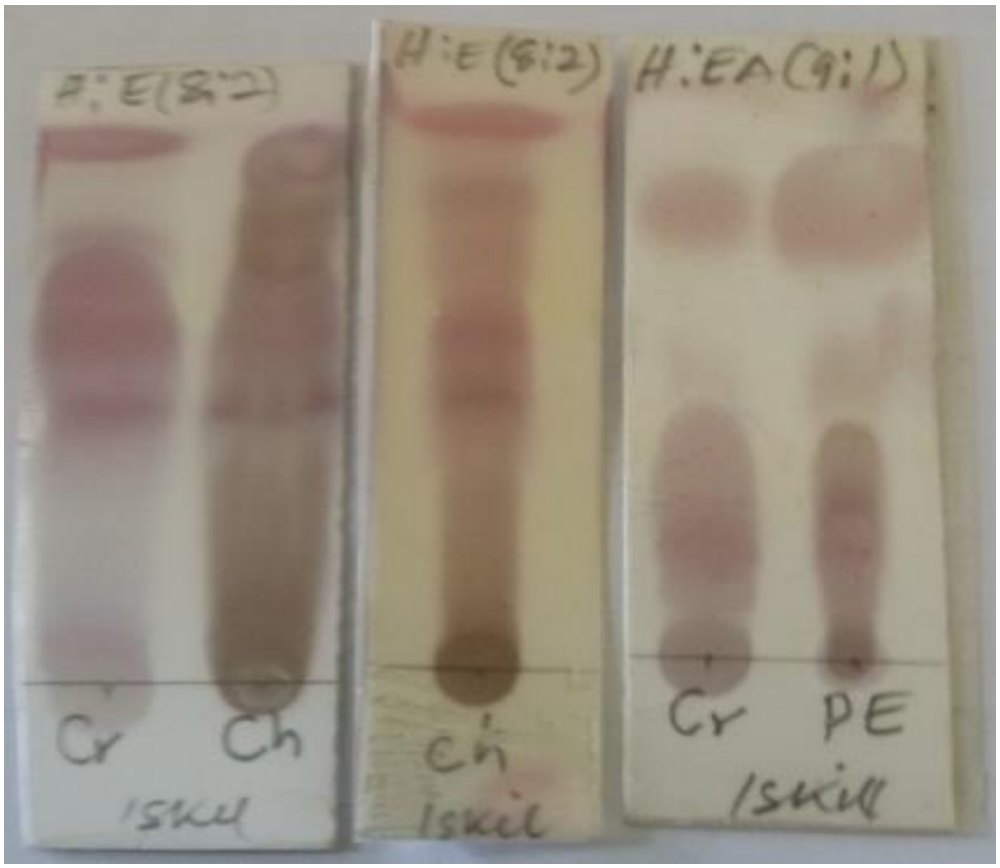


Plate 3.2d: TLC plates with different compound after spraying with P-anisaldehyde and heated in an oven at 105^{o}C for 5 minutes

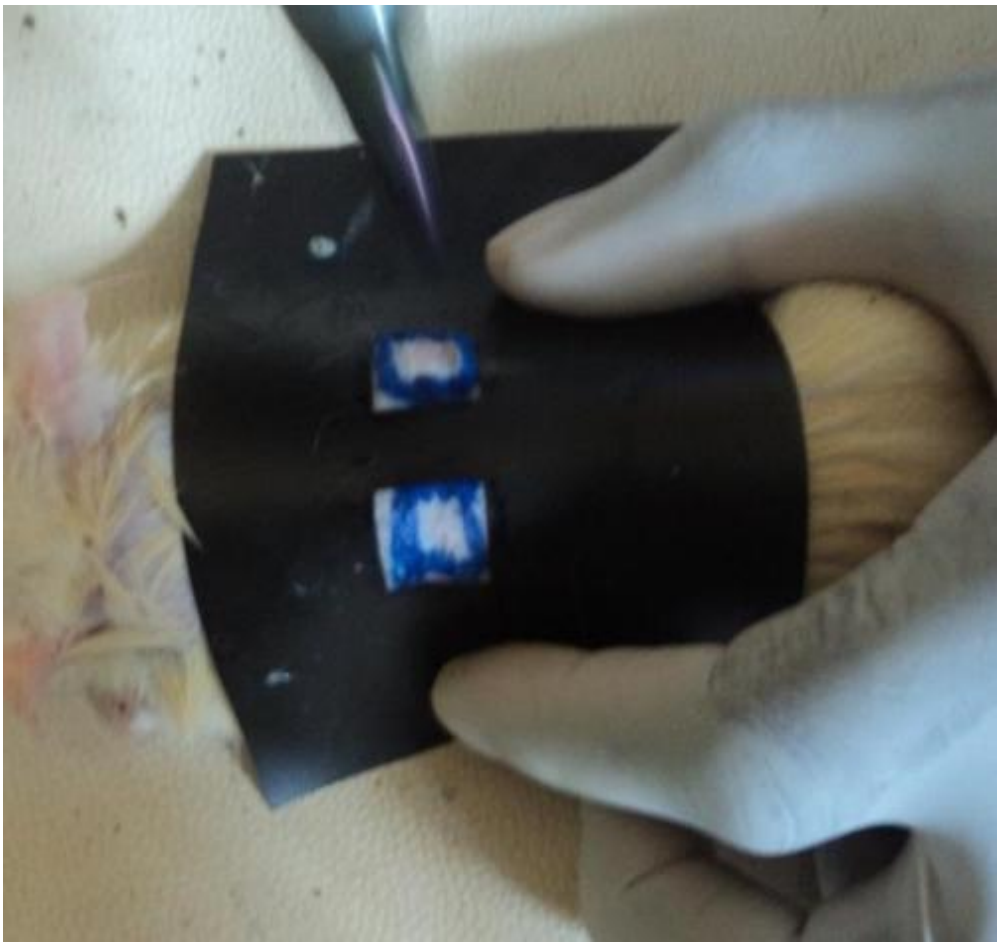


Plate 3.3a: cleared dorsal surface of skin marked with a sterile square shaped (15x15 millimeter) stencil (arrow)



Plate 3.3b: Two wounds created on each animal.

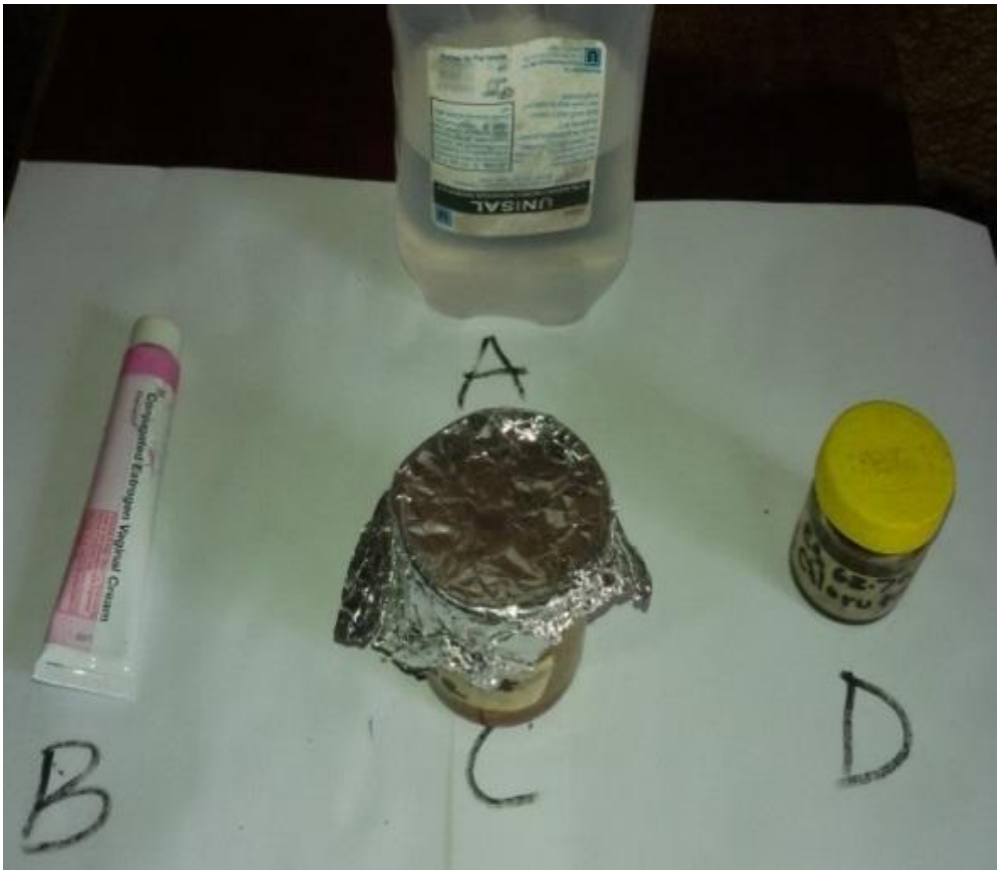


Plate 3.3c Normal saline (A), oestrogen cream (B), soybean crude-ethanolic extract (C) and Soybean-chloroform extract (D) applied topically to the wounds of rats



Plate 3.3d: Separate caging of the animal with paper beddings during the experiment

3.9.1 Gross evaluation of wound healing

Gross evaluation: Following wound formation, granulation tissue development, progress of epithelialization, exudate, scab formation, wound bed appearance, presence of pus, necrotic tissue, colour, and odour were visually evaluated and photographs were taken.

Wound Contraction: Wound contraction was evaluated by wound tracing method on days 0, 4, 8, 12, 16 and 20 after removing the crusts/scab. Wound area of each lesion on each evaluation day was done by tracing the perimeter of the wound onto a sterile transparent paper with a sterile pencil. The transparent paper was laid on the wound surface, smoothed, and held flat and immobilized by an assistant while the examiner traced out the wound area. The tracing was then placed on a metric grid (graph paper) and the number of squares counted to determine the area.

Percentage wound closure was calculated according to Moreira *et al.* (2015) thus;

$$\frac{\text{Initial wound size- Specific day wound Size}}{\text{Initial wound Size}} \times 100$$

3.9.2 Histopathologic evaluation of wound

Full thickness skin biopsy was harvested on day 4, 8, 12 and 16. Three animals per group were selected at random on each day and elliptical biopsy was from one of the wounded site on each animal. Thereafter, sampled biopsy site of the wound was sutured.

Histological procedure: Skin samples from both the wound and comparable adjoining normal skin was fixed in 10% neutral-buffered formalin. The tissues were mechanically and biochemically stabilized in neutral buffered formalin (10% formaldehyde in phosphate buffered saline (PSB) pH 7.0). The samples were then immersed in multiple baths of progressively more concentrated ethanol to dehydrate the tissue, followed by a

clearing agent, "xylene" and finally immersed in hot molten paraffin wax (impregnation). During this 12 to 16 hour process, paraffin wax replaced the xylene. The tissue was sectioned in 5μm using a microtome. These sections were placed on a glass slide for staining. The section on the slide was dewaxed in xylene and rehydrated in graded concentration of ethanol before staining. The staining method involved application of the basic dye hematoxylin, which coloured basophilic structures with blue-purple blue, and alcohol-based acidic eosin Y, which colors eosinophilic structures as bright pink. Masson green trichrome was also used for collagen evaluation (Oryan *et al*., 2010). Then, all slides were photographed on the computer screen using a light microscope (Leica, Japan) with a digital color camera attachment.

Abramov *et al.* (2007) histologic scoring system (Modified Greehalgh's scoring system) was used to score the histologic samples in terms of granulation development, collagen accumulation, acute inflammatory cells, chronic inflammatory cells, angiogenesis and fibrosis as shown in table 3.1.

Table 3.1: Histological scoring of the granulation tissue in ovariectomized albino rats ((Abramov *et al.*, 2007)

Wound Healing Parameters		**Scoring**			
		0	**1**	**2**	**3**
(A)	Granulation development	None or immature	Low amount	Moderate degree of maturation	Mature
(B)	Collagen accumulation	None	Low amount/mild	Moderate	High amount/dense
(C)	Acute inflammatory cells	None	Scant/<10 cells	Moderate/10-25 cells	Abundant/>25 cells
(D)	Chronic inflammatory cells	None	Scant/<10 cells	Moderate/10-25 cells	Abundant/>25 cells
(E)	Angiogenesis	None	< 5 vessels	6-10 vessels	>10 vessels
(F)	Fibroblast density	None	Low amount/mild	Moderate	High amount/dense

(A): The degree of granulation tissue maturation was determined by the shape and alignment of the fibroblasts, while mature fibroblasts were thin and usually arranged in compacted parallel layers, immature fibroblasts were stellate-shaped and less organized.

(B): The degree of network of collagen fiber deposistion was accessed

(C): Acute inflammation was defined as the presence of neutrophils and as such amount of neutrophils were counted

(D): Chronic inflammation was defined as the presence of plasma cells and monocytic cells and these cells were counted

(E): Angiogenesis was defined as the formation of new blood vessel from the existing ones and as such amount of blood vessels in the granulation tissue were counted

(F): Mature and immature fibroblast were identified and counted in 10 microscope field to determine their density.

3.9.3 Estimation of antioxidant enzymes and stress marker of granulation tissue.

Antioxidant enzymes of granulation tissue including superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and oxidative stress marker; malondialdehyde (MDA) were estimated in wet granulation tissue homogenate (Rahman *et al*., 2017).

a. Superoxide dismutase (SOD):

The reaction mixture was prepared by mixing 0.5 ml of granulation tissue homogenate, 1 ml 50mM sodium carbonate, 400 ml of 25 mM NBT (Nitro Blue Tetrazolium) and 200 ml 0.1 mM EDTA (Ethylene diamine tetraacetic acid), simultaneously control was prepared without tissue homogenate. The reaction was initiated by the addition of 400ml of 1mM hydroxylamine hydrochloride solution. The change in absorbance was recorded at 560 nm. Units of SOD activity were expressed as the amount of enzyme required to prevent the reduction of NBT by 50% (Rahman *et al*., 2017).

b. Catalase (CAT):

The assay mixture contained 1.95 ml of phosphate buffer (0.0 05 M, pH7.0), 1.0 ml (0.019 M) H_2O_2 and 5 ml of granulation tissue homogenate. The mixture was incubated at room temperature for 2 min. The catalase activity was calculated by the change of the absorbance at 240 nm (Rahman *et al*., 2017)

c. Reduced Glutathione (GSH):

An aliquot of 1 ml of granulation tissue homogenate (10%, w/v) was precipitated with 1 ml of sulphosalicylic acid (4% w/v). The samples were kept at 4^{o}C for 1 h and then centrifuged at 3500G for 15 min at 4^{o}C. The reaction mixture in a total volume of 3 ml consisted of 100 ml supernatant, 2.7 ml 0.1 M phosphate buffer (pH 7.4) and 200 ml of DTNB (40 mg dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.4) The optical density of the yellow colour developed was measured at 412 nm (Rahman *et al*., 2017).

d. Estimation of Malonaldehyde (MDA) concentration:

To the mixture of 500 ml 20% granulation tissue homogenate in 0.15M KCl, 100 ml of 0.2 mM ferric chloride solution was added to initiate the peroxidation at 37 ^{o}C for 30 min. The reaction was stopped by adding 2 ml of ice cold mixture of 0.25 N HCl containing 15% TCA (Trichloroacetic acid), 0.30% TBA (Thiobarbituric acid) and 0.05% BHT (Butylated hydroxytoluene). The reaction mixture was heated at 80 C for 60 min, the samples were cooled and centrifuged at 3500G for 15 min and the optical density (O.D.) of the supernatant was measured at 532 nm. Lipid peroxidation was expressed as malon-dialdehyde (MDA) equivalents in nanomoles per milligram of protein (Rahman *et al*., 2017).

3.9.4 Immunohistochemistry of Bcl-2 protein.

Five µm thick sections were cut from the specimens and placed on poly-L-lysine-coated slides. They were then dewaxed in xylene and rehydrated in graded alcohol. The endogenous peroxidase activity was consumed by immersing the sections in 0.1% hydrogen peroxide in absolute methanol for 20 minutes. Non-specific binding was blocked

by incubating the slides in 20% fetal calf serumin PBS for 20 minutes. A monoclonal antibody directed against Bcl-2 oncoprotein was obtained from Elabscience Biotechnology Inc. Texas USA. Before application of the primary antibody, the sections were heated in a microwave oven in 10 mM citric acid monohydrate, pH 6.0 for 3 minutes. After one hour of incubation with the primary antibody (dilution1:50) a biotinylated secondary anti-mouse antibody was applied followed by the HRP-biotin-peroxidase complex. The color was developed by adding diaminobenzidine and mounted. Negative control was prepared by omitting the primary antibody while normal skin was used for positive control.

Quick score was used for immunohistochemical evaluation of bcl-2 and brownish cytoplasmic staining was interpreted as positive for bcl-2. According to this score, intensity and percentage of positive staining of cells were evaluated. Intensity of the staining was scored as follows; 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong). Percentage of the positive cells were scored as 0 (no staining), 1 (10 <%), 2 (10- 50%), 3 (51-80%), and 4 (80%>). For each case, the values of the two parameters (percentage of the positive cells and predominant intensity) were multiplied, resulting in possible scores from 0 to 12 to obtain Ouick score (Ozer *et al.*, 2012).

3.10 Data Analysis

All data were analyzed using Graph pad prism version 5.0 and results were presented as mean and standard error (M±SEM). Both progression and decline in Bcl-2 quick score and histopathological score, wound areas and antioxidant assay among groups were assessed using Two-way Analysis of Variance (ANOVA) followed by Bonferroni post-hoc test. Values of $p<0.05$ was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Laboratory Preparation and Evaluation of Soybean.

4.1.1 Preliminary phytochemical studies of Soybean extracts

The result of preliminary phytochemical screening of both the ethanolic and chloroform Soybean extract using Thin Layer Chromatography revealed that soybean-ethanolic extract contained steroids, tritarpens, phenol, alkaloid and flavonoids while soybean-chloroform extract contained steroids, tritarpens, alkaloid and flavonoids (Table 4.1).

4.2 Wound Healing Properties of Soybean Extracts

4.2.1 Gross evaluation of the wound healing

On the third day of wound creation, 85% of the square shaped wound created had transformed into irregular circular shaped wounds. Scabs were also formed from the wound exudates which were not very firmly attached to the wound bed. It was observed that scabs on Normal saline treated wounds were very sticky, firmly attached to the wound beds and gum to the wound bed during debridement while scabs from other wounds were easily detached (Plate 4.1a, b, c, d).

Table 4.1 Phytochemical Screening of Soybean Extracts used in this study

Phytochemicals	Soybean ethanolic extract	Soybean chloroform extracts
Cardiac glycosides	_	_
Saponins	_	_
Streiods and Triterpenes	+	+
Flavonoids	+	+
Tannins	_	-
Alkaloids	+	+
Anthraquinones	_	_
Phenol	+	_

+ = Present, -=Absent

4.2.2 Wound contraction

The percentage wound contraction obtained in this study is as shown in Table 4.2. Normal saline control rats were 15.00 ± 0.82% to 64.56 ± 0.70% at day 4 to day 12 of the study while complete epithelization and healing was observed on day 18.25 ± 0.25. The Oestrogen-cream treated rats showed increased in rate of wound contraction from 19.64 ± 2.39% on day 4 to 90.29 ± 0.75% on day 12 and 100% at day 14.25 ± 0.25 as shown in Table 4.2. The percent rate of wound contraction in rats, treated topically with Soybean ethanolic extract, was from 20.25 ± 1.33% to 89.28 ± 0.95% on day 4 to day 12 and 100% wound contraction was observed on day14.00 ± 0.00. Similarly, the Soybean chloroform animals group had wound contraction of 19.73 ± 1.69% at 4th day and 79.43 ± 1.07% at 12th day and complete epithelisation was observed at 16.25 ± 0.25 day. At day 4, the maximum wound contraction was observed in the Soybean ethanolic extract treated group (20.25 ± 1.33%) while the minimum value was recorded with the normal saline treated wounds (15.00 ± 0.82%). At day 12, the maximum wound contraction was observed in the oestrogen cream treated group (90.29 ± 0.75%) while the minimum value was recorded with the normal saline treated wounds (64.56 ± 0.70%) (Plate 4.1 a-p).

Table 4.2 Mean percentages wound contraction (%) on days 4, 8, 12, 16 and 20 of wound healing in ovariectomised rats.

