A STUDY OF HEPATOPROTECTIVE EFFECTS OF *CURCUMA XANTHORRHIZA* AND *IPOMOEA AQUATICA* ON THIOACETAMIDE-INDUCED LIVER CIRRHOSIS IN RATS

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2013

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2013

UNIVERSITI MALAYA

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Name of Degree: PhD

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ABSTRACT

In this work, an attempt was made to study the hepatoprotective effects of ethanol extracts of *Curcuma xanthorrhiza* and *Ipomoea aquatica* which are traditionally used for liver complications.

The preliminary work involved an *in vitro* screening of free radical scavenging of both plants together with silymarin as an agent known to have prevention effect on cirrhosis. In order to study the hepatoprotective and possible mechanism of actions, thioacetamide was used to induce liver cirrhosis in rat model. For each plant extract experiment, 5 groups of rats were assigned as normal control, cirrhotic control, silymarin control, low dose and high dose plant extract. After two months of the experiment, the animals were sacrified and blood and tissues samples were collected for biochemical and histological studies. The parameters included serum liver biomarkers, ALP, AST, ALT, total protein, albumin and bilirubin; liver tissue histological examination; liver tissue Masson's trichome staining and immunohistochemical staining of α-SMA; liver tissue antioxidant, CAT, SOD; liver tissue lipid peroxidation maker, MDA; serum proinflammatory and profibrotic cytokines TNF-α, TG-Fβ1; liver tissue NF-κB. Then, both plant extracts were subjected to fractionation using column chromatography and the obtained fractions were screened for hepatoprotective in thioacetamide-induced hepatotoxicity normal liver cell line (WRL-68). From each plant extract, the fraction which was most effective in WRL-68 was subjected to LCMS to identify the available compounds. From the LCMS results of the fractions which exhibited best hepatoprotective results, violaxanthin from *Ipomoea aquatica* and xanthorrhizol from *Curcuma xanthorrhiza* were selected for further study. The selection of these compounds was based on the available information from the previous studies which

could be used to infer that these compounds may be the main contributor in the hepatoprotective activity of the fraction and plant extract as whole.

The results demonstrated that ethanol crude extracts of *Curcuma xanthorrhiza* and *Ipomoea aquatica* possess hepatoprotective activities *in vivo* against thioacetamide-induced liver cirrhosis which was proven in biochemical and histopathological findings. In the *in vitro* screening, *Curcuma xanthorrhiza* showed higher free radical scavenging than silymarin, however, the free radical scavenging of *Ipomoea aquatica* was less than silymarin. Nevertheless, both plant extracts significantly increased antioxidant enzymes and reducing lipid peroxidation, which suggest that both plants exhibit hepatoprotective activity, at least in part, by improving endogenous antioxidant status. In addition to that, both extracts exhibited antinflammatory properties to which might have inhibited fibrosis progression through the down regulation of proinflammatory and profibrotic cytokines which lead to the decrease in HSC activation and therefore decreased ECM deposition. This was proven by liver tissue α-SMA and Masson's trichome staining.

The hepatoprotective activities of both plant crude extracts were confirmed *in vitro* against thioacetamide induced cell damage in WRL-68. Furthermore, separated fraction 5 from *Curcuma xanthorrhiza* and fraction 11 from *Ipomoea aquatica* exhibited the highest hepatoprotective activities against thioacetamide induced cell damage in WRL-68. Similar to the animal study, both crude extracts, their fractions and selected compounds improved antioxidant enzymes and inhibited lipid peroxidation. The selected compounds were more effective than their crude extracts in protecting cell damage and improving antioxidant status. In fact, violaxanthin was more effective than silymarin.

These results suggest that both plant extracts are hepatoprotective and the possible mechanism of action is likely to be through improving endogenous antioxidant status,

decreasing proinflammatory and profibrotic cytokines, to which xanthorrhizol and violaxanthin may be the main contributors.

ABSTRAK

Dalam ujikaji ini, percubaan telah dibuat untuk mengkaji kesan hepatoprotektif bagi ekstrak etanol temu lawak dan kangkung yang secara tradisinya digunakan untuk komplikasi hati. Kerja-kerja awal yang terlibat dalam ujian *in vitro* menunjukan kadar pemerangakapan radikal bebas bagi kedua-dua tumbuhan ini bersama-sama dengan silymarin sebagai agen, telah diketahui mempunyai kesan pencegahan pada sirosis. Dalam usaha untuk mengkaji mekanisme hepatoprotektif dan tindakbalas mungkin, thioacetamide telah digunakan untuk mendorong sirosis hati dalam model tikus. Bagi setiap eksperimen ekstrak tumbuhan, 5 kumpulan tikus telah diketogorikan sebagai kawalan normal, kawalan cirrhotic, kawalan silymarin, ujian dos rendah dan ujian dos tinggi extrak tumbuhan. Selepas dua bulan eksperimen, haiwan tersebut dibunuh dan sampel darah dan tisu dikumpul untuk kajian. Parameter termasuk biomarkers hati serum, ALP, AST, ALT, jumlah protein, albumin dan bilirubin; pemeriksaan histologi tisu hati; pewarnanan Masson trichome tisu hati itu dan pewarnaan immunohistochemical terhadap SMA α-; antioksidan tisu hati, CAT, SOD; agen peroksidaan pembuat lipid tisu hati, MDA; serum proinflamasi dan sitokin profibrotik TNF-α, TG-Fβ1; tisu hati NF-κB. Kemudian, kedua-dua ekstrak tumbuhan telah mejalani langkah farksinasi dengan menggunakan kromatografi turus dan fraksi-fraksi yang diperoleh telah manjalani ujian hepatoprotective dengan thioacetamid-penyebab hepatoksisiti bagi sel hati normal (WRL-68). Bagi setiap ekstrak tumbuhan, fraksi yang paling berkesan terhadap sel WRL-68 akan menjalani ujian LCMS untuk mengenal pasti sebatian yang tersedia. Daripada keputusan LCMS fracksi-fraksi yang mempunyai aktiviti hepatoprotektif berpunca kehadiran sebatian dikenali violaxanthin dari kangkung dan xanthorizol dari temu lawak, yang seterusnya telah dipilih untuk kajian lanjut. Pemilihan sebatian ini adalah berdasarkan maklumat yang didapati daripada kajian sebelumnya yang boleh digunakan untuk membuat kesimpulan bahawa sebatian

ini mungkin menjadi penyumbang utama dalam aktiviti hepatoprotif bagi fraksi-fraksi dan ekstrak tumbuhan secara keseluruhan. Keputusan bagi ekstrak etanol mentah temulawak dan kangkung menunjukan ianya mempunyai aktiviti hepatoprotektif dalam ujian *in vivo* terhadap thioacetamide penyebab sirosis hati yang telah dibuktikan dalam penemuan biokimia dan histopathologi. Di dalam ujian in-vitro, temu lawak menunjukkan kadar pemerangkapan radikal bebas yang lebih berbanding silymarin. Walaubagaimanapun, kadar pemerangkapan radikal bebas oleh kangkung adalah kurang daripada silymarin. Kedua-dua ekstrak menunjukan peningkatan enzim antioksidan dan pada masa yang sama mengurangkan peroksidaan lipid, yang mencadangkan bahawa kedua-dua tumbuhan mempamerkan aktiviti hepatoprotetif, dengan meningkatkan tahap antioksidan endogen. Di samping itu, kedua-dua ekstrak menujukan sifat antinflammasi yang mungkin telah menghalang perkembangan fibrisis melalui down-regulation proinflamasi dan profibrotik sitokin yang membawa kepada penurunan kadar pengaktifan HSC dan seterusnya menurun pemendapan ECM. Ini telah dibuktikan oleh keadaan tisu hati α-SMA oleh pewarnaan Masson trichome. Aktiviti hepatoprotetif bagi kedua-dua ekstrak mentah tumbuhan telah disahkan dalam ujian in-vitro terhadap kerosakan sel thioacetamide teraruh dalam WRL-68. Tambahan pula, fraksi 5 daripada temu lawak dan fraksi 11 dari kangkung mempamerkan aktiviti hepatoprotektif tertinggi terhadap kerosakan sel thioacetamide teraruh dalam WRL-68. Serupa dengan kajian haiwan, kedua-dua ekstrak mentah, pecahan fraksi dan sebatian terpilih boleh meningkatkan enzim antioksidan dan menghalang peroksidaan lipid. Sebatian tulen yang dipilih adalah lebih berkesan daripada ekstrak mentah tumbuhan dalam memainkan peranan untuk melindungi daripada kerosakan sel dan meningkatkan tahap antioksidan. Malah, violaxanthin adalah lebih berkesan daripada silymarin. Kesimpulannya, bahawa kedua-dua ekstrak tumbuhan ini mempunyai kesan hepatoprotektif dan faktor kemungkinan mekanisma tindakabalas adalah mungkin

melalui penigkatan tahap antioksidan endogen, pengurangan proinflamasi dan profibrotik sitokin, dimana xanthorrhizol dan violaxanthin adalah penyumbang utama.

ACKNOWLEDGEMENT

First of all, a very deep thankful to ALLAH (S.W.T) that helped me to carry out this project satisfactorily. I would sincerely like to extend my heartfelt gratitude and appreciation to my supervisor, Prof Dr. Mahmood Ameen Abdulla for the encouragement, exceptional ideas, and tireless optimism that facilitate the completion of the research work and in the preparation process of this thesis. I am also pleased to express deep appreciation to my co-supervisor Ass Prof Dr. Salmah Ismail for her support, guidance and technical advice throughout this work. There are many people who deserve to be acknowledged for their support and continued encouragement in the course of my years of study. I am taking this opportunity to express my most humble appreciation and gratitude to all staffs and students of Immunology Laboratory, Department of Molecular Medicine and Faculty of Medicine. My thanks and appreciations are also expressed to staffs of the Faculty of Medicine Animal House for the care and supply of rats and to the University of Malaya for their financial support, Grant No. PVO42-2011A and UM/MOHE High Impact Research Grant (HIR Grant No.F000009-21001). Last but not least I would like to thank my parents, wife, kids and all close relatives for their endless support and encouragement that have kept me going on.

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviations	**Description**
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATCC	American type culture collection
C. xanthorrhiza	*Curcuma xanthorrhiza*
CAT	Catalase
CCl4	Carbon tetrachloride
CT	Computerized tomography
CYP2E1	Cytochrome P450 2E1
DMSO	Dimethyl sulfoxide
DPPH	α, α-diphenyl-β-picryl-hydrazyl radical scavenging assay
ECM	Extracellular matrix
FBS	Fetal bovine serum
GPx	Glutathione peroxidase
H&E stain	Hematoxylin-eosin stain
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HD	High dose
HSC	Hepatic stellate cells
I. aquatica	*Ipomoea aquatica*
I.P	Intraperitoneal
LC-MS	Liquid chromatography-mass spectrometry

LD	Low dose
LD50	Lethal dose 50
MDA	Malondialdehyde
MMPs	Matrix metaloproteinases
MRI	Magnetic resonance imaging
MTT	3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide assay
°C	Degree celcius
P value	Level of significance
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
ROS	Reactive oxygen species
Rpm	Revolution per minute
SD	Sprague Dawley
SEM	Standard error of the mean
SOD	Superoxide dismutase
TAA	Thioacetamide
TGF-β	Transforming growth factor-beta
TIMPs	Tissue inhibitors of metaloproteinases
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor-alpha
TP	Total protein
UV	Ultraviolet
WHO	World health organization
α-SMA	Alpha-smooth muscle actin

CHAPTER I

INTRODUCTION

1.1 Introduction

Liver is the largest organ in the human body that performs various interrelated functions. In an adult of 70 kg, liver weighs approximately 1.7 kg (Arias, *et al.*, 1988) Liver located at the right side of the abdominal cavity just below the diaphragm and divided into two unequal lobes by the falciform ligament. These lobes made up of numerous lobules that include plates of liver parenchymal cells (hepatocytes), hepatic venules, branches of hepatic artery and sinusoids (Cunningham & Van Horn, 2003). Apart from parenchymal cells and hepatocytes, liver also include stellate cells, endothelial cells, kupffer cells and bile duct epithelial cells. Hepatocytes get the blood containing absorbed, digested or secreted substances by the gastrointestinal tract, pancreas, and spleen from the portal vein. Oxygenated blood, on the other hand, delivered through the hepatic artery (Cunningham & Van Horn, 2003). Liver is the essential prerequisite of the human body without the normal function of which one might not survive. Liver carryout several critical functions such as:

- Metabolism of proteins, carbohydrate and lipids.
- Production of clotting factors.
- Storage of vitamins (vitamin A, D and B12) and iron and glycogen.
- Secretion of bile (Heidelbaugh & Bruderly, 2006).

Liver also protects the body against the foreign substances by detoxifying, neutralizing and eliminating the drugs and other xenobiotics (Samuel, *et al.*, 2012).

Since liver is the critical organ of the body for maintaining overall health and detoxification, disorders to this organ remain one of the serious health problems (Samuel, *et al.*, 2012). The main causes of the liver diseases in the developed countries include viral-induced and excessive alcohol consumption. Whereas the primary causes in the developing countries are hepatitis B and C viruses, parasitic disease, environmental toxins, and hepatotoxic drugs (chemotherapeutic agents, certain antibiotics, high doses of paracetamol, carbon tetrachloride (CCl_4) and thioacetamide (TAA) (Schuppan & Afdhal, 2008). In the Western countries liver cirrhosis is the ninth leading cause of death (Kim, *et al.*, 2002).

Liver cirrhosis regarded as the ending stage of chronic liver disease in which chronic inflammation of liver results in a substantial accumulation of scar tissue in the liver. Regardless of the cause of liver diseases, most of the chronic liver diseases carry the potentiality to develop to this stage, and it tends to be stable or unstable, with varied presentations (Al-Attar, 2011). Progression to cirrhosis results in the liver structural and functional alteration as a results that normal tissue replaced with scar tissue, obstructing the flow of blood all through the organ and thus accumulation of toxins within the body. Accumulation of harmful toxins can initiate various other complications, making it a critical stage of chronic liver diseases which can results in liver failure accompanied with an impaired metabolic function, bleeding tendency, hepatic encephalopathy, portal hypertension and hepatocarcinoma. Chronic liver damage initiates specific cellular response, which regulated by a number of cytokines leading to increased deposition of extracellular matrix proteins (Li & Friedman, 1999). Imbalance between formation and degradation of extracellular matrix proteins result in alteration of the liver architecture of the liver which brings about serious pathological and physiological effects. Cirrhosis, histologically, known as a widespread hepatic process characterized by the transformation of normal liver structural design into abnormal fibrosis and nodules.

Several research studies have revealed that oxidative stress play a primary role in the etiopathogenesis of fibrosis, an important component of cirrhosis in chronic liver diseases (Poli, 2000; Shimizu, *et al.*, 1999). Reactive oxygen species (ROS) have a primary role in the initiating as well as in the progression of disease of the liver. In response to toxic injury, increased production of ROS results in cellular membranes lipid peroxidation, and DNA and protein oxidation, which causes cellular injury (Bremer, *et al.*, 1994; Loguercio & Federico, 2003). Lipid peroxidation, which means the oxidative degradation of lipids, is a multifarious and natural destructive process (Tribble, *et al.*, 1987). The biological systems incorporate protective arrangements that protect them from the harmful effects of ROS. This includes superoxide dismutase (SOD) and catalase (CAT) (Zhao, *et al.*, 2011). However, enhanced lipid peroxidation leads into tissue injury and failure of the antioxidant defence mechanisms to prevent the formation of excess free radicals. These ROS mainly generated from the mitochondria of hepatocytes, the activated Kupffer cells (macrophages), and also the infiltrating neutrophils (Bremer, *et al.*, 1994; Loguercio & Federico, 2003). They exaggerate the condition by initiation of the activation of nuclear factor-kappa B (NF-κB) and thus the activation of inflammatory cytokines, adhesion molecules and chemokines which further promote the production of ROS (Bremer, *et al.*, 1994; Loguercio & Federico, 2003). Inflammation lead to the release of the cytokine transforming growth factor β (TGFβ) which stimulate extracellular matrix (ECM) production by activation of quiescent hepatic stellate cells (HSCs) (Bataller & Brenner, 2005b; Friedman, 2008). During the fibrogenic process, HSCs along with inflammatory cells release and also respond to TGFβ (Gressner, *et al.*, 2002). TGFβ steadily upregulates the synthesis and accumulation of the primary ECM (Arthur, 2000; Gressner, *et al.*, 2002).

Although liver cirrhosis is a global health issue and one of the most frequent occurring diseases with high mortality and high morbidity, currently, the medicinal management

is inadequate and often have side effects (Rao, *et al.*, 2006). Moreover, most of the available therapies tackle the symptoms and not preventing the progression of fibrosis process, which eventually develop into cirrhosis (Franklin, 1995). On account of the absence of effective therapy, a great number of scientific studies searching for hepatoprotective constituents from natural sources continued to be conducted (Adnyana, *et al.*, 2001; Huang, *et al.*, 2010).

The application of medicinal plants for the treatment of different disorders extended ancient civilizations (Wadud, *et al.*, 2007). The usage of traditional medicinal plants in treating various disorders is in practice even when modern healthcare are readily available. According to the World Health Organization (WHO) estimation, in the developing countries about 80% of individuals in general use traditional medicine (Rahim & Khan, 2006). In developing countries, in which communicable diseases are endemic, and hygiene and healthcare services are insufficient, medicinal plant used as antibacterial, antiviral, antioxidant, ant inflammatory, antiulcer, antidiabetic and anticancer. In view of serious side effects as a results of synthetic agents and absence effective therapy in the modern medicine, a good number of medicinal plants increasingly used to treat liver disorders (Dhiman, *et al.*, 2012). These recommended medicinal plants for the treatment of liver disorder have been in use for long time and claimed to produce significant results (Sanmugapriya & Venkataraman, 2006). Some of those which have scientifically studied for various liver disorders have exhibited promising results. For example, for the treatment of chronic viral hepatitis glycyrrhizin exhibited promising results whereas Silymarin demonstrated potential in the prevention of cirrhosis (Stickel & Schuppan, 2007). Silymarin, which derived from *Silybum* marianum, is a well known hepatoprotective agent. It exhibits considerable protection in laboratory animal models of different liver cirrhosis inducer agents, including thioacetamide (TAA).

Several chemicals such as chloroform, iodoform, carbon tetrachloride and acetaminophen has been used to induce liver cirrhosis in experimental animals (Weber, *et al.*, 2003; Weiler-Normann, *et al.*, 2007). However, TAA has an advantage over other liver damage chemicals. It induces hepatic fibrosis resembling the human hepatic fibrosis (Wong, *et al.*, 2012). Hence, TAA widely employed to induce liver cirrhosis in the experimental animals.

From prospective reliable drug viewpoint, it is obviously beneficial to conduct studies in medicinal plants using laboratory model systems searching for safe and reliable liver-protective therapy. Hepatoprotective studies, particularly, of the medicinal plants possess antioxidants and antinflammatory properties may lead to a discovery of new drug, which can prevent or even treat the cirrhosis by blocking the progression of fibrosis process. In this study, an attempt was made to scientifically study the hepatoprotective effects of *Curcuma xanthorrhiza* and *Ipomoea aquatic* in thioacetamide-induced liver cirrhosis in rats.

1.2 Objectives

1.2.1 General

To evaluate hepatoprotective and antioxidant activities of *Curcuma xanthorrhiza* and *Ipomoea aquatica* then identify their possible mechanisms of action and active constituents.

1.2.2 Specific

1) To prepare ethanol extracts of *Curcuma xanthorrhiza* and *Ipomoea aquatica*, and conduct acute toxicity study for both extracts.

2) To evaluate hepatoprotective activities of *Curcuma xanthorrhiza* and *Ipomoea aquatica* ethanol crude extracts against thioacetamide-induced liver cirrhosis in rats (*in vivo*).

3) To evaluate the antioxidant activities of the ethanol crude extracts of the two plants (*in vitro)* and in rats liver homogenate samples (*in vivo*).

4) To fractionate both crude extracts and screen the fractions for hepatoprotective against thioacetamide-induced WRL-68 normal liver cell line (*in vitro*).

5) To identify the possible mechanism of action of both two plants

6) To identify the active constituents of the plant extracts proven to have hepatoprotective activities.

CHAPTER II

LITERATURE REVIEW

2.1 Liver

Liver is the largest glandular organ in human body which performs various interrelated functions. It is on the right hand side of the abdominal cavity, just below the diaphragm and covered by visceral peritoneum as well as dense irregular connective tissue layer. It is reddish brown in color and weighs around 1.7 kg in an adult of around 70 kg (Arias, *et al.*, 1988). Liver is divided into four lobes, right, left, caudate and quadrate. The large right lobe and a small left lobe are separated by the falciform ligament and located superiorly. The unique nature of liver is the ability of regenerating its own tissue within few weeks to its original size (Highleyman & Franciscus, 2012).

Each liver lobe is made up of numerous lobules which is about the size of sesame and contains plates of liver cells or also known as hepatocytes which are organized in irregular, branching, connected plates around the central vein. The large space in the liver lobule through which the liver receives the blood is known as sinusoid. The small space between the sinusoids and the hepatocytes are known as space of Disse. These sinusoids are partly present along with stellate endothelial cells also known as Kupffer's cells. Bile is partially a digestive secretion and partially an excretory product of hepatocytes present in liver. The bile secreted by liver cells passes through the bile capillaries to the small bile ducts which are merged together to form left and right hepatic ducts (Cunningham & Van Horn, 2003).

Hepatocytes or the liver cells occupy around 78% of the tissue volume and non-hepatocytes such as stellate cells, endothelial cells and kupffer cells accounts for 6.3% of the total tissue volume (Gumucio, *et al.*, 1996). Hepatocytes arranged in plates of single cell thick are responsible for most of liver's synthetic and metabolic activities.

Since both surface areas of hepatocyte are exposed to the blood flowing towards the hepatic vein within the space of Disse, they extract the toxins and nutrients. Kupffer's cells are macrophages that phagocytes bacteria, worm, worn out red as well as white blood cells and toxic substances. It also breakdown heme into bilirubin which is the principle bile pigments. Hepatic stellate cells (HSC) are found in space of Disse and during the liver injury they transform into cells that produce collagen leading to liver fibrosis (Cunningham & Van Horn, 2003).

The total blood flow to the liver is around 1300 mL per minute. Hepatic artery and portal vein supply the oxygenated and deoxygenated blood into to the liver sinusoids, respectively. Portal vein supply the blood containing enterally absorbed digested or secreted substances by gastrointestinal tract, pancreas, and spleen to the liver. Whereas hepatic arteries supply oxygenated blood to the liver (Cunningham & Van Horn, 2003).

2.1.1 Functions of liver

Liver is an essential and a vital organ of human body, which carryout around 500 bodily essential functions required for the survival of human. It plays a key role in the metabolic process of carbohydrates, proteins and lipids, digestion, secretion of bile and also in the body's immune defense. It synthesizes blood proteins, stores body nutrients, and generates energy remove toxins from the blood.

2.1.1.1 Metabolism of carbohydrate, proteins and lipids

Liver carries out several metabolic functions to generate the energy required by the body and regulates the production, storage as well as release of carbohydrates, proteins and fats. In case of carbohydrates, the consumed carbohydrates are partly converted into energy by liver and partly converted into glycogen and stored. During fasting or stress liver converts glycogen to energy by the process known as glycogenolysis or gluconeogenesis. Liver is responsible for the oxidative deamination and transamination in order to generate substrates for both amino acid synthesis and carbohydrate

metabolism. Through the urea cycle liver excretes the nitrogen in the form of urea. Liver synthesizes around 80% of the cholesterol in the body from acetyl-CoA and remove it from circulation. Liver also helps in synthesis, storage and export of triglycerides. Liver also converts excess of amino acids from the digested food into fatty acids which is later converted to ketones to generate energy by liver (Highleyman & Franciscus, 2012).

2.1.1.2 Secretion of bile juice

Bile is a reddish yellow or brownish liquid secreted (800-1000 mL/day) by liver cells everyday and bilirubin is the principle pigment present in bile. Bile behaves like a detergent to emulsify and breakdown of lipid globules into suspension of droplets followed by their digestion and absorption. Liver removes bilirubin, cholesterol, toxins and other wastes through the bile (Heidelbaugh & Bruderly, 2006).

2.1.1.3 Synthesis and storage

Liver synthesize and store several binding proteins which bind and transport vitamins, minerals, hormones and fats. Several clotting factors such as fibrinogen, prothrombin and other clotting factors such as VII and VII were also synthesized and stored in liver. Liver also stores various nutrients such as vitamin A, D, B9 and B12 (Highleyman & Franciscus, 2012).

2.1.1.4 Detoxification and removal of toxic substances

Liver plays a vital role in the detoxification of pleasant substances such as alcohol, organic solvents, pesticides, heavy metals and more importantly various consumed drugs. These substances were delivered to the sinusoids and then area of Disse of liver through the portal vein where in the enzymes present in hepatocytes (cytochrome P-450 enzymes) metabolize them and excreted in the bile. Liver is responsible for the destruction and removal of worn out erythrocytes. Moreover, liver also process and

excretes the toxic byproduct such as ammonia and some excess sex hormones (Saleem, *et al.*, 2010).

2.1.2 Liver diseases

Since the liver is the important organ of our body, for maintaining overall health and detoxification, any disorder to this organ remain one of the serious health issues (Samuel, *et al.*, 2012). Around hundred liver diseases are developed due to liver damage, and it is extremely difficult to early warning symptoms of liver disorders. If it is not treated in the early stages it will leads to liver fibrosis, cirrhosis and finally liver damage and death (Ao, *et al.*, 2009). Liver diseases such as hepatocellular carcinoma, viral hepatitis, alcoholic and non-alcoholic steatosis are the most common liver associated and prevalent diseases in the world that are all closely associated with jaundice (Akanitapichat, *et al.*, 2010). Also, liver cirrhosis is the ninth top rated cause of mortality in Western countries (Kim, *et al.*, 2002).

2.1.2.1 Viral hepatitis

Hepatitis refers to the inflammation of the liver. Viral hepatitis is caused by the virus which are known as hepatitis A, B. C, D and E. consumption of food and fluids contaminated with hepatitis A and E virus causes hepatitis A and E, but it is not life threatening (Sembulingam & Sembulingam, 2004). However, hepatitis B, C and D are serious diseases, which may lead to cirrhosis and liver cancer (Kobayashi, *et al.*, 2002; Loguercio & Federico, 2003). These are caused by blood transfusion from infected donors, sharing infected person needles, inheritance from mother during parturition and also unprotected sexual activity with an infected individual. During the early stage of viral hepatitis, liver have the ability to tolerate and resists damage caused to liver cells by regenerating liver cells and hence compensates the liver damage caused by a virus. This phase is called as compensated liver disease during which liver is still able to continue all functions normally. However, as the time elapse liver lose its ability to

regenerate liver tissues and hence its normal functions are altered and this phase is called as decompensated liver disease (Chen, *et al.*, 2006).

2.1.2.2 Jaundice

Jaundice is represented by the yellow pigmentation of the skin, mucous membrane as well as deeper tissues due to the increased amount of bilirubin in the blood circulation. Jaundice are of hemolytic jaundice caused due to the destruction of red blood cells, hepatocellular jaundice caused due to the damage of liver cells and obstructive jaundice caused due to the obstruction of bile flow from biliary system (Chen, *et al.*, 1994; Sembulingam & Sembulingam, 2004).

2.1.2.3 Alcoholic liver diseases

Alcoholic liver diseases include alcoholic fatty liver, alcoholic hepatitis and alcoholic cirrhosis which may occur alone or together in the same patient. Chronic consuming alcohol results in the development of scar tissue in the lobules of liver followed by cirrhosis and finally leads to the development of alcoholic liver diseases (Cunningham & Van Horn, 2003; Ishak, *et al.*, 2006). Fatty liver is the very common and least harmful alcoholic liver disease which occurs within few days of heavy consumption of alcohol. During this liver swells slight to large proportions due to the accumulation of in the cytoplasm of liver cells. Heavy consumption of alcohol also lead to acute hepatitis, chronic hepatitis, which may leads to cirrhosis followed by death. Alcoholic cirrhosis is always life threatening and characterized by the development of nodules of hepatic tissues followed by the growth of scar tissues, which finally ends with liver cirrhosis (Hoek, 1994; Ishak, *et al.*, 2006).

2.1.2.4 Hemochromatosis

It is the most common genetic disease in United States of America, characterized by the presence of the higher level of iron in the body. High amount of iron accumulated in the body enhances oxidative stress by increasing the level of free radicals in the body. During hemochromatosis, the excess amount of iron present in the body is absorbed by various organs mainly by the liver. Hemochromatosis also co-exists along with other liver disorders making the matters much worse in those patients (Bassett, *et al.*, 1986; Cogswell, *et al.*, 1998).

2.1.2.5 Hepatocellular carcinoma

Hepatocellular carcinoma is the most common liver malignant caner in the world (90% of all tumors). Viral hepatitis often leads to cirrhosis which further ends with liver cancer. Liver cancers can be detected by combinations of serologic tests, imaging studies and biopsies. If liver tumors are formed it can be removed by surgery as the liver has the ability regenerate itself to its full size but it is not possible in patients with liver cirrhosis. In these patients liver transplantation is conducted (Beasley, 1988; Chen, *et al.*, 2006; El-Serag, *et al.*, 2003).

2.2 Cirrhosis

Liver cirrhosis regarded as the end stage of chronic liver disease in which scar tissue replaces the normal tissue and thus alters the liver structure and function. Regardless of the cause of liver damage, most chronic liver diseases progress from mild inflammation, to more severe inflammation, to fibrosis, and finally to cirrhosis. Hepatic fibrosis during which healthy liver tissue is replaced by the scar tissue is the wound healing process which leads to the accumulation of extracellular matrix (ECM) due to chronic liver injury (Schuppan, *et al.*, 2001). Hepatic fibrosis is associated with the oxidative stress, chronic inflammation, increased inflammatory cytokines and transforming growth

factors (Brenner, 2009). Liver cirrhosis is commonly preceded by hepatitis coupled with fatty liver. If diagnosed and the underlying cause is eliminated at this stage, the changes are still completely reversible (Detlef & Nezam, 2008). Continue to form scar tissue is a potentially life-threatening condition as it alters the structure of the liver and consequently obstruct the flow of blood. As the liver continue to loss the normal liver tissue, the processing and handling of nutrients, drugs, hormones and toxins declines. At the same time, the synthesis of proteins together with other substances produced by the liver is reduced (Schuppan & Afdhal, 2008). Because of the fact that liver function declines, water might accumulate in the abdomen and legs. Reduction in proteins necessary for blood clotting results in easy bruising and bleeding. The detoxification process of drugs and toxins is also slowed down by cirrhosis which leads to the accumulation toxins in the blood. Jaundice develop and present itself in the later stages of cirrhosis as a result of the accumulation of bile pigment which is passed by the liver into the intestines. Liver cirrhosis is mainly classified into three different major forms. The most common form is called *Laennec's* cirrhosis and also known as alcohol-induced cirrhosis. *Laennec's* cirrhosis caused by chronic alcoholism together with malnutrition. It is the most commonly encountered form which is characterized by forming fibrosis predominantly around central veins as well as portal areas. *Postnecrotic* is another form of cirrhosis which is also known as micronodular cirrhosis. *Postnecrotic cirrhosis is caused by* acute viral hepatitis especially hepatitis C which leads to broad bands of scar tissue or as a results of drug-induced massive hepatic necrosis. *Biliary* cirrhosis is a rarer form of cirrhosis than the preceding forms. *Biliary* cirrhosis occurs as a result of scarring damage of bile ducts and lobes of the liver which results from chronic biliary blocking and infection (cholangitis).

2.2.1 Pathology

Liver cirrhosis regarded as the ending stage of chronic liver disease in which chronic fibrosis leads to the accumulation of scar tissue and therefore structural and functional alteration. Fibrosis is a reversible wound healing process which takes place virtually in all chronic liver damage. Despite the fact that the wound healing response generally starts when hepatocyte damaged, the total response may considerably extends (Baldo, *et al.*, 2008). Fibrosis, thereby, is a physiologic mechanism, which happens to be beneficial in the beginning, but then develops into pathological in the event that chronic hepatocellular injury persist (Jarnagin, *et al.*, 1994; Meyer, *et al.*, 2005; Minagawa, *et al.*, 2004). The damage to the hepatocytes generates a cascade of events (Holstege, *et al.*, 2005; Svegliati Baroni, *et al.*, 2003). These particular events bring in onset and mobilization of several inflammatory cells that launch cytokines that not only result in expansion of the totally response, but at the same time may bring in indirectly or even directly activation of HSC (Friedman, *et al.*, 1985). Among the events that take place when liver is injured include alteration in cellular response and ECM deposition in the space of Disse and sinusoid subendothelial. These alteration results in losing sinusoidal endothelial fenestrae, hepatocyte microvilli and activation of Kuppfer cell which further activate HSC (Figure 2.1). The main source of extracellular matrix deposition is HSC, regardless of whether other cell types as an example portal fibroblasts are likely involved (Bataller & Brenner, 2005a). Hepatic stellate cells lie within the perisinusoidal space (or simply space of Disse) between the endothelial wall surface of the sinusoid and the vascular face of the hepatocytes (Geerts, 2001). The HSC's generally activated by cytokines that includes tumor necrosis factor alpha (TNFα), tumor growth factor beta (TGFβ) and platelet derived growth factor (PDGF) which can be released in response to liver damage. At the same time, other cytokines such as interleukin-10 (IL-10) are release and have the opposite action of HSC which

degradation of ECM. Once activated, HSC's secrete cytokines which includes tumor growth factor beta (TGFβ1), platelet derived growth factor PDGF, endothelin (ET-1) and monocyte chemotactic protein 1(MCP-1). These cytokines are directly involved in fibrogenesis and each of the play different roles. For instances, MCP-1 involved in chemotaxis, PDGF and ET-1 in proliferaton of HSC whereas TGFβ1 is a growth factor for the connective tissue (Realdon, *et al.*, 2004). In addition to being caused by increased synthesis of ECM, liver fibrosis also caused from imbalance between production and degradation of matrix proteins. Activated HSCs also increase release of tissue inhibitors of metalloproteinases (TIMPs) which prevent matrix metaloproteinases (MMPs) to degrade excessive matrix collagen. The process is driven by signalling pathways mediated by proinflammatory cytokines mainly involves including TNFα and also TGFβ1 (Wang, *et al.*, 2006). The most accumulated ECM involved in fibrosis are the interstitial collagens, types I, and III (Schuppan, 1990). In all different chronic liver disease, the progression of fibrosis is a step by step and gradually process starting from minimal fibrosis limited to in and within the portal tracts causing periportal stellate fibrous accumulates and extension (periportal or zone one fibrosis) (Marcellin, *et al.*, 2006). As the process gets progressively worse over time, fibrous tissues increase to the nearby mesenchymal arrangements with the development of fibrous septa. Then it progressively grows into the lobules towards the central veins (zone three) with septa development. Finally, when almost all of the central veins or portal tracts interconnected, bridge fibrosis generated, and annular fibrosis surrounding nodules of the liver cells, cirrhosis occur ((Z.D. Goodman & K.G. Ishak, 1995; Zachary D Goodman & Kamal G Ishak, 1995; Scheuer, *et al.*, 2005).

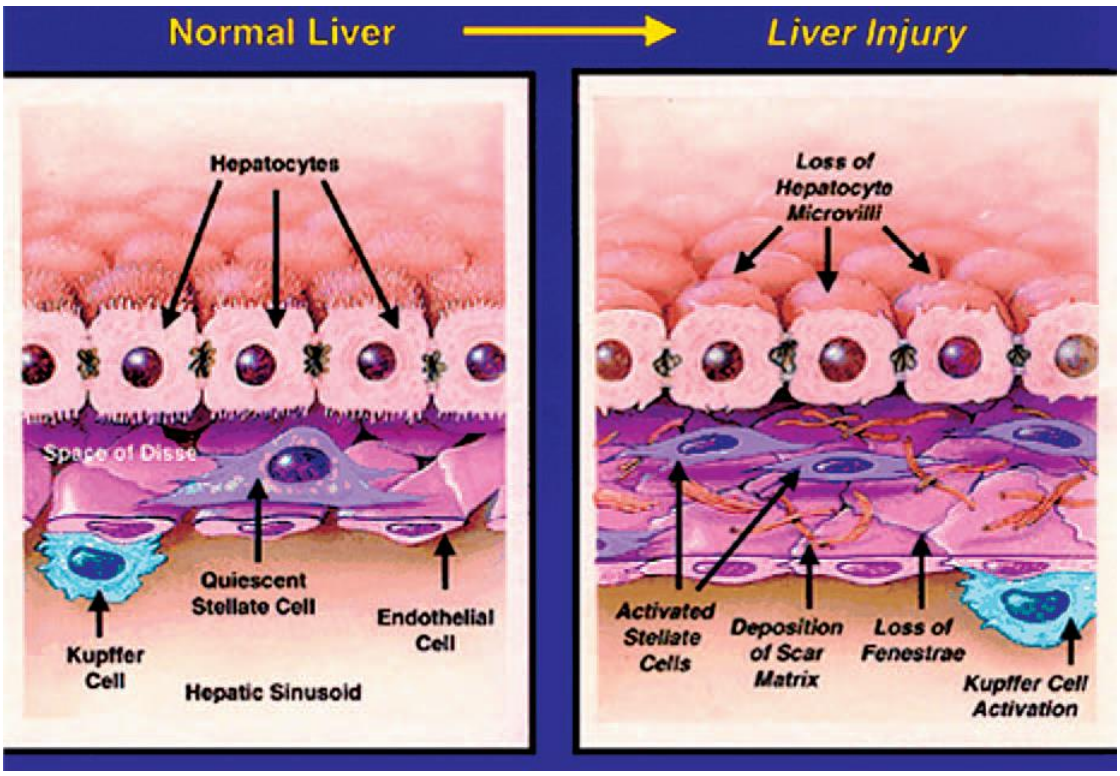

Figure 2.1**:** Diagram shows normal and damaged liver with four major cells (hepatocytes, endothelial cells, Kupffer cells and stellate cells) (Adapted from (Moreira, 2007)).

2.2.2 Etiologies of liver cirrhosis

Normally any chronic insult or damage to the liver will leads to inflammation, fibrosis and cirrhosis. Etiologies of liver cirrhosis are as follows:

Most common causes

- Excessive consumption of alcohol (60 to 70 %)
- Chronic hepatitis B and C (10 %)
- Hemochromatosis (5 to 10 %)
- Nonalcoholic fatty liver disease (10 %t) – commonly resulting from obesity
- Biliary obstruction (5-10 percent)

Less common causes

- Toxins and drugs such as amiodarone, alpha methyldopa, paracetamol,.

- Genetic metabolic diseases such as amino acid disorder (tyrosinemia), bile acid disorder, lipid disorder (abetalipoproteinemia), porphyria and Wilson's disease.
- Infections such as brucellosis, congenital syphilis, schistosomiasis, echinococcosis etc.
- Idiopathic and miscellaneous such as granulomatous liver disease and portal fibrosis.

(Friedman & Schiano, 2004; Heidelbaugh & Bruderly, 2006)

2.2.3 Symptoms of liver cirrhosis

More or less forty percent of individuals with liver cirrhosis have no symptoms, and the diseases revealed during routine laboratory tests or during the course of radiographic studies. Hence liver cirrhosis is considered as a asymptomatic disease mostly until decompensation of liver take place. Following are the symptoms of liver cirrhosis:

- Jaundice (observed in skin and/or eyes)
- Bleeding of gastrointestinal
- Osteoporosis
- Oagulopathy
- Pruritus
- Anorexia and weight loss
- Fatigue,
- Increased abdominal girth
- Changes in mental status
- Weakness

Each one of these clinical findings is the consequence of deteriorated hepatocellular function with or even without physical obstruction secondary to cirrhosis (Heidelbaugh & Bruderly, 2006).

2.2.4 Complications of cirrhosis

The major complications of liver cirrhosis includes ascites, hepatic encephalopathy, portal hypertension, variceal bleeding, bacterial peritonitis and hepatorenal syndrome (Friedman & Schiano, 2004; Jalan & Hayes, 2000; Runyon, 2004). Ascites is referred to as the pathological increased accumulation of fluid in the peritoneal cavity and around 85% patients with ascites have cirrhosis (Runyon, 2002). Hepatic encephalopathy is caused due to acute or chronic liver diseases, and it is characterized by slight and sometimes intermittent changes in memory, concentration, personality and reaction times (Heidelbaugh & Bruderly, 2006). Development of portal hypertension is very common in patients with liver cirrhosis irrespective of the etiology of cirrhosis. It occurs as a result of increased resistance to portal blood flow as a result of narrowing, scarring and compression of liver sinusoids (Heidelbaugh & Bruderly, 2006). Around 50% of patients with liver cirrhosis develop varices and variceal bleeding and it occurs around 10-30 percent per year (Cales, *et al.*, 1990). Variceal bleeding is defined as bleeding from an esophageal varix during the time of endoscopy or the presence of substantial esophageal varices with blood in the stomach (Jalan & Hayes, 2000). Hepatorenal syndrome is defined as functional renal failure in liver cirrhotic patients in the absence of intrinsic renal disease. Patients with this syndrome exhibit higher sodium and water retention, renal vasoconstriction leading to reduced} renal blood flow, glomerular filtration rate and also urinary output (Heidelbaugh & Bruderly, 2006). Hepatorenal syndrome occurs in patients with severe hepatic disorders, however, patients with biliary cirrhosis are relatively protected.

2.2.5 Diagnosis of liver cirrhosis

2.2.5.1 Physical examination of liver cirrhosis

Various physical examinations provide the useful information regarding liver cirrhosis and its treatment (Heidelbaugh & Bruderly, 2006) which are as follows:

- Asterixis
- Ascites
- Abdominal wall vascular collaterals
- Clubbing and hypertrophic osteoarthropathy
- Anorexia, fatigue, weakness and weight loss
- Jaundice
- Palmar erythema
- Splenomegaly
- Hepatomegaly
- Gynecomastia
- Testicular atrophy
- Spider angiomata
- Dupuytren's contracture
- Terry's nails and Muehrcke's nails
- Fetor hepaticus- a sweet, pungent breath odor
- Kayser Fleischer ring-brown-green ring of copper deposition within the cornea
- Cruveilhier-Baumgarten murmur - a venous hum in the patient developed portal hypertension

2.2.5.2 Laboratory evaluation of liver cirrhosis

Liver function tests were conducted routinely to diagnose cirrhosis. Though these tests may not reflect exactly with hepatic function, interpreting these biochemical parameters along with physical examination studies and radiographic studies provides the clinical

picture of various liver disorders (Heidelbaugh & Bruderly, 2006). Liver function is generally assessed by static tests and dynamic tests. Common tests, which were included in the standard liver panels, are serum enzyme aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (AP), gamma glutamyl transferase, serum bilirubin, serum albumin, complete blood count with platelets and prothrombin time tests (Dufour, *et al.*, 2000; Heidelbaugh & Bruderly, 2006). Estimation of ALT is the most cost effective test conduct to identify metabolic and drug induced hepatotoxicity nevertheless its use is confined since it is unable to predict the degree of inflammation and severity of fibrosis (Dufour, *et al.*, 2000). Dynamic tests such as clearance of indocyanine green, bromosulfopthalein, caffeine test and aminopyrine tests provide hidden hepatocellular dysfunction (Paxian, *et al.*, 2003). Dynamic tests are related to the ability of the liver to eliminate or metabolize particular substances (Sakka, 2007).

Liver enzymes

When hepatocellular plasma membrane is damaged by hepatotoxins, various serum enzymes located in the cytosol are released into the blood stream these enzymes reveal the extent of hepatocellular necrosis (AST and ALT) or cholestasis (AP). In the animal experimental models the liver damage induced by carbon tetrachloride, paracetamol, thioacetamide and other hepatotoxicin were assessed by the determination of levels of liver enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Dobbs, *et al.*, 2003; Xu, *et al.*, 2002). AST is a cytosolic enzyme present mainly in the liver where as ALT is a mitochondrial enzyme released from heart, liver, skeletal muscles and kidney hence; both these enzymes are sensitive markers of hepatocellular damage. These two enzymes play a major role in the metabolic process of amino acid by catalysing reversible transfer of the alpha amino group. The elevated level of these liver enzymes in serum represents the hepatocellular leakage together with the losing

functional integrity of liver cell membrane (Zimmerman & Seeff, 1970). Estimation of these serum marker enzymes provide the useful information about the extent and type of hepatocellular damage (Mitra*, et al.,* 1998). Normally ALT levels are high in patients who are suffering from viral hepatitis, alcoholic hepatitis, hepatic necrosis, cirrhosis and intrahepatic cholestasis. AST levels are also elevated by 10 to 200 fold in patients with viral hepatitis and hepatic necrosis.

Alkaline phosphatase (AP) is one more important enzyme normally determined to evaluate the liver function test. It is produced by tissues like liver, bone, intestine, bile ducts, kidneys and placenta and excreted through the bile and responsible for the hydrolysis of organic phosphate esters (Sakka, 2007). Increased production of AP by hepatic parenchymal cell and defective hepatic excretion lead to an elevated level of serum alkaline phosphatase which indicates the hepatobiliary disease. Determination of the amount of this enzyme provides the clinical information regarding the disease occurring in the liver or in the bile duct (Sakka, 2007).

Serum protein

Liver possess the ability to synthesize various proteins such as albumin, fibrinogen, transferrin, haptoglobin and prothrombin out of this albumin is the major protein synthesised and secreted into the blood (Kind & King, 1954). Total protein was measured to diagnose and treat various diseases of liver, kidney and also other metabolic disorders. A significant decrease in the level of protein such as albumin can be caused by factors, including deterioration of liver function, sever necrosis of the liver and hepatic resistance to insulin (Ezzat, *et al.*, 1989; Rao, 1995). Similarly, hepatotoxins also causes the reduction levels of both total protein as well as albumin. This decrease is caused by the injury caused to the endoplasmic reticulum of liver (Recknagel, *et al.*, 1989). Drugs with hepatoprotective activity generally increase the protein and also

albumin levels indicating the stabilisation of the endoplasmic reticulum, resulting in protein production (Mondal, *et al.*, 2005).

Serum bilirubin

Measurement of total bilirubin amount in the blood stream also provides information related to status and function of hepatic cells. Bilirubin is a metabolic product of the breakdown of haemoglobin and it is conjugated by the liver and excreted in bile. Normally serum bilirubin level is elevated due to the over production or decreased conjugation or decreased uptake by the liver or due to the bile ducts blockage (Mondal, *et al.*, 2005). However, if drugs under screening possess hepatoprotective activity, it will have a tendency to bring down serum bilirubin level in the liver damage model indicating the effectiveness of the drug in the normal functional status of the liver (Singh, *et al.*, 2003).

Liver biopsy

Liver biopsy was considered only when serologic and radiological evaluation failed to confirm the diagnosis. Liver biopsy is carried out through laparoscopic, transjugular, percutaneous, open operative or CT guided fine needle approaches. Liver biopsy provides 80-100 percent of accuracy of liver cirrhosis diagnosis and this method is very sensitive and specific. The accuracy of this method mainly depends on the sampling method employed and number and size of the histology samples collected. It also helps in the treatment of liver diseases (Abdi, *et al.*, 1979; Heidelbaugh & Bruderly, 2006).

Indocyanine green and bromosulfophthalein clearance

It is an infrared absorbing fluorescent agent that is completely removed by the liver into the bile without going through enterohepatic recirculation (P Faybik & H Hetz, 2006; P. Faybik & H. Hetz, 2006). Indocyanine green (ICG) clearance from the blood depends

on the live blood flow, parenchymal cellular function and biliary excretion. The normal value of ICG plasma disappearance rate (PDR) is over 18%/min and mortality related to liver dysfunction is higher in patients with ICG-PDR under 8%/min. Moreover, ICG elimination kinetics was commonly considered before setting of liver surgery (P. Faybik & H. Hetz, 2006; Sakka, 2007). Similarly, bromosulfophthalein (BSP) is also extracted and cleared by the liver. Less than 10% of BSP is retained in healthy individuals in comparison with patients with liver disorders (Chopra & Griffin, 1985).

Caffeine test

Caffeine physiologically metabolised by the liver to various metabolites such as theobromine, theophylline and parxanthine. Hepatic dysfunction was evaluated on the basis of metabolite/caffeine ratio. In patients with liver dysfunction elimination of caffeine takes longer time than the healthy volunteers (Jodynis-Liebert, *et al.*, 2004).

2.2.5.3 Radiographic evaluation

Radiographic studies were conducted to detect ascites, hepatocellular carcinoma, hepatosplenomegaly, hepatic vein thromboses and portal vein thromboses (Heidelbaugh & Bruderly, 2006). Abdominal ultrasonography provides useful information regarding gross appearance of the liver and blood in portal and hepatic veins. The presence of hepatic multinodulars, irregularities and ascites are easily diagnosed by ultrasonography. Magnetic resonance imaging (MRI) and computed tomography (CT) accurately demonstrates about nodularity, lobular atrophy and hypertrophy, ascites, varices, hepatocellular carcinoma and hence helps in determining the severity of cirrhosis (Heidelbaugh & Bruderly, 2006).

2.2.6 Cirrhosis treatment

Medical treatments for chronic liver diseases which include cirrhosis are in general problematic. In spite of the fact that liver cirrhosis is a global health issue and one of the most frequent occurring diseases with high mortality and high morbidity, currently, the medicinal management is inadequate and often have side effects (Rao, *et al.*, 2006). In addition to serious side effects, for instance, corticosteroids, colchicines, penicillamine and interferon are associated with possibly risk of relapse. Moreover, the available therapies tackle the symptoms and not preventing the progression of fibrosis process, which eventually develop into cirrhosis (Franklin, 1995). Some of these treatments, for instance, corticosteroids, colchicines, penicillamine and interferon are associated with possibly risk of relapse. Eventually, when the complications can no longer be managed by the available treatment and the liver is severely damage then liver transplant become the only option. Due to the reason that there is no effective and safe therapy, a great number of scientific studies searching for hepatoprotective constituents from natural sources continued to be conducted (Adnyana, *et al.*, 2001; Huang, *et al.*, 2010). In many cases the research on medicinal plant is guided from a traditional use or from undocumented treatment modalities.

2.2.7 Liver cirrhosis and medicinal plants

Medicinal plants for the treatment of different disorders extended ancient civilizations (Wadud, *et al.*, 2007). The usage of traditional medicinal plants in treating various disorders is in practice even when modern healthcare are readily available. According to the World Health Organization (WHO) estimation, in the developing countries about 80% of individuals in general use traditional medicine (Rahim & Khan, 2006). In developing countries in which communicable diseases are endemic, and hygiene and healthcare services are insufficient, medicinal plant used as antibacterial, antiviral, antioxidant, antinflammatory, antiulcer, antidiabetic and anticancer. In view of serious

side effects as a results of synthetic agents and absence effective therapy in the modern medicine, a good number of medicinal plants increasingly used to treat liver disorders (Dhiman, *et al.*, 2012). These recommended medicinal plants for the treatment of liver disorder have been in use for long time and claimed to produce significant results (Sanmugapriya & Venkataraman, 2006). Only a few of these have been adequately studied. These include *Silybum marianum* (milk thistle), *Camellia sinensis* (green tea), *Curcuma longa* (turmeric), , *Chelidonium majus* (greater celandine), and *Allium sativa* (garlic) (Luper, 1998). In general the constituents of the medicinal plant extract posse anti-inflammatory, liver protective and immune stimulating activities. The plant extracts exhibit ability to stimulate the production of hepatocyte for the repairing of liver. There are evidence that plant derived antioxidant is effective against fibrosis process. Silymarin derived from Silybum marianum is the most effective hepatoprotective natural product. It has been found to have potential in the prevention of cirrhosis (Stickel & Schuppan, 2007) Silymarin reduce the accumulation of hepatic collagen and exhibits considerable protection in laboratory animal models of different liver cirrhosis induced agents, including thioacetamide (TAA). Medicinal plant regarded as important source of antioxidant to which its secondary metabolites has potent free radical scavengers. Silymarin as well as its constituent silibinin possess important antioxidant activity (Pietrangelo, *et al.*, 1995).

2.3 Thioacetamide induced hepatotoxicity model

Thioacetamide (figure 2.2) was initially used to control the decay of oranges and further used as a fungicide (Childs & Siegler, 1945). It is a selective hepatotoxin and widely used to induce acute and chronic liver injury in rats and other experimental animals depending on the dose and duration of administration (Hung, *et al.*, 2005; Wang, *et al.*, 2004). Thioacetamide induces rat liver cirrhosis with biochemical and morphological

observations, resemble that of human liver cirrhosis and therefore serves as a suitable animal model for studying human liver fibrosis and cirrhosis (Fan & Weng, 2005).Oxidative stress and inflammation have been reported to contribute to the pathogenesis of TAA liver damage in animal model (Lin, *et al.*, 2009). The appropriate dose of TAA to induce liver damage in experimetal animals through intraperitoneal route of administration is reported to be 200 mg/kg (Chen, *et al.*, 2012). Its long-term administration induces hepatic fibrosis in rats, and causes the development of cirrhosis associated with an increased extent of lipid peroxidation (Kadir, *et al.*, 2011). Following administration, TAA is metabolised by the mixed function of oxidase system to acetamide and TAA-S-oxide ((Ahmed, *et al.*, 2008; Bastway Ahmed, *et al.*, 2010). Then TAA-S-oxide is metabolised, at least in part, by cytochrome P-450 monoxygenases to, a very highly reactive compound, TAA-S-dioxide (Wang, *et al.*, 2006). The binding of TAA-S-dioxide to tissue macromolecules induce hepatic necrosis and oxidative stress (Túnez, *et al.*, 2005). Besides, TAA produces oxidative stress, equally by both increased generation of ROS and also by reduction of cellular oxidative defences system. Studies have also revealed that liver cells intoxicated with TAA tend to be more susceptible to the cytotoxic effects of TNF-α along with other cytokines. It is well established that mitochondria has an essential role in the apoptotic response, therefore functional alteration in mitochondrial following TAA treatment lead to cell death through necrosis or apoptosis (Hoek & Pastorino, 2002).

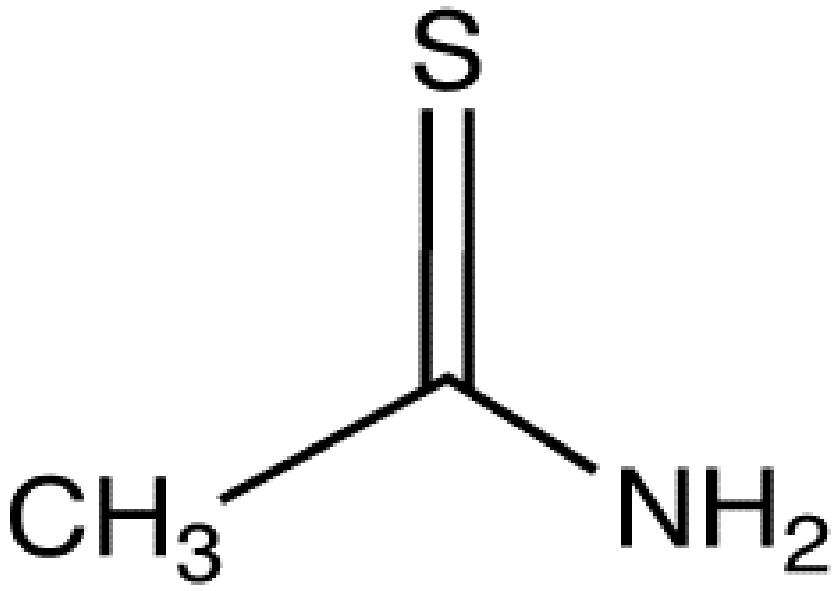

Figure 2.2 Chemical structures of thioacetamide (adapted from (Muñoz, *et al.*, 1991)).

2.4 *In vivo* and *in vitro* model used for hepatoprotective

2.4.1 *In vivo* models

In hepatoprotective studies, *in vivo* experiments are the most important applications employed to study liver damage and the response to the treatment. The animal model is prepared by chemical-inducing liver damage along with the plant extract treatment or after the plant treatment has started for a period of time. The liver damage animal model (rat or mice) produced by using toxic chemical dosage or prolonged dosage of hepatotoxin mimic any type natural liver damage occur in liver diseases. In hepatoprotective studies, the effectiveness of the plant extracts are studied against hepatotoxin which include thioacetamide (TAA), rifampicin, beta galactosamine, carbon tetrachloride (CCl4), paracetamol and alcohol. Among these, the most used chemicals to induce liver cirrhosis are CCl4, paracetamol and TAA, and each of them has different mechanism of inducing liver damage. Thioacetamide liver cirrhosis animal model is found to be more reliable and convenient to perform (Kreft, *et al.*, 1999). Generally, the plant extract studied for its effectiveness in hepatoprotective is

administrated in advance or alongside the toxin, and if possess hepatoprotective activity it should be able to prevent or reduce the damage on the liver. The assessment of liver damage is carried out by means of biochemical parameters which include serum levels of AST, ALT, AP, LDH, GGT, total protein, albumin and bilirubin (Rahman, *et al.*, 2001; Schuppan, *et al.*, 2003). In addition to that, histological parameters which include apparent necrosis signs and cirrhosis, and also the ration of liver size to the body weight confirm the biochemical findings.

2.4.2 *In vitro* model

In vitro experiments working with liver cell line may be more advantageous in terms of processing large number of samples, providing more reproducible results and also less costly as compared to the animals experiments. For the purposes of identification of active constituents separated from a known hepaprotective plant crude extract, a large number of the fractions need to be screened. Using animal model for the purpose of screening large number of sample may not be convenient when considering the time involved together with expenses. Cell lines can be applied to study cellular functions and provide important interactions information within it, for instance, metabolism (Gebhardt, 2000). The cell lines used in researches are mainly obtained from the cell banks that maintain the cell conditions which include American Type Culture Collection (ATCC). Generally, these cell lines preserve most of the features that can be found in the primary cell lines and are documented and identified for particular experimental applications. For the hepatoprotective studies, liver cell damage is induced by the use of hepatotoxin to which the effectiveness of the treatment (for example, plant extract) can be evaluated. The effectiveness of the treatment can be evaluated by means of parameters which include cell viability and oxidative stress (Bladier, *et al.*, 1997; Huang, *et al.*, 1999).

2.5 Silymarin

Silymarin, derived from *Silybum marianum* (milk thistle), is a well known hepatoprotective agent. Silymarin comprise three flavonolignan isomers, silybin, silychristine and silydianin. The mixture exhibits considerable protection in laboratory animal models of different liver cirrhosis inducing agents. Silybin appear to be the primary active constituents that deliver the effectiveness of silymarin (Kshirsagar, *et al.*, 2009). Silymarin continues to be used to tackle toxic liver condition and as a supporting therapy for persistent active hepatitis and also liver cirrhosis (Flora, *et al.*, 1998).

It has been reported that silymarin suppress the deposition of collagen in fibrotic-induced animal models. Silymarin is a free radical scavenger with properties including antioxidant, antinflammatory and antifibrotic. It prevents the binding of the hepatotoxin to the hepatocyte receptor and reduction of glutathione oxidation levels in the liver. It enhances the generation of hepatocyte by stimulating ribosomal RNA polymerase and therefore synthesis of protein. Silymarin exhibits considerable protection in laboratory animal models of different liver cirrhosis inducing agents, including thioacetamide (TAA) (Dixit, *et al.*, 2007; Ghosh, *et al.*, 2010; Pradhan & Girish, 2006).

2.6 Antioxidant activity

2.6.1 Free radicals and reactive oxygen species

Free radicals are highly unstable chemical substances that have unpaired electrons in the external electron orbit. They continually try to find electrons to pair with the intention achieve stability, hence they grab the electrons from macromolecules particularly DNA, proteins, carbohydrates and lipids. The molecules from which the electron is stolen become free radical and therefore steal electron from another molecule and therefore result in an uncontrolled chain reaction which alter and damage the function leading various diseases (Valko, *et al.*, 2006). The free radicals are categerised into

reactive nitrogen species and reactive oxygen species (ROS). The most important free radicals in the human body is ROS which represent any free radical associated with oxygen-centered free radical with two unpaired electrons in external shell. The free radicals may arise exogenously or endogenously. The generation of free radicals endogenously takes place during the generation of energy in the mitochondria, and in the course of nutrient metabolism. Reactive oxygen species (ROS), particularly, in the liver cells also produced by a group of enzymes collectively known as cytochrome P450 mixed–function oxidases. Small amount of ROS is generated during the cytochrome P450 enzyme catalyzing process for the reason that oxygen is involved in reaction. The amount of ROS produced remarkably depending on the compound undergoing degradation and the cytochrome P450 molecule involved. The most known active cytochrome molecule in the production of ROS is called P450 2E1 (CYP2E1) (Lieber, 1996). The exogenous source free radicals are the environmental contaminants which include toxic chemicals, smoking, radiation, pesticides, air pollution and organic solvents (GülçİN & KüfrevİoğLu, 2001). Free radicals take part in a number of physiological processes and also human diseases that include arteriosclerosis, cancer, alcoholism and liver injury, ischaemia, Parkinson syndrome, arthritis, aging and toxin induced reaction (Willcox, *et al.*, 2004). The increase in the amount of free radical amounts found during liver injury is caused by enhanced generation of free radicals together with reduced scavenging capability of the cells.

2.6.2 Oxidative stress in liver cirrhosis

Oxidative stress is regarded as imbalance between the increased generation of ROS and antioxidant (endogenous) defense system that might result in cell damage. This imbalance oxidation of biomolecules results in dysregulation of signal transduction and gene expression and thereafter cell necrotic and/or apoptotic (Dröge, 2002). In the liver oxidative stress contributes to the development and pathological findings of cirrhosis

and ultimately hepatocellular carcinoma irrespective of the cause and acts as a link between liver damage and fibrosis (Halliwell, 1999; Zhu, *et al.*, 2012). Fibrosis of the liver as a result of long-term ethanol intoxication is usually with indices of increased oxidation of polyunsaturated membrane lipids. Oxidative stress is responsible in the initiation and progression of the activation of TGF-β, HSC and collagen accumulation in the fibrogenesis induced by chemical (Kim, *et al.*, 2000). During oxidative stress the aldehydes produced from membrane lipid peroxidation assault extracellular and even cellular proteins in the hepatocytes and promote the accumulation of collagen in the inflamed cells (Albano, 2006).

2.6.3 Antioxidant and liver cirrhosis

Antioxidants are compounds which have the ability to scavenge, dispose and prevent the generation of free radicals, or challenge their actions. Antioxidants offer protection to body against free radical assaults which may result in diseases which include liver cirrhosis (Hasani-Ranjbar, *et al.*, 2009). They adopt four mechanisms of scavenging free radical. The mechanisms of scavenging include reducing the potency of ROS, donating electron to the free radical, removing the ROS initiator or by chelating the free radical. Plant derived antioxidants have demonstrated to possess potent hepatoprotective and antifibrotic agents. At the same time, in patients with alcoholic liver disease lipid peroxidation has been found be considerably high which is coupled with decline in antioxidant (Shinde & Ganu, 2009). There is growing interest nowadays to plant materials which have bioactive properties, particularly, antioxidant properties. There is a wide range of compounds in the plant materials that possess antioxidant properties and these include polyphenolic, terpenoids and tocopherols. Recently, a number of hepatoprotective, antinecrotic and antinflammatory drugs have revealed, as a part of their mechanism, to possess antioxidant properties. In order to maintain redox homeostasis, all cells are armed with different types of cellular antioxidant enzymes

together with substances that can potentially battle against ROS. Antioxidant enzymes, take for instance, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) each perform a specific role in scavenging free radicals and therefore as a whole protecting against cell damage. Along the line of the antioxidant enzymes, vitamin C and E protect the cells by preventing lipid peroxidation. In cases where the antioxidant defense is overwhelmed by oxidant, antioxidant supplement can be used to assist in reducing oxidative damage (Bergendi, *et al.*, 1999).

2.6.4 Evaluation of antioxidant activity

There are number of *in vitro* and *in vivo* assays employed to determine antioxidant capacity of plant based extract. The *in vitro* methods, ABTS and DPPH, can be used to measure the activity of the antioxidant of plant extract or pure isolated compound. These methods are known to be highly sensitive. Never the less, it is recommended to use more than one assay to determine the antioxidant activity of plant extract due to the complex nature of phytochemicals (Chanda & Dave, 2009). Together with the *in vitro* assays conducted on plant extracts, there are several *in vivo* cellular enzymes and substances that might be accomplished on tissue homogenate of the experimental animal model which include CAT, SOD and MDA, which determine the lipid peroxidation levels (Pietrangelo, *et al.*, 1995)

2.7 *Curcuma xanthorrhiza*

2.7.1 Botanical description of *C. xanthorrhiza*

C. xanthorrhiza (figure 2.3) is a medicinal herb belongs to the family Zingiberaceae of genus Curcuma with a synonym of *Curcuma javanica* found in various tropical regions of the world. *C. xanthorrhiza* is one of the extensively studied with various vernacular names such as temo labak (Indonesia), temu lawas (Malaysia), wan chakmotluk (Thailand) and temu lawak (Javanese). This medicinally valued rhizome is commonly

found wild as well as cultivated in Java, Malaysia, Thailand, and Philippines, and occasionally in India also. Several species of Zingiberaceae family are commonly found in damp, shaded parts of hill slopes and lowlands in Peninsular Malaysia. *Curcuma xanthorrhiza* rhizome is one of the important herb belongs to this family. Outer region of *C. xanthorrhiza* rhizomes are dark to reddish brown and interior have orange-red in colour. *C. xanthorrhiza* is commonly found in thick, moist, fertile and humus rich soils (Wardini & Prakoso, 1999) and possesses an aromatic, pungent odour with bitter taste (Devaraj, *et al.*, 2010).

Figure 2.3 *Curcuma xanthorrhiza* rhizoma

2.7.2 Traditional uses of *C. Xanthorrhiza*

Traditionally, temu lawak has been used as food and medicinal purposes. Young stems, rhizomes and inflorescences of *C. xanthorrhiza* are consumed either raw or after cooking as food. Rhizomes were cooked to prepare juice and used as soft drink (Husein, *et al.*, 2009; Lin, *et al.*, 1996). The juice is also used to treat indigestion and rheumatism

(Ruslay, *et al.*, 2007). *Curcuma xanthorrhiza* as a traditional folk medicine 'Jamu' in Indonesia used to treat malaria, dysentery, cancer (Yamada, *et al.*, 2009), to relieve uterus pain and to relieve abnormal menstruation symptoms. *C. xanthorrhiza* rhizomes are traditionally used for the treatment of various conditions which include liver disorders, abdominal complaints, fever, constipation, bloody diarrhea, haemorrhoids, skin infection, acne vulgaris and smallpox. It is also used as a galactogogue and to reduce the uterine inflammation after the child birth (Wardini & Prakoso, 1999).

2.7.3 Phytochemistry of *C. xanthorrhiza*

Rhizome of *C. xanthorrhiza* contains several phytoconstituents such as terpenoids, monoterpenoids, sesquiterpenoids and curcuminoids. The important sesquiterpenoids present are α and β-curcumene, xanthorrhizol, α and β-turmerone, arturmerone, germacrone, β-sesquiphellandrene, and curzerenone (Uehara, *et al.*, 1992). Essential oils present in *C. xanthorrhiza* rhizome constitute around 65% of the total bioactive components (Yasni, *et al.*, 1994). Xanthorrhizol is the main sesquiterpenoid which make up 51% of the total essential oils (Chean, *et al.*, 2006; Chen, *et al.*, 2006)). It has been reported that *ar*-curcumene, xanthorrhizol, α- and β-curcumene, curzerenone, and germacrone are the main constituents in the essential oil obtained by hydrodistillation of *C. xanthorrhiza* Roxb (Zwaving & Bos, 1992). Several non phenolic diarylheptanoids such as trans, trans-1,7-diphenyl-2,3-heptadien-4-one, 1,7-diphenyl-5-hydroxy-(l*E*)-1.heptene and 1,7-diphenyl-5-hydroxy-(l*E*,3*E*)-1,3-heptadiene was isolated from the hexane extract of *C. xanthorrhiza* rhizomes cultivated in Thailand (Claeson, *et al.*, 2007). Even some phenolic diaryheptanoids such as demethoxycurcumin, bisdemethoxycurcumin, 1-(4-hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-(1E,6E)-1,6-heptadien3,5dione (Masuda et al, 1992), 5-hydroxy-7-(4-hydroxyphenylt1-phenyl-(l*E*)-l-heptene, 7-(3,4-dihydroxy phenyl)-5-hydroxy-l-phenyl-(1*E*)-l-heptene were also isolated from *C. xanthorrhiza* rhizomes.

Curcumin and its analogues such as hexahydrocurcumin, octahydrocurcumin, dihydrocurcumin, l-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-6-hepten-3,5-dione (Uehara, *et al.*, 1992) and 3'-demethoxycyclocurcumin were also reported from *C. Xanthorrhiza* (Yamada, *et al.*, 2009). *C. xanthorrhiza* rhizome also contains saponins, flavonoids and tannins..

A new LC-MS method was developed to analyse and identify the bioactive compounds from the aqueous extracts of *C. xanthorrhiza* (Ruslay, *et al.*, 2007). These researchers then reported that curcuminoids such as bis-demethoxycurcumin, demethoxycurcumin and curcumin are the potent antioxidant components present in the ethyl acetate fraction of aqueous extracts of *C. xanthorrhiza*. Earlier, a rapid capillary zone electrophoresis method for the separation as well as identification of three main curcuminoids, demethoxycurcumin, and bis-demethoxycurcumin, from *C. xanthorrhiza* Roxb was also developed (Lechtenberg, *et al.*, 2004).

2.7.4 Previous scientific studies of *C. xanthorrhiza*

C. xanthorrhiza rhizome has been extensively studied to evaluate its therapeutic efficacy. *C. xanthorrhiza* extract as well as *xanthorrhizol* showed significant and dose dependent decrease in the expression of matrix metalloproteinase-1, and increased the type-1 procollagen levels in UV-irradiated human skin fibroblasts suggesting it as a potential skin anti-aging candidate. An anti-inflammatory study revealed the potent anti-inflammatory potency of methanolic extract of *C. xanthorrhiza* in 12-*O*-tetradecanolyphorbol-13-acetate (TPA) induced inflammation as well as 7,12-dimethylbenz[*a*]anthracene (DMBA induced number of tumors in mouse explaining its anticancer potency (Park, *et al.*, 2008).

Hepatoprotective activity of aqueous extract of *C. xanthorrhiza* prepared by decoction method in male Wistar albino rats intoxicated with *β*-D-galactosamine was evaluated (Lin, *et al.*, 1996). The results of this study revealed that *C. xanthorrhiza* extract at a

dose of 100 mg/kg could significantly reduce the elevated serum transaminases and reduced the severity of liver damage induced by *β*-D-galactosamine. Another study of hepatoprotective was conducted using ethanolic extract of *C. xanthorrhiza* against ethanol induced liver toxicity in Sprague-Dawley rats (Devaraj, *et al.*, 2010). The results of the study demonstrated that oral administration of 500 mg/kg extract could significantly reduce the elevated serum transaminases and protein emphasizing that the plant could be an important source of hepatoprotective agent.

Oral acute toxicity of *C. xanthorrhiza* showed no any toxic symptoms even at the maximum dose used (5 g/kg) (Devaraj, *et al.*, 2010). The plant extract also demonstrated peripheral and central analgesic activity at the dosage of 200 and 400 mg/kg in both phases of formalin induced pain model (Devaraj, *et al.*, 2010). An *in vitro* antioxidant study of acetone extract revealed the potent antioxidant activity of *C. xanthorrhiza* in both thiobarbituric acid and thiocyanate assay methods (Jitoe, *et al.*, 1992).

Oral administration of *C. xanthorrhiza* at the dose of 2, 3 and 4g/kg in male rabbits resulted in significant increase in fat excretion through the lipid metabolism by liver also decreased cholesterol, triglyceride and LDL concentrations. In addition, *C. xanthorrhiza* sample also significantly increased the HMG-CoA reductase activity (Wientarsih, *et al.*, 2002). Contrary to this a recent study revealed that ethanolic extract of *C. xanthorrhiza* only decreased LDL concentration when given orally at a dose of 25 mg/kg in male Wistar rats (Sukandar & Nurdewi, 2012). However in combination with aqueous extract of *Guazuma ulmifolia* at a dose of 12.5 mg/kg significantly decreased total cholesterol and LDL level (Sukandar & Nurdewi, 2012).

Earlier, study reported the significant decrease in the concentration of serum triglycerides and phospholipids as well as liver cholesterol and also increased the serum HDL cholesterol in rats treated with *C. xanthorrhiza* extract (Yasni, *et al.*, 1993). The

study also reported that curcuminoids of *C. xanthorrhiza* does not have role in the obtained hypolipidemic effect and hence concluded that active constituents other than curcuminoids are responsible for the found activity. Another study reported the significant hypolipidemic activity through the inhibition of hepatic triglyceride secretion by two phenolic diarylheptanoids isolated from *C. xanthorrhiza.*

Potent antimicrobial activity of ethanolic as well as ethyl acetate extract of *C. xanthorrhiza* rhizome against *Staphylococcus aureus, Bacillus cereus* and *Escherichia coli* have been reported (Husein, *et al.*, 2009). *C. xanthorrhiza* extract antibacterial effects on *Streptococcus mutans* bioflims revealed the strong bactericidal activity and inhibition of acidogenesis rendering it has a new source for the prevention of dental caries (Kim, *et al.*, 2008). A study reported two fold increase in the estrogenic activity of *C. xanthorrhiza* aqueous ethanolic extract in yeast two hybrid system (Siri & Incamnoi, 2008).

2.7.5 Important pharmacological studies of xanthorrhizol

Xanthorrhizol is the main and bioactive sesquiterpenoid found only in *C. xanthorrhiza* Roxb but not in any other species of Curcuma (Husein, *et al.*, 2009). Several positive findings regarding its therapeutical potency have been investigated. Xanthorrhizol exhibited the hepatoprotective activity on cisplatin-induced hepatotoxicity through its ability to regulate the DNA-binding action of nuclear factor-kB (NF-kB) (Kim, *et al.*, 2005) and by the phosphorylation of c-Jun N-terminal Kinases (JNKs) in a dose-dependent manner (Hong, *et al.*, 2005). Topical application of xanthorrhizol possess the ability to inhibit the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced acute inflammation in mouse ear by the inhibiting the expression of ornithine decarboxylase, cyclooxygenase-2 and inducible nitric oxide synthase through the NF-kB and mitogen-activated protein kinases.

Protective effect of xanthorrhizol on cisplatin-induced nephrotoxicity in ICR male mice was carried out (Kim, *et al.*, 2005). In this study, it was reported that xanthorrhizol at a dose of 200 mg/kg significantly reduced the elevated levels of blood urea nitrogen, serum creatinine and kidney weight. Moreover, the same study also revealed that the nephroprotective effect of xanthorrhizol is more efficacious than curcumin at the same tested dose (200 mg/kg).

A study was carried out to evaluate the antioxidant and anti-inflammatory activity of xanthorrhizol in hippocampal neurons and cultured microglial cells. The results of this study showed that xanthorrhizol could reduce the expression of cyclooxygenase-2 as well as inducible nitric oxide synthase and the release of proinflammatory cytokines like TNF-α and, interleukin-6 in microglial cells activated by lipopolysaccharide (Lim, *et al.*, 2005). The same team of researchers also reported the potent neuroprotective activity of xanthorrhizol on glutamate induced neurotoxicity in murine hippocampal HT22 cells through the inhibition of lipid peroxidation (Lim, *et al.*, 2005).

In a study conducted to evaluate the growth inhibitory effect of xanthorrhizol in HCT116 human colon cancer cells, it was reported that xanthorrhizol dose dependently exerted antiproliferative effect and apoptosis by triggaring DNA fragmentation, release of cytochrome c and by activation of caspases (Kang, *et al.*, 2009). Xanthorrhizol show antiproliferative effect on human hepatoma cells HepG2 through the activation of suppressor p53 and down regulation of antiapoptic Bcl-2 and Bcl-X_L protein expression. (Handayani, *et al.*, 2007; Tee, *et al.*, 2012). In addition, xanthorrhizol also induces apoptosis of cervical cancer cell line HeLa cells via the up-regulation of tumour suppressor protein p53 and the pro-apoptic protein Bax (Ismail, *et al.*, 2005). Xanthorrhizol in combination with curcumin exhibited synergistic growth inhibitory effect on MDA-MB-231 human breast cancer cells through apoptosis induction (Cheah, *et al.*, 2009).

Xanthorrhizol also found to inhibit the formation of tumour nodules in the mouse lung tissue and intra-abdominal tumour mass formation through the attenuation of expression of cyclooxygenase-2, matrix metalloproteinase-9 and phosphorylated extracellular signal regulated kinase.

With regards to anticandidal activity of xanthorrhizol, a compound isolated from methanolic extract of *C. xanthorrhiza* was studied against six Candida species and reported to have potent anticandidal activity against all the species with MIC concentration of 1.0-25 mg/L (Rukayadi, *et al.*, 2006). Further study conducted revealed that the found anticandidal activity is through the modification of external morphology, deformation and protrusions on the surface of the Candida cells (Rukayadi & Hwang, 2007). Xanthorrizol isolated from *C. xanthorrhiza* also exhibited potent anti-*Malassezia* activity expressed in terms of minimum inhibitory concentration (MIC) against two Malassezia species with the MIC value of 0.25 to 1.25 µg/mL (Rukayadi & Hwang, 2007). A study regarding the antibacterial effects of *C. xanthorrhiza* extract on *Streptococcus mutans* bioflims revealed the strong bactericidal activity and inhibition of acidogenesis rendering it has a new source for the prevention of dental caries (Kim, *et al.*, 2008). Other researchers revealed the potent antibacterial activity of xanthorrhizol against Streptococcus species which cause dental caries. Xanthorrhizol and its methylation derivative exhibited strong inhibition of platelet activating factor receptor binding (Jantan, *et al.*, 2004). It has been reported that two fold increases in the estrogenic activity of *C. xanthorrhiza* aqueous ethanolic extract in yeast two hybrid systems.

2.8 *Ipomoea aquatica*

2.8.1 Botanical description of *Ipomoea aquatica*

Ipomoea aquatic (figure 2.3) is one of the 400 species under the genus *Ipomoea* L. belongs to the family Convolvulaceae and distributed throughout tropical climatic countries. It is indigenous to China but also distributed throughout India, Sri Lanka, Africa, Australia and Tropical Asia. It is also well-known by different names including aquatic morning glory, kangkong, water spinach, water convolvulus, swamp cabbage (Prasad, *et al.*, 2008). It grows wildly in India but cultivated commercially in Malaysia, Singapore, Indonesia Taiwan, China and Hong Kong where it is consumed as an edible leafy vegetable (Prasad, *et al.*, 2008; Wills, *et al.*, 1984).

Ipomoea aquatica is an annual or perennial aquatic plant normally found floating or trailing in fresh water ponds, marshes, and in ditches also found in muddy stream banks as creepers (Rao & Vijay, 2002). Leaves, petiole and stems of this plant are green in color with white or pale purple color flowers. Leaves are ovate to elliptic in form with an acute tip and sagittae base having a length of 6-15 cm. Leaves are present on long, fleshy petiole arising from the nodes of the lateral branches. Flowers are of 2-2.5 cm, axillary in shape present either singly or in group of 3-7 on the lateral branches and fruit is in the capsule form (Edie & Ho, 1969).

Leaves of this plant possess high nutritive value for human, fish and grazing animals and also reported to have medicinal value (Hu, *et al.*, 2010). Since the amount of essential amino acids present in *I. aquatica* is comparable to that of soyabean or egg it can be utilized as a source of food supplement. Currently, *Ipomoea aquatic* leaves were extensively used to purify waste water (Hu, *et al.*, 2008)

Figure 2.3 *Ipomoea aquatica* plants (adapted from (Prasad, *et al.*, 2008))

2.8.2 Traditional uses of *I. aquatica*

Though *I. aquatica* is extensively used as leafy vegetable due to its high nutritive value its traditional uses will not be neglected. In Ayurveda and homeopathic medicine it is used to various disorders including the disorders of liver, oxidative stress, nose bleed and high blood pressure (Prasad, *et al.*, 2005). Traditionally, juice of this plant is used in liver disorders, eye disease (Jain & Verma, 1981), gastric and intestinal disorders (Westphal, 1993) and in constipation (Samuelsson, *et al.*, 1992) and, buds are used in the treatment of leprosy, leucoderma and fever (Mamun, *et al.*, 2003).

In traditional medicine, *Ipomoea aquatica* have also been used to treat certain nervous conditions with sleeplessness as well as head-ache and also the plant has a anxiolytic effect (Prasad, *et al.*, 2008). According to the Sri Lankan indigenous medicine it possesses insulin like activity (Jayaweera, 1980). In particular juice of this vegetable is commonly used as an emetic to treat opium and arsenic poisoning (Kapoor & Kapoor, 1980).

2.8.3 Phytochemistry of *I. aquatica*

Preliminary phytochemical analysis *of I. aquatica* plant extract has shown the presence of phenols, flavonoids, tannins, saponins, steroids and alkaloids (Vasu, *et al.*, 2009; Yadav & Agarwala, 2011). *Ipomoea aquatica* is a rich source of vitamin C, soluble proteins and sugars (Hu, *et al.*, 2010), essential amino acids such as aspartic acid, glycine, alanine, leucine, organic acids like citric acid, oxalic acid, malic acid (Wills, *et al.*, 1984).

Several carotenes and pigments from *I. aquatica* were separated using thin layer chromatography, column chromatography and high performance liquid chromatography and to identify *β*-carotene, *β*-cryptoxanthin, lutein, lutein epoxide, violaxanthin and neoxanthin by comparing the absorption spectra and retention time with reference standards (Chen, *et al.*, 1991). Later , a new HPLC method developed to separate and identify these carotenes and pigments along with *cis*-lutein and, *cis*-*β*-carotene from *I. aquatica* (Chen & Chen, 1992). Several other carotenes such as zeaxanthin, anthraxanthin and luteoxanthin were also identified from this plant (Wills & Rangga, 1996). Further down the line the provitamin A carotenoid content was determined using a validated HPLC method and the variation in the carotenoid content of *I. aquatica* was reported according to its maturity and origin (Hulshof, *et al.*, 1997).

The separation of essential oils from the leaves and stems of *I. aquatica* was carried out using Likens Nikerson apparatus that identified 58 volatile components some important components are phytol, palmitic acid, alpha humulene, n-hexacosane and bis(2-ethyl-hexyl) subacetate (Kameoka, *et al.*, 1992). *I. aquatica* found to posses several phenolic compounds such as procatechuic acid, p-hydroxybenzoic acid, vanillic acid and salicylic acid (Leela & Rao, 1994) and flavonoids such as 7-*O*-*β*-D-glucopyranosyl-dihydroquercetin-3-*O*-*α*-D-glucopyranoside (Prasad, *et al.*, 2005). Other study reported the presence of quercetin (205 mg/kg of dry weight) in the acid hydrolysed aqueous

ethanolic extract of *I. aquatica* (Miean & Mohamed, 2001). Several other flavonoids such as quercetin, luteolin, apigenin, myricetin, quercetin-3'-methyl ether, quercetin-4'-methyl ether and anthocyanins were also reported from *I. aquatica* (Chu, *et al.*, 2000; Daniel, 1989).

2.8.4 Previous scientific studies

The wide distribution and easy availability attracted the attention of various researchers to evaluate its therapeutic efficacy. A study evaluated antioxidant activity of *Ipomoea aquatica* leaf and stem ethanolic and waters extract using DPPH assay, reducing power method and ferric thiocyanate method, and antiproliferative activity in human lymphoma NB4 cells (Huang, *et al.*, 2005). The results of this study revealed the highest DPPH radical scavenging activity, reducing power and significant total phenolic content in the ethanolic stem extract whereas highest antiproliferative activity was observed with water extract of stem with an EC_{50} value of 661.4 ± 3.36 µg dry matter/mL (Huang, *et al.*, 2005). Using Soxhlet extraction method, serial extraction of *I. aquatica* leaf was extracted and followed by the determination of antioxidant activity in which both methanolic as well as aqueous extract showed significant antioxidant activity. Recently, three carotenoids namely violaxanthin, lutein and *β*-carotene were separated by HPLC-MS and then evaluated for their antioxidant potency (Fu, *et al.*, 2011). The result revealed that *β*-carotene possess highest DPPH radical scavenging activity whereas, violaxanthin significantly prevented the lipid peroxidation.

A study reported the potent antioxidant activity of *I. aquatica* in DPPH, trolox equivalent antioxidant capacity as well as oxygen radical absorbance capacity assays and also mentioned that this plant extract also possess antitumor activity on mouse myeloma cell line P388 (Shekhar, *et al.*, 2011). Further in the study conducted by the same team of researchers the plant extract revealed the antimutagenic activity in *Salmonella Typhimurium* TA98 and antimicrobial activity against *Pseudomonas*

aeroginosa and *Escherichia coli* bacteria. Crude methanolic extract of *I. aquatica* possess moderate cytotoxic activity against Hep-2 (human larynx epithelial carcinoma) and A-549 (human small cell lung carcinoma) cell lines followed by the column fraction and the isolated bioactive compound 7-*O*-*β*-D-glucopyranosyl-dihydroquercetin-3-*O*-*α*-D-glucopyranoside (Prasad, *et al.*, 2005).

Ipomoea aquatica is also known to posse hypoglycemic activity and study was conducted using boiled whole extract of *I. aquatica* in normal male Wistar rats. In this study it was reported that single dose of the extract (3.4g/kg) significantly reduced the serum glucose level (33%) in comparison with the multiple dose of the extract (25%) (Malalavidhane, *et al.*, 2000). Based on this result, the same authors conducted an oral hypoglycemic activity of finely shredded leaves and stem of *I. aquatica* in streptozotocin-induced male Wistar diabetic rats and boiled whole extract in human diabetic patients. The result of this study revealed the significant decreased fasting blood glucose levels in diabetic rats and also in type 2 diabetic patients with a 29.4% decrease in the serum glucose concentration (Malalavidhane, *et al.*, 2003). The hypoglycemic efficacy of *I. aquatica* extract was reported to be comparable with that of tolbutamide in lowering blood glucose level (Malalavidhane, *et al.*, 2001). In the same line, another study evaluated the effect of *I. aquatica* aqueous as well as dichloromethane/methanolic extracts on the glucose absorption in rat intestinal preparation and reported that aqueous extract significantly decreased the intestinal glucose absorption (Sokeng, *et al.*, 2007).

In the evaluation of antimicrobial and anti-inflammatory activities of methanolic and aqueous extracts of *I. aquatica* leaf has been reported that methanolic extract possess potent antimicrobial activity against *Bacillus subtilis Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Proteus vulgaris* compared to aqueous extract. In addition they also reported that, the anti-inflammatory activity exhibited by

both extracts at a dose of 200 mg/kg was almost equal to the activity shown by indomethacin. It is also reported to inhibit prostaglandin synthesis (Tseng, *et al.*, 1992) and moderate antinematocidal activity against pine wood nematode (Mackeen, *et al.*, 1997).

Methanolic extract of *I. aquatica* at dose of 200 and 400 mg/kg significantly decreased the concentration of total cholesterol, total lipid, free fatty acids, phospholipids and triglycerides in plasma as well as in liver and, kidney in hyperlipidemic Swiss albino rats (Sivaraman, 2010). Ethanolic extract of *I. aquatica* leaf at a dose of 200 and 400mg/kg body weight effectively reduced the free and total acidity in aspirin induced gastric ulcer model in Swiss albino rats through the its cytoprotective effect (Sivaraman, 2010).

Diuretic activity of this plant has also been reported in a study in which Swiss albino mice treated with methanolic extract of *I. aquatica* at a dose of 250 and 500 mg/kg body weight. The result proved that *I. aquatica* possess potent and dose dependent diuretic activity, and the found activity was higher than the reference drug furosemide (Mamun, *et al.*, 2003). Positive findings regarding the anxiolytic activity of *I. aquatica* extracts in elevated pus maze, light dark apparatus and hole board apparatus has also been reported (Khan, *et al.*, 2011).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals

All chemicals used in the study were of analytical grade and purchased from Sigma-Aldrich. Thioacetamide (TAA, Sigma-Aldrich, Switzerland) was used as an inducer for liver cirrhosis and silymarin (International Laboratory, USA) was used as a standard drug in the experiment. 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium persulphate, 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Vitamin-C, gallic acid, silymarin were purchased from Sigma Chemicals, Germany.

3.2 Plant materials

Fresh plant materials of *C. xanthorrhiza* (vulture No. KLU 11022) and *I. aquatica* (vulture No. KLU 041845) were purchased from the Ethno Sdn Bhd, Selangor Malaysia, and identified by comparison with the Voucher specimen deposited at the Herbarium of Rimba Ilmu. Separately, *C. xanthorrhiza* rhizome (cut into small pieces) and *I. aquatic* leaves were first washed with tap water and followed rinsing with distilled water. The plant materials were then air dried for 7 days.

3.3 Extraction of *Curcuma xanthorrhiza* rhizome and *Ipomoea aquatica* leaves

The dried *C. xanthorrhiza* rhizome and the dried leaves of *I. aquatica* were powdered using electrical blander. 100 grams of each powdered plant material then was extracted in 900 ml of 95% ethanol at 25°C for 72 hours. The suspensions were gently mixed, from time to time, to allow the powdered materials fully dissolved in the ethanol. The ethanol extract was filtered through cheese cloth and then by filter paper (Whatman No. 1) and evaporated under reduced air pressure using Buchi rotary evaporator to obtain

the percentage yield crude-dried extract of 5.4 and 18.4%, (w/w) for *C. xanthorrhiza* and *I. aquatica* samples, respectively.

3.4 *In vitro* antioxidant of plant extracts

In vitro antