WOUND HEALING POTENTIAL OF COPPER (II) BIS [N'- (5-CHLORO-1H-INDOL-3-YL) METHYL NICOTINIC HYDRAZIDE] ON EXPERIMENTALLY INDUCED EXCISION WOUNDS IN RATS

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ABSTRAK

Memandangkan kepentingan luka dalam kesihatan manusia mengisytiharkan keperluan menemui dan membangunkan agen drug baru yang berkesan. Cl-indole-nicotinik adalah sebatian Schiff asas dengan menjanjikan potensi struktur dalam mempercepatkan penyembuhan luka. Tujuan kajian ini adalah untuk menyiasat kesan-kesan Cl-indole-nicotinik kesan ke atas penyembuhan luka dan ketoksikan akut pada tikus Sprague Dawley serta aktivitinya sebagai antimikrobial dan faktor antioksidan.

Hirisan manual teraruh kulit pada kawasan leher punggung empat kumpulan tikus SD lelaki dirawat selama 10 hari dengan sebatian dos tinggi (50 mg/kg), sebatian dos rendah (25 mg/kg), CMC 5%, dan gel Intrasite untuk menilai sifat perubatan sebatian. Superoksida Dismutase (SOD) dan Malondialdehyde (MDA) ujian menandakan sifat antioksidan enzim dan tahap peroksidaan lipid (LP) dalam tisu homogenates disediakan selepas membuang kawasan luka dirawat.

Kesan ketoksikan akut Cl-indole-nicotinik dicirikan dengan memantau enam kumpulan tikus SD jantan dan betina (dos rendah: 0.5g/kg, dos tinggi: 1g/kg, dan kawalan: 10% Tween-20 kumpulan) untuk dua minggu selepas administrasi oral sebatian. Pemeriksaan histologi buah pinggang dan hati, dan mengesahkan parameter-parameter biokimia serum darah dilakukan berikutan mengorbankan tikus pada hari ke 14.

Penilaian *in vitro* potensi antioksidan sebatian adalah dijelaskan oleh ujian-ujian DPPH dan FRAP. Aktiviti antimikrob Cl-indole-nicotinik ditentukan terhadap bakteria gram positif dan gram negatif dipilih berdasarkan kaedah penyebaran cakera dan keputusan mengesahkan ketidakcekapan sebatian mengenai kes itu. Kepekatan perencatan minimum (MIC) sebatian juga dikenalpasti.

Hasil dari pengalaman pembaikan luka telah mengisytiharkan sifat penyembuhan diterima sebatian yang lebih tinggi daripada gel Intrasite dan meningkatkan tahap SOD bersama-sama dengan kepekatan sebatian. Pemeriksaan histologi pada organ-organ dalaman tikus menunjukkan tiada gejala malignan, yang sedang mempertimbangkan sebatian sebagai tidak toksik. Keputusan mengesahkan ketidakcekapan sebatian mengenai aktiviti antibakteria tetapi menunjukkan potensi antioksida *in vitro* sebatian dalam keadaan tertentu. Disebabkan keputusan yang diperolehi menunjukkan kesan positif dalam tahap yang berbeza dalam kebanyakan ujian yang dipilih dalam kajian ini, Cl-indole-nicotinik boleh dianggap sebagai faktor yang berpotensi terutamanya dalam kes penyembuhan luka yang menawarkan keperluan pengetahuan lebih lanjut mengenai kuprum (II) Schiff asas sebagai agen bioaktif.

ABSTRACT

Considering the importance of wounds in human health proclaims the necessity of discovering and developing new effective drug-to-be agents. Cl-indole-nicotinic is a Schiff base compound with promising structural potentials in accelerating healing of wounds. The purpose of present study is to investigate Cl-indole-nicotinic effects on wound healing and acute toxicity on Sprague Dawley rats as well as its activity as an antimicrobial and an antioxidant agent.

The manually induced dermal incision on dorsal neck area of four groups of male SD rats were treated for 10 days with high dose compound (50 mg/kg), low dose compound (25 mg/kg), CMC 5%, and Intrasite gel to assess the medical nature of the compound. The superoxide dismutase (SOD) and malondialdehyde (MDA) tests signified the enzymatic antioxidant properties and level of lipid peroxidation (LP) in tissue homogenates prepared after removing the treated wound area.

The acute toxicity effect of Cl-indole-nicotinic was characterized by monitoring six groups of male and female SD rats (low dose: 0.5g/kg, high dose: 1g/kg, and control: 10% Tween-20 groups) for two weeks after oral administration of the compound. Histological examination of kidney and liver, and verifying biochemical parameters of blood serum were done following sacrificing rats on day 14.

In vitro evaluation of antioxidant potential of the compound is clarified by DPPH and FRAP tests. Antimicrobial activity of Cl-indole-nicotinic was determined against selected gram positive and gram negative bacteria based on disk diffusion method and results affirm compound's inefficiency regarding the case. The minimum inhibitory concentration (MIC) of the compound is also distinguished.

The outcomes from wound repair experience declared acceptable healing nature of the compound that was higher than Intrasite gel and increasing level of SOD along with compound's concentration. Histological examination on rats' inner organs showed no symptom of malignancy, which was considering the compound as non-toxic. Results affirm compound's inefficiency regarding antibacterial activity but expressing *in vitro* antioxidant potency of the compound in some extent. Since the obtained results showed positive effects in different levels in most of selected tests in this study, Cl-indole-nicotinic could be presumed as a potential factor especially in case of healing wounds which offers the need of more knowledge and research about copper (II) Schiff bases as bioactive agents.

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Last but not the least, heartfelt respects to my ever supporting family and my sincere friends whom always encouraged me to carry on despite all hopeless moments I experienced. Thank you all.

“ To my loving parents and a wonderful brother “

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BHI	Brain Heart Infusion
BHT	Butylated Hydroxytoluene
CMC	Carboxymethylcellulose
DM	Diabetes Melitus
DMSO	Dimethyl Sulfoxide
DW	Distilled Water
ECM	Extracellular Matrix
ED_{50}	Median Effective Dose
ESR	Electron Spin Resonance
EtOH	Ethanol
HPLC	High-performance Liquid Chromatography
LP	Lipid peroxidation
MDA	Malondialdehyde
MDCK cells	Madin Darby Canine Kidney cells
MRSA	Methicillin Resistant *Staphylococcus aureus*
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PBS	Phosphate-buffered Saline
PMN	Polymorphonuclear
PUFA	Polyunsaturated Fatty Acids
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species

S.E.M.	Standard Errors of the Mean
SOD	Superoxide dismutase
TBRAS	Thiobarbituric Acid Reactive Substances
TEAC	Trolox Equivalent Antioxidant Capacity assay
TGFβ	Transforming Growth Factor beta

UNITS

IU/L	International unit per liter
mg	Miligram
mL	Milliliter
mM	Milli mole
°C	Degree Centigrade
µ	Micron
µL	Microliter

CHAPTER ONE

INTRODUCTION

1.0 INTRODUCTION

1.1 The importance of the proposed research

In modern age, biomedical science has supported developing researches about wound healing factors considering their safety, efficacy, and utilization. Despite advanced cellular and molecular understanding of biologic procedure in wound repair and improvements in pharmaceutical drug industries, the limitation in availability of drugs capable of stimulating wound repair is still remarkable (Epstein et al., 1999; Kumar et al., 2007). This research study tries to unfold the innate potentials of a chemical compound [1H-indol-3-yl methyl nicotinic hydrazide] – Copper (II) specially in case of healing wounds, to examine the probable opportunity of introducing a new efficient agent to be improved as a new drug in field of wound care.

1.2 Wound healing background

Wounds were with humankind from the beginning of history. A surgically induced wound aged as old as 100 centuries has been recorded in texts; skull trepanations and fingers amputations are few testimonies. In 5th century BC, surgical instruments had been made to facilitate wound healing; later in 6th century, Hippocrates wrote of dry dressing and stressing suturing. During Roman Empire, Celsus defined acute inflammation in four signs: redness, swelling, heat and pain; He described acute wound as an injury that kills patient rapidly or being healed rapidly (Robson et al., 2001). Differentiation between wound and ulcer lead to the explanation of chronicity. Stated by Celsus, ulcers being healed weakly, they are infected, irritated, and secreting pus.

Avicenna, the proficient scientist of 10th century, has presented organized and clear medical knowledge of the time in his book, Canon, the most famous medical textbook known

as an encyclopedia of medicine. Later on, in 13th century, suturing techniques and factors to be noticed in healing procedure (i.e. hair, salve, oil) described by Bruno da Longoburgo. By the Renaissance, Ambrose Paré, suggested the wound care and promoting treatments which are used today (Robson et al., 2001).

1.3 Wound terminology

Any physical disruption in integrity and functional structure of the skin is called wound or injury. Wounds cause cuts and opening of large segments of the skin, the primary defense barrier against any external agent, which results in dynamic functionality deficiency, disability, and death in patients (Epstein et al., 1999; Kumar et al., 2007). Created intentionally or accidentally or as a disease side effect, wounds cause the fractured area undergo bleeding, vessels shrinkage, coagulation, complement system activation and inflammation (Robson et al., 2001).

1.4 Classification of wounds

The clinical classification of wounds is based on length of healing which divides them into acute and chronic wounds (Robson et al., 2001). Acute wounds get healed shortly but chronic wounds show difficulty in healing and they may never get recovered. As mentioned by Baranoski and Ayello (2007) in their book, Bates-Jensen and Wethe have defined acute wounds as "a disruption in the integrity of the skin and the underlying tissue that progress through the healing process in a timely and uncomplicated manner". Those injuries and traumas undergo surgeries and recover by primary intention are ranked as typical acute wounds (Baranoski et al., 2007).

Assented by the Wound Healing Society, chronic wound is defined as "wounds that pass through the repair process without restoring anatomic and functional results"; they are not healing in an orderly sequence and specify by prolonged convalescence. Chronic wounds

are dependent to many specific environmental factors including age, diabetes, perfusion and immune competence aspects which interfere in the inflammation response in patients. Aged people having low immune capacity are mostly subjected to chronic wound and show unrelated responses to pathologic factors. The full thickness tissue loss is the specific characterization of chronic wound (Baranoski et al., 2007).

Another classification differentiates wounds based on depth and nature of the wound, introducing: Open wounds (penetrating, incised, lacerating, crushed) and Close wounds (hematoma, abrasions, contusions); also separation on severity, recommends Simple and Complex wounds; simple wound has just refer to skin injuries while complex wounds show underlying tissue damages, tendons, etc. (Sudha Bhargavi et al., 2011).

1.5 How are wounds healed?

1.5.1 Definition of healed wound

The "healed wound" refers to an injury in which: 1) the connective tissue has completely repaired and re-epithelialization generated and 2) the tissue has regained its anatomic structure and activity without requiring diagnosis and dressing anymore (Enoch et al., 2008). The healing process starts with granulation and ends in scar formation. Complete healing is only achieved when the disrupted tissue is sealed with collagen. For an optimal healing and minimizing tissue damage, nutrition, moist environment, and oxygenation should be considered (Kumar et al., 2007).

Knowing about the healing biology and how to induce the skin to be repaired is a goal in wound healing process which also helps to design and produce new drugs and perform more effective wound care technics.

1.5.2 Physiology of wound repair

Wound healing process is considered as series of successive physiological events, starting exactly after injury occurs and continues until the injured tissue obtains its functionality. Wound healing is a dynamic interactive process (Robson et al., 2001); self-motivated physiological process (Rajasekaran et al., 2004); a complex, well-orchestrated phenomenon (Sudha Bhargavi et al., 2011), involving different cell types, soluble intermediates and extracellular matrix. Although wound healing process occurs in all organs in the body, but some tissues include liver, eye and skeletal tissues are undergo specific healing process and require more discussion (Robson et al., 2001). Biochemical, cellular, molecular and physiological phenomena are involved in the process of wound healing to result in reformation of connective tissue and regaining the functional status (Al-Bayaty et al., 2012) however the recovered tissue would never restore its initial appearance and strength to function perfectly (Fonder et al., 2008). Interrelation of coagulation, inflammation, chemotaxis, deposition and differentiation of ECM, fibroplasia, cell division, epithelialization, neovascularization, contraction and remodeling events create the whole process of healing (Enoch et al., 2008; Robson et al., 2001). Intermediates involved in healing process include platelets, cytokines, growth factors, inhibitors and metalloproteinases (Enoch et al., 2008).

The normal 3-14 days healing period is related to many peripheral conditions such as nutrition, immune system, circulation, etc. Any mechanical forces should be avoided in this period (Fonder et al., 2008). Countless factors can interrupt the normal wound healing; diseases such as diabetes, vascular insufficiency, neurologic defect, chronic disease, nutritional deficiency, advanced age as well as pressure, infection, and edema known as peripheral factors impair wound healing (Fonder et al., 2008).

Cell-cell and cell-matrix interaction as well as different cell lineages and tissues cooperation are essential to precede the normal healing process in a chronological order. The primary goal of wound healing is rapid wound closure and tissue's aesthetical and mechanical state retrieving (Epstein et al., 1999). In the continuous healing operation, cells undergo biological changes in order to assist hemostasis, conflict infection, deposit a matrix, in-site migration, create new blood vessels and final closure (Baranoski et al., 2007). Any mutations in any of the healing factors or stages launch the healing process to chronicity, delay in healing and facing difficulties in closure of the wound; this is how a chronic wound occurs.

1.5.3 Types of healing

(a) Primary (first) intention is seen in clean incisions, surgical cuts and wounds with approximated edges that can be treated by suturing, glues, and tapes. The missing content of epithelial cells in the basement membrane and underlying connective tissue reconstitute shortly. Wound closure occurs rapidly in the first 12-24 hours as a result of proper harmony of healing phases (Enoch et al., 2008).

(b) Delayed primary healing happens in infected wounds that are not strong enough to be closured immediately. These wounds will remain open for 3-4 days in order to defend against contaminants; Immune system will help healing through inflammatory phagocytic cells to clean the area. The wound edges may approximated after few days and the tissue may retain its normal functionality (Enoch & Leaper, 2008).

(c) Secondary intention occurs in wounds with separated edges; tissue loss is common and reconstruction may not happen by epithelialization singly; as a result, ingrowth of granulation tissue, ECM reposition, and collagen repose

occurs followed by wound contraction and epithelialization leading to full-thickness wound closure. Potent myofibroblasts having structural properties and soft muscle cells are leaders in this type of wound healing, appearing 3 to 21 days after injury. However, slow healing, an unsightly scar, and restriction of movement are common outcomes for the affected tissue. Major trauma, burns and laparotomy are few examples of this case (Enoch & Leaper, 2008; Sudha Bhargavi, Amaresh Kumar, & Ranjith Babu, 2011).

1.5.4 Orderly healing stages

In a highly organized process, few vital sequential stages of repair occur in a wounded site including inflammation (0–3 days), cellular proliferation (3–12 days) and remodeling (3–6 months) (Fonder et al., 2008; Kumar et al., 2007). As a time-limited procedure, overlapping of phases happen during the process, though each phase remains distinct in terms of time (Baranoski et al., 2007)

1.5.4.1 Inflammation

The humoral and cellular inflammatory phase acts as a primary shelter against external invaders such as bacteria. The immediate response to an injury is hemostasis. By losing skin integrity, the cascade of coagulation initiates. The first reaction is to prevent bleeding which occurs by presence of platelets as source of mediators in the wounded area. The aggregation of platelets creates a clot, which helps stop bleeding. The insoluble blood clot containing fibrin fibers, fibronectin, vitronectin, and thrombospondin also provides a temporary extracellular matrix for migration of other cells and reestablishes hemostasis (Enoch et al., 2008; Epstein et al., 1999).

Growth factors released by platelets and other chemotactic signals attract phagocytic white blood cells to phagocytize bacteria infecting the area early after injury; this helps early closure of the acute wound. Neutrophils and monocytes depart from blood circulation to the injury site in response to surface changes of endothelial cells in capillaries (Fonder et al., 2008; Martin, 1997). Beside clearing the early contaminants by releasing degrading enzymes and free radicals derived from oxygen, neutrophils are recently considered as sources of pro-inflammatory cytokines to activate local fibroblasts and keratinocytes (Enoch et al., 2008; Martin, 1997). Few days later, these cells themselves as well as other redundant in the wound area may be extruded by macrophages while stimulating the phagocytic action by binding to ECM proteins through their integrin receptors (Epstein et al., 1999). Tissue growth factors such as transforming growth factor beta (TGFβ) which are necessary for repair process are released from Macrophages as plentiful reservoirs (Robson et al., 2001). Macrophages play a key regulatory role in healing the wound; without macrophages, the healing is severely impaired (Martin, 1997) leading to poor debridement of the wound, postponed fibroblast proliferation, incomplete angiogenesis, and poor fibrosis (Enoch et al., 2008). The main goal of inflammatory stage is to clear the wound of any infection so little is done according to the normal process of wound healing beyond this stage (Enoch et al., 2008).

Based on type of cells involved and time and duration of response, inflammatory stage is divided into early and late phases, each distinguish as follow:

Early phase (days 1-2): activation of classical complement cascade, infiltration of neutrophils in response to chemoattractants within 24-48 hours after injury, phagocytic activity of polymorphonuclear leukocytes (PMN) against bacteria, and other foreign particles, migration and proliferation of epithelial cells along the dermis, depositing components of basement membrane (Enoch et al., 2008).

Late phase (days 2-3): phenotypic changes done to monocytes to create macrophages as the most important cells in late phase (within 48-72 hours), phagocytosis, primary production of growth factors responsible for the proliferation, releasing proteolytic enzymes (e.g. collagenase); collagen fibers are apparent at the margins of the wound (Enoch et al., 2008).

1.5.4.2 Proliferation

The total proliferation phase includes epithelial regeneration, fibroplasia, collagen formation, wound contraction, and neovascularization. Vascular and cellular interactions results in formation of granulation tissue and epithelial repair. Epithelial regeneration is considered as a crucial event in wound healing as it maintains underlying tissue against bacteria and extrinsic bodies (Sebaceous, 2009). Epithelial cells originated from skin appendages such as hair follicles and sebaceous glands in neighbor area or from the wound edges, start moving to the wound space few hours after injury. Differentiation of epithelial cells and dissolution of links results in separation of dermis and basement membrane and allows the movement of epithelial cells. Receptors on the surface of epithelial cells facilitate their attachment to the ECM protein consisting of fibrin and fine collagen fibers made by fibroblasts. Epithelial migration leads to closure of the full-thickness open wound and separation of desiccated eschar from viable tissue. (Epstein et al., 1999; Robson et al., 2001). End of epithelialization is characterized by monolayer keratinocytic coverage over the bare surface of the wound and epidermal laminate rebuilt over a basal lamina layer from the inward margins of the wound (Martin, 1997).

The process of fibroblasts migration, ECM deposition and formation of granulation tissue and new blood vessels starts on day three after injury and progresses for next two to four weeks (Enoch et al., 2008). In few days, fibroblasts are major cell type found in the

granulation tissue. Whilst macrophages detraction, other cells including fibroblasts, endothelial cells, and keratinocytes start secreting growth factors to stimulate continual proliferation, protein production, and angiogenesis. Fibroblasts appear one day earlier than endothelial cells, attracted to platelet-derived growth factors and TGFβ, proliferate and produce fundamental protein constructors (i.e. fibronectin, hyaluronan, collagen, and proteoglycans) of a new extracellular matrix, that supports the ingrowth of the tissue as a substantial factor in repair activity. Fibroblasts substitute the extracellular fibrin/fibronectin matrix created during inflammatory stage with new collagen-rich granulation tissue. Collagen, a rope-like triple helix, known as the most abundant protein in the body provides solidity and integrity to all tissues. Optimal healing is indicated by formation of granulation tissue characterized by soft, pink, granular gross perspective (Enoch et al., 2008; Robson et al., 2001).

1.5.4.3 Remodeling (maturation)

Extracellular matrix‘s synthesis is symmetrical with formation of granulation tissue; remodeling of ECM and collagen becomes stables after three weeks (Enoch et al., 2008) restoring only 20% of tissue’s final strength by this time (Epstein et al., 1999). In this stage, the total wound healing activity slows down; reduction in number of mature fibroblasts happens after fibroblasts final differentiation and apoptosis; blood flow falls and angiogenesis steps down; thick collagen bundles are created by merging thin fibers; wound contraction occurs to reach the smallest wound volume (Robson et al., 2001). However, the newly formed tissue can just regain 80% of its initial strength (in terms of collagen fibers) (Enoch et al., 2008).

1.6 Objectives of this study

This research study aims to:

1. Evaluate the antimicrobial activity of the compound *in vitro*.
2. Study antioxidant activity of the compound *in vitro.*
3. Determine the acute toxicity study of the compound *in vivo.*
4. Evaluate wound healing potential of the compound on experimentally induced excision wounds in rats (*in vivo*).
5. Evaluate the antioxidant properties of the compound on wound homogenates (*in vivo*).

CHAPTER TWO

REVIEW OF RELATED LITERATURE

2.0 REVIEW OF RELATED LITERATURE

2.1 Compounds as drugs

In search for cure of illnesses, man has tried several clinical trials (chewing roots, herbs, barks and berries) over thousand years (Silverman, 2004).Traditional remedies and discovery by chance, has built the history of the drugs, later followed by understanding the ingredients of plants, extracts, cells, synthesizing the small molecules and identifying the bioactive molecules with promising role in pharmacology. There is always need for efficient cheap raw material to offer to the drug industry (Nair et al., 2006). Combinatorial chemistry has provided the technological field, benefited from usage of chemical compounds to generate diverse and efficient drugs, although most of the products had natural origin. Typically, the chemical modification is done on active natural products to improve the detected properties (Silverman, 2004).

Medicinal chemistry also talks about integration of chemicals and compounds to medical science and their improvement into functional therapeutic medicines (Silverman, 2004). Drugs are used to treat, cure, diagnose, relieve and prevent disease (Silverman, 2004). The principal keys in drug discovery are the drug's selectivity, efficacy, safety (non-toxic and orally bioavailable), metabolic stability (long half-life). Any compound fulfilling these requirements would have the chance to be developed and clinically tested.

The acquisition of a drug/medicine is target selective and directly related to the disease type with the primary goal of antibacterial and bactericidal effect. As microorganisms are evermore competing for enough space and nutrients to proliferate, they are getting adapted to their environment and modify their potentials against external agents such as drugs to survive. It is also related to microbe characteristics, frequency and type of the agent used and societal

and technological changes that proceeds transmission of drug resistant organisms (Cohen, 1992).

Antimicrobial resistancy leads to higher health-care costs, mortality and morbidity (Cohen, 1992). Formation of the microbial resistancy to many drugs enhances the need of designing new and effective drugs today. The increase in occurrence of some disease such as deep mycosis , arises the need of screening new broad-spectrum and low-toxic medicine (Lv et al., 2006). Modified pharmacological and toxicological properties have been observed in many drugs applied in form of metallic complexes (Khattab, 2005). Compounds which coordinate with metal ions, perform different properties based on the structure of ligand and the nature of the metal ion (Nair et al., 2006). Coordination compounds, those that give complex ions by dissolution instead of releasing the ions they are made up of, are able to bind to metals. The characteristic properties of these compounds are related to the nature of the metal ion they are bound to, and the ligand type. Metal complexes are of human interest due to their vast fields of application. The importance of coordination compound is that they are found in living systems (Nair et al., 2006).

Certain metal-ligand complexes show biological activities and simulate drug-like molecules. The complex generates an organic pharmacophore by coordinating to the organic ligand (Beshir et al., 2008). The metal complexes with Schiff bases as ligands have been playing an important role in the development of coordination chemistry as a whole (Tumer et al., 2007). As stated by Yilmaz & Cukurovali (2003) in their study, Schiff base, cyclobutane and thiazole functions in any compound molecules make it suitable for further chemical modifications and useful ligand in coordination chemistry (Yilmaz et al., 2003).

2.1.1 Schiff base-Copper complexes biological studies

Named after Hugo Schiff, Schiff bases as intermediates, play a key role in bioactive compound synthesizing, displaying anticancer, antibacterial and antifungal properties (Rosu et al., 2010) and in development of coordination chemistry (Bhowmik et al., 2011). This biological activity might be because of *in vivo* interactions with the potential donors of biological heterocycle (Mughrabi et al., 2011). Schiff bases, of old chemistry reactions, are basically combinations of amines and carbonyl (Gulcan et al., 2012) with vast aldehyde and ketone functionalities and their of interested transition metal (II) complexes (Geeta et al., 2010). As defined by IUPAC (International Union of Pure and Applied Chemistry (Compendium of Chemical Terminology), 2012),"Schiff bases are Imines bearing a hydrocarbyl group on the nitrogen atom RHC=NR' (R' ≠ H) considered by many to be synonymous with azomethines".

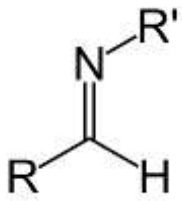

Figure 2.1: General structure of Schiff base

The coordination of Schiff base and metal is through the imine nitrogen and oxygen group placed on the carbonyl compound; while it is believed that the Schiff base provides the possibility of rearrangement in presence of metal ion or H+ (Bhowmik et al., 2011). Beside the biological features, the preparative accessibility, tunable electronic properties and structural variability of Schiff bases has made them operational in systematic reactivity studies (Geeta et al., 2010). Amongst all metal complexes, Copper (II) Schiff base derivatives

are frequently evaluated for their functional properties. In comparison to free ligands, metal chelates generally show higher antibacterial activity due to increase in cell permeability (Nair et al., 2006). To increase the activity and rate of inhibition in Schiff base-metal complexes, it is required to increase the concentration as well (Tumer et al., 2007).

As reported by Gulcan et al. (2012), a new Schiff base ligand containing pyrimidine ring and Cu(II) has shown an effective and selective antibacterial activity against a spore-forming gram positive bacteria (*B.cereus*) and few other bacteria. Pyrimidine importance in studies is because of its frequent availability in living systems and its wide range of biological activities: antitumor, antifungal, antipyretic, antibacterial (Gulcan et al., 2012). A comparison between pyrazolone-based Schiff base ligand and its Cu(II) complex done by Rosu et al. (2011) declares that the metal complex presents more growth inhibitory effect against certain microorganisms than free ligand.

Beshir et al. (2008) studied cell migration inhibitory potential of a Cu(II) complex of Schiff base product of condensation of benzyl dithiocarbazate and acetylpyridine and the strong cell motility prevention of the compound proved. Cell migration is a basic vital issue in many pathological events including wound healing, angiogenesis, inflammation, immune functions, and tumor metastasis. The study has been done through scratch-wound assay specifically on human breast cancer and colorectal carcinoma cell lines as well as MDCK cells, the most sensitive cell monolayer to the compound - $[Cu_2Cl_2(L^2)_2]$- used , which displayed to prohibit cell migration selectively (Beshir et al., 2008). Type equation here.

As thiosemicarbazide-based Schiff base complexes have demonstrated therapeutic impression in many research studies, Hossain et al. (1996) and his collaborators investigated the bioactivity of acetylpyridine Schiff bases of N-substituted thiosemicarbazides -copper(II)

complex and they concluded that the complex has weaker fungitoxicity on certain fungus strains compared to free ligand or nystatin as positive control (Hossain et al., 1996).

Another novel derivative of thiosemicarbazides-Cu (II) complex has been utilized by Cukurovali and his fellows (2002) beside two other chemical functional groups which were considered biologically active as they potentially have wide range of antiparasite, antibacterial, antidepressant and herbicide efficacy. However, the two metal complexes of the Schiff base ligands studied, exhibited weak or no bacterial growth inhibition (Cukurovali et al., 2002).

Recently, the efficacy of a new Schiff base copper complex family (CuP1) containing two pyridine and one imine group has been evaluated by Chakraborty et al. (2010). Pyimpy – the tridentate ligand – expected and finally expressed to be able to act as a strong anticancer agent (Chakraborty et al., 2010).

2.1.2 Biological activity of Copper element (Cu)

Known as an essential trace element and transition metal, Copper is involved in many biochemical and biological processes in our body (Tennant J. et al., 2002) and it shows synergistic activity with the drug (Lv et al., 2006). Cu is found in all living cells, in form of oxidized [Cu(II)] and reduced [Cu(I)] molecule (Tapiero H. et al., 2003) and is water soluble. It represents fungicidal and bacteriostatic properties in low concentration and is somehow vital in transportation of oxygen and electrons (Berg, 1994).

Copper not only acts as a cofactor in basic redox activities, but its presence is necessary to some proteins to function accurately in many physiological functions such as oxidative phosphorylation, superoxide dismutase (antioxidant) and oxidation of iron. However, excess level of copper intake results in production of adverse hydroxyl radical (Tennant J. et al.,

2002) and can invest toxicological activities (renal malfunction and lipid profile changes) (Chen Z. et al., 2006). Copper(II) complexes having square planar molecular geometry involve in enzymatic catalysis for hydrogenases and oxidases enzymes (Roat-Malone, 2007).

It has been thought for many years that Cu(II) has the bactericide potential itself, but studies have explained that the credit goes to the activated oxygen molecule on the surface of metal copper and Cu activity itself in killing bacteria is weak (Lv et al., 2006). It is also proven that Cu combined with a drug acts more strongly while the nitrogen donor heterocyclic ligand is present. An existing example of nitrogen donor is pyridine, which examined by Lv et al. (2006) in form of Cu(II) complex of valine-derived Schiff base with two nitrogen donor of pyridine and CON and the experiment declared the ability of the compound as a wide spectrum drug against pathogenic fungi and resistant bacteria.

Copper chelating is considering a remedy for neurodegenerative disorders such as Parkinson, Alzheimer, and Creutzfeldt-Jakob. Excess level of Cu has been employed as copper chelators in treatment of some human cancers like prostate, breast, colon, and lung cancers, as an anti-angiogenic agent. Cooperating in cancer cells, copper chelators with copper salts have been detected to affectively inhibit proteasome activity and induce apoptosis (Tapiero H. et al., 2003).

2.2 Free radicals and antioxidants

Simply defining, atoms with unpaired electrons in their nucleus are called free radicals. These chemical species form as by products, accidentally or intentionally during some processes like phagocytosis. Oxygen derivatives (hydroxyl radical $OH^{\bullet}$, superoxide anion $O_2{}^{\bullet-}$), hydrogen peroxide (H_2O_2), and certain transition metals are important free radical reactants in aerobic cells (Celil Uslu et al., 2003). Free radicals are continuously produced in

the body either through respiratory chain in mitochondria or from external sources like radiation, cigarette smoke, metabolized drugs or UV light (Södergren, 2000). Free radicals are more reactive than non-radicals and are able to convert a non-radical to radical molecule by donating or taking an electron; this may cause the generation of radical reaction chain in the body (Södergren, 2000).

Increasing the chemical reactivity of a substance and inducing oxidative stress is the result of unbalanced formation and elimination of free radicals. Oxidative stress is a precursor of serious diseases such as of neoplasia, diabetes mellitus, acute pancreatitis, inflammation processes, ischemia and atherosclerosis and acts through seriously damaging vital components of the cell specially lipids, proteins, nucleic acids and suchlike biomolecules (Vanco J. et al., 2004). Reactive free radicals cause cell death and tissue injury through attacking cell components and oxidizing biomolecules inside the cell. Mostly, lipid deterioration of cell membrane and organelles leads to lipid peroxidation (Celil Uslu et al., 2003). Free radicals are not always unfavorable; they play physiological functions purposely e.g. phagocytic cells kill bacteria and fungi by secreting superoxide anion radicals and hydrogen peroxide (Södergren, 2000).

Talking about free radicals brings up the antioxidant issue. Quoted by Dejian et al. (2005) in their review paper, the biological definition of antioxidant is "synthetic or natural substances added to products to prevent or delay their deterioration by action of oxygen in air ". It is also described as "enzymes or other organic substances, such as vitamin E or β-carotene, that are capable of counteracting the damaging effects of oxidation in animal tissues" in biochemistry and medicine. In general, antioxidants are health protecting factors, able to trap free radicals and prevent degenerative disease by forbidding oxidative mechanism

(Prakash A., 2001). Vegetables, fruits, whole grains, and generally plants rich in vitamin C, carotenes, vitamin E, and phenolic acids are sources of antioxidants in nature.

The interest about the antioxidants arises from the ability of preventing the formation of free radicals and preparing a defense system to hamper fat and other food stuffs' decadence in the body (Molyneux, 2004). The defense system of the body against free radicals includes antioxidant enzyme and molecules include glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, etc.(Celil Uslu et al., 2003), and non-enzymatic antioxidants such as antioxidant enzyme cofactors, oxidative enzyme inhibitors (cyclooxygenase), and transition metal chelators (Dejian Huang et al., 2005). Based on the location in the body, antioxidants can be divided into intracellular group containing enzymatic antioxidants responsible in making substrates less reactive and extracellular group including urate and proteins serving as metal ion chelators. Bilirubin, albumin and urate are instant scavengers (Södergren, 2000). The mechanism through which an antioxidant acts is to donate an electron to the unpaired atom to neutralize the free radical and repair the cell damage (Figure 2.2).

The copper rich enzyme Cu, Zn-superoxide dismutase (Cu,Zn-SOD) is known as a very important free-radical scavenger. Although human body is self-sustaining in defense against free radicals, but administration of synthetic or natural antiradical compounds may help to promote the antioxidative processes in the body (Vanco J. et al., 2004).

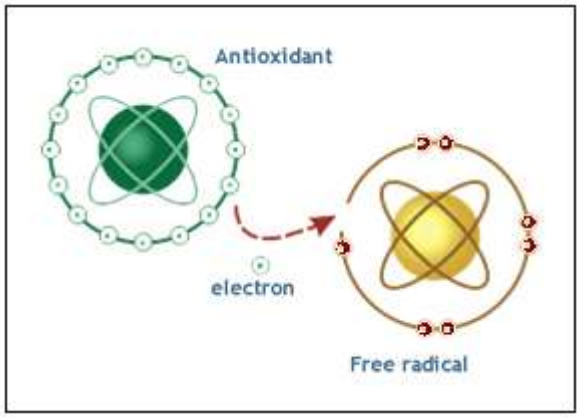

Figure 2.2: Schematic free radical and antioxidant actions. (picture is recreated from https://www. readanddigest.com/free-radicals-the-friendly-foes/)

2.2.1 Antioxidant role in healing wounds

Reactive oxygen species (ROS) are known as harmful agents that threaten cells and tissues and interfere the wound healing process (Kumar et al., 2007). Many reactive oxygen and nitrogen centered species are found in the wound area (Rajasekaran et al., 2004). During the process of wound healing, free radicals are produced at the wounded site through acute inflammation response. Numerous white blood cells are attracted to the signals sent from the wound area after injury occurs. Neutrophils doing respiratory activity and non-phagocytic cells involving in non-phagocytic NADPH oxidase mechanism release free radicals (Kumar et al., 2007). Defending against ROS, Free radical scavenging enzymes (FRSE) with cytoprotective role are able to eliminate, detract and deactivate ROS/RNS and thereupon manage to regulate the wound healing process. Free radicals and imbalances between oxidants and antioxidants cause oxidative stress which effects the cell by DNA breakage, enzyme inactivation and lipid peroxidation where all lead to impaired wound healing procedure (Kumar et al., 2007; Rajasekaran et al., 2004). Oxygen containing free radicals have found much interesting in biological systems recently as they can induce ageing and chronic disease

consisting cancer, rheumatoid, malaria, stroke, neurodegenerative diseases and others (Aliyu et al., 2009; Odukoya et al., 2005).

Antioxidants as scavengers, control the removal of free radicals when an injury occurs, however any disease condition such as diabetes can disrupt this control. As stated by Rajasekaran (2004), in diabetes melitus (DM), high level of ROS followed by high oxidative stress delays the healing in patients. The production of ROS in DM is due to glucose autoxidation and mitochondrial malfunction (Rajasekaran et al., 2004).

The potency of antioxidants as powerful therapeutic agents to prevent free radical related tissue damages is agreed. In this case, compounds with antioxidant activity can be applied as drugs on the top of wound areas. Significantly, plant extracts containing tannins, flavonoids, catechin, anthocyanin and phenols are known as active free radical scavengers (Kumar et al., 2007; Nooman A. Khalaf et al., 2008).Treatment of complex diseases such as Alzheimer, atherosclerosis, diabetes and stroke is the major goal of antioxidant-based drugs studies (Nooman A. Khalaf et al., 2008).

2.2.2 Antioxidant evaluation

Different methods has been designed to assess the antioxidant efficacy of the substances (both natural and synthetic products) due to controlling free radicals (Molyneux, 2004). The drawback in detection and measurement of free radicals is their short life span (Södergren, 2000). The antioxidant capacity of foods, biological substances and serums are compared to each other relying on technical skills and equipment support, mostly based on chemiluminescence assays and oxygen radical absorbance capacity assay (Prakash A., 2001). Another analytical method, electron spin resonance (ESR), is based on determination of antioxidants ability in identifying particular free radicals such as the superoxide anion radical ($O_2^{\bullet}$), the hydroxyl radical ($OH^{\bullet}$), the peroxyl radical ($ROO^{\bullet}$) and DPPH radical which

provides a more stable molecule to be detected. ESR method is more accessible as no specific equipment is needed (Prakash A., 2001; Södergren, 2000). MDA (malondialdehyde) is also a method of estimating the lipid peroxidation of a substance in biological systems in presence of antioxidants (Prakash A., 2001). As individual methods evaluate the antioxidant activity of a substrate based on chemical reactions, the methods generally categorize in two groups: 1) assays based on single electron transfer (ET) , 2) assays based on hydrogen atom transfer (HAT), both measuring the radical scavenging ability (Dejian Huang et al., 2005). Of most popular ET based assays are FRAP, DPPH, TEAC and Cub(II) reduction capacity assay, all contain an oxidant (probe) snatching the electron and antioxidant in the reaction (Dejian Huang et al., 2005). The color change in the reaction is related to both the probe and the antioxidant concentration and determines the end of reaction.

2.2.2.1 DPPH assay

DPPH is a current popular method, introduce by Marsden Blois (1958), which is based on trapping 1,1-diphenyl-2-picrylhydrazyl stable free radical (Molyneux, 2004; Prakash A., 2001). Known as fast, simple, accurate and cost-effective technique (Dejian Huang et al., 2005; Prakash A., 2001), DPPH assay qualifies the total antioxidant capacity of solid/liquid substance or complex substances which help understanding the functional property of the substance (Prakash A., 2001). Stated by Dejian et al., 2005, DPPH is noticed as commercially available organic nitrogen radical having UV visible absorption and monitoring by spectrophotometer easily. The specific feature of DPPH is that despite other free radicals, dimerization of the molecule is inhibited as the reserve electron is wandering all around the molecule, not localizing. Mixing with any substance able to donate a hydrogen atom, DPPH molecule transforms to reduced configuration, changing the deep violet color to light yellow (Molyneux, 2004). The concentration of the substance causing reduction in 50 percent of the

initial DPPH activity has concerned as $\boldsymbol{EC_{50}}$ value (Efficient Concentration) or $\boldsymbol{IC_{50}}$ (Inhibitory Concentration) by Brand-Williams and his group in 1995. A drawback about $\boldsymbol{EC_{50}}$ is that this parameter appears the lower while a substance has the higher antioxidant activity (Molyneux, 2004). $\boldsymbol{EC_{50}}$ value is measured by color change.

2.2.2.2 FRAP assay

FRAP which stands for Ferric Reducing Antioxidant Assay (Dejian Huang et al., 2005) or Ferric Reducing Ability of Plasma (Benzie et al., 1996) is a method based on reduction of ferric iron to ferrous at low pH . The oxidant is the color producing ferric salt, Fe(III)-2,4,6-tripiridyl-s-triazine named TPTZ (Dejian Huang et al., 2005). The values are obtained by absorbance comparison of test samples to those having known concentration of ferrous ion. The strong blue color development proves the presence of the reductant antioxidant. The test is rapid, automated and inexpensive, accessible using commonly found equipment with reproducible results in different concentration (Benzie et al., 1996).

2.2.3 Enzymatic antioxidants

2.2.3.1 Superoxide dismutase

Former member of a group of metalloproteins, discovered and renamed by Irwin Fridovich and Joe McCord in 1969. Superoxide dismutase (SOD) is found in dermis and epidermis supporting the healthy fibroblast production, and is an antioxidant enzyme, reducing cell damages occurred by superoxide free radicals. Superoxide (O_2^-), widely distributed and the most common in mammalian tissue, is removed by SOD. The reduction of superoxide anions to hydrogen peroxide takes place in presence of superoxide dismutase. SOD defeats toxic effects of oxygen radicals and is capable of protecting cells against cancer and aging (Chakraborty P. et al., 2009; McCord et al., 1969). It is proven that SOD inhibition and

presence of superoxide radicals influences cell malignancy; however, SOD inhibition results in O2- assemblage in cell and consequent free radical-mediated damages which helps destroying cancer cells by induce apoptosis (Huang et al., 2000). SOD acts as both antioxidant and anti-inflammatory factor.

Human body is dependent to two types of SOD: copper/zinc (Cu/Zn) SOD and manganese (Mn) SOD that help absorption of copper, zinc, and manganese ions. Cu/Zn SOD protects cellular cytoplasm and Mn SOD keeps mitochondria safe against free radicals.

The topical form of SOD helps to reduce facial wrinkles, scar tissue, heal wounds and burns, hyperpigmentation, and protect against harmful UV rays. Treatment of some diseases such as burn injuries, corneal ulcers, prostate problems, inflammatory diseases, arthritis, and damage from long-term exposure to smoke and radiation has benefited the use of SOD ("Antioxidants - superoxide dismutase,").

An experiment to determine the SOD activity of [N,N'-bis(2-pyridyl-phenyl) methylene-1,4-butanediamine]-(N)-copper (II) synthetic Schiff base compound done by (Liu et al., 1996)expressed high SOD activity of compound *in vitro*.

2.3 Lipid peroxidation

Defined as oxidation of lipids rich in carbon-carbon double bonds (Devasagayam et al., 2003), Lipid peroxidation (LP) naturally occurs in human body by phagocytes or/and by the effect of several reactive oxygen species (Mylonas et al., 1999). Fatty acids with higher double bounds in number are more delicate to lipid peroxidation. Polyunsaturated fatty acids (PUFAs) found in biological membranes are targets to ROS such as hydrogen peroxide and hydroxyl radical to be oxidized; the free radical-mediators or second messengers are toxic by-products generated consequently (Södergren, 2000). As biological membrane is a fundamental part of many cell organelles including mitochondria, Golgi apparatus,

endoplasmic reticulum, lysosomes, etc., lipid peroxidation is strongly noxious to cells' functionality and survival (Devasagayam et al., 2003) which proceeds to serious tissue damage; Kidney damage, asthma, Parkinson's disease, atherosclerosis, and preeclampsia are few examples (Mylonas et al., 1999). Invasion to phospholipid bilayer of cell membranes containing PUFAs during lipid peroxidation mainly interferes its barrier functionality (Devasagayam et al., 2003).

LP initiates by grabbing a hydrogen atom from a methylene group of PUFAs and converting to lipid hydro-peroxides containing a carbon atom with an unpaired electron. Generation of the new carbon-centered free radical followed by creating a peroxyl fatty acid radical through reacting with molecular oxygen, starts a self-propagating chain reactions (Marnett, 1999; Södergren, 2000). The process stops while a free radical reacts to another one and a non-radical molecule is created. Antioxidant, vitamin E, SOD, and catalase are factors able to accelerate termination stage and defend cells against free radicals.

According to stability of hydro-peroxide molecule, high temperature and transition metal ions (iron and copper ions) are the factors that can motivate its catalysis; secondary lipid peroxidation products such as aldehydes (e.g. malondialdehyde) are yielded by this decomposition (Södergren, 2000).

2.3.1 Malondialdehyde

During last forty years, malondialdehyde (MDA) has been known as lipid peroxidation marker (Jentzsch et al., 1996). To detect LP, MDA and TBRAS (thiobarbituric acid reactive substances) are most popular assays based on measurement by TBA (Södergren, 2000). MDA is a major product of LP, exposing carcinogenic features (Marnett, 1999). MDA's biomarker potency is due to easily reacting with thiobarbituric acid (TBA) to form a pink color

chromogen (Devasagayam, Boloor, & Ramasarma, 2003). Although the method is fast and simple, its prominent drawback is non-specificity (Södergren, 2000).

2.4 Acute toxicity

Acute toxicity is described as noxious effects of a test substrate applied in single dose or multiple exposures, occurring within 24 hours from starting point up to 14 days (short duration of time). The distinction between acute and chronic toxicity is in several numbers of exposures to lower doses of a substance in a long period of time (months to year) in chronic toxicity (International Union of Pure and Applied Chemistry (Compendium of Chemical Terminology), 2012). Adverse effect is defined as "any effect that results in functional impairment and/or biochemical lesions that may affect the performance of the whole organism or that reduce the organ's ability to respond to an additional challenge" (Walum, 1998).

Human accidental exposures (e.g. factory accidents), laboratory animal use, and *in vitro* testing methods give some information about acute toxicity of a chemical which helps understanding its biologic activity and mechanism of action (IUPAC, 2006; Walum, 1998). To produce, handle and use different chemicals, it is important to manage the risk of utilization and identify the hazard by determining the acute toxicity of the chemical (Walum, 1998).

To classify chemicals toxicologically, LD_{50} or LC_{50} values are determined. Based on the route of the consumption, they are defined as the median lethal dose (LD : oral, dermal) or median lethal concentration (LC : inhalation) of a chemical to cause death in 50 percent of the treated animals in a duration of time when running an acute toxicity test. It should be noticed that the value for a certain compound may vary among individual laboratories due to differences in protocols, animal strains, caging, etc. (Walum, 1998). LD_{50} has been considered universally in many international fields of testing acute toxicity during last thirty years. Formerly, acute toxicity and LD_{50} determination were basically done for industrial chemicals

toxicological assortment with a governmental requirements worldwide. However, recommended by the Organization for Economic Co-operation and Development (OECD), nowadays it is noticed as the index of labeling orally intaking substances, describing a substance as: very toxic, < 5 mg/kg body weight; toxic, > 5 < 50 mg/kg; harmful, >50<500 mg/kg; and no label, > 500 < 2000 mg/kg (Walum, 1998).

The endpoint in the acute toxicity test is the loss of half number of the test animals during two weeks observation.

2.5 Antimicrobial activity

Any substance able to kill or inhibit microorganisms is known to have antimicrobial potency. These agents may either kill the microorganism (microbiocide) or inhibit the growth of it (microbiostatic). Microorganisms include bacteria, fungi, parasites, and viruses. If used to get rid of infectious factors on non-living surfaces, the substance is called disinfectant. Wide ranges of chemical and natural compounds are used as antimicrobials.

Appropriate antimicrobial drug use has unquestionable benefit (Lalitha, 2005). Plants have been old traditional remedies in treatment of diseases and inhibition of pathogenic microorganisms' growth. Copper alloys have also shown potentials to destroy microorganisms (Michels et al., 2005; Noyce et al., 2006). The use of poisons and heavy metals as bacteriostatic agents had been more popular in previous decades. However, the bacterial resistance to many antibiotics and drugs is related to non-complete inhibition of bacteria, allowing them to survive and become resistant through genotypic changes.

Infectious wounds have always been troublesome to patients suffering from chronic wounds. Acute wounds usually contain skin flora (*Diphteroids*, *Staphylococcus*) but as chronic wounds occupy vast skin area with deep cracks and slots, exudates, necrotic tissues, pus and eschar, they prepare a desirable environment for many pathogenic microorganisms to

live. The prevalent bacteria in chronic wounds are *Pseudomonas, Proteus mirabilis, Staphylococcus, Escherichia coli, Streptococcus, Bacteroides* and *Corynebacteria* species. Anaerobic bacteria are also very common as the non-oxygenated space is available for them in deep crevices of the chronic wound (Baranoski et al., 2007).

Methods to determine antimicrobial activity should be easy, speedy, cost effective, and reproducible. A variety of laboratory technics has been designed to measure *in vitro* sufficiency of antimicrobial agents. Different antimicrobial susceptibility testing methods include diffusion, dilution, and diffusion-dilution (Lalitha, 2005). The agar dilution method is recommended by CLSI to specify the antibiotics quantitatively. Broth dilution is also suggested to determine inhibitory activity (CLSI, 2008; Klančnik et al., 2010). The minimal concentration of antimicrobial to inhibit or kill the microorganism can be determined through dilution susceptibility testing methods (Lalitha, 2005).

A short review on antibacterial activity of Schiff-base compounds proclaims studies in this case. Gulcan et al., (2012) reported efficacious function of a Schiff base-copper (II) complex compound containing a pyrimidin ring against *Bacillus cereus* (ATCC 7064), *E. coli* (ATCC 4230), and other gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, *S.aureus* ATCC 25923, and *Micrococcus luteus* ATCC 9345) with MIC values in range of 20-40 μg (Gulcan et al., 2012).

In an experiment concluded by Rosu et al. (2011), 4-aminoantipyrine Schiff base and its copper complex showed an inhibitory effect (MIC values in range 128–512 g/mL) on the growth of the tested strains.

A broad spectrum antibacterial activity of Cu(II) of valine-derived Schiff base has been suggested by Lv et al.,(2006) evaluated by a serial dilution sensitivity test against both chosen Gram-positive and Gram-negative bacteria.

2.5.1 MIC (Minimum Inhibitory Concentration)

As a Globally accepted test, MIC is considered as gold standard in determination of antibacterial potency of any substance. Defined by Andrews (2001) in her study, MIC is the lowest concentration of a drug that will inhibit the visible growth of an organism after overnight incubation. Mentioned in Manual of Antimicrobial Susceptibility Testing (Coyle, 2005), "MIC inhibits a given bacterial isolate from multiplying and producing visible growth in the test system". Besides helping to certify resistance, MIC is also considered as a research tool to qualify new antimicrobials' activity *in vitro* (Andrews, 2001). It can be determined by broth or agar media, though broth micro dilution is the most popular method. Antimicrobials are tested in log2 serial dilutions (two fold) (Lalitha, 2005). The advantage of MIC is that just very small scale of antimicrobial agent would be enough to run the test and little preparation required keeps test turnaround time low; however prolonged incubation time leads to higher MIC appearance.

2.6 Promising structural potentials of the present compound

In this study, the compound contains nicotinohydrazide and indole groups, where nicotinic acid and nicotinamide are traditional materials used in drugs, with their unusual antibacterial spectrum (Khattab, 2005); Nicotinamide is the amide of nicotinic acid (vitamin B3 / niacin); Nicotinic acids are derivatives of pyridine which have a carboxy group. Figure 2.3 represents nicotinamide structure.

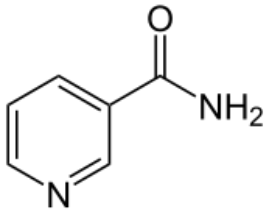

Figure 2.3: Nicotinamide structure

Hydrazides are a class of organic compounds in organic chemistry sharing a common functional group characterized by a nitrogen to nitrogen covalent bond with four substituents with at least one of them being an acyl group (International Union of Pure and Applied Chemistry (Compendium of Chemical Terminology), 2012; IUPAC, 2006). Carboxyilic acid hydrazides and their derivatives has been used to solve environmental problems in technology and chemistry (Odunola et al., 2002). Indole derivatives also reveal some antioxidant, anti-allergic and antiulcer potentials (Mughrabi et al., 2011). Individual features of different compounds and chemical groups guide to evaluate the biological properties, specifically wound healing latent ability of undertaken compound Copper(II) bis [N'-((5-chloro-1H-indol-3-yl) methylene) nicotinohydrazide] briefly called Cl-indole-nicotinic in the present study. The compound and its chemical structure is shown in Figures 2.4 and 2.5 below:

Figure 2.4: Cl-indole-nicotinic's appearance

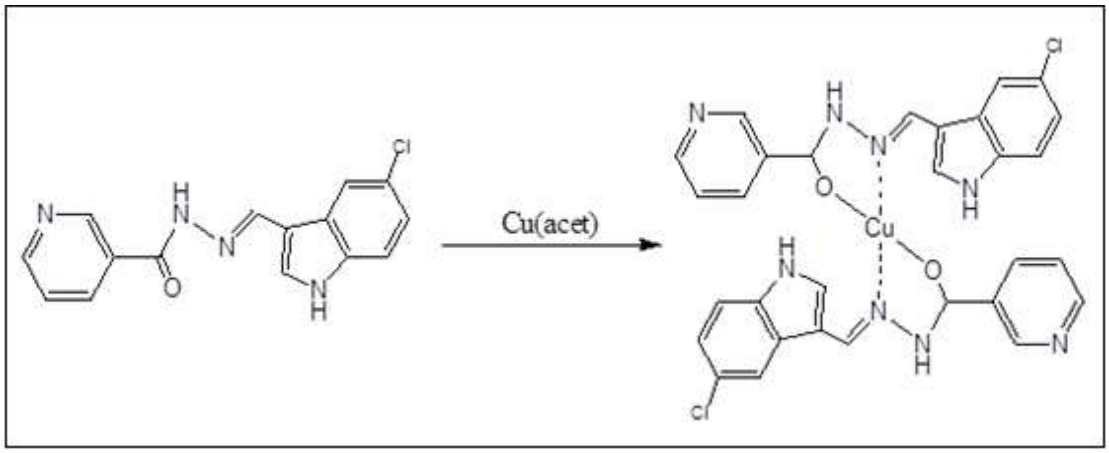

Figure 2.5: Cl-indole-nicotinic's chemical structure

CHAPTER THREE

DESIGN, MATERIALS AND PROCEDURE

3.0 DESIGN, MATERIALS AND PROCEDURE

3.1 Materials

The materials used in this study to perform the tests are shown in Table 3.1.

Table 3.1: List of materials used in this study.

	Etahnol 95%
	Formaldehyde 37-40%
	GILL'S Haematoxylin
	Alcoholic Eosin 1%
	2,4,6-tripyridyl-s-triazine (TPTZ)
	Sodium acetate trihydrate
	Glacial acetic acid
	Ferric chloride ($FeCl_3.6H_2O$)
	2,2-diphynyl-2-picrylhydrazyl hydrate (DPPH)
	Ascorbic acid
	BHT
	Gallic acid
	Quercetin
	Distilled water
	Antibiotic disks (vancomycin & gentamicin)
	Müeller-Hinton agar
	BHI broth
	10%-Tween 20
	Intrasite gel
	CMC (carboxymethyl cellulose)
	Ketamine
	Xylazine
	Lignocaine HCl
	Etahnol 75%
	PBS (phosphate buffer solution)
	TBA acetic acid
	TBA sodium hydroxide (NaOH
	HPLC-grade water

3.2 Equipments

The equipment used in this study are as listed in Table 3.2.

Table 3.2: List of equipment used in this study.

	Microplate reader Power Wave X 340 Software: KC Junior Programme
	Class II biohazard safety cabinet NU 425-400E, Nuaire™, USA
	CO2 water-jacketed incubator Nuaire™ IR autoflow
	Homogenizer machine Wisemax, HG-15A
	Tissue processing machine
	Inverted microscope CK-40 Olympus

3.3 Methodology of antibacterial activity

The antibacterial potential of the compound were evaluated against three Gram positive bacteria including *Staphylococcus aureus* (MRSA), *Bacillus subtilis*, *Staphylococcus epidermidis*, and three Gram negative bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* through disk diffusion assay.

The compound stock solution was prepared in one concentration: 50mg compound / mL DMSO. The 6mm in diameter paper disks, sterilized primarily and put in a sterile Petri dish (Lalitha, 2005) were loaded with 10 µL, 20 µL, and 30 µL of stock solution (50mg/mL) and left to become dry. Disks loaded with DMSO besides two antibiotic disks vancomycin and gentamicin were applied respectively as negative and positive controls.

The broth culture made up of few numbers of well-isolated colonies of each bacteria in a BHI broth was incubated at 37 °C for 2-6 hours to achieve the turbidity of the 0.5

McFarland standard with the absorbance of 0.008 to 0.10 at 625 nm wavelength (Lalitha, 2005). The prepared broth cultures then spread on the surface of Müeller-Hinton agar as described by Lalitha (2005), followed by distribution of the prepared control and sample disks on the surface of inoculated agar plate and overnight incubation in 37°C. Müeller-Hinton agar brings the advantage of tolerable batch-to-batch reproducibility for susceptibility testing and satisfactory growth of most nonfastidious pathogens.

The results were collected by measurement of the diameter of the zones of complete inhibition and interpreted by referring to Zone Diameter Interpretative Standards and equivalent Minimum Inhibitory Concentration Breakpoints of the NCCLS M100-S17 (CLSI, 2007) .

3.3.1 MIC evaluation

The test was run based on broth dilution method as explained by Lalitha (2005). The amount of 100 µL of stock solution of 10 mg compound/mL DMSO was serially diluted for eight times in micro well transparent plate loaded primarily with 50 µL BHI broth, followed by addition of 10 µL of each bacterial suspension to the wells separately. After an overnight incubation, the agar dilution of each diluted broth (on Müeller-Hinton agar) showed the growth and ingrowth of the specific bacteria in specific concentration which is the MIC value.

3.4 Methodology of antioxidant assay *in vitro*

3.4.1 DPPH assay

This test is based on trapping 1,1-diphenyl-2-picrylhydrazyl stable free radical (Molyneux, 2004; Prakash A., 2001). Measurement is done with maximal absorption at 515 nm. The absorbance detraction as the reason of reducing the free radical, results in color

change from deep purple to yellow in final stage, and determines antioxidant activity of the sample. The procedure is as follow:

1. Sample preparation and dilution :
    (a) 3mg of the compound was dissolved in 1mL of DMSO.

    (b) Serial dilution of sample solution was prepared by loading each of six eppendorf tubes by 0.5mL of DMSO, then adding 0.5 mL of the prepared sample into the first tube, mixing and transfering 0.5 mL of the solution into the second tube, then continue same process till the last tube.

2. Control preparation :

    (a) Four different controls in this test were ascorbic acid (1mg), quercetin (1mg), gallic acid (1mg) and BHT (2mg). All controls were dissolved in 1mL of DMSO individually and in separate eppendorf tubes, then all should be diluted 5 times, same as samples. The use of controls helps to check if the procedure is done correctly or not. The blank (negative control) is DMSO.

    (b) Ascorbic acid stock were diluted ten times (×10) before preparing standard solution which means mixing 100µL of prepared ascorbic acid stock with 900 µL of distilled water (DW). The blank (negative control) is distilled water.

3. Standard preparation:

    Ascorbic acid was used as standard in this test. After initial ten times dilution in DW, different A-F standard dilutions were prepared based on DPPH kit protocol, providing final 0, 5, 10, 15, 20, 2µM/mL concentrations to help plotting the standard curve.

4. Reagent preparation :

   Each 1 mg of DPPH was dissolved in 25mL of ethanol 95% or methanol. The assay was performed as reported by (Gerhauser et al., 2003)

5. Performing the assay :

   5 µL of blank (DMSO), standards, and sample dilutions were put respectively in polystyrene flat-bottom translucent 96-well plate, triplicate; and then 195µL of reagent solution is added in each well and incubated in 37°C in dark place for 20 minutes. The final concentrations after several dilutions are 75 mg/mL, 37.5 mg/mL, 18.75 mg/mL, 9.4 mg/mL, 4.7 mg/mL and 2.35 mg/mL respectively. Eventually, the absorbance is read in 515 nm wavelength by microplate reader (Power wave X340).

6. Calculation:

   The free scavenging activity i.e. percentage inhibitory of DPPH was expressed by the chemical synthesis and calculated through hereunder equation:

   DPPH radical scavenged (%) = (OD blank - OD sample) / (OD blank) X 100 %

3.4.2 FRAP assay

The ferric reducing activity of samples is measured based on reduction of ferric iron to ferrous at low pH (Benzie et al., 1996; Dejian Huang et al., 2005). The FRAP value is obtained by comparing the absorbance change in 593nm between test samples and standards containing known concentration of ferrous ions. The procedure is explained as follow:

1. Sample preparation:

   3 mg of the compound was dissolved in 1 mL DMSO in eppendorf tube.

2. Control preparation:

Ascorbic acid, gallic acid, quercetin, and BHT were used as controls in this test.

(a) 0.001g of Ascorbic acid (vitamin C) is dissolved in 1 mL of distilled water (DW), but as Ascorbic acid is a strong antioxidant, it should be diluted ten times (×10) before use.

(b) 0.001g of other controls was dissolved in 1mL DMSO.

3. Standard preparation :

The amount of 0.0278 g of FeSO4 was dissolved in 10 mL DW, and then standard stocks are prepared in eppendorf tubes (A-F) as mentioned in test instruction.

4. Reagent preparation :

The final reagent was prepared in three steps as described in Table 3.3. After preparing each reagent separately, they should be mixed in a dark container to avoid color change in light. The end solution is in orange color.

5. Performing the assay:

10 µL of each prepared standards, controls and samples were put in polystyrene flat-bottom translucent 96-well plate, triplicate; and then 300 µL of the prepared reagent was added to each well and the absorbance is read at minute 0 and minute 4 in 593 nm wavelength by micro-plate reader (Power wave X340).

Table 3.3: Reagent preparation for FRAP test.

Acetate buffer	*Total Volume : 25 mL*

300 mM/L pH 3.6	$NaAc,C_2H_3NaO_2.3H_2O$	Acetic acid glacial, $C_2H_4O_2$	DW
	0.0775 g	0.4 mL	24.6 mL
10 mM/L **TPTZ** in 40mM HCl	*Total Volume : 2.5 mL*		
	TPTZ	HCl 1M	DW
	0.00781 g	0.1 mL	2.4 mL
$FeCl_3.6H_2O$ 20 mM	*Total volume : 2.5 mL*		
	$FeCl_3$	DW	
	0.0135 g	2.5 mL	

[Note: TPTZ= Fe(III)-2,4,6-tripiridyl-s-triazine].

3.5 Methodology of acute toxicity

3.5.1 Experimental animals

Thirty Sprague Dawley adult male and female rats were obtained from the experimental animal house, Faculty of Medicine, University of Malaya with Ethic No. PM 27/07/2014 MR (R) for acute toxicity operation.

The animals were fasted overnight (food but not water) prior dosing; this helps elimination of food inside the gastrointestinal tract and better absorption of the testing compound. All animals were fed orally with vehicle (10% Tween-20) once only. Animals were divided into three groups (five male and five female rats in each group), labeled as control, low dose and high dose groups. Animals were fasting overnight. Single oral administration of 500mg/kg and 1000mg/kg compound was executed on treatment groups respectively. The control group was treated with 10%-Tween 20. Clinical and toxicological symptoms were observed during 30 minute, 2, 4, 24, and 48 hours after treatment. Any following behavioral changes and mortality were also recorded for a period of two weeks. The survived animals were sacrificed on the 15th day. Hematological, serum biochemical, and

liver and kidney histological parameters were determined following standard methods. The aim of acute toxicity test is to find LD_{50} and make a decision on safe dose of compound that is not toxic to animals during treatment. The confident dose will be chosen according to the result of the test.

3.5.2 Procedure design

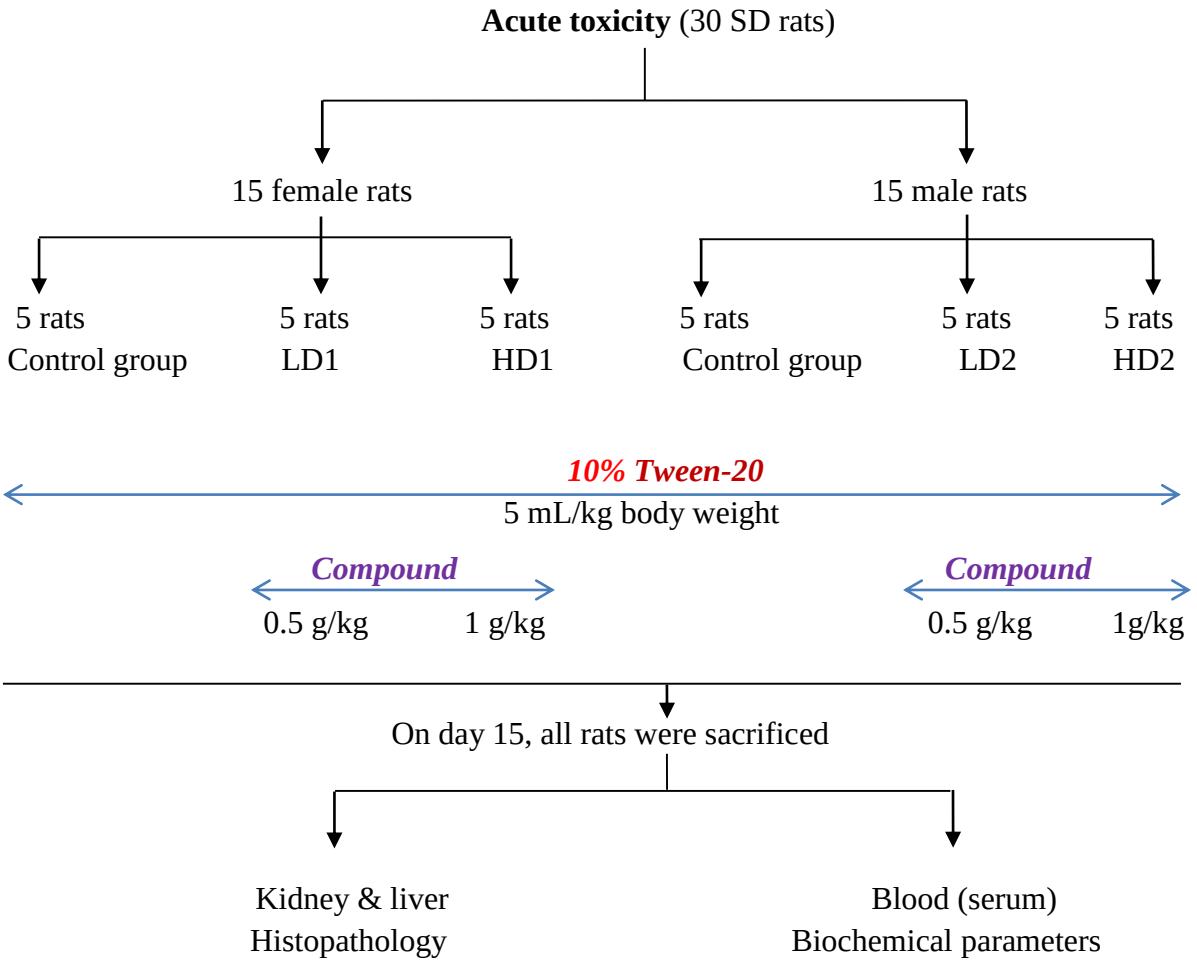

Figure 3.1: Acute toxicity procedure. LD1: Low dose, group1; LD2: Low dose, group2; HD1: High dose, group1; HD2: High dose, group2.

3.6 Applied materials and usage methodology for wound healing

3.6.1 Lignocaine HCl (2%, 100 mg/5 mL)

The local anesthesia was purchased from experimental animal house, Faculty of Medicine, University Malaya. 1 mL Lignocaine was injected subcutaneously.

3.6.2 Intrasite gel

Intrasite gel was purchased from University Malaya, Medical Center Pharmacy, and used as positive control. Intrasite gel (a trade mark for Smith and Nephew Ltd) is a colorless transparent hydrogel containing CMC (carboxymethyl cellulose) polymer (2.3%) that is able to donate fluid to the wound together with propylene glycol (20%) which has bacteriostatic properties. Moreover, propylene glycol acts as a moisturizer and preservative, which prevents the gel from drying out, and improves handling. Intrasite gel is applicable in deep and shallow wounds, promoting efficient autolytic debridement, providing moist environment for tissue and rehydrate necrotic tissue (Williams, 1994). It is also designed for wounds that are granulating and epithelializing; besides, the optimum moist wound management environment during the later stages of wound closure is provided. The non-adherent and harmless features of Intrasite gel makes it ideal to be used in every stage of the wound management process (Williams, 1994).

Therefore, 0.2 mL of Intrasite gel was applied topically twice daily to the wound of Group 2 rats (positive control group).

3.6.3 Experimental animals

Sprague Dawley adult male rats were obtained from the experimental animal house, Faculty of Medicine, University of Malaya with Ethic No. PM 27/07/2014 MR (R) to perform wound healing experiment.

Twenty rats were divided randomly into 4 groups of 5 rats. Each rat averagely weighted between 250-300 g and was housed separately (one rat per cage). The animals were maintained on standard pellet diet and tap water. All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health.

3.6.4 Experimentally induced wounds

The animals were anesthetized with Ketamine (5 mg/kg, IP injection) and Xylazine (2 mg/kg, IP injection). The skin shaved by electrical clipper, disinfected with 70% alcohol, and 1 mL of Lignocaine HCl was injected. An area of uniform 2.00 cm in diameter (circular area = 3.14 cm^2) on the nape of the dorsal neck of all rats was marked using a round seal and the excision induced. The skin kept constant during the procedure to avoid incision of the muscle layer and tension of skin. The wound area was measured immediately by placing a transparent tracing paper over the wound and tracing it out. The tracing paper then placed on milimetric graph sheet, the squares were counted, and the area was recorded as described by (Abdulla et al., 2011).

3.6.5 Topical application of compound

Four groups of animals were treated as described below:

Group 1: negative control group: wounds dressed with 0.2 mL of CMC 5%, twice daily.

Group 2: drug reference (positive control) group: wounds dressed with 0.2 mL as a thin layer of Intrasite gel twice daily until wound healed.

Group 3: treated group – low dose (25 mg/kg body weight) compound: wounds dressed with 0.2 mL of compound dissolved in CMC 5% twice daily until wounds healed.

Group 4: treated group - high dose (50 mg/kg body weight) compound: wounds dressed with 0.2 mL of compound dissolved in CMC 5% twice daily until wounds healed.

The estimation of wound closure area of each animal were assessed by tracing the wound on days 1, 5, and 10 post-wounding surgery using transparency paper and a permanent marker under light ketamine and xylazine anesthesia as described by (Nayak et al., 2006). The recorded wound areas were measured using a graph paper. The percentage of wound healing on selected days was determined (Abdulla et al., 2011). All animals were sacrificed on day 10 post surgery.

3.6.6 Wound closure estimate

The rate of healing in wounded area was assessed through tracing wounds manually by transparent and millimetric paper sheets and permanent marker under general stupor (Ketamine and Xylazine) on day 1^{st}, 5^{th}, and 10^{th} after inducing wounds.

3.6.7 Histological evaluation of wound tissues

The skin specimen of wounded area was removed on day 10 post surgery after sacrificing animals, and fixed in 10% buffered formalin and processed by paraffin tissue processing machine. Sections with 5 µ (micron) thickness were taken and the smears provided were stained by H&E staining method (refer to appendix A) to facilitate microscopic observation.

3.6.8 Tissue homogenation

The removed wounded skin of each rat weighed separately and put in PBS (specimen weight × 10 = PBS volume), and tissue homogenate prepared with aid of homogenizer machine (Wisemax, HG-15A). The suspension then centrifuged in 4°C, 3500 rpm, 20 minutes, and the supernatant removed and kept in eppendorf tubes in -80°C to be used later.

3.7 Methodology of antioxidant measurement in granulation tissue

3.7.1 SOD

The Sod activity is measured according to the method of Beyer and Fridovich (Beyer Jr et al., 1987). The metalloenzyme SOD catalyzes the dismutation of superoxide anion to hydrogen peroxide and molecular oxygen, which forms a decisive antioxidant defense mechanism in cells (Malmström et al., 1975). The enzyme activity is evaluated through measurement of inhibition capacity of the photochemical reduction of nitroblue tetrazolium (NBT). Presence of SOD forbids the reduction of NBT due to formation of peroxide. The reaction is shown in Figure 3.2.

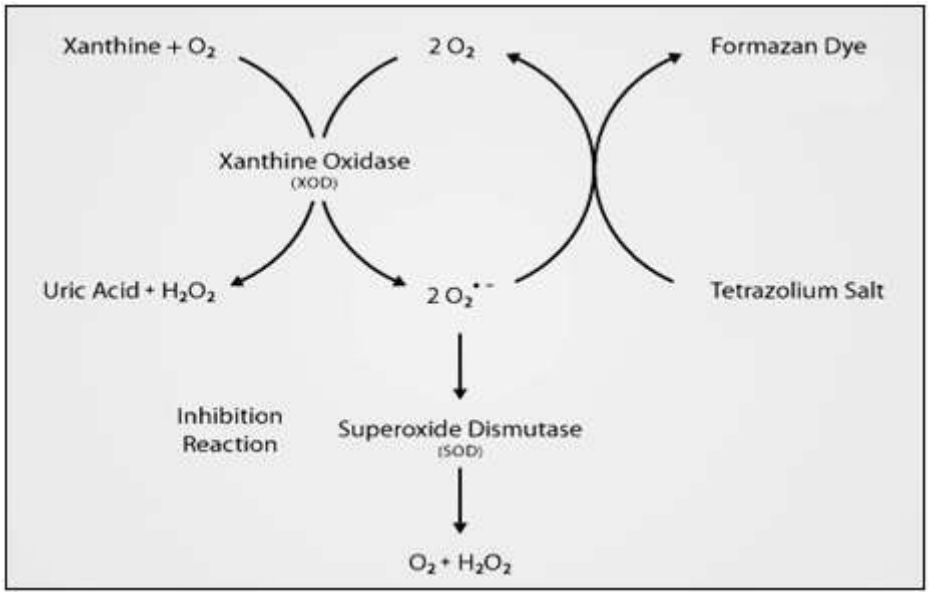

Figure 3.2: Schematic Superoxide dismutase reaction (the figure is retrieved from Cayman SOD assay kit, item No.706002)

Tissue homogenates provided initially were samples of this test. The following procedure and materials used were based on instruction of Cayman chemical company Superoxide dismutase assay kit, item No. 706002:

1. Reagent preparation:

   Different reagents applied in this test are presented in Table 3.4.

Table 3.4: Reagent preparation for SOD test.

Reagents → / ↓	**Radical detector**	**Xanthine oxidase**
Assay buffer (diluted 10× with HPLC grade water)	50 µL + 19.95 mL	
Sample buffer (diluted 10× with HPLC grade water)		50 µL + 1.95 mL

2. Standard preparation:

   After dilution of 20 µL of SOD standard with 1.98 mL of diluted sample buffer to provide SOD stock, the solution is distributed to separate eppendorf tubes (A-G) preparing test standards, following the kit instruction booklet.

3. Performing the assay:

   The total volume of 230 µL containing 200 µL of diluted Radical detector, 10 µL of samples/standards, and 20 µL of diluted Xanthine oxidase is distributed in each micro well.

   The reaction initiates by addition of Xanthine oxidase. The covered plate is incubated on a shaker at room temperature for 20 minutes. Absorbance was measured at 440 nm by microplate reader, power wave X340.

4. Calculation:

   The linear regression of the standard curve was used to calculate SOD activity. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical.

3.7.2 Lipid peroxidation measurement in wound tissue homogenate

Lipid peroxidation level was estimated by determination of malondialdehyde (MDA) using the thiobarbituric acid reactive substances (TBARS) test. The tissue homogenates prepared and stored previously are used as test samples. The materials used and test procedure were based on Cayman chemical company TBARS assay kit, item No.10009055:

1. Color reagent preparation:
   (a) Concentrated TBA acetic acid was diluted (5×) with HPLC-grade water.
   (b) TBA sodium hydroxide (NaOH) solution was diluted (10×) with HPLC-grade water.

(c) The final color reagent was prepared by mixing 50 mL of diluted TBA-acetic acid, 50 mL of diluted TBA-NaOH, and addition of 530 mg Thiobarbituric acid (TBA) in powder form.

2. Colorimetric standard preparation:

(a) TBA malondialdehyde stock solution of 125 µM was made by dilution of 250µL of MDA standard with 750µL water.

(b) Numbers of eight standards (A-H) were prepared in next step, following the kit instruction.

3. Performing the assay:

100 µL of sample/prepared standard, 100 µL of SDS solution, and 4mL of prepared color reagent were mixed gently in an appropriate 5 mL vial, capped properly, and put in boiling water (95-100°C) for 1 hour. Putting vials in ice bath immediately after boiling for 10 minutes helps to stop the reaction. Centrifugation was done at 1600×g, 4°C, 10 minutes. 150µL of each vial's content, in duplicate, was loaded into transparent 96-well plate (colorimetric version) and the absorbance was read at 530-540 nm.

3.8 Statistical analysis

All values are reported as mean ± S.E.M. The statistical significance of differences among groups was assessed using one-way ANOVA in SPSS, that compares the means between the groups we are interested. Values of $p < 0.05$ were considered significant (Park, 2003).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.0 RESULTS AND INTERPRETATIONS

4.1 Antibacterial activity and MIC

The disk diffusion test to assess compound's antibacterial potential proved the resistance of all selected bacteria to the prepared concentrations of the compound as no growth inhibition zone was observed after overnight incubation of the bacterial cultures. As shown in Figure 4.1, all chosen gram-positive and gram-negative bacteria offered resistance to the tested compound (Cl-indole-nicotinic). The results indicate that the chosen concentration of the compound was not effective on growth inhibition of the selected bacteria and the bacteria showed resistant to the compound. The bacteriostatic activity of the compound might be achieved through trial and error, considering other different types of bacteria and other concentrations of the compound.

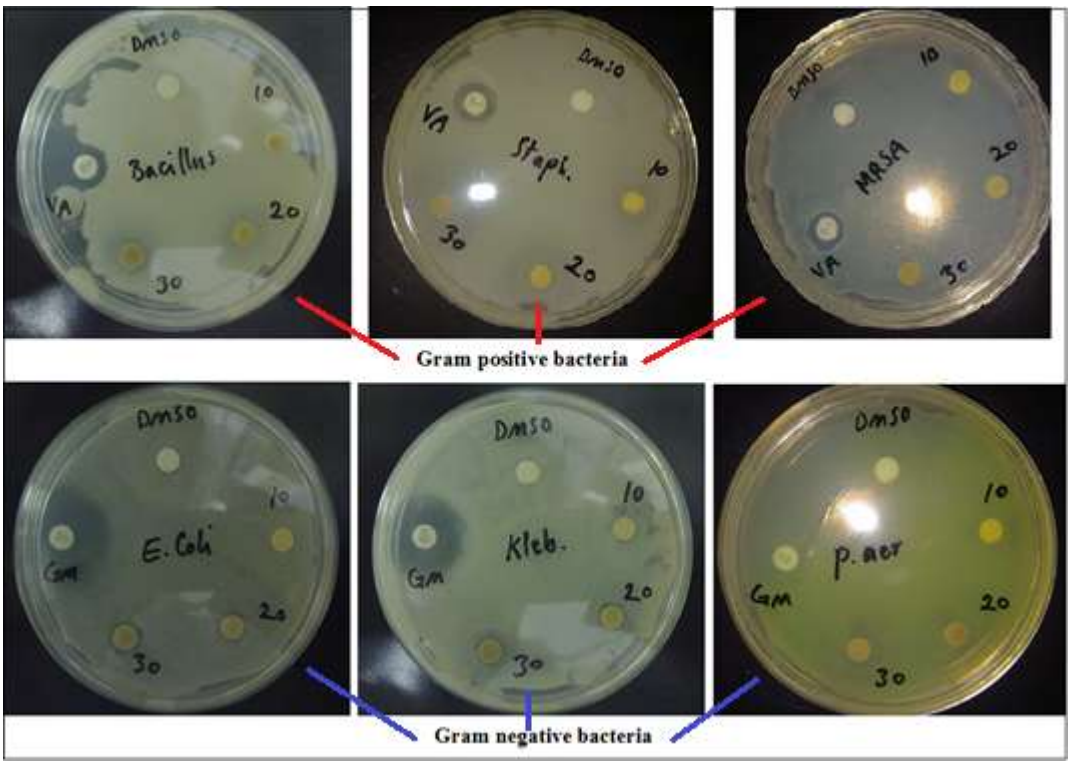

Figure 4.1: Antibacterial potential of the compound assessed against selected Gram positive (*Bacillus subtilis*, *Staphylococcus epidermidis, and Staphylococcus aureus* (MRSA)) and Gram negative bacteria (,*Escherichia coli, Klebsiella pneumoniae, and Pseudomonas*

aeruginosa) (left to right order). [Note: VA= vancomycin, GM= gentamicin, DMSO= Dimethyl Sulfoxide ,(10,20,30)= 10 µL, 20 µL. 30 µL].