	Normal saline (%)	**Oestrogen cream** (%)	**Soybean ethanolic extract** (%)	**Soybean chloroform extract** (%)
Day4	15.00 ± 0.82^{a}	19.64 ± 2.39	20.25 ± 1.33	19.73 ± 1.69^{b}
	(13.04-16.67)	(14.35-25.20)	(16.81-23.23)	(15.18-22.55)
Day 8	32.67 ± 0.88^{a}	54.16 ± 1.39^{b}	50.45 ± 1.34^{bc}	44.54 ± 1.34^{c}
	(30.77-35.00)	(51.05-56.85)	(48.42-54.40)	(42.56-48.48)
Day 12	64.56 ± 0.70^{a}	90.29 ± 0.75^{b}	89.28 ± 0.95^{b}	79.43 ± 1.07^{c}
	(63.77-66.67)	(89.02-92.30)	(86.52-90.80)	(76.47-81.48)
Day 16	80.23 ± 0.51	-	-	100 ± 0.75
Day 20	-	-	-	-

^{abc} Means in the same row with different superscript alphabets were significantly different ($p<0.05$)

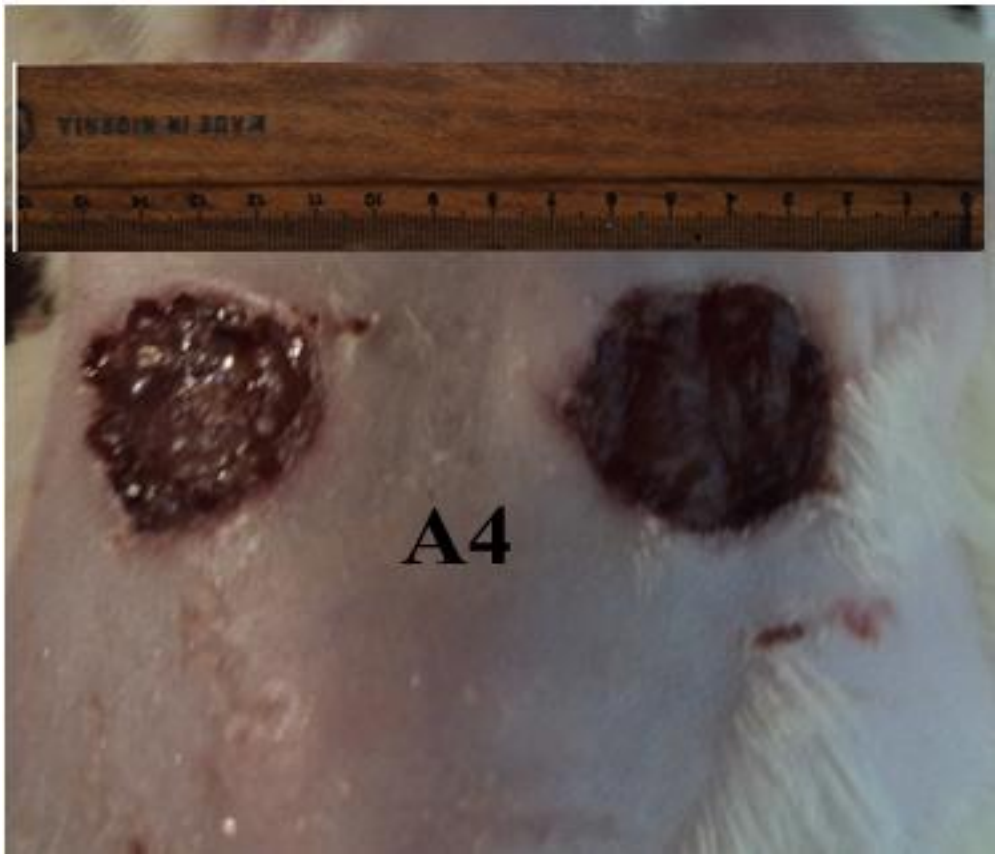


Plate 4.1a: Wound treated with normal saline. Transformation of all the wounds into irregular circular wounds at day 4. The wounds were covered with dry scab

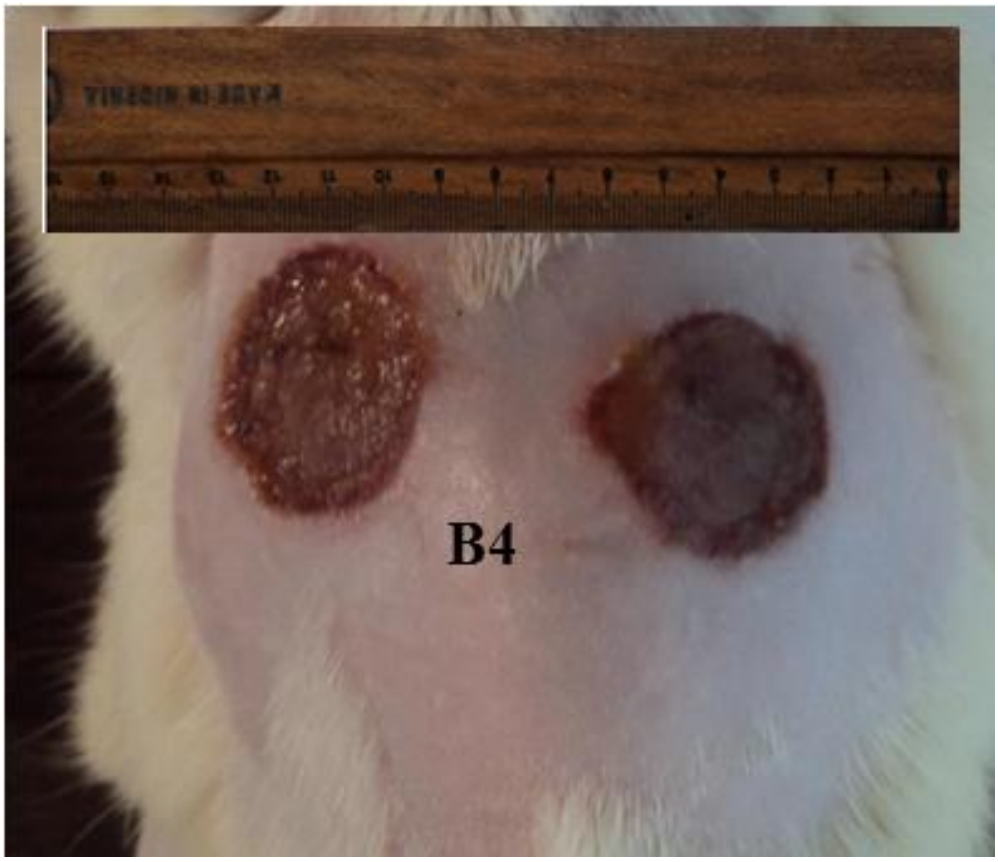


Plate 4.1b: Wound treated with oestrogen cream. Transformation of all the wounds into irregular circular wounds at day 4. The wounds were covered with dry scab.

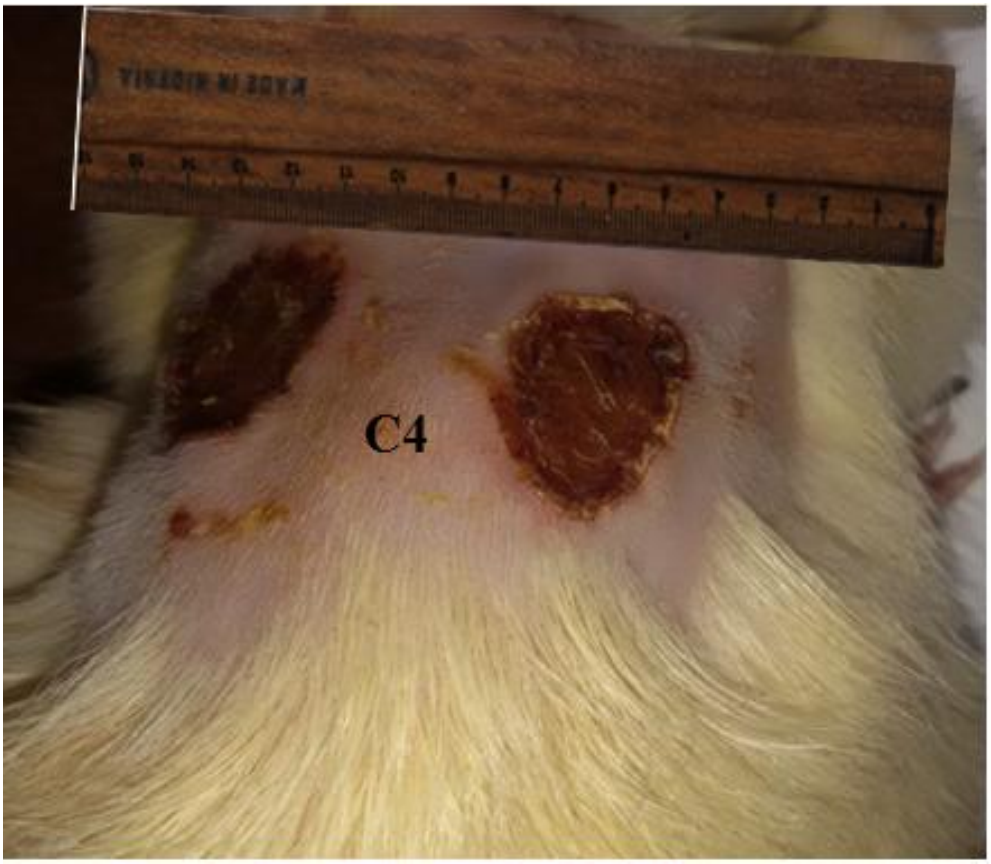


Plate 4.1c: Wound treated with soybean ethanolic extract. Transformation of all the wounds into irregular circular wounds at day 4. The wounds were covered with dry scab.

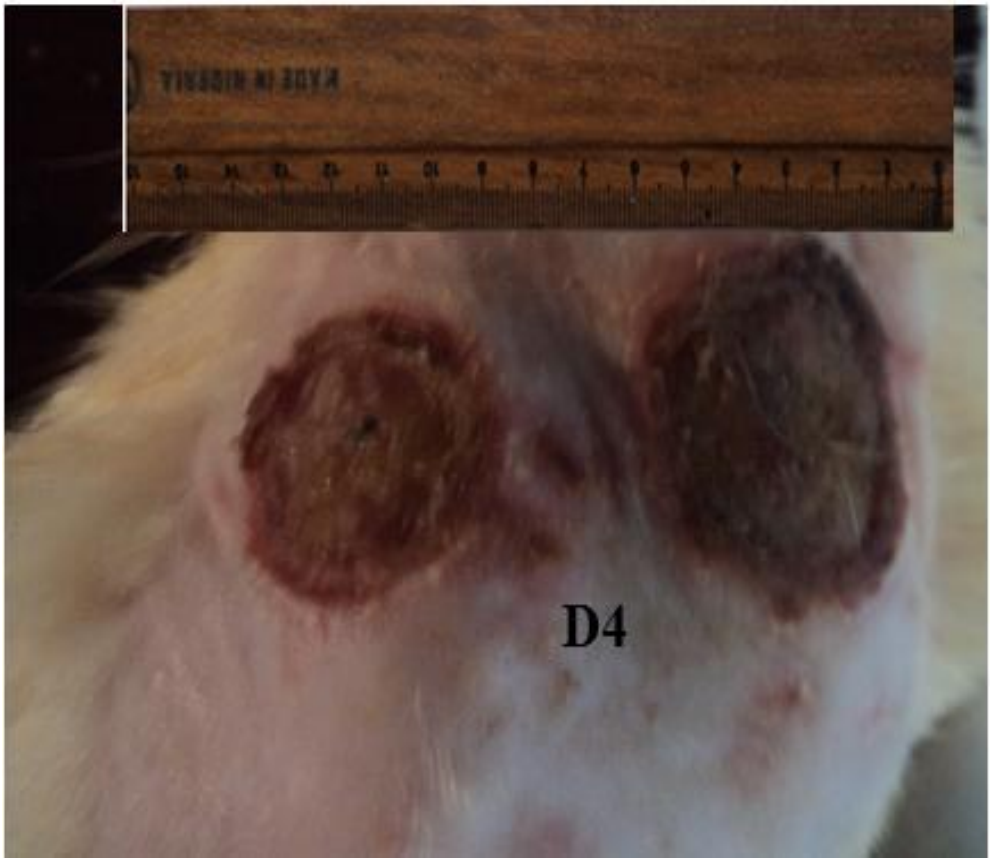


Plate 4.1d: Wound treated with soybean chloroform extract. Transformation of all the wounds into irregular circular wounds at day 4. The wounds were covered with dry scab



Plate 4.1e: Wound treated with normal saline. Reduced rate of wound epithelisation and contraction with inflammatory response at day 8. The wounds were covered with wet scab.

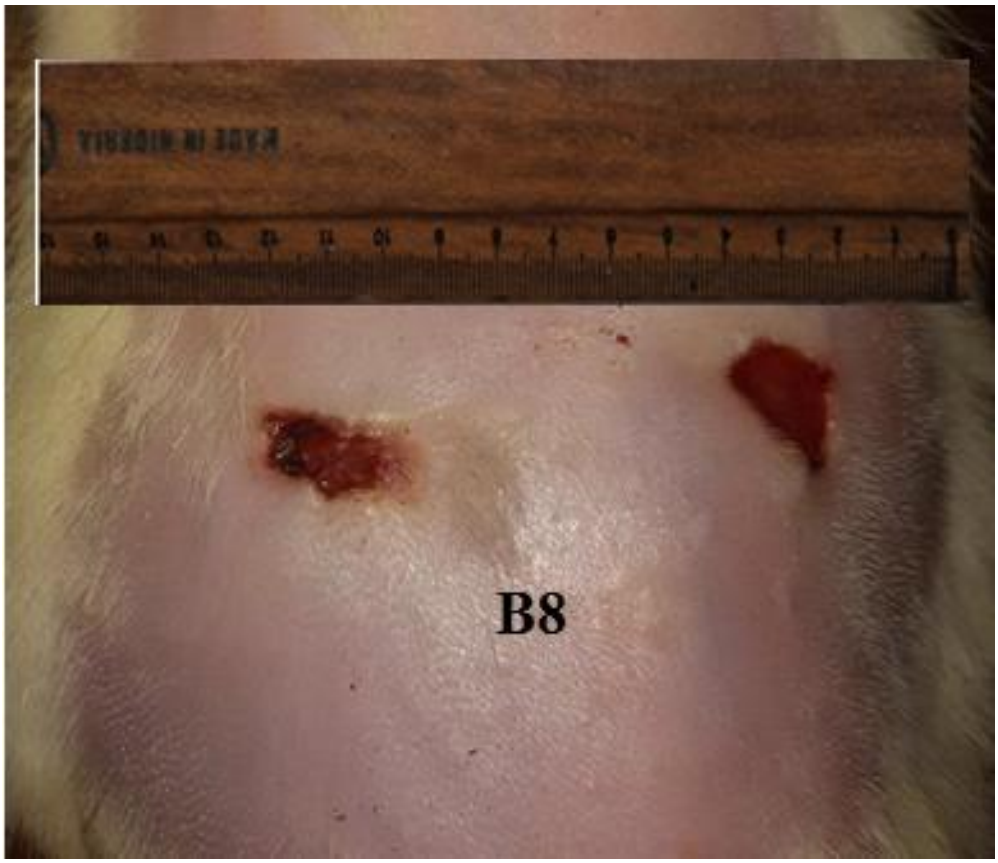


Plate 4.1f: Wound treated with oestrogen cream. There was increased rate of wound epithelisation and contraction. The wound reduced to half of the initial size at day 8.

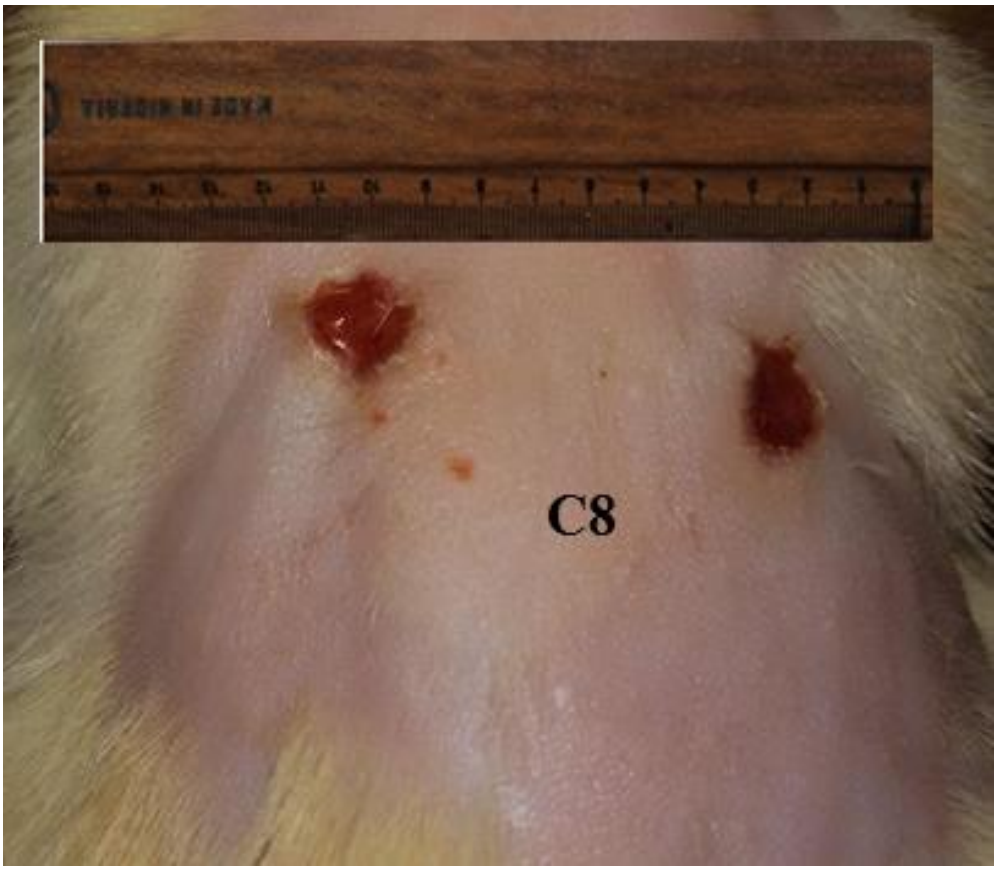


Plate 4.1g: Wound treated with soybean ethanolic extract. There was increased rate of wound epithelisation and contraction. The wound reduced to half of the initial size at day 8.

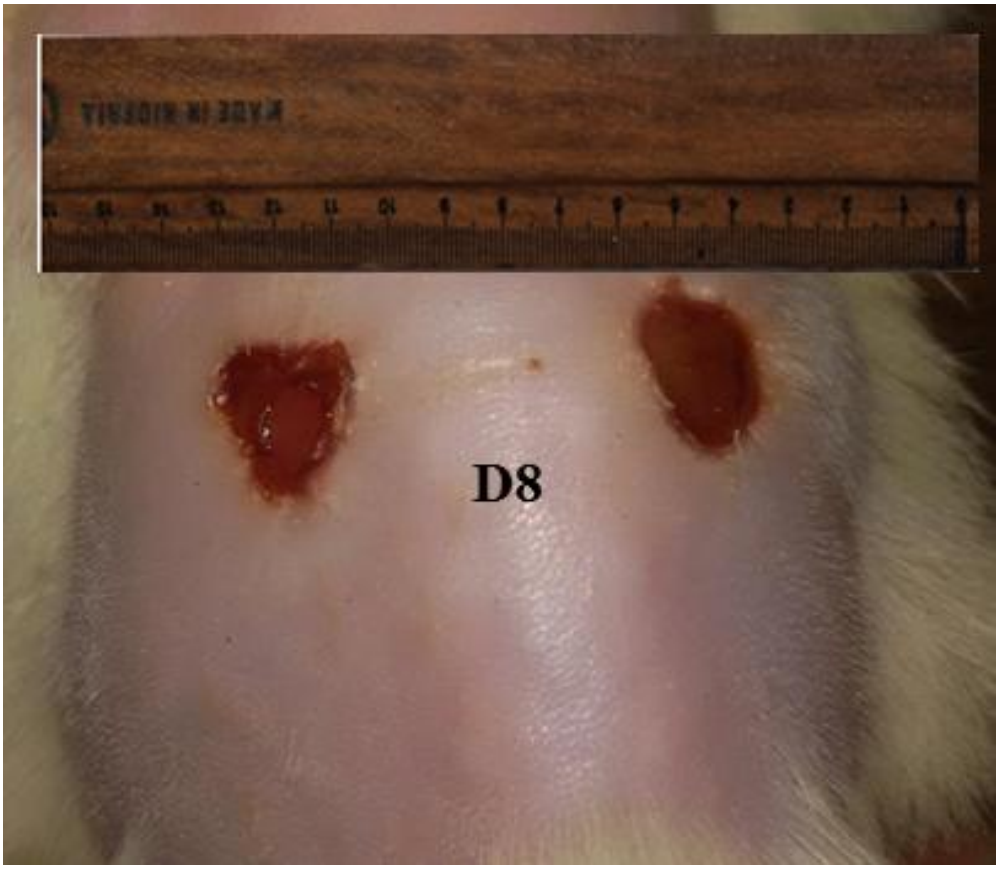


Plate 4.1h: Wound treated with soybean chloroform extract. There was increased rate of wound epithelisation and contraction. The wound reduced to half of the initial size at day 8.



