GASTROPROTECTIVE EFFECT, WOUND HEALING AND ANTI-DIABETIC POTENTIAL OF TWO NOVEL DICHLORO AND DIBROMO SCHIFF BASE DERIVATIVES IN SD RATS

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GASTROPROTECTIVE EFFECT, WOUND HEALING AND ANTI-DIABETIC POTENTIAL OF TWO NOVEL DICHLORO AND DIBROMO SCHIFF BASE DERIVATIVES IN SD RATS

ABSTRACT

Schiff base derivatives have displayed to possess a broad spectrum of pharmaceutical and biological properties. The present study encompasses an *in vitro* and *in vivo* study to evaluate the gastroprotective activity, wound healing potential and anti-diabetic effect of the bromine and chlorine Schiff base derivatives. 2,2'-[1,2-cyclohexanediylbis (nitriloethylidyne)] bis [4-chlorophenol] (CNCP) and 2,2'-[1,2-cyclohexanediylbis (nitriloethylidyne)] bis [4-bromophenol] (CNBP) were synthesized through Schiff base reaction by applying related ketones and diamines as initiators. CNCP and CNBP exhibited strong ferric reducing antioxidant power (FRAP) and possess mild DPPH radical scavenging activity. The safety of these compounds was verified through acute toxicity study exerted no signs of toxicity at 100 and 200 mg/kg. The gastroprotective effect of CNCP and CNBP were investigated against ethanol-induced gastric ulcer in SD rats. The study was performed with normal, ulcer control (5ml/kg of 10% tween 20), treatment group (10 and 20 mg/kg of CNCP and CNBP, respectively) and a reference group (omeperazole, 20 mg/kg). Vast shallow haemorrhagic injury of gastric glandular mucosa was observed in the ulcer group compared to the CNCP and CNBP-treated animals. Histological evaluations showed that these compounds possess stomach epithelial defense effect with an observation of reduction in gastric ulceration, edema and leucocytes penetration of submucosal stratum. Immunostaining of gastroprotective analysis exhibited over-expression of HSP70 protein and down-expression of Bax protein in CNCP and CNBP-treated groups. The gastric protein analysis of treated rats with CNCP and CNBP showed low levels of malondialdehyde (MDA) and high activity of prostaglandin E2 (PGE2), superoxide dismutase (SOD) and catalase (CAT). In the evaluation of wound healing potential, SD rats (male) were subjected into six groups

(n=6); negative control (gum acacia), positive control (intrasite gel), and the treatment group (CNCP and CNBP, respectively). Topical treatment on wounded rats with CNCP and CNBP, showed a significant increase of wound closure percentage compared to the negative control. Histological evaluation of the skin sections showed granulation tissues contained more proliferating fibroblast, collagen deposition, angiogenesis, and less inflammatory cells in CNCP (10 mg/ml) and CNBP (20 mg/ml)-treated groups compared to the normal rats. In the treated groups with CNCP and CNBP, the SOD, CAT, and glutathione peroxidase (GPx) activities were found significantly higher, however, the MDA level was shown to be lower than the negative control. At the molecular level, CNCP (10 mg/ml) and CNBP (20 mg/ml) improved wound healing process via down-regulation of Bax and up-regulation of Hsp70 protein. The anti-diabetic potential of CNCP and CNBP were evaluated in the STZ-NA-induced type 2 diabetic animal model. Interestingly, increased serum insulin and a significant reduction in fasting blood glucose levels were observed in normal and treated diabetic rats with CNCP and CNBP. Furthermore, the histological observations of the liver, kidney and pancreas of diabetic rats treated with CNCP and CNBP illustrated a significant improvement in structural degeneration. In conclusion, the findings of the present study suggest that CNBP and CNCP are safe to consume and hold great promise for use as preventive and curative agent for anti-diabetic, gastric ulcer and wound healing.

Keywords: Schiff base derivatives, gastroprotective activity, wound healing, HSP70 protein, anti-diabetic

KAJIAN GASTROPROTEKTIF, PENYEMBUHAN LUKA, ANTI-DIABETIK OLEH DUA JENIS DERIVATIF DICHLORO DAN DIBROMO SCHIFF BES KE ATAS TIKUS SD

ABSTRAK

Derivatif-derivatif Schiff bes mempunyai pelbagai jenis aktiviti biologikal dan farmaseutikal. Penilitian ini merangkumi kajian aktiviti gastroprotektif, potensi penyembuhan luka dan efek anti-diabetik oleh kompleks Schiff bes terbitan bromin dan klorine secara *in vitro* dan *in vivo*. 2,2'-[1,2-cyclohexanediylbis (nitriloethylidyne)] bis [4-chlorophenol] (CNCP) dan 2,2'-[1,2-cyclohexanediylbis (nitriloethylidyne)] bis [4-bromophenol] (CNBP) telah disediakan melalui kaedah sintesis dengan terbitan keton dan diamin. CNCP dan CNBP menunjukkan keupayaan aktiviti antioksida FRAP yang tinggi dan aktiviti DPPH yang sederhana. Hasil penilitian ujian toksisiti akut terhadap CNCP dan CNBP mengesahkan bahawa kedua Schiff bes ini selamat digunakan dan tidak toksik walaupun pada dos tertinggi iaitu 200 mg/kg. Selanjutnya, kajian aktiviti gastroprotektif telah dijalankan ke atas tikus SD yang telah diinduksi dengan ulser perut melalui etanol. Kajian ini telah dijalankan ke atas kumpulan kawalan (normal), ulser (5 ml/kg, 10% tween 20), rawatan (masing-masing 10 dan 20 mg/kg CNCP atau CNBP) dan rujukan (omeprazole, 20 mg/kg). Hasil penilitian menunjukkan terdapat kecederaan hemoragik pada lapisan mukosa kelenjar gastrik ke atas kumpulan ulser berbanding dengan kumpulan tikus yang telah dirawat dengan CNCP dan CNBP. Hasil penilaian histologi pula, menunjukkan bahawa CNCP dan CNBP berkesan dalam merangsang penyembuhan luka dengan mengurangkan saiz luka ulser, edema, meningkat pertumbuhan semula tisu melalui penetrasi leukosit ke atas stratum submukosa. Hasil analisis immunostaining dalam kajian gastroprotektif menunjukkan keupayaan CNBP atau CNCP dalam meningkatkan protein HSP70, dan menurunkan protein Bax berbanding kumpulan ulser (kawalan). Tikus yang dirawat dengan CNCP dan CNBP

menunjukkan peningkatan terhadap aktiviti superoxide dismutase (SOD), catalase (CAT), and prostaglandin E2 (PGE2) yang tinggi dan aktiviti malondialdehyde (MDA) yang rendah berbanding kumpulan kawalan (negatif). Seterusnya, di dalam kajian potensi penyembuhan luka, tikus jantan jenis SD telah dibahagikan kepada 6 kumpulan (n=6); kumpulan kawalan (gam acacia), kawalan positif (gel intrasit), dan kumpulan yang dirawat dengan CNCP dan CNBP. Rawatan topikal dengan CNBP atau CNCP ke atas luka tikus jenis SD menunjukkan keberkesanan CNBP dan CNCP dalam pengecutan dan penyembuhan luka secara signifikan berbanding kumpulan kawalan. Berdasarkan penilitian histologikal ke atas kulit tikus yang telah dirawat (CNCP dan CNBP) menunjukkan granulasi tisu mengandungi banyak sel fibroblast, pemendakan kolagen, angiogenesis dan jumlah sel inflamatori yang lebih sedikit berbanding kumpulan yang tidak dirawat. Tikus yang dirawat dengan CNCP dan CNBP menunjukkan aktiviti SOD, CAT, dan glutathione peroxidase (GPx) yang lebih tinggi dan aktiviti MDA yang rendah berbanding kumpulan kawalan (negatif). Pada ujikaji molekular, CNCP (10mg/kg) dan CNBP (20 mg/kg) didapati berupaya mempercepatkan proses penyembuhan luka melalui regulasi penurunan protein Bax dan peningkatan protein HSP70. Berikutnya, kajian potensi anti-diabetik CNCP dan CNBP telah dijalankan ke atas model haiwan diabetes jenis 2 yang telah diinduksi dengan STZ-NA. Peningkatan serum insulin dan penurunan kadar glukosa dalam darah berpuasa yang ketara diperhatikan ke atas tikus kawalan (normal) dan juga yang telah dirawat dengan CNCP dan CNBP. Pemerhatian histologi ke atas tikus yang dirawat dengan CNCP dan CNBP pula, menunjukkan terdapat peningkatan yang ketara dalam degenerasi struktur hati, ginjal dan pankreas. Kesimpulan dari hasil penemuan dalam kajian ini, menunjukkan potensi tinggi CNCP dan CNBP sebagai agen antiulser, merawat penyembuhan luka, anti-diabetik dan selamat untuk digunakan.

Kata Kunci: Derivatif Schiff bes, Kajian gastroprotektif, penyembuhan luka, protein HSP70, anti-diabetik

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LIST OF SYMBOLS AND ABBREVIATIONS

<	:	Less than
>	:	Greater than
µl	:	Microliter
µm	:	Micrometer
µM	:	Micromole
AEU	:	Animal experimental unit
ALP	:	Alkaline phosphatase
ALT	:	Alanine transaminase
ANOVA	:	Analysis of variance
AST	:	Aspartate transaminase
Bax	:	Bcl2-associated X Protein
Bcl-2	:	B-cell lymphoma 2
CAT	:	Catalase
CB	:	Conjugated bilirubin
cm	:	Centimeter
CNBP	:	2, 2-[1, 2-cyclohexanediylbis(nitriloethylidyne)]bis(4-bromophenol)
CNCP	:	2, 2-[1, 2-cyclohexanediylbis(nitriloethylidyne)] bis(4-chlorophenol)
CO_2	:	Carbon dioxide
COX-1	:	Cyclo-Oxygenase-1
DM	:	Diabetes mellitus
DM2	:	Diabetes mellitus type 2
DMSO	:	Dimethyl sulfoxide
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
DPX	:	Dibutyl phathalate xylene

EC-SOD	:	Extracellular SOD
EGF	:	Epidermal growth factor
FGF	:	Fibroblast growth factor
FRAP	:	Ferric reducing antioxidant power
g	:	Gram
GGT	:	G-Glutaml transferase
GI	:	Gastrointestinal
GK	:	Goto-Kakizaki
GLUT-2	:	Glucose transporter-2
GPX	:	Glutathione peroxidase
GU	:	Gastric ulcer
GWM	:	Gastric wall mucus
H & E	:	Hematoxylin and eosin stain
H. pylori	:	*Helicobacter pylori*
H2	:	Histamine-2
H_2O_2	:	Hydrogen peroxide
H2RA	:	H2-receptor antagonists
HGF	:	Hepatocyte growth factor
HD	:	High dose
HOCl	:	Hypochlorous acid
HRP	:	Horseradish peroxidase
HSP70	:	Heat shock protein 70
HSPs	:	Heat shock proteins
IC_{50}	:	Inhibitory concentration at 50%
IDDM	:	Insulin-dependent diabetes mellitus
IM	:	Intramuscular

iNOS	:	Nitric oxide synthase
IP	:	Intraperitoneal
Kg	:	Kilogram
LD	:	Low dose
M	:	Molar
MDA	:	Malondialdehyde
mg	:	Milligram
Min	:	Minutes
mm	:	Millimeter
mmol	:	Millimole
MPO	:	Myeloperoxidase
MT	:	Masson's trichrome
NA	:	Nicotinamide
NAD	:	Nicotinamide adenine dinucleotide
NIDDM	:	Non-insulin dependent diabetes mellitus
nm	:	Nanometer
NO	:	Nitric oxide
NSAIDs	:	Non-steroidal anti-Inflammatory drugs
O_2^-	:	Superoxide anion radicals
OECD	:	Organization for Economic Co-operation and Development
ºC	:	Degree Celsius
PAS	:	Periodic acid Schiff
PBS	:	Phosphate buffered saline
PDGF	:	Platelet-derived growth factor
PGE_2	:	Prostaglandins E_2
PGs	:	Prostaglandins

PPI	:	Proton-pump inhibitor
PUFA	:	Unsaturated fatty acid
ROS	:	Reactive oxygen species
SD	:	*Sprague Dawley*
SEM	:	Standard error mean
SOD	:	Superoxide dismutase
STZ	:	Streptozotocin
TB	:	Total bilirubin
TGF-ß	:	Transforming growth factor-beta
TP	:	Total protein
TPA	:	12-O-tetradecanoylphorbol 13-acetate
TPTZ	:	2,4,6-tripyridyl-s-triazine
VEGF	:	Vascular endothelial growth factor

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CHAPTER 1: INTRODUCTION

1.1 Research background

Nowadays the risk of gastric attacks significantly increased due to exposure of human to many noxious agents and chemicals (Eldemerdash, 2017; Chaturvedi et al., 2007). Chronic gastritis can be asymptomatic (He et al., 2016; Peiffer et al., 2020) or seen in non-steroidal anti-Inflammatory drugs (NSAID)-provoked ulcers, the upper gastrointestinal hemorrhage or perforation might be the initial clinical symptom of disease. Bleeding is the most frequent and severe complication of peptic ulcers (Xi et al., 2016). Perforation of the gastric and duodenal walls is less frequent than bleeding, with an incidence rate of about seven to ten per 100,000 (Gisbert & Pajares, 2003).

There are different types of ulcer in the gastrointestinal tract. Esophageal ulcer can happen due to long term usage of NSAIDs and smoking and its common symptom are usually acid reflux. Peptic ulcer also causes the digestive tract to be affected due to various factors such as bacterial infection (Amandeep et al., 2012b). In general, gastric ulcer appeared to be resultant from the damage or injured gastrointestinal tract mucosa (Sunil et al., 2012). Among the different types of ulcers, gastric ulcer and duodenal ulcer are the most common to occur (Ahmed & Belayneh, 2019). Nearly 90% of the world population suffers from peptic ulcer (Azamthula, 2020). Peptic ulcer syndrome has been a major threat to the world's population over the past two centuries, with an elevated morbidity and mortality rates (Padmavathi & Shampalatha, 2012). *Helicobacter pylori* infection is one of the predominant causes of peptic ulcer disease. *H. pylori* and non-steroidal anti-inflammatory drugs (NSAIDs) may reduce the activity of defensive factors such as mucin, bicarbonate and prostaglandins, which may lead to peptic ulcer (Golbabapour et al., 2013c; Indran et al., 2008; Wasman et al., 2011). Inducing agents due to life style such as alcohol intake, spicy food, stress, some

aggressive factors or long term anti-inflammatory drugs consumption may exposed the stomach to ulcer (Abdulla et al., 2009; Al Batran et al., 2013a; Mahmood et al., 2010b). Those factors may cause lesions in the stomach which provide suitable conditions for further development of peptic ulcer. Gastric ulcer occurs frequently in human compared to other types of ulcers due to exposure of some aggressive factors. Internal aggressive factors such as pepsin, hydrochloric acid or refluxed bile could destroyed the bicarbonate mucus barrier which act as protective elements in stomach and led to higher chances in developing gastrointestinal ulcers (Abood et al., 2014). Ulcer treatment is now mostly targeting the harmful consequences of offensive acid secretion and producing safer and cytoprotective alternative drugs, which protects the stomach wall from damaging agents without influencing acid secretion or neutralizing the acidity of the stomach (Mei et al., 2013).

Different kind of medical treatments have been produced to disport gastric acid secretion for the betterment of mucosal defense (Malfertheiner et al., 2009). The popular approved drugs are the H2-receptor antagonist and the proton pump inhibitors (PPIs) such as omeprazole identified to help prevent gastric acid secretion by blocking H^+ K^+ in the parietal cell's ATPase (Malfertheiner et al., 2009). The degree of ulcer healing usually is dependent on the effectiveness of the drug used to curtail gastric acid secretion. Other drugs such as misoprostol, sucralfate and bismuth salts have been targeted to enhance ulcer healing by providing for mucosal repair and mucosal reinforcement. Yet, the function and usage of these drugs is limited because of their reported side-effects (Malfertheiner et al., 2009). By identifying one of the causes in ulcerogenesis (*Helicobacter pylori* (*H. pylori*)), the researchers concluded that a great deal of alternative drug regimens play an important role in ulcer healing by increasing antimicrobial and mucosal effects (Chan & Leung, 2002; Pohl et al., 2019). Gastric

disorders like hyperacidity and ulcers require treatment for a prolonged period. However, use of drugs for such a period may alter various normal physiological functions of the body i.e., they may influence pharmacokinetic parameters of other concomitantly used drugs by inhibiting drug metabolizing enzymes. Even though many synthetic drugs are available in the clinical practice, the researchers still in search of antiulcer compounds from natural resources (Bucciarelli et al., 2010). Some chemical compounds, such as phenolic, flavonoids, metallic and nonmetallic organic derivatives and heterocyclic, Schiff bases, possessed anti gastric ulcer activity (Munawar et al., 2018; Sumbul et al., 2011). In literature reviews, vast quantities of chemical compounds were synthesized in chemistry laboratories and were revealed to have an array of biological property for example the prevention of peptic ulcer (Dhiyaaldeen et al., 2014; Gwaram et al., 2012; Ketuly et al., 2013; Nazarbahjat et al., 2016; Salama et al., 2016).

Wound happens as a response to an injury and leads to the change in the skin integrity as well as the normal appearance of the skin (Bonifant & Holloway, 2019). But wound may occur in other tissues of the body because of various factors, such as physical, chemical, thermal, microbial, or immunological tissue trauma (Kujath & Michelsen, 2008). Therefore the natural process of wound healing goes under four phases of the cellular processes to restore the integrity of the skin. The four phases are known as coagulation, inflammatory response, proliferation and remodeling phases (Barreto et al., 2014; Tottoli et al., 2020). Accurate sequence and time frame for these phases and also the proper interplay of the factors which are interfere in these phases can lead to a successful wound cure (Martin & Nunan, 2015; Mobley et al., 2011). A complex procedure, including some series of reactions and interactions among cells and “mediators”, such as inflammatory mediators and cellular interactions growth are involved in would healing process (Mobley et al., 2011; Rodrigues et al., 2019). In the

coagulation which is also known as a hemostasis phase in which the platelet provides blood clotting. During inflammatory phase, inflammatory cells phagocyte debris therefore proliferation, arrangement of fibrous tissue and angiogenesis arise throughout the propagation stage. Finally collagen formation occurs in the remodeling phase which leads to the formation of the scar tissue (Rodrigues et al., 2019; Velnar et al., 2009).

In spite of recent attempts in the field of wound healing, only 1%-3% of the available drugs are partially successful in completely healing wounds. Hence, this highlights the imposing requirement of this study and the search for new compounds for wound closure, ant-inflammatory and antimicrobial activity (Moghadamtousi et al., 2015; Zielins et al., 2015). Some chemical compounds, such as phenolic, flavonoids, metallic and nonmetallic organic derivatives and heterocyclic, and Schiff bases have been able to show wound repair activity (Majtan, 2014; Oliveira et al., 2016; Padhye et al., 2009; Zhao et al., 2017). Recently, researches have focused on bioactivity effects of the Schiff bases, including its use as an anti-skin ulcer healing agent (Padhye et al., 2009; Saremi et al., 2019a; Zhao et al., 2017). It is noted that Schiff bases are excellent antiaging, skin whitening, skin repair agents which act via some same ways to accrue and speed up would healing (Gupta, 2010).

The prevalence of diabetes mellitus (DM) has increased tremendously, worldwide. DM is a serious global metabolic disorder known for leading to impaired healing of the wounds. Complications arising from diabetes have become serious public health issues (Brem & Tomic-Canic, 2007). Many studies have showed that diabetic patients suffer from wounds that do not easily heal (Falanga, 2005). The risk of lower extremity amputation because of injury is 15-46 times higher in diabetic patients. Wounds in diabetic patients usually are stuck in the inflammatory phase, characterized by continuous influx of neutrophils that release cytotoxic enzymes, free radicals, and

inflammatory mediators causing extensive collateral damage to surrounding tissue. These destructive processes disrupt healing in wounds of diabetics and delay their repair. Diabetes as the most frequent endocrine disorder categorized into three major types: type 1 or juvenile diabetes/insulin-dependent, type 2 or diabetes mellitus (DM), which commonly known as diabetes, or gestational diabetes (World Health Organization, 2019). Type 1 diabetes is the result of inadequate production of pancreatic islet β-cell mass with dysfunctionality which makes patients dependent to insulin injection for sustaining their life (Pathak et al., 2019). Decreasing insulin secretion and its sensitivity (insulin resistance) have significantly accountability for the disease pathogenesis, but, the trend for insulin resistance may not always be detected (Zhao et al., 2017). Type 2 diabetes is identified by high blood glucose level which causes impaired function of pancreatic insulin secretion and/or defected sensitivity of the targeted cells to insulin (Nandhini et al., 2019; Rad et al., 2018). A multitude of factors, such as, lack of physical exercise, obesity, genetic, and unhealthy diets have been known to be responsible for causing diabetes type 2 (Lega & Lipscombe, 2020). Also the role of free radicals in pathogenesis of such disease is extremely significant (Padhye et al., 2009). Numerous strategies and medications discovered and established in order to prevent diabetes; however, the management of this disorder has remained profoundly unsuccessful (Sarkar et al., 2020). There are many available treatments to manage diabetes, such as hypoglycemic agents, phenformin, troglitazone, rosigitazone and repaglinide which are capable of controlling hyperglycemia, but, some undesirable adverse-effects are reported with such drugs (Gupta, 2010; Khursheed et al., 2019). In the realm of pharmacological studies, specific diet encompassing antioxidant rich food show highly effective hypoglycemia. Schiff bases with different substitutions have shown outstanding role in treating diabetes. For instance, metformin-3-hydroxyflavone (Gupta, 2010), copper (II)-donor Schiff base complexes with ligands of aldimine or

ketimine (Chukwuma et al., 2020; Padhye et al., 2009), Substituted benzenediol Schiff bases (Choudhary et al., 2011), all are reported as promising new anti-diabetic agents. Particularly, it is noted that bromine (Lin & Liu, 2012; Niizato et al., 2002) and chlorine (EL-Hashash et al., 2015; Mahmoud et al., 2016; Shukla et al., 2019) substituted complex of chemical drugs can exhibit excellent effects against diabetes by lowering glucose level in body.

Schiff bases compounds from the reaction of amine with ketones derivatives which were discovered for the first time by Hugo Schiff (1864). It is interesting that Schiff bases have regularly used as chelating ligands in organic chemistry (Roth et al., 2006). Chelation of such compounds with some metals (Golbabapour et al., 2013b; Hegedus, 1999; Uneyama, 2008), halogens (Plech et al., 2013; Plech et al., 2011), oxygen (Silva et al., 2011), have demonstrated to have beneficial applications as restorative drugs and possess many biological activates, such as , anti-bacteria and anti-fungal (Kannan & Ramesh, 2006; Raju et al., 2011; Silva et al., 2011; Sultan et al., 2020), anti-viral (Chen et al., 2019; Munawar et al., 2018), gastroprotective activity (Salga et al., 2017; Saremi et al., 2020), anti-diabetic (Rahim et al., 2020; Torabi et al., 2018; Vančo et al., 2008), antioxidant (Bakır & Lawag, 2020; Batra & Chawla, 2018), anti-inflammation (Ayaz et al., 2019; Murtaza et al., 2017), anti-tumors (Chen et al., 2020; Faghih et al., 2018), antimicrobial (Rollas et al., 2002), anti-pain (Dave & Bansal, 2013), anticancer (Holla et al., 2003; Kuz'min1½ et al., 2000; Mahal et al., 2019), and herbicidal (Yilmaz & Cukurovali, 2003). Besides, Schiff bases are considered as important class of compounds due to their utilization as starting materials in the synthesis of industrial products (Asadia et al., 2011). There are numerous reaction paths to synthesize Schiff bases (Warren, 1977). The common pathway is an acid catalyzed concentration reaction of amine and ketone derivatives in ethanol (O'Donnell, 2019). In the current study, a

new synthetic route was used to synthesize a series of different compounds. The method that was applied includes *in situ* method in which the compound was synthesized directly in a one pot synthesis method.

Schiff base compounds are chemically stable in cold diluent acid as well as alkaline solutions. However, these compounds are easily destroyed by excessive heat or at boiling temperature (Asif, 2014). Schiff base derived compounds are synthetic heterocyclic compounds, have attracted enormous interest of pharmaceutical researchers due to their vast potential in numerous applications in pharmacology (More et al., 2019). Schiff bases have been described as promising compounds and not only effective in battling against wide range of diseases but they also improves and speed up wound healing. A great number of synthetized chemical with wound healing properties have been reported (Al-Bayaty & Abdulla, 2012; Dhiyaaldeen et al., 2014; Mughrabi et al., 2011). There were adequate reports of the activity of bromine and chlorine substituted complex of Schiff bases against gastric ulcer, wound healing, and anti-diabetic activity in rats (Dhiyaaldeen et al., 2014; Haider et al., 2018; Hajrezaie et al., 2012b; Jaiganesh & Subramanian, 2017; Mustafa et al., 2009b). Although, there were limited reports on the dichloro and dibromo activities, but the gastroprotective effect, wound healing activity and anti-diabetic potential of these derivatives and its underlying mechanism remains unclear. Hence, the objective of the current study is to investigate the gastroprotective effect, wound healing and anti-diabetic potential of 2,2'-[1,2-cyclohexanediylbis (nitriloethylidyne)] bis [4-chlorophenol] (CNCP) and 2,2'-[1,2-cyclohexanediylbis (nitriloethylidyne)] bis [4-bromophenol] (CNBP).

1.2 Hypothesis of the present research

1. The present study of CNCP and CNBP Schiff base derivatives may be regarded as promising drugs for prevention of gastric ulcer, acceleration of wound healing and hypoglycemic effects.
2. Schiff base derivatives (CNCP and CNBP) will decrease the oxidative stress, thus, preventing ulcer and enhanced wound healing and hypoglycemic effect.

1.3 Research objectives

1.3.1 General objective

The aim of the present study is to evaluate the gastroprotective effect, wound healing and anti-diabetic potential of two novel Schiff base derivatives (dichloro -CNCP and dibromo -CNBP) in SD rats.

1.3.2 Specific objectives

The specific objectives of the present study are:

1. To determine the antioxidant activity of CNCP and CNBP Schiff base derivatives, *in vitro*.
2. To investigate the acute toxicity, gastroprotective activity and wound healing of CNCP and CNBP Schiff base derivatives in SD rats.
3. To evaluate the anti-diabetic effect of the Schiff base derivatives and histology in streptozotocin-NA-induced diabetic rats.

CHAPTER 2: LITRATURE REVIEW

2.1 Schiff bases compounds and their biological activities

Compounds that contains the azomethine group (-CH=N-), also known as Schiff bases often used as a chelating ligands in organic chemistry due to their physic-chemical properties (Roth et al., 2006). Schiff bases with donors (N, O, S, etc) have formation similarities with neutral biological systems and because of the presence of the imine (N=CH-) group which are utilized in elucidating the mechanism of transformation and conversion of rasemination reaction in the biological system (Ghames et al., 2006). Figure 2.1 illustrates the general scheme of the formation of Schiff base.

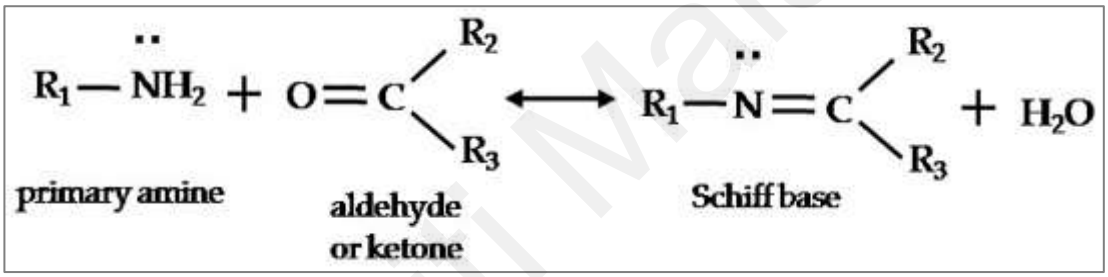

Figure 2.1: General scheme of the formation of Schiff base. Adopted from (Abu-Dief & Mohamed, 2015)

Furthermore, Schiff bases are considered as "privileged ligands" because they are easily prepared by condensation between the aldehydes and imines (More et al., 2019; Yoon & Jacobsen, 2003). Due to their ability to form complexes with different transition of metals, it acts as catalysts for many different reactions (Cozzi, 2004). In addition, Schiff bases are known to be dioxygen carriers and have been studied as potential reagents for oxygen separation and transportation (Gaballa et al., 2007). A great deal of information regarding the potential of biological properties of synthetic Schiff bases has surfaced during the last decade (Liu & Hamon, 2019; Przybylski et al., 2009; Ren et al., 2002; Wu et al., 2020; Yilmaz & Cukurovali, 2003). It is noted that

new chlorine substituted metformin compounds and bromine substituted complex of Schiff bases exhibit excellent biological activities in rats (Narayana Reddy et al., 2012; Siddiqui et al., 2014). The new chlorine substituted metformin compounds are more potent in reducing plasma glucose levels in comparison with metformin which can be due to the effect of chlorine atom in chlorine pump and insulin resistance of β-cells in order to regulate insulin secretion (Quintão et al., 2016). Basic functions of bromine including the digestion of carbohydrates, fats and, also activates pepsin production in gastritis with low acidity (Moore et al., 1940; Obembe et al., 2016).

In recent literature it is found that several authors have reported the various bioactivities of synthetic Schiff bases including their bioactivity in gastroprotective effect (Hajrezaie et al., 2012b; Ketuly et al., 2013). Evaluating research on the acute toxicity of several Schiff bases has revealed that the compounds have no toxic effects on experimental animal models even at higher concentrations of 2 to 5 g/kg (Ibrahim et al., 2016; Salga et al., 2012). The biological potential uses and function of Schiff bases has been investigated previously to be useful in many applications (Kumar et al., 2010; Przybylski et al., 2009) such as for antibacterial and antifungal properties (Kannan & Ramesh, 2006; Raju et al., 2011), anti-diabetic (Torabi et al., 2018; Vančo et al., 2008), antitumor (Jesmin et al., 2010; Ren et al., 2002), anti-proliferative (Chaviara et al., 2004; Illán-Cabeza et al., 2008; Kuz'min1½ et al., 2000), anticancer (Holla et al., 2003), herbicidal (Yilmaz & Çukurovali, 2003), analgesic and anti-inflammatory activities ,(Singh et al., 2007; Yilmaz & Çukurovali, 2003), gastroprotective effect (Ketuly et al., 2013), anti-allergic (Lebaut et al., 1999), as well as a potential wound healing agent (Mughrabi et al., 2011).

2.2 Gastric ulcer

Deep lesions or holes in the lining of the stomach that has been corroded by digestive juices which are secreted by the stomach cells are defined as gastric ulcer or peptic ulcer. They perforate deep into the mucosa of the gastrointestinal tract and create ulcer serogenic lesions (Ren et al., 2019). These are subsequently called duodenal or intestinal ulcer when formed in the lining of the duodenum and together is known as peptic ulcer. Most of the time these ulcers are present as deep necrotic erosions passing into the mucosal layers and muscularis mucosae (Tarnawski et al., 2001). The term "peptic ulcer" is broadly used indicating the ulcers found in the digestive tract of the stomach or duodenum (Amandeep et al., 2012a). The disease is viewed as the condition where the mucosal lining of the gastrointestinal (GI) tract is neutralized when large amount of acid and pepsin are secreted (Chaudhari et al., 2017). Sodium bicarbonate is expressed broadly by the pancreas to digest food. But the normal amount of sodium bicarbonate is not enough for food digestion when the amount of acid produced is substantially large (Bray et al., 2020). The disruption in the normal equilibrium between the aggressive factors (i.e. *H.pylori*, NSA1Ds), acid, and pepsin, ingested drugs and bile salts (Remmele & Möller, 1996) and protective barriers of the stomach in the lumen such as epithelial cells, mucosal barrier (mucus secretion), blood flow, endogenous protective agents (prostaglandins and epidemic growth factors, bicarbonate), and cellular regeneration (Lohr & Field, 1992) can affect the mucosal integrity and induce gastric lesions (Sung et al., 2010). During the natural chewing and mastication of food digestion, acid and pepsin are triggered upon food consumption through the destructive and defensive mechanisms which also protect and prevent the excoriated mucosa by secreting the defensive factors (Bashah, 2009). Pathogenesis of peptic ulcer disease represents in Figure 2.2.

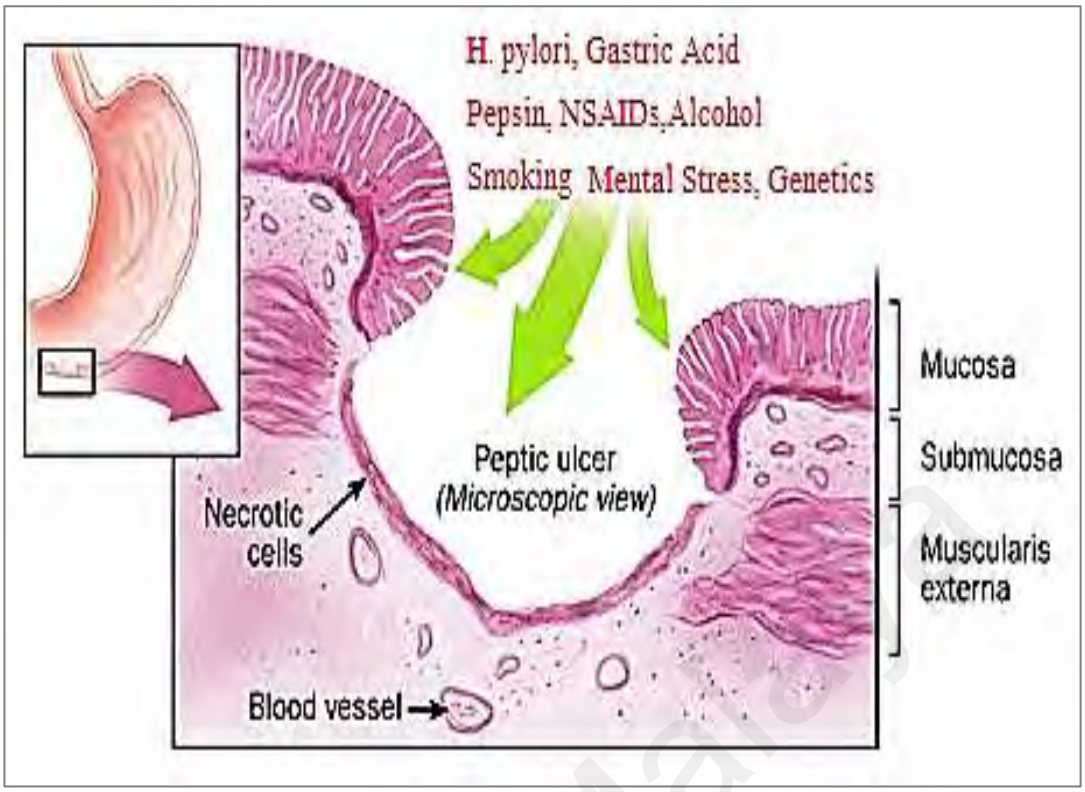

Figure 2.2: Pathogenesis of peptic ulcer disease adopted from (https://www.hopkinsmedicine.org)

When food product enters the stomach via the oesophagus, the stomach also secretes aggressive factors such as hydrochloric acid (pH 1.0) and pepsin to process food product, which is known as chime (Chang & Leung, 2014). Upon the intake of food chime, in the case of stomach with an uninterrupted protective mechanism, the mucus activates a defensive mechanism against acid and pepsin to protect the surface mucosa from damaged. The gastrointestinal mucosa defensive system that acts against the secreting aggressive factors is mainly composed of functional, hormonal and neuronal factors. Functional factors consist of alkaline mucus secretions, microcirculation and motility while hormonal factors are nitric oxide, prostaglandin (Repetto & Llesuy, 2002), epidermis growth factors (endogenous protective agents), mucosa barrier, mucus secretion, parietal cell, blood flow, cell regeneration (Jones, 2006), and gastric mucosa glycoprotein, all contribute to the mucosal protection. Some studies have identified heat shock proteins topically like HSP70 that have response to protect the intracellular

mucosa-lining (Tsukimi & Okabe, 2001). Moreover, HSP60 molecule has been implicated to act as an inflammatory triggering clement during the course of mucosal protection (Dukay et al., 2019; Vorobjova et al., 2001). Mucosal protective barrier can reduce the oxygen free radical damages in the mucus by its antioxidant properties depending on the gel structure of the mucus and the layer thickness covering the mucosal surface (Fishman et al., 2013; Laine et al., 2008).