The results indicate that the chosen concentration of the compound was not effective on growth inhibition of the selected bacteria and the bacteria showed resistant to the compound. The bacteriostatic activity of the compound might be achieved through trial and error, considering other different types of bacteria and other concentrations of the compound.

4.2 MIC value

There was no MIC value outcome in this test as the growth of each selected bacteria were observed in all concentrations provided by two-fold microwell dilution in this test.

4.3 Antioxidant activity *in vitro*

4.3.1 DPPH

Detecting antioxidant potential of Cl-indole-nicotinic via DPPH assays led us to the results declaring that higher concentrations of this compound show higher inhibition percent of DPPH radical, which means higher antioxidant activity (refer to Appendix D). The compound's IC_{50}estimation is very close to that of BHT (Table 4.1).

Table 4.1: IC_{50} value determined by DPPH assay.

Comparing IC_{50} value between control groups and the compound		**IC_{50}** (µg/mL)
Controls	Gallic acid	1.17
	Ascorbic acid	2.57
	Quercetin	4.22
	BHT (butylated hydroxytoluene)	50
Sample	Compound: Cl-indole-nicotinic	57.48

4.3.2 FRAP

The FRAP value derived from the data collected from FRAP assay demonstrates that Cl-indole-nicotinic compound has almost one third the activity of the control factor BHT (Figure 4.2 and Appendix D). The results declare that Cl-indole-nicotinic is weak in term of antioxidant potency and showed a very low antioxidant activity through FRAP test.

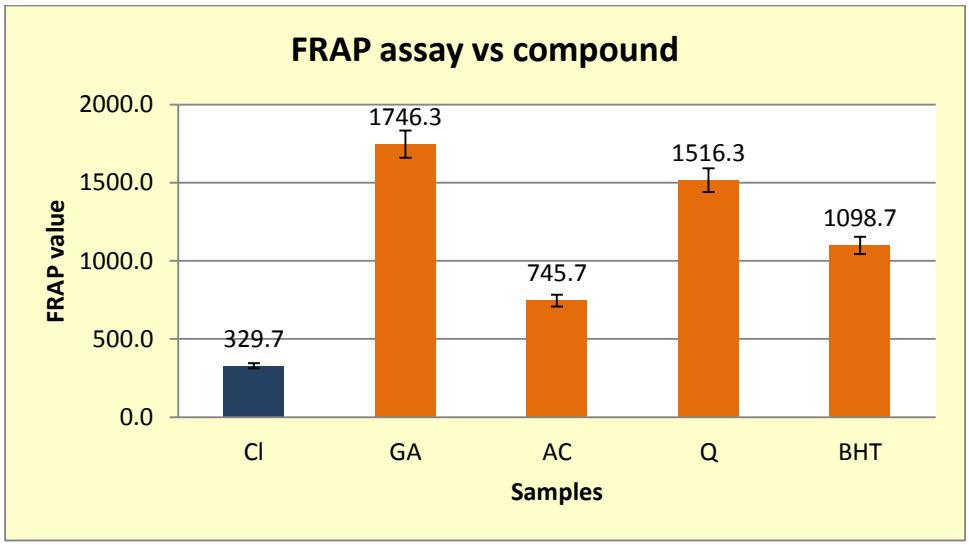

Figure 4.2: Cl-indole-nicotinic's antioxidant activity determined by FRAP assay comparing to selected controls. Values are expressed as mean ± S.E.M. [Note: GA= gallic acid; AC= ascorbic acid; Q= quercetin; BHT= butylated hydroxytoluene, and Cl= Cl-indole-nicotinic].

4.4 Acute toxicity study

Animals treated with Cl-indole-nicotinic at dosage of 500 mg/kg and 1000 mg/kg survived during the period of two weeks and showed no physiological symptomatic distress. Moreover, clinical observations, serum biochemistry, and histopathology data collected after sacrificing them, proved the lack of any significant differences between control and treated groups. Results were compared between same genders (male to male, female to female) as females are expected to be more sensitive in case of acute toxicity. Absence of any detected drug related toxicity supports to conclude that oral administration of Cl-indole-nicotinic in

rats is secure even at the highest dose investigation and Cl-indole-nicotinic is safe to be used as drug base after further investigations in future. The oral median lethal dose (LD_{50}) for the male and female rats was greater than 1 g/kg body weight.

4.4.1 Body weight observation

In two-week duration of treatment, animals' body weight was registered and no anorexia, weakness, and weight loss recorded. Overall, male rats gained more body weight comparing to female rats, which is a normal outcome between genders. Treated animals showed natural growth and no significant result achieved (Table 4.2).

Table 4.2: Physiological result and mean body weight of rats in acute toxicity test. Values are expressed as mean ± S.E.M. There is no significant difference among the groups.

Groups	***No. of rats***	***Body weight***	
		Day 1	*Day 14*
Control, female	5	170.6 ± 2.09	178.23 ± 1.87
LD1	5	169.4 ± 6.50	178.43 ± 4.71
HD1	5	177.6 ± 4.11	186.77 ± 3.33
Control, male	5	187 ± 1.84	196.3 ± 1.5
LD2	5	198 ± 4.98	211.2 ± 4.55
HD2	5	177.6 ± 1.94	187.64 ± 1.46
Occurrence of mortality	Observation within 24 hrs, 48 hrs & 14days • All the rats survived during the two-week period. • No physiological symptom and behavioral change observed.		

[Note: LD1= low dose (0.5g/kg), female; LD2= low dose, male; HD1= high dose (1g/kg), female; HD2= high dose, male. Control groups= just vehicle].

4.4.2 Histological evaluation in acute toxicity test

The study of animals' inner body organs' structure (liver and kidney) after processing and H & E staining expressed no structural difference and no phenotypically transformation comparing between control group and test groups. All the cells appeared healthy and no tissue malignancy in term of cells observed (Figures 4.3 and 4.4), which can be considered as non-toxic nature of Cl-indole-nicotinic in the selected concentrations (0.5g/kg and 1g/kg).

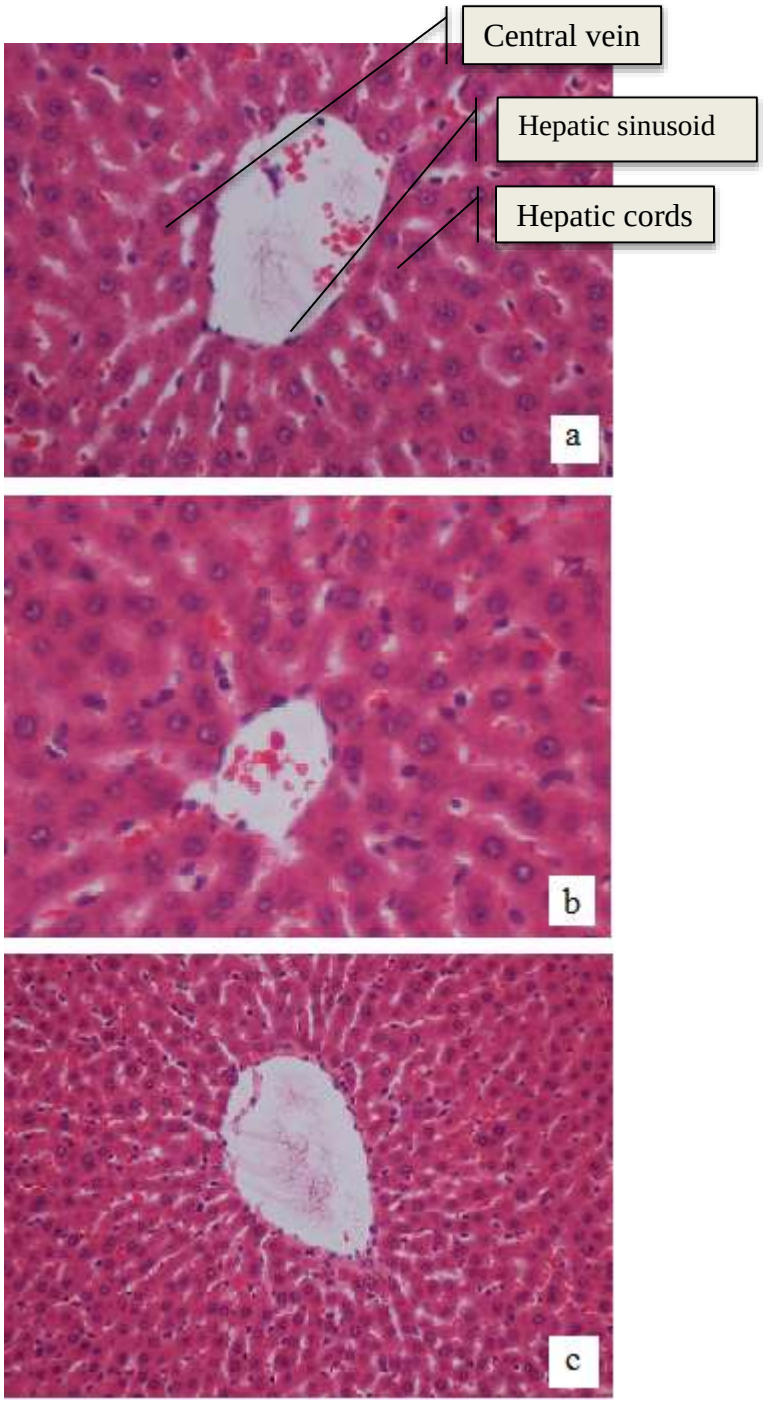

Figure 4.3: Structural appearance of histological sections of liver: (a) vehicle (10% Tween-20); (b) LD (0.5g/kg); (c) HD (1g/kg). (H&E stain 20x)

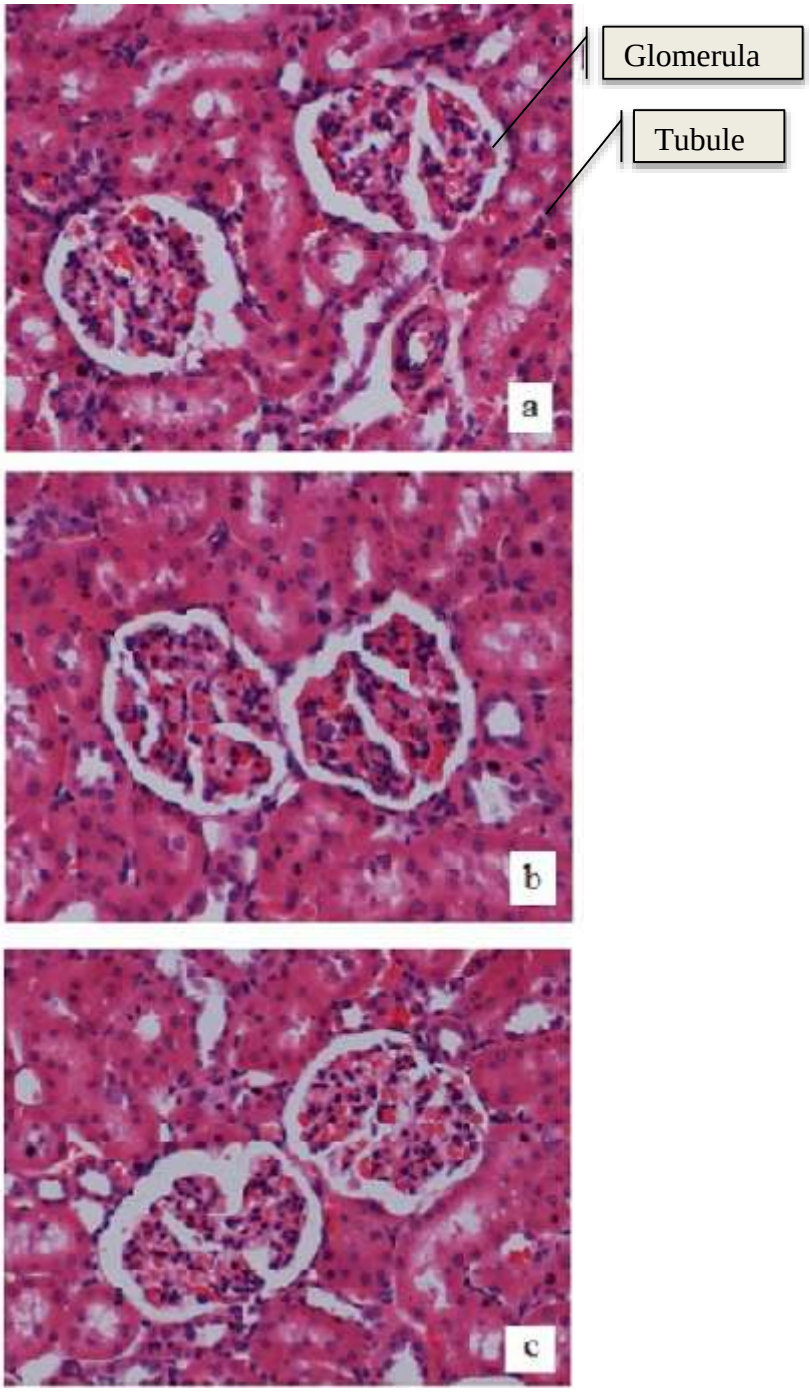

Figure 4.4: Structural appearance of histological sections of kidney: (a) vehicle (10% Tween-20); (b) LD (0.5g/kg); (c) HD (1g/kg). (H&E stain 20x)

4.4.3 Serum biochemistry

Biochemical parameters of renal function, liver function, and lipid profile that talk about state of kidney, liver, and level of lipids in serum respectively were detected in treated animals' blood serum after administration of the compound and sacrificing them, because any fluctuation in level of these parameters in the body can consider to be related to a disease. Renal function test can assess the excretory function of the kidney and talks about kidney's role in renal physiology. Liver function test also gives information about healthy operation of the liver.

The results were compared between control group (just treated with vehicle: 10% Tween-20) and experiment groups (LD= 0.5g/kg, HD= 1g/kg). Comparison between groups is done separately between genders (males to males and females to females). As the results show, although some results are higher than normal range, but no significant result obtained based on the data presented in Tables 4.3 (A,B,C), which is analyzed through One-way ANOVA in SPSS. High results are because the error will be less when the sample size is great, ant it effects the result; this happened to some of the parameters such as Urea; by the way, detecting no side effect in rats and recording no mortality as well as having no significant differences among the result, makes the achievements trustable and worthy.

Table 4.3 A: Renal function of acute toxicity test: LD1: low dose (0.5g/kg), female; LD2: low dose, male; HD1: high dose (1g/kg), female; HD2: high dose, male. Control groups: just vehicle (10%-Tween 20). Values are expressed as mean ± S.E.M. There is no significant difference between the groups.

	Sodium	***Potassium***	***Chloride***	***Carbon Dioxide***	***Anion Gap***	***Urea***
Control, female	141.66 ± 1.01	4.13 ± 0.76	105.88 ± 1.11	19.37 ± 0.61	20.8 ± 0.55	7.93 ± 0.31
Control, male	139.00 ± 0.7	4.5 ± 0.28	110.10 ± 1.46	22.82 ± 1.2	23.03 ± 0.99	7.20 ± 0.86
LD1	134 ± 2.95	5.34 ± 0.87	96.4 ± 1.6	18.22 ± 1.91	25.4 ± 1.16	9.74 ± 0.24
LD2	143± 3.56	4.98 ± 0.16	103.2 ± 3.52	22.1 ± 0.87	22.6 ± 0.81	6.44 ± 0.36
HD1	135.6 ± 1.91	5.38 ± 0.43	98.6 ± 1.69	20.36 ± 0.91	22.2 ± 0.86	8.68 ± 1.56
HD2	139 ± 1.26	5.7 ± 1.07	99.8 ± 2.13	18.74 ± 2.05	26.2 ± 1.88	6.32 ± 0.42
Normal Range & Unit	136-145 mmol/L	3.6-5.2 mmol/L	100-108 mmol/L	21.0-30.0 mmol/L	10-20 mmol/L	2.5-6.4 mmol/L

Table 4.3 B: Liver function of acute toxicity test: LD1: low dose (0.5g/kg), female; LD2: low dose, male; HD1: high dose (1g/kg), female; HD2: high dose, male. Control groups: just vehicle (10%-Tween 20). Values are expressed as mean ± S.E.M. There is no significant difference between the groups.

	Liver Function Test				
	Total Protein	**Albumin**	**Globulin**	**Total Bilirubin**	**Conjugated Bilirubin**
Control, female	58.33 ± 1.15	10.17 ± 0.27	48.07 ± 1.2	2.00 ± 0.00	1.05 ± 0.00
Control, male	59.23 ±1.15	9.13 ± 0.93	47.5 ± 0.89	2.17 ± 0.16	1.07 ± 0.53
LD1	60.4 ± 2.15	12.4 ± 0.67	48 ± 1.51	2 ± 0.0	1.2 ± 0.2
LD2	60.6 ± 1.66	13.2 ± 0.48	47.4 ± 1.12	2 ± 0.0	1 ± 0.0
HD1	64.2 ± 2.47	14.2 ±0.58	50 ± 2.07	2.6 ± 0.4	1 ± 0.0
HD2	59.6 ± 1.5	13.4 ± 0.4	46.2 ± 1.15	2.2 ± 0.2	1 ± 0.0
Normal Range	64-82	35-50	23-35	3-17	0-3
& Unit	g/L	g/L	g/L	Umol/L	Umol/L

Table 4.3 B, continue... Values are expressed as mean ± S.E.M. There is no significant difference between the groups.

	Liver Function Test			
	ALP	**Alanine Amino-transferase**	**AST**	**G-Glutamyl Transferase**
Control, female	124.83 ± 9.10	44.27±1.64	169.33 ± 6.14	3.67 ± 0.56
Control, male	153.10 ± 12.14	50.23 ± 1.52	166.5 ± 5.01	3.17 ± 0.05
LD1	180.8 ± 108.47	53.8 ± 2.47	275.4 ± 29.18	3 ± 0.0
LD2	180.6 ± 7.97	75.2 ± 22.61	184.4 ± 25.42	3 ± 0.0
HD1	110 ± 47.45	52.6 ± 7.14	266.8 ± 26.28	7 ± 4.0
HD2	227.2 ± 5.22	72.6 ± 5.92	274.4 ± 21.51	3.6 ± 0.4
Normal Range & Unit	50-136 IU/L	30-65 IU/L	15-37 IU/L	15-85 IU/L

Table 4.3 C: Lipid profile of acute toxicity test: LD1: Low dose (0.5g/kg), female; LD2: Low dose, male; HD1: High dose (1g/kg), female; HD2: High dose, male. Control groups: just vehicle (10%-Tween 20). Values are expressed as mean ± S.E.M. There is no significant difference between the groups.

	Lipid Profile			
	Triglyceride	***Total Cholestrol***	***HDL Cholestrol***	***LDL Cholestrol***
Control, female	0.4 ± 0.0	1.28 ± 0.1	2 ± 0.0	0.14 ± 0.0
Control, male	0.42 ± 0.05	1.3 ± 0.04	1.83 ± 0.66	0.14 ± 0.02
LD1	0.48 ± 0.73	1.3 ± 0.0	1.12 ± 1.99	0.1 ± 0.05
LD2	0.4 ± 0.44	1.3 ± 0.0	1.16 ± 0.03	0.01 ± 0.0
HD1	0.44 ± 0.24	1.3 ± 0.0	0.92 ± 0.02	0.15 ± 0.0
HD2	0.48 ± 0.37	1.34 ± 0.04	1.21 ± 0.06	0.15 ±0.01
Normal Range & Unit	<1.7 mmol/L	<5.2 mmol/L	>1.10 mmol/L	<2.59 mmol/L

4.5 Wound healing experiment

Considerable dermal healing indications observed in wound of animals treated with Cl-indole-nicotinic and reference control (Intrasite gel) comparing to negative control receiving just vehicle. The wounded area in day 5 after inducing the excision was obviously smaller and the resultant lesion area on day 10 was little consequently (Figure 4.5).

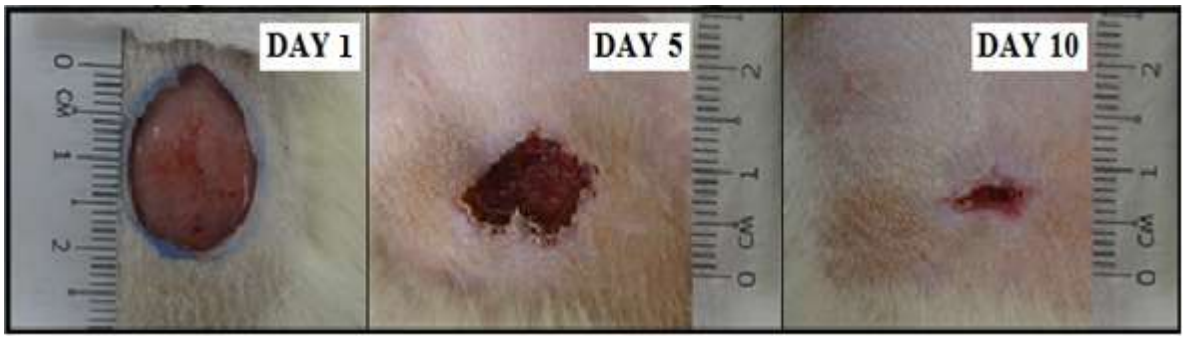

Figure 4.5: Gross necropsy of the wounded skin on day 1, 5, and 10.

In case of body weight, the results express the normal body growth of animals during treatment which indicates on healthiness of the animals after compound application and their normal physiological behavior of the body (Table 4.4)

Table 4.4: Mean body weight of male rats' dermal incisions after treatment. Values are expressed as mean ± S.E.M. There is no significant difference between the groups.

Type of wound dressing	Body weight (g)		
	Day 1	Day 5	Day 10
CMC	289 ± 6.87	307± 9.24	310 ± 9.5
Intrasite gel	301 ± 14.45	315 ± 19.2	321 ± 18.32
Cl-indole-nicotinic (25 mg/kg)	342.8 ± 13.23	336.2 ± 16.65	352.8 ± 17.47
Cl-indole-nicotinic (50 mg/kg)	293.5 ± 37.51	307 ± 35.19	320.25 ± 31.87

[Note: CMC= Carboxymethylcellulose]

4.5.1 Wound closure rate

The rate of wound healing was monitored during 10 days treatment and the percentage of healing and wound closure were recorded and calculated as shown in Figure 4.6 and Table 4.5. Results show that the total closure rate of wounds dressed with Cl-indole-nicotinic is comparable to that of Intrasite gel which is a commercial drug for skin problems. The upward trend of healing which resulted in more than 90 percent closure rate supports the efficacy of the compound for recovering wounds.

Table 4.5: Percentage of wound closure rate in male rats' dermal incisions after treatment. Values are expressed as mean ± S.E.M. There is no significant difference between the groups.

Animal groups	**No. of animals**	**Type of wound dressing**	**wound closure after 10 days (%)**
1	5	CMC	56.5 ± 1.13
2	5	Intrasite gel	89.24 ± 0.53
3	5	Cl-indole-nicotinic (25 mg/kg)	91.12 ± 2.62
4	5	Cl-indole-nicotinic (50 mg/kg)	96.1 ± 0.34

[Note: CMC= Carboxymethylcellulose]

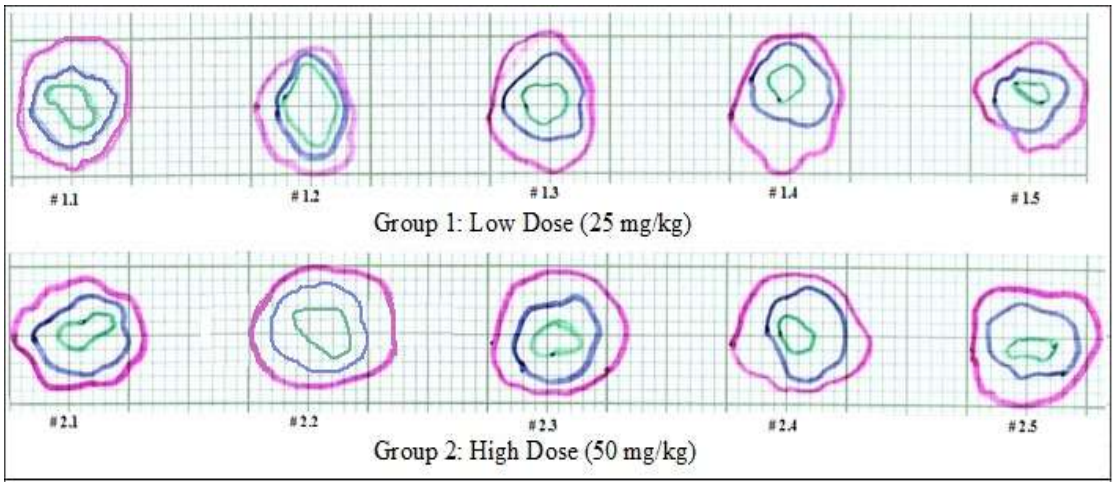

Figure 4.6: The manually recorded rate of wound healing in experimental rats on day1st, 5th, and 10th post-surgery.

4.5.2 Histology of wounds after treatment

Histopathological examination of the wounds treated with Cl-indole-nicotinic showed less scar area and the thin outer layer of epidermis as a proof of new skin regeneration and wound recovery (Figures 4.7 and 4.8). The incomplete healing area is observed in wound of rats treated by CMC (negative control group); wounds dressed with intrasite gel recovered more, however Cl-indole-nicotinic was the most effective in healing wounds comparing among the groups. The granulation tissue in healed wound contained few inflammatory cells, and more collagen and proliferating blood vessels comparing to negative control group (Figures 4.9 and 4.10).

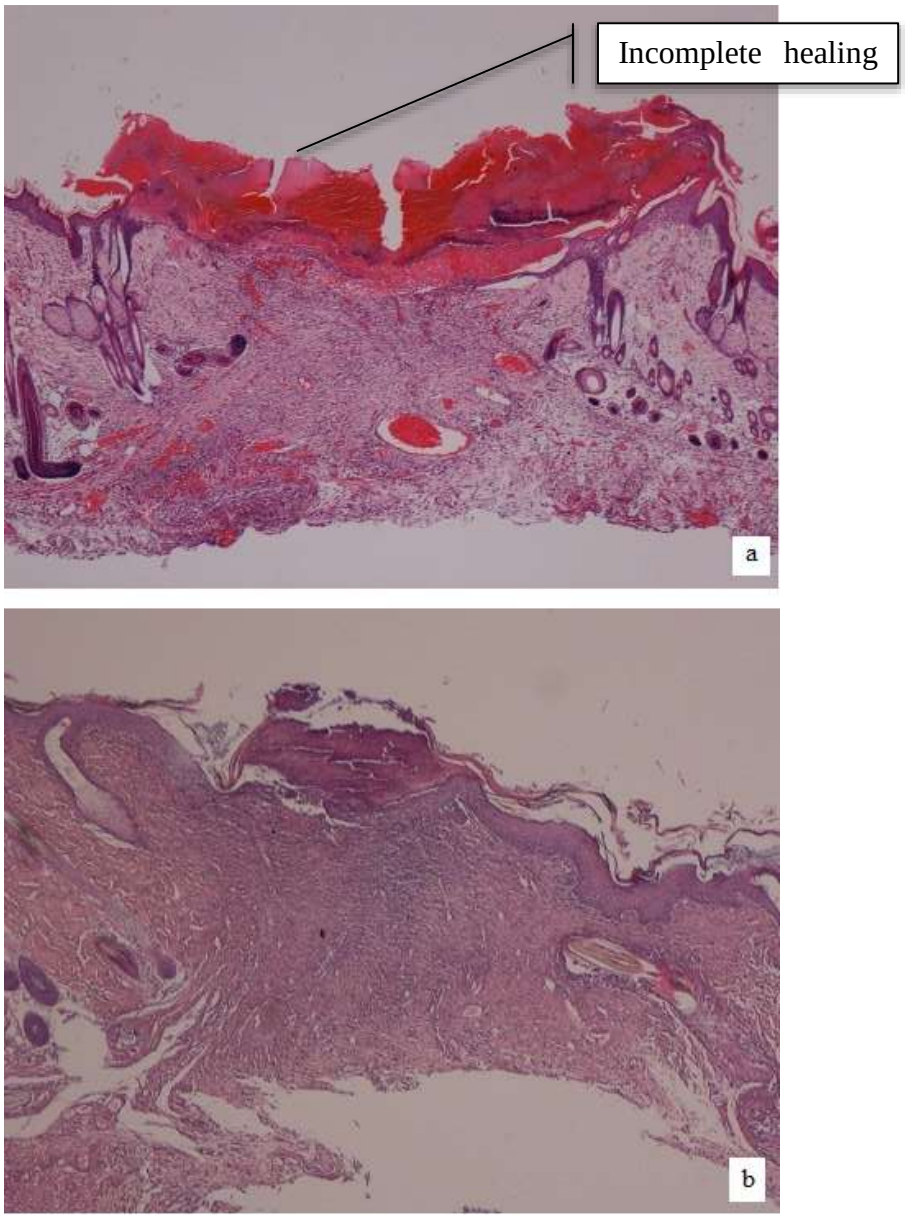

Figure 4.7: Histological section of wounds dressed with 0.2 mL CMC (a) and Intrasite gel (b) on day 10 post surgery showing incomplete wound closure (H & E stains 20x).

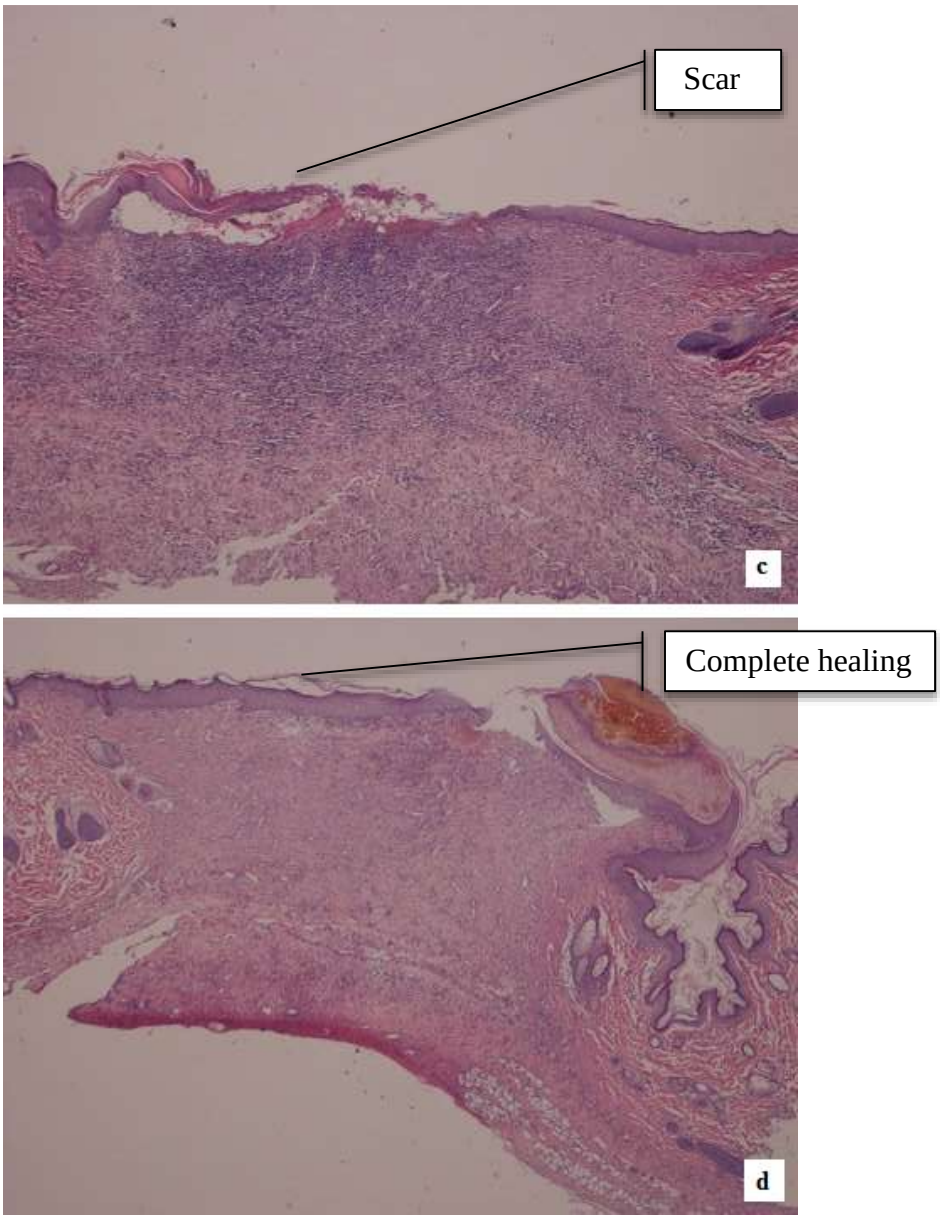

Figure 4.8: Histological section of wounds dressed with 0.2 mL Cl-indole-nicotinic (25mg/kg) (c), and Cl-indole-nicotinic (50mg/kg) (d), on day 10 post surgary showing effective wound healin activity of the compound. (H & E stains 20x).

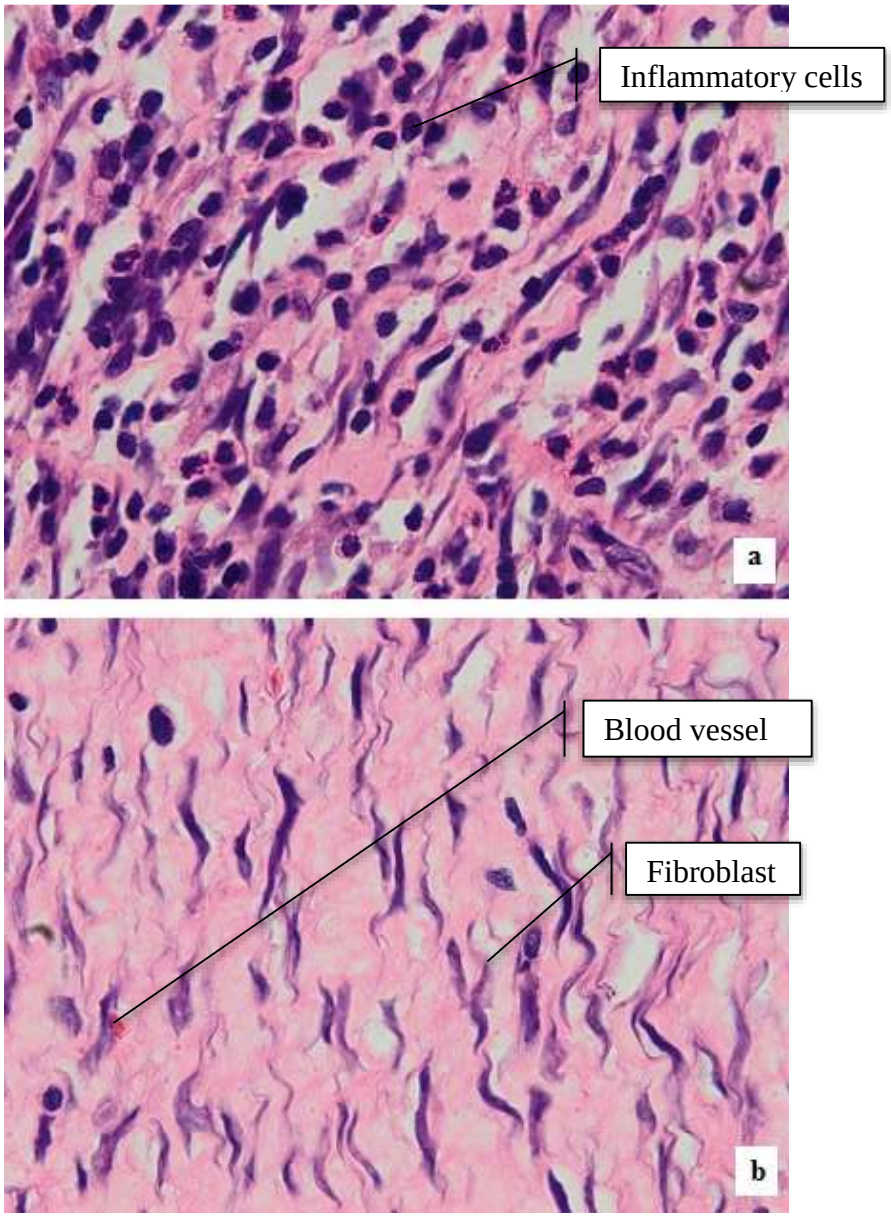

Figure 4.9: Histological sections of wounds dresse with 0.2 mL CMC (a), and Intrasite gel (b), on day 10 post surgary. (H & E stain 100x).

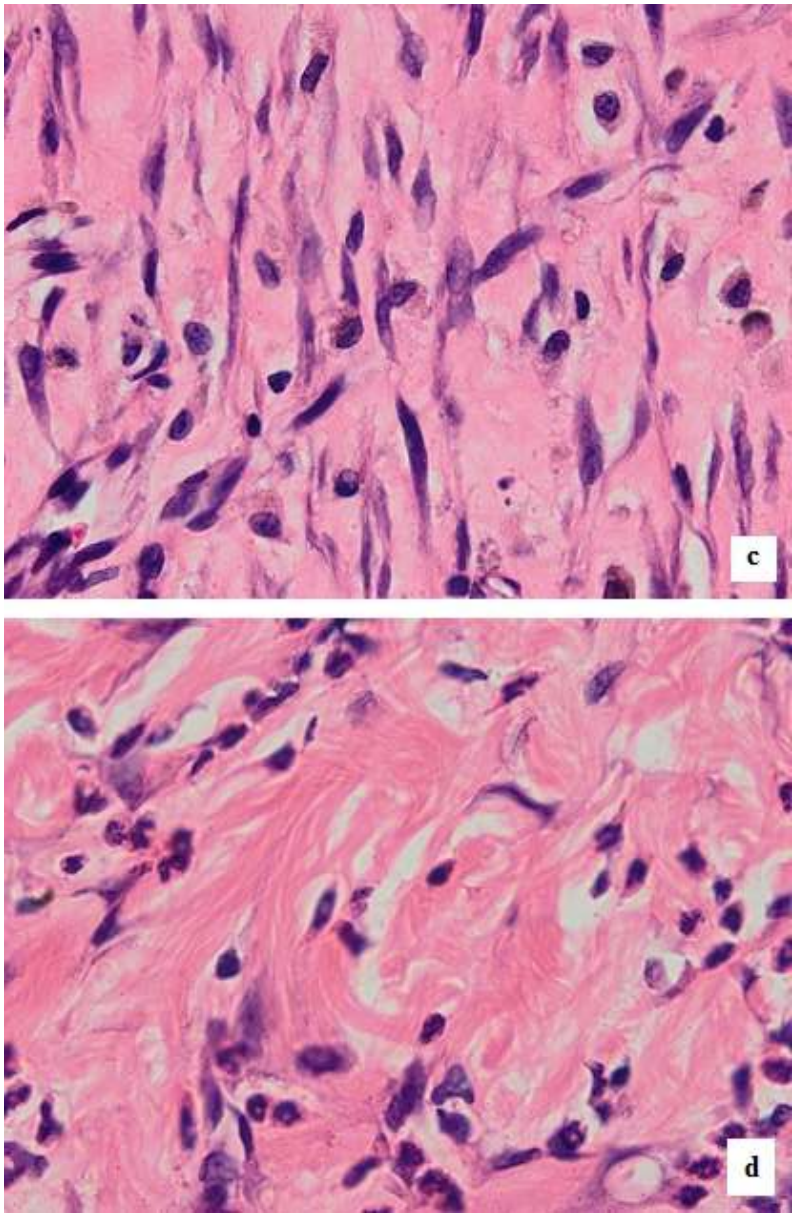

Figure 4.10: Histological sections of wounds dresse with 0.2 mL Cl-indole-nicotinic (25mg/kg) (c), and Cl-indole-nicotinic (50mg/kg) (d), on day 10 post surgary. (H & E stain 100x).

4.6 Antioxidant activity determination in tissue homogenate

4.6.1 Superoxide dismutase

Detecting antioxidant activity in tissue homogenates led us to the results that show both low dosage and high dosage of Cl-indole-nicotinic tested in this experiment has antioxidant potency and higher concentration of Cl-indole-nicotinic have higher activity against free radicals (Figure 4.11 and Appendix D). Although bothe selevted concentrations of the compound show low antioxidant activity, but it is still achieved that Cl-indole-nicotinic's potency is dose-dependant and shows higher activity in higher concentrations. As superoxide dismutase enzyme is present in dermis and epidermis and accelerates the rate of healing as a scavenger, and wounded skin of the rats dressed with the compound shows SOD activity, it introduces the possibility of use of Cl-indole-nicotinic as a drug in case of the wounds. No significant result obtained among groups in this test.

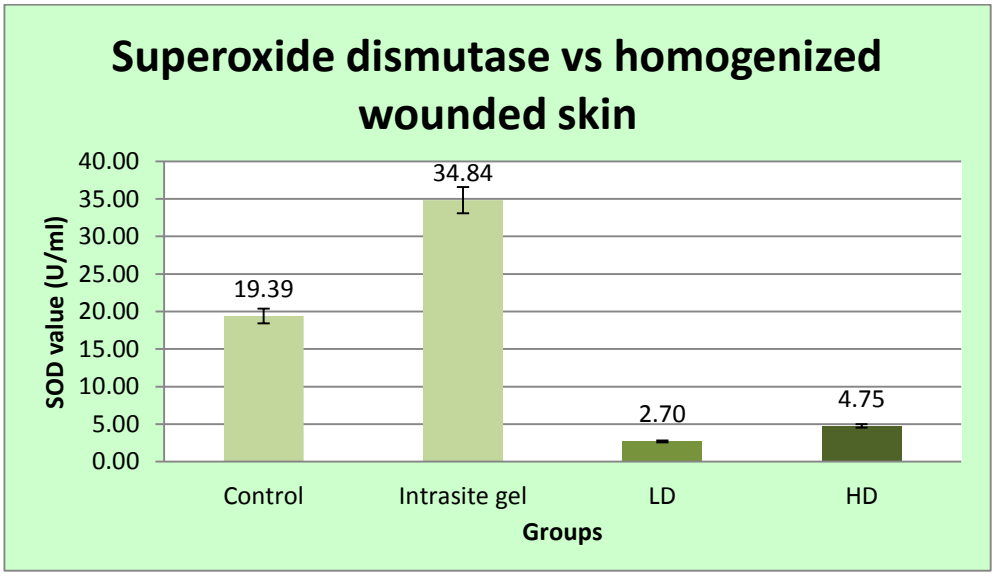

Figure 4.11: SOD value of homogenized skin in treated rats. Group1 = negative control = no treatment; Group2 = reference control = Intrasite gel; Group3 = low dose (25mg/kg); Group4 = high dose (50mg/kg). Values are expressed as mean ± S.E.M. There is no significant difference among the groups.

4.6.2 Lipid peroxidation (MDA)

Detecting MDA value in tissue homogenates led us to achieve close levels of MDA in wound of rats treated by Cl-indole-nicotinic (in both concentrations tested in this experiment) comparing to those treated with Intrasite gel (reference standard control group). As marker of Lipid peroxidation in tissue, the least MDA level indicated the most Lipid peroxidation inhibition activity. The results obtained declare that the inhibitory activity of Cl-indole-nicotinic is comparable to intrasite gel, and the wound of rats treated with low dose compound (25mg/kg) showed the lowest lipid peroxidation activity among the groups in this test (Figure 4.11 and Appendix D). It should be noticed that the MDA value is expected to decrease along with increasing the concentration of the compound, which is not achieved between selected concentrations of the compound in this test. No significant result obtained in the test.

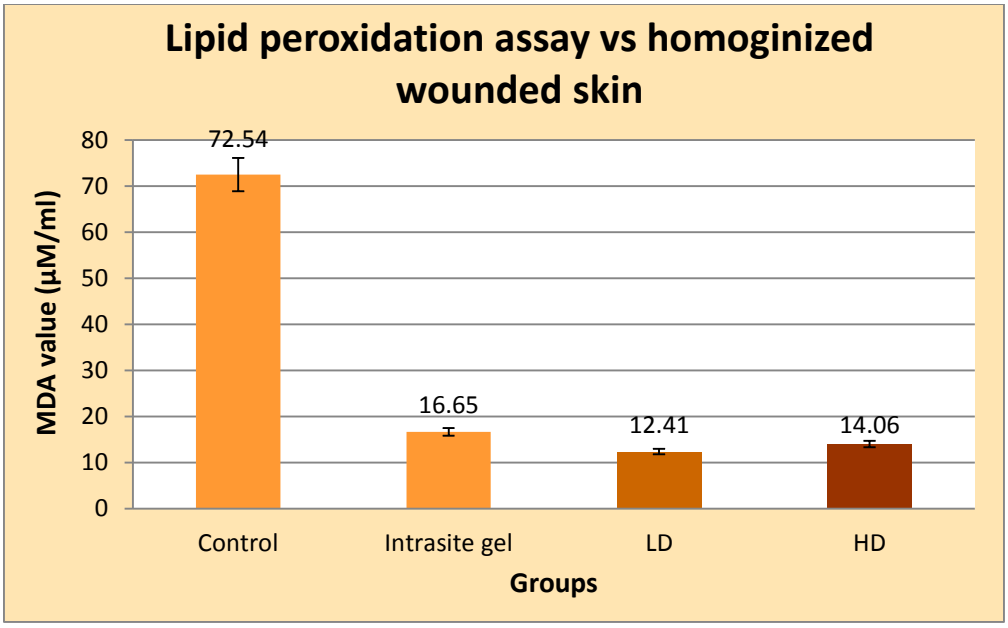

Figure 4.12: MDA value of homogenized skin in treated rats. Group1 = negative control = just vehicle (CMC); Group2 = reference control = Intrasite gel; Group3 = low dose (25mg/kg); Group4 = high dose (50mg/kg). Values are expressed as mean ± S.E.M. There is no significant difference among the groups.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS

AND SUGGESTIONS FOR FUTURE WORK

5.1 DISCUSSION, CONCLUSION AND SUGGESTIONS FOR FUTURE WORK

5.2 Discussing the current study

Wound healing is the occurrence of several complex interactive processes that helps the injured connective tissue resynthesizes and regains its strength and functionality. The healing process starts by triggering the clotting cascade in blood vessels to prevent both bleeding and entry of microorganisms, followed by local vasodilation caused by prostaglandins and consumption of debrides through phagocytic activity of macrophages. This inflammation stage continues to produce immature collagen by fibroblasts and fibrins, formation of extracellular matrix and granulation tissue, and augmentation of epithelial cells. The wound contraction begins in this proliferative phase. The mature and organized collagen that refunds the tissue strength appears gradually after few months during the next stage, remodeling. The end-point in healing process is to transform the wound area to the possible smallest scar (Enoch et al., 2008; Richardson, 2004; Robson et al., 2001).

Based on the results achieved in this study, the topical application of the chemical compound, Cl-indole-nicotinic, precipitated the rate of healing in the wounded area notably. Histological status of the wounds after repair states the proper regeneration of the skin layers, collagen fibers, and angiogenesis.

The epidermis layer is the uppermost layer of the healthy skin; a barrier made up of stratified squamous epithelial cells with an undulating appearance, which covers the two other sub layers of the skin, dermis and subcutis (New Zealand Dermatological Society, 2010). The presence of numerous capillaries that invade the granulation tissue is important in transmitting growth factors (Martin, 1997). Proper angiogenesis supports the healing process by circulating nutrients and oxygen in the injured area.

Microscopic observation of the healed area in tested animals showed the premiere formation of epidermis layer as a sign of healing and regeneration of the skin layer, comparing to control groups. It should be noticed that during this study, no sign of restlessness, itching and scratching of the wounds was observed in the animals treated by Cl-indole-nicotinic and dermal lesions were monitored regarding infection. Any infected animal was excluded from the test.

The rate of healing of the skin is promoted in presence of antioxidants. Antioxidants are known as free radical scavengers that support the healing process by reducing the harmful effects of free radicals in the injured site and prevent impaired healing (Kumar et al., 2007; Rajasekaran et al., 2004). Superoxide dismutase, which is an antioxidant enzyme present in dermis and epidermis and malondialdehyde as biomarker of Lipid peroxidation, were of value in this study.

The evaluation of superoxide dismutase in tissue homogenates prepared from animals' wound area after topical application of 25mg/kg and 50mg/kg concentrations of Cl-indole-nicotinic indicates the slight antioxidant activity of the compound, increasing by concentration addition. It is considering that Cl-indole-nicotinic shows more antioxidant activity in higher concentration, as for the higher dosage of the compound used in this study, SOD activity was greater compering to the lower dosage of the compound.So, although the low SOD activity of Cl-indole-nicotinic, its SOD potency is dose dependant.

The MDA value obtained in this study declares that Cl-indole-nicotinic is able to inhibit lipid peroxidation process. In case of malondialdehyde detection, obtaining less MDA value for higher concentrations is favorable, where in this study application of both concentrations of the compound show close results.

In vitro evaluation of antioxidant activity was done through DPPH and FRAP tests. DPPH gives the total antioxidant capacity of the compound (Prakash A., 2001) and FRAP test measures the antioxidant activity of the compound by reducing ferric iron to ferrous (Benzie et al., 1996). Based on the IC_{50} value of Cl-indole-nicotinic which was obtained from DPPH test results, and FRAP test outcomes, the *in vitro* antioxidant activity of the compound in selected concentration (3mg/mL DMSO) is very close to BHT which is known as an antioxidant agent. Although Cl-indole-nicotinic possesses weak antioxidant activity, it might be stronger in higher concentrations.

While talking about wounds, the type of dressing and cleanness of the injured site should be considered. Many opportunistic bacteria can infect the blemished skin and interfere in the healing process. To investigate the potential of Cl-indole-nicotinic as an antibacterial factor to be used as a dressing material, six different bacteria were selected: *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Bacillus subtilis.* As most of them are parts of skin flora, the probability of causing infection during skin injury was considered in selecting these bacteria. Among all six, *Bacillus subtilis* is the spore forming bacterium, more resistant to bacteriostatic and bactericide agents; *Pseudomonas aeruginosa* is a versatile bacterium enable to infect damaged tissues; *Klebsiella pneumoniae* is known as important pathogen that can cause disease in surgical wounds; *Staphylococcus epidermidis* infects the devices placed in the body by surgery; *Staphylococcus aureus* is a common cause of skin infections where the emergence of antibiotic-resistant forms of pathogenic *S.aureus* (e.g. MRSA) is an epidemic problem in clinical medicine; and *E.coli* as one of the most famous bacteria that applies in many researches as a reference bacterium.

The elected bacteria are also known as infectious opportunistic bacteria, getting resistant to routine drugs used nowadays. Therefore, it is reasonable to discover and substitute new and efficient drugs having antimicrobial potency.

Determining the activity of Cl-indole Nicotinic against selected bacteria through disk diffusion method as described by Lalitha 2005, illustrates the privation of antibacterial potency of the compound in selected concentrations (50mg compound/mL DMSO) against the selected bacteria in this study.

The minimum inhibitory concentration of Cl-indole Nicotinic was not achieved in this study as the prepared concentrations of the compound didn't inhibit the growth of selected bacteria after two-fold dilution.

The orally administration safety of Cl-indole-nicotinic was carried out by evaluating toxicological effects of the compound in experimental rats to choose the safe dose in human body. The application of unsafe and toxic material may cause biochemical lesions, organ malfunctioning (Walum, 1998), haematological and gastrointestinal noxious effects.

The selected median lethal dose (LD50) of Cl-indole-nicotinic in this study was 1mg/kg, suggesting the non-toxic nature of the compound and safe dose for oral administration. The compound considers non-toxic if no mortality and adverse effects seen during two-week running the acute toxicity test (International Union of Pure and Applied Chemistry (Compendium of Chemical Terminology), 2012).

The biochemical values of blood serum were in normal range and comparable to control animals, and histological status of the kidney and liver showed normal and healthy structure of the tissue and no significant changes comparing to control groups were observed. The total acute toxicity results indicates the favorable nature of Cl-indole-nicotinic for oral intake and its non-poisonous property.

5.3 Conclusion

Based on the total outcomes of current study, it is concluded that Cl-indole Nicotinic (Copper (II) bis [N'-5-Chloro-1H-indol-3-yl methyl nicotinic hydrazide]) is effective in healing wounds as the wounds dressed with this compound showed significant rate of closure and healing and smaller scar area comparing to negative control group. Moreover, the antioxidant activity of the compound *in vitro* and *in vivo* is dose dependent and might reveal better results at higher doses.

The compound does not induce any malignancy to tissues and cells histologically and is considered as non-toxic material.

5.4 Future work suggestions

Better results might be achieved by choosing other concentrations of Cl-indole-nicotinic compound in the same or different tests by trial and error. Running the tests using higher number of animals in each group might lead to better consequences.

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PUBLICATION

One manuscript has been submitted to “Molecules” journal.

APPENDICES

Appendix A: Hematoxylin and Eosin Staining Protocol

Modified from UNC Histopathology Protocol
Updated 9/12/03 – Reggie Hill

1. **Deparaffinize** in Xylene I and II and III (each 5 minutes)

2. **Rehydrate**

    a) EtOH 100% (twice, each 3 minutes)

    b) EtOH 95% (twice, each 3 minutes)

    c) EtOH 70% (3 minutes)

3. Rinse in distilled water (5 minutes)

4. Stain in hematoxylin (6 minutes)

    (Filter before each use to remove oxidized particles)

5. Rinse in running tap water (20 minutes)

6. **Decolorize** in acid alcohol (1 second)

    (Can go up to 3 seconds, Longer =Lighter / Discard after each use)

7. Rinse well in tap water (5 minutes)

8. Immerse in Lithium Carbonate (3 Seconds)

    (Longer time = floating tissue)

9. Rinse in tap water (5 minutes)

10. Counterstain in Eosin (15 seconds)

11. **Dehydrate**

    a) EtOH 95 % (twice, each 3 minutes)

    (Discard after each use)

    b) EtOH 100 % (twice, 3 minutes)

12. Clear in Xylene I and II (each 5 minutes)

13. Mount with Cytoseal in fume hood

Preparation:

- **Stock Solutions – Eosin:**

  Stock – 1% aqueous Eosin-Y

  Stock – 1% aqueous Phloxin B

- **Working Solutions – Eosin:**

  100mL stock Eosin

  10 mL stock Phloxin B

  780 mL 95% Ethanol

  4 mL glacial Acetic Acid

- **Working Solution: - Hematoxylin**

  Harris Hematoxylin, Sigma, HHS-32, 1 Liter

- **Working Solution: - Lithium Carbonate 1.36%**

  Lithium Carbonate, 47g

  dH_2O, 3500 mL

- **Working Solution: - 0.25% Acid Alcohol**

  95% Ethanol, 2578 mL

  dH_2O, 950mL

  HCL, 9mL

Appendix B: Definitions

(Alphabetically sorted)

- **Aldehydes:** Compounds RC(=O)H , in which a carbonyl group is bonded to one hydrogen atom and to one R group.
- **BHT:** Butylated Hydroxytoluene ; A crystalline phenolic antioxidant, $C_{15}H_{24}O$, used to preserve fats and oils, especially in foods, cosmetics, and pharmaceuticals; Antioxidant for food, animal feed, petroleum products, synthetic rubbers, plastics, animal and vegetable oils, soap; also an antiskinning agent in paints and inks.
- **CLSI:** Clinical and Laboratory Standards Institute: Formerly NCCLS: An international, interdisciplinary, non-profit, non-governmental organization composed of medical professionals, government, industry, healthcare providers, educators, etc.
- **CMC:** or cellulose gum; A cellulose derivative with carboxymethyl groups (-CH2-COOH) bound to some of the hydroxyl groups of the glucopyranose monomers that make up the cellulose backbone. It is often used as its sodium salt, sodium carboxymethyl cellulose; it is used in the manufacture of processed foods as a stabilizing and emulsifying agent and in medicine as a laxative.
- **DMSO:** Dimethyl sulfoxide; A colorless hygroscopic liquid obtained from lignin, used as a penetrant to convey medications into the tissues.
- **Gallic acid:** 3,4,5-trihydroxybenzoic acid: A white crystalline acid C7H6O5; an astringent-tasting plant compound that is aromatic and water-soluble. It is grouped with phenolic compounds and has antifungal, antiviral, antioxidant properties. It may also have anticancer activities.

- **HPLC:** High performance liquid chromatography: Lab instrumentation; A highly sensitive analytic method in which analytes are placed at high pressure–500-1500 psi in a chromatography column to separate them, allowing highly specific identification.
- **Imines:** Compounds having the structure RN=CR2 (R = H, hydrocarbyl). Thus analogues of aldehydes or ketones, having NR doubly bonded to carbon; aldimines have the structure RCH=NR, ketimines have the structure R'2C=NR (R' ≠ H). Imines include azomethines and Schiff bases. Imine is used as a suffix in systematic nomenclature to denote the C=NH group excluding the carbon atom.
- **IP injection:** Intra-peritoneal injection: is the injection of a substance into the peritoneum (body cavity). IP injection is more often applied to animals than to humans. In animals, IP injection is used predominantly in veterinary medicine and animal testing for the administration of systemic drugs and fluids due to the ease of administration compared with other parenteral methods.
- **Ketones:** Compounds in which a carbonyl group is bonded to two carbon atoms R2C=O (neither R may be H).
- **MRSA:** Methicillin-resistant *Staphylococcus aureus:* a bacterium responsible for several difficult-to-treat infections in humans. MRSA is any strain of *Staphylococcus aureus* that has developed, through the process of natural selection, resistance to beta-lactam antibiotics, which include the penicillins (methicillin, dicloxacillin, nafcillin, oxacillin, etc.) and the cephalosporins. MRSA is especially troublesome in hospitals, prisons, schools, and nursing homes, where patients with open wounds, invasive devices, and weakened immune systems are at greater risk of infection than the general public.
- **MDCK cells:** Madin Darby Canine kidney cells; a polarized immortalized epithelial cell line.

- **PBS:** Phosphate buffer saline: A handy buffer to have around, especially for biological applications.
- **Quercetin:** A yellow powdered crystalline compound produced synthetically or occurring as a glycoside in the rind and bark of numerous plants, used medicinally to treat abnormal capillary fragility; a bioflavonoid (or flavonoid), which is a type pigment found in almost all herbs, fruits, and vegetables; one of the most powerful and effective herbal anti-inflammatory, and antioxidant supplements on the market today.

Appendix C: Data analyzed by SPSS/ One way ANOVA in acute toxicity test

Descriptive (Renal function)

		No.	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Lower Bound		
SODIUM	LDF	5	134.8000	6.61060	2.95635	126.5919	143.0081	124.00	142.00
	LDM	5	143.0000	7.96869	3.56371	133.1056	152.8944	133.00	155.00
	HDF	5	135.6000	4.27785	1.91311	130.2883	140.9117	131.00	140.00
	HDM	5	139.0000	2.82843	1.26491	135.4880	142.5120	136.00	142.00
	TOTAL	20	138.1000	6.25679	1.39906	135.1717	141.0283	124.00	155.00
CHLORIDE	LDF	5	96.4000	3.57771	1.60000	91.9577	100.8423	92.00	102.00
	LDM	5	103.2000	7.88670	3.52704	93.4074	112.9926	93.00	115.00
	HDF	5	98.6000	3.78153	1.69115	93.9046	103.2954	94.00	103.00
	HDM	5	99.8000	4.76445	2.13073	93.8842	105.7158	95.00	107.00
	TOTAL	20	99.5000	5.47242	1.22367	96.9388	102.0612	92.00	115.00
POTASSIUM	LDF	5	5.3400	1.96672	.87955	2.8980	7.7820	4.00	8.80
	LDM	5	4.9800	.36332	.16248	4.5289	5.4311	4.50	5.50
	HDF	5	5.3800	.96281	.43058	4.1845	6.5755	4.50	6.90
	HDM	5	5.7000	2.40624	1.07610	2.7123	8.6877	4.50	10.00
	TOTAL	20	5.3500	1.52471	.34094	4.6364	6.0636	4.00	10.00
CO2	LDF	5	18.2200	4.28100	1.91452	12.9044	23.5356	10.60	20.60
	LDM	5	22.1000	1.95320	.87350	19.6748	24.5252	20.10	24.80
	HDF	5	20.3600	2.03789	.91137	17.8296	22.8904	17.40	22.90
	HDM	5	18.7400	4.58399	2.05002	13.0482	24.4318	11.80	23.10
	TOTAL	20	19.8550	3.51904	.78688	18.2080	21.5020	10.60	24.80

Descriptive (Renal function, continue)

		No.	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Lower Bound		
ANION GAP	LDF	5	25.4000	2.60768	1.16619	22.1621	28.6379	24.00	30.00
	LDM	5	22.6000	1.81659	.81240	20.3444	24.8556	20.00	25.00
	HDF	5	22.2000	1.92354	.86023	19.8116	24.5884	20.00	25.00
	HDM	5	26.2000	4.20714	1.88149	20.9761	31.4239	22.00	33.00
	TOTAL	20	24.1000	3.12713	.69925	22.6365	25.5635	20.00	33.00
UREA	LDF	5	9.7400	.54129	.24207	9.0679	10.4121	9.40	10.70
	LDM	5	6.4400	.78294	.35014	5.4678	7.4122	5.40	7.50
	HDF	5	8.6800	3.49457	1.56282	4.3409	13.0191	4.90	12.40
	HDM	5	6.3200	.95760	.42825	5.1310	7.5090	5.20	7.30
	TOTAL	20	7.7950	2.28300	.51049	6.7265	8.8635	4.90	12.40
CREATININ	LDF	5	38.0000	6.89202	3.08221	29.4424	46.5576	26.00	43.00
	LDM	5	29.8000	3.70135	1.65529	25.2042	34.3958	27.00	36.00
	HDF	5	48.0000	14.88288	6.65582	29.5205	66.4795	32.00	71.00
	HDM	5	35.0000	9.00000	4.02492	23.8250	46.1750	22.00	46.00
	TOTAL	20	37.7000	11.08389	2.47843	32.5126	42.8874	22.00	71.00

Descriptive (Liver function)

		No.	Mean	Std. Deviation	Std. Error	95% Confidence Interval for		Minimum	Maximum
						Lower Bound	Lower Bound		
TOTAL PROTEIN	LDF	5	60.4000	4.82701	2.15870	54.4065	66.3935	53.00	66.00
	LDM	5	60.6000	2.60768	1.16619	57.3621	63.8379	58.00	64.00
	HDF	5	64.2000	5.54076	2.47790	57.3202	71.0798	55.00	69.00
	HDM	5	59.6000	3.36155	1.50333	55.4261	63.7739	56.00	65.00
	TOTAL	20	61.2000	4.29933	.96136	59.1879	63.2121	53.00	69.00
ALBUMIN	LDF	5	12.4000	1.51658	.67823	10.5169	14.2831	10.00	14.00
	LDM	5	13.2000	1.09545	.48990	11.8398	14.5602	12.00	15.00
	HDF	5	14.2000	1.30384	.58310	12.5811	15.8189	13.00	16.00
	HDM	5	13.4000	.89443	.40000	12.2894	14.5106	13.00	15.00
	TOTAL	20	13.3000	1.30182	.29110	12.6907	13.9093	10.00	16.00
GLOBULIN	LDF	5	48.0000	3.39116	1.51658	43.7893	52.2107	43.00	52.00
	LDM	5	47.4000	2.50998	1.12250	44.2834	50.5166	45.00	51.00
	HDF	5	50.0000	4.63681	2.07364	44.2426	55.7574	42.00	53.00
	HDM	5	46.2000	2.58844	1.15758	42.9860	49.4140	43.00	50.00
	TOTAL	20	47.9000	3.41668	.76399	46.3009	49.4991	42.00	53.00
TOTAL BILIRUBIN	LDF	5	2.0000	.00000	.00000	2.0000	2.0000	2.00	2.00
	LDM	5	2.0000	.00000	.00000	2.0000	2.0000	2.00	2.00
	HDF	5	2.6000	.89443	.40000	1.4894	3.7106	2.00	4.00
	HDM	5	2.2000	.44721	.20000	1.6447	2.7553	2.00	3.00
	TOTAL	20	2.2000	.52315	.11698	1.9552	2.4448	2.00	4.00

Descriptive (Liver function, continue)

		No.	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Lower Bound		
CONJUGATED BILIRUBIN	LDF	5	1.2000	.44721	.20000	.6447	1.7553	1.00	2.00
	LDM	5	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	HDF	5	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	HDM	5	1.0000	.00000	.00000	1.0000	1.0000	1.00	1.00
	TOTAL	20	1.0500	.22361	.05000	.9453	1.1547	1.00	2.00
ALP	LDF	5	180.8000	242.55453	108.47368	-120.3712	481.9712	45.00	612.00
	LDM	5	180.6000	17.84096	7.97872	158.4475	202.7525	156.00	203.00
	HDF	5	110.0000	106.12021	47.45840	-21.7657	241.7657	46.00	297.00
	HDM	5	227.2000	11.69188	5.22877	212.6826	241.7174	216.00	244.00
	TOTAL	20	174.6500	129.22208	28.89493	114.1722	235.1278	45.00	612.00
AST	LDF	5	53.8000	5.54076	2.47790	46.9202	60.6798	49.00	62.00
	LDM	5	75.2000	50.55888	22.61062	12.4229	137.9771	45.00	165.00
	HDF	5	52.6000	15.97811	7.14563	32.7606	72.4394	35.00	77.00
	HDM	5	72.6000	13.24009	5.92115	56.1603	89.0397	61.00	88.00
	TOTAL	20	63.5500	27.36974	6.12006	50.7406	76.3594	35.00	165.00
GGT	LDF	5	275.4000	65.26714	29.18835	194.3601	356.4399	236.00	390.00
	LDM	5	184.4000	56.85772	25.42754	113.8018	254.9982	149.00	285.00
	HDF	5	266.8000	58.76819	26.28193	193.8297	339.7703	200.00	362.00
	HDM	5	274.4000	48.10717	21.51418	214.6671	334.1329	238.00	342.00
	TOTAL	20	250.2500	65.76343	14.70515	219.4718	281.0282	149.00	390.00

Descriptive (Lipid profile)

		No.	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Lower Bound		
TRI-GLYCERIDE	LDF	5	.4800	.16432	.07348	.2760	.6840	.30	.70
	LDM	5	.4000	.10000	.04472	.2758	.5242	.30	.50
	HDF	5	.4400	.05477	.02449	.3720	.5080	.40	.50
	HDM	5	.4800	.08367	.03742	.3761	.5839	.40	.60
	TOTAL	20	.4500	.10513	.02351	.4008	.4992	.30	.70
TOTAL CHOLESTRO	LDF	5	1.3000	.00000	.00000	1.3000	1.3000	1.30	1.30
	LDM	5	1.3000	.00000	.00000	1.3000	1.3000	1.30	1.30
	HDF	5	1.3000	.00000	.00000	1.3000	1.3000	1.30	1.30
	HDM	5	1.3400	.08944	.04000	1.2289	1.4511	1.30	1.50
	TOTAL	20	1.3100	.04472	.01000	1.2891	1.3309	1.30	1.50
HDL	LDF	5	3.0220	4.46456	1.99661	-2.5215	8.5655	.68	11.00
	LDM	5	1.1620	.07530	.03367	1.0685	1.2555	1.09	1.28
	HDF	5	.9260	.05320	.02379	.8599	.9921	.87	1.00
	HDM	5	1.2180	.15106	.06756	1.0304	1.4056	.98	1.34
	TOTAL	20	1.5820	2.22330	.49715	.5415	2.6225	.68	11.00
LDL	LDF	5	.1020	.11584	.05181	-.0418	.2458	.02	.30
	LDM	5	.0180	.00837	.00374	.0076	.0284	.01	.03
	HDF	5	.1520	.07085	.03169	.0640	.2400	.07	.24
	HDM	5	.1540	.03435	.01536	.1113	.1967	.12	.20
	TOTAL	20	.1065	.08573	.01917	.0664	.1466	.01	.30

Descriptive (Body weight)

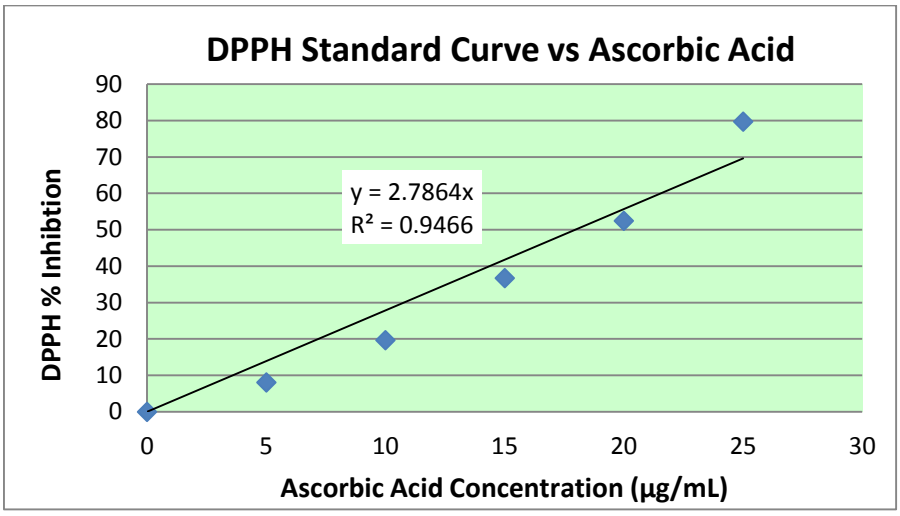
		No.	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Lower Bound		
DAY 1	LD	5	342.8000	29.58378	13.23027	306.0669	379.5331	316.0	390.0
	HD	5	293.5000	75.01778	37.50889	174.1300	412.8700	197.0	379.0
	Total	10	320.8889	56.77245	18.92415	277.2497	364.5281	197.0	390.0
DAY 5	LD	5	336.2000	37.23842	16.65353	289.9624	382.4376	304.0	395.0
	HD	5	307.0000	70.37045	35.18522	195.0249	418.9751	237.0	399.0
	Total	10	323.2222	52.79389	17.59796	282.6412	363.8032	237.0	399.0
DAY 10	LD	5	352.8000	39.06021	17.46826	304.3003	401.2997	311.0	406.0
	HD	5	320.2500	63.73578	31.86789	218.8321	421.6679	271.0	409.0
	Total	10	338.3333	50.79862	16.93287	299.2861	377.3806	271.0	409.0

LD: Low Dose / HD: High Dose

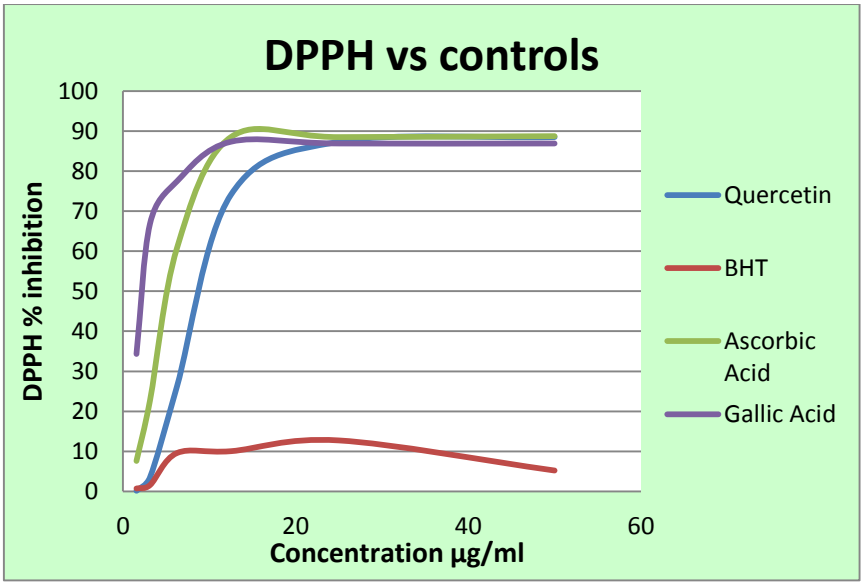
LDF: Low Dose Female / LDM: Low Dose Male

HDF: High Dose Female / HDM: High Dose Male

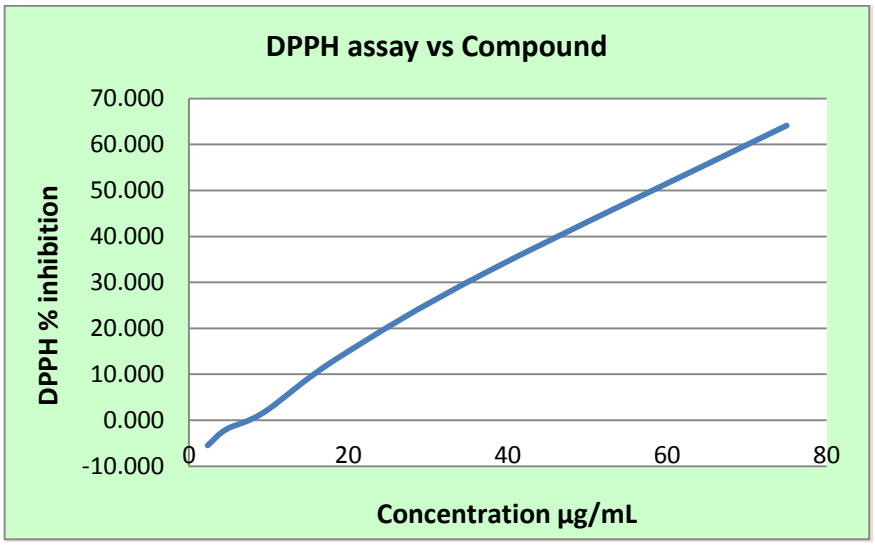
Appendix D: Standard curves



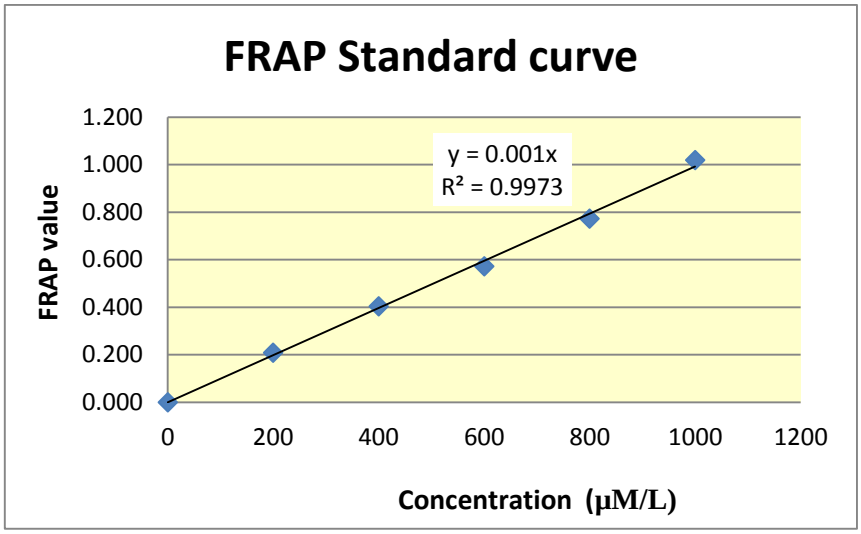
DPPH standard curve. Values are expressed as mean ± S.E.M.



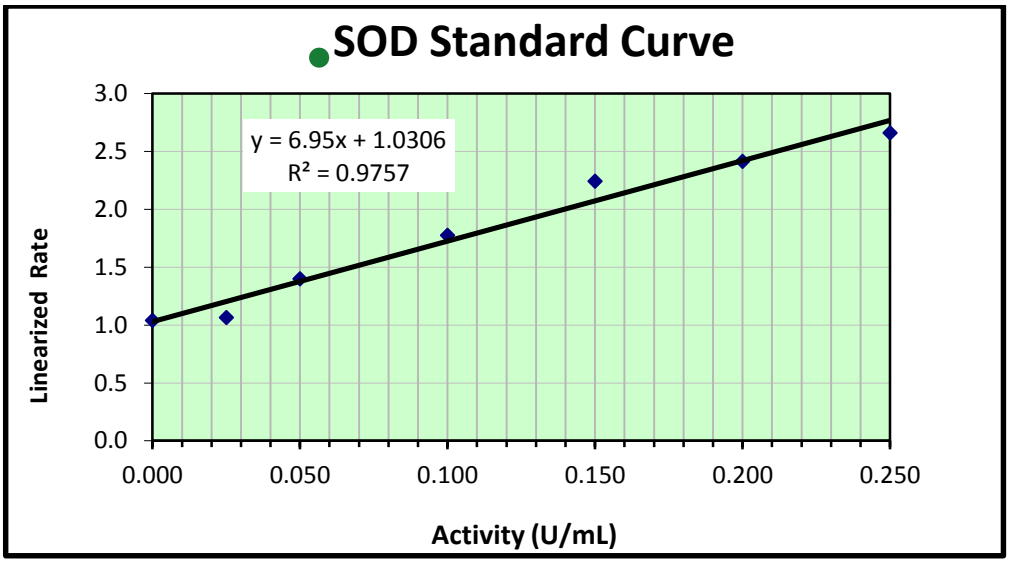
DPPH assay of selected controls. Values are expressed as mean ± S.E.M.



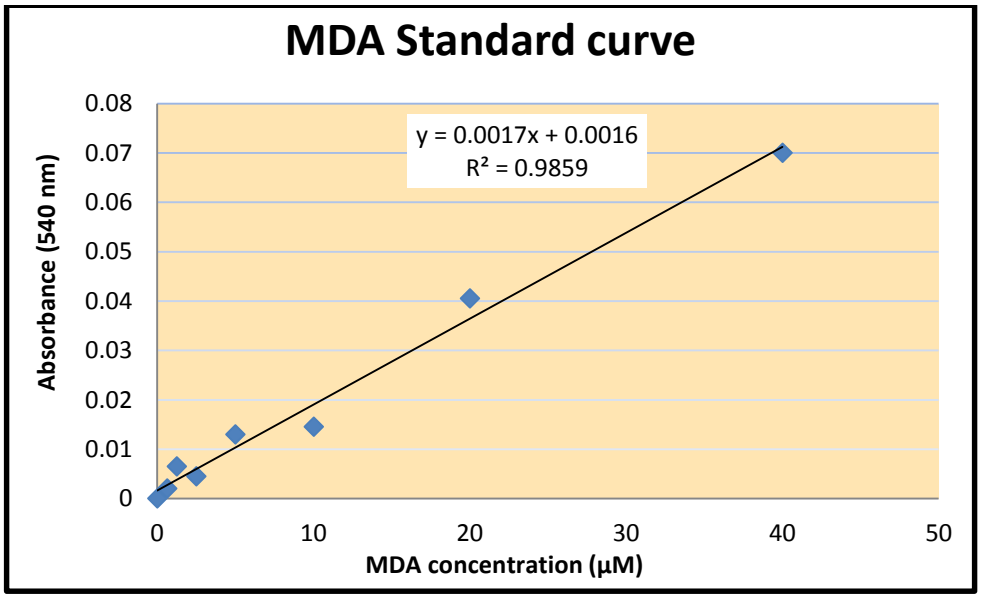
Cl-indole-nicotinic's antioxidant activity determined by DPPH assay. Values are expressed as mean ± S.E.M.



FRAP standard curve. Values are expressed as mean ± S.E.M.



SOD standard curve. Values are expressed as mean ± S.E.M.



MDA standard curve. Values are expressed as mean ± S.E.M.