Plate 4.1i: Wound treated with normal saline. Slow rate of wound epithelisation and contraction at day 12. The wounds covered with a thin scab.

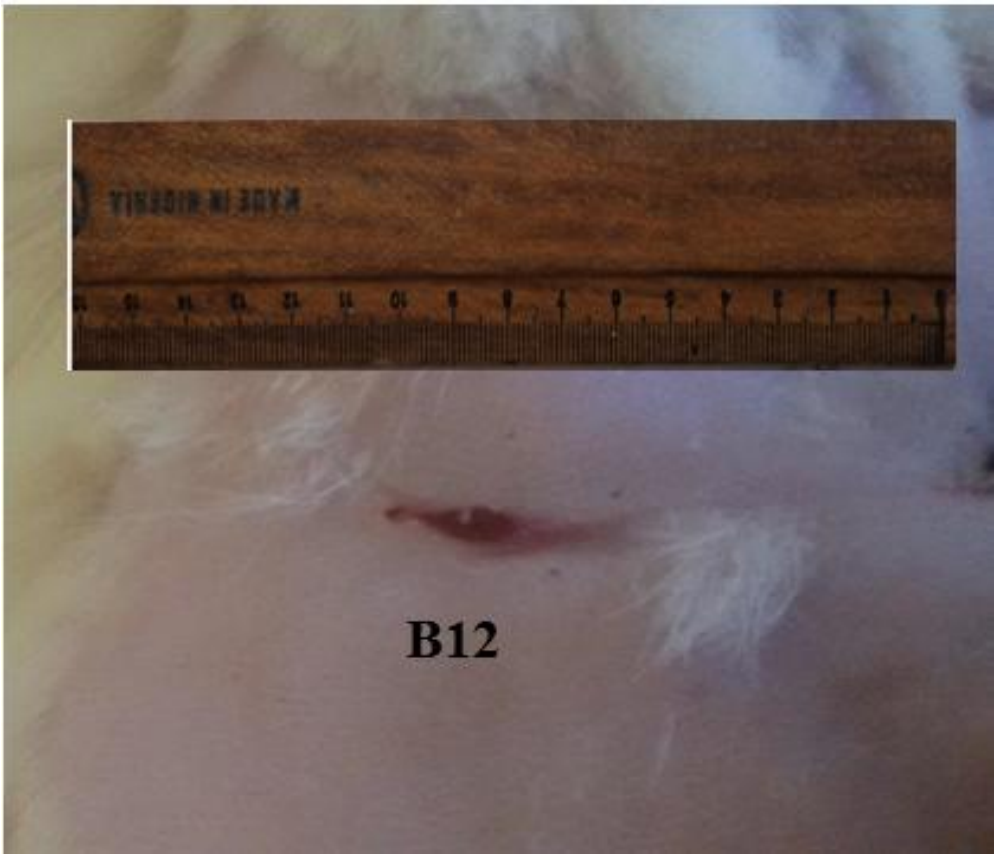


Plate 4.1j: Wound treated with oestrogen cream. Faster rate of wound epithelisation and contraction at day 12. The wounds covered with a thin scab.



Plate 4.1k: Wound treated with soybean ethanolic extract. Faster rate of wound epithelisation and contraction at day 12. The wounds covered with a thin scab.

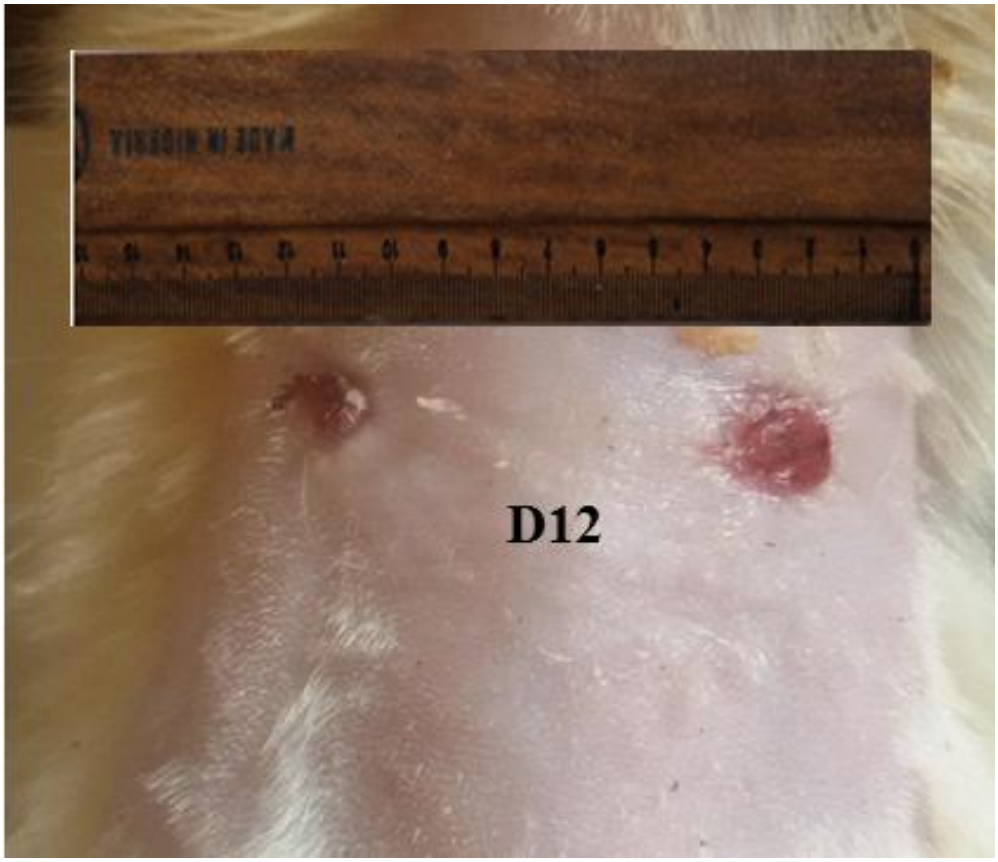


Plate 4.1l: Wound treated with soybean chloroform extract. Faster rate of wound epithelisation and contraction at day 12. The wounds covered with a thin scab.

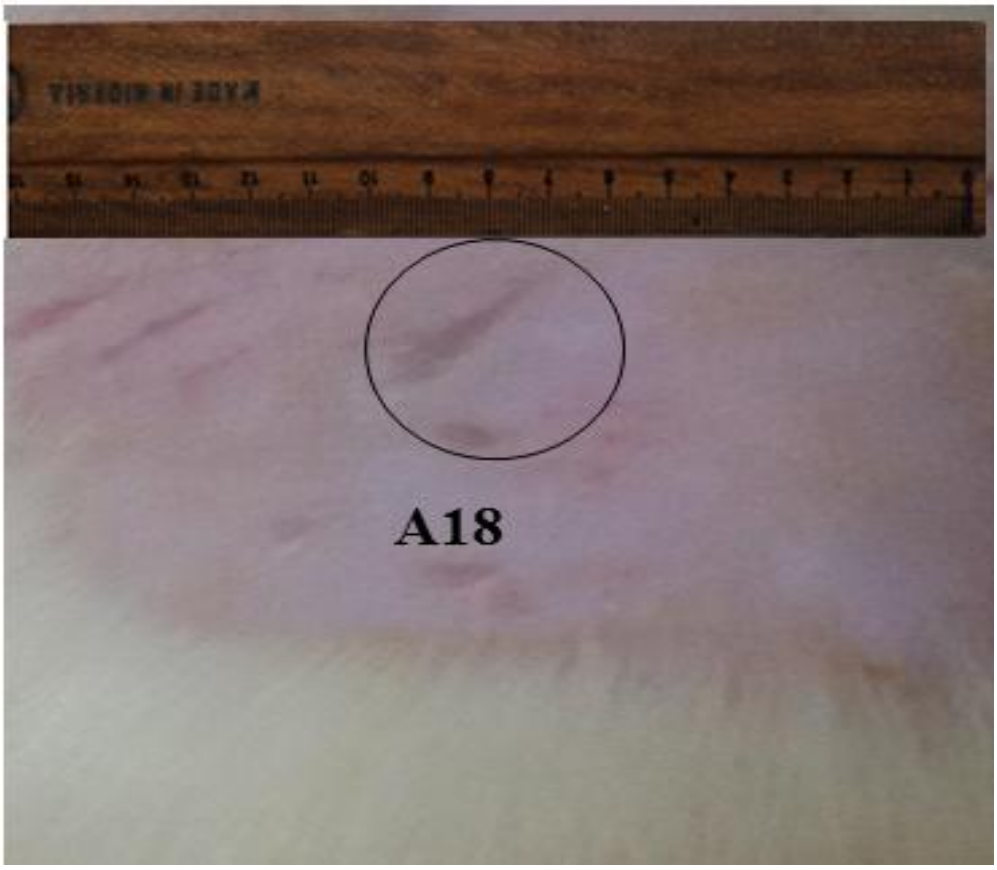


Plate 4.1m: Wound treated with normal saline. The scar formed on the healed wound on day 18 (circled).



Plate 4.1n: Wound treated with oestrogen cream. The scar formed on the healed wound on day 14 (circled).

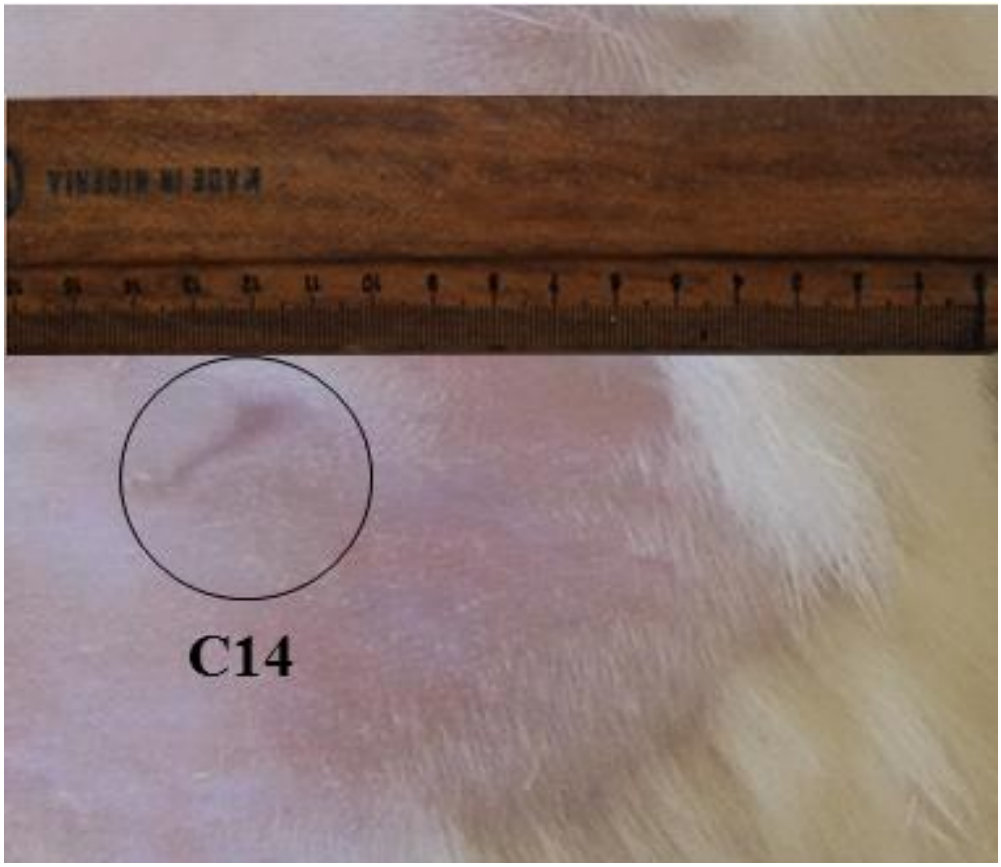


Plate 4.1o: Wound treated with soybean ethanolic extract. The scar formed on the healed wound on day 14 (circled).

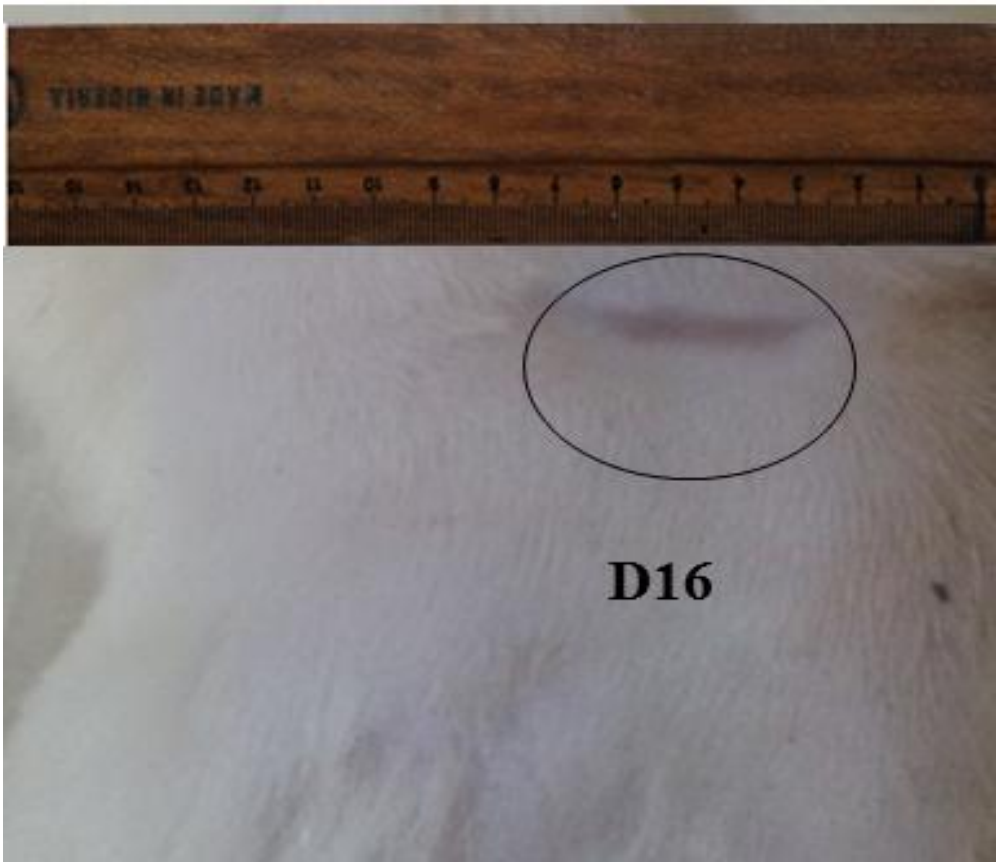


Plate 4.1p: Wound treated with soybean chloroform extract. The scar formed on the healed wound on day 16 (circled).

4.2.3 Histopathology of granulation tissue of skin wound in ovariectomised rats used in the study

Plate 4.2a-h, shows the histopathological sections while Table 4.3 shows the histopathological scoring of the granulation tissue components for the 4th, 8th, 12th and 16th days of wound healing in control rats as well as treated groups. Overall result indicated that normal saline treated wound showed minimal cellular infiltration, fibroblasts density, neovascularization and collagen deposition, while it had the highest inflammatory cells infiltration throughout the sampling periods. On the other hand oestrogen cream and Soybean-ethanolic extract treated groups had the highest cellular infiltration, fibroblasts density, angiogenesis and collagen deposition with low inflammatory cells infiltration throughout the sampling periods.

On day 4, normal saline and soybean-chloroform extract treated group showed lowest granulation tissue development with scanty to moderate amount of collagen fiber, blood vessel proliferation and fibroplasia while there was moderate to abundant amount of acute inflammatory cells which were largely made up of neutrophils. However, in oestrogen cream and soybean-ethanolic treated groups, there were scanty inflammatory cells infiltration with high granulation tissue development, moderate collagen deposition, numerous blood vessels and moderate to abundant fibroblast density.

On day 8, normal saline and Soybean-chloroform treated groups showed loosely laid collagen fibers with irregular pattern with few proliferating blood capillaries and lesser fibrous tissue at the wound site while there were moderate amount of acute and chronic inflammatory cells. Meanwhile 8th day oestrogen cream and soybean-ethanolic treated

wound showed cluster of collagen fibers parallel to the wound region with proliferating fibroblasts, moderate blood vessels and scanty inflammatory cells.

The histology of wound area on day 12 after surgery showed that wounds dressed with the oestrogen cream and soybean-ethanolic extract showed comparatively less scar width at wound closure compared to the normal saline and Soybean-chloroform group. In all the groups, except the normal saline control group, inflammatory cell infiltration was less intense. In Soybean-ethanolic extract treated groups and the oestrogen cream treated group, comparatively, more collagen, fibroblasts and blood capillaries were seen.

In both groups B (oestrogen cream treated) and C (Soybean-ethanolic acid treated), the granulation tissues contained comparatively less inflammatory cells and more collagen, fibroblast, and blood proliferating capillaries than the normal saline and soybean-chloroform treated group.

Table 4.3. Histopathology of granulation tissue of wounds in ovariectomised rats used for the study.

Treatment group/biopsy day	Granulation development	Collagen	Acute inflammatory cells	Chronic inflammatory cells	Angiogenesis	Fibroblast density
A/Day4	1.00 ± 0.58	0.33 ± 0.33	2.33 ± 0.33	0.33 ± 0.33	0.33 ± 0.33	0.67 ± 0.33
B/Day4	2.00 ± 0.00	1.33 ± 0.33	1.33 ± 0.33	1.33 ± 0.33	1.33 ± 0.33	2.00 ± 0.58
C/Day4	1.33 ± 0.33	1.00 ± 0.58	1.33 ± 0.33	1.00 ± 0.58	1.67 ± 0.33	1.67 ± 0.33
D/Day4	1.00 ± 0.00	0.67 ± 0.33	2.00 ± 0.00	1.00 ± 0.58	0.67 ± 0.33	1.33 ± 0.33
A/Day8	1.67 ± 0.33	0.67 ± 0.33	2.00 ± 0.00	1.67 ± 0.33	1.00 ± 0.00	1.67 ± 0.33
B/Day8	2.33 ± 0.33	1.33 ± 0.33	1.00 ± 0.57	1.33 ± 0.33	1.67 ± 0.33	2.00 ± 0.58
C/Day8	2.33 ± 0.33	1.00 ± 0.58	0.67 ± 0.33	1.00 ± 0.58	1.33 ± 0.33	1.67 ± 0.88
D/Day8	1.67 ± 0.33	1.00 ± 0.00	1.67 ± 0.33	1.33 ± 0.33	1.67 ± 0.33	1.33 ± 0.33
A/Day12	1.67 ± 0.33	1.33 ± 0.33	1.33 ± 0.33	0.67 ± 0.33	1.00 ± 0.58	1.67 ± 0.33
B/Day12	2.67 ± 0.33	2.00 ± 0.00	0.67 ± 0.33	0.00 ± 0.00	1.33 ± 0.67	2.00 ± 0.00
C/Day12	2.33 ± 0.33	2.33 ± 0.33	0.33 ± 0.33	0.33 ± 0.33	1.67 ± 0.88	2.00 ± 0.00
D/Day12	2.00 ± 0.00	1.67 ± 0.33	1.00 ± 0.58	0.67 ± 0.33	1.67 ± 0.67	2.67 ± 0.33
A/Day16	1.33 ± 0.33	1.33 ± 0.33	1.33 ± 0.33	0.67 ± 0.33	0.33 ± 0.33	1.67 ± 0.33
B/Day16	2.67 ± 0.33	2.67 ± 0.33	0.67 ± 0.33	0.33 ± 0.33	2.00 ± 0.00	2.33 ± 0.33
C/Day16	2.33 ± 0.33	2.67 ± 0.33	0.33 ± 0.33	0.00 ± 0.00	2.33 ± 0.33	2.33 ± 0.33
D/Day16	1.67 ± 0.33	1.67 ± 0.33	1.00 ± 0.00	0.33 ± 0.33	1.00 ± 0.58	2.00 ± 0.00

[abc] Means in the same row with different superscript alphabets were significantly different ($p<0.05$)

KEY: A; Normal saline, B: oestrogen cream, C: Soybean ethanolic extract, D: Soybean chloroform extract.