2.3 Gastroprotective factors

2.3.1 Mucus

The mucus which constitutes of clear elastic, adherent, viscous gel composing of 95% water and 5% glycoprotein's is known as the main properties of gastrointestinal mucosa (Lathe et al., 2008; Repetto & Llesuy, 2002). As one of the primarily lines of defence, gastric mucosa, is governed by the gastric epithelium that performs as a bather through high resistant mechanisms in tandem with occurrence of disturbances in the acid-alkaline balance. Such protection is directed mainly by local gastric and neuro-hormonal regulation of mucosal defence mechanisms. With these mechanisms, the mucosa is able to tolerate certain levels of hydrochloric acid environment, osmolarity and temperatures (Werther, 2000). Surface epithelial cells will allow secretions of prostaglandins, heat shock proteins, mucus and bicarbonates factors and together with its construction by the means of tight junctions, prevents back-release of acid and pepsin (Ham & Kaunitz, 2008). Destructive agents such as pepsin and hydrochloride acid diffusion into the mucosa can rupture the gastric mucosal blood veins and causes ulceration and bleeding (Wallace et al., 2000). Surface epithelial cells secrete heat shock proteins in conditions of oxidative stress, acidic environment and in the presence of necrotizing agents (Tanaka et al., 2007). Factors including volume, osmotic pressure, pH, and infuscate calorie induce the gastric mucosa

discharges in the stomach (Gyires, 2005). When damage to the mucosal lining is caused, mucous integrity is altered thus restoration of superficial ulcerated gastric tissues and natural healing is prevented (Wallace et al., 2000).

2.3.2 Cytoprotection

Naturally, on a daily basis, the gastro-alimentary lining is exposed to attacks that are either from secreted gastric acids or from free radicals liberated by neutrophils and monocytes (Giefer et al., 2020). This could resulted in oxidative damage to the mucosal lining via protein oxidation and lipid peroxidation consequently leading to ulcerogenesis (Abdelwahab, 2013; Rajakrishnan et al., 2020). Hence, the mucosal lining always protects the gastrointestinal tract against these aggressive chemical attacks. The term "cytoprotection" was coined by Robert et al, 1979 to describe the balance between the renewal and death of the damaged or aged cells for integrity of the gastric mucosa. To achieve viable and efficient mucosal cell proliferation, it is important to maintain normal mucosal blood flow. Prostaglandins have been reported to enhance ulcer healing by stimulating the release of growth factors such as epidermal growth factor (EGF), hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) from the gastric mucosa (Brzozowski et al., 2001; Mao & Huang, 2019). On the other hand, as previously described by literature, cyclo-oxygenase-1 (COX-1) and cyclo-oxygenase-2 (COX-2) are important factors in the maintenance of mucosal blood flow and the biosynthesis of prostaglandin. In fact, COX-2 is known to regulate the production of vascular endothelial growth factor (VEGF) by gastric fibroblasts (Kalish et al., 2013; Takeuchi & Amagase, 2018), thus playing an important role in the enhancement of gastric ulcer (GU) healing (Syam et al., 2009). Previous studies have suggested that COX-2 is produced in the gastric mucosal lesions, therefore, inhibition of COX-2 results in an observable impaired ulcer healing (Mizuno et al., 1997). Studies have

shown that gastric fibroblasts with a high expression of COX-2 and VEGF could greatly enhance the ulcer healing process (Kalish et al., 2013; Sheng et al., 2020).

Gastric mucosa shows a high rate of renewal, with reported mucosal surface exfoliation into the gastric lumen every 3 to 5 days under normal physiological conditions (Targosz et al., 2012). The continuous secretion, renewal and viscoelastic nature of the mucosal layer efficiently protects the lining from peptic erosion by providing a kind of lubricant and an ion diffusion barrier that curtails the diffusive effects of gastric acid with subsequent neutralization of the released acid by mucosal bicarbonates (Carlson et al., 2018; Kalish et al., 2013). Heat shock proteins (HSPs) are also important and significant cytoprotection factors. HSPs are a group of genetically conserved proteins which serve as molecular chaperons, and which functions by ensuring accurate and functional folding or refolding of bioactive proteins. They were first reported by (Ritossa, 1962) who observed the HSPs in *Drosophila melanogaster* chromosomes subsequent to applying heat treatment on them. This group of proteins are said to be expressed in stress related conditions in order to protect the cells against the stress (Hassan et al., 2019; Targosz et al., 2012) and are located in almost every cellular compartment. HSPs are important proteins in the maintenance of cell integrity during normal cellular growth as well as during pathophysiological conditions. The over-expression of HSPs may shelter multiple cellular compartments and avert protein damage from any oxidative stress. In particular, the cytosolic 70-kDa molecular chaperon (HSP70) is reported to be expressed in response to environmental changes such as heat, oxidants, and various viral and bacterial infections (Bolhassani & Agi, 2019; Otaka et al., 2007). HSP70 has an important function in preventing damage and aiding cellular repair process after injury (Otaka et al., 2007). Previously, Ishihara et al. (2011) studied the simulation of gastric ulcer healing using HSP70; the authors observed that orogastric administration of recombinant HSP70 accelerates ulcer healing.

They also established that the induction of HSP70 protects rats against damage by the ethanol-induced gastric mucosal (Park et al., 2008). Several genes such as the Bcl-2-associated X Protein (Bax) and B-cell lymphoma 2 (Bcl-2) regulate the balance between apoptosis and cellular proliferation in gastric injury and repair (Zhang et al., 2017). The Bax gene is a proliferative suppressor gene that encodes Bax protein, which promotes apoptosis (Bowen et al., 2006; Elmore, 2007). Qiao et al. (2011) reported that the down-regulation of the Bax protein is correlated with the healing and restoration of gastric ulcer.

Usually, the current approach in the chemotherapeutic management of gastric ulcer works by i- targeting the inhibition of gastric acid secretion or neutralization of the acid via reaction with antacids (e.g. aluminium hydroxide, magnesium hydroxide), thereby increasing gastric protection through increased epithelial cell proliferation; ii -inhibiting cellular apoptosis via the 'cytoprotection' effect resulting in an effective ulcer healing process (Asmaz & Berrin, 2020; Sen et al., 2009). It has been reported that the most common approach to ulcer management is the use of proton pump inhibitors (PPIs), histamine-2 (H2) receptor antagonists and antacids that are employed to neutralize the gastric acid, and antimicrobial drugs that are directed at eliminating *H. pylori* infection (Abdelwahab, 2013; Martinsen et al., 2019).

2.4 Gastric ulcer risk factors

2.4.1 *Helicobacter pylori*

Ninety percent of the duodenal and most gastric ulcers, not brought by aggregative factors such as non-steroidal anti-inflammatory drugs (NSAID) and acids (de Boer & Driessen, 1995) are mainly induced by the colonization of *Helicobacter pylori* (*H. pylori*). This bacterium is known as the etiologic agent in gastritis and peptic ulcer disease outcomes in adults and children (Atiyah, 2020; Wong et al., 2005). Ulcer

complications are reported as indirect markers of ulcer prevalence (Lanas & Chan, 2017; Uyub et al., 1994); however reoccurrences of gastric ulcer disease reduces significantly with *H. pylori* eradication in the gastric ulcer patients (Ford et al., 2004; Tongtawee et al., 2019). The ulcer forming bacterium has been present for a long time throughout human population (Majumdar & Atherton, 2006). The *H. pylori* infection rate is significantly relative different factors such as, racial differences and admixture (Raj et al., 2001; Tongtawee et al., 2019); ethnicity, genetic composition, age, environmental restriction factors in bacteria transmission, poor childhood living hygiene, low socio-economic levels, and high population (Mendall et al., 1992; Raj et al., 2001); However, there had been a decline in the rate of infection by *H. pylori* bacterium in gastric ulcer disease and duodenal ulceration among the western Europe and United States (U.S.) population. An estimated 40% of the adults get infected by the bacterium in developed countries and even less the number of children that have been infected in the second half of the 20th century. On the contrary, the *H. pylori* infection has indicated to be very common with more than 80% prevalence rates found in developing countries and a high prevalence of this is among the immigrant population (Raj et al., 2001). Nevertheless, a preliminary research has examined an exceptional low frequency of *H. pylori* prevalence among patients with ulcerative gastric lesions in Terengganu, a state in Malaysia especially among the Malay majority (Raj et al., 2001) that appears to be the lowest rate reported among studies conducted around the world. The study revealed that among the Malaysian population, 69.4% of the patients were Malays, 24.2% Chinese. 2.4% Indian, 2.4% Kampuchean, and 1.6% or Thai ethnic origin (Raj et al., 2001). It is critical to mention that the results were non-specific to *H. pylori* or other ulcer forming agents and generally report the overall percentage of peptic ulcer among

different racial groups; further examination were taken to identify the lesion origin and that clearly introduced Malay inhabitants had lower propensity to *H. pylori* infection and further suggesting NSAIDs the main causative peptic ulceration contributor in the population (Raj et al., 2001). Also, the findings in the United States have proven low trends of *H. pylori* infection, as low as 30-50%, in patients with duodenal ulcer (Jyotheeswaran et al., 1998; Sprung & Apter, 1998). It is tempting to mention here that the findings showed that the higher number of infected Malays that were diagnosed were from cohorts who had inter-marriages with non-Malays, and that strongly support the postulate of bacteria transmission among races. The method of transmission of the bacterium is via person to person in populated communities than through water or food particle infected with the pathogen through contact to faces and is known likely to be the oro-oral rather than faecal-oral route (Luman et al., 2002; Zamani et al., 2017). No significant animal or environmental condition as a reservoir has been detected (Majumdar & Atherton, 2006). Studies suggest that not all the *H. pylori* infections can lead to peptic ulcer; it is considerably dependent on the environmental factors (e.g. smoking), host genetic susceptibility and bacteria virulence form of a strain that is more competent to developing ulcers (Ansari & Yamaoka, 2020; Majumdar & Atherton, 2006).

2.4.2 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are valuable medication against inflammation used also as analgesics and antipyretics for arthritis and broad range of medical conditions (Amandeep et al., 2012a; Bindu et al., 2020). They are the most commonly prescribed and widely used type of medication for pain globally (Lanas et al., 2005) but have been reported as being ulcerogenic (Bajaj et al., 2020; Wolfe et al.,

1999). In contrast to the valuable sedative and anti-inflammation properties provided by NSAIDs, these drugs have unfortunately proven to exert harmful effect on the gastric mucosa (Bhat et al., 2020). Studies have suggested toxicity and side-effects of increased nonspecific administration of these drugs are capable of producing ulcer induction (Castellsague et al., 2012; Higham et al., 2002) in the gastrointestinal tract as a result of damaged mucous, interrupted repair mechanism as well as bleeding (Higham et al., 2002; Wallace et al., 2000). Gastric mucosa protects stomach by high resistant mechanisms against many injurious agents and keeps organ from injuries depending on the number of mucosal lining secretions against the noxious factor, the speed and quality of repairing after the damage is caused (Laine et al., 2008). However, when the protective physiological response is impaired by harmful agents, mucosal lesion can develop due to administration of NSAID (Colucci et al., 2018; Fornai et al., 2011). These drugs not only alter the secretion pathway by elimination of the toxic factors, but also impede healing of an injured gastric lining through many systemic mechanisms (Bindu et al., 2020; Musumba et al., 2009). Previous studies have shown the effects of NSAIDs persisting for long periods, even after termination of drug administration (Lichtenberger et al., 2006).

2.4.3 Epidemiology

Gastric ulcer currently is one of the most prevalent human suffering today (Flaskerud, 2020) . The multi factor intestinal disease forms when the intrinsic luminal damaging factors and protective gastro-duodenal mucus are not balanced and can reoccur in presence of ulcerogen (Fock & Ang, 2010). The perforation is among the most chronic digestive tract diseases that became a major concern to cause morbidity and mortality (Møller et al., 2011). Large numbers of people are diagnosed with gastric ulcer worldwide and a considerable number of approximately 500,000 people are

suffering from gastritis disorder annually in the U.S. (Ramakrishnan & Salinas, 2007). *H. pylori*, is believed to be the main cause of gastritis and many gastric cancers (Seeley, 2003). Chances of acquiring cancer are high, when the peptic ulcer causes gastrointestinal bleeding due to perforation of the mucosal lining (Aabakken, 2008; Yuan et al., 2020). Earlier, people experiencing stress, anxiety and spicy food were believed to be more sensitive in developing gastric ulcer (Amandeep et al., 2012). Stress can cause a high rate of gastric secretion (15 times more than normal amount) between meals that leads to a very acidic chyme into the duodenum (Seeley, 2003). A study has reported the psychological factors such as stress in association with prevalence of peptic ulcer up to 30% to 65% (Levenstein, 2000). Acute psychological stress contributes to mucosal ulceration by reduced blood circulation and gastric mucin production (Livingston, 2001; Zhao et al., 2020). Conventional wisdom suggests the causative factors as prolonged anxiety, emotional stress, haemorrhagic surgical shock, bums and trauma, diet, smoking, or alcohol due to extended acid production in the stomach (Murison, 2001). Alcohol administration contributes to generation of ulcer when tissue damages and cell death occurs due to reactive oxygen derived free radical species (ROS) and gastric mucous deterioration by neutrophil infiltration that complicates the course of ulcer healing (Suzuki et al., 1998; Takeuchi, 2012). ROS species are proven candidates as the pathogenesis of gastric damage (Nagai et al., 2020; Rao et al., 2000). Weight-loss, loss of appetite, nausea, bloating and vomiting and black stool (as a sign of intestinal bleeding) are some of the symptoms of gastric ulcer (Amandeep et al., 2012). Gastric ulcers can also occur in the case of duodenal refluxed chyme into the pylorus (Segregur et al., 2019). Bile salt components of the reflux have detergent effect that can lower the mucosal protection and integrity against acid and bacteria (Seeley, 2003). But principal agent believed to impair the mechanism of gastric mucus defense is the infection caused by *H. pylori* bacteria and administration of NSAIDs, indometacin and

similar medicines (Rao et al., 2000). A person's diet is an important factor in creating a ground for *H. pylori* infection to develop an ulcer; extra hot temperature of the drinks (62°C) reduce the thickness of the mucus lining of the stomach and therefore, less protection from mucus that predisposes an infected individual by *H. pylori* for bacterial invasion to create ulcer (Seeley, 2003). also it is studied that *H. pylori* infection controls secretion of gastric acid (Miyata et al., 2019; Smolka & Backert, 2012) reduced mucosal integrity and ulcer development is studied in the acute phase of infection when acid secretion is impaired or increased relative to the mucosal and alkaline protection (Malfertheiner, 2011; Ommurugan & Rao, 2019). This bacterium is now known as the most apparent cause of peptic ulcer disease (Schöttker et al., 2012).

The United States population has been reported for 1% infection rate from *H. pylori* per year. The bacterium infects 30% of the people in their 30's, and 80% of those in their 80's. Higher percentage of the population in the developing countries have had the bacterium at the age of 25 and older that may be due to high rate of stomach cancer in some of the third world countries (Hunt et al., 2011). Still, very little is understood about *H. pylori* and how people become infected by this bacterium. Additionally, with such high number of infection incidence, it is not clear why the ulcer development only occurs in a tiny fraction of those infected (Rizzato et al., 2019; Suzuki et al., 2012). Only about 15% of the majority of the infections by this bacterium will actually develop a gastric ulcer (Majumdar & Atherton, 2006). It is assumed that aggravating factors including stress and a person's diet can alter the bacteria in the infected individuals to develop gastric ulcer (Eslami et al., 2019; Seeley, 2003); However, the rise and fall and mechanism of the peptic ulcer has yet remained controversial.

2.5 Drugs used in treatment of gastric ulcer

Mucosa of the stomach is naturally being protected from damage or injuries by prostaglandin secretions released from the GI tract (Périco et al., 2020; Seeley, 2003). Synthetic prostaglandins (analogues) can supplement this resistance as well as preventing the gastrointestinal tract from the excess acid. Wide use of this medication is not recommended as it can cause nausea and diarrhoea (Bevec et al., 2012). Demulcents and antacids are other types of drugs used for gastric lesions treatment; demulcents protect the small intestine and gastric mucus. Antacids have remained popular as provisional sedatives of stomach problems (Wada et al., 2019). They play role in interfering with acid secretion pathway (Awaad et al., 2013), as it is known that stomach has a low resistance towards its own acid. United States spends near to $1 billion each year on antacids (Seeley, 2003). Studies have reported undesirable side-effects of these drugs and drug interaction upon administration for gastric ulcer treatment (Lavanya et al., 2019). However, consumption of natural compounds is well documented (Ardalani et al., 2020; Devi et al., 2007; Kuna et al., 2019). Recently, plant extracts with promising results have attracted many researchers as an alternative source for drug discovery (Chanda et al., 2011; Shinde et al., 2019) Studies demonstrated that antacid treatment does eradicate the ulcer, but 50% incidence of relapse is within 6 months administration of antacids and 95% of occurrence with individuals after 2 years of treatment (Seeley, 2003).

Gaviscon and Gelusil are prescribed for treatment of heartburn and gastro-esophagal ulcer that are accompanied by low or inability in thiamine, phosphate and vitamin A absorption. Amphogel is an aluminium hydroxide and acts as antacid that medicates the stomach excess acid by rapid decrease of acid in the stomach; it treats the symptoms

of heartburn, and acid digestion and is documented to cause nausea and vomiting (Escott-Stump, 2008). Proton-pump inhibitor (PPI) treatment is considered as one of the main therapies for most gastrointestinal diseases (Alqasoumi et al., 2009) but excessive ingestion of these drugs result in abdominal pain, nausea and vomiting (Sumi et al., 2020; Whitney & Rolfes, 2012). Some classes of the drugs protect stomach as acid control agents by decreasing the acid production such as bismuth and gastrointestinal agents as sucralfate that are associated with hypophosphatemia, constipation and diarrhoea (Whitney & Rolfes, 2012). Studies using antibiotic therapy along with bismuth and ranitidine have resulted in rapid healing of duodenal ulcers (74%) and high number of gastric ulcer elimination (95%) within 2 months of medication. One study indicated 8% reoccurrence rate when treating with antibiotic in comparison with 86% relapse in controls that demonstrates a dramatic fall in the incidence of relapse (Seeley, 2003).

Other acid agent controllers are H2-receptor antagonists (H2RA) that inhibit stimulation of HCl secretion in the stomach by binding to histamine receptors and block their effect on parietal cells which in turn raise the creatinine level and cause constipation, diarrhoea and confusion in elderly people (Escott-Stump, 2008; Wallace & Sharkey, 2011). H2 antagonists are mainly used for dyspepsia treatment although they have lost their popularity by the more operative proton pump inhibitors such as omeprazole, pantoprazole and esomeprazole (Eriksson et al., 1995; Safavi et al., 2016) that directly prevent HCI secretion (Seeley, 2003).

2.6 The role of antioxidant activity in anti-ulcer

Any molecule able to reduce the oxidation of other molecules and which protects the body against free radicals may be called an antioxidant (Ali et al., 2020). The ability of antioxidants to inhibit the production or scavenge the

produced ROS is said to be pertinent in the protection of gastric mucosa or tissue wound healing against various aggressive or necrotic agents, and augments the defence systems of the body against several diseases (AlRashdi et al., 2012a). Thus, the effectiveness of a therapeutic drug in the management of peptic ulcer or wound healing is partly owed to its anti-oxidative power, which is the ability of the said bioactive compound/drug to scavenge for the generated oxidative radicals species and inhibit the oxidation reactions caused by those free radicals (Chen et al., 2016). A number of methods which have been employed in evaluating the antioxidant activity of bioactive compounds in both *in vitro* and *in vivo* analyses are reported in literature. Among the *in vitro* assays that are available utilizes calculation of 2, 2-diphenyl-l-picrylhydrazyl (DPPH) (Ghasemzadeh et al., 2010; Stoilova et al., 2007) and Ferric reducing antioxidant power (FRAP) (Benzie & Szeto, 1999). The DPPH assay is a colorimetric analysis, popularly employed to quantify the power of the compound's ability to scavenge for free radicals. It is said to rely on the ability of the test compound to scavenge the unstable paramagnetic free radical that accepts electrons (e^-) or proton (H^+) radicals, thus becoming a stable diamagnetic molecule (Buijnsters et al., 2002; Milardović et al., 2006). The antioxidant compound reduces DPPH resulting in the formation of a purple-blue colouration that is usually quantified by spectro-photometric scanning at a wavelength of 541 nm (Buijnsters et al., 2002). On the other hand, the antioxidants reducing power of ferric (FRAP) to ferrous ions leads to the formation of a quantifiable spectro-photometric complex described as ferrous-tripyridyltriazine (Odukoya et al., 2005).

Some current studies have revealed that the ulcer healing potential of several Schiff bases and their derivatives can be attributed to their potent antioxidant

potential (Buldurun et al., 2020; Mughrabi, 2012; Salga et al., 2012). The use of experimental animal models such as rats and mice provides an excellent opportunity for evaluating the *in vivo* anti-oxidative properties of several compounds (Golbabapour et al., 2013b; Hajrezaie et al., 2012b). The superoxide dismutase (SOD) catalyzes the detoxification of superoxide radical anion (O_2^-) into the formation of stable oxygen and the less pernicious hydrogen peroxide (H_2O_2). SOD has been reported to be the first line of antioxidant defence in an animal's body (AlRashdi et al., 2012a). The hydrogen peroxide that is formed is said to be converted to water by the catalysis of catalase (CAT) in the lysosomes or glutathione peroxidase (GPx) in the cellular mitochondria (AlRashdi et al., 2012a). Based on genetic expression variants, three distinct isoforms of SOD have been reported i.e. SOD1, SOD2, and SOD3 (Zelko et al., 2002). In particular, SOD3 (extracellular SOD; EC-SOD) is mostly located in the extracellular matrix. The enzyme contains a heparin-binding domain that binds to connective tissues and contributes easily to providing an important defence mechanism against superoxide anions in the extracellular matrix microenvironment (Lookene et al., 2000). Furthermore, EC-SOD is said to participate in the suppression of induced cutaneous inflammation due to the production of 12-O-tetradecanoylphorbol-13-acetate (TPA) and hyperplasia (Ha et al., 2006). It also protects EC-SOD transgenic mice against 7,12-dimethylbenz (a) and anthracene (DMBA)/TPA-induced tumor (Kim et al., 2005). Kim et al. (2011) elucidated the EC-SOD molecular mechanism of action, attributing its anti-angiogenic and anti-inflammatory effects to the down-regulation of the expression of angiogenic factors and pro-inflammatory mediators, and a concurrent up-regulation of the expression of anti-angiogenic factors and anti-inflammatory cytokines. The use of SOD to assess *in vivo* the antioxidant activity of bioactive chemicals including

Schiff bases has been reported (Chen et al., 2003; Lam et al., 1989; Panda, 2012; Sankarganesh, 2020). AlRashdi et al. (2012a) reported a marked decrease in superoxide anion radicals (O_2^-) in ulcerogenic stomach homogenates upon treatment with *Jasminum sambac*. The maintenance and integrity of gastric mucosal function is reported to critically depend on the status of micro circulation (do Amaral Tafner et al., 2017; Whittle et al., 1990). Hence, microvascular vasoconstriction or removal of endogenous vasodilators results in gastric ulcer due to mucosal erosion. It has been reported that lipid peroxidation results in the excessive production of myeloperoxidase (MPO) within the neutrophil leukocytes (AlRashdi et al., 2012a). The produced MPO catalyses the formation of toxic hypochlorous acid (HOCl) from hydrogen peroxide, which consequently leads to cell membrane damage (AlRashdi et al., 2012a). The end-products of lipid peroxidation is the generation of malondialdehyde (MDA), an organic compound that is used as a marker for oxidative stress in tissues (Dursun et al., 2009). Lipid peroxidation is an important pathophysiological factor in several diseases including peptic ulcer (Bandyopadhyay et al., 2001; Zatorski, 2017). It has been documented that DNA bases react with MDA produced by lipid peroxidation, and this induces mutagenic lesions (Marnett, 1999). In ulcerogenesis, several studies have reported an increase in gastric MDA level in ulcer control groups and a subsequent decrease upon treatment with bioactive compounds (AlRashdi et al., 2012a; Mahmood et al., 2010b).

2.7 Wound healing

2.7.1 Physiology of the wound healing process

Skin ulcerogenic conditions are usually associated with lesions that normally result in wounds. The healing of these wounds is a complex biological process

that involves the integration of inflammation, mitosis, angiogenesis, synthesis and remodelling of the extracellular matrix (Komi et al., 2020). Healing in an acute wound is characterized by four distinct phases namely: hemostasis, inflammation, proliferation and remodelling. The healing process in this kind of wound is reported to be very orderly and efficient (Barreto et al., 2014; Diegelmann and Evans, 2004; Martin & Nunan, 2015). Knowing the process of tissue response to injury is mandatory in understanding the underlying mechanisms involved in pathologic conditions like fibrosis and chronic non-healing ulcers (Nikoloudaki et al., 2020). The human body can accommodate a variety of injuries, such as penetrating trauma, burn trauma, blunt trauma and ulcerogenic lesions. These bodily insults invoke a series of orderly events that are involved in the healing response, such as the mobility of platelets and inflammatory cells to the wound site. The presence of these cells in the wound site incurs the production of cytokines and growth factors that signal the influx of connective tissue cells and a new blood supply (Diegelmann & Evans, 2004).

There are four basic responses that can occur following tissue injury i.e normal repair, excessive healing, deficient healing and regeneration (Maruyama et al., 2020). In a typical response, during the tissue repair process the response re-establishes equipoise between scar formation and its remodelling. However, there is a sharp contrast between the pathological response and normal repair response during the tissue repair process. For example, too much deposition of connective tissue results in excessive healing, which in turn incurs an altered structure, loss of remodelling and, thus, loss of function (Angeles et al., 2002; Maruyama et al., 2020). Fibrosis, strictures, adhesions and contractures are examples of excessive healing, while keloids and hypertrophic scars on the skin are examples of fibrosis

(Bock & Mrowietz, 2002; Jones et al., 2019). Contraction is part of the normal process of healing but, if excessive, it becomes pathologic and is known as contracture. Deficient healing is the opposite of fibrosis; the condition exists due to insufficient deposition of connective tissue matrix resulting in poor wound closure or even breakage of the tissue (Bernasconi & Nyström, 2018). Chronic non-healing ulcers are examples of deficient healing (Nedelec et al., 2000). After the loss of tissue structure and function as a result of injury, the organism, especially in lower forms of life such as the salamander and crab, has a sophisticated system that can replace the structure almost exactly as it was before the injury in a process described as regeneration (Diegelmann & Evans, 2004; Velnar et al., 2009). Due to evolutionary hurdles, mammals and higher animals have lost this capacity and can only replace a limited amount of damaged tissue through the process of regeneration (Sandoval & Maden, 2020). Organs such as the liver, epidermis and to some extent, nerves are said to be only partially regenerated in humans following injury (Diegelmann & Evans, 2004; VandenBosch & Reh, 2020).

Collagen is said to be among the most prominent and abundant proteins in animals accounting for 30% of the total protein content of the human body. Collagen is needed for the repair of tissue injury. It is said to be deposited by fibroblasts, a kind of connective tissue cell (Al-Bayaty & Abdulla, 2012). Collagen is known to provide strength, integrity and structure in normal tissue. Upon cellular disruption as a result of injury, collagen is needed to repair the defect and restore anatomic structure and function (Liu et al., 2020). However, collagen deposition has to be precise and specific, as too much collagen deposition during wound healing usually results in the loss of the normal

anatomical structure of the site, resulting in fibrosis. Conversely, insufficient deposition of collagen during wound healing results in weak tissue regeneration and the wound site may dehisce or open up (Wang & Li, 2009).

2.7.2 Wound healing phases

The normal healing response begins the moment the tissue is injured resulting in the spillage of blood components into the site (Birbrair et al., 2019). The blood platelets are stimulated to discharge clotting factors, critical growth factors and cytokines such as platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-ß) upon contact with the exposed collagen and the other extra cellular matrix. This results in the occurrence of the haemostasis phase, followed by the phagocytosis of the neutrophils (Enoch & Leaper, 2008). The released macrophages continue the synergistic phagocytotic cleaning of the wound area and secretion of PDGF and TGF-ß that causes the inflammation of the wound area. This is described as the inflammatory phase (Diegelmann & Evans, 2004).

Once the wound site has been cleaned out, the proliferative phase sets in by migrating fibroblasts and depositing a new extracellular matrix resulting in epithelisation of the wound area. The new collagen matrix becomes interwoven and cross-linked into an organized anatomical structural frame work during the final remodelling phase (Diegelmann & Evans, 2004)

Unfortunately, during pathologic conditions such as non-healing pressure skin ulcers, the systematic orderly process of wound healing is totally lost, and the ulcerogenic wounds are locked in a state of chronic inflammation due to over infiltration of neutrophils coupled with associated ROS and destructive enzymes (Dunnill et al., 2017). In such conditions, healing is expected to proceed after the

inflammation is successfully controlled. On the other hand, excessive matrix deposition and an increased density of mast cells result in poor remodelling of the wound area due to incurred fibrosis (Diegelmann & Evans, 2004).

2.7.3 Role of anti-oxidative activity and lipid peroxidation in the wound healing process

As mentioned earlier, physiological stress worsens the ulcerogenic situation, hence controlling stress could influence the amelioration of the disease. Due to the ability of antioxidants to inhibit the oxidation reaction caused by free radicals and to help scavenge some of these radicals, antioxidants have the direct effect of reducing stress in many pathologic situations. Several evaluative methods (DPPH and FRAP) that have been used to assay the antioxidant activity of different compounds *in vitro* have been reported in a few literatures (Golbabapour et al., 2013a; Halabi et al., 2014; Ismail et al., 2012; Zhang et al., 2020). These methods have been described in section 2.6, previously. Several studies have demonstrated the beneficial effects of antioxidants in ulcerogenic situations (Abdulla et al., 2010; Biradar et al., 2010; Ofusori et al., 2019). Using FRAP, Rodriguez-Naranjo et al. (2012), Çetinkaya et al. (2012), and Sethi et al (2020) evaluated the ferric antioxidant potential of different compounds. In successful wound treatment, O_2^- have been reported to be significantly reduced in the wound area, while SOD activity is described as being highly expressed (Bilgen et al., 2019). In the wound healing process, another important antioxidant enzyme is CAT, a homotetrameric oxidoreductase that catalyzes the decomposition of toxic hydrogen peroxide to oxygen molecule and water (Abdel-Mageed et al., 2012). It has been revealed that CAT is among the important factors in the prevention of inflammation and apoptosis (Floyd et al., 2001; Hajji et al., 2019). Abdel-Mageed et al. (2012)

evaluated the wound healing activity of CAT encapsulated in flexible sugar ester vesicles, and observed a significant influence of CAT activity on *in vivo* wound healing. Recently, spectrophotometric analysis at a wavelength of 560 nm was used by Sahreen et al. (2011) to assay the activity of SOD in quantifying the *in vivo* antioxidant activity of the methanolic extract of *Carissa opaca* leaves. The researchers observed a significant decrease in SOD activity upon treatment with the extract.

Among the major causes of free radical mediated injury in tissues is the lipid peroxidation of fatty acids that occur mostly in the membrane's phospholipids (Catalá, 2013). The peroxidation reaction of lipids is reported to destroy to a large extent the physicochemical properties of lipid bilayers, leading to severe cellular damage (Catalá, 2013). Lipid peroxidation cellular damage is reported to lead to the impairment of the wound healing process due to reduced expression of vascular endothelial growth factor (VEGF) in the affected tissues (Altavilla et al., 2001; Odukoya et al., 2012; (Shaikh-Kader et al., 2019). The peroxidation reaction is reported to be triggered either by the abstraction of hydrogen or the addition of an oxygen radical, which results in oxidative damage to the fatty acids (Repetto et al., 2012; Singh et al., 2020). In this process, the poly unsaturated fatty acid (PUFA) is reported to be more susceptible to lipid peroxidation than the saturated fatty acid (Repetto et al., 2012). This is because the unsaturated methylene double bonds provide a critical site of attack by weakening the C-H bond, resulting in hydrogen reduction during the peroxidation reaction (Repetto et al., 2012). This reduction in hydrogen resulted in the formation of a carbon radical which undergoes molecular rearrangement to form diene, which takes up O_2 to produce peroxyl radicals. The produced peroxyl radicals are further reported

to abstract potential hydrogen atoms from another PUFA, thus, looping the chain reaction process (Repetto et al., 2012).

Aldehydic compounds such as MDA are among the end products of this peroxidation. MDA has been shown to comprise both mutagenic and cytotoxic properties (Ma et al., 2019; Mateos et al., 2005). Its concentration is normally found to be elevated in various diseases related to free radical damage (Odukoya et al., 2012), thus becoming a biochemical marker for the lipid peroxidation that is quantified spectrophometrically in biomedical research (Mateos et al., 2005). In addition, the use of the MDA assay as an *in vivo* biomarker for oxidative stress in the rat model has been reported (Al-Bayaty & Abdulla, 2012; Kaczmarczyk-Sedlak et al., 2019, Mateos et al., 2005).

2.8 Diabetes and its complications

Diabetes mellitus over the years has been ranked as one of the leading cause of mortality (Khan et al., 2020; Wild et al., 2004). This disorder was primarily regarded as the deficit of glucose homeostasis (Oguntibeju, 2019) as a result of deficiency or flaws in the body's chemical messenger secretions and the pathway of the insulin action or both of these, that brings about a metabolism breakdown and damage in production of glucose and lipids as well as proteins (American Diabetes Association, 2010). In recent years, diabetes has been classified as type 1 (DM1) and type 2 (DM2) and also gestational diabetes. These causes the impaired function of the pancreatic islets, β-cell abnormalities, and are characterized either by the death of the pancreatic islet cell for type 1 and accelerated apoptosis of cells in type 2 diabetes (Eizirik et al., 2020). The worldwide occurrences and prevalence rate of diabetes has been approximated to be around 6.4%, and documented in 2010 to be affecting more than 285 million adults and its forecast

for 2030 has been estimated to be approximately 7.7% and affecting more than 439 million adults globally (Tamrakar et al., 2011).

Malaysia has been ranked as among the top 10 in the world for the estimation of diabetes prevalence in 2010 and 2030 at a rate of 11.6% and 13.8%, respectively among its population (Shaw et al., 2010; Sharoni & Wu, 2012). According to the figures collected in 2006 during the National Health and Morbidity Survey III, 15.5% of Malaysian general population is afflicted with chronic illnesses, and diabetes mellitus has been touted to be the second that is afflicting the population. Statistics show that four present (4%) are affected with DM out of 15.5% chronic illness of the population in the country (Amal et al., 2011). As a chronic metabolic disorder, diabetes mellitus has been documented to affect all other major body organs that eventually lead to severe complications that may result in considerably high morbidity rates as well as untimely mortality (Anichini et al., 2020). Clinical, pre-clinical and epidemiologic research has indicated a linear relationship between oxidative stress and inflammation in the development of DM2 and its related difficulties (Díaz-Gerevini et al., 2019; Donath & Shoelson, 2011; Hirsch & Heck, 2019; Incani et al., 2020).

2.9 Classification of diabetes mellitus

The World Health Organization has classified and recognized the types of diabetes mellitus as: type 1, type 2, and gestational diabetes which occurs primarily during pregnancy (World Health Organization, 1999). Type 1 diabetes mellitus comes about when the body produces insufficient or no insulin at all due to an autoimmune or non-immune damage to the insulin-producing β-cells of the islets of Langerhans (Hettiarachchi et al., 2004). In the case for type 2 diabetes mellitus, it occurs due to the incapability of the insulin that is available in the

body to work and carry out its function efficiently (Tamarai et al., 2019). It may involve multiple organs and includes abnormal amount of insulin secretions as well as peripheral tissue (muscle, adipose tissue and hepatic) insulin resistance (World Health Organization, 1999). Type 1 diabetes is classified as an insulin-dependent diabetes (IDDM) or as juvenile diabetes, whereas type 2 diabetes is acknowledged as non-insulin dependent diabetes (NIDDM) (Rughani et al., 2020; World Health Organization, 1999). Type 1 DM is usually diagnosed at the childhood stage, type 2 in middle age or late adult life and gestational diabetes during pregnancy (World Health Organization, 1999).

2.10 *In vivo* studies on animal models

The present study undertakes manipulating the accessible biological systems to help us understand the precise and in depth mechanism of targeted candidates for further drug development and enhancement. Animal models preclinical studies and research plays an important function in ascertaining and optimising the effectiveness and safe use of the biological substances. It has been recognized that for preliminary and initial screenings, animal models are altogether sensitive and receptive to be used in determining and validating the efficacy and safe use of natural biological products (Hoa et al., 2009).

2.11 Type1 diabetes in animals

In most of the recent diabetes research that has been carried out, rat models are frequently used to determine and study the possible utilization and benefits of natural biological products for management and supervision of diabetes disease progression (Saleh et al., 2020). Animals that have been induced chemically to manifest diabetes like symptoms are considered the most conventional and standard replica for mimicking type 1 or insulin dependent diabetes mellitus

(IDDM) because the chemicals selectively destroys or eliminates only the β-cells of the pancreas, leading to a decline in insulin synthesis (Ahmed, 2006; KunduSen et al., 2011) However, the incomplete loss of the β-cells mass and persistent hyperglycemia that occurs may result in insulin resistance which is a characteristic and typical feature of non-insulin-dependent diabetes mellitus (NIDDM) (Olivares et al., 2017; Shima et al., 1998). Streptozotocin (STZ) which has been classifies as a cytotoxic substance that is utilized traditionally to induce diabetes like symptoms and hyperglycemia in model animals by choosing and discriminately destroying the β-cells (Al-Awar et al., 2016; Masiello et al., 1998). STZ, which is a 1-methyl-l-nitrosourea that is affixed to the carbon-2 location causes β-cells necrosis and stimulates "experimental or chemically induced diabetes" in animal models (Al-Awar et al., 2016; Thulesen et al., 1997). The glucose moiety of STZ permits for the partisan inflow of STZ into β-cells, done probably via the glucose transporter-2 (GLUT-2) and causes the alkylation of DNA (Jin et al., 2009). STZ is an alkylating agent and triggers the breakage of DNA strands and this induces the activation of poly-ADP-ribose synthetase followed by the depletion of nicotinamide adenine dinucleotide (NAD) (Virág & Szabó, 2002).

This NAD reduction is also mediated by strong immunogenic response of cytokines, T-helper cells and macrophages (Nagy et al., 2020). Once activated, macrophages produces an array of free radicals, nitric oxide (NO) as well as IL-6 and IL-10, which has been found to activate the inducible form of nitric oxide synthase (iNOS), thus causing an amplified production of NO within the β-cells. The probable problems that may occur and are well documented with the use of STZ due to its lethal effects is not only limited to the damages that occur to the

pancreatic β-cells, but is also known to cause damage to the renal system, oxidative stress syndrome, inflammation, and endothelial dysfunction (Hyun et al., 2010).

2.12 Type 2 diabetes in animals

Animal models usually are induced with type 2 diabetes, by administering an appropriate dose of nicotinamide (NA) before the administration of the STZ, in which the NA wields an incomplete form of protection against the detrimental cytotoxic effects of STZ (Noshahr et al., 2020; Masiello et al., 1998; Szkudelski, 2012; Yang & Wright Jr, 2002). Vitamin B3 (Nicotinamide) is a water-soluble vitamin and a poly-ADP-ribose synthetase inhibitor. NA shelters the functionality and function of the β-cells by protecting the level of NAD's and proinsulin from deteriorating over time marginally (Lenzen, 2017). This in turn enriches the energy status in the ischemic tissues and demonstrates protective antioxidant effects. The process then further leads to metabolic improvement via the inhibition of the mass apoptosis of β-cell by partially reversing the inhibition of insulin secretions to arrest or halt the complete aggravation of β-cells damage following the dispensation of the STZ dose (Shima et al., 1998). This subsequently help establish and produce an experimental diabetic syndrome in rats that looks as if it is closely related to the human type-2 diabetes (DM2) than the other existing models and currently used for example neonatally STZ-injected rats, Goto-Kakizaki (GK) rats. The condition displayed with the use of NA, has a number of characteristics that is akin to DM2, and is exemplified by stable hyperglycemia levels, glucose intolerance, and drastically transformed glucose-stimulated insulin secretions (Like & Rossini, 1976).

2.13 Treatments available for diabetes mellitus patients

The deferral or delaying of diabetes affliction can be attained by consuming low-calorie diet intake, having recurring physical activity, maintaining normal body weight and shunning tobacco use (Eriksson & Lindgärde, 1991; Katz & Meller, 2014; Knowler et al., 2002; Kosaka et al., 2005; Ramachandran et al., 2006; Tuomilehto et al., 2001). However, patients only realise that they have diabetes once the symptoms have manifested or has been aggravated (Kelkar et al., 2019). Controlling one's diet is essential in preventing hyperglycemia (Israili, 2011). Drugs that comprise of anti-diabetic effects have also been documented to reduce hyperglycemia. The majority of anti-diabetic drugs utilised nowadays are "secret-agogues" which facilitates insulin discharge via the direct action on the K^+-ATP channel of the pancreatic beta cells or "sensitizers" that decreases the output of liver glucose and improves the uptake of glucose by the peripheral tissue or the enhanced production of mRNAs of insulin dependent enzyme (Kapinya et al., 2008). For instance, glibenclamide which is a well-known secretagogue helps to decrease blood glucose by invigorating the existing β-cells in the pancreas to secrete additional insulin. Metformin, which has been classified as an insulin-sensitizing drug plays a role by conquering insulin resistance in type 2 diabetes (Peters, 1995; Sekar et al., 2019). A mixture of sulfonylureas with insulin-sensitizers and insulin therapy will be prescribed if therapy with sulfonylurea fails (Turner et al., 1999). Type 1 diabetes eventually ends up with insulin therapy because functional β-cells are no longer available for insulin production (Dirr et al., 2020). As with the evidence conducted by many studies most drugs have their own side-effects and sulfonylureas are known to cause body weight to increase and hypoglycemia (Costello & Shivkumar, 2019; Eriksson et al., 2016; Van Staa et al., 1997).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Drugs, chemicals and reagents

The drugs, chemicals and reagents that were used in the study are as follows: Ethanol (95%), tween 20 (10%), concentrated formalin (38-40%), dibutylphthalate polystyrene xylene (DPX), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Merck (Germany). Sodium dihydrogen phosphate monohydrate, sodium citrate, and sodium chloride were obtained from Sigma-Aldrich (Germany). Xylazine, ketamine, nicotinamide, glibenclamide, formalin, phosphate buffer saline (PBS), and hematoxylin & eosin were purchased from Sigma-Aldrich (UK). Streptozotocin was purchased from Merck Millipore (USA). Acacia Arabic gum, sodium pyruvate/L, glucose/L, L-glutamine, 2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride (FeCL3.6H2O), sodium acetate trihydrate, glacial acetic acid, ferrous sulfate heptahydrate (FeSO4.7H2O), periodic acid Schiff (PAS), gallic acid (GA), and ascorbic acid (AA) were approached from Sigma Aldrich (USA). Omeprazole was purchased from pharmacy of Universiti Malay Medical Centre (UMMC).

3.1.2 Kits

Some of the commercially available kits used in this study included:

Malonialdehyde (MDA): from Cayman Chemical Co., USA, cat. #10009055

Catalase (CAT): from Cayman Chemical Co., USA, cat. #707002

Superoxide dismutase (SOD): from Cayman Chemical Co., USA, cat. #706002

Prostaglandin E2 (PGE2): from Cayman Chemical Co., USA, cat. #703202

Glutathione peroxidase (GPx): from Cayman Chemical Co., USA, cat. #703202

Rat Insulin ELISA kit: from ER1113, Wuhan Fine Biological Technology Co., Ltd., China

3.1.3 Equipment

Automated tissue processor, Leica Tissue Microtome (Leica, Germany), Homogenizer (DAIHAN Sci., Seoul, Korea), Jouan C312 centrifuge (Santa Fe Springs, CA, USA), one-touch glucometer (Accu-Chek Performa, Roche, Mannheim, Germany), hot air oven (Venticell, MMM, Einrichtungen, Germany), Rotofix 32 refrigerated centrifuge (Hettich Zentrifugen, Germany), and SPSS version 24 (SPSS Inc, Chicago, IL, USA). Power wave X 340 ELISA plate reader from BIO-TEK instruments (Winooski, VT, USA).

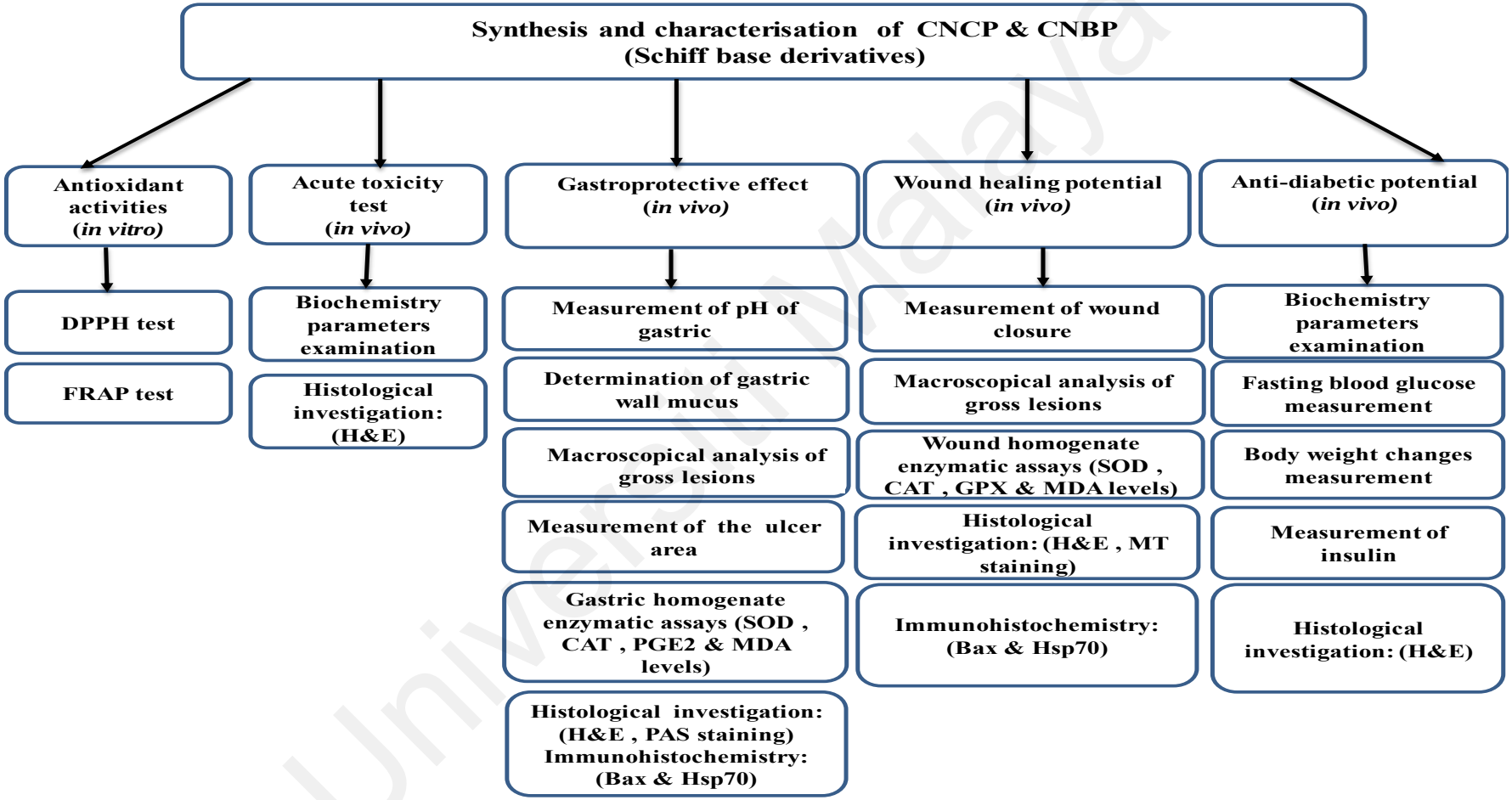

Figure 3.1: Flowchart of the research methodology

3.2 Schiff base derivatives

Two Schiff base derivatives namely 2, 2-[1,2-cyclohexanediylbis (nitriloethylidyne)] bis(4-chlorophenol) (CNCP) (Figure 3.2) and 2,2-[1,2-cyclohexanediylbis (nitriloethylidyne)]bis(4-bromophenol) (CNBP) (Figure 3.3) were obtained from Prof. Dr. Hapipah Mohd Ali (Department of Chemistry, Faculty of Science, Universiti Malaya, Malaysia).

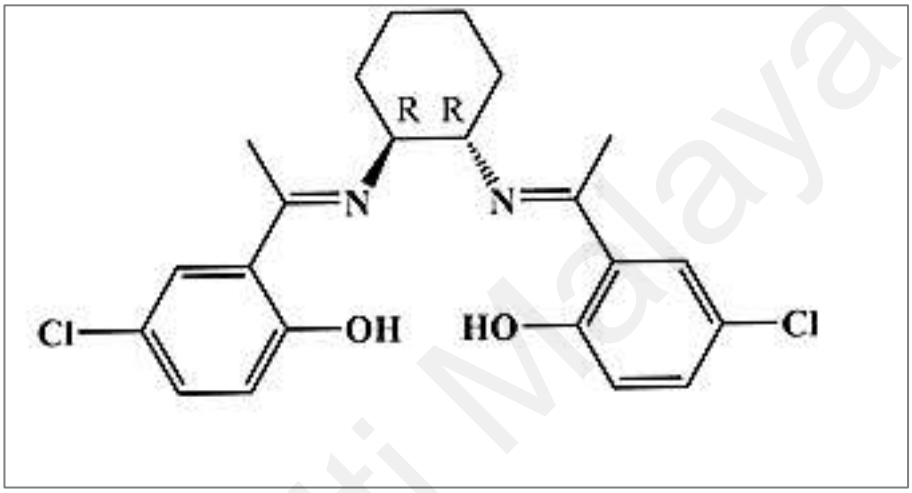

Figure 3.2: Chemical structure of Schiff base CNCP

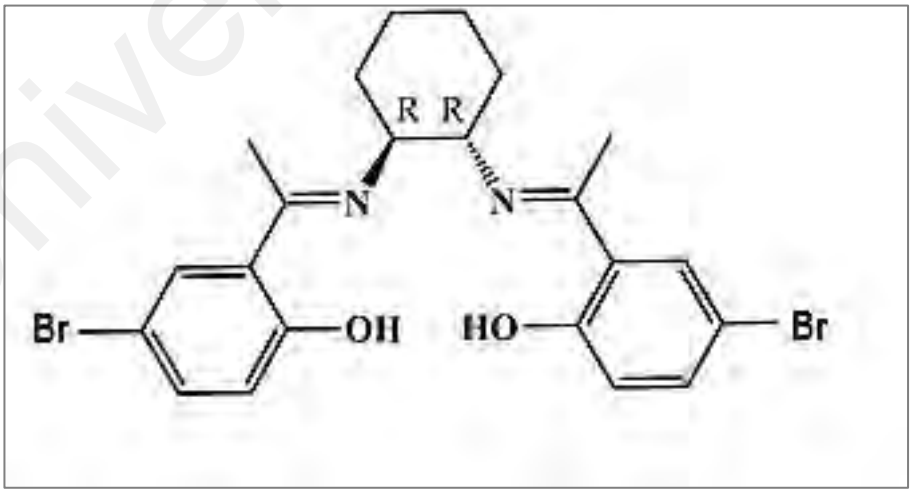

Figure 3.3: Chemical structure of Schiff base CNBP

3.3 Synthesis and characterizations of Schiff base derivatives

3.3.1 2,2'-[1,2-cyclohexanediylbis(nitriloethylidyne)]bis[4-chlorophenol] (CNCP) Schiff base derived

The Schiff base derivative of 2, 2-[1,2-cyclohexanediylbis (nitriloethylidyne)] bis (4-chlorophenol) was prepared as previously described by Yaul et al. (2013). A solution of trans-1,2-diaminocyclohexane (3.0 g, 26.27 mmol) in methanol (80 ml) was reacted by 5-chloro-2-hydroxyacetophenone (8.96 g, 52.54 mmol) under reflux for 6 hours. Following cooling to room temperature, a yellow hard crystal was formed. This crystal was washed with methanol, filtered and finally dehydrated with phosphorus pentoxide. The final product was recrystallized from ethanol to provide CNCP (8.82 g, 80%) as yellow crystal (Figure 3.4). m.p. 228-230 °C.IR [KBr]: 3500 cm^{-1} (OH), 3010 cm^{-1} ($CH_{aromatic}$), 2937, 2861 cm^{-1} ($CH_{aliphatic}$), 1609 cm^{-1} (C=N), 1565 cm^{-1}(C=C), 1256 cm^{-1}(C-N). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.34 (d, 2H, ^{3}J= 2.6 Hz, 2x Ar-H), 7.16 (dd, 2H, ^{3}J= 8.8 Hz, 2x Ar-H), 6.79 (d, 2H, ^{3}J= 8.8 Hz, 2x Ar-H), 3.85 (dt~m_c, 2H, 2x CH-N), 2.25 (s, 6H, 2x CH_3), 1.9 (t, 4H, ^{3}J= 9.5 Hz, 2x CH_2-CH), 1.67 (p~m_c, 2H, CH_2), 1.48 (p~m_c, 2H, CH_2).^{13}C NMR (100 MHz, $CDCl_3$): δ 170.17 2x (C=N), 162.28 2x (Ar-OH), 132.40, 127.90 2x (CH_{Ar}) 121.84 2x (Ar-Cl), 120.09 2x (CH_{Ar}), 119.9 2x ($\underline{C}_{Ar}$-CN), 63.27 2x (CH-N), 32.32, 24.19 2x (CH_2CH_2), 14.55 2x (CH_3).

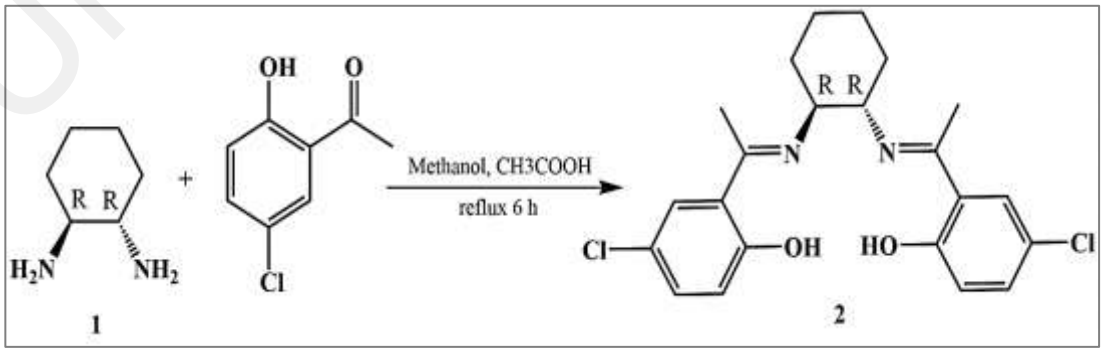

Figure 3.4: Synthesis of the Schiff base derivative of 2,2'-[1,2-cyclohexanediylbis (nitriloethylidyne)]bis[4-chlorophenol] (CNCP)

3.3.2 2,2'-[1,2-cyclohexanediylbis(nitriloethylidyne)]bis(4-bromophenol) (CNBP) Schiff base derived

The Schiff base derivative of 2, 2'-[1, 2-cyclohexanediylbis (nitriloethylidyne)] bis (4-bromophenol) (CNBP) was prepared according to Yaul et al. (2013). A solution of trans-1,2-diaminocyclohexane (2.5 g, 21.9 mmol) in methanol (70 ml) was reacted with 5-bromo-2-hydroxyacetophenone (9.42 g, 43.8 mmol) in the presence of catalytic amount of acetic acid under reflux condition for 6 hours. After cooling to ambient temperature, a yellowish green solid was formed via filtering, washed with methanol, and finally dried over phosphorus pentoxide (Figure 3.5). The yellowish green crystals were recrystallized from ethanol to obtain CNBP (8.45 g, 76%), m.p. 220-222 °C. IR [KBr]: 3500 cm^{-1} (OH), 3020 cm^{-1} ($CH_{aromatic}$), 2940, 2860 cm^{-1} ($CH_{aliphatic}$), 1605 cm^{-1} (C=N), 1560 cm^{-1}(C=C), 1256 cm^{-1}(C-N); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.60/(7.48) (d, 2H, ^{3}J= 2.4 Hz, 2x Ar-H), 7.30/(7.29) (dd, 2H, ^{3}J= 8.9 Hz, 2x Ar-H), 6.77/(6.75) (d, 2H, ^{3}J= 8.9 Hz, 2x Ar-H), 4.60/(3.85) (m_c, 2H, 2x CH-N), 2.32/(2.25) (s, 6H, 2x CH_3), 1.9 (t~m_c, 4H, 2x CH_2-CH), 1.79-1.57 (m_c, 2H, CH_2), 1.48 (p~m_c, 2H, CH_2).^{13}C NMR (100 MHz, $CDCl_3$): δ 170.16 (169.95) 2x (C=N), (163.01) 162.83 2x (Ar-OH), 135.24 (135.16), 130.87 (130.70) 2x (CH_{Ar}) 120.82 (120.78) 2x ($\underline{C}_{Ar}$-CN) 120.62 (120.51) 2x (CH_{Ar}), 108.79 (108.57) 2x (Ar-Br), 63.21 (59.61) 2x (CH-N), 32.33 (29.81), 24.19 (22.29) 2x (CH_2CH_2), 14.57 (14.52) 2x (CH_3).

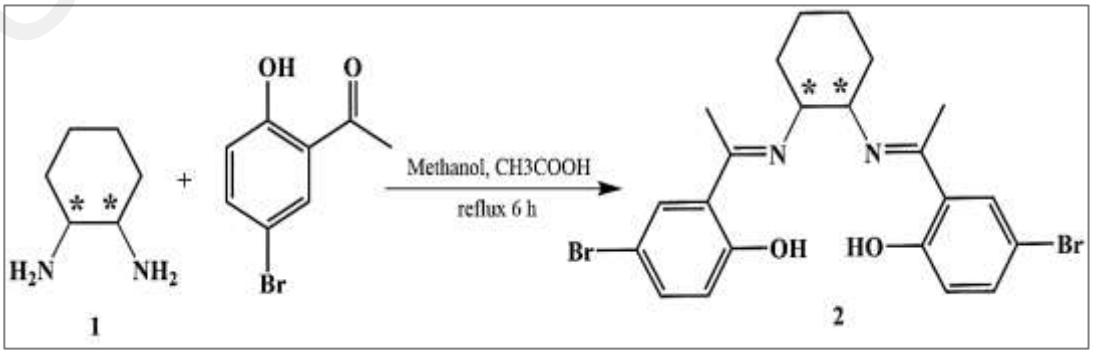

Figure 3.5: Synthesis of the Schiff base derivative of 2,2'-[1,2-cyclohexanediylbis (nitriloethylidyne)] bis (4-bromophenol) (CNBP)

3.4 *In vitro* study

3.4.1 2,2-diphenyl-1-picrylydrazyl (DPPH) radical scavenging activity

The CNCP and CNBP were examined for the scavenging activity to the steady free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Merck) taking ascorbic acid as a reference (Qader et al., 2012). A total of 0.6 ml DPPH radical solution (0.004 g of DPPH reagent in 100 ml methanol) was added to 100 µl of diluted stock solution (DPPH reagent), followed by incubation in the dark for 25 min (at room temperature). Lastly the absorbance was recorded at 517 nm (each 20 min for 2 hours). The % DPPH slowdown was estimated by the addendum formula where A_0 is the absorbance of the control and A_1 is the absorbance of the specimen:

$$\textbf{DPPH scavenging activity}\ (\%) = (\mathbf{A_0} - \mathbf{A_1}) / (\mathbf{A_1}) \times \mathbf{100}$$

3.4.2 Ferric reducing antioxidant power (FRAP) test

The ferric dropping activity of Schiff base derivatives was investigated. This test is a famous technique for calculating the total antioxidant power (Griffin & Bhagooli, 2004). Reduction of the ferric tripyridyl triazine (Fe III TPTZ) complex to its ferrous form (intense blue color) at low pH can be monitored by measuring the absorbance change at 593 nm (0 and 4 min). 300 mmol/l sodium acetate buffer, pH 3.6 (100 ml H_2O_2, 1.6 ml glacial acetic acid, 0.31 g sodium acetate trihydrate) and ferric chloride were added (20mM) to the complex followed by addition of tripyridyl triazine (TPTZ; 0.0625 g in 40 mM HCl) and then mixed in a ratio of 10:1:1 to prepare of FRAP reagent. 10 µl of both compounds were added to 300 µl of FRAP reagent. Gallic acid and ascorbic acid were used as the control. The FRAP value was estimated by the addendum formula where Y is absorbance of the spectrophotometer:

$$\mathbf{FRAP} = \frac{(\mathbf{0-4\,min\ \Delta A593\ nm\ of\ test\ sample})}{(\mathbf{0-4\,min\ \Delta A593\ nm\ of\ test\ standard})} \times [\mathbf{standard}]\ (\mu\mathbf{M}) \times \mathbf{Y} \times \mathbf{1000}$$

3.5 *In vivo* study

3.5.1 Experimental animals and ethical statement

One hundred and fifty healthy *Sprague Dawley* (SD) rats (6-8 weeks old, 200-250 g) were purchased from the animal experimental unit (AEU), Faculty of Medicine, Universiti Malaya. The SD rats were housed in plastic cages under standardized environments (23 ± 2°C, 12 hours light/ 12 hours dark cycle) and permitted to access free to water, *ad libitum* and standard chow pellets. The rats were acclimatized for 24 h before the experiments without any access to food. The experimental processes including the protocols in this study were approved by the Ethics Committee of the Research Centre and in accordance with the recommendations of the Universiti Malaya;

Council on Animal Care Guidelines for the proper care and use of laboratory animals (Ethic no. 2015-09-11/BMS/R/MAA).

3.5.2 Acute toxicity test

The acute oral toxicity study was conducted according to the "fixed dose" method of OECD (Organization for Economic Co-operation and Development) Guideline No.420 (OECD, 2002). The experimental design of the acute toxicity study is shown in the Table 3.1. Thirty female SD rats were divided equally into five groups: a control group and four treatment groups. Overnight fasted rats were administered orally with low dose (100 mg/kg) and high dose (200 mg/kg) of CNCP and CNBP in 10% Tween 20, respectively. The control group and compounds (CNCP and CNBP) were administered in a single dose at a rate of 5 ml/kg. The control group received only the vehicle (10% Tween 20). Female rats were chosen because it is the most sensitive gender (especially for systemic toxicity) to see the effect of treatment (OECD, 2001; Lipnick et al., 1995). After administration of compounds, the animals were kept under observation at least once during the first 30 min, 2 h, 3 h, 24 h and daily for 14 days for any behavioral changes, toxicological symptoms or mortality (AlRashdi et al., 2012a; Mahmood et al., 2011). At the end of the experiments on the 15 days, the rats were then anaesthetized with ketamine (30 mg/kg) and xylazine (3 mg/kg) through an intramuscular (IM) injection and the blood samples were collected via cardiac puncture for serum biochemistry analysis. Then, the rats were euthanized with an overdose of xylazine and ketamine (Shakir et al., 2014). The liver and kidney were collected and preserved for histopathological study.

Table 3.1: Experimental design of acute toxicity of CNCP and CNBP

Species		*Sprague Dawley* rats		
Age	Weight	6-8 weeks	200-250 g	
Number of animals		6 of female rats per dose level, for each compounds		
Dosage		**Vehicle group** 10% tween 20	**Low dose groups** 100 mg/kg CNCP 100 mg/kg CNBP	**High dose groups** 200 mg/kg CNCP 200 mg/kg CNBP
Observation period		14 days		

3.5.3 Induction of gastric ulcer

The experimental design of gastric ulcer of the compounds is shown in Table 3.2. The animals were divided into seven groups of six rats. After an overnight fasting (food but not water), the normal and ulcer control groups received 5ml/kg of 10% Tween 20. The reference group was given 20 mg/kg omeprazole (5 ml/kg) and the experimental groups were administrated with low and high doses of CNCP and CNBP (10 and 20 mg/kg, respectively). All the treatments were fed to the rats via oral gavage. In order to induce stomach injury, the other six groups, except the normal group, were exposed to oral gavage of absolute alcohol after one hour. After additional hour, all stomachs were immediately removed for further analysis by euthanasia of the animals with an overdose of xylazine and ketamine (AlRashdi et al., 2012a, 2012b; Marhuenda et al., 1993).

Table 3.2: Experimental design of gastric ulcer of CNCP and CNBP

Group	Normal control	Ulcer control	Reference control	Low dose CNCP	High dose CNCP	Low dose CNBP	High dose CNBP
Number of rats	6	6	6	6	6	6	6
Oral administration (5ml/kg)	10% tween 20	10% tween 20	20 mg/kg Omeprazole	10 mg/kg	20 mg/kg	10 mg/kg	20 mg/kg
After 1 hour							
Oral administration (5ml/kg)	10% tween 20	Absolute alcohol	Absolute alcohol	Absolute alcohol	Absolute alcohol	Absolute alcohol	Absolute alcohol

3.5.3.1 Assessment of gastric fluid acidity

The stomachs were opened along the greater curvature and the gastric juices were collected in the separate labeled tubes. The contents of each stomach was centrifuged at 3000 rpm for 10 min and the supernatant was assessed for pH measurement via digital pH meter titration, using 0.1 N NaOH solution (Ketuly et al., 2011).

3.5.3.2 Determination of gastric wall mucus (GWM)

Gastric wall mucus (GWM) determination was done based on the method developed by Corne. (1974). The glandular portions of the stomachs were firstly weighed and immediately mixed with 10 ml of 1% (w/v) Alcian blue staining solution (0.16 M of sucrose solution and 0.5 ml, sodium acetate, pH 5). After 2 hours, the excessive dye was washed away by two times rinse with 10 ml of 0.25 M of sucrose. Then, the Alcian blue dye attaching to the stomach wall mucus was completely removed by incubation with 10 ml of 0.5 M of magnesium chloride for 30 min. About 4 µl of the product was mixed with 4 ml of ethyl ether and shaken for 2 min, followed by centrifugation at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 598 nm and the amount Alcian blue extracted from 1 g of glandular stomach tissue was calculated using the formula as described previously (AlRashdi et al., 2012a).

3.5.3.3 Measurement of the ulcer area

The length and width (mm) of each haemorrhagic lesions of the animals were measured using a planimeter (10 × 10 mm^2=ulcer area) individually under a dissecting microscope with a magnification of 1.8X. The sum of the areas of the lesions for every stomachs was used for calculating the ulcer area (UA) (Bardi et al., 2011). The UA was calculated using the following formula (Robert et al., 1984).

$$(\mathbf{UI}\ \%) = \frac{\mathbf{UA\ of\ negative\ control - UA\ of\ treated}}{\mathbf{UA\ of\ negative\ control}} \times \mathbf{100}$$

Where **UI** is the ulcer inhibition.

3.5.4 Antioxidants activity of ethanol-induced gastric ulcers in SD rats

3.5.4.1 Gastric homogenate preparation

The tissues were homogenized according to a method described by Sidahmed et al. (2013a). Homogenization of tiny segments of glandular portion of each stomach was done in 50 mM PBS (pH=7.2) at 4°C with a teflon homogenizer, followed by centrifugation at 4500 rpm for 15 min. The supernatant was used for measuring the proteins concentration, activities of catalase (CAT) and superoxide dismutase (SOD), the levels of malondialdehyde (MDA) and prostaglandin E2 (PGE2).

3.5.4.2 Measurement of stomach's proteins concentration

Protein concentration of the homogenate stomach tissues (1 mg/ml) was measured according to the Biuret reaction (Gornall et al., 1949).

3.5.4.3 Superoxide dismutase (SOD) activity assay

The activity of gastric SOD was evaluated using superoxide dismutase assay kit from Cayman Chemicals. This assay was performed based on the instruction which was provided by the manufacturer. SOD activity was expressed as U/mg protein, where one unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radicals. 10 µL of the sample was mixed with 200 µL of diluted radical detector in 96-well plate and the reaction was started by adding 20 µL of diluted xanthine oxidase and after 20 min of incubation, the absorbance was read at 440-460 nm.

3.5.4.4 Catalase (CAT) activity assay

The activity of the gastric's CAT was evaluated using the catalase assay kit from Cayman Chemicals. 20 µL of the samples were mixed with 20 µL of catalase followed by addition of 20 µL of dilute hydrogen perioxide to start the reaction. After 20 min incubation, the reaction was terminated by adding 30 µL of diluted potassium hydroxide at room temperature for 10 min. 10 µL of catalase potassium periodate was added to the samples and left for another 5 min incubation, before reading the absorbance at 540 nm. The base of the assay is the reaction of CAT with methanol leading to production of H_2O_2 formaldehyde which is measured colorimetrically using 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazol (purpald) as the chromogen. CAT activity was expressed in U/mg protein, which one of the units can be defined as the amount of enzyme that causes formation of 1.0 nmol of formaldehyde per min at 25°C.

3.5.4.5 Prostaglandin E2 (PGE2) level assay

The level of gastric PGE2 was assayed using prostaglandin immunoassay kit. The PGE2 was assayed in pre-coated 96-well plate with the antibody which was specific for

rat's PGE2. The gastric PGE2 level was interpolated from the standard curve of the serially diluted stock solution (300 pg/ml) and the absorbance was read at 450 nm.

3.5.4.6 Membrane lipids peroxidation (MDA) level assessment

Malondialdehyde **(**TBARS) assay kit was applied to measure MDA levels (mmol/g protein). 100 µl was pipetted from each sample and mixed with 100 µl of SDS solution and 4 ml of the color reagent. The samples were kept in a water bath at 100°C for one hour, followed by immediate transferring to ice bath to halt the reaction for 10 min. It was then centrifuged at 1,600 × g and 4°C and the absorbance was read at 532 nm.

3.5.5 Histology of gastric epithelium

3.5.5.1 Routine hematoxylin and eosin staining

Phosphate buffered formalin (10%) was used to fix specimens of the stomach's wall at ambient temperature. Then samples were subject to tissue-processing (dehydration, clearance, and infiltration with paraffin) on a tissue-processing machine, followed by paraffin-embedding. The stomach tissues were sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin (H&E) for further histological study (Abdulla et al., 2010).

3.5.5.2 Evaluation of gastric mucosal glycoproteins

To observe gastric epithelial mucus secretion and also to evaluate alteration in either acidic or basic glycoproteins, the sections of the stomach's walls (glandular portion) were stained with periodic acid Schiff (PAS) stain (Nordin et al., 2014).

3.5.6 Evaluation of immunohistochemically stain of Bax and HSP70

Commercial immunohistochemical reagents (Dakocyomation, USA) was used to perform immunohistochemical staining of Bax and HSP70 proteins according to the literature findings (Hajrezaie et al., 2012a). Concisely, hot air oven was used to heat the

tissue section slides at 60°C for approximately 25 min. Xylene was then used to de-paraffinize the sections and alcohol was used for rehydration. Then by boiling the samples in 10 mM sodium citrate buffer in a microwave, the antigens were recovered. 0.03% hydrogen peroxide containing sodium azide was used to block the production of endogenous peroxidase. Using a washing buffer the tissue sections were washed gently, then incubated for 15 min with HSP70 (1:500) (monoclonal, Abcam cat #ab2787, USA) or Bax (1:200) (polyclonal, Abcam cat #ab7977, USA) biotinylated primary antibodies. The tissue sections were rinsed with washing buffer and placed in the buffer bath. Finally, the tissue sections were placed in a humidified chamber.

Consequently, the sections were incubated 15 min in Streptavidin-HRP (streptavidin conjugated to horseradish peroxidase in PBS containing an anti-microbial agent). After which, the tissue sections were rinsed gently using the wash buffer and placed in the buffer bath before being incubated for 5 min with DAB-substrate-chromogen. The sections were then again gently rinsed and counterstained with hematoxylin for 5 sec, then dipped in weak ammonia (0.037 M/L) 10 times, and finally rinsed with distilled water and then mounted with cover slips. Immuno-histochemical staining of positive sections results is seen as brown coloration under light microscope.

3.5.7 Excision wound model

The acute wound healing activity of CNCP and CNBP was tested on uninfected excisional wounds of SD rats. The animals (n=6) were randomly divided into six groups. Each of the rats was anaesthetized with general anesthesia ketamine (30 mg/kg, 100 mg/ml) and xylazine (3 mg/kg, 100 mg/ml) by intramuscular (IM) injection. The hair at the dorsal region of rats was cleanly shaved with an electrical clipper. Then, the skin area was sterilized with 70% alcohol and anaesthetized with local anaesthetic 2% lignocaine. An oval shaped wound was ear-marked and then a uniform wound in the

size of 500 mm^2 with 2 mm depth was cut from the nape of dorsal neck of each rat using a sterile surgical scissors (Figure 3.6). All procedures were carried out carefully with no constant tension to the skin, avoiding any damage to the muscle layer. The instruments used were disinfected with alcohol (70%) after procedures. Following the creation of the injuries, they were left open and the area of the wounds was measured by using transparent paper on the same day (day 0) (Rollas et al., 2002). Then, the wounded rats were treated with the samples as follows:

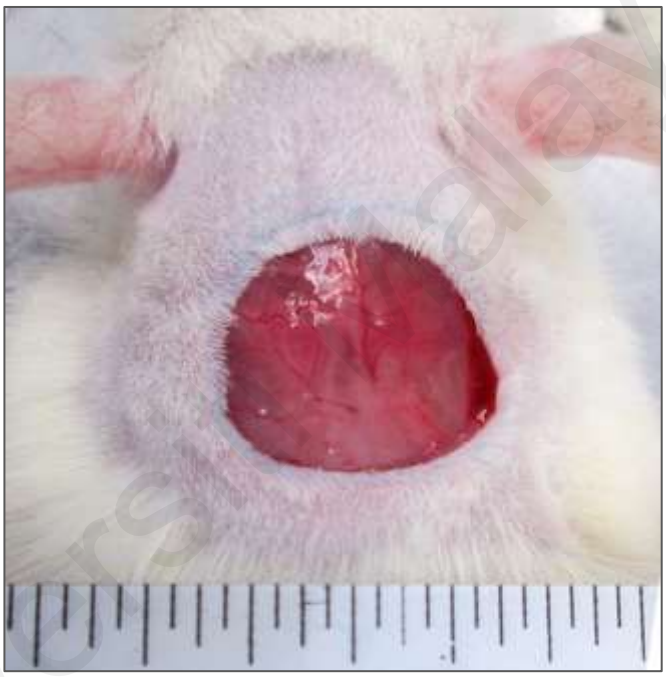

Figure 3.6: Excisional skin wound on day 0, before starting treatments

Group 1: Vehicle control group was treated with 0.2 ml of gum acacia in normal saline

Group 2: Positive control group was treated with 0.2 ml of intrasite gel

Group 3: Testing group was treated with 0.2 ml of CNCP (10 mg/ml, LD)

Group 4: Testing group was treated with 0.2 ml of CNCP (20 mg/ml, HD)

Group 5: Testing group was treated with 0.2 ml of CNBP (10 mg/ml, LD)

Group 6: Testing group was treated with 0.2 ml of CNBP (20 mg/ml, HD)

All treatments were administrated with the samples twice a day, after 24 hours of wound creation (Mahmood et al., 2010a). The wounds were monitored daily throughout the works and animals were euthanized after day 10.

Table 3.3: Experimental design of wound healing of CNCP and CNBP

Wound induction						
Group	Vehicle control	Reference control	Low dose CNCP	High dose CNCP	Low dose CNBP	High dose CNBP
Number of rats	6	6	6	6	6	6
Applied topically (0.2 ml)	Gum acacia	Intrasite gel	10 mg/ml	20 mg/ml	10 mg/ml	20 mg/ml
Duration (days)	10	10	10	10	10	10

3.5.7.1 Wound closure measurement

Wound closure area (mm^2) of the skin of each animal was measured by checking the wounds on days 5 and 10, using a permanent marker and transparent papers. Percentage of wound contraction of the healings were determined by calculating the percentage of the wound decrease from the original wound using the following formula (Hajiaghaalipour et al., 2013).

$$\%\ \textbf{Wound closure} = \frac{\textbf{Wound area on day 0} - \textbf{Wound area on day n}}{\textbf{Wound area on day 0}} \times \textbf{100}$$

n = number of days (5 and 10) that the healed area was read

3.5.8 Histological evaluation of the healed wounds

The rats were euthanized, using an overdose of ketamine (30 mg/kg, 100 mg/ml) and xylazine (3 mg/kg, 100 mg/ml) on day 10. Specimens of the skin from the healed wounds and the surrounding tissues were cut from each rat. The healed wound area (granulation tissue) was slowly soaked into 10% formalin buffer for using tissue processing machine. Each tissue (5μm thickness) was then stained for microscopic analysis by hematoxylin & eosin (H & E) and Masson trichrome (Majtan et al., 2013).

3.5.8.1 Masson's Trichrome stain

Masson's trichrome staining techniques was conducted according to manufacturer's protocol (Dakocytomation, USA) and used to evaluate the collagen in 5 μm sections of healed skin. The stained sections were examined under a light microscope to observe the morphology of fibroblasts, collagen deposition, angiogenesis and epithelisation. The stain preparations and techniques are described in more detail in Appendix C.

3.5.9 Immunohistochemistry

Immunohistochemistry analyses of Bax and Hsp70 were carried out as the procedure described in the published study (Hajrezaie et al., 2012b). The wound sections slides were heated at 60°C with hot air oven for approximately 25 min, followed by deparaffinization, using xylene and then rehydration with alcohol. The resulted samples were boiled in 10 mM sodium citrate buffer for recovering the antigens. Hydrogen peroxide (0.03%) containing sodium azide was applied for blocking production of endogenous peroxidase. The wound tissue sections were gently washed with washing buffer followed by incubation with HSP70 (1:500) (monoclonal, Abcam cat #ab2787, USA) or Bax (1:200) (polyclonal, Abcam cat #ab7977, USA) biotinylated primary antibodies for 15 min. The wound sections were then rinsed with the wash buffer and put in buffer bath and then placed in humidified chamber. The resulted sections were

incubated in Streptavidin-HRP (streptavidin conjugated to horseradish peroxidase in PBS composing of anti-microbial agent) for 15 min. The tissue sections were then rinsed with wash buffer and put into buffer bath followed by incubation with DAB-substrate-chromogen for 5 min. Washing the wound sections were continued with hematoxylin for another 5 sec followed by dipping in weak ammonia (0.037 M/L) for 10 times, and finally rinsed with distilled water. The slides were then observed under light microscope which showed brown color specks presenting positive immunohistochemically staining.

3.5.10 Tissue homogenate preparation

The tissue specimens were excised from the wounds after 10 days of the surgery, homogenized in 1.15% calcium chloride (1:5 w/v) using a Teflon homogenizer. Homogenate was processed on ice at concentration of 10% (w/v) in potassium phosphate buffer (50 mM, pH 7.8) composing of mammalian protease inhibitors. The homogenized tissues are centrifuged at 4500 rpm and 4 $^{\circ}$C for 15 min.

3.5.10.1 Enzymatic activities

Activities of GPx, SOD and CAT were measured as prescribed in the manufactured kits (Cayman, USA).

3.5.10.2 Lipid peroxidation

Lipid peroxidation (MDA levels) was determined in wound tissue homogenate using the TBA reaction (Wills, 1966). TBA reaction measures the level of MDA as the product of lipid peroxidation, results of which is expressed as nmol of MDA formed/mg protein.

3.5.11 Type 2 diabetes induction

Type 2 diabetes induced in the animal model was performed based on the protocol by Arya et al. (2012). The experimental design of anti-diabetic effect of CNCP and CNBP is shown in Table 3.4. STZ was dissolved freshly in 0.05 M citrate buffer (pH 4.5) whilst nicotinamide was dissolved in normal saline. Briefly, male SD rats were fasted overnight before injection with a single intraperitoneal (i.p) injection of nicotinamide (210 mg/kg) for 15 min followed by STZ (60 mg/kg). After 48 hours, the blood glucose level was analyzed using a glucometer taken from the tail vein of rats. The animals with higher than 200-mg/dl of fasting blood glucose were considered diabetic and were used for this experiment (Bedia et al., 2006). The diabetic SD rats were randomly assigned into 7 groups and treated with CNCP and CNBP as shown in Table 3.4. The blood glucose levels as well as body weights were measured weekly. On day 45, the testing animals were then anaesthetized with ketamine (30 mg/kg) and xylazine (3 mg/kg) through an intramuscular (IM) injection and the blood samples were collected via cardiac puncture for biochemical parameters analysis. Then, the rats were euthanized with an overdose of xylazine and ketamine. The liver, kidney and pancreas were collected and preserved for histopathological study.

The rats were assembled into seven groups (each group had six animals) and oral gavaged with CNCP and CNBP for 45 days as follows:

Group 1: Normal (vehicle) control rats (fed with 10% tween 20)

Group 2: Diabetic control rats (fed with 10% tween 20)

Group 3: Diabetic control reference rats (fed with glibenclamide (600 µg/kg)

Group 4: Diabetic rats 1 (treated with CNCP (10 mg/kg, LD))

Group 5: Diabetic rats 2 (treated with CNCP (20 mg/kg, HD))

Group 6: Diabetic rats 3 (treated with CNBP (10 mg/kg, LD))

Group 7: Diabetic rats 4 (treated with CNBP (20 mg/kg, HD))

Table 3.4: Experimental design of anti-diabetic effect of CNCP and CNBP

Induction of Diabetes mellitus type 2, except normal control group							
Group	Normal control	Diabetic control	Reference control	Low dose CNCP	High dose CNCP	Low dose CNBP	High dose CNBP
Number of rats	6	6	6	6	6	6	6
Oral administration (5ml/kg)	10% tween 20	10% tween 20	(600 µg/kg) Glibenclamide	10 mg/kg	20 mg/kg	10 mg/kg	20 mg/kg
Duration (days)	45	45	45	45	45	45	45

3.5.12 Biochemical analysis

3.5.12.1 Biochemical parameters examination

The clotted blood samples were separated by centrifugation at 2500 rpm for 15 min. The blood serum was assessed spectrophotometrically using an automated standardized technique based on the procedure described in the laboratory manual of the Central Diagnostic Laboratory, Universiti Malaya Medical Centre for evaluating renal and liver function, as well as lipid profile.

3.5.12.2 Fasting blood glucose measurement

At day 1, 7, 15 and 45, concentrations of the fasting blood glucose were determined using an Accu-check Advantage II Glucometer and compatible blood glucose test strips. The percentage of variation of glycaemia was calculated based on the formula below; where X was considered separately for day 7, 15 or 45 (Küçükgüzel et al., 2005).

$$\% \textbf{ Variation of glycaemia } = \frac{\textbf{Day X} - \textbf{Day 1}}{\textbf{Day 1}} \times \textbf{100}$$

3.5.12.3 Body weight changes measurement

Body weight (g) of the rats was measured by a digital weight scale before and after the experiments. The body weight changes were calculated via the following formula:

$$\frac{\textbf{Body weight (Day 45)} - \textbf{Body weight (Day 1)}}{\textbf{Body weight on Day 1}} \times \textbf{100}$$

3.5.12.4 Measurement of insulin

The assay to measure insulin level was based on the sandwiched ELISA principle using a commercially available ELISA assay kit (Rat Insulin ELISA kit). Different serials concentrations (0.2, 0.5, 1, 2, 5, 10 ng/ml) of rat insulin standards, control and also samples at equal volume of 10 µl were added into each well. 80 µl detecting antibody was added to each well and then the plate incubated at an ambient temperature for 2 hour on a micro-titer plate shaker with a speed of 400-500 rpm. After incubation, the solution was aspirated from the wells and any residuals were removed by gently tapping and then rinsed with the prepared washing buffer. 100 µl of enzyme solution was added to every well followed by a half hour incubation period at room temperature on a micro-titer plate shaker with moderate shaking. Washing buffer was used to wash the coated wells and then a 100 µl of substrate solution was added to each well followed by shaking on micro-titer plate shaker for 15 min. At this stage, after adding 100 µl stop solutions, the blue color observed in the standard wells changed to yellow color. The absorbance was read at 450 nm within 5 min.

3.5.13 Histological study of diabetic tissues

3.5.13.1 Routine hematoxylin and eosin staining

The samples of kidney, liver and pancreas tissues were fixed in 10% buffered formalin, and then were administered in paraffin tissue-processing device, followed by being embedded in paraffin blocks. The pieces (5μm) were stained by hematoxylin and eosin (H&E) which was considered as standard staining to assess tissue architecture. The stained samples were used for microscopic analysis (Rollas et al., 2002).

3.6 Statistical analysis

All data are accessible as mean ± SEM. Differences among the experimental groups were determined by one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons using SPSS version 24. Values of $^{*}p < 0.05$ were considered as significant.

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CHAPTER 4: RESULTS

4.1 Antioxidant properties of CNCP and CNBP evaluated by DPPH assay

The free radical scavenging activity of CNCP and CNBP were evaluated. As shown in Figure 4.1, the highest scavenging effect was observed in CNCP followed by CNBP with IC_{50} values of 29 µg/ml and 30.86 µg/ml, respectively. However, the standard (ascorbic acid: 8.84 µg/ml) revealed better scavenging ability than these compounds. The lower value of IC_{50} signify a stronger ability of the compounds to act as DPPH scavenger, as lesser scavengers were required to achieve 50% scavenging reaction. Hence, CNCP has a better ability to scavenge the stable DPPH radical than CNBP.

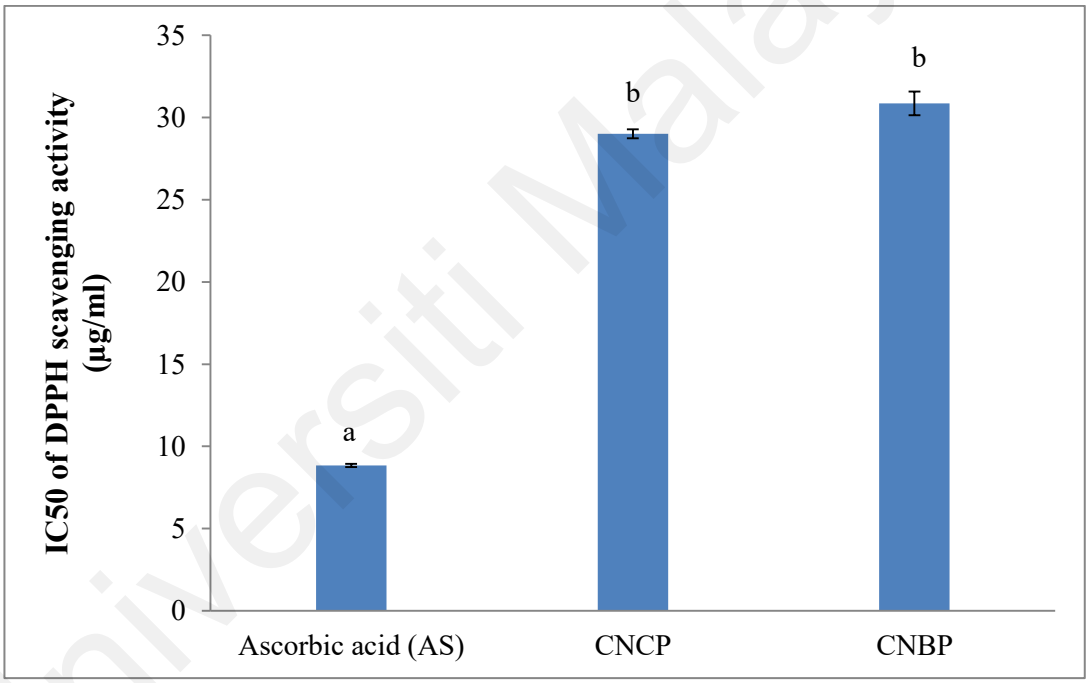

Figure 4.1: The IC_{50} of DPPH scavenging activity of CNCP and CNBP together with the standard ascorbic acid (AS). All the values were analysed as Mean ± SEM (n=3). Ascorbic acid (AS) were used as standard. Different letters indicate significant differences between groups at $p< 0.05$.

4.2 Antioxidant properties of CNCP and CNBP evaluated by FRAP assay

The antioxidant capacity of CNCP and CNBP were evaluated by the ability of the antioxidants in the compounds to reduce ferric iron (Fe^{+3}) to ferrous form (Fe^{+2}). As shown in Figure 4.2, the FRAP values of CNCP and CNBP (536.9 ± 11.89 and 628.8 ± 19.33 µmol Fe (II)/g), in comparison with ascorbic acid (973.7 ± 3.48 µmol Fe (II)/g) and gallic acid (2,373.8 ± 84.70 µmol Fe (II)/g, is quit lower.

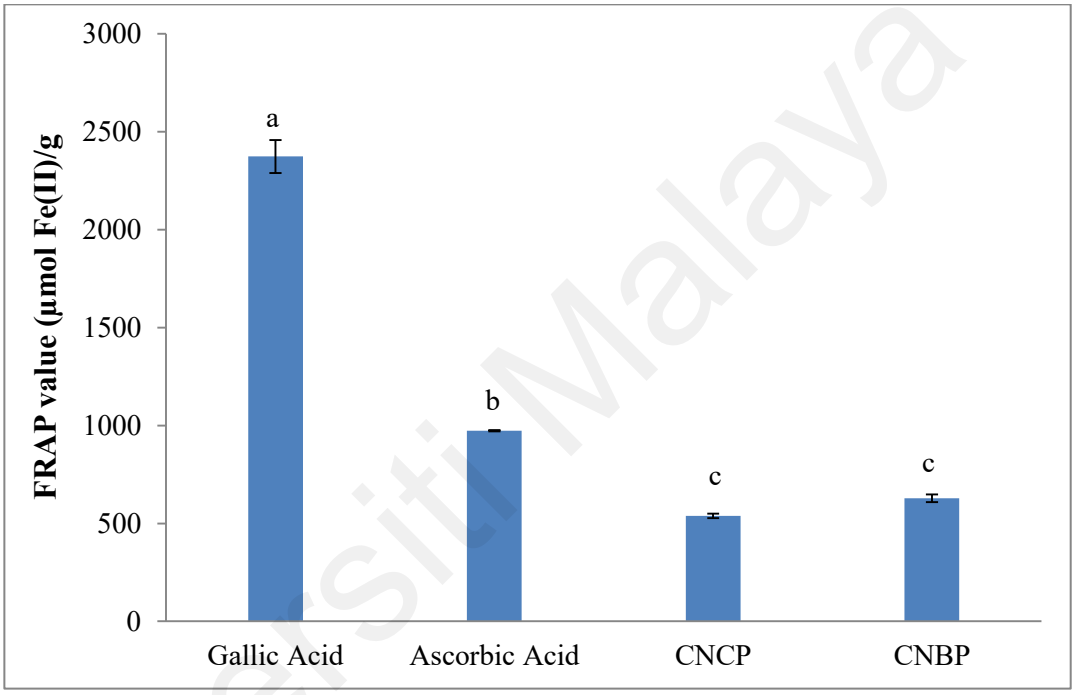

Figure 4.2: Comparison of the FRAP value of CNCP and CNBP with the reference standards (ascorbic acid and gallic acid). The data are represented as the mean ± SEM (n=3). Different letters indicate significant differences between groups at $p< 0.05$.

4.3 Evaluation of acute toxicity

In the current study, SD rats treated with CNCP and CNBP at a dosage of 100 and 200 mg/kg, respectively, demonstrated no sign of acute toxicity. There are no mortality recorded, all SD rats survived and showed normal behavior during 14 days of the study. The effect of CNCP and CNBP on renal function, liver function and lipid profile of the SD rats are listed in Table 4.1 and 4.2. The animals did not show any sign of

abnormalities in physiological feature or behavioral. Histological examination of the liver, kidney, and serum biochemical analysis in treated groups showed that there was no significant differences compared with those of the control group (Figure 4.3 and 4.4).

Table 4.1: Effects of 100 mg/kg and 200 mg/kg of CNCP on renal function, liver function (A and B), and lipid profile (C) of SD rats

A) Effects of CNCP on renal function of SD rats (n=6)

Animal groups	Renal function test						
	Sodium (mM/l)	Pottasium (mM/l)	Chloride (mM/l)	CO_2 (mM/l)	Anion (mM/l)	Urea (mM/l)	Creatinine (µM/l)
Control (10% tween 20)	144.050 ± 0.33	4.50 ± 0.15	103.85 ± 0.28	25.02 ± 0.91	18.00 ± 0.93	7.01 ± 0.75	36.37 ± 1.92
CNCP (100 mg/kg)	143.63 ± 0.25	4.56 ± 0.36	104.66 ± 1.14	27.35 ± 1.18	15.97 ± 1.50	6.43 ± 0.84	37.33 ± 1.37
CNCP (200 mg/kg)	141.68 ± 0.56	4.59 ± 0.18	102.01 ± 1.21	28.05 ± 0.73	16.84 ± 1.84	7.62 ± 1.07	38.05 ± 1.72

B) Effects of CNCP on liver function of SD rats (n=6)

Animal groups	Liver function test								
	TP (g/l)	Albumin (g/l)	Globulin (g/l)	TB (µM/l)	CB (µM/l)	ALP (IU/l)	ALT (IU/l)	AST (IU/l)	GGT (IU/l)
Control (10% tween 20)	61.17 ± 0.98	40.09 ± 0.49	21.67 ± 0.56	2.00 ± 0.15	1.05 ± 0.10	150.65 ± 0.71	49.35 ± 5.71	169.63 ± 2.94	1.93 ± 0.21
CNCP (100 mg/kg)	59.05 ± 0.47	32.37 ± 1.65	19.06 ± 1.92	2.01 ± 0.17	1.04 ± 0.08	147.97 ± 9.78	48.05 ± 4.62	167.39 ± 3.61	2.02 ± 0.02
CNCP (200 mg/kg)	60.01 ± 2.05	38.03 ± 0.95	22.11 ± 1.26	2.02 ± 0.15	1.01 ± 0.12	143.81 ± 2.73	49.33 ± 3.27	170.13 ± 4.03	2.00 ± 0.02

C) Effects of CNCP on lipid profile of SD rats (n=6)

Animal groups	Lipid profile analysis			
	Triglyceride (mM/l)	Total cholesterol (mM/l)	HDL Cholesterol (mM/l)	LDL Cholesterol (mM/l)
Control (10% tween 20)	0.33 ± 0.03	1.43 ± 0.15	1.45 ± 0.06	0.81 ± 0.10
CNCP (100 mg/kg)	0.31 ± 0.03	1.33 ± 0.13	1.39 ± 0.05	0.65 ± 0.10
CNCP (200 mg/kg)	0.39 ± 0.05	1.30 ± 0.22	1.38 ± 0.04	0.73 ± 0.11

Abbreviation: TP (Total protein), TB (Total bilirubin), CB (Conjugated bilirubin), ALP (Alkaline phosphatase), ALT (Alanine transaminase), AST (Aspartate transaminase), GGT (G-Glutaml transferase). All values are displayed as mean ± SEM. No significant differences were detected between the groups. The significant value was considered at $^{*}p < 0.05$.

Table 4.2: Effects of 100 mg/kg and 200 mg/kg of CNBP on renal function, liver function (A and B), and lipid profile (C) of SD rats

A) Effects of CNBP on renal function of SD rats (n=6)

Animal groups	Renal function test						
	Sodium (mM/l)	Pottasium (mM/l)	Chloride (mM/l)	CO_2 (mM/l)	Anion (mM/l)	Urea (mM/l)	Creatinine (µM/l)
Control (10% tween 20)	144.33 ± 0.27	4.43 ± 0.10	104.33 ± 0.27	24.00 ± 0.47	17.67 ± 0.54	6.73 ± 0.30	34.33 ± 0.54
CNBP (100 mg/kg)	141.67 ± 0.54	5.10 ± 0.33	104.67 ± 1.52	27.67 ± 1.09	14.33 ± 1.36	7.30 ± 0.29	38.67 ± 0.72
CNBP (200 mg/kg)	141.00 ± 0.47	5.63 ± 0.19	103.33 ± 0.98	28.33 ± 1.09	15.67 ± 0.27	6.50 ± 0.60	36.33 ± 1.91

B) Effects of CNBP on liver function of SD rats (n=6)

Animal groups	Liver function test								
	TP (g/l)	Albumin (g/l)	Globulin (g/l)	TB (µM/l)	CB (µM/l)	ALP (IU/l)	ALT (IU/l)	AST (IU/l)	GGT (IU/l)
Control (10% tween 20)	60.33 ± 0.27	40.33 ± 0.27	22.00 ± 0.47	1.83 ± 0.14	0.90 ± 0.15	119.00 ± 2.94	53.67 ± 1.19	126.67 ± 2.13	1.00 ± 0.00
CNBP (100 mg/kg)	59.33 ± 0.72	37.33 ± 0.54	22.00 ± 0.47	2.00 ± 0.00	0.95 ± 0.19	137.67 ± 0.27	54.67 ± 1.09	122.33 ± 3.60	1.67 ± 0.02
CNBP (200 mg/kg)	60.33 ± 1.36	38.67 ± 1.19	21.67 ± 0.54	2.00 ± 0.00	0.89 ± 0.21	143.00 ± 3.68	68.33 ± 3.31	151.00 ± 0.82	1.67 ± 0.27

C) Effects of CNBP on lipid profile of SD rats (n=6)

Animal groups	Lipid profile analysis			
	Triglyceride (mM/l)	Total cholesterol (mM/l)	HDL Cholesterol (mM/l)	LDL Cholesterol (mM/l)
Control (10% tween 20)	0.40 ± 0.00	1.27 ± 0.07	0.40 ± 0.01	0.52 ± 0.04
CNBP (100 mg/kg)	0.30 ± 0.00	1.34 ± 0.05	0.46 ± 0.01	0.86 ± 0.04
CNBP (200 mg/kg)	0.33 ± 0.03	1.30 ± 0.09	0.41 ± 0.04	0.75 ± 0.05

Abbreviation: TP (Total protein), TB (Total bilirubin), CB (Conjugated bilirubin), ALP (Alkaline phosphatase), ALT (Alanine transaminase), AST (Aspartate transaminase), GGT (G-Glutaml transferase). All values are displayed as mean ± SEM. No significant differences were detected between the groups. The significant value was considered at $^{*}p < 0.05$.

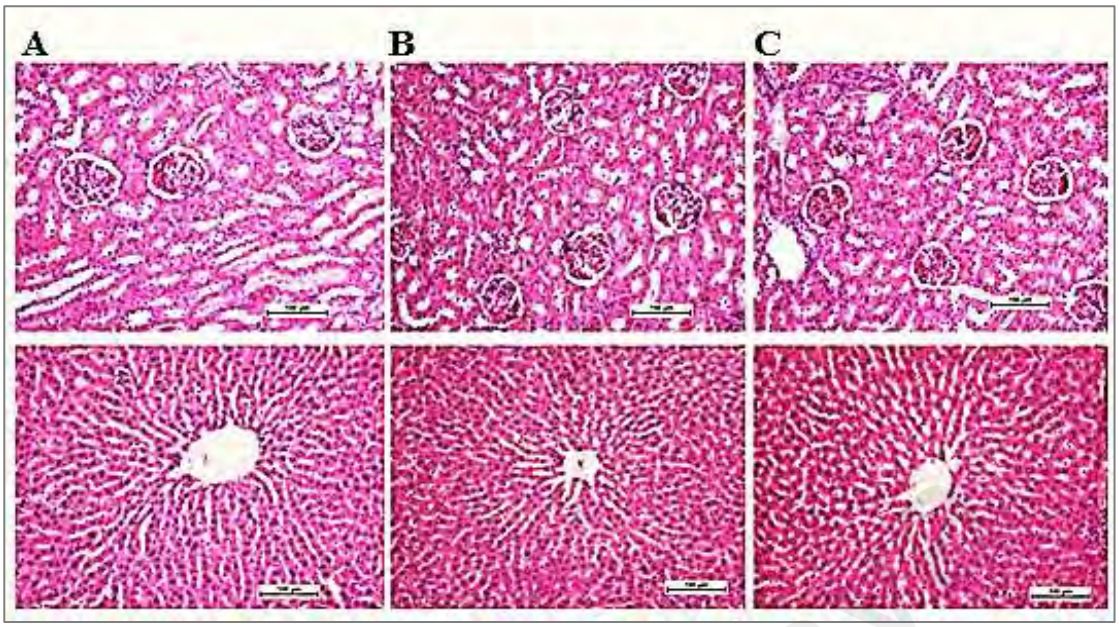

Figure 4.3: Histological sections in acute toxicity effect of CNCP (H&E staining). Histological sections of kidney (upper row) and liver (lower row). (A) Vehicle control (10% tween 20), (B) CNCP (100 mg/kg), and (C) CNCP (200 mg/kg) in SD rats. There is no structural difference was detected among the CNCP-treated and control groups. Scale bar: 100 µm, (n=6).

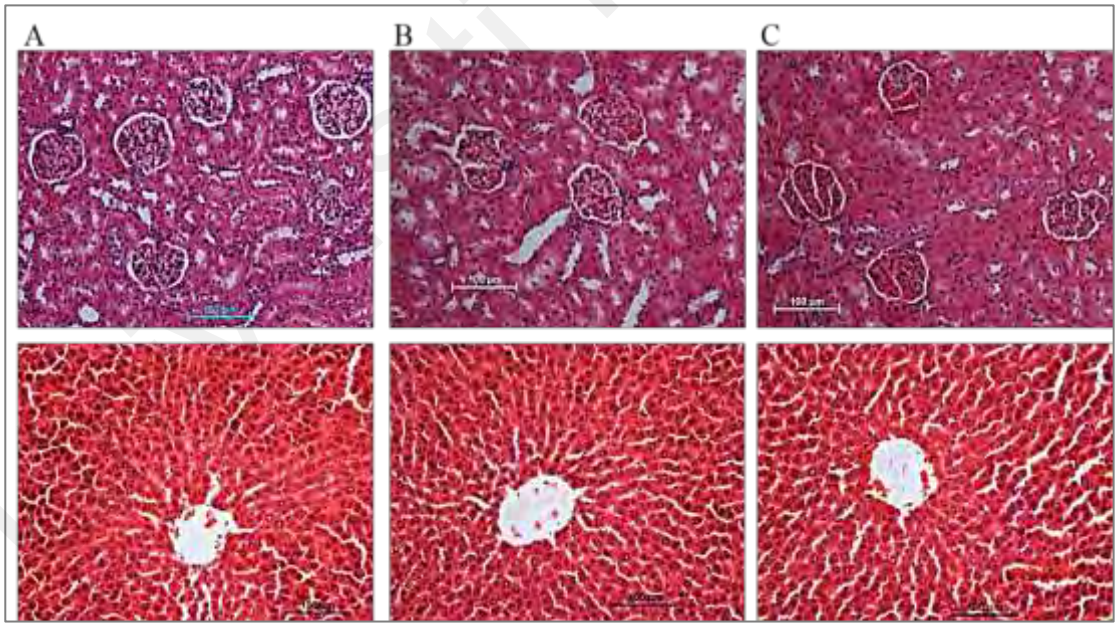

Figure 4.4: Histological sections in acute toxicity effect of CNBP (H&E staining). Histological sections of kidney (upper row) and liver (lower row). (A) Vehicle control (10% tween 20), (B) CNBP (100 mg/kg), and (C) CNBP (200 mg/kg) in SD rats. There is no structural difference was detected among the CNBP-treated and control groups. Scale bar: 100 µm, (n=6).

4.4 Gastroprotective experiment of CNCP and CNBP

4.4.1 Effect of CNCP and CNBP on the pH of gastric secretion and gastric wall mucus

The pH values of both compounds were found to be significantly ($^{*}p < 0.05$) higher in the treated groups (n=6) when compared to that in the ulcer groups (Figure 4.5). Pre-feeding of the treated rats with CNCP (HD) and CNBP (HD) (n=6), significantly increased gastric wall mucus (GWM) compared with that of the ulcerated group (n=6) (Figure 4.6).

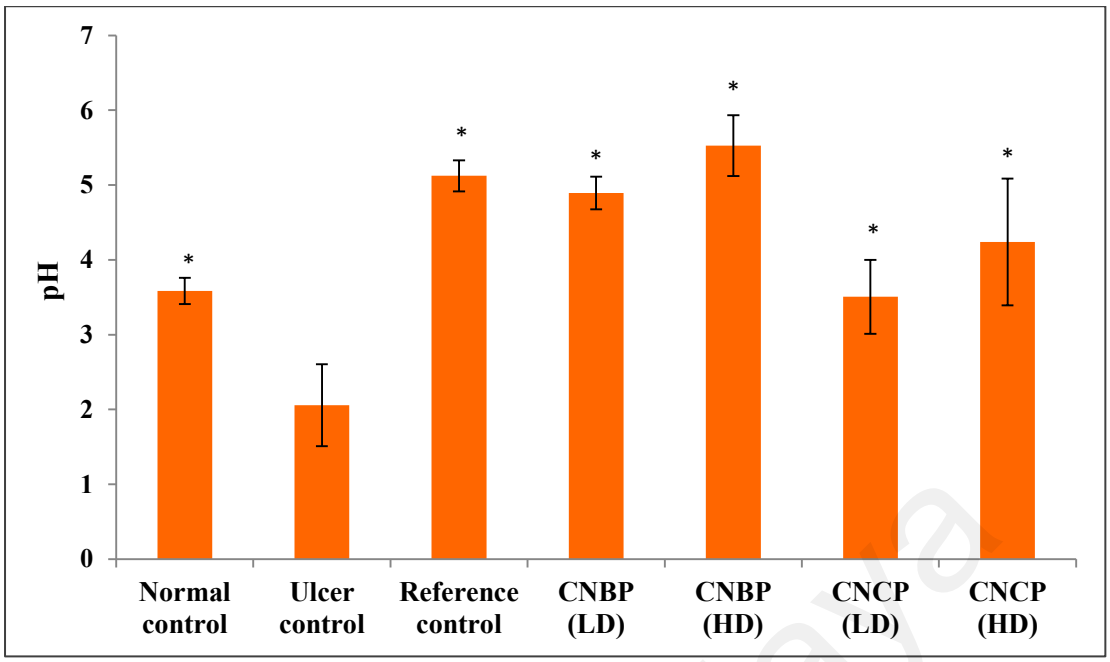

Figure 4.5: Effect of CNCP and CNBP on pH. Reference group was given 20 mg/kg omeprazole, low dose (LD; 10 mg/kg) and high dose (HD; 20 mg/kg) of CNCP and CNBP. Data are presented as the mean ± SEM, (n=6), *p< 0.05.

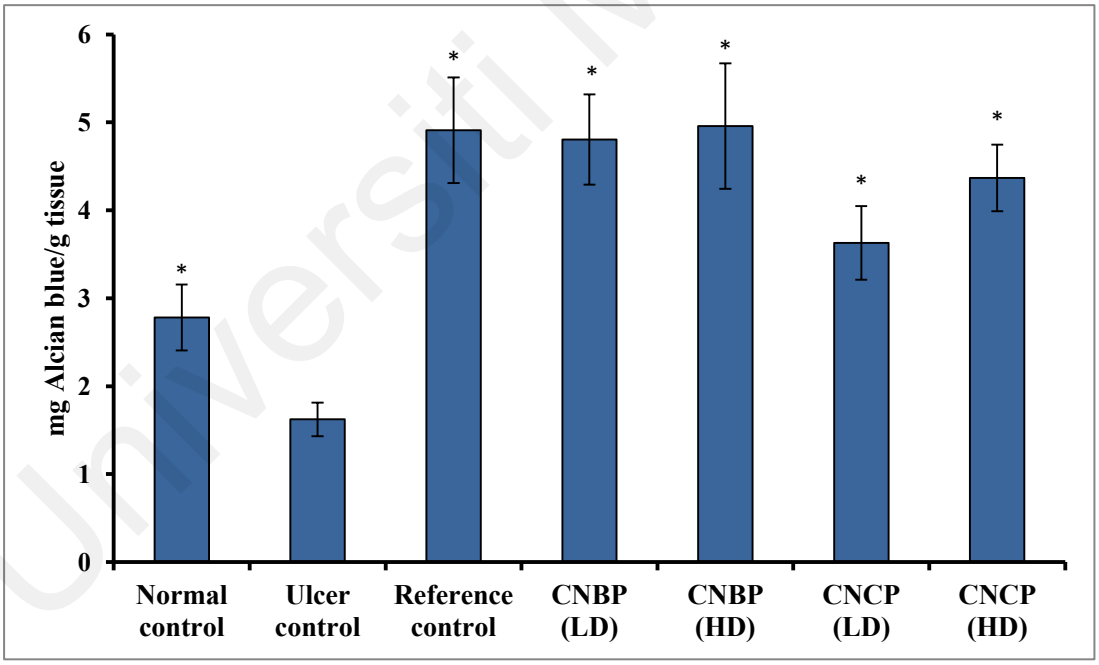

Figure 4.6: Effect of CNCP and CNBP on GWM. Alcian blue binding capacity is defined as GWM. Reference group was given 20 mg/kg omeprazole, low dose (LD; 10 mg/kg) and high dose (HD; 20 mg/kg) of CNCP and CNBP. Data are presented as the mean ± SEM, (n=6), *p< 0.05.

4.5 Effect of CNCP and CNBP on gastric mucosa

The gross images of the gastric mucosa in ethanol-induced gastric ulcer in SD rats pre-treated with 10 and 20 mg/kg of CNCP and CNBP, respectively, showed remarkable decrease in the red severe ulcerated bands, which coexisted with acute inflammation when it was compared with that of the ulcer control animals (Figure 4.7 and 4.8). The effect of CNCP and CNBP on the ulcer area induced by ethanol is depicted in Figure 4.9. In fact, this appearance was observed in the ulcer area measured as the percentage of inhibition. However, CNCP and CNBP pre-fed with both LD and HD could drastically reduce ulcer area and the percentage of inhibition compared with the ulcer group, and high dose of the both compounds showed the highest reduction of ulcer area (Figure 4.9).

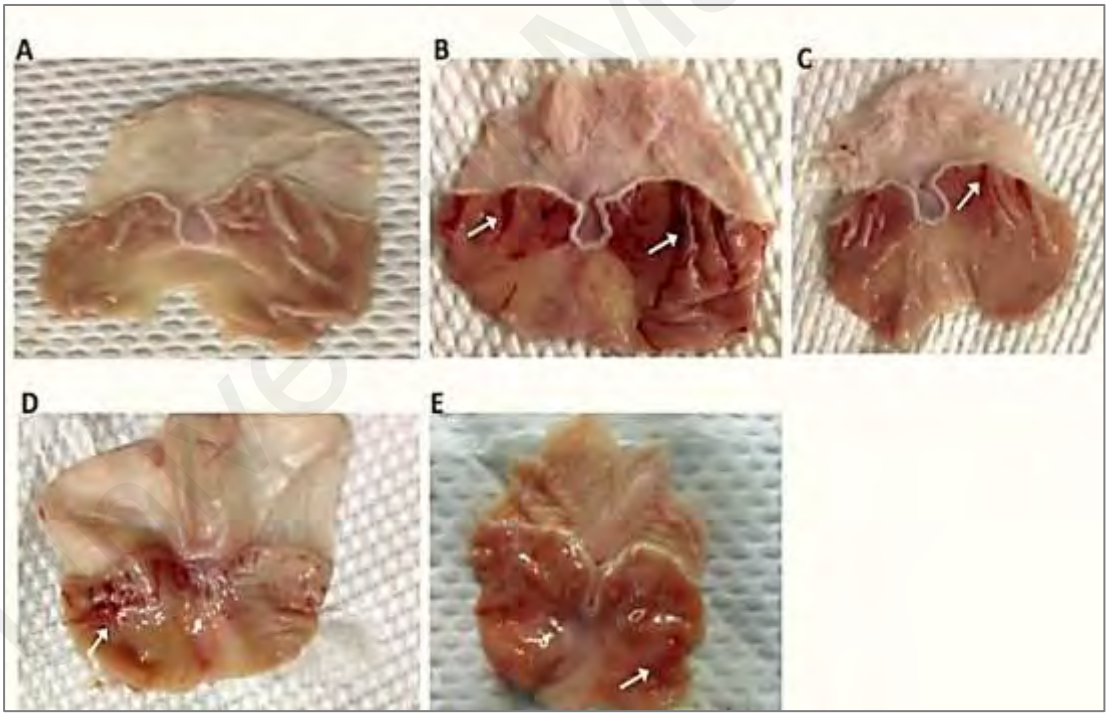

Figure 4.7: Effect of CNCP on gross images of ethanol-induced gastric injury in SD rats (n=6). A) Normal control group (10% tween 20). B) Ulcer control group (absolute ethanol) exhibiting extraordinary acute haemorrhagic ulceration (white arrow). C) Reference control group (omeprazole, 20 mg/kg) exhibiting mild injury. D & E) Stomachs pre-fed with CNCP at low dose (LD, 10 mg/kg) and high dose (HD, 20 mg/kg) respectively showing obvious reduction in the gastric lesions.

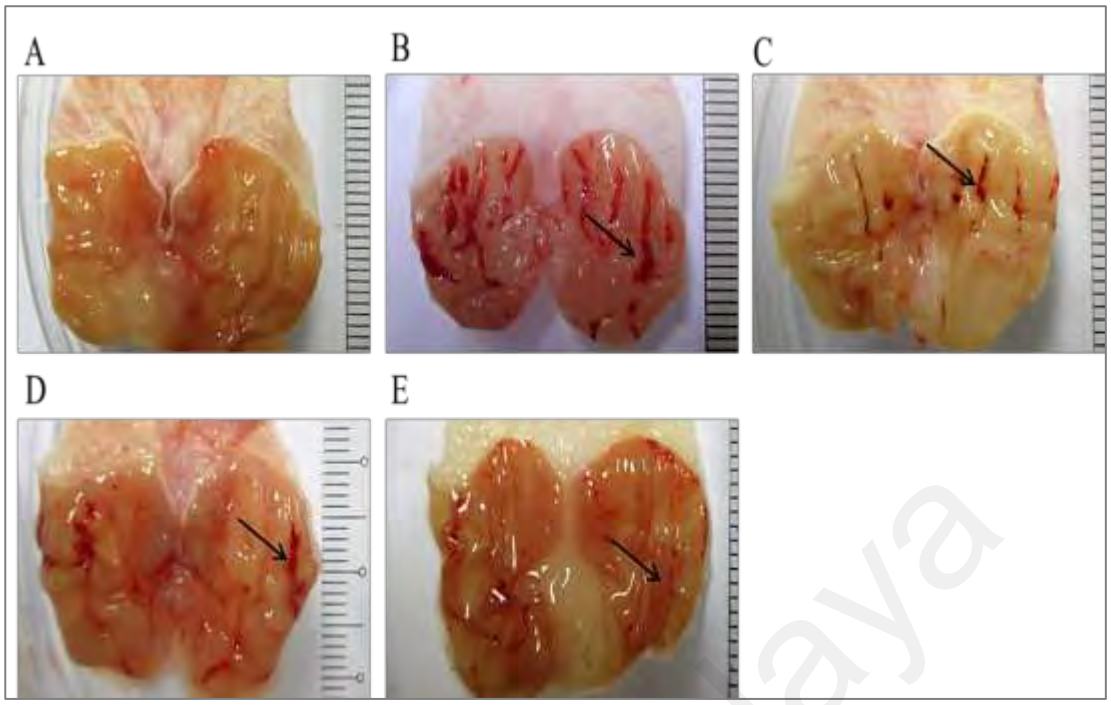

Figure 4.8: Effect of CNBP on gross images of ethanol-induced gastric injury in SD rats (n=6). A) Normal control group (10% tween 20). B) Ulcer control group (absolute ethanol) which exhibiting extraordinary acute haemorrhagic ulceration (black arrow). C) Reference control group (omeprazole, 20 mg/kg) indicates mild injury. D & E) Stomachs pre-fed with low dose of CNBP (LD, 10 mg/kg) and high dose (HD, 20 mg/kg), respectively show obvious reduction in the gastric lesions.

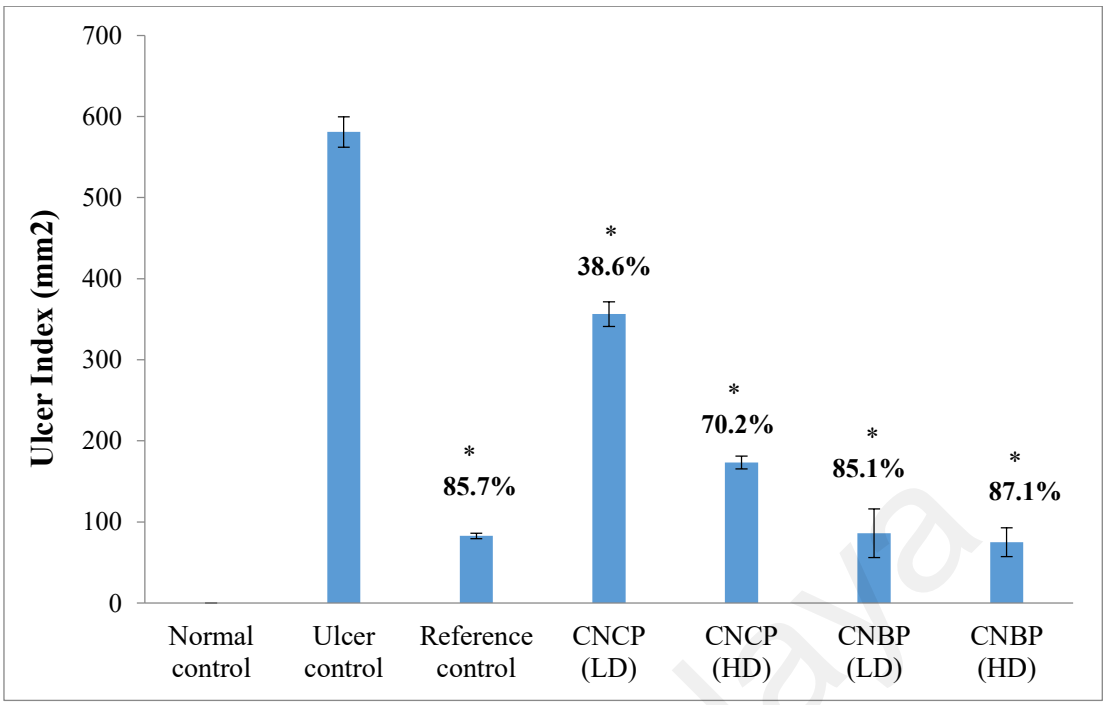

Figure 4.9: The ulcer area of CNCP and CNBP. The rats, pre-fed with CNCP and CNBP significantly decreased the ulcer area when compared to ulcer control group. Values are presented as Mean ± SEM (n=6). Significant differences are considered as *p< 0.05. Percentage (%) of ulcer inhibition of the reference and experimental groups are indicated above the bars.

4.6 Protein concentration in gastric homogenate

Table 4.3 shows the protein concentration in gastric homogenate of pre-fed SD rats with CNCP and CNBP. The protein level in gastric homogenate were notably increased when treated with high dose of CNCP and CNBP in comparison with the ulcer control group (n=6) (Table 4.3).

4.7 Antioxidant activities of stomach homogenate

The effects of CNCP and CNBP on endogenous antioxidant enzymes, SOD, and CAT are listed in (Table 4.3). The ulcer control group exhibited significant reductions of SOD and CAT activities in comparison with normal control group were found. Stomach of the rats treated with high dose (20 mg/kg) of the both compounds could

significantly ($^{*}p< 0.05$) increase those activities when compared with the ulcer control group (Table 4.3).

4.8 MDA and PGE2 levels of gastric tissue homogenate

The animals which were pre-fed with high dosage of CNCP and CNBP respectively, showed significant attenuation in the gastric MDA level compared to the ulcer control groups (n=6) which were given only absolute ethanol. However, the gastric level of PGE2 indicated higher value in CNCP (HD) and CNBP (HD) -pre-fed rats (n=6) than that in the ulcer control group (Table 4.3).

Table 4.3: Effect of the CNCP and CNBP on the stomach homogenate endogenous antioxidant enzymes activities and, MDA and PGE2 levels, and protein concentration

Animal groups	SOD	CAT	MDA	PGE2	Protein concentration
	(U/mg protein)	(U/mg protein)	(nmol/g protein)	(ng/mg protein)	(mg/ml tissue)
10% tween 20 (Normal control)	**17.15 ± 0.90***	**82.51 ± 2.17***	**62.31 ± 2.90***	**3.08 ± 0.11***	**9.00 ± 0.11***
Absolute EtOH (Ulcer control)	**4.04 ± 0.47**	**18.70 ± 0.01**	**146.80 ± 0.15**	**1.01 ± 0.04**	**5.12 ± 0.40**
Omeprazole (20 mg/kg)	**20.07 ± 0.50***	**100.34 ± 0.56***	**82.64 ± 3.52***	**2.95 ± 0.06***	**7.71 ± 0.88***
CNCP (LD)	**13.54 ± 0.65***	**54.95 ± 4.57***	**128.81 ± 8.06**	**2.10 ± 0.22***	**6.49 ± 0.01**
CNCP (HD)	**19.21 ± 0.85***	**70.73 ± 4.55***	**110.48 ± 5.15***	**2.50 ± 0.13***	**7.32 ± 0.06***
CNBP (LD)	**12.22 ± 0.58***	**42.45 ± 2.48***	**112.48 ± 7.47***	**2.59 ± 0.24***	**6.03 ± 0.17**
CNBP (HD)	**19.12 ± 1.14***	**68.30 ± 2.88***	**101.01 ± 7.67***	**2.88 ± 0.15***	**6.72 ± 0.15***

All values (in triplicate) are expressed as the mean ± SEM (n=6). Significant at $^{*}p< 0.05$ compared to ulcerated group. EtOH: ethanol, LD: low dose, HD: high dose.

4.9 Effect of CNCP and CNBP on histological evaluation of gastric lesions

4.9.1 H & E staining

Images of tissue sections stained with H&E are shown in (Figure 4.10 and 4.11). Absolute ethanol causes gastric lesions and significant damage to the epithelial cell of gastric mucosa. In addition, the deep lesions (yellow arrow) showed obvious necrosis of the mucosa together with extensive edema (blue arrow) and remarkable inflammation (black arrow) in the ulcer group of SD rats. CNCP (HD) and CNBP (HD) could protect the gastric mucosa of animals with sign of reduction of the ulcer lesions (Figure 4.10 and 4.11).

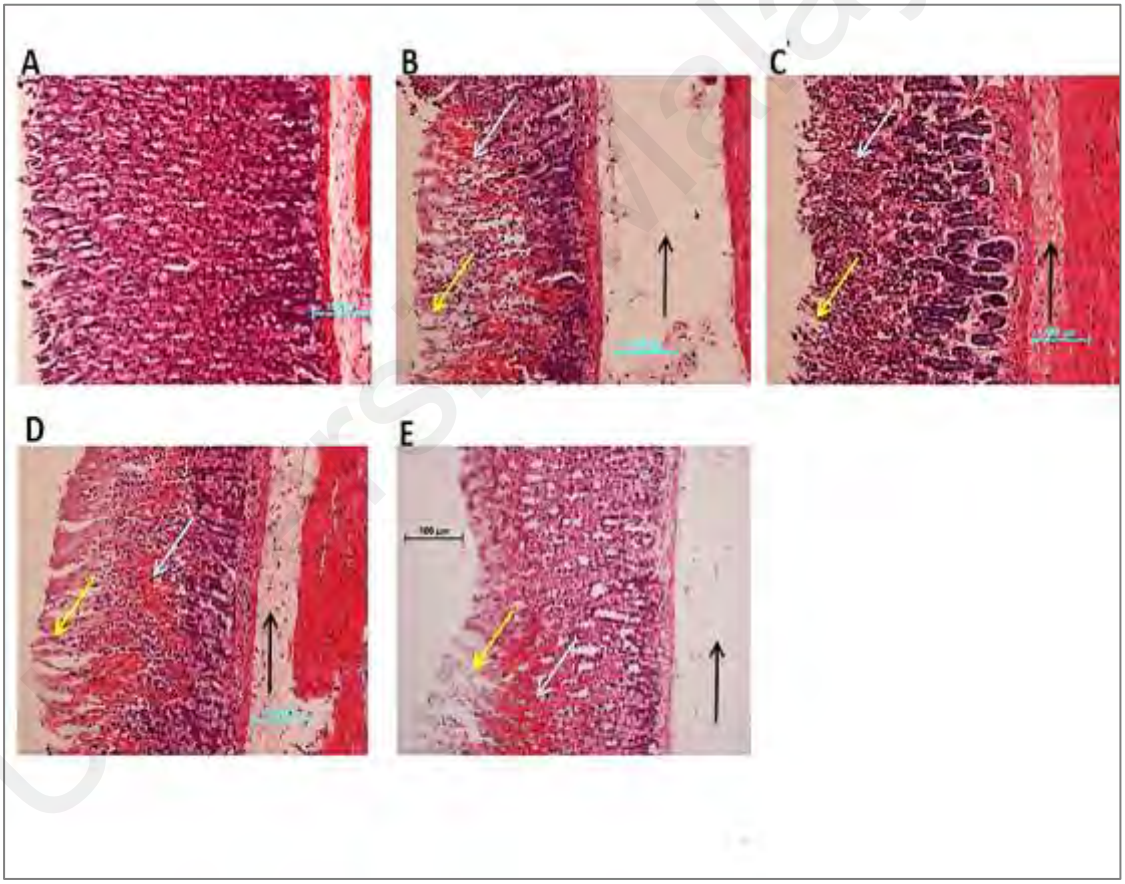

Figure 4.10: Effect of CNCP on the histology of gastric epithelium in ethanol-induced gastric mucosal damage in SD rats (n=6). A) Normal control group. B) Ulcer control group, stomach displayed severe mucosal injury (yellow arrow) along with deep necrosis (blue arrow), edema and inflammation of submucosal layer (black arrow). C) Reference control stomach (omeprazole, 20 mg/kg) presenting mild mucosal injury. D & E) experimental animals' stomachs pre-fed with CNCP showing reduced mucosal damage, while the high dose of CNCP (E) showing better gastroprotective effect than the low dose (D). Scale bar: 100 µm. (H&E staining)

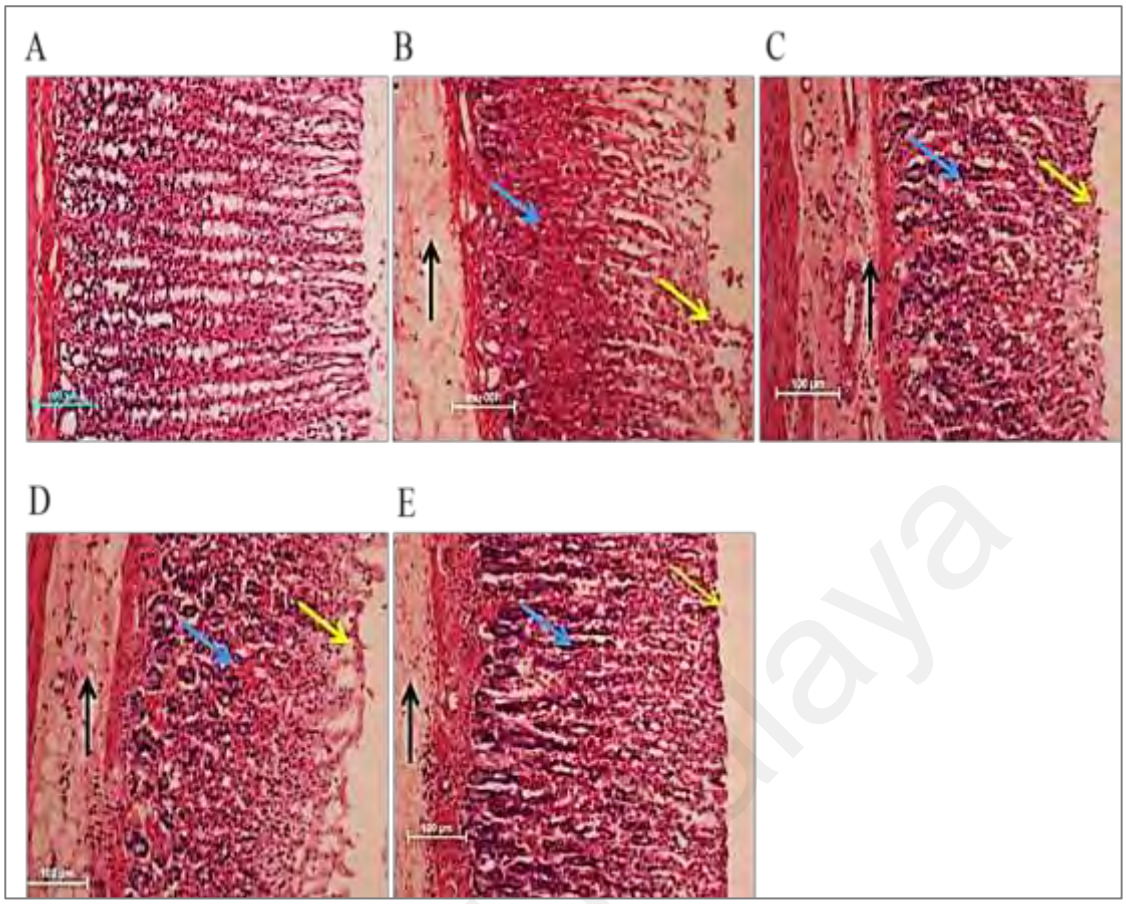

Figure 4.11: Effect of CNBP on the histology of gastric epithelium in ethanol-induced gastric mucosal damage in SD rats (n=6). A) Normal control group. B) Ulcer control stomach displayed severe mucosal injury (yellow arrow) along with deep necrosis (blue arrow), edema and inflammation of submucosal layer (black arrow). C) Reference control stomach (omeprazole, 20 mg/kg) presenting mild mucosal injury. D & E) experimental animals' stomachs pre-fed with CNBP showing reduced mucosal damage, while the high dose of CNBP (E) showing better gastroprotective effect than the low dose (D). Scale bar: 100 µm. (H&E staining)

4.9.2 PAS staining

As depicted in (Figure 4.12 and 4.13), PAS staining showed reduction of gastric mucosal secretion in the ulcer group. The positive PAS staining of the mucosal lining of the stomach in CNCP and CNBP high dose-treated groups showed higher level of mucosal glycoproteins (magenta color) than those of the ulcer groups. These findings suggested that CNCP and CNBP possess significant gastroprotective activity.

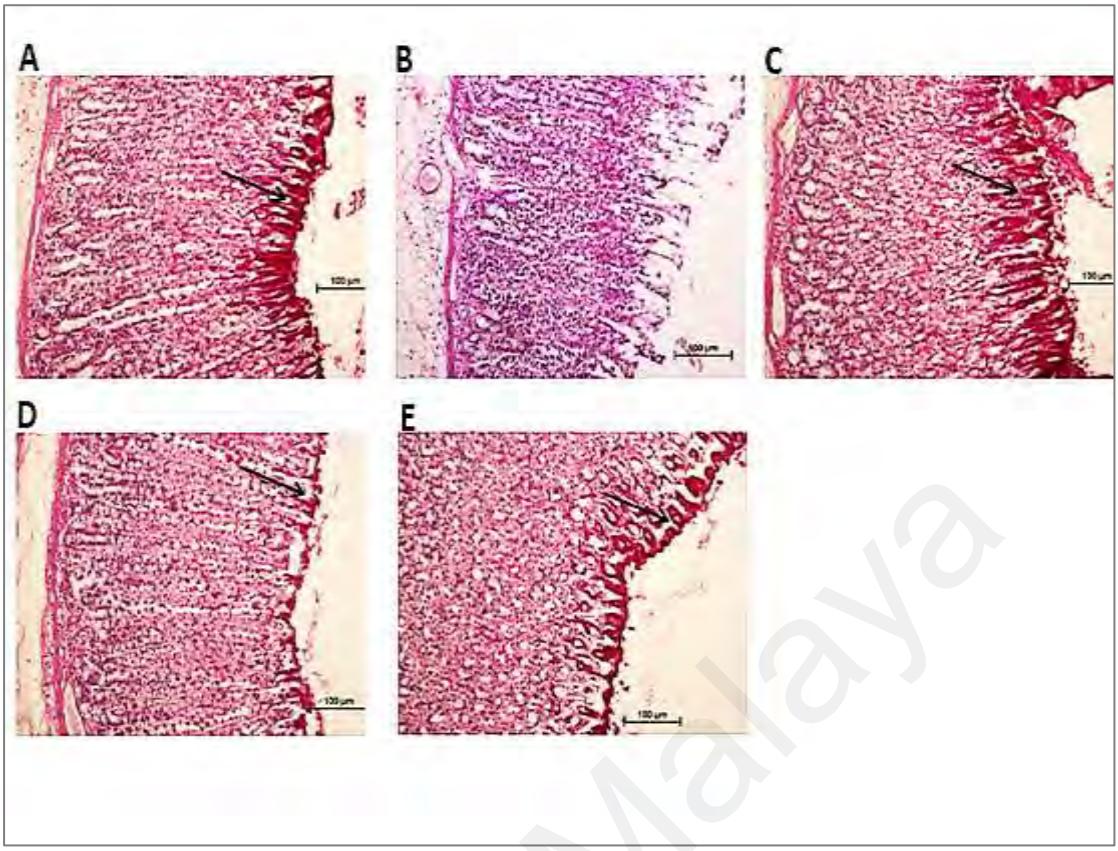

Figure 4.12: Effects of CNCP on PAS staining of gastric glycoproteins secretion in ethanol-induced stomach damage in SD rats (n=6). A) Normal control group exhibiting normal magenta color (black arrow) of gastric mucus glands. B) Absence of PAS staining from the mucosa of ulcer control group exhibiting severe mucosal injuries. C) Reference group exhibiting intense PAS stain. D & E) Experimental groups fed with the low dose (10 mg/kg) and high dose (20 mg/kg) of CNCP respectively, displayed intense up-take of PAS stain. The high dose of CNCP (E) showing more intense PAS staining than the low dose (D). Scale bar: 100 µm. (PAS staining)

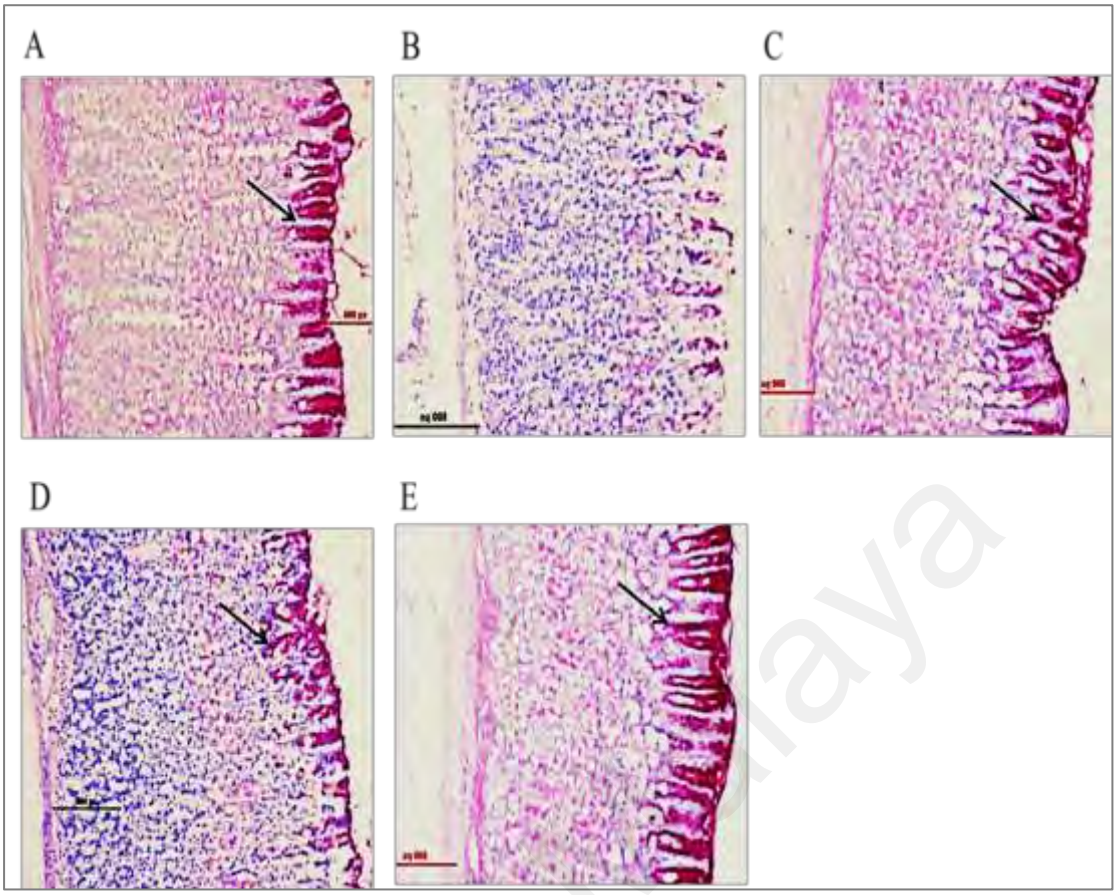

Figure 4.13: Effects of CNBP on PAS staining of gastric glycoproteins secretion in ethanol-induced stomach damage in SD rats (n=6). A) Normal control group exhibiting normal magenta color (black arrow) of gastric mucus glands. B) Absence of PAS staining from the mucosa of ulcer control group exhibiting severe mucosal injuries. C) Reference group exhibiting intense PAS stain. D & E) Experimental groups were fed with the low dose (10 mg/kg) and high dose (20 mg/kg) of CNBP, respectively exhibited intense up-take of PAS stain. The high dose of CNBP (E) is showing more intense PAS staining than the low dose (D). Scale bar: 100 µm. (PAS staining)

4.10 Effect of CNCP and CNBP on immunohistochemically staining of gastric mucosal Bax and HSP70

4.10.1 Effect of CNCP on the protein expressions of HSP70 and Bax

Images of tissue sections subject to immunohistochemical staining for the Bax and HSP70 are shown in (Figure 4.14 and 4.15). Brown staining indicates presence of Bax and Hsp70 proteins expression. In CNCP HD-treated rats, over-expression of HSP70 was observed compared with the ulcer control group (Figure 4.15). Staining for the Bax

protein showed that ethanol could induce injury and apoptosis in the stomachs with overexpression of Bax, while pre-treatment with high dose of CNCP caused down-regulation of Bax expression (Figure 4.14).

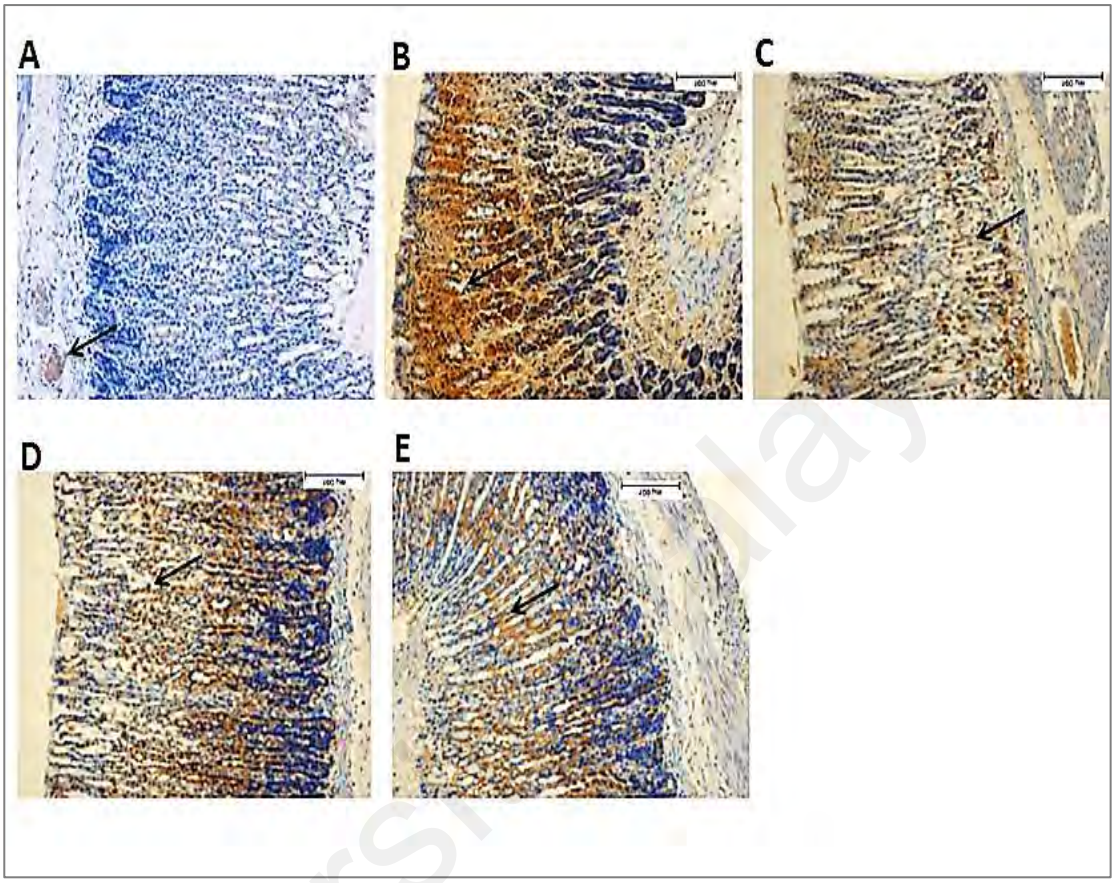

Figure 4.14: Effect of CNCP on immunohistochemical staining of Bax protein expression in ethanol-induced gastric epithelial damage in SD rats (n=6). A) Normal control group. B) Ulcer control group exposing up-regulated Bax protein (black arrow). C) Reference control group revealing clear down-regulation of Bax protein. D & E) Experimental group pre-treated with the low dose (10 mg/kg) and high dose (20 mg/kg) of CNCP, respectively shows down-regulation of Bax protein. Scale bar: 100 µm.

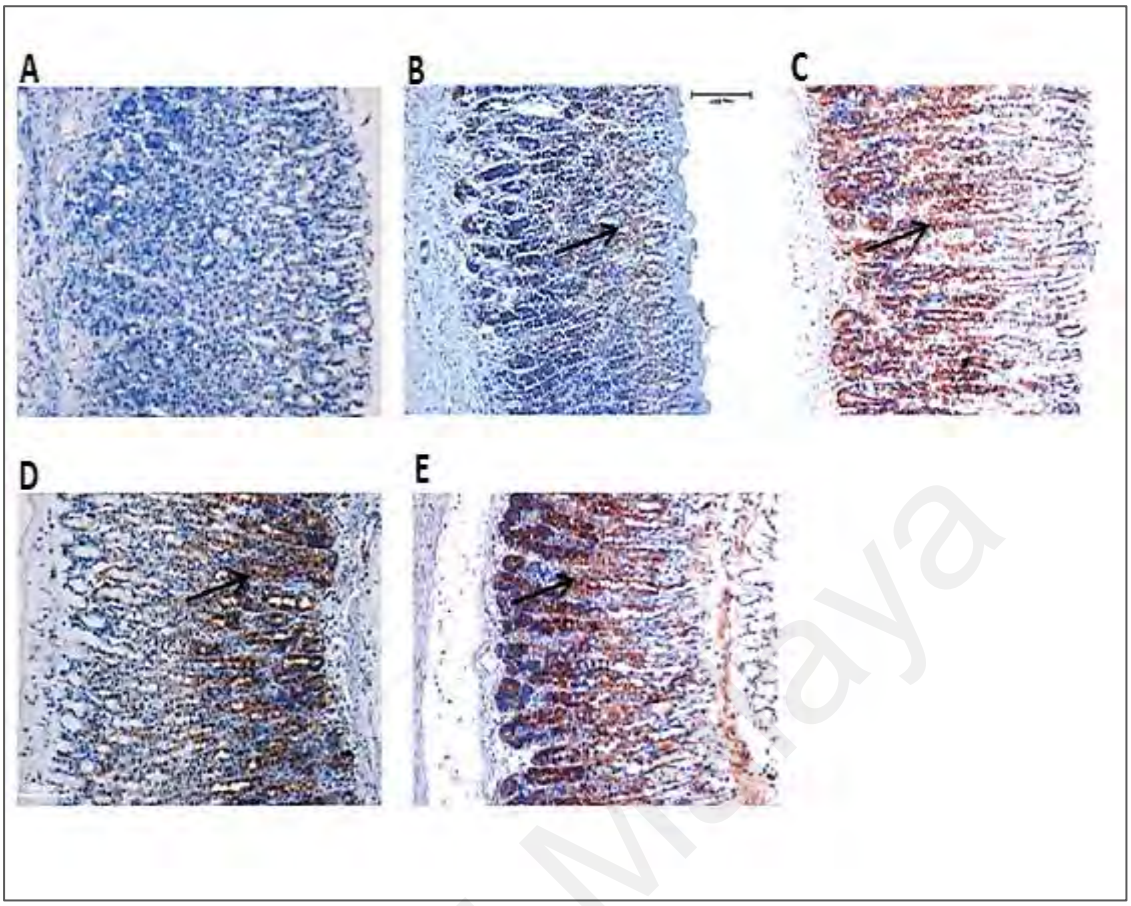

Figure 4.15: Effect of CNCP on the expression of HSP70 protein of gastric mucosa in ethanol-induced stomach ulcer in SD rats (n=6). A) Normal control group. B) Ulcer control group expressed less staining of HSP70 protein (black arrow). C) Reference group demonstrated obvious up-regulation of HSP70 protein. D & E) Experimental groups pre-fed with CNCP (10 and 20 mg/kg respectively) exposing expression of HSP70 protein in the high dose group better than the low dose group. Scale bar: 100 µm.

4.10.2 Effect of CNBP on the protein expressions of HSP70 and Bax

Images of tissue sections subject to immunohistochemical staining for the Bax and HSP70 are shown in (Figure 4.16 and Figure 4.17). In the CNBP HD-treated rats, over-expression of HSP70 was noticed compared with the ulcer control group (Figure 4.17). Immunohistochemical staining of Bax protein showed that ethanol could induce injury and apoptosis in the stomachs with overexpression of Bax, while pre-treatment with CNBP (HD) caused down-regulation of Bax expression (Figure 4.16).

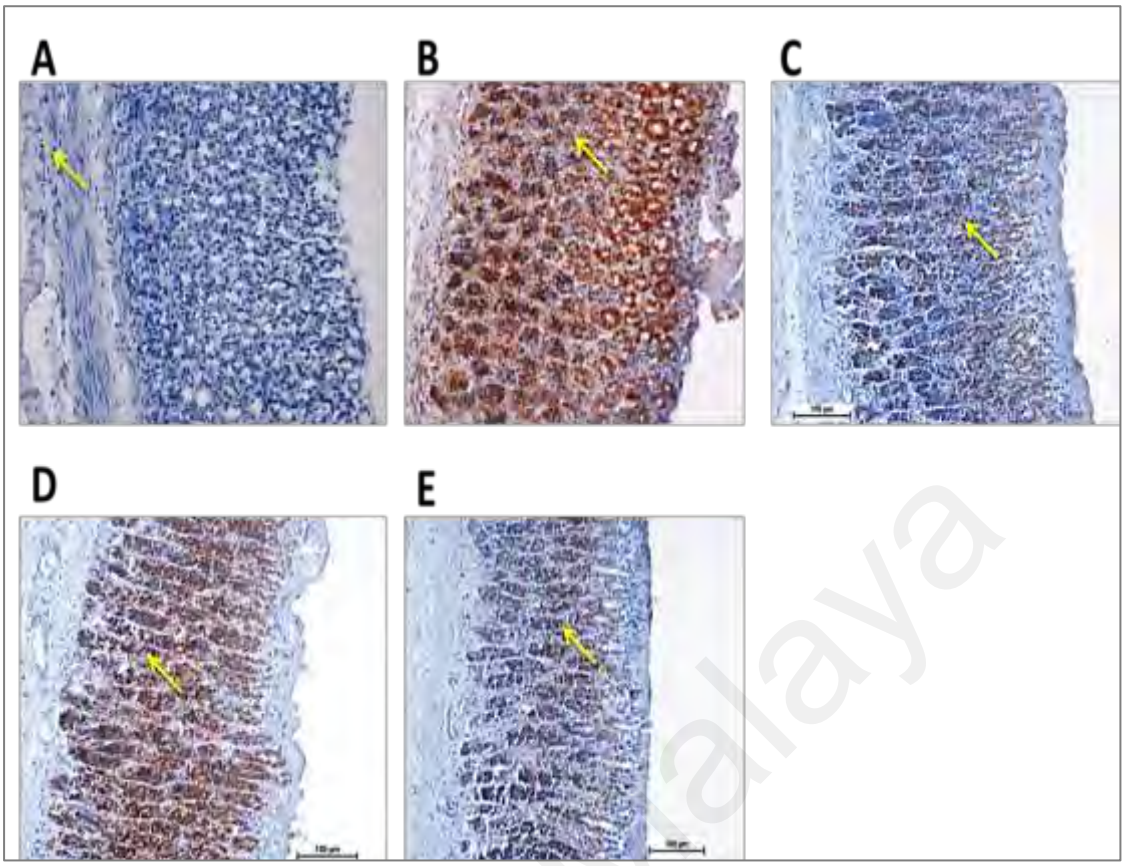

Figure 4.16: Effect of CNBP on immunohistochemical staining of Bax protein expression in ethanol-induced gastric epithelial damage in SD rats (n=6). A) Normal control group. B) Ulcer control group exposing up-regulation of Bax protein (yellow arrow). C) Reference control group revealing clear down-regulation of Bax protein. D & E) Experimental group pre-treated with the low dose (10 mg/kg) and high dose (20 mg/kg) of CNBP respectively showing down-regulation of Bax protein. Scale bar: 100 µm.

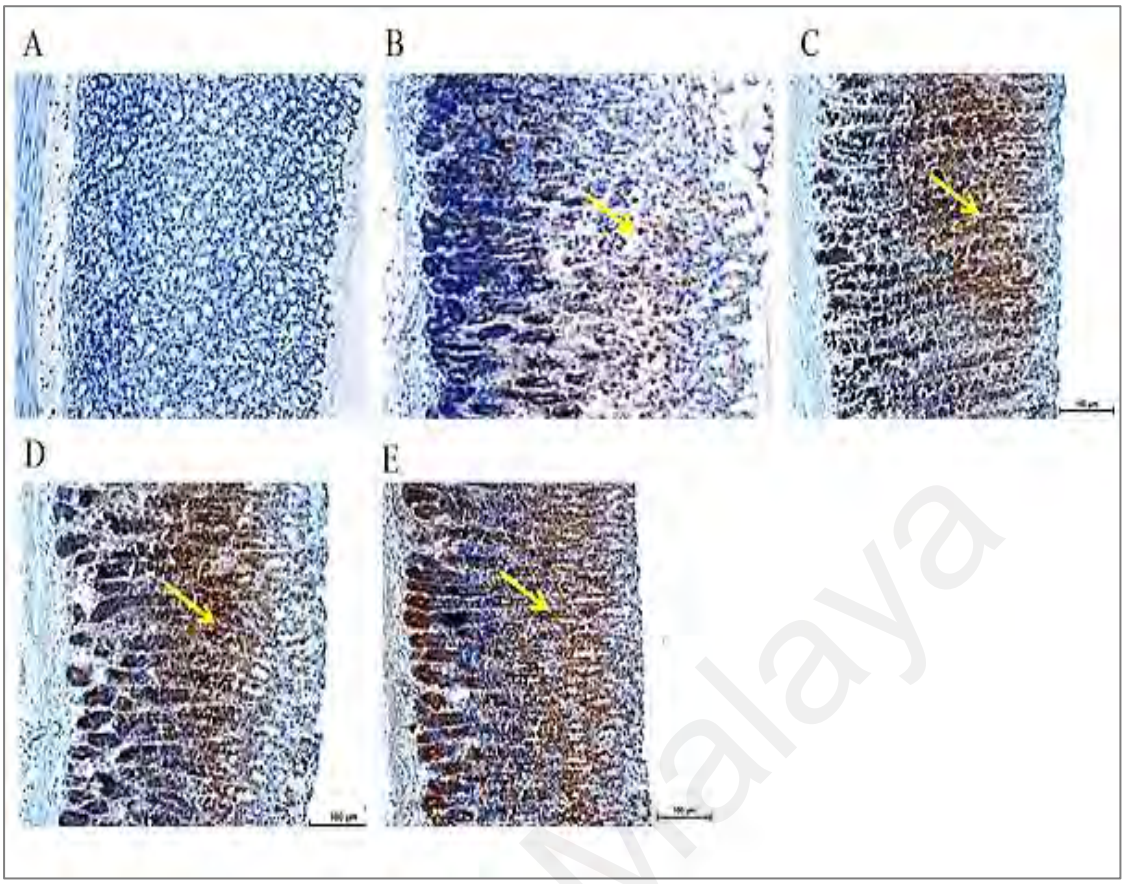

Figure 4.17: Effect of CNBP on the expression of HSP70 protein of gastric mucosa in ethanol-induced stomach ulcer in SD rats (n=6). A) Normal control group. B) Ulcer control group expressed less HSP70 protein (yellow arrow). C) Reference group demonstrated obvious up-regulation of HSP70 protein. D & E) Tested groups pre-fed with CNBP (LD & HD) are exposing expression of HSP70 protein in the high dose group more effective than the low dose group. Scale bar: 100 µm.

4.11 Wound healing experiment of CNCP and CNBP Schiff base derivatives in SD rats

4.11.1 Effect of CNCP and CNBP on wound closure

Percentage of wound closure of CNCP and CNBP are shown in Figure 4.18. Every collection on day 5 of the wound healing showed some dissimilarity in the speed of injury reduction, while significant increase in percent wound closure was observed in positive control (intrasite gel: 75.29 %) animals, CNCP (LD: 68.34 %) and CNBP (HD: 64.84 %) treated group when compared to the untreated control group (vehicle). As observed in day 10 (Figure 4.18), the percentage of wound closure in treated group of low dose (94.80 %) and high dose (94.90 %) of CNBP are in good comparison to the

intrasite gel (96.55 %). There was not much difference of percentage of wound closure in the treated group with low dose of CNCP in comparison to the group treated with intrasite gel and CNBP. Based on the representation of macroscopic appearance, Figure 4.19 and Figure 4.20 are shown the curing of excisional lesion on day 10 after surgery. The treatment with intrasite gel, CNCP (LD) and CNBP (HD) represented remarkable reduction of the wound area compared with the vehicle control group (gum acacia).

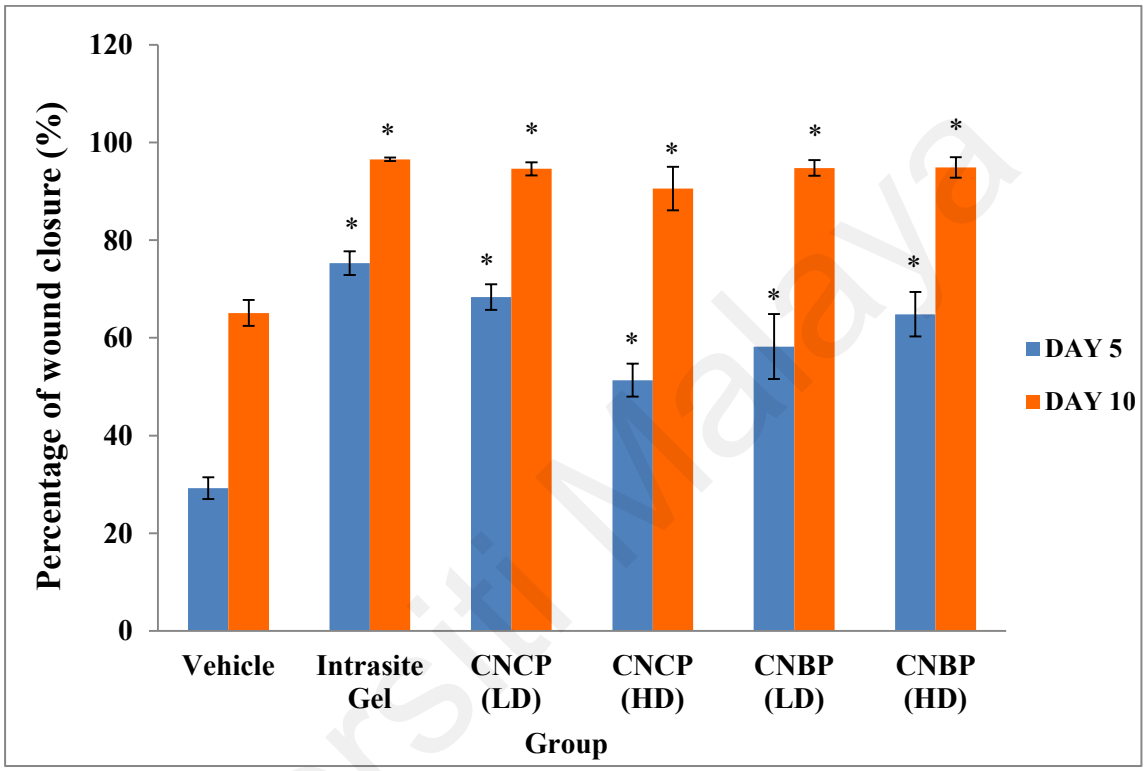

Figure 4.18: Effect of topical treatment with CNCP and CNBP on percentage (%) of wound closure in SD rats. Values are expressed as means ± SEM. (n=6). Significance was defined as *p< 0.05 compared to vehicle (gum acacia).

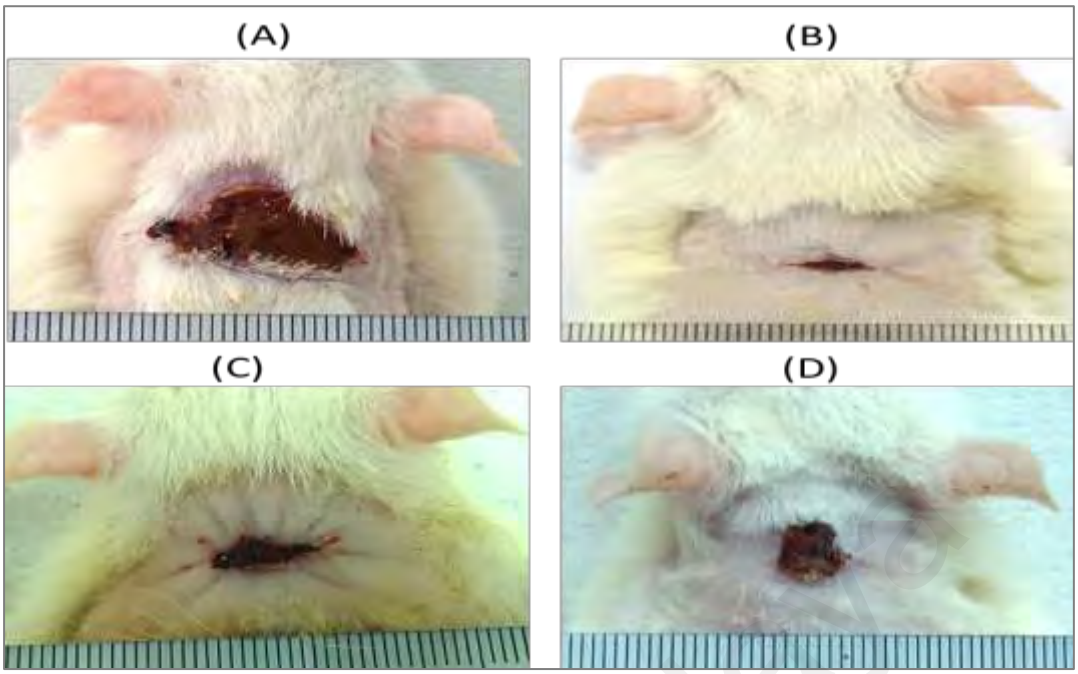

Figure 4.19: Effects of CNCP on macroscopic appearance of excision lesion in SD rats on day 10 (n=6). Rats were topically treated (0.2 ml) with (A) Gum acacia group showing incomplete wound healing, (B) Intrasite gel showing wound almost healed, (C) low dose of CNCP (10 mg/ml) showing wound healing, but not complete and (D) high dose of CNCP (20 mg/ml) showing wound healing, but not complete.

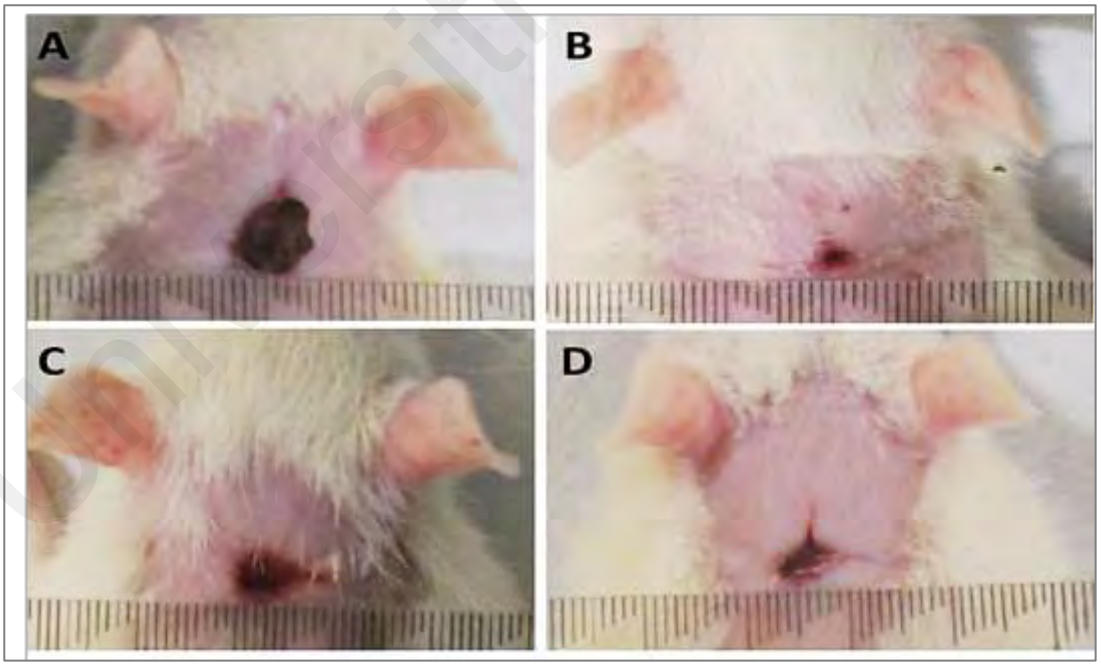

Figure 4.20: Effects of CNBP on macroscopic appearance of excision lesion in SD rats on day 10 (n=6). Rats were topically treated (0.2 ml) with (A) Gum acacia group showing incomplete wound healing, (B) Intrasite gel showing wound almost healed, (C) low dose of CNBP (10 mg/ml) showing wound healing, but not complete and (D) high dose of CNBP (20 mg/ml) showing wound healing, but not complete.

4.12 Histopathology and immunohistochemistry analysis of injured tissues dressed by CNCP and CNBP

4.12.1 H & E staining

Histology studies of the wound tissue samples were carried out on day 10 (Figure 4.21 and 4.22). The results of the H & E staining confirmed that the scar width at wound closure of rats treated with CNCP (LD), CNBP (HD), and intrasite gel were noticeably lesser than those treated with gum acacia (Figure 4.21 and Figure 4.22). CNCP and CNBP at both concentrations, particularly CNCP (10 mg/ml) and CNBP (20 mg/ml), exhibited extremely close up outline in contrast to the vehicle control group, on day 10. In addition, the granulation tissue contained more collagen and fibroblasts, and less inflammatory cells in wounds treated with low dose of CNCP (10 mg/ml), high dose of CNBP (20 mg/ml), and intrasite gel compared to the wounds treated with gum acacia.

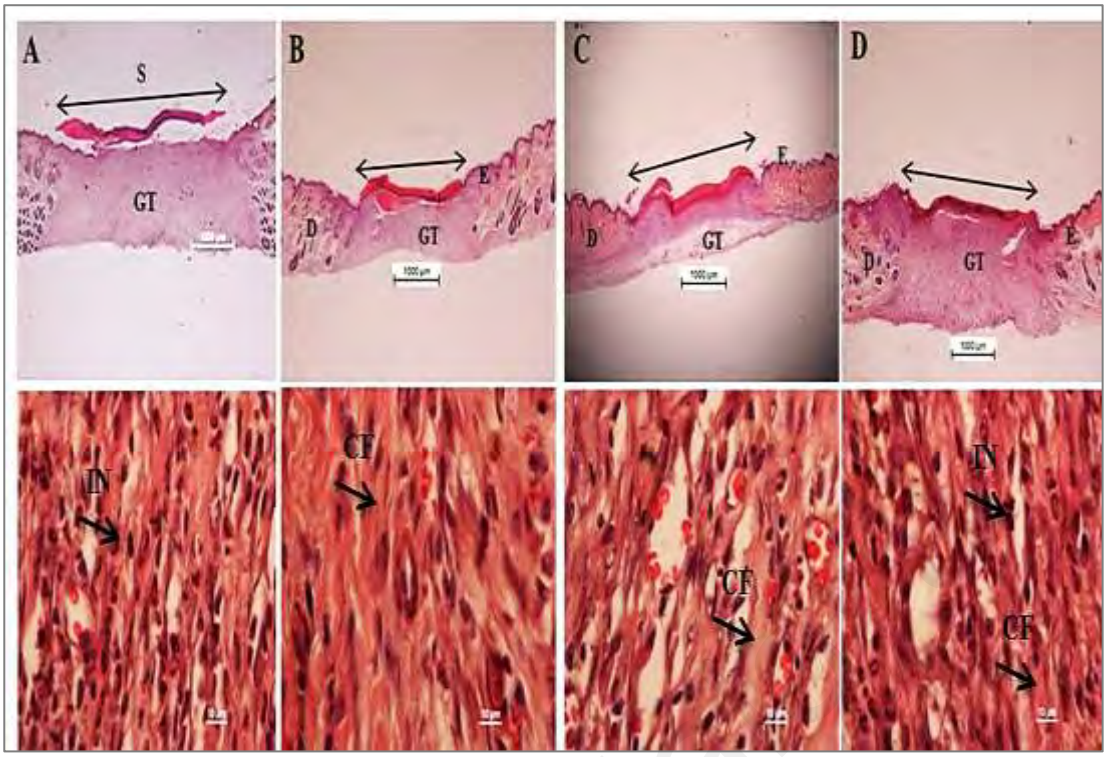

Figure 4.21: Effect of CNCP on histopathological examination view of wound healing excision. (H & E staining). The arrow showed epithelialization. S-Scar width; E-Epidermis; D-Dermis; GT-Granulation tissue. At day 10 of curing, lesion tissues were development from (A) Gum acacia group shows incomplete wound healing enclosure and few degree and alignment of collagen (CF) and minimum inflammatory cells (IN), (B) Intrasite gel, shows significant wound closure, more collagen deposition and minimum inflammatory cells, (C) CNCP (10 mg/ml) and (D) CNCP (20 mg/ml) administrated rats demonstrate wound closure significantly, extra collagen and fewer inflammatory cell in granulation tissue. Scale bar: 1000 µm and 10 µm, (n=6).

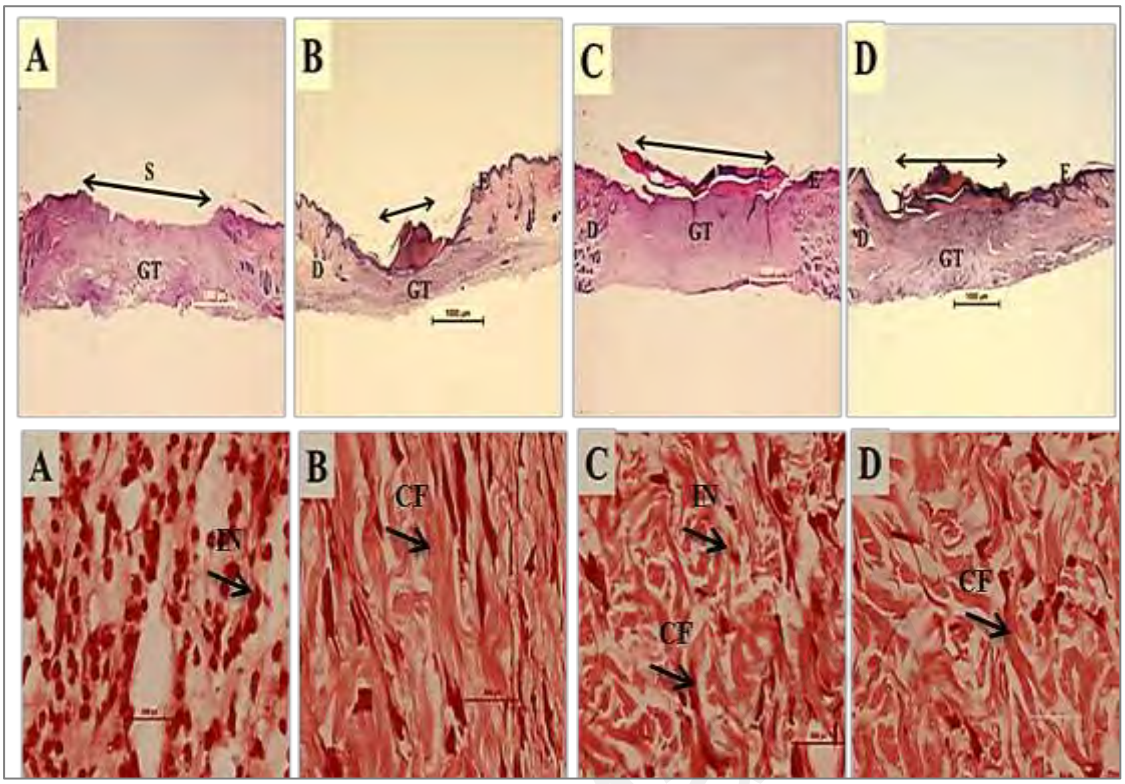

Figure 4.22: Effect of CNBP on histopathological examination view of wound healing excision. (H & E staining). The arrow showed epithelialization. S-Scar width; E-Epidermis; D-Dermis; GT-Granulation tissue. At day 10 of curing, lesion tissues were development from (A) Gum acacia group shows incomplete wound healing enclosure and few degree and alignment of collagen (CF) and minimum inflammatory cells (IN), (B) Intrasite gel, shows significant wound closure, more collagen deposition and minimum inflammatory cells, (C) CNBP (10 mg/ml) and (D) CNBP (20 mg/ml) administrated rats demonstrate wound closure significantly, extra collagen and fewer inflammatory leucocytes in granulation tissue. Scale bar: 1000 µm and 10 µm, (n=6).

4.12.2 Masson trichrome staining

Masson trichrome staining was used to examine dermis maturation and organization of collagen fibbers (Figure 4.23 and Figure 4.24). In the vehicle (gum acacia) control group, poor collagen production was observed. The sprinkled gathering of inflammatory cells responses comprised macrophages and neutrophils having incompetent adjust collagen fibers, exhibited undeveloped tissue granulation in the vehicle control group (gum acacia). On the contrary, the amount of fibroblast and collagen deposition in

wounds treated with low dose of CNCP (10 mg/ml), high dose of CNBP (20 mg/ml), and intrasite gel increased.

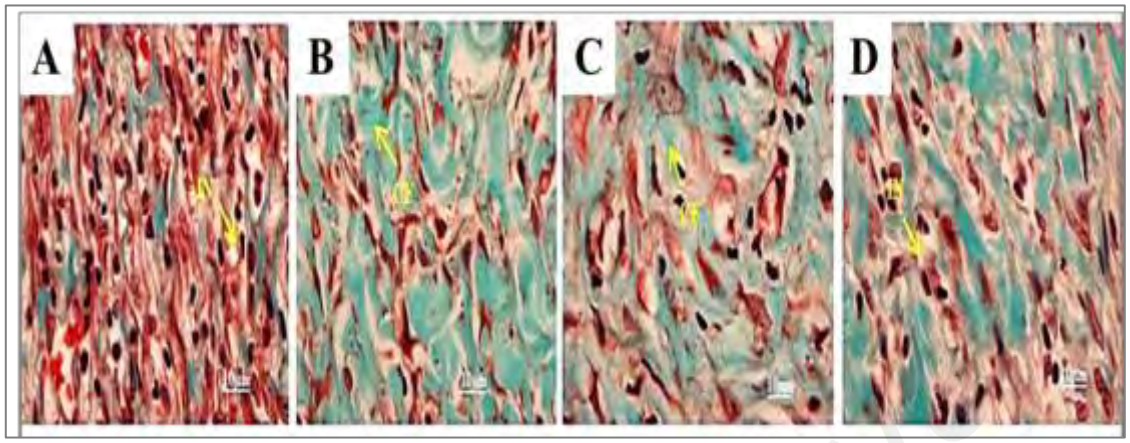

Figure 4.23: Effect of CNCP on histology analysis (Masson trichrome) of wounded tissues at day 10 post-operation. The rat's grouping includes (A) Gum acacia group shows incomplete wound healing enclosure and few degree and alignment of collagen (CF), (B) Intrasite gel group demonstrates almost complete wound closure, more collagen deposition and smaller number of inflammatory cells (IN), (C) CNCP (10 mg/ml) shows significant wound closure and (D) CNCP (20 mg/ml) express wound closure. In both LD and HD treated groups, additional collagen fibbers and few inflammatory cells are observed. Scale bar: 10 µm, (n=6).

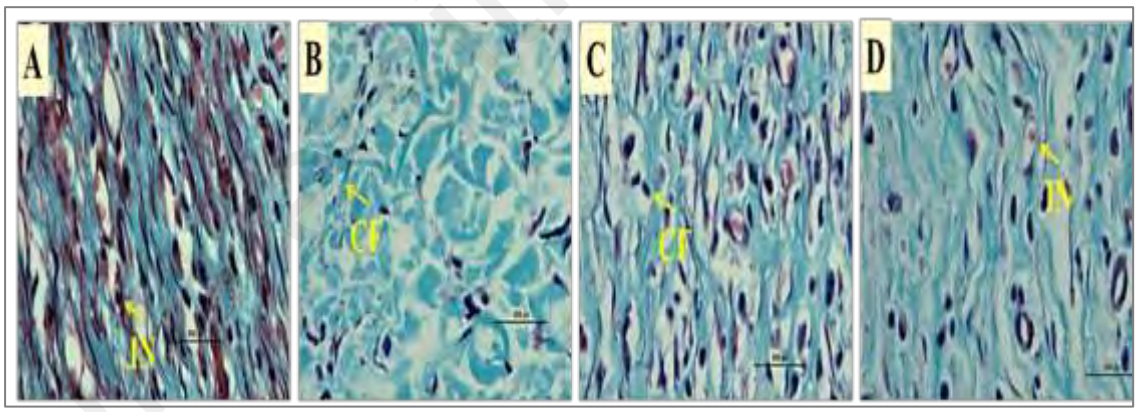

Figure 4.24: Effect of CNBP on histology analysis (Masson trichrome) of wounded tissues at day 10 post-operation. The rat's grouping includes (A) Gum acacia group shows incomplete wound healing enclosure and few degree and alignment of collagen (CF), (B) Intrasite gel group demonstrates almost complete wound closure, more collagen deposition and smaller number of inflammatory cells (IN), (C) CNBP (10 mg/ml) express wound closure, (D) CNBP (20 mg/ml) shows wound closure. In both LD and HD treated groups, additional collagen fibbers and few inflammatory cells are observed. Scale bar: 10 µm, (n=6).

4.12.3 Immunohistochemistry staining (HSP70 & BAX proteins)

Immunohistochemistry analysis of Bax & Hsp70 proteins expression in the wound tissues sample (on day 10) was also carried in this study (Figure 4.25 and 4.26). Brown staining is illustrating Bax and Hsp70 proteins expression. Topical application of CNCP (LD), CNBP (HD), and intrasite gel displayed wound healing of the skin. The immunohistochemical analysis in the wound healed tissues samples were further confirmed with the down-regulation of Bax proteins which indicated a normal part of homeostasis of the wound healing process in the tissue cells. This expression level of Bax protein and induction of apoptosis is representing scar removal. However, the vehicle control (gum acacia) of rat's wounds showed up-regulation of Bax protein. Immunohistochemical analysis of the wounds healed with CNCP (LD), CNBP (HD), and intrasite gel demonstrated up-regulation of Hsp70 protein, however, down-regulation of this protein was observed in the vehicle control group (gum acacia). The finding suggested that a low dose of CNCP and a high dose of CNBP accelerate the curing process of the wound.

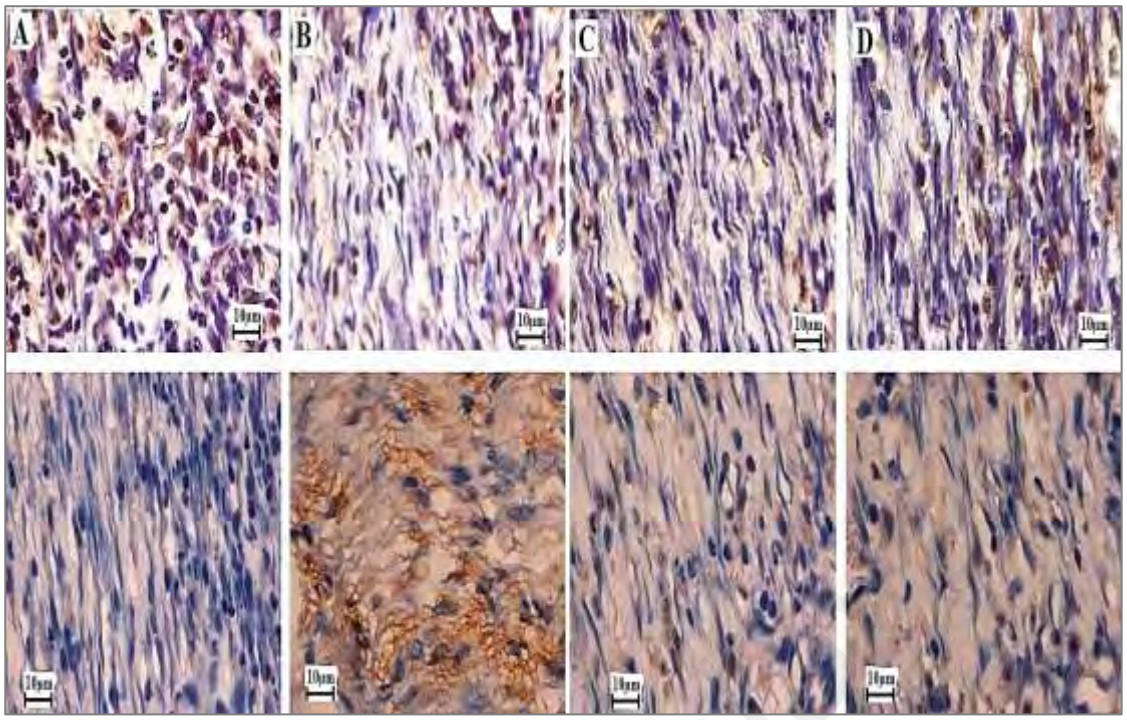

Figure 4.25: Effect of CNCP on immunohistochemical staining of wound tissue fragment for Bax & Hsp70 proteins (n=6). SD rat treated with topical application of (A) Gum acacia group, (B) Intrasite gel group, (C) CNCP (10 mg/ml) and (D) CNCP (20 mg/ml) after 10 days. Immuno-positivity showing down-regulation of Bax and up-regulation of Hsp70 in treated groups and intrasite gel (B-D) Scale bar: 10 µm, Bax (first row) & Hsp70 (second row).

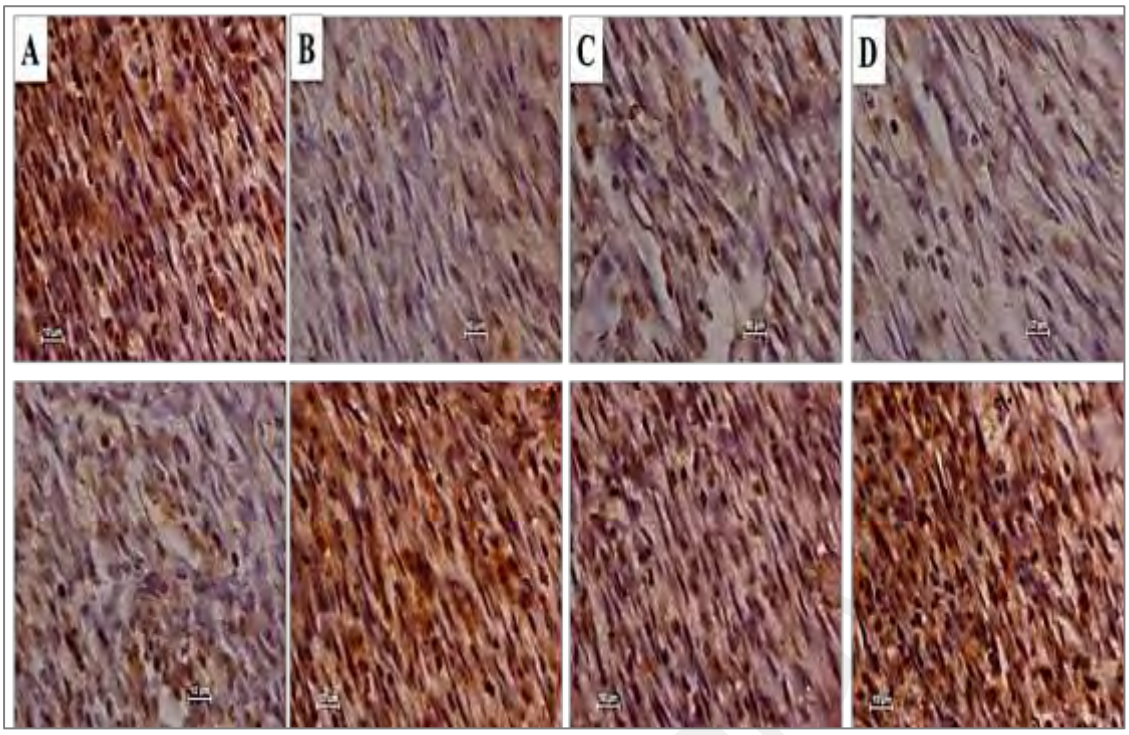

Figure 4.26: Effect of CNBP on immunohistochemical staining of wound tissue fragment for Bax & Hsp70 proteins (n=6). SD rat treated with topical application of (A) Gum acacia group, (B) Intrasite gel group, (C) CNBP (10 mg/ml) and (D) CNBP (20 mg/ml) after 10 days. Immuno-positivity showing down-regulation of Bax and up-regulation of Hsp70 in treated groups and intrasite gel (B-D) Scale bar: 10 µm, Bax (first row) & Hsp70 (second row).

4.13 Effect of CNCP and CNBP antioxidants enzyme activity and MDA levels in wound tissues

The effects of CNCP and CNBP on endogenous antioxidant enzymes (SOD, CAT, and GPx) and MDA levels are illustrated in Figure 4.27. It can be obviously observed that there are alterations in the SOD activity of the injured tissue after application of both compounds and intrasite gel to the wound area. Topical treatment with the compounds CNCP (LD), CNBP (HD), and intrasite gel caused increasing in activity of the enzymes in the lesion tissue homogenates, and therefore, it can be deduced protective method supporting the abrasion curing route in the rats. The CAT activity was significantly high in those animals treated with CNCP (LD), CNBP (HD), and in the positive control group (intrasite gel). The improved CAT action in the injured tissue

is a defensive reply to oxidative harm to hasten the cuts therapeutic course. Treatment with CNCP (LD) and CNBP (HD) caused considerable rise in the activity of GPx enzyme in the tissue homogenates in contrast to the vehicle control group (gum acacia).

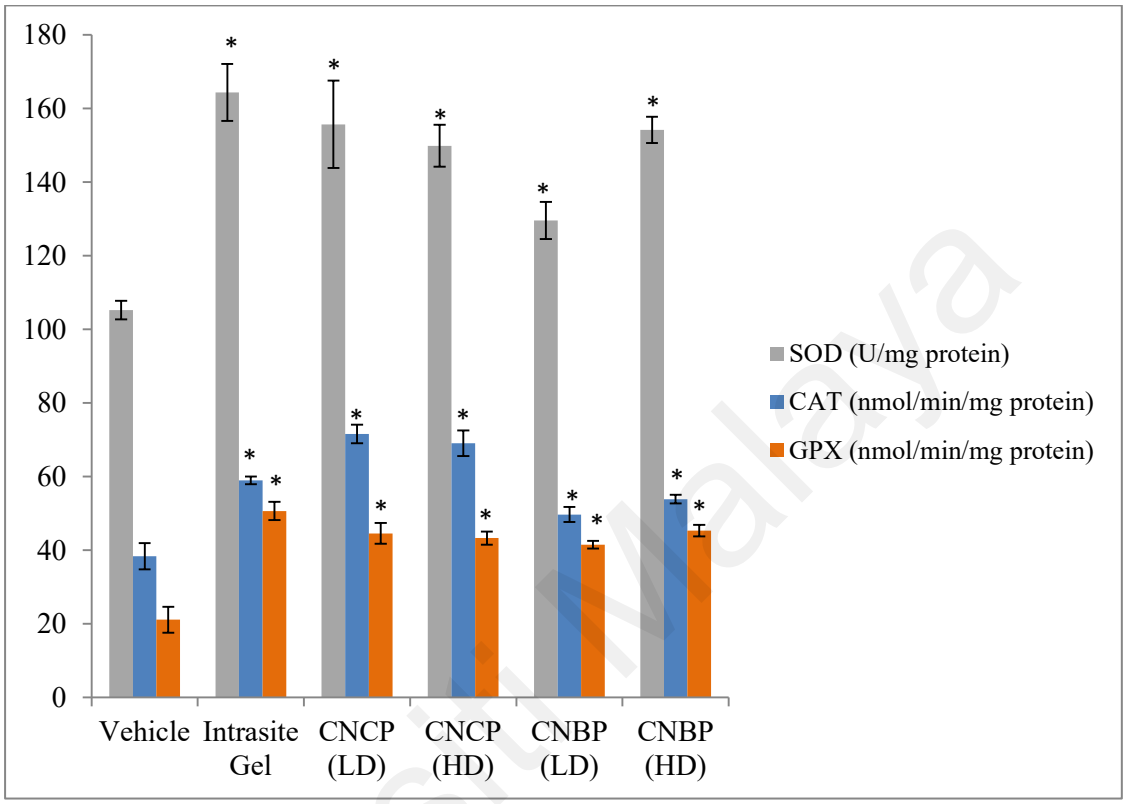

Figure 4.27: Effect of CNCP and CNBP on antioxidant enzymes (CAT, SOD) and GPx level in tissue homogenate. Data is articulated as mean ± SEM, (n=6). *p< 0.05 was considered significant compared to gum acacia.

4.14 Effect of CNCP and CNBP on MDA level in wound tissues

Both doses of CNCP and CNBP, particularly CNCP (LD) and CNBP (HD) animals, and also the positive control group (intrasite gel) show significant reduction of MDA level when compared to the vehicle group (gum acacia) (Figure 4.28). This result suggested that CNCP and CNBP dealing at low and high dosage clearly decreased the lipid peroxidation in the tissues homogenate.

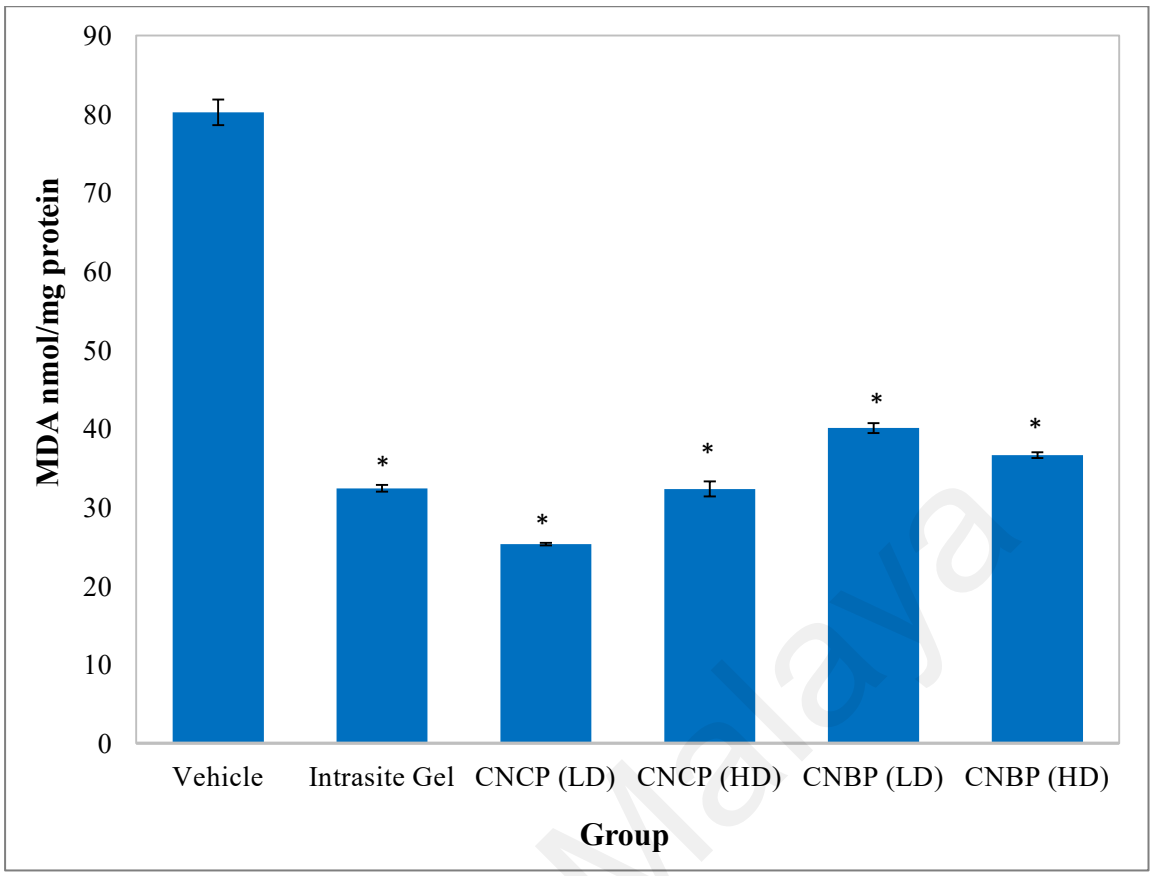

Figure 4.28: Effect of CNCP and CNBP on MDA level in wound tissue homogenate in SD rats. Vehicle treated-cluster illustrates increased level of MDA, while treated wound with intrasite gel, CNCP and CNBP demonstrates significantly reduced MDA level in wound homogenates. Data are accounted as means ± SEM, (n=6). *p< 0.05 compared to gum acacia.

4.15 Type 2 diabetic mellitus experiment of CNCP and CNBP

4.15.1 Biochemical parameters

As listed in Table 4.4, total protein and albumin levels in the diabetic control group were significantly declined when compared to the normal control group. Both CNCP (low dose, LD) and CNBP (high dose, HD) increased total protein amounts as well as albumin levels. CNCP (LD) showed highest increase in the total protein amount and also in the albumin levels versus the diabetic controlled animals. Table 4.4 lists the values which are associated with the effect of both CNCP and CNBP (LD and HD) on aspartate transaminase (AST), alanine aminotransferase (ALT), and also alkaline phosphatase (ALP) activity in the serum of normal, treated, and untreated diabetic rats. As observed, the enzymes activities in the serum of untreated diabetic rats were significantly increased; while AST, ALT, and ALP activity in the serum of CNCP (LD) and CNBP (HD)-treated animals showed noticeable decrease in 6 weeks of post treatment therapy.

Effects of the both compounds (LD & HD) as well as glibenclamide which was used as a reference control for serum triglyceride, total cholesterol, and high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol level in the treated, untreated diabetic normal, and also control rats is listed in Table 4.4. The serum triglyceride, total cholesterol, and LDL cholesterol levels in the untreated diabetic rats were remarkably increased when compared to those of the normal control rats; however, HDL cholesterol level in the untreated diabetic rats showed totally reversed effects. The serum triglyceride, total cholesterol, and LDL cholesterol levels were significantly reduced by treatment with either glibenclamide or CNCP (LD) and CNBP (HD), while, HDL cholesterol level were increased compared to that of the untreated diabetic rats

after 6-week treatment period. Evidently, it can be postulated that continuous treatment with the both compounds could help decrease the lipid parameters in diabetic rats.

Table 4.4: Effect of CNCP, CNBP, and glibenclamide on biochemical parameters in total protein, albumin, liver function and lipid profile of normal and STZ-NA-induced diabetic rats after 45 days of treatment

	Normal Control	Diabetic Control	Glibenclamide	CNCP (LD)	CNCP (HD)	CNBP (LD)	CNBP (HD)
Total protein (g/dl)	8.20 ± 2.66	5.20 ± 1.46	7.10 ± 2.07*	6.60 ± 2.29*	6.20 ± 2.02*	5.90 ± 1.00*	6.50 ± 1.20*
Albumin (g/dl)	5.40 ± 1.76	3.20 ± 2.56	4.80 ± 1.25*	4.67 ± 2.70*	3.76 ± 1.90*	4.37 ± 0.33*	4.60 ± 0.56*
Liver function test							
Alkaline phosphatase (U/l)	69.60 ± 2.99	110.40 ± 2.10	76.60 ± 2.86*	77.20 ± 2.13*	78.80 ± 2.91*	79.33 ± 2.18*	78.77 ± 2.06*
Aspartate transaminase (U/l)	55.00 ± 2.23	92.60 ± 2.72	61.00 ± 2.95*	67.60 ± 2.34*	79.60 ± 2.86*	82.33 ± 2.09*	68.67 ± 2.05*
Alanine aminotransferase (U/l)	46.80 ± 2.68	77.40 ± 2.05	52.80 ± 2.34*	60.40 ± 2.82*	67.80 ± 2.90*	66.00 ± 2.24*	62.00 ± 2.16*
Lipid profile							
Triglyceride (mmol/L)	1.56 ± 0.10	2.65 ± 0.15	1.59 ± 0.07*	1.62 ± 0.17*	1.96 ± 0.31*	1.84 ± 0.15*	1.64 ± 0.24*
Total cholesterol (mmol/L)	2.36 ± 0.17	3.23 ± 0.15	2.57 ± 0.17*	2.43 ± 0.38*	2.64 ± 0.12*	2.60 ± 0.10*	2.48 ± 0.48*
HDL Cholesterol (mmol/L)	1.88 ± 0.12	1.00 ± 0.20	1.73 ± 0.03*	1.52 ± 0.05*	1.42 ± 0.15*	1.46 ± 0.6*	1.51 ± 0.9*
LDL Cholesterol (mmol/L)	0.23 ± 0.11	0.61 ± 0.10	0.26 ± 0.13*	0.28 ± 0.34*	0.32 ± 0.17*	0.31 ± 0.15*	0.29 ± 0.27*

All values are presented as mean ± SEM, compared to diabetic control groups (n = 6). *$p < 0.05$ is considered as significant.

4.15.2 The Effect of CNCP and CNBP on blood glucose

In Figure 4.29, the level of blood glucose in the treated group is assessed against those in the diabetic control group. The diabetic control group showed higher glucose levels in comparison to the normal control group. According to percentage decrease of blood glucose level after using each treatment at different doses at day 45, the treatments (CNCP (LD) and CNBP (HD)) and glibenclamide could decrease glucose level which were obtained with the administration of the both compound treatments, especially astounding find was that glucose levels was markedly decreased in those rats treated with CNCP (LD) (Figure 4.29). It was noticed that blood glucose levels in diabetic rats was significantly decreased after being treated with CNCP (LD), CNBP (HD) as well as glibenclamide from day 21 to 45. However, the doses of the both compounds produced significant reduction in the blood glucose level post day 21 and showed highest lessening rate at day 45 in comparison with the diabetic control group. According to Table 4.5, the highest percentage of variation of glycaemia is seen at day 45 which belonged to CNCP (LD, -31.28 ± 12.80). Therefore, the findings confirmed that both compounds (CNCP and CNBP) decrease blood glucose levels in diabetic rats.

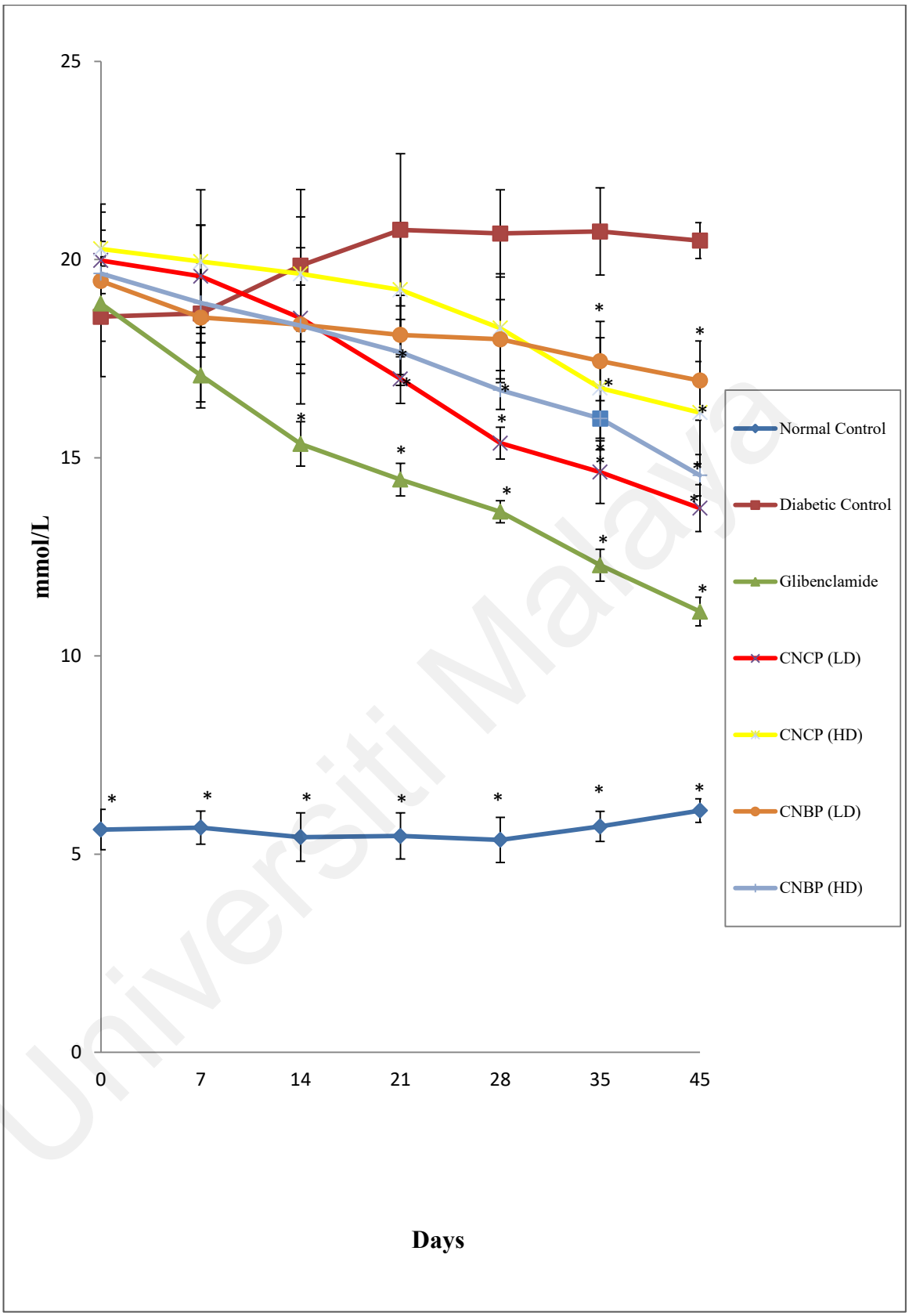

Figure 4.29: Fasting blood glucose levels in STZ-NA-induced diabetic and control rats for 45 days. All values are recorded as Mean ± SEM (n=6), compared to diabetic control groups. *$p < 0.05$ is considered as significant compared to diabetic control.

Table 4.5: Percentage of variation of glycaemia ± SEM

Test samples	Dose	Day 7	Day 14	Day 21	Day 28	Day 35	Day 45
Normal Control	–	**0.89 ± 2.06***	**-3.38 ± 4.38***	**-3.70 ± 6.20***	**-4.63 ± 7.44***	**1.42 ± 9.24***	**8.54 ± 11.27***
Diabetic Control	–	**0.43 ± 2.00**	**6.95 ± 4.76**	**11.80 ± 6.81**	**11.31 ± 7.95**	**11.58 ± 9.22**	**10.34 ± 10.26**
Glibenclamide	**600 (µg/kg)**	**-9.58 ± 2.49***	**-18.74 ± 4.88***	**-23.50 ± 6.63***	**-27.79 ± 8.26***	**-34.94 ± 10.12***	**-41.13 ± 12.14***
CNCP (LD)	**10 (mg/kg)**	**-2.00 ± 2.06***	**-7.31 ± 4.14***	**-14.96 ± 6.45***	**-23.07 ± 8.67***	**-26.73 ± 10.64***	**-31.28 ± 12.80***
CNCP (HD)	**20 (mg/kg)**	**-1.58 ± 2.34***	**-3.11 ± 4.25***	**-5.08 ± 6.02***	**-9.87 ± 7.86***	**-17.32 ± 10.23***	**-20.37 ± 12.63***
CNBP (LD)	**10 (mg/kg)**	**-4.73 ± 2.39***	**-5.65 ± 4.20***	**-6.99 ± 5.96***	**-7.55 ± 7.66***	**-10.38 ± 9.52***	**-12.90 ± 12.5***
CNBP (HD)	**20 (mg/kg)**	**-3.77 ± 1.79***	**-6.72 ± 3.43***	**-10.13 ± 5.02***	**-14.96 ± 6.82***	**-18.63 ± 8.45***	**-25.90 ± 10.97***

All values are presented as mean ± SEM, compared to diabetic control groups (n = 6). *$p < 0.05$ is considered as significant compare to diabetic control. The negative value (−) demonstrated a decrease in glycaemia.

4.15.3 The Effect of CNCP and CNBP on body weight changes

The diabetic rats treated with different concentrations of low dose of CNCP (LD, 10 mg/kg) and high dose of CNBP (HD, 20 mg/kg), respectively caused an increase in body weight when the weights were compared with the untreated diabetic rats. At day 14, no significant difference was found in body weight between the rats treated with both concentrations of CNCP and CNBP. However, at day 28, the rats treated with CNCP (LD) and CNBP (HD) showed an increase in weight gain, compared to diabetic rats. Furthermore, at day 45, those rats receiving low doses of CNCP gained significant body weight (287.50 ± 13.69 gram) (Figure 4.30). With regards to the physical appearance of eye colour, the changes after feeding the rats with CNCP (LD) and CNBP (HD) at 45 days, the eye colour of those animals treated as well as those animals fed with glibenclamide which were found to be completely normal when compared to the untreated diabetic rats who exhibited white eyes syndrome after 45 days. Their lenses also showed obvious lesions of pigmentary degeneration. Besides that the untreated diabetic rats were detected to have thin and rough fur which was followed by very obvious fur loss.

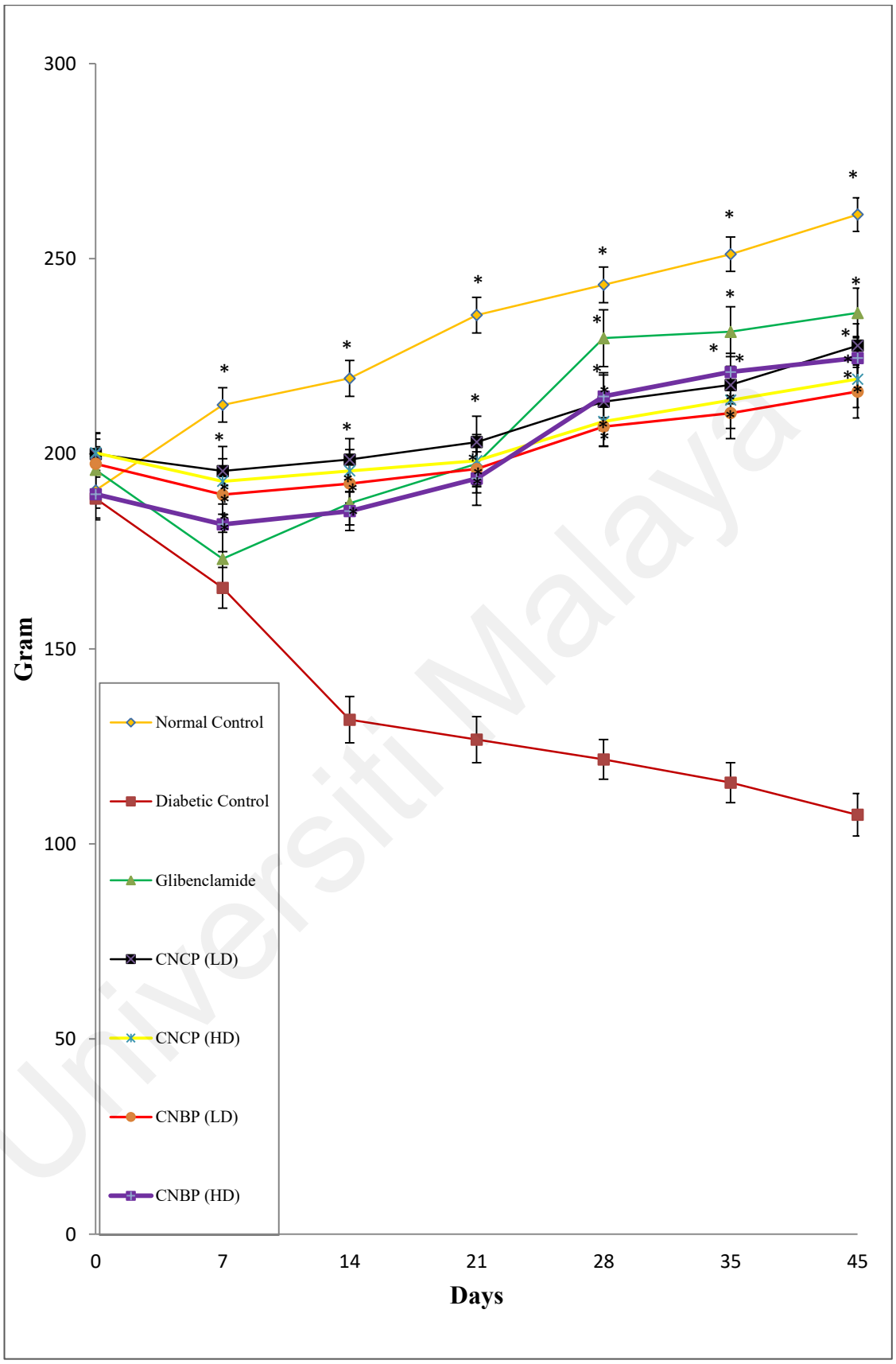

Figure 4.30: Body weight changes of STZ-NA-induced diabetic and control rats for 45 days. All values are recorded as Mean ± SEM (n=6), *p< 0.05 is considered as significant compared to diabetic control group.

4.15.4 Measurement of insulin

Figure 4.31 illustrated the effects of CNCP and CNBP on insulin serum level of STZ-NA diabetic and normal rats. Serum insulin readings in the diabetic control group were noted to be significantly declined when compared to the normal control group after 45 days treatment period. The treated rats with CNCP (LD) and CNBP (HD) fed groups showed high increase of the serum insulin level when compared to the diabetic control group. In addition, the highest increase of the serum insulin belongs to the CNCP (LD, 28.98 ± 1.52) fed groups when compared to the diabetic control group (6.56 ± 1.31). The findings suggested that there was an observation of improvements in glycemic control by CNCP (LD) and CNBP (HD) in the diabetic rats.

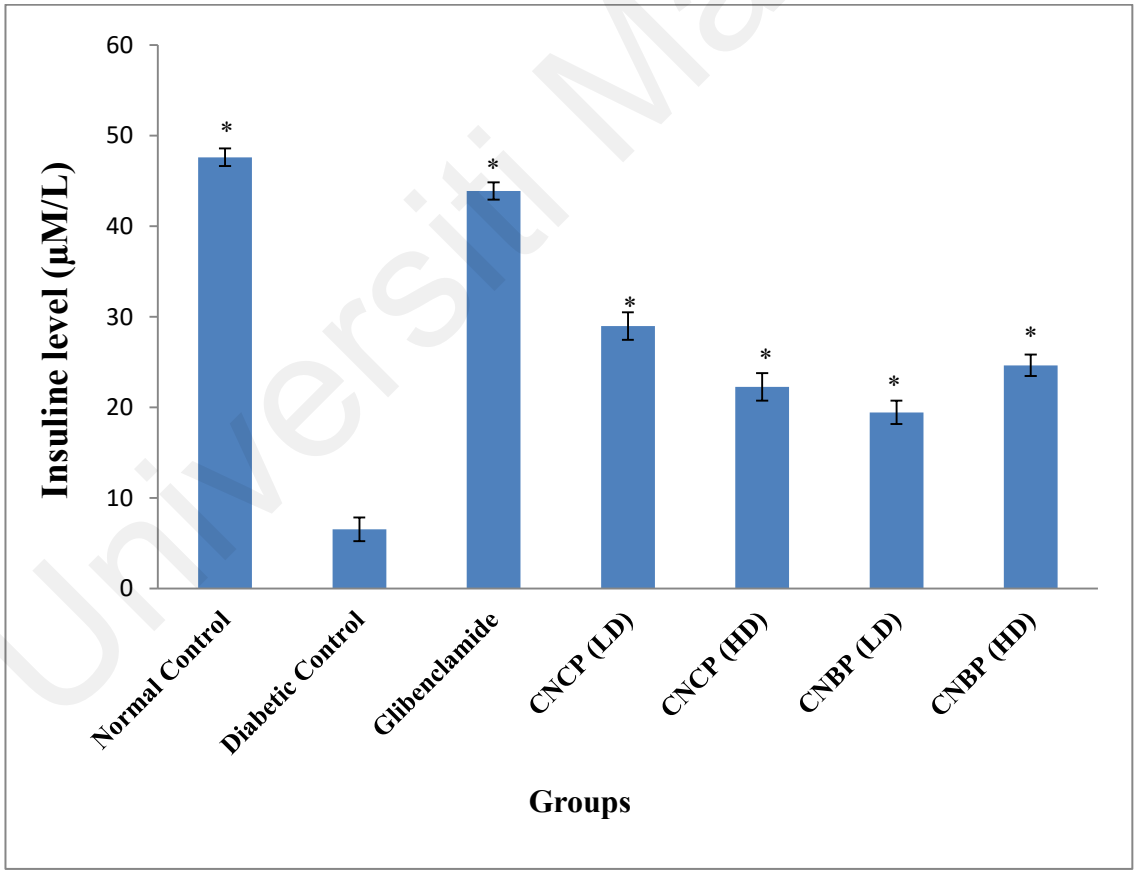

Figure 4.31: Effects of CNCP and CNBP on serum insulin level of STZ-NA diabetic rats in comparison with diabetic control rats after 45 days treatment. Data are presented as means ± SEM (n = 6). Significant difference compared to diabetic control (*p< 0.05).

4.16 Effect of CNCP and CNBP on histological evaluation

4.16.1 Histological investigations of the liver

As depicted in Figure 4.32, the tissue sections of the liver were stained with haematoxylin and eosin. Picture A shows the normal control group rats showed normal hepatic architecture with normal hepatocyte morphology, organized hepatic cell (Hc) cords, and portal vein (Pv) with sinusoidal cords (S). Picture B shows that the diabetic control group had manifested severe pathological changes, such as disordered hepatic cords, focal necrosis, congestion in the portal vein, infiltration of lymphocytes, increase of Kupffer cells (Kc), and also dilated sinusoids (S). Picture C shows the glibenclamide in the reference group showed positive effect on diabetic rats' pathological changes. Picture D shows that in the CNCP (LD) treated group, the hepatic architecture of liver was similar to that of the normal hepatic architecture and was characterized by normal central vein and hepatocytes with no fatty changes and containing mild RBC infiltration. Picture E and F clearly illustrated CNCP (HD) and CNBP (LD) caused sinusoid remained dilated with RBC and central vein in an orderly manner in the liver of the diabetic rats. Finally, picture G demonstrates that CNBP (HD) caused a small amount of inflammatory cell infiltration on diabetic rat liver.

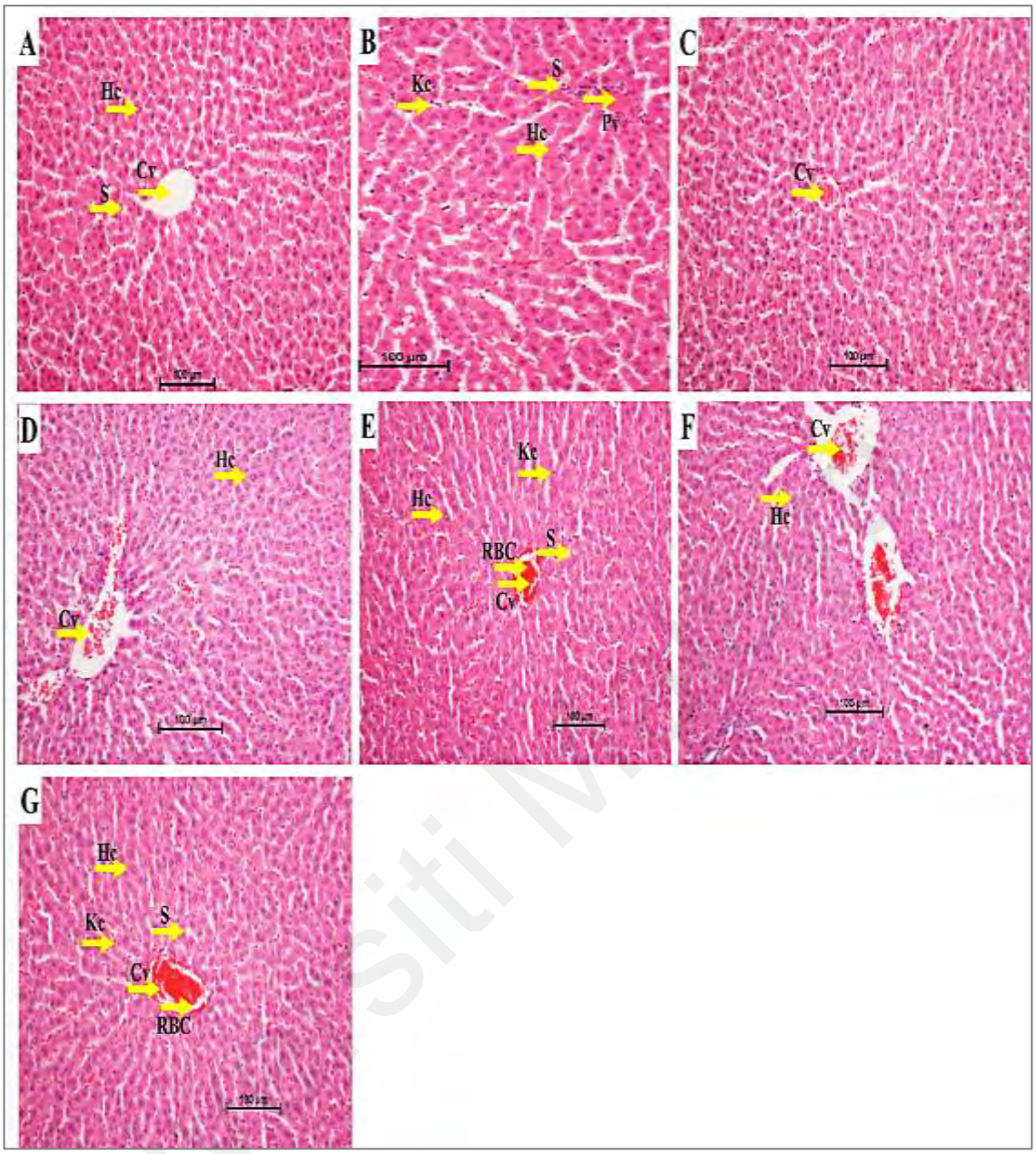

Figure 4.32: H & E staining of liver tissues of SD rats (n=6). (A) Normal control, (B) Diabetic control, (C) Reference control (Glibenclamide), (D) CNCP (10 mg/kg), (E) CNCP (20 mg/kg), (F) CNBP (10 mg/kg) and (G) CNBP (20 mg/kg). Picture A shows that had a normal hepatic architecture. Picture B shows that diabetic control resulted in severe pathological changes, such as increase in Kuppfer cells (Kc), and dilation sinusoids (S). Picture D shows the highest effect obtained by CNCP (10 mg/kg) treatment on the liver of diabetic rat. The hepatic architecture was similar to the normal hepatic architecture (C). Scale bar: 100 µm. Abbreviations: Hepatic cell (Hc), Portal vein (Pv), Central vein (Cv).

4.16.2 Histological investigations of the kidney

Figure 4.33 indicates rat kidney sections stained with haematoxylin and eosin. As shown in picture A, normal control rat kidneys showed normal glomerulus which was surrounded by Bowman's capsule, distal convoluted tubules and proximal with no inflammatory changes. Also, normal capillaries (CP), and normal podocytes (Pc) were observed in kidney sections of the control group. On the contrary, picture B depicts that kidney sections of the diabetic control rats contained degenerated glomeruli which was infiltrated by inflammatory cells and basement membrane thickness. The proximal convoluted tubule showed edematous changes with deposition of mucopolysaccharide and hyaline substances. Furthermore, it contained severe pathological damages including mesangial expansion and glomerular hypertrophy which could cause loading of the Bowman's capsule space and adhesion of capillaries to the wall. Picture C shows that glibenclamide (reference group) showed positive effect on diabetic rats' pathological changes. As shown in picture D and G, CNCP (LD) and CNBP (HD) showed no clear histopathological abnormalities or decreased expansion of glomerular and mesangial matrix in the renal sections. Both pictures E and F show the effect of CNCP (HD) and CNBP (LD) on diabetic rat's kidney which verified the moderate changes of the kidney tissue.

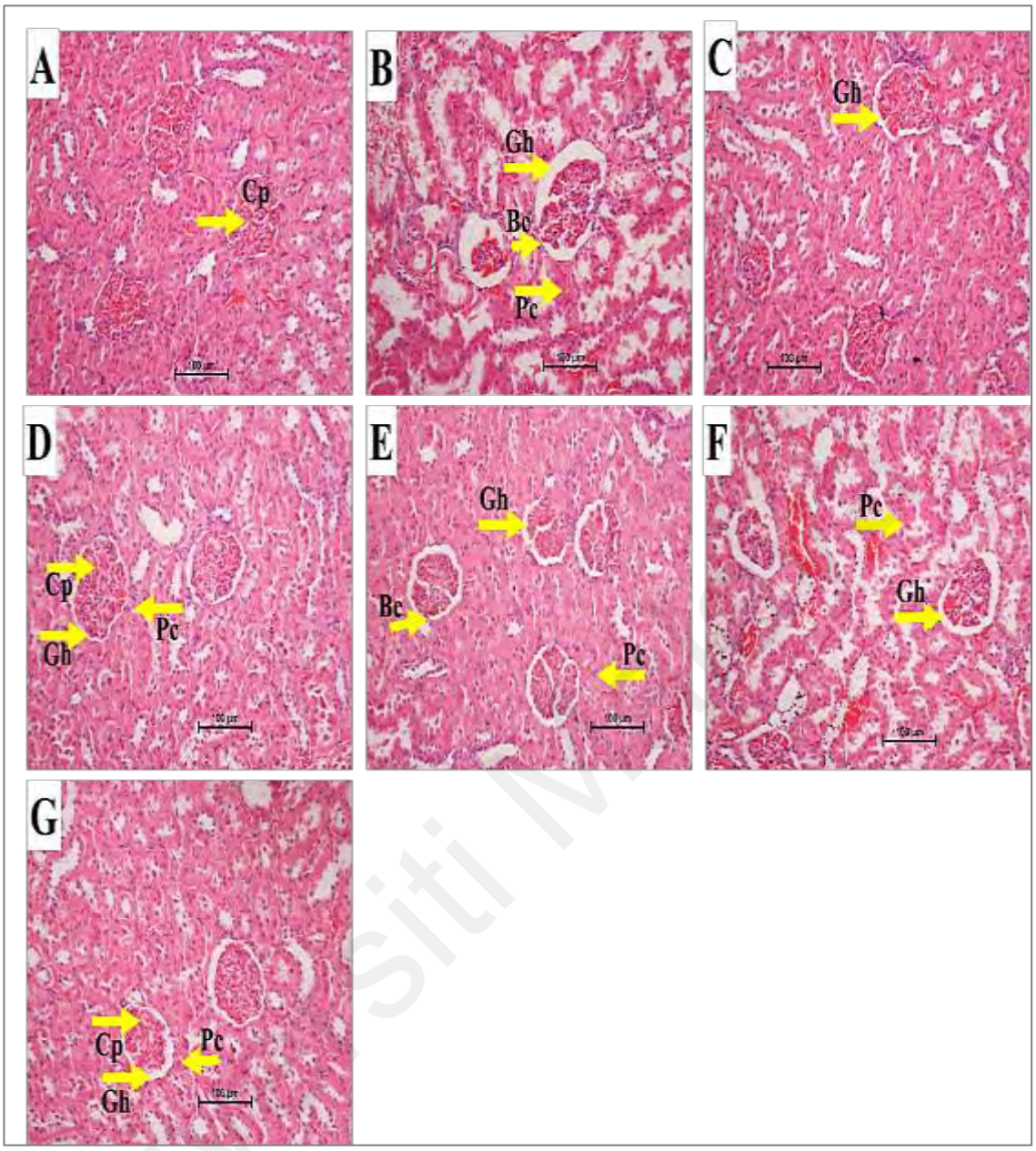

Figure 4.33: H & E staining of kidney tissues of SD rats (n=6). A) Normal control, (B) Diabetic control, (C) Reference control (Glibenclamide), (D) CNCP (10 mg/kg) and (E) CNCP (20 mg/kg), (F) CNBP (10 mg/kg) and (G) CNBP (20 mg/kg). In diabetic rats (B), there is a significant damage to the glomeruli (hypertrophy, hypercellularity, tubules dilatation and atrophy) compared to the normal control (A). Treatment of CNCP (10 mg/ml) and CNBP (20 mg/ml) attenuated the kidney alteration (D and G) in diabetic rats, which are comparable with the reference control (C) on the STZ-NA-induced pathological changes. Scale bar: 100 µm. Abbreviations: Bowman's capsule (Bc), Podocytes (Pc), Capillaries (CP), Glomerular hypertrophy (Gh).

4.16.3 Histological investigations of the pancreas

Staining results of the rat's pancreas sections with hematoxylin and eosin are shown in Figure 4.34. In picture A, the normal control group's pancreatic section shows normal islets of Langerhans, normal pancreatic structure, and normal acini tissues. Picture B shows disorganization of structure of the endocrine and exocrine cells with some damages, necrotic pancreatic acini and islet shrinkage. As shown in picture C, the reference control group exhibits the most interesting aspect of examined pancreatic sections which shows normal islets of Langerhans. In picture D and G, effect of CNCP (LD) and CNBP (HD) on diabetic rat's liver demonstrated that these factors could much better promote cell survival and also provide a much healthier structure. Effects of the CNCP (HD) and CNBP (LD) on the diabetic rat pancreas (picture E and F) display moderate restoration of the pancreatic cells.

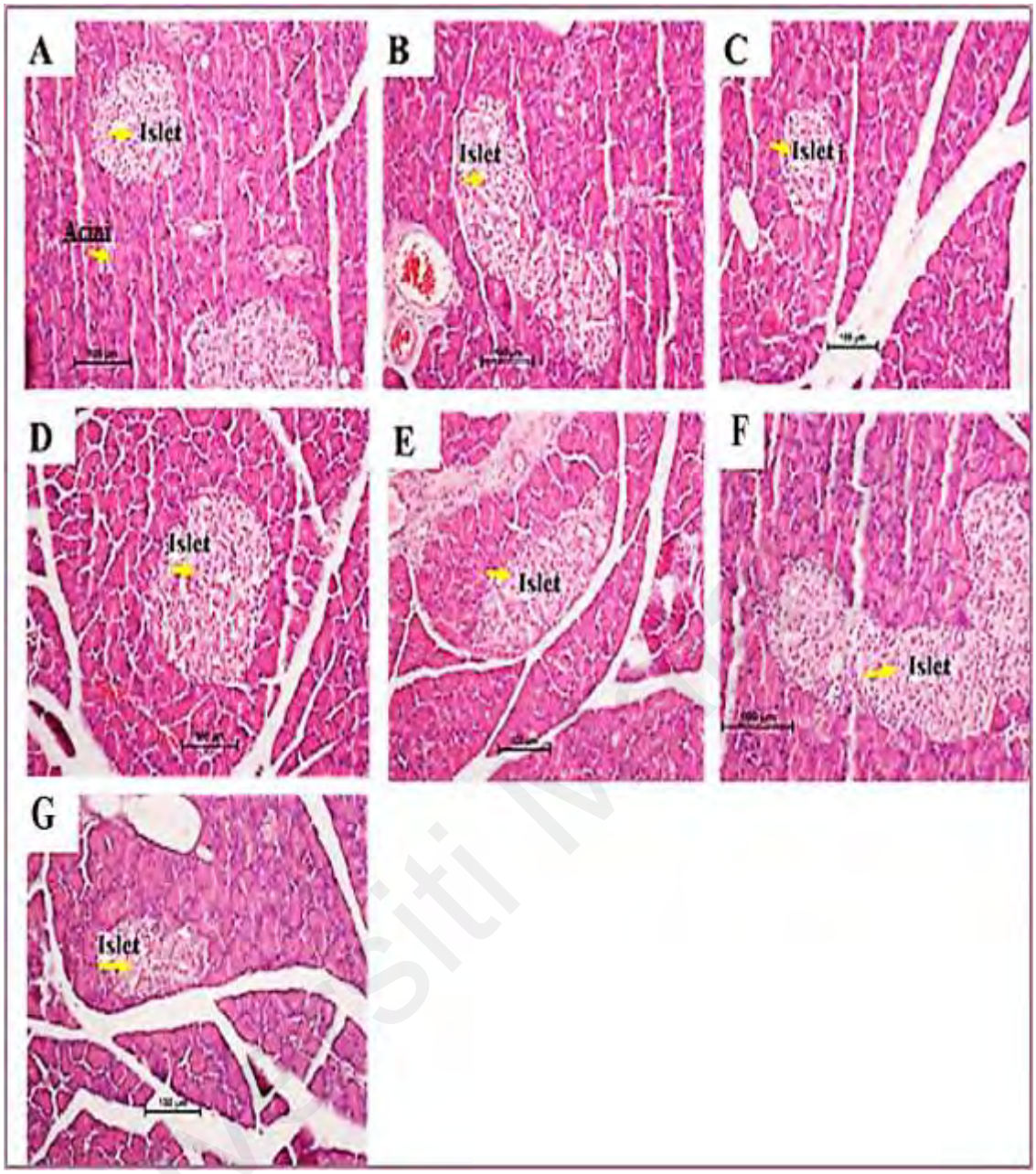

Figure 4.34: H & E staining of pancreas tissues of SD rats (n=6). A) Normal control, (B) Diabetic control, (C) Reference control (Glibenclamide), (D) CNCP (10 mg/kg) and (E) CNCP (20 mg/kg), (F) CNBP (10 mg/kg) and (G) CNBP (20 mg/kg). In picture (A), the normal control pancreatic section showed normal islets of Langerhans. Picture (B) diabetic control shows a disorganisation of the pancreatic structure. The treatment of reference control (Glibenclamide) in picture C showed normal islets of Langerhans. In Picture (D), the effect of CNCP (10 mg/kg) on diabetic rat's pancreas demonstrates that these factors better promoted the cells and provides a more healthy structure and shows a prominent hyperplastic islet and improved islet morphology. Scale bar: 100 µm.

CHAPTER 5: DISCUSSION

5.1 Novel Schiff base derivatives (CNCP and CNBP)

The structures of both compounds which were used as the key intermediates for the biological activities were confirmed by spectroscopic methods. The spectra's are given in Appendix A and B.

^{1}H NMR of the chemical structure of 2, 2-[1,2-cyclohexanediylbis (nitriloethylidyne)] bis(4-chlorophenol) (CNCP) shows two doublet signals of four aromatic protons at about δ 7.34 and δ 6.79 ppm as well as a double doublet signal of two aromatic protons at about 7.16 ppm. The protons for two groups of CH-N show double triplet to multiple signals appear at about 3.85 ppm. The protons of the CH3 groups appear as singlet signal at 2.25 ppm. While the rest protons of cyclohexane ring show triplet to multiple signals at about 1.9, 1.67 and 1.48 ppm respectively.

^{13}C NMR spectra shows the signals of the two C=N at about δ 170.17 ppm. The spectra also show the presence of the two carbons signals attached to hydroxyl groups at 162.28 ppm. The tertiary carbons of the aromatic rings appear at about 132.40, 127.90 and 120.09. Whereas, quaternary carbon, CAr-Cl and CAr-CN at 121.84 and 119.9 ppm respectively. The CH-N carbons were found δ 63.27. The positions of cyclohexane carbons methylene at δ 32.32 and 24.19 and methyl carbons were located around δ 14.55 ppm. ^{13}C NMR is used to assign the carbon skeleton, applying the PENDANT (Polarization Enhancement Nurtured during Attached Nucleus Testing) experiment to distinguish carbons attached to varying numbers of H. In PENDANT spectra, carbons attached to odd numbers of protons (methyl, CH3; methine, CH) appear as negative signals, whereas those attached to even numbers of protons (methylene, CH2; quaternary, C) appear as positive signals.

^{1}H NMR of the chemical structure of 2,2-[1,2-cyclohexanediylbis (nitriloethylidyne)]bis(4-bromophenol) (CNBP) shows two doublet signals of four aromatic protons at about δ 7.34 and δ 6.79 ppm as well as a double doublet signal of two aromatic protons at about 7.16 ppm. The protons for two groups of CH-N show double triplet to multiple signals appear at about 3.85 ppm. The protons of the CH3 groups appear as singlet signal at 2.25 ppm. While the rest protons of cyclohexane ring show triplet to multiple signals at about 1.9, 1.67 and 1.48 ppm respectively

^{13}C NMR spectra shows the signals of the two C=N at about δ 170.17 ppm. The spectra also show the presence of the two carbons signals attached to hydroxyl groups at 162.83 ppm. The tertiary carbons of the aromatic rings appear at about 135.24, 130.87 and 120.82. Whereas, quaternary carbon, CAr-Br and CAr-CN at 120.62 and 108.79 ppm respectively. The CH-N carbons were found δ 63.21. The positions of cyclohexane carbons methylene at δ 32.33 and 24.19 and also methyl carbons were located around δ 14.57 ppm.

5.2 *In vitro* evaluation of Schiff base derivatives (CNCP and CNBP)

5.2.1 Antioxidant properties of CNCP and CNBP

Excessive oxidative stress and normal organ functions have been proven to give rise to ROS like free radicals (Kumar & Rawat, 2013) and elevated levels of free radicals is believed to cause damage to the lipid structure, proteins and DNA. This in turn can lead to several disease pathogenicity's which also include hindered ulcer and wound healing (Kumar & Rawat, 2013). And on the other spectrum, antioxidants are acknowledged to help provide protection against cellular impairment or destruction caused by any oxidative stress and to help boost the body's defence systems against degenerative diseases (Ahmad et al., 2013). The decrease of oxidative stress owning to the action of antioxidants is said to be central for therapeutic anti-ulcer efficacy (Mahmood et al.,

2011), and for the protection of the gastric mucosa against various necrotic agents (Sidahmed et al., 2013a). Several studies have shown that, Schiff base compounds and their by-products do possess antioxidant activity which has healing and beneficial abilities (Abou-Hussein & Linert, 2014; Chen et al., 2003).

This work assessed the antioxidant action of two Schiff bases derivatives; CNCP and CNBP. These results confirmed and concurred with other researchers (Chen et al., 2003; Raweh et al., 2013) that Schiff bases may possess potential antioxidant activity. Antioxidant action and detection of CNCP and CNBP were performed via DPPH and FRAP assays. Studies have demonstrated that the metabolism of ethanol in the body could produce ROSs, such as superoxide anions and/or hydroperoxyl leading to gastrointestinal ulcer in varying degrees (Gwaram et al., 2012; Ismail et al., 2012). Investigations into the DPPH free radical scavenging activity unearthed that both compounds possessed antioxidant free radicals foraging power. The probable cause for the moderate DPPH values observed may be explained by the location and existence of the Schiff base phenolic hydroxyl and the amido groups that significantly plays a role on the antioxidant prowess of the Schiff bases (Guo et al., 2005; Raweh et al., 2013). The effect of DPPH activity by the Schiff base phenolic hydroxyls is inversely correlated to the attributes of the hydroxyl groups affixed to the phenolic rings in the compounds. It has been reported that improved electron density of the aromatic ring due to the electron providing substituents and the probable redox effects that enables the compounds to be more responsive towards electrophilic assaults. This then has a significant impact on the absorption and neutralization of free radicals, the quenching of singlet and triplet oxygen, as well as the disintegration of peroxides (Gwaram et al., 2012; Khaledi et al., 2011).

Ferrous ion has been discovered to be chelated by, when experiment was carried out to evaluate FRAP action of the compounds. The anti-oxidant properties are documented to be linked with the reducing power of a compound and are a noteworthy indicator of any anti-oxidant ability. When the compounds are reduced, thus serves to show that they are electron donors which may decrease the oxidized intermediates of the lipid peroxidation process; hence functioning as primary and secondary antioxidants. A paper by published in 1996, has documented that the FRAP assay is centered on the notion that at lower pH, the reduction of the ferric occurs, which results in the formation of chromatic ferrous-tripyridytriazine compound (Benzie & Strain, 1996). This test is deemed as a reliable technique for analysing the antioxidant power of many therapeutic compounds (Gwaram et al., 2012). The examined and documented FRAP activity of these Schiff bases (CNCP and CNBP) may be related to escalation in the delocalization of the π-electron surrounding the phenolic ring causing an increase in the density of the electron, and resulting in reduced ferric ion (Gwaram et al., 2012). Similar observations of increased activity of FRAP in Schiff bases have been described in many acclaimed papers (Parmar et al., 2015; S. M. Salga et al., 2011).

5.3 *In vivo* evaluation of Schiff base derivatives

5.3.1 Acute toxicity study

Severe toxicity studies are the primary step in assessing and the examination of toxicological chemicals or pharmaceutical substrates (Andersen & Krewski, 2009). International guidelines in the pharmaceutical and chemical industry in present times require the use of rodents as models in any safety evaluation and testing (Danneman et al., 2012). It is stated in the guidelines that for short-term toxicity studies both rats and mice can be used as outlined in the Food and Drug Administration Guidelines for Toxicological Principles for the Safety Assessment of Food Ingredients (Redbook,

2000). The outcome of this present study shows valid scientific proof for establishing the safe dosage levels of two novels Schiff based derivatives (CNCP and CNBP) through an acute toxicity study using rats. Acute toxicity is also known as the toxicity caused by any substance when administered to animals, by means of one or more than one dose. The toxicity needs to be observed and checked regularly for 14 days after administrating pharmaceutical substance or compounds. Complete clinical precursors, deaths, time of onset, duration, and reversibility of the toxicity must be documented within 15 days (Bidlack, 2002). In this present study, the female rats were oral gavaged with CNCP and CNBP suspension via two doses of 100 mg/kg and 200 mg/kg in single dose at a rate of 5 ml/kg. After administration of compounds, the animals were kept under observation daily for 14 days for any behavioral changes, toxicological symptoms or mortality. The nonappearance of any indication of toxicity in addition to the occurrence of death during the experimentations advocates that the compounds at 100 and 200 mg/kg were harmless and non-toxic to use. In addition to no changes in the body weight, nil hepatic or renal toxicity in the rats administered with CNCP and CNBP (Danneman et al., 2012). Further analysis of histological examinations using hematoxylin and eosin staining also verified that there were no nephrocellular or hepatocellular injuries in the rats.

5.3.2 Gastroprotective effect of CNCP and CNBP

Gastric ulcer is the deterioration of extensive necrotic lesion encompassing whole mucosal vastness and muscularis mucosa (Malfertheiner et al., 2009) and can be developed in any imbalanced conditions in the gastric system. Such undesirable conditions can happen when the mucosal defensive system is affected by some detrimental factors, such as, high acidity, infection, and other factors on the luminal ‘surface of stomach’ (Padmavathi et al., 2012). It causes puncture or/and acute

haemorrhagic ulcer; however, there are some internal restrictive factors, such as PGE2, mucus secretion, and bicarbonate synthesis, which can inhibit ulcer progression. Despite the major role of defensive system against any ulcer in the body, some imbalanced conditions between self-protective agents and external or internal damaging factors, including extra stomach acid, *H. pylori* infection and its proteolytic enzymes, ethanol consumption, cigarettes and etc. lead to peptic ulcer formation (AlRashdi et al., 2012a).

It has also been found that generation of free radicals, which are initiated by such damaging factor, plays key roles in the formation and progression of the disease (Ketuly et al., 2013). Some previous studies showed direct correlation between suppression of extra acid production in the stomach and the effective treatments (Malfertheiner et al., 2009; Saremi et al., 2019b). Due to the crucial role of extra acid neutralization in stomach by lowering the activation of acid producer pumps and the prominent role of oxidants in the production of extra acidity, antioxidants can play key roles in inhibiting gastric ulcer and the relative diseases (Malfertheiner et al., 2009; Saremi et al., 2019b). In the present study, CNCP (HD) and CNBP (HD) were found to increase the generation of epithelial cells, which could drastically increase protein concentration in the gastric secretions of the pre-treated animals. Several factors can contribute to the formation of ethanol-induced gastric wounds, such as gastric mucus reduction and over production of free radicals. Such factors, individually or maybe together, lead to lipid peroxidation, resulting in the damage of surface layer of mucosal epithelium (Al Batran et al., 2013b). CNCP (HD) and CNBP (HD) showed significant effect against acute haemorrhagic lesions of gastric mucosa induced by ethanol.

It was found that gastroprotective activity of CNCP (HD) and CNBP (HD) could be due to its ability for maintaining stomach mucus discharge, leading to decrease of mucosa volume and increase of surface mucosal area. Both compounds at high doses were found to have significant gastroprotective effect via increasing both pH and mucus

and decreasing sub-mucosal edema and submucosal inflammation. Such findings were in agreement with several other studies, which evaluated the gastroprotective and anti-ulcer activity of several novel synthetic compounds (Golbabapour et al., 2013b; Ibrahim et al., 2012). It was found that gastric mucus functions, such as inhibition of over production and secretion of acid and pepsin, were considered as notable defensive factors against any injuries of gastrointestinal tract (AlRashdi et al., 2012b). In the present study, it was observed that CNCP (HD) and CNBP (HD) could considerably increase the stomach mucus secretion and decrease the acidity of stomach contents. Additionally, the compounds could increase the glycoprotein of glandular portion of surface epithelium, which was confirmed by PAS staining. Similarly, the results of some previous studies demonstrated significant incline of gastric mucus secretion in rats pre-treated with different synthetic compounds (Golbabapour et al., 2013b; Hashim et al., 2012; Ketuly et al., 2013; Mustafa et al., 2009a). The findings showed that gastroprotective effect could be resulted from the preservation of mucus secretion. Progression of gastric mucosal damage could be developed by stress, causing drastic increase in stomach's acid secretion and leading to mucosal layer damage, so it could not be protected by suppressing mucus production (M. Abdulla et al., 2010; Gornall et al., 1949).

CNCP and CNBP-pre-treated animals showed considerable decline in MDA level compared with ulcer control group animals, which can be caused by the capability of the compounds to increase the activation of the antioxidant enzymes. Likewise, reduction of MDA level and induction of activities of SOD and CAT were suggested to be the most important factors associated with the gastroprotective effect of drugs (Ibrahim et al., 2016). It was also confirmed in the study that SD rats fed with CNCP (HD) and CNBP (HD) showed significant increase in both SOD and CAT activities, which were coupled with the reduction of gastric MDA level. The results were in

agreement with those reports indicating that the activities of SOD and CAT were increased in the synthetic compounds-treated rats (Golbabapour et al., 2013a; Halabi et al., 2014; Ketuly et al., 2013; M. S. Salga et al., 2011). Some previous studies suggested that PGE2 can be in activated when free radicals are increased, leading to mucosal damage. Significant role of PGE2 in regulating mucus secretion from the stomach walls has been proved and found to possess defensive activity indifferent gastric wound models (Brzozowski et al., 2005; Golbabapour et al., 2013c). Takeuchi et al. (2014) found the mechanism of action of prostaglandins in motivating mucus production and bicarbonate discharging. This could sustain mucosal blood circulation, leading to the protection of epithelial cells from any damage induced by some inflammatory elements, such as cytotoxins. Hajrezaie et al. (2012b) found that the gastroprotective action of prostaglandins against stomach mucosal damage could be initiated by the preservation of gastric mucus productions. Based on the results of the current study, it is noticed that enhancement of the mucosal level of PGE2 in CNCP and CNBP-treated rats can partly support the gastroprotective effect of the compounds.

Ibrahim et al. (2016) noticed that the HSP70 protein level was increased in response to either stress or high generation of ROS. In fact, the ethanol-induced ROS generally can damage proteins, causing partial unfolding and aggregation. CNCP (HD) and CNBP (HD) could protect gastric tissues via up-regulation of HSP70, which inhibits tissues from aggregation. In addition, Singh et al. (2016) suggested that HSP70, as an important apoptosis regulator protein, can regulate both adaptive and internal immune reactions. In fact, the HSPs family, particularly HSP70, can interplay with both up- and down-stream apoptotic mediating elements against stress condition via cytoprotection. In peptic ulcer, for instance, it was found that HSP70 inducers could protect gastric mucosa against ethanol-induced injury through rehabilitation of HSP70 expression in gastric wall cells in animal model. Moreover, HSP70 protein possesses conservative

effect on usual construction of proteins and also shows excellent ability to abolish toxic remains of cell lysis (Kim et al., 2006). Similarly, up-regulation of HSP70 protein in the testing animals showed that CNCP (HD) and CNBP (HD) possessed gastroprotective effect by enhancing HSP70 expression in gastric tissues of the treated rats.

The pro-apoptotic protein, Bax, as a member of Bcl-2 family, is associated with the regulation apoptosis through mitochondrial damages (Reed, 1997; Salama et al., 2013). In fact, ethanol can trigger the induction of apoptosis in gastric epithelium by overexpression of pro-apoptotic proteins, such as Bax and/or down-expression of anti-apoptotic bodies, like Bcl-2 (Kerr et al., 1972). Bax protein was found to be down-regulated and HSP70 up-regulated in the gastric tissues of treated SD rats administered with CNCP (HD) and CNBP (HD) when compared with those in the ulcer control group. The present findings were in agreement with the results of some previous studies indicating that induction of HSP70 protein along with suppression of Bax protein in animals can cause protection of gastric mucosa against damages induced by ethanol (AlRashdi et al., 2012b; Ismail et al., 2012; Ketuly et al., 2013; Sidahmed et al., 2013b).

In summary, both compounds are safe for use and these have significant gastroprotective potential via the protection of stomach epithelium against ethanol-induced damage. CNCP (HD) and CNBP (HD) could drastically enhance the activities of SOD and CAT, while it could retard the level of MDA in stomach tissue homogenates and could remarkably elevate the level of PGE2. The significant increase of GWM in CNCP and CNBP-treated rats suggested the protective capacity of the compounds. Reduction of haemorrhagic mucosal lesions in stomach mucosal epithelium and decrease of edema and leukocytes penetration of sub-mucosal layers were also observed in CNCP and CNBP-treated animals. CNCP (HD) and CNBP (HD) could increase glycoprotein contents, and moreover it could cause up-regulation of HSP70

protein and down-regulation of Bax protein, indicating its effectiveness in regulating apoptosis. The present study provides convincing evidence that CNCP (HD) and CNBP (HD) possessed gastroprotective activity in gastric ulcer models via the pathways, leading to the increase of the levels of mucus and PGE2, as well as the activities of SOD and CAT.

5.3.3 Wound healing potential of CNCP and CNBP

Wound healing is an extremely intricate, multi-factor series of a process which is connected to the numerous cellular and biochemical activity. Oxidative tensions and free radicals are involved in developing any type of wound. The repair of injury is multifaceted procedure that re-establishes and helps damaged tissues to turn back to its ordinary condition. This is done by starting the propagation of fibroblast, as well as collagen filaments and angiogenesis in granulation tissue, and helps to scar development, injury narrowing, and epithelialization (Kuipers et al., 1995). CNCP (LD) and CNBP (HD) could significantly accelerate the rate of wound closure compared to the vehicle (gum acacia). Histology of granulation tissue in healed wounds by CNCP (LD) and CNBP (HD) showed more fibroblasts proliferation, collagen synthesis, and few inflammatory cells infiltration according to the histology results which show that the compounds cause faster curing injury in the wound area than the vehicle control group. Wound repairing is possibly resulted from up-regulation of collagen (Abdulla et al., 2010) and blood vessel capillaries in granulation tissues causing enhanced blood circulation in the injured locations and so supplying higher oxygen as well as necessary nutrients for successful healing progression and re-epithelization (Dhiyaaldeen et al., 2014). Therefore, improvement of cell proliferation and angiogenesis can be considered as core factors for successful wound healing (increased wound tensile strength and accelerated process) (Abood et al., 2015; Hajrezaie et al., 2015). The current findings support the studies conducted by Mughrabi et al (2011), El-Ferjani et al (2016), and

Saremi et al (2019a), showing that the wounds treated with novel Schiff bases derivatives (CNCP and CNBP) contained fewer inflammatory cells and more collagen in granulation tissue in rat models.

The production of excessive ROS at injury sites causes activation of pro-apoptotic proteins, such as Bax which can rail cell apoptosis. Activation of such proteins has direct correlation with lowering frequency of healing process of wound, and therefore, down-regulation of such proteins in the wound area contributes to accelerate healing process (Moulin et al., 2004; Rai et al., 2005). In this work, the immunostaining of the wound cut segment show up-regulation of Bax protein in the vehicle control group and indicating that the lesion curative progression is motionless at the inflammatory stage. On the other hand, those wounds treated with CNCP (LD) and CNBP (HD) or intrasite gel illustrate significant down-regulation in Bax protein, signifying that the wound curing time is at the subsequent step by blocking and slowing the onset of necrosis cell and improves vascularity (Rouhollahi et al., 2015). Bax, an input protein is related to apoptosis during mitochondrial injury. Apoptosis is known as the major probable function in cellular removal in the diverse stages of remedial injury (Brown et al., 1997). Heat shock proteins (Hsps) are critical factors which can positively involve in cell proliferation as well as collagen synthesis and moderating inflammatory responses (Bellaye et al., 2014) causing improvement of wound healing process (Kovalchin et al., 2006; Rouhollahi et al., 2015; Tsukimi et al., 2001). Among Hsps family, Hsp70 plays key role to speed up wound healing by ameliorating protein homeostasis and cell survival as well as declining rate of protein denaturation and protecting the cells from oxidative stress (Atalay et al., 2009; Rouhollahi et al., 2015; Song et al., 2014). In the current study, up-regulation of Hsp70 protein in the wounds treated with CNCP (LD)

and CNBP (HD) indicated that the compounds induce expression of Hsp70 in the injured tissue and accordingly improved wound healing progression.

Neutrophils are the first cells which appear in the wound sites and start recruiting inflammatory process leading production of ROS in the wound area. ROS is considered as detrimental interference in the wound healing process (Babior, 2000; Soneja et al., 2005), hence, the antioxidant enzymes are activated in the wound area to reduce and remove ROS and help improvement of the healing (Judith et al., 2010; Sen et al., 2002). Assessment of activity of antioxidant enzymes, such as SOD and CAT as well as GPx in granulation tissues is suggested as important factors to evaluate the wound process. In the inflammation phase, macrophage numbers are increased and contribute to digestion and phagocytosis of wound debris which lead to produce the excessive ROS formation in the wound area (Bereznicki, 2012; Guo & DiPietro, 2010; Tellechea et al., 2010). Furthermore, excessive ROS induces lipid peroxidation, leading to damage the cells, causing an increase in MDA levels in the wound site. As a matter of fact, MDA destructs the functions of collagen and fibroblast metabolism, endothelial cells, and keratinocyte capillary permeability (Odukoya et al., 2012).

In this study, the wounds which were treated by CNCP (LD) and CNBP (HD) shows significant raise in SOD, CAT and GPx activities and extensively decline proportion of injury retrenchment and lipid peroxidation (MDA) which are evaluated in the vehicle control group. The rise of both SOD and GPx enzymatic activities enhances hunting of superoxide, hydrogen peroxide, hydroxyl and lipid peroxyl radicals which can be increased in wounds (Rouhollahi et al., 2015). Furthermore, the increase of CAT activity may change harmful peroxyl radicals into protected material, such as water which is safer for cells (Targosz et al., 2012). CNCP (LD) and CNBP (HD) showed decrease in MDA level along with improving the viability of collagen fibers in

comparison with the vehicle control group (gum acacia). This most likely reflects the raise in lipid oxidation in the injured tissue leading creation of free oxidative radicals or reducing antioxidant defence activity, or both (Musalmah et al., 2001; George et al., 2014). The findings demonstrated that any treatment with capability of preventing lipid peroxidation might improve viability of collagen fibers by increasing their strength, accelerating circulation, inhibiting cell damage and increasing DNA synthesis.

5.3.4 Anti-diabetic potential of CNCP and CNBP

It is a well-known fact that diabetes can be a major killer worldwide and based on the estimation, 387 million people across the world suffer from this disease (Group, 2014), hence, the need of assessing and garnering targeting approaches for effective therapeutics is increasing. Among the array of natural products and chemical agents, Schiff base compounds are of those candidates which has drawn researchers' attention in recent years, due to its outstanding biological activities (Ndagi et al., 2017). Moreover, generally, both metals and some chemical elements, such as bromine, chlorine, oxygen and hydrogen, are regarded as vital components of cells chosen by nature, investigators attempt to substitute such elements to other available agents such as Schiff based compounds to obtain new combinations with higher efficacy in treating various diseases (Tao et al., 2015; Verma et al., 2018), such as diabetic diseases (Mahmoud et al., 2016; Shukla et al., 2019; Torabi et al., 2018). When chlorine is substituted with other anti-diabetic agents, it can promote anti-diabetic activities. For instance, metformin as a strong and famous anti-diabetic drug can be substituted with chlorine atoms, such as metformin P-chlorophenoxyacetic (Lara, 2017) and metformin hydro-chloride (Hasan et al., 2013; Narayana Reddy et al., 2012). It is shown that new chlorine substituted metformin compounds are more potent in reducing plasma glucose levels in comparison to metformin which can be due to the effect of chlorine atom on

chlorine pump and insulin resistance of β-cells that regulates insulin secretion (Narayana Reddy et al., 2012; Siddiqui et al., 2014). CNCP at lower dose (LD) showed highest activity to reduce hyperglycaemia after six weeks of treatment in comparison with CNCP (HD) and CNBP (HD and LD). Moreover, both compounds showed beneficial pharmacological effect on diabetic, liver functional test, lipids profile, and renal function.

Diabetes disease produces disturbances in lipid profiles, particularly causes of increased lipid peroxidation, which is one of the most essential culprits for developing diabetes mellitus (Lu, 1999). Free radical production lead to oxidative stress enhancement which plays an outstanding role in pathogenesis and progression of the diabetic disease (Kakkar et al., 1995). Moreover, lipid peroxidation is suggested to be a factor associated with hepatotoxicity when the activity of serum marker enzymes such as AST, ALT, and ALP are significantly increased (Janani et al., 2009). With regards to normalized serum lipids profile, CNCP (LD) shown to boost the levels of the enzymes markers (AST, ALT, and ALP) back to the normal value. This result suggests that CNCP (LD) could be beneficial compound in preventing hepatocellular damage and tissue necrosis via suppression of gluconeogenesis; while, the other compounds showed sufficient activity but at lower levels. Antioxidant properties in both Schiff base compounds might also help restore liver damages (Asadi-Samani et al., 2015). Also, CNCP and CNBP (Saremi et al., 2019b) showed acceptable antioxidant activity, but CNCP with lower IC_{50} than CNBP was found to be more potent. Due to higher antioxidant activity of the CNCP rather than CNBP, the difference between lowering enzyme activates between these compounds could be reasonable.

It is well-known that hyperlipidemia is a typical feature during hyperglycemia in diabetes. As shown in the current study, total cholesterol and also LDL cholesterol

levels increased significantly in serum of the diabetic control animals; whereas the level of HDL cholesterol was found to be decreased. In contrast, it was found that successive administration of CNCP (LD) could reduce total cholesterol and LDL cholesterol levels and increase HDL cholesterol which could suggest that CNCP (LD) possessed noticeable hypolipidemic effect. It is generally accepted that during oxidative stress, ROS cause to oxidation unsaturated lipids existing in external layer of LDL or in lipid bilayer of bio membrane. The oxidation lead accumulating secondary products of free radical oxidation, namely, malondialdehyde (MDA) (Kumskova et al., 2014). Antioxidant agents, including chemical compounds, such as Schiff bases with high antioxidant activity are able to decrease LDL level and reversely increase HDL in blood by suppressing free radicals (Mabuza et al., 2019; Sykuła et al., 2018; Torabi et al., 2018). Hence, it can be suggested that hypolipidemic effect of CNCP (LD) could be associated with higher antioxidant activity when compared with CNBP (Kumskova et al., 2014).

As depicted in Figure 4.29, both CNCP (LD) and CNBP (HD) could decrease the elevated blood glucose level in diabetic control group and helped the glucose levels turned back to the normal range values, but, CNCP (LD) was found to be more potent than CNBP (HD). As the matter of fact, it can be implied that antidiabetic effect of the novel Schiff base compounds might exhibit an oxidation state dependent effect on blood glucose levels; however, the chlorine substituted compound having higher antioxidant activity could be more active in lowering blood glucose levels (Xie et al., 2014). So far, insulin as the only pancreatic β-cell hormone is identified as the main hormone to lower blood glucose levels (Brezar et al., 2011). Hyperglycaemia in diabetes disease is regarded as the main symptom caused by lack of insulin and/or insulin resistance (Ke et al., 2009). Based on these facts, it is anticipated that both Schiff bases, especially, CNCP (LD) could decrease glucose levels in the blood maybe via increasing either

regeneration of pancreatic islets or increasing insulin release in the STZ-NA-induced diabetic rats (Mabuza et al., 2019; Schuit et al., 2001).

In the histopathology study, the diabetic control group showed several pathological changes and were listed in the results section. In the diabetic liver, unorganized hepatocytes, granular degeneration, micro-vesicular vacuolization and necrosis were the marked changes which have been observed (Zhou et al., 2008). Accomplished efforts are made for finding out and trying to decipher the main molecular mechanisms which are responsible for β-cell death in diabetes (Thomas & Kay, 2000) and based on several mouse models (Kurrer et al., 1997; O'brien et al., 1997), apoptosis is likely as the main cause of such destruction. In the current study, severity of hepatic injuries in SD rats is reduced with treatment of CNCP (LD). Motshakeri et al. (2014) showed that STZ-NA-induced diabetic rodents showed small islets, including degenerated cells and increased β-cells, which was in agreement with those findings obtained by Roat et al. (2014). In the present work, similarly, the pancreas tissues of diabetic rats could reduce the amount of islets, degeneration of β-cells, hydropic degeneration, clumping of β-cells, pyknosis, and necrosis, which caused changes in cells morphology due to STZ-NA-induced partial damages in some β-cells (Figure 4.34). Furthermore, islet cells might be restored by CNCP (LD), which means that treatment was capable to recover these degenerated cells. Unlike CNBP, the administration of increased individual dose of CNCP administered to diabetic animals showed a comprehensive reversible effect. This finding implied that CNCP (10 mg/kg) and CNBP (20 mg/kg) treated groups compared to the groups treated with the higher dose of CNCP (20 mg/kg) and CNBP (10 mg/kg) indicated better function on the cells. One of those key complication that is connected with diabetes is diabetic nephropathy (DN) which can be accompanied with expansion of mesangial cells, as hallmark of diabetic rats (Matsubara et al., 2006), accumulation of

extracellular matrix protein, thickening of glomerular and tubular basement membranes, tubulointerstitial fibrosis, glomerulosclerosis, renal endothelial dysfunction, albuminuria, proteinuria, and also reduction in glomerular filtration rate (Balakumar et al., 2009). In the current study, the diabetic SD rats control group displayed acute swelling of cells, hydropic degeneration of tubules, widening of Bowman's space, glomerular atrophy, congestion of capillaries, and tubular necrosis. Drastic pathological changes were also observed in focal mesangial matrix expansion, proteinuria, and also significant increase in albuminuria compared to the normal control group. CNCP (LD) could ameliorate mesangial expansion, proteinuria, and albuminuria which demonstrated that CNCP at 10 mg/kg in comparison with CNBP at both doses could be the best and more beneficial treatment dose for alleviating histological injuries.

5.3.5 Limitation of this study

There are some limitations in these studies, firstly is the time-framed and lack of funding for further research. Secondly, some rats develop resistance to Streptozocin-nicotinamide induced diabetes and therefore the rats' blood glucose level was checked again on day 7th before deciding for another injection at the same dosage. The reason for doing so was because the lack of rats meanwhile the demands of Sprague Dawley rats among researcher in Animal House, Faculty of Medicine was high and to maintain the quality of rats used throughout the study. However, if the rats had failed to become hyperglycemia after two times injection, that rats excluded from being a sample.

CHAPTER 6: CONCLUSION

In conclusion, Schiff base derivative are compounds which exhibited Effective and promising effects in field of several biological properties. In present research, we examined two Schiff base derivatives (CNCP and CNBP) for their potential of gastroprotective effects, wound healing properties, and anti-diabetic compared to the appropriate standard drugs (omeprazole, intrasite gel, and glibenclamide, respectively) in SD rats. *In vivo* acute toxicity of the study showed that CNCP and CNBP did not show any mortality or obvious sign of abnormality in rats, representing safety characterization of both compounds. The FRAP and DPPH tests demonstrated the potential antioxidant activity of CNCP and CNBP.

The present study elucidated the gastroprotective effect of CNCP (HD) and CNBP (HD) against ethanol-induced gastric lesions. The gastroprotective mechanism of CNCP and CNBP may be suggested via increasing SOD, CAT activities, which in turn suppressed gastric acidity and prevented destruction of gastric mucus wall (GMW). Moreover, there was notable reduction in MDA and enhancement in PGE2 levels upon intake of the CNCP and CNBP. In histological analysis, decrease of haemorrhagic mucosal region in gastric wall together with reduction or inhibition of edema and leukocytes infiltration of sub-mucosal layers was also observed. PAS staining reduction induced by ethanol was reversely increased by CNCP and CNBP pre-treatment and also caused an increase in glycoprotein content. Immunohistochemistry analysis of gastric homogenate elicited critical role of Hsp70 up-regulation and down-regulation of Bax protein in rats. This study provided histological evidences on gastroprotective property of both compounds and also suggested that these compounds could preserve of gastric mucus secretion and enhance antioxidant activities of CAT, SOD.

In the wound healing experiment, CNCP and CNBP at both concentrations, particularly at 10 mg/ml and 20 mg/ml, respectively revealed noticeable wound healing properties. The results of histology showed less scarring of the wound area on day 10 after wound creation. Hunting of free radicals and reactive oxygen increased narrowing of the cut and arrangement of angiogenesis, along with collagen, fibroblasts deposition, few inflammatory cells, and capillaries in granulation tissue when compared to the wounds of rats treated with gum acacia (vehicle). CNCP and CNBP caused high endogenous antioxidant enzymes (SOD, CAT and GPx) and reduced MDA in wound tissue homogenates which helped healing. Immunohistochemistry which was used in the study demonstrated down-regulation of Bax and up-regulation of Hsp70 proteins in CNCP and CNBP treated group when compared to rats treated with gum acacia. The findings suggest that CNCP and CNBP are safe reagents both of which can be considered potential drugs for wound healing application.

In summary, although CNCP and CNBP at both doses showed some antidiabetic effect in STZ-NA-induced diabetic rat model, CNCP (LD) could possibly exert the highest anti-hyperglycaemic effect. CNCP (LD) was the most successful compound compared to CNCP (HD) and both doses of CNBP in decreasing insulin level and showed higher protective effect on liver, kidney, and pancreatic β-cells. These findings propose that CNCP, a chlorine substitute Schiff base was an excellent candidate for treating diabetic complications. However, bromine substitute of the same Schiff base showed acceptable antidiabetic effect. In summary, the study shows new Schiff base derivatives showed anti-diabetic, anti-hyperglycaemic and anti-hyperlipidemic effects which could be associated to their antioxidant activity in diabetic rats.

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