Histology score keys: Granulation development, Collagen accumulation and fibroblast density (0; None, 1; Low amount, 2; moderate, 3; high amount); Acute and chronic inflammatory cells (0; None, 1; Low amount/<10cells, 2; moderate/10-25cells, 3; high amount/>25cells); Angiogenesis (0; None, 1; < 5 vessels 2; 6 -10 vessels 3; >10 vesels)

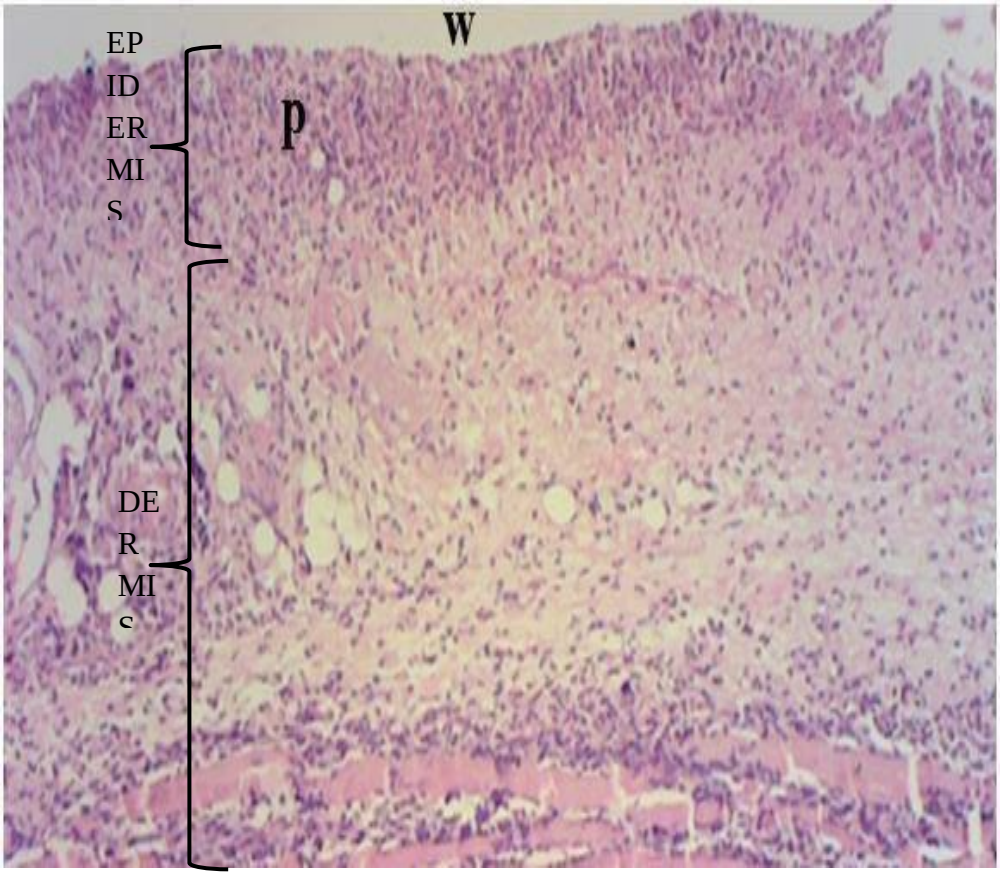


Plate 4.2a: Micrograph of the granulation tissue harvested from the wounds treated with normal saline on day 4 post wounding. w (wound area) and p (mixed type inflammatory cells). H&E staining, Mg x 100.

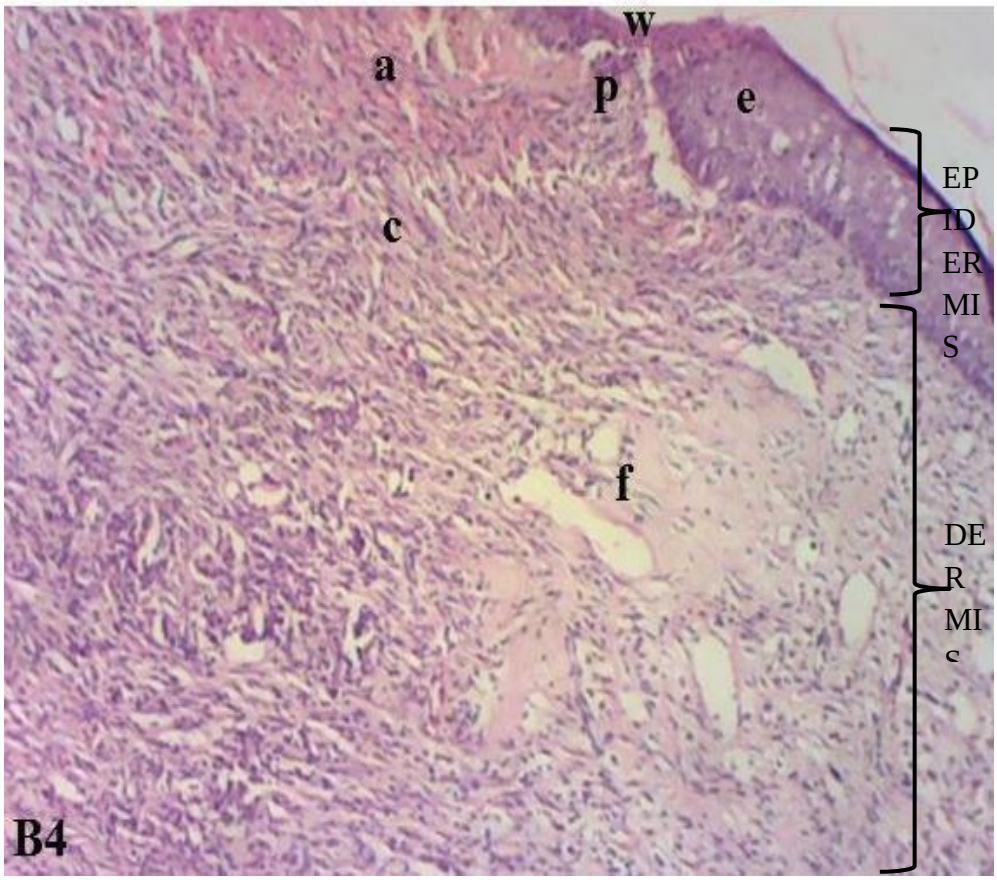


Plate 4.2b: Micrograph of the granulation tissue harvested from the wounds treated with oestrogen cream on day 4 post wounding. w (wound area), p (mixed type inflammatory cells), a (angiogenesis), c (collagen fibers), e (epidermis with keratinocyte migrating into the wound) and f (fibroblast). H&E staining, Mg x 100.

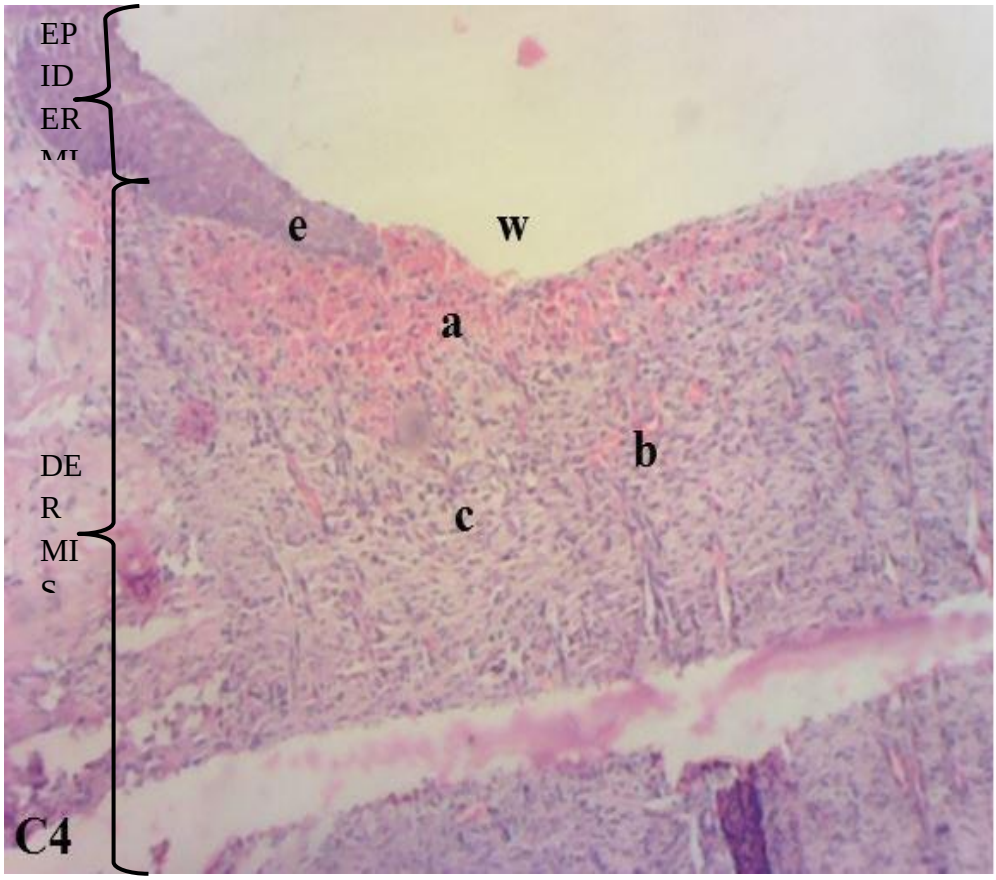


Plate 4.2c: Micrograph of the granulation tissue harvested from the wounds treated with soybean ethanolic extract on day 4 post wounding. w (wound area), a (angiogenesis), b (blood vessel), c (collagen fibers) and e (epidermis with keratinocyte migrating into the wound). H&E staining, Mg x 100.

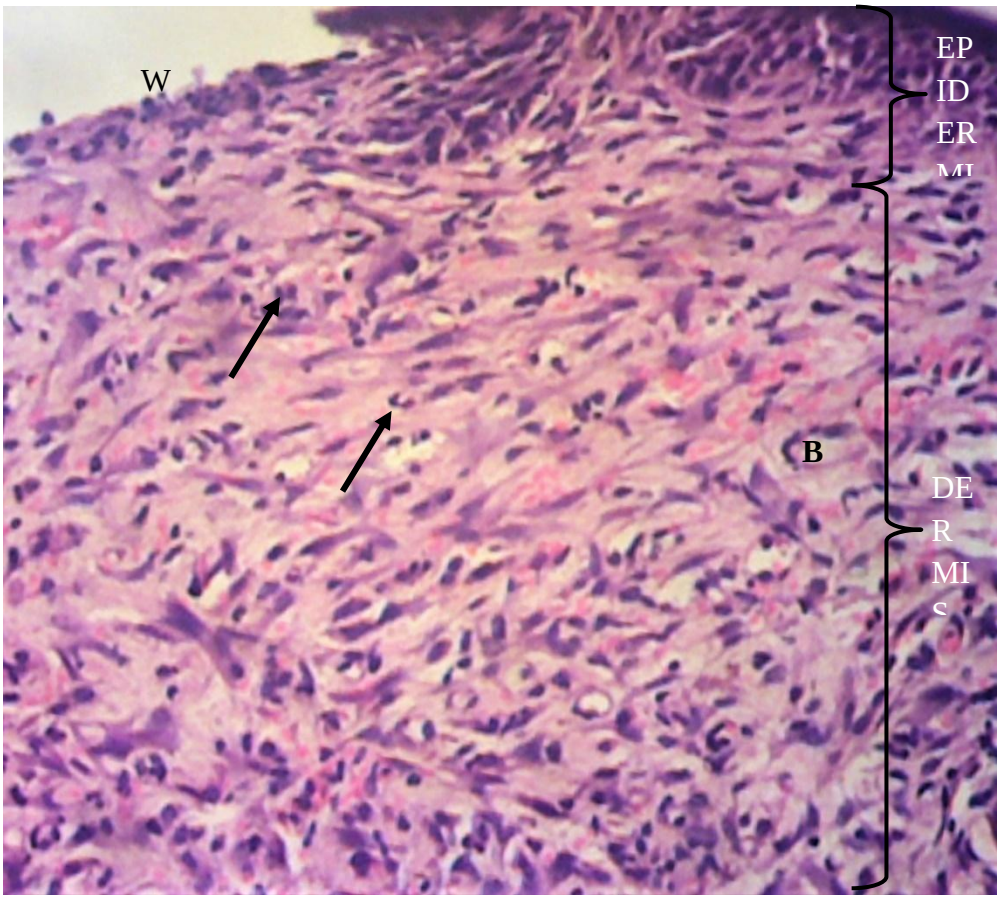


Plate 4.2d: Micrograph of the granulation tissue harvested from the wounds treated with soybean chloroform extract on day 4 post wounding. w (wound area), mixed type inflammatory cells (black arrows), and b (blood vessel). H&E staining. Mg x 100

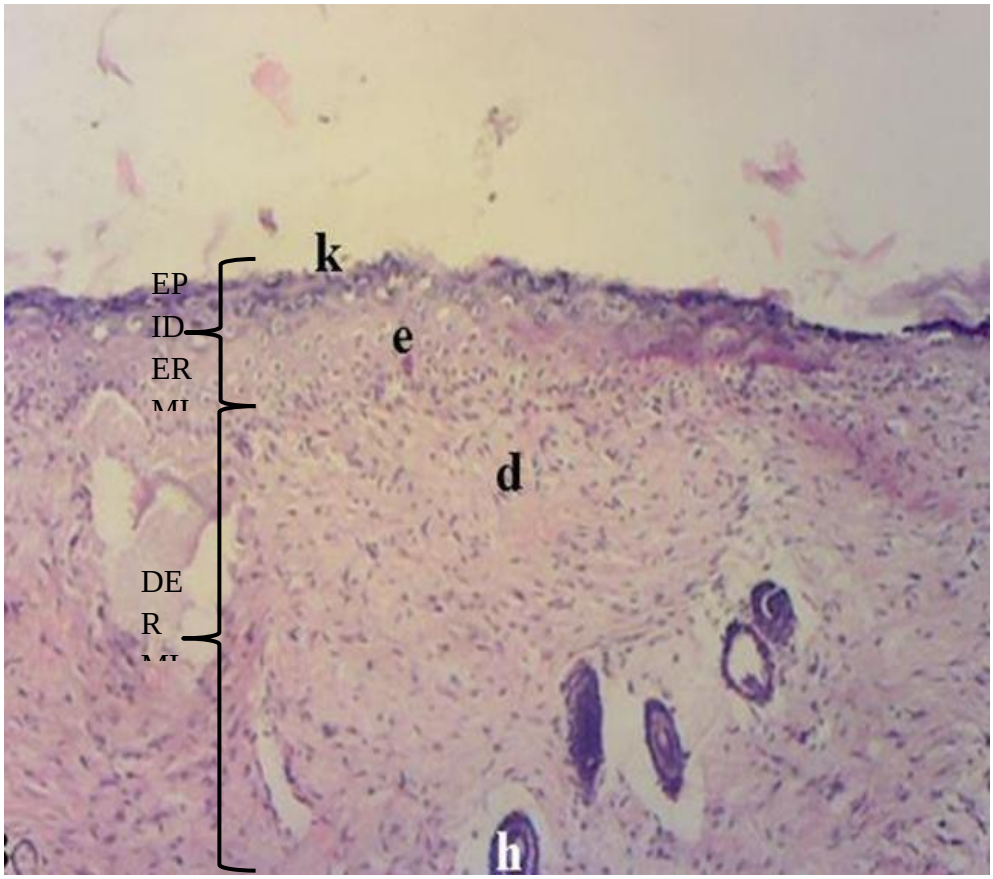


Plate 4.2e: Micrograph of the granulation tissue harvested from the wounds treated with normal saline on day 18 post wounding. k (moderate keratin deposition on the epidermis), d (dermis composed of loose collagen fibers) and h (hair follicles. H&E staining, Mag x 100.

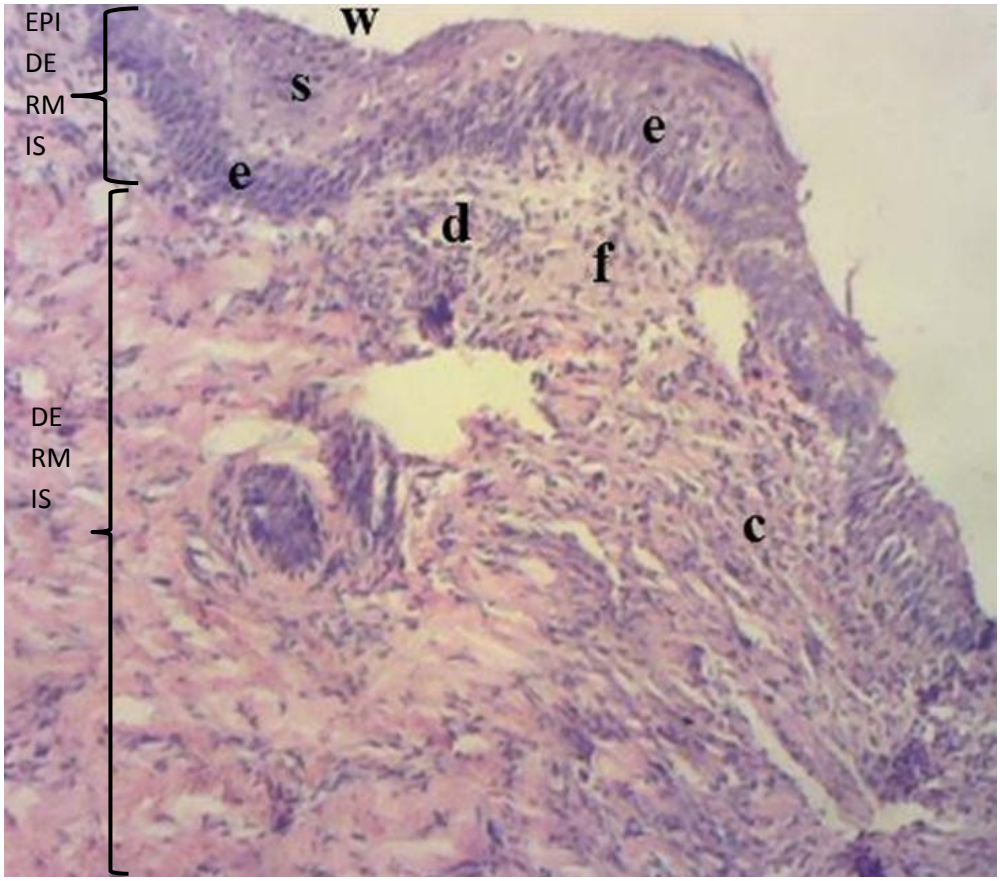


Plate 4.2f: Micrograph of the granulation tissue harvested from the wounds treated with oestrogen cream on day 14 post wounding. w (wound area), e (complete epithelisation of the epidermis), s (scar tissue), d (dermis), c (collagen fibers) and f (fibroblast). H&E staining. Mag x 100.

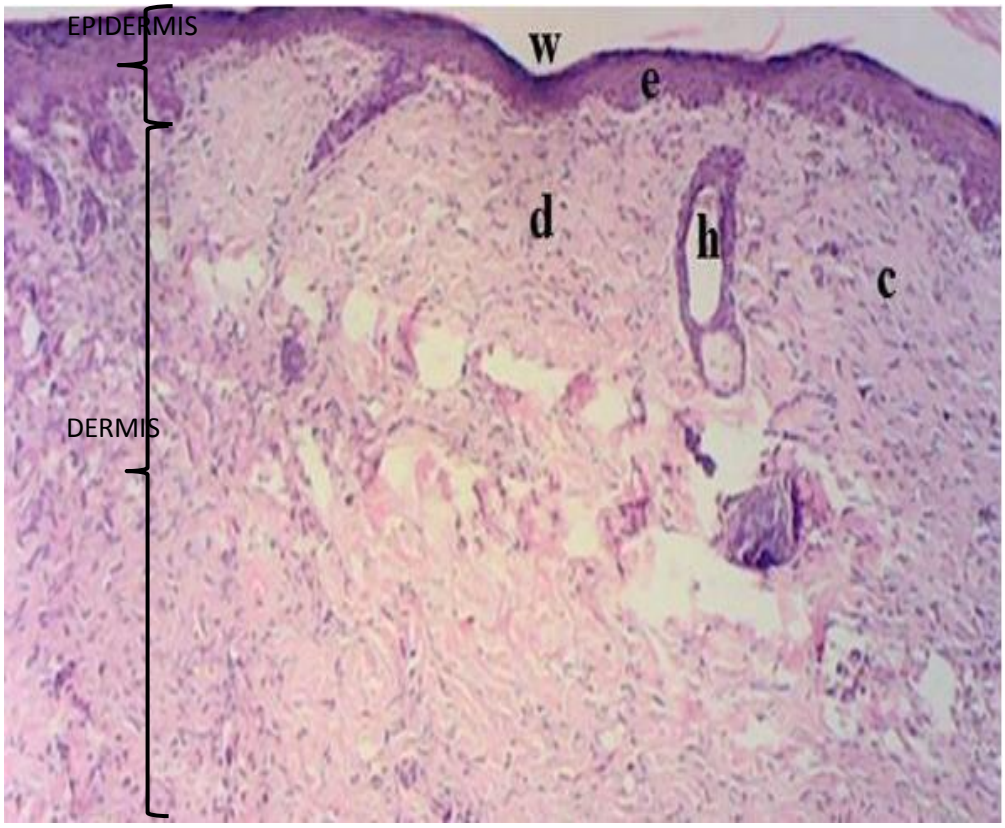


Plate 4.2g: Micrograph of the granulation tissue harvested from the wounds treated with soybean ethanolic extract on day 14 post wounding. w (wound area), e (complete epithelisation of the epidermis), d (dermis composed of loose and dense collagen fibers 'c') and h (hair follicles. H&E staining. Mg x 100.

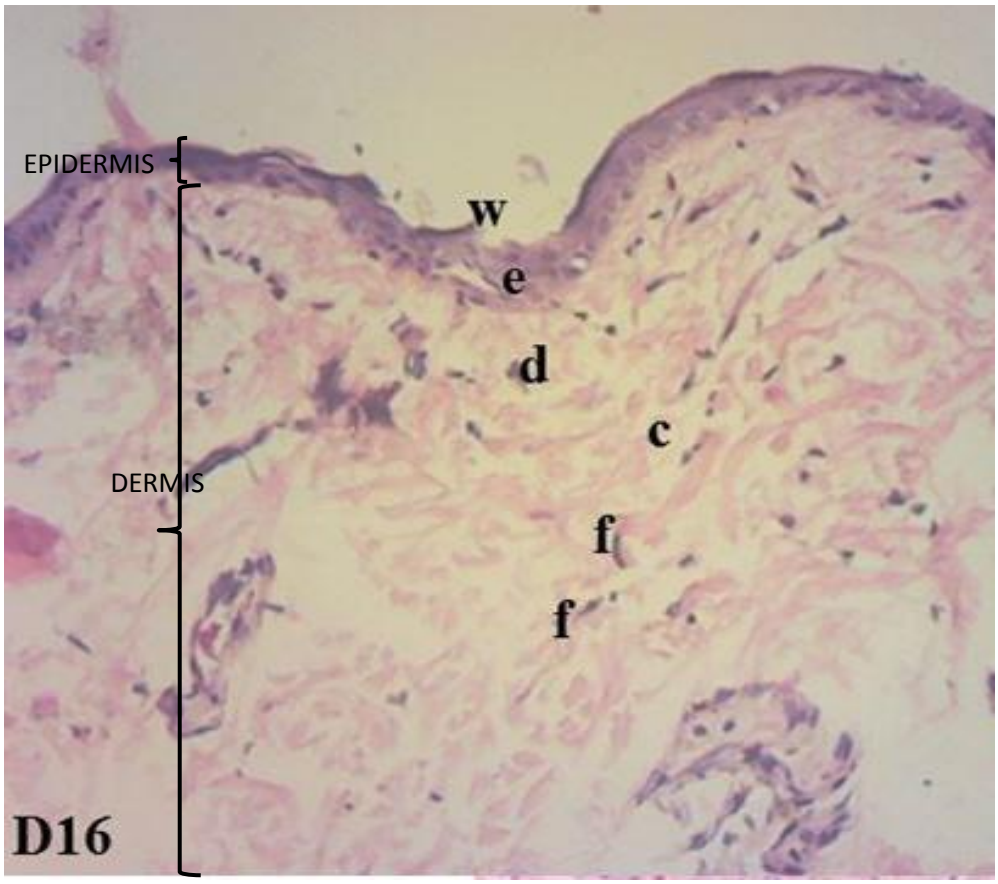


Plate 4.2h: Micrograph of the granulation tissue harvested from the wounds treated with soybean chloroform extract on day 16 post wounding. w (wound area), e (complete epithelisation of the epidermis), c (collagen fibers) f (fibroblat), d (dermis). H&E staining, Mg x 100.

4.3 Antioxidant Enzymes and Oxidative Stress Marker Expression in Granulation Tissue of ovariectomised rats used for the study

4.3.1 Reduced glutathione (GSH) concentration (µg/ml)

The mean value of reduced glutathione (GSH) expression in granulation tissue of all groups on day 4, 8, 12 and 16 is shown in figure 4.1. There was a general increase in GSH expression in granulation tissue of treated groups (71.50 ± 4.16 µg/ml to 77.43 ± 1.23 µg/ml) as compared to normal saline control group (63.5 ± 0.5 µg/ml) on day 4. The GSH level measured in granulation tissue was highest on day 8 in the estrogen cream treated (84.67 ± 2.34 µg/ml) and soybean ethanolic extract treated (82.27 ± 1.63 µg/ml) groups while the normal aline treated group had the lowest level (56.90 ± 8.09 µg/ml). There was a decline in GSH activity in estrogn cream, soybean ethanolic extract and soybean chloroform extract (66.87 ± 3.32 µg/ml, 60.37 ± 4.04 µg/ml, 51.17 ± 5.47 µg/ml respectively) treated groups on day 12 post-wounding, with normal saline (45.77 ± 0.43 µg/ml) treated groups having lowest GSH activity. There was no much difference in the GSH activity of both the oestrogn cream, soybean ethanolic extract and soybean chloroform extract treated groups (60.30 ± 1.91 µg/ml, 61.90 ± 3.82 µg/ml, 58.33 ± 3.74 µg/ml respectively) and Normal saline control group (57 ± 0.06 µg/ml) on day 16 post wounding.

4.3.2 Malonaldehyde (MDA) concentration (µmols/mg protein)

The mean value of malonaldehyde (MDA) concentration in granulation tissue of all groups on day 4, 8, 12 and 16 is shown in figure 4.2. The oestrogen cream, soybean ethanolic extract and soybean chloroform extract treated groups (77.37 ± 7.88 µmols/mg protein, 90.13 ± 3.32 µmols/mg protein, 86.60 ± 6.99 µmols/mg protein respectively) had lowest

MDA concentration on day 4 while normal saline treated group had a high MDA concentration in granulation tissue (180.97 ± 10.0 µmols/mg protein). On day 8 post wounding the MDA concentration was 117.18 ± 8.77 µmols/mg protein, 129.66 ± 7.0 µmols/mg protein, 133.83 ± 32.06 µmols/mg protein respectively in the oestrogen cream, soybean ethanolic extract and soybean chloroform extract treated groups while it was 180.43 ± 10.2 µmols/mg protein in the normal saline treated. There was a reduction the MDA concentration in oestrogen cream, soybean ethanolic extract and soybean chloroform extract treated groups on day 12 (210.27 ± 16.10 µmols/mg protein, 193.33 ± 17.55 µmols/mg protein, 227.13 ± 21.49 µmols/mg protein respectively) compared to normal saline control group (282.87 ± 0.09 µmols/mg protein). Both the oestrogen cream (89.00 ± 10.98 µmols/mg protein) and soybean ethanolic extract treated groups (74.80 ± 7.45 µmols/mg protein) had a lower MDA concentration on day 16 compared to soybean chloroform extract (104.50 ± 5.27 µmols/mg protein) and normal saline (132.97 ± 1.32 µmols/mg protein) treated groups.

4.3.3 Superoxide dismutase (SOD) activity (mg/mL)

The mean value of Superoxide dismutase (SOD) concentration in granulation tissue of all groups on day 4, 8, 12 and 16 is shown in figure 4.3. There was general increase expression of Superoxide dismutase (SOD) in granulation tissue in both normal saline control and treated groups throughout the period of sampling. The oestrogen cream treated group had persistently higher activity of Superoxide dismutase (SOD) enzyme in granulation between day 4 (36.74 ± 0.18 mg/mL) to day 16 (30.9 ± 0.64 mg/mL) of wound healing compared to other groups. Soybean ethanolic extract treated group had a high SOD activity on day 12 (33.98 ± 1.41 mg/mL) and day 16 (32.80 ± 0.52 mg/mL) while Soybean

chloroform extract treated group had a high SOD activity on day 4 (31.85 ± 1.77 mg/mL) and day 12 (30.99 ± 0.58 mg/mL) compared to the normal saline treated group which had the lowest SOD activity ranging from 28.08 ±2.28 mg/mL on day 4 to 24.53 ± 1.01 mg/mL on day 16 post wounding.

4.3.4 Catalase (CAT) activity (µg/ml)

The mean value of Catalase (CAT) concentration in granulation tissue of all groups on day 4, 8, 12 and 16 is shown in figure 4.4. There was a increase in CAT activity in granulation tissue of oetrogen cream, soybean ethanolic extract and soybean chloroform extract treated group on day 4 (25.80 ± 2.13 µg/ml, 23.63 ± 1.19 µg/ml, 22.49 ± 3.81 µg/ml respectively) which decreases gradually to 21.17 ± 0.84 µg/ml, 22.93± 0.64 µg/ml and 20.85± 5.41 µg/ml respectively on day 16 for oetrogen cream, soybean ethanolic extract and soybean chloroform extract treated group compared to the normal aline control group which had a low SOD activity on day 4 (22.77 ± 1.96 µg/ml) and high SOD activity on day 16 (22.37 ± 2.45 µg/ml).

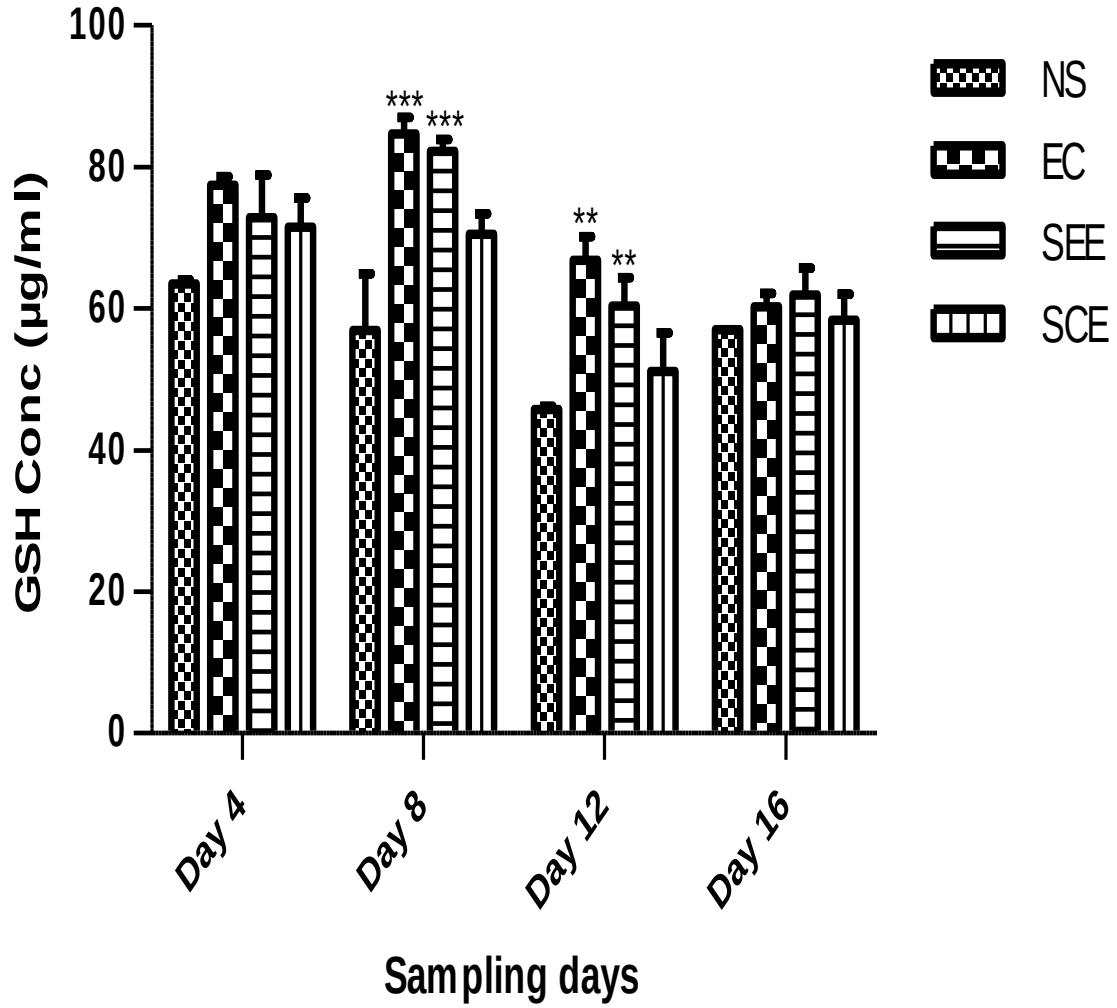


Figure 4.1: Biochemical estimation of reduced glutathione (GSH) activity in granulation tissue samples harvested from healing wounds treated with different agents

KEY:

***Mean value is statistically significant at $p<0.001$

**Mean value is statistically significant at $p<0.01$

NS: Normal Saline; EC: Estrogen cream; SEE: Soybean ethanolic extract; SCE: Soybean Chloroform extract

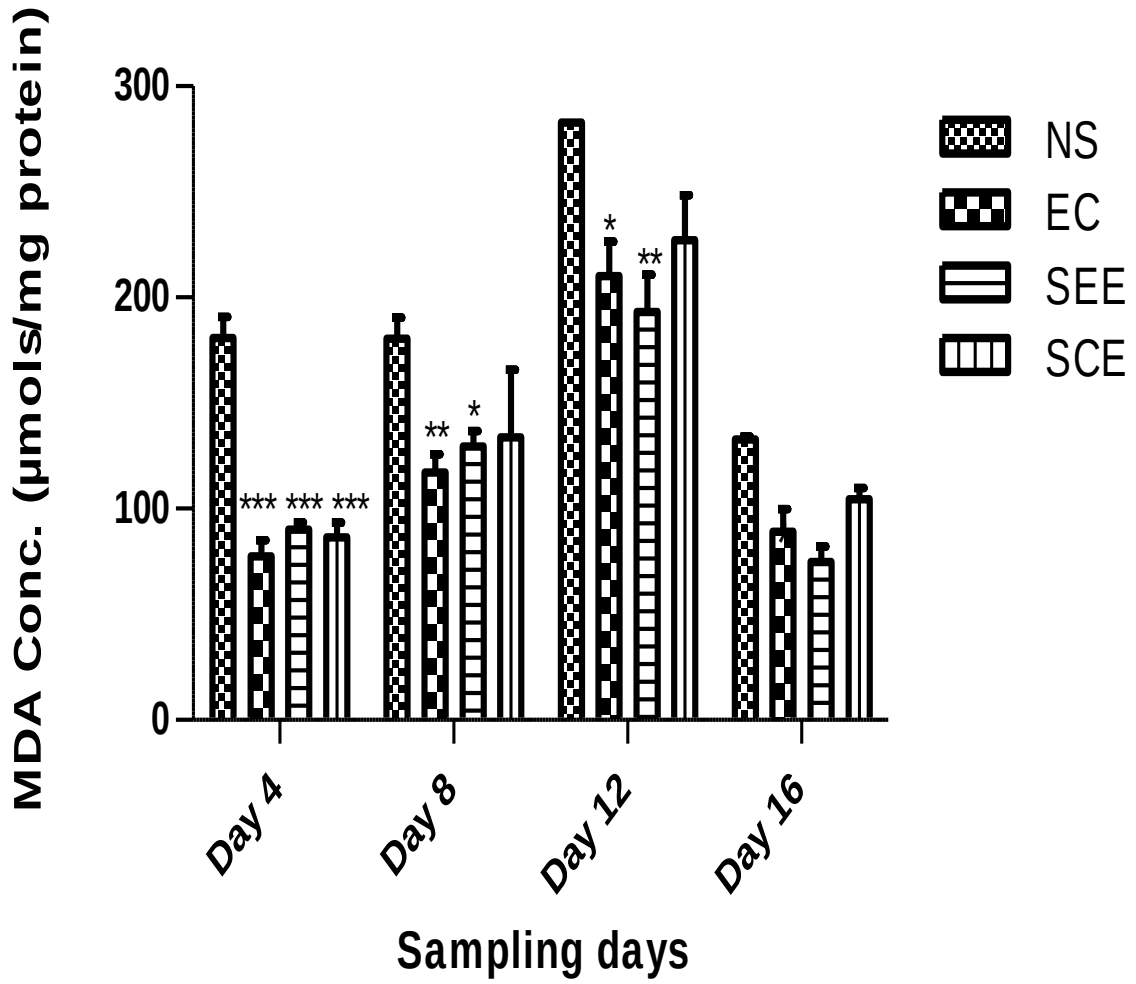


Figure 4.2: Biochemical estimation of malonaldehyde (MDA) concentration in granulation tissue samples harvested from healing wounds treated with different agents

KEY

*Mean value is statistically significant at $p<0.05$

**Mean value is statistically significant at $p<0.01$

***Mean value is statistically significant at $p<0.001$

NS: Normal Saline; EC: Estrogen cream; SEE: Soybean ethanolic extract; SCE: Soybean Chloroform extract

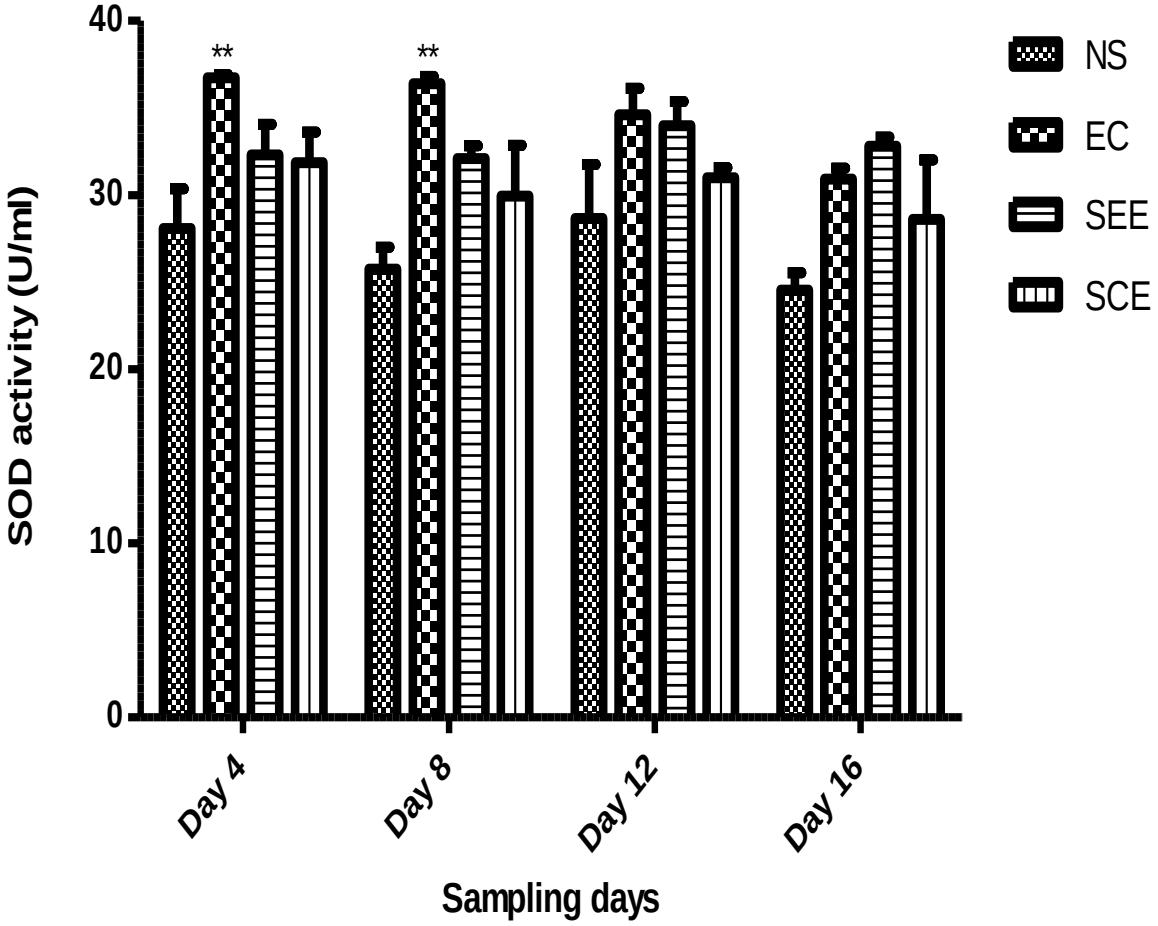


Figure 4.3: Biochemical estimation of Superoxide dismutase (SOD) activity in granulation tissue samples harvested from healing wounds treated with different agents.

KEY

**Mean value is statistically significant at $p<0.01$

NS: Normal Saline; EC: Estrogen cream; SEE: Soybean ethanolic extract; SCE: Soybean Chloroform extract

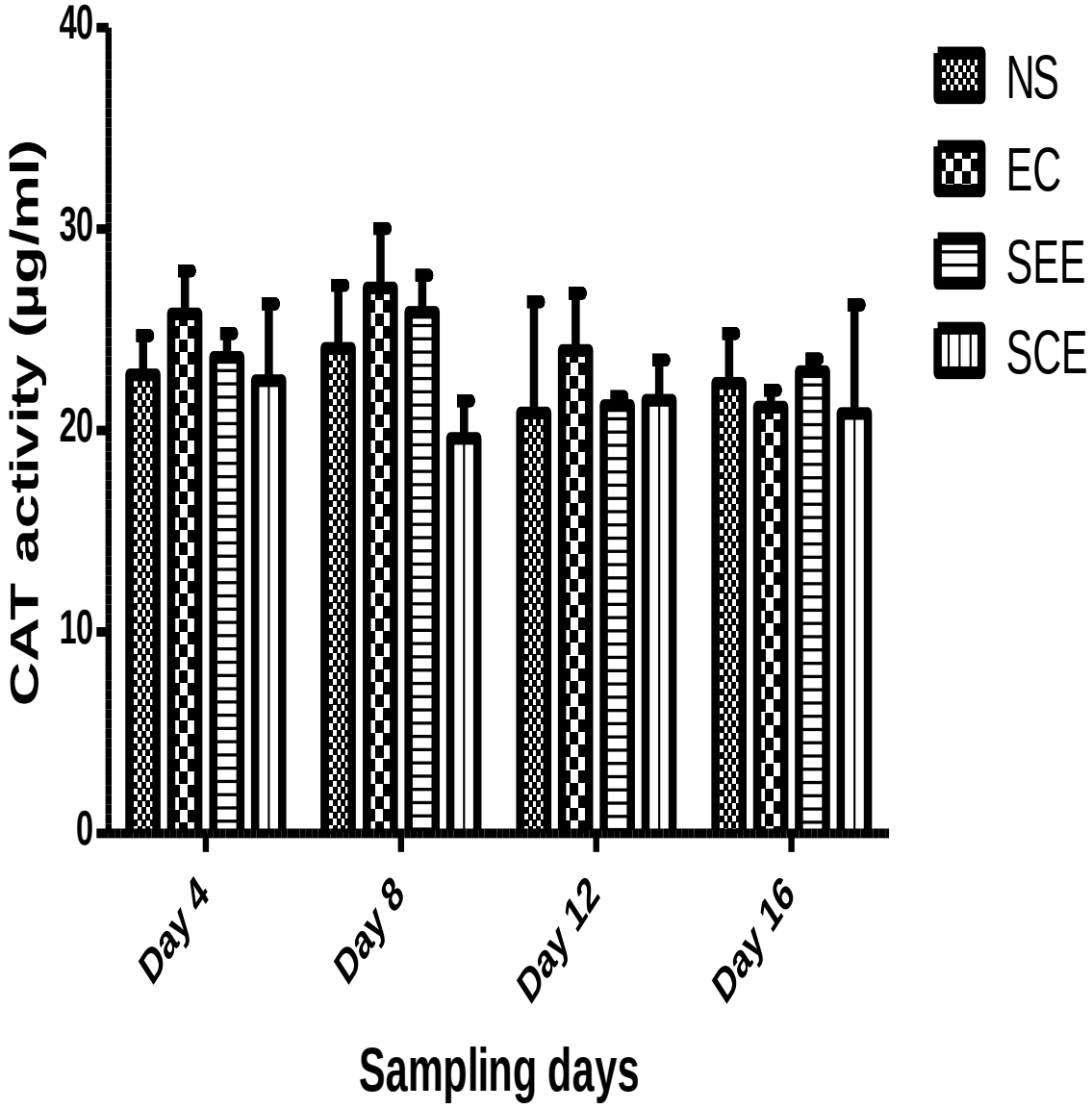


Figure 4.4: Biochemical estimation of Catalase (CAT) activity in granulation tissue samples harvested from healing wounds treated with different agents.

KEY

NS: Normal Saline; EC: Estrogen cream; SEE: Soybean ethanolic extract; SCE: Soybean Chloroform extract

4.4 Immunohistochemistry of Bcl-2 Anti-apoptotic Protein day 4, 8 and 12 of Wound Healing in Ovarictomised rats used for the study.

The mean quick score value of Bcl-2 staining obtained in this study is shown in Plate 4.3a-h. The Bcl-2 staining and its expression as protooncogene and anti-apoptotic protein were observed in granulation tissue of all treated rats wounds between day 4 and day 12 of the wound healing corresponding to the proliferative stage of wound healing. The mean quick score obtained for Bcl-2 immunostaining was on day 4 and 12 of wound healing was 5.3 ± 0.67and 2.0 ± 0.0; 10.0 ± 1.0 and 4.3 ± 0.88; 8.0 ± 1.0 and 4.0 ± 1.15; 5.3 ± 0.67 and 2.67 ± 0.67 for normal saline, oestrogen cream, soybean-ethanolic extract and soybean-chloroform extract treated groups respectively. Maximum mean quick score value of Bcl-2 staining was observed on day 8 and these were 6.3 ± 1.45, 11.0 ± 1.0, 10.0 ± 1, 8.0 ± 1.0 in normal saline, oestrogen cream, soybean-ethanolic extract and soybean-chloroform extract treated groups respectively. On day 4, 8 and 12 of wound healing, the normal saline treated group had the minimum mean quick score value of Bcl-2 staining while oestrogen cream treated group had the maximum mean quick score value of Bcl-2 staining.

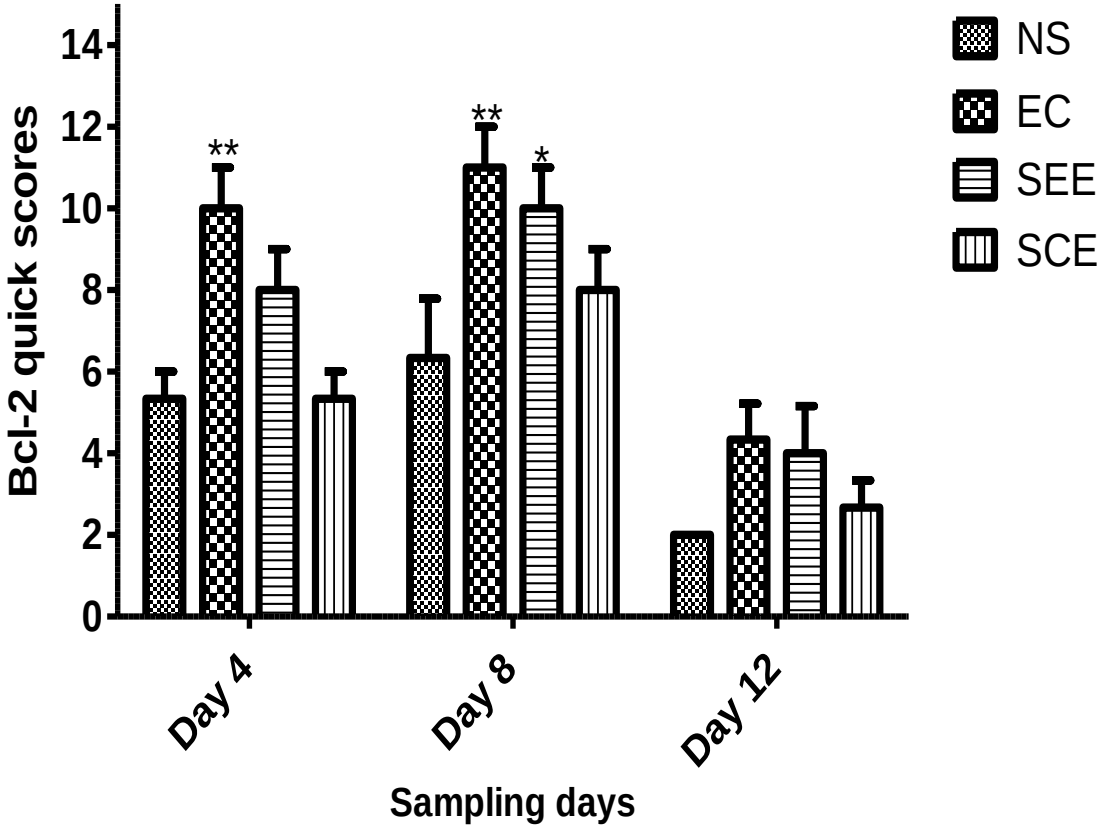


Figure 4.5. Mean Quick score value Bcl-2 anti-apoptotic protein immunostaining in granulation tissue tissue samples harvested from healing wounds treated with different agents

KEY

**Mean value is statistically significant at $p<0.01$

*Mean value is statistically significant at $p<0.05$

NS: Normal Saline; EC: Estrogen cream; SEE: Soybean ethanolic extract; SCE: Soybean Chloroform extract

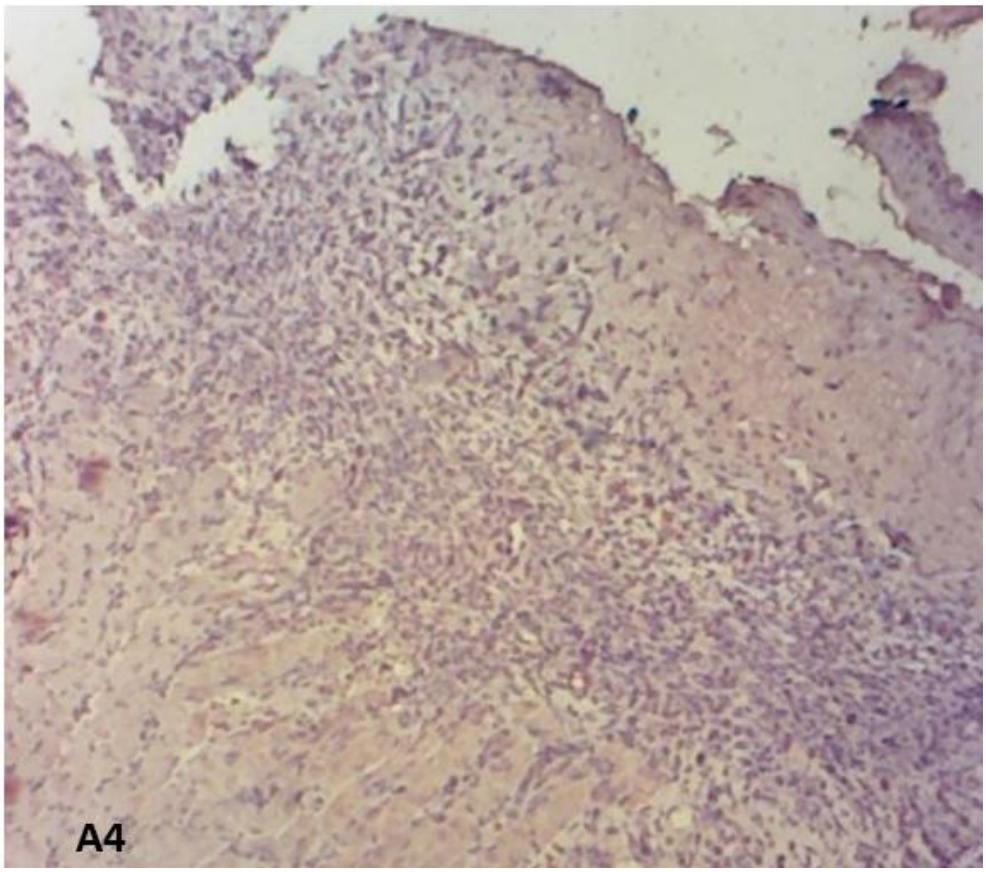


Plate 4.3a: Immunohistochemical localization and expression of the bcl-2 (antiapoptotic protein) on day 4. There is reduced intensity and percentage of cell that expres the protein Mgx100

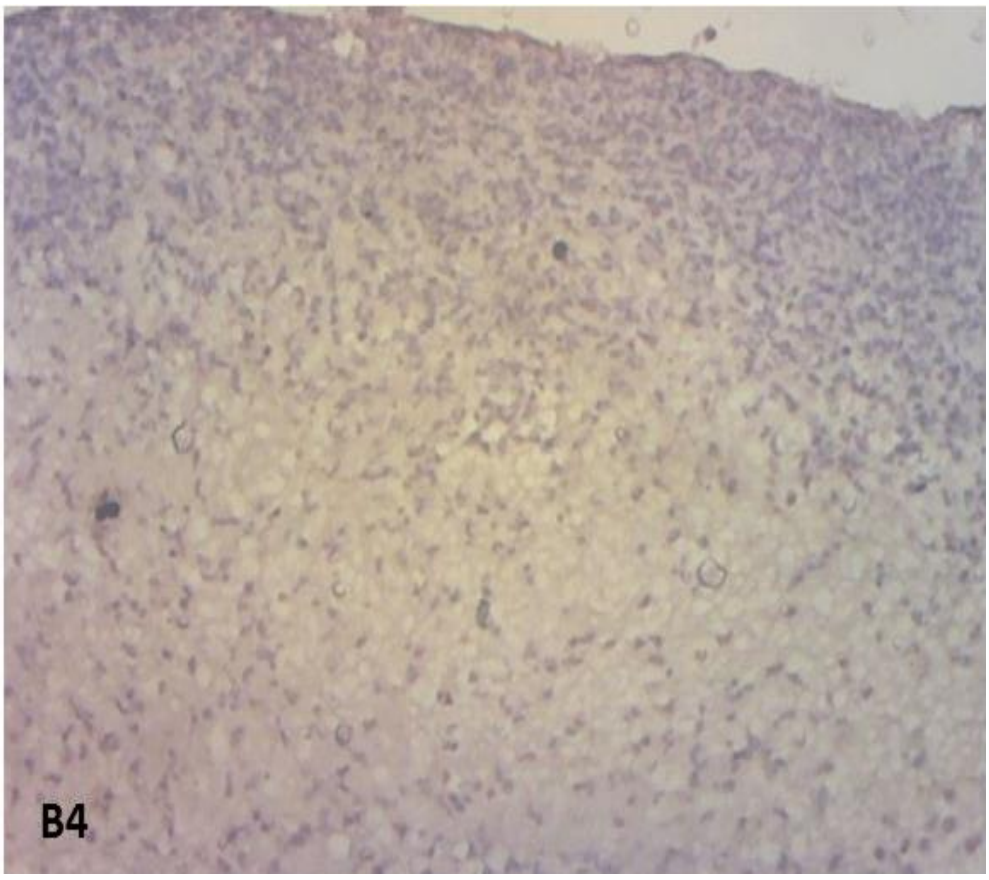


Plate 4.3b: Immunohistochemical localization and expression of the bcl-2 (antiapoptotic protein) on day 4. There is high intensity and percentage of cell that express the protein. Mgx100

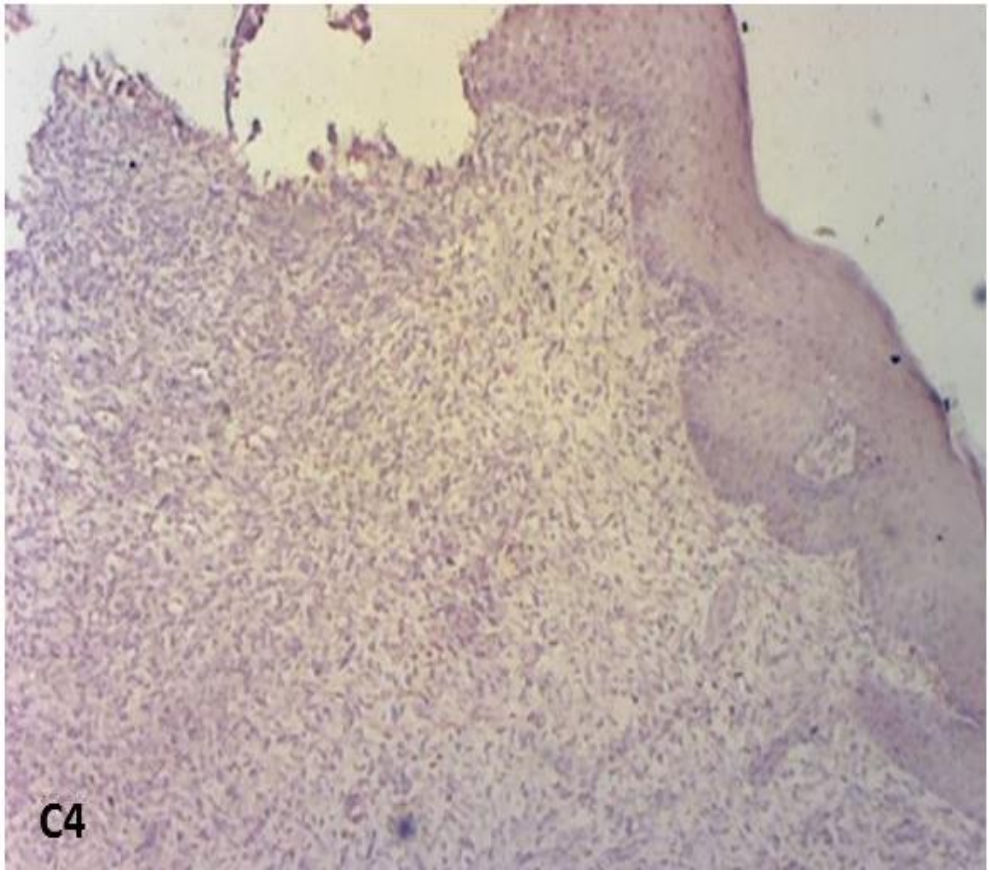


Plate 4.3c: Immunohistochemical localization and expression of the bcl-2 (antiapoptotic protein) on day 4. There is high intensity and percentage of cell that express the protein. Mgx100

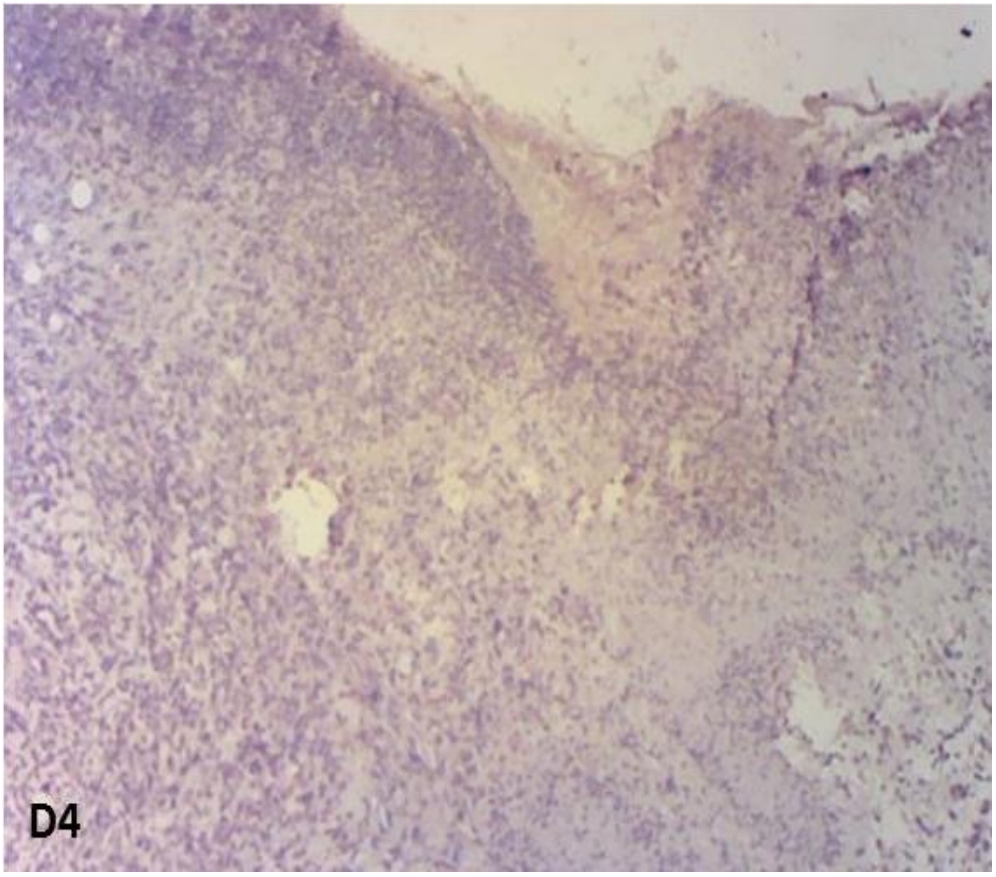


Plate 4.3d: Immunohistochemical localization and expression of the bcl-2 (antiapoptotic protein) on day 4. There is moderate intensity and percentage of cell that express the protein. Mgx100

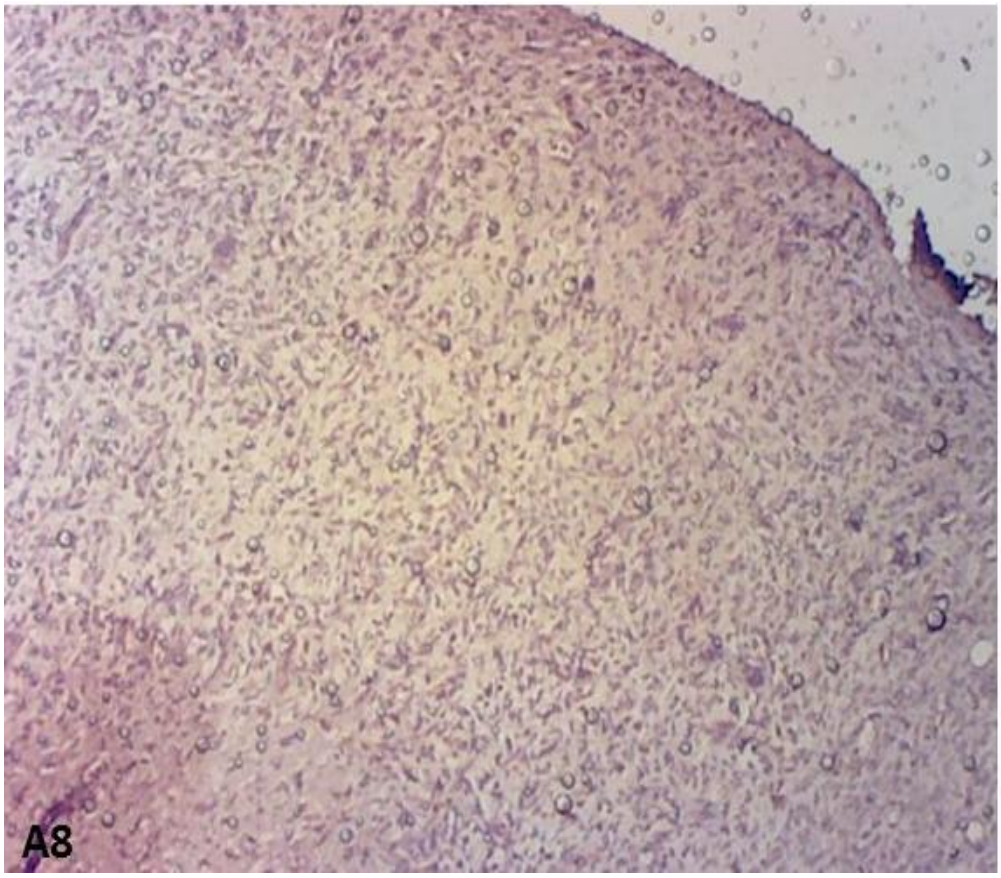


Plate 4.3e: Immunohistochemical localization and expression of the bcl-2 (antiapoptotic protein) on day 8. There is reduced intensity and percentage of cell that express the protein. Mgx100

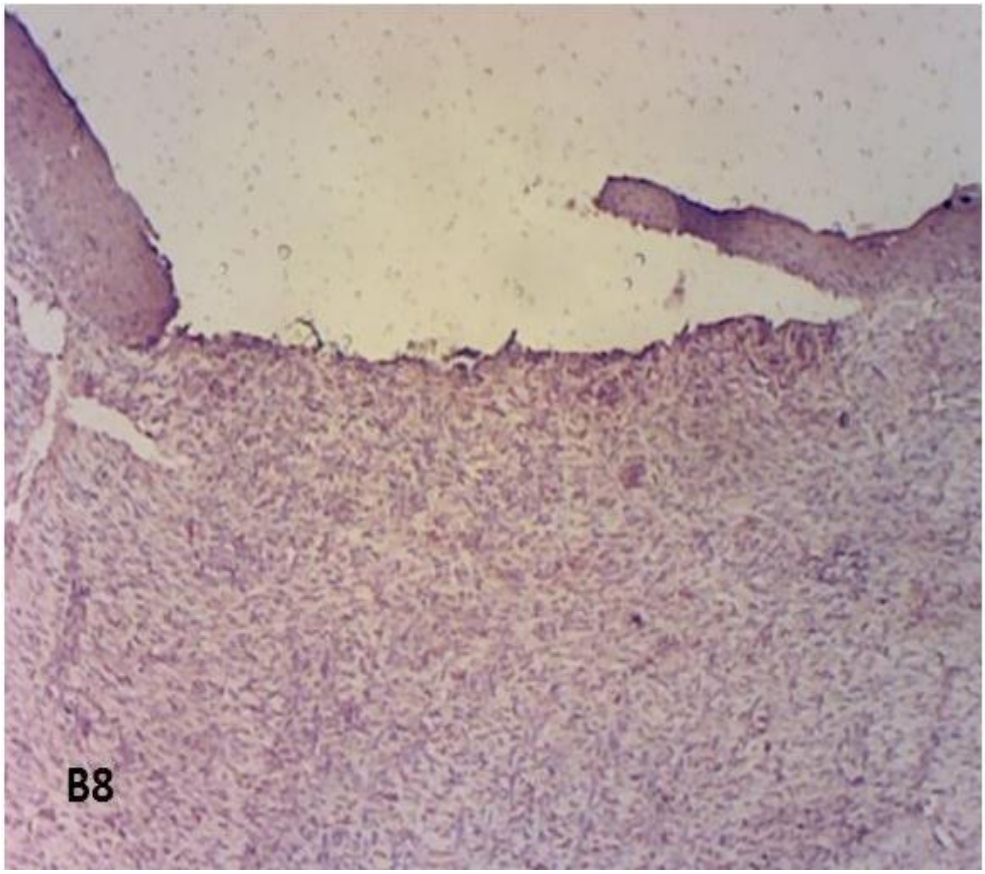


Plate 4.3f: Immunohistochemical localization and expression of the bcl-2 (antiapoptotic protein) on day 8. There is higher intensity and percentage of cell that express the protein. Mgx100

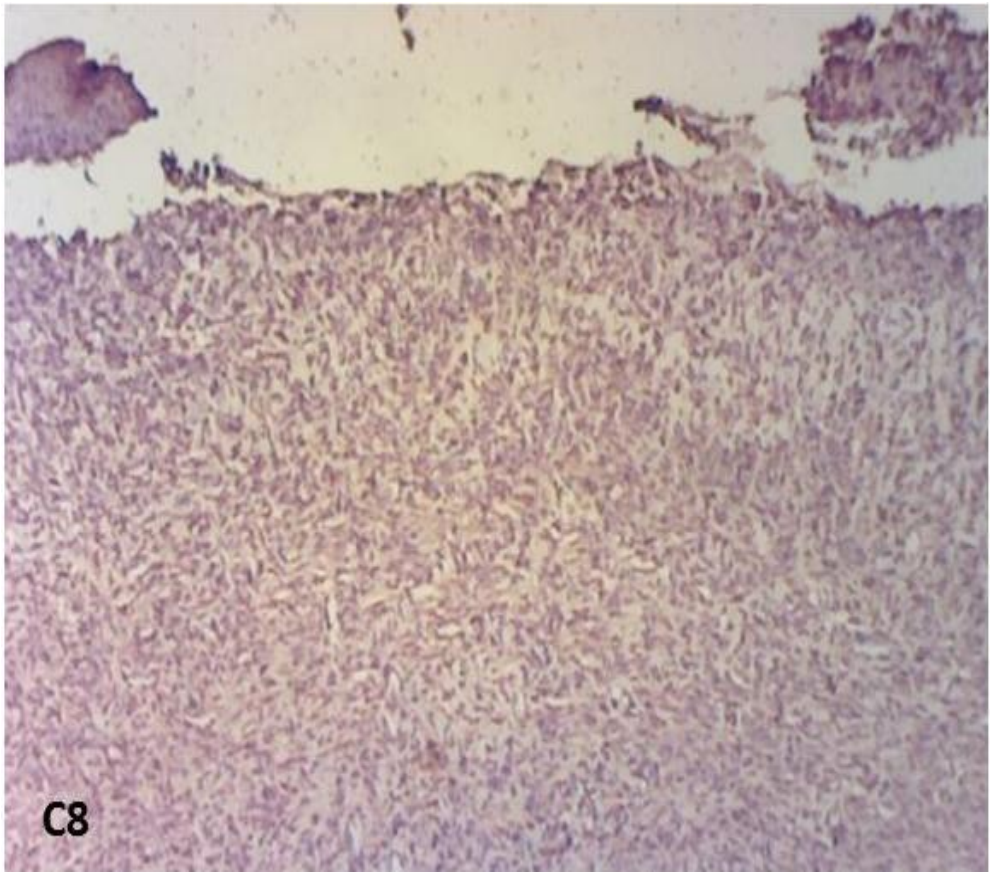


Plate 4.3g: Immunohistochemical localization and expression of the bcl-2 (antiapoptotic protein) on day 8. There is higher intensity and percentage of cell that express the protein.Mgx100

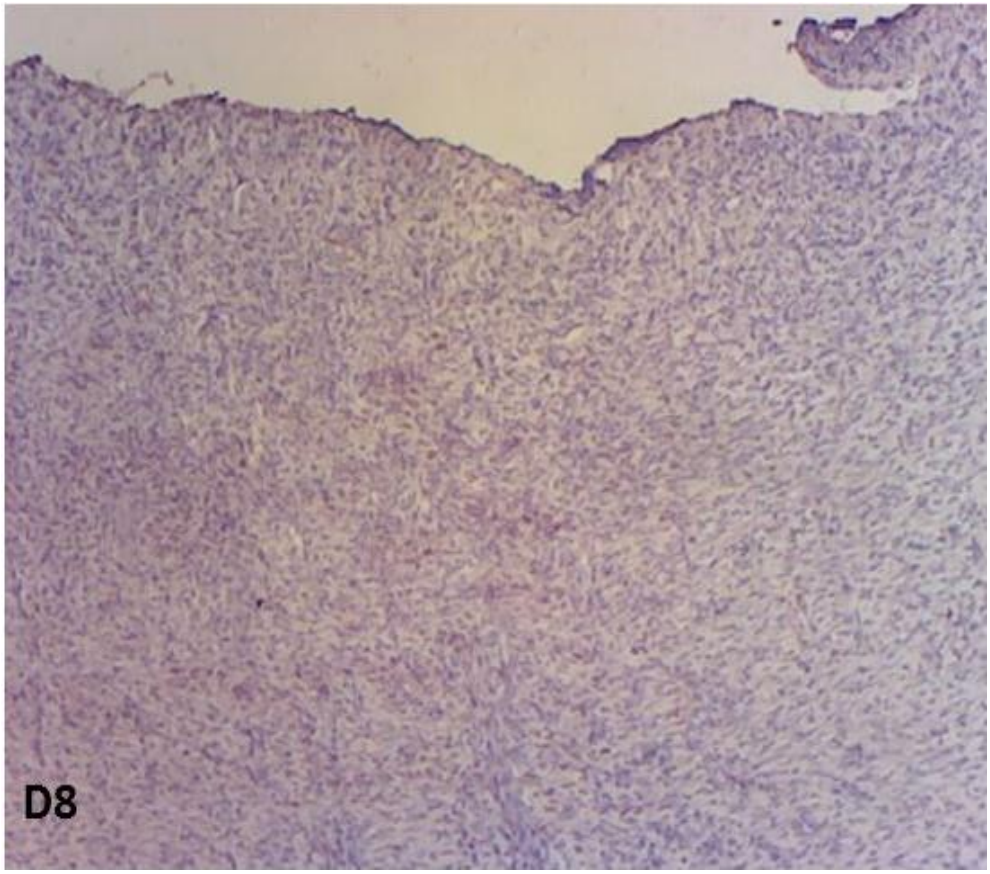


Plate 4.3h: Immunohistochemical localization and expression of the bcl-2 (antiapoptotic protein) on day 8. There is high intensity and percentage of cell that express the protein. Mgx100

CHAPTER FIVE

5.0 DISCUSSION

This study revealed that phytochemical components present in soybean included: steroids, tritarpens, phenolic acid, alkaloid and flavonoids in the crude ethanolic extract while chloroform fraction contains steroids, tritarpens, alkaloid and flavonoids. Several authors have similarly reported large number of bioactive phytochemicals including phenolic acids, flavonoids, isoflavones, saponins, phytosterols and sphingolipids for soybean (Gutierrez *et al.*, 2004; Luthria *et al.*, 2007; Lee *et al.*, 2008; Kumar *et al.*, 2010; Cho *et al.*, 2013; Lee *et al.*, 2015; Alghamdi *et al.*, 2018; Lee *et al.*, 2018). Some reported phytoconstituents of Soybean including saponins, phytosterols and sphingolipids etc. were not found in the soybean extracts used in this study. This could be due to the solvent used for the extraction as well as the techniques of phytochemical screening. The difference in the phytochemical constituent of the ethanolic and choloroform extract of soybean could be due to difference in the polarity of the two solvents. It is widely accepted that a solvent of similar polarity to the solute will properly dissolve the solute and the polarity, from least polar to most polar, of a few common solvents are such that: Hexane < Chloroform < Ethylacetate < Acetone < Methanol < Water (Altemimi *et al.*, 2017). Thus, chloroform is non-polar while ethanol is more polar. Meanwhile, most of the phytoconstituents of soybean are better extracted by highly polar solvent such as ethanol and methanol at various concentrations (Altemimi *et al.*, 2017). Flavonoids, proanthocyanidins and phenolic acids belong to the group of polyphenolic compounds and currently, there is intense interest in polyphenolic phytochemicals (Choi *et al.*, 2003). Currently, four different families of phenolic compounds produced by plants are considered as

phytooestrogen: the isoflavonoid, stilbenes, lignans and coumestans (Cornwell *et al.*, 2004) and soybean is the main source of phytooestrogen isoflavones group, namely as genistein and daidzein (Cederroth and Nef, 2009; Dhaubhadel, 2011; Teekachunhatean *et al.*, 2013). The excellent wound healing activity of soybean extract found in this study was due pharmacological activities of phytochemicals in soybean especially phenols and flavonoids and this is similar to several earlier reports (Kumar *et al.*, 2010; Lim, 2012; Lee *et al.*, 2018; Alghamdi *et al.*, 2018). Available researches have shown that soybean phytochemicals such as polyphenolic-isoflavones have been shown to have many beneficial effects on the appearance and structure of skin (Skovgaard *et al*., 2006; Izumi *et al*., 2007; Draelos *et al*., 2007; Accorsi-Neto *et al*., 2009) through their selective oestrogen receptor modulator (SERM) activities (Cederroth and Nef, 2009).

Similar to the reports of Ashcroft *et al.* (1997) and Hardman *et al.* (2005) in ovariectomised (ovx) untreated rats, gross evaluation of the wound healing in this study showed delayed healing, with increased inflammation and reduced granulation tissue development in the normal saline control rats. This could be due to reduced systemic estrogen in the ovx rats. It has been reported that under conditions of reduced systemic ooestrogen production, skin tends to undergoes structural and functional damage leading to skin ageing, impaired wound healing and skin cancer (Calleja-Agius *et al.*, 2007; Mukai *et al.*, 2016; Horng *et al.*, 2017; Wilkinson and Hardman, 2017). Meanwhile both topical and systemic application of oestrogen has been well researched as an excellent wound healing agent (Stevenson and Thornton, 2007; Archer, 2012; Thornton, 2013; Midgley *et al.*, 2016; Mukai *et al.*, 2016; Brufani *et al.*, 2017; Pepe *et al.*, 2017; Leblanc *et al.*, 2017; Wilkinson and Hardman, 2017; Chenu *et al.*, 2017).

Also this study showed that there was delayed wound healing in oestrogen deprived rats considering wound contraction, granulation and cell proliferation, which are shown in Tables 4.2 and 4.3. The oestrogen cream treated wound had the highest rate of wound contraction and re-epithelization ($p<0.05$) as compared to the normal saline control group. Similar to findings in this study, several reports have shown that application of synthetic oestrogen in oestrogen-deprived/deficient rats improved the rate of wound healing and scarring (Leblanc *et al.*, 2017; Wilkinson and Hardman, 2017; Chenu *et al.*, 2017).This is probably the reason why the oestrogen cream treated group had the fastest rate of wound contraction and epithelisation as compared to the normal saline treated group which have insufficient systemic oestrogen. However, both the oestrogen cream and soybean extract treated groups healed at the same time with slight difference in their rate wound contraction which was not statistically significant ($p>0.05$). The comparative improvement of wound contraction and epithelisation could be due to the presence of phenol and flavonoid compounds which are potent phytooestrogen in the soybean extract used for this study. Horng *et al.* (2017) reported that oestrogen action on wound healing and regulation of skin function and development was due to abundant expression of oestrogen receptors (ERα and ERβ) in skin. The Soybean extract was able to heal wound comparatively with oestrogen due to presence of phenolic compound which has the affinity and ability to bind oestrogenic receptors (ERα and ERβ) (Kupier *et al.*, 1997; Kupier *et al.*, 1998). A number of studies have included soy isoflavones from soybean extract alone or in combination with other agents and showed positive effects on skin appearance and composition (Skovgaard *et al.*, 2006; Draelos *et al.*, 2007; Izumi *et al.*, 2007; Accorsi-Neto *et al.*, 2009), wound healing (Shevchenko and Santin, 2014), skin regeneration (Meikle *et al.*,

2012), decrease photoaging of the skin (Polito *et al.*, 2012) and skin cancers prevention (Polito *et al.*, 2012; Waqas *et al.*, 2013; Waqas *et al.*, 2014).

At day 4, the mean percentage wound contraction in oestrogen cream and soybean extract treated wound was significantly higher ($p<0.05$) than the normal saline treated group. This was due to reduced inflammation and higher granulation tissue, collagen deposition, neoangiogenesis and fibrolasia as indicated by the histopathology (Table 4.3) in the treated group as compared with normal saline group. There was an increase in inflammatory response in the normal saline treated group due to increase acute inflammatory cell infiltration (especially neutrophils) and reduced chronic inflammatory cell (macrophages) infiltration. This could be due to oestrogen deprivation in the normal saline control group as compared to other groups treated with oestrogen and soybean extract which contains phytooestrogen. Similar to this findings, reports have shown that both oestrogen and soybean isoflavone have anti-inflammatory activity through reduction of neutrophil influx (Ashcroft *et al.*, 1999; Emmerson, 2010; Mukai *et al.*, 2016), increased macrophage infiltration, (Ashcroft *et al.*, 2003b) and down-regulation of pro-inflammatory cytokines, including TNF-α, Interleukin (IL)-1β and IL-6 (Hassan *et al.*, 2010; Zhang *et al.*, 2010; Pfeilschifter *et al.*, 2002). Also higher granulation tissue formation, collagen deposition, neoangiogenesis and fibroplasia observed in the oestrogen and soybean extract treated groups as compared to the normal saline treated group is similar to their reported characteristics when applied on wound (Marini *et al.*, 2010a; Uyar *et al.*, 2014). Similar to this study, Martin (1997) reported that in enhanced wound healing, dermal fibroblasts usually start to proliferate after injury around 3-5 days post-wounding and begin to migrate into the wound provisional matrix. Oestrogen is important for the proliferation, migration,

and differentiation of fibroblasts (Brufani *et al.*, 2017) to achieve wound contraction, which is primarily mediated by ER (Billon *et al.*, 2017) and this is the reason why there was increased fibroblast density in the oestrogen and soybean extract treated group as compared to the normal saline control group. Meanwhile soybean extracts was found to instigated the synthesis of collagen and elastin, and to boost the correct assembly of new elastin fibers, providing a complete protection and restoration to the dermal extracellular matrix (Heinrich *et al.*, 2016).

At day 8, there was statistical significance ($p<0.05$) difference in the rate of wound contraction between the oestrogen and soybean extract treated groups and normal normal saline control group. Likewise the rate of wound contraction was more than half the initial size in the soybean ethanolic extract and oestrogen treated groups while soybean chloroform extract and normal saline control group had less than 50% rate of wound contraction. This indicated that soybean ethanolic extract and oestrogen cream had a comparable effect on wound healing due to the presence of abundant isoflavone 'a phytooestrogen' in the soybean extract. The reduced activity of soybean chloroform extract compared to soybean ethanolic extract could be due to presence of high amount of phenolic compound "isoflavone' in the soybean ethanolic extract. This is because ethanol a polar solvent extract more polar compound than the chloroform (Altemimi *et al.*, 2017). Significant rate of wound contraction was further demonstrated by presence of moderate granulation tissue development with increased fibroblast density, angiogeneis and collagen deposition while inflammatory reaction was suppressed in the oestrogen and soybean ethanolic extract treated group. This histopathology result is characteristics of wound

treated with oestrogen, SERM like phytooestrogen (soybean isoflavone) as documented by Brufani *et al.* (2017), Marini *et al*. (2010a) and Uyar *et al.* (2014).

Day 12 of the wound healing process was classical in that the rate of wound contraction was significantly higher ($p<0.05$) in the oestrogen and soybean ethanolic extract treated group compared to the normal saline and soybean chloroform treated group. Although, not significantly different ($p>0.05$), inflammatory reaction was suppressed with none to less acute and chronic inflammatory cells in the oestrogen and soybean ethanolic extract treated groups compared to the normal saline and soybean chloroform treated group. This further show that soybean ethanolic extract (containing phytooestrogen 'isoflavone') like oestrogen have anti-inflammatory activity as previously reported (Kumar *et al.*, 2010; Lim, 2012; Lee *et al.*, 2018; Alghamdi *et al.*, 2018). Meanwhile the observed increased inflammation with moderate acute and chronic inflammatory cells in the normal saline and soybean chloroform treated group could be due to oestrogen deficiency as reported by several similar studies (Ashcroft *et al.*, 1997; Vegeto *et al.*, 1999; Ashcroft *et al.*, 1999; Vegeto *et al.*, 2001; Hardman *et al.*, 2008; Horng *et al.*, 2017). This is because oestrogen is known to reduce the number of wound neutrophils, (Ito *et al.*, 1995; Mukai *et al.*, 2016; Emmerson, 2010), directing activities of monocytes and macrophages, (Gulshan *et al.*, 1990; Harkonen and Vaananen, 2006; Murphy *et al.*, 2009) and down-regulating expression of pro-inflammatory cytokines (Zhang *et al.*, 2010). Also in vivo Ovx mice have increased macrophage infiltration to wounds, indicating a role for oestrogen in the control of excessive inflammation (Ashcroft *et al.*, 2003b). While the wounds in both the oestrogen and soybean ethanolic extract treated groups healed on day 14 post wounding, the soybean chloroform extract and normal saline treated groups had their wound healed

on day 16 and day 18 respectively. The delayed wound healing in the soybean chloroform extract and normal saline treated group could be due to oestrogen deficiency which led to increased inflammation, reduced granulation tissue development, collagen deposition and fibroblast density as observed in the histopathology results.

Over production of reactive oxygen species (ROS) results in oxidative stress thereby causing cytotoxicity and delayed wound healing and elimination of ROS could be an important strategy in healing of chronic wounds (Schäfer and Werner, 2008; Hameedaldeen *et al.*, 2014). Therefore, estimation of antioxidants like GSH, SOD, and CAT in granulation tissues is relevant because the antioxidants have been reported to hasten wound healing by decreasing the free radicals (James *et al.*, 2001; Sharifi *et al.*, 2012). This study on the antioxidants and MDA status revealed that oestrogen and soybean ethanolic extract had significant antioxidant activity. There was significantly increased GSH activity measured in the granulation tissue on day 8 ($p<0.001$) and day 12 ($p<0.01$) in oestrogen cream (84.67 ± 2.34, 66.87 ± 3.32 respectively) and soybean ethanolic extract 82.27 ± 1.63, 60.37 ± 4.04 respectively) compared to all other groups. This is in contrast to to report of Kılıç *et al.*, (2013) who reported that GSH level was found to be similar between treated and control group in rabbit. High GSH activity in the oestrogen cream and soybean ethanolic extract shows that both agents have comparable high antioxidant activity and this could be the reason while the wounds in these groups healed faster as the ROS generation was controlled. At the end of day 4, MDA concentration in granulation tissue was significantly ($p<0.001$) lower in the oestrogen (77.37± 7.88), soybean ethanolic extract (90.13 ± 3.32) and soybean chloroform extract (86.60 ± 6.99) treated groups compared to normal saline control group (180.97 ± 10.0) which had a very high MDA concentration in

the granulation tissue. Meanwhile, estrogen cream and soybean ethanolic extract had a significantly ($p<0.05$) lower MDA concentration on day 8, 12 and 16 compared to soybean chloroform extract and normal saline control group. This indicated that oestrogen and soy isoflavone 'a phytoetrogen' which is a phenolic compound have ability to reduce lipid peroxidation and oxidant generation with resultant promotion of wound healing. Higher concentration of MDA was observed on day 12 in all the groups and this could probably be due to reduced oestrogen, as there was reduced frequency of topical application of both the oestrogen cream and soybean extract. There was increased SOD activity in the soybean extracts (both ethanolic and chloroform) and oestrogen treated groups compared to normal saline treated group in all the sampling days. Although it was only the oestrogen cream treated group that had a significant ($p<0.05$) higher SOD activity in granulation tissue on day 4 and day 8. The increased expression of SOD (a ROS-scavenging enzymes) that we observed after skin injury seems to be in contrast to findings of other investigators (Shukla *et al.*, 1997), who found reduced SOD activities in wound tissue between days 2 and 7 after cutaneous injury in rats. Meanwhile, The CAT concentration in granulation tissue of treated groups increased and decreased haphazardly from day 4 to day 16 of wound healing. However, the oestrogen cream treated group had persistently higher expression of CAT enzyme in granulation tissue when compared to the Soybean extract (both ethanolic and chloroform) treated groups. This is in contrast to Steiling *et al.*, (1999) who reported that expression of catalase was not altered by skin injury. Nutritional supplements with isolated soy protein attenuates post-burn oxidative stress and inflammation, leading to improved wound healing in burn patients (Babajafari *et al.* 2017). Estradiol and soy isoflavone have been shown to be potent antioxidant activities (Sakthivelu *et al.*, 2008;

Duenas *et al.*, 2012; Lee and Cho, 2012; Polito *et al.*, 2012; Georgetti *et al.*, 2013; Waqas *et al.*, 2013; Waqas *et al.*, 2014; Alghamdi *et al.*, 2018;). The antioxidant and oxidative stress lowering activity of oestrogen and soybean phytooestrogen is due to their ability to lower free radicals and peroxide production by mitochondria (Borrás *et al.*, 2010) through up-regulation of nuclear gene expression of antioxidant enzymes which are directed to mitochondria (Sastre *et al.*, 2002; Borras *et al.*, 2003; Vina *et al.*, 2006). Also it has been suggested that the antioxidant action of oestrogens and phytooestrogen is due to their interaction with oestrogen receptors in cells which eventually lead to the activation of mitogen activated protein kinases (MAPK) and nuclear factor kappa B (NFκB) (Borras *et al.*, 2005; Vina *et al.*, 2006; Surico *et al.*, 2017). Reactive oxygen species (ROS) that arise after cutaneous injury may worsen the healing process and induce apoptosis (Hameedaldeen *et al.*, 2014) and the utilization of antioxidants has been considered as an effective therapeutic approach in wound healing (James *et al.*, 2001; Sharifi *et al.*, 2012). This could be the reasons why the oestrogen and soybean extract treated group healed faster than normal saline controlled group, since there was reduced free radical oxidative tissue damage due to presence of higher antioxidant enzyme. Thus, both oestrogen cream and soybean extract have comparable protective cellular antioxidant effects and improved wound healing.

Apoptosis has been identified as the mechanism of cell death in a number of degenerate diseases. Apoptosis occurs through several independent pathways that are initiated by diverse extracellular (via death receptor) and intracellular factors (Şener *et al.*, 2015). The intrinsic pathway centers on the mitochondria, which contain key apoptotic factors (Şener *et al.*, 2015). One of the major regulators of the intrinsic pathway is the pro- and antideath

member, the Bcl-2 family. Bcl-2 regulates mitochondrial physiology and caspase activation (Lippens *et al.,* 2009). In the present study, Bcl-2 protein expression was detected as apoptotic markers. The level of expression of Bcl-2 anti-apoptotic proteins in the wound tissue of oestrogen and soybean extract treated rats was significantly ($p<0.05$) greater than that in the wound tissue of normal saline control rats. This shows that there was decreased cellular apoptosis in the wound treated with either oestrogen or soybean extract compared to the normal saline control wound. Oestrogen as well as genistein 'a soy isoflavone, has been shown to prevent cellular apoptosis (Borrás *et al.,* 2010). The mechanism of apoptosis prevention by estradiol as well as phytooestrogen (soy isoflavone) was found to be via prevention the release of cytochrome c by cellular mitochondria (Borrás *et al.,* 2010). A critical function of mitochondria is the control of cell viability via the apoptotic process and the release of cytochrome c is a key sub-cellular event to unleash cellular apoptosis (Borrás *et al.,* 2010). Also Choi *et al.* (2003) have demonstrated that antiapoptotic activity of flavonoids (which is abundant in soybean extract) appears to be mediated at the mitochondrial bcl-2 and bax gene level. They showed that flavonoids prevent apoptosis by enhancing the expression of bcl-2 antiapoptoyic protein and inhibiting the expression of bax proapoptotic protein and the cleavage and activation of caspase-3 (Choi *et al.,* 2003).

The expression of Bcl-2 protein was significantly ($p<0.05$) upregulated at the end of day 4 and 8 in oestrogen and soybean extract treated wounds when compared with those in the control wounds. This period corresponded to the proliferative phase of wound healing where several cellular arcades were recruited including keratinocyte, fibroblast and endothelial cell for wound healing. Increased expression of Bcl-2 protein on day 4 and 8

indicated a significant reduction in rate of cellular apoptosis during that period. This is supported by research of Farahani and Kloth, (2008) who reported that the rate of wound closure is associated with the extent of apoptosis in the wound: an increased rate of wound closure is associated with a reduced extent of apoptosis. Also, Bhan *et al.* (2013) asserted that upon injury, Bcl-2 expression usually increase, in order to allow cellular proliferation to occur. Meanwhile, significant ($p<0.05$) peak expression of Bcl-2 proteins was found on day 8 in oestrogen and soybean extract treated wounds. This is similar to the reports of Şener *et al.*, (2015) who observed that Bcl-2 was markedly increased in wound granulation tissue on day 7 after the administration of melatonin. In clean uncontaminated wounds, inflammatory cells underwent apoptosis starting as early as 12h after wounding (Brown *et al.*, 1997; Greenhalgh, 1998; Wu and Chen, 2014) and peaked at the 4th day after wounding and slowly decreased afterwards. Desmouliere *et al.* (1995) reported that apoptosis in myofibroblasts began on day 12, peaked at 20day and resolved by 60day. This could be the reason why there was decreased expression of Bcl-2 antiapoptotic protein in the granulation tissue of both the oestrogen and soybean extract treated wounds as well as the normal saline treated wounds. This probably occur to allow evacuation of cells involves in granulation tissue formation to prevent abnormal wound healing such as excessive collagen deposition seen in keloid or hypertrophic scar. The effect of soybean extract was essentially comparable to those of estradiol and therefore our results indicate that soybean extract was as effective as estradiol to protect cellular apoptosis during wound healing. Soy protein has recently received attention for a variety of its potential health benefits, including enhanced skin regeneration and has been found to be safe and cost-effective alternative to oestrogen (Ahn *et al.* 2018).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study has established the following about the comparative use of oestrogen cream and Soyebean extract in management of cutaneous wounds in ovariectomised albino rats:

I. The phytochemicals present in Soybean extract include steroids, tritarpens, phenol, alkaloid and flavonoids and were responsible for its wound healing activities.

II. There was a significant ($p<0.05$) increase in the percentage wound contraction of estrogen cream, soybean ethanolic extract, and soybean chloroform extract day 12 post wounding (90.29 ± 0.75%, 89.28 ± 0.95% and 79.43 ± 1.07% respectively) compared to normal saline (64.56 ± 0.70%) treated group, however; there was no significant difference ($p>0.05$) in the rate of wound contraction in estrogen cream treated wounds and Soybean ethanolic extract treated wounds

III. Wound treated with oestrogen cream and soybean ethanolic extract healed on the same day (day 14 post wounding) while soybean chloroform extract and normal saline treated wound healed on day 16.25 ± 0.25 and 18.25 ± 0.25 respectively.

IV. Histopathological studies revealed that comparatively, both oestrogen cream and Soybean ethanolic extract promote formation of healthy granulation tissue, collagen deposition, fewer and regulated inflammatory cells infiltration, fibroblast proliferation and formation of new blood vessels.

V. Oestrogen cream and soybean ethanolic extract treated groups had a significant ($p<0.001$) increase in GSH level (84.67 ± 2.34 µg/ml, 82.27 ± 1.63 µg/ml respectively) on day 8 post wounding compared to normal saline (56.9 ± 8.09 µg/ml) treated group.

MDA level reduced significantly ($p<0.001$) on day 4 post wounding in oestrogen cream (77.37 ± 7.88 µmols/mg protein), soybean ethanolic extract (90.13 ± 3.32 µmols/mg protein), and soybean chloroform extract (86.60 ± 6.99 µmols/mg protein) treated groups compared to normal saline (180.97 ± 10.02 µmols/mg protein). Thus, oestrogen cream and soybean ethanolic extract improve wound healing by stimulating antioxidant enzyme production and reduction of oxidative stress.

VI. There was a significant ($p<0.05$) increased expression and mean quick score of Bcl-2 antiapoptotic protein in oestrogen cream (11.00 ± 1.00) and Soybean ethanolic extract (10.00 ± 1.00) treated group on day 8 post-wounding compared to normal saline (6.33 ± 1.45) treated group indicating improved wound healing via reduced cellular apoptosis during the proliferative phase of wound healing.

In conclusion, this study demonstrated that topical treatment of cutaneous wound with soybean ethanolic extract had comparable efficacy to oestrogen in the management of wound in ovariectomized wistar albino rats.

6.2 Recommendation

- That topical Soybean ethanolic extract be used daily in management of cutaneous wound.
- Research should be carried out to develop soybean extract gel or dermal patch for application during wound healing.
- Further study should be carried out to determine the toxicity and minimum dose that would be required for optimal wound healing

- Studies should be done to determine the outcome of long term administration of soybean extract on skin and other body tissue

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Appendices

Appendix 1: Rate of Wound Contraction in each animal sampled during the experiment

Area of wound contraction (mm^2) in both treated and control group					Percentage wound contraction (%) in both treated and control group				
Normal saline					Normal saline				
	0	4	8	12		0	4	8	12
1	250	210	136	49	1	-	16	32.24	63.97
2	230	200	130	47	2	-	13.04	35	63.84
3	245	205	138	50	3	-	14.29	32.68	63.77
4	234	195	135	45	4	-	16.67	30.77	66.67
Oestrogen cream					Oestrogen cream				
	0	4	8	12		0	4	8	12
1	245	192	91	7	1	-	21.63	52.6	92.3
2	230	197	85	8	2	-	14.35	56.85	90.59
3	230	190	93	10	3	-	17.39	51.05	89.25
4	250	187	82	9	4	-	25.2	56.15	89.02
Soybean ethanolic extract					Soybean ethanolic extract				
	0	4	8	12		0	4	8	12
1	243	194	98	10	1	-	20.16	49.48	89.80
2	240	190	98	9	2	-	20.83	48.42	90.8
3	254	195	89	12	3	-	23.23	54.40	86.52
4	238	198	100	10	4	-	16.81	49.49	90.00
Soybean chloroform extract					Soybean chloroform extract				
	0	4	8	12		0	4	8	12
1	245	198	102	24	1	-	19.18	48.48	76.47
2	250	195	112	22	2	-	22.00	42.56	80.36
3	235	182	102	21	3	-	22.55	43.96	79.41
4	224	190	108	20	4	-	15.18	43.16	81.48

Appendix 2: Day 4. Histological Scoring (HandE and Trichrome Green)

Sample	Granulation development	Collagen accumulation	Acute inflammatory cells	Chronic inflammatory cells	Angiogenesis	Fibroblast density
A14	1	0	3	0	1	1
A24	0	1	2	1	0	1
A64	2	0	2	0	0	0
B14	2	1	2	2	1	1
B24	2	2	1	1	2	2
B34	2	1	1	1	1	3
C14	2	2	1	1	1	1
C24	1	0	2	0	2	2
C64	2	1	1	2	2	2
D24	1	0	2	0	1	1
D34	1	1	2	1	1	1
D64	1	1	2	2	0	2

Appendix 3: Day 8. Histological Scoring (HandE and Trichrome Green)

Sample	Granulation development	Collagen accumulation	Acute inflammatory cells	Chronic inflammatory cells	Angiogenesis	Fibroblast density
A38	2	1	2	2	1	1
A68	1	0	2	1	1	2
A48	2	1	2	2	1	2
B28	2	2	0	1	2	3
B48	3	1	1	2	2	1
B68	2	1	2	1	1	2
C38	3	2	0	0	2	3
C48	2	1	1	2	1	0
C68	2	0	1	1	1	2
D68	2	1	2	2	1	2
D38	1	1	1	1	2	1
D48	2	1	2	1	2	1

Appendix 4: Day 12. Histological Scoring (HandE and Trichrome Green)

Sample	Granulation development	Collagen accumulation	Acute inflammatory cells	Chronic inflammatory cells	Angiogenesis	Fibroblast density
A212	1	1	2	1	1	2
A412	2	1	1	1	2	2
A512	2	2	1	0	0	1
B212	3	2	1	0	2	2
B512	3	2	0	0	0	2
B412	2	2	1	0	2	2
C212	3	3	0	1	0	2
C412	2	2	0	0	3	2
C512	2	2	1	0	2	2
D212	2	1	0	1	3	3
D512	2	2	2	0	1	2
D412	2	2	1	1	1	3

Appendix 5: Day 16. Histological Scoring (HandE and Trichrome Green)

Sample	Granulation development	Collagen accumulation	Acute inflammatory cells	Chronic inflammatory cells	Angiogenesis	Fibroblast density
A216	1	1	1	1	0	2
A516	2	2	1	1	1	1
A416	1	1	2	0	0	2
B516	2	3	1	0	2	3
B416	3	3	1	0	2	2
B216	3	2	0	1	2	2
C216	3	2	1	0	2	2
C416	2	3	0	0	2	2
C516	2	3	0	0	3	3
D216	2	1	1	1	0	2
D416	1	2	1	0	2	2
D516	2	2	1	0	1	2

Appendix 6: Mean concentration of antioxidant enzyme and oxidative stress marker in granulation tissue on day 4, 8 and 16.

GSH CONC (µg/ml)				
	A	B	C	D
Day 4	63.5 ± 0.5	77.43 ± 1.23	72.87 ±5.96	71.50 ± 4.16
Day 8	56.90 ± 8.09	84.67 ± 2.34	82.27 ± 1.63	70.56 ± 2.90
Day 12	45.77 ± 0.43	66.87 ± 3.32	60.37 ±4.04	51.17 ±5.47
Day 16	57 ± 0.06	60.30± 1.91	61.90 ± 3.82	58.33 ± 3.74

MDA Conc. (µmols/mg protein)				
	A	B	C	D
Day 4	180.97 ± 10.0	77.37± 7.88	90.13 ± 3.32	86.60 ± 6.99
Day 8	180.43 ± 10.2	117.18 ±8.77	129.66 ±7.0	133.83 ± 32.06
Day 12	282.87 ± 0.09	210.27 ±16.1	193.33± 17.55	227.13 ± 21.49
Day 16	132.97 ± 1.32	89.00 ± 10.98	74.80 ± 7.45	104.50 ± 5.27

SOD Activity (U/ml)				
	A	B	C	D
Day 4	28.08 ±2.28	36.74 ± 0.18	32.31± 1.74	31.85 ± 1.77
Day 8	25.75 ± 1.26	36.39 ± 0.42	32.10 ± 0.72	29.95 ±2.90
Day 12	28.65 ± 3.09	34.6 ± 1.53	33.98 ± 1.41	30.99 ± 0.58
Day 16	24.53 ± 1.01	30.9 ± 0.64	32.80 ± 0.52	28.60 ±3.42

CAT (µg/ml)				
	A	B	C	D
Day 4	22.77 ± 1.96	25.80 ± 2.13	23.63 ± 1.19	22.49 ± 3.81
Day 8	24.10 ± 3.13	27.09 ± 2.95	25.89 ± 1.84	19.61 ± 1.86
Day 12	20.87 ± 5.55	23.99 ± 2.84	21.26 ± 0.45	21.50± 2.01
Day 16	22.37 ±2.45	21.17 ± 0.84	22.93± 0.64	20.85± 5.41

Appendix 7: Day 4. Quick Scores for Bcl-2 expression in granulation tissue

Sample	INTENSITY (A)				PERCENTAGE (B)					Quick Score
	0 (nostain)	1 (weak)	2 (Moderate)	3 (strong)	0 (no staining)	1 (<10)	2 (10-50)	3 (51-80)	4 (>80)	(AXB)
A14				@			@			6
A24			@				@			4
A64				@			@			6
B14				@				@		9
B24				@				@		9
B64				@					@	12
C14				@				@		9
C24			@					@		6
C64				@				@		9
D24			@				@			4
D64			@					@		6
D14			@					@		6

Appendix 8: Day 8. Quick Scores for Bcl-2 expression in granulation tissue

Sample	INTENSITY (A)				PERCENTAGE (B)					Quick Score
	0	1	2	3	0	1	2	3	4	(AXB)
	(no staining)	(weak)	(Moderate)	(strong)	(no staining)	(<10)	(10-50)	(51-80)	(>80)	
A38			@					@		6
A48			@				@			4
A68				@				@		9
B38				@				@		9
B48				@					@	12
B68				@					@	12
C38				@					@	12
C48				@				@		9
C68				@				@		9
D48				@				@		9
D38				@				@		9
D68				@			@			6

Appendix 9: Day 12. Quick Scores for Bcl-2 expression in granulation tissue

Sample	INTENSITY (A)				PERCENTAGE (B)					Quick Score
	0	1	2	3	0	1	2	3	4	(AXB)
	(no staining)	(weak)	(Moderate)	(strong)	(no staining)	(<10)	(10-50)	(51-80)	(>80)	
A212			@			@				2
A412			@			@				2
A512		@					@			2
B112				@		@				3
B312			@				@			4
B512				@			@			6
C212				@			@			6
C412			@			@				2
C512			@				@			4
D212			@			@				2
D412			@			@				2
D512			@				@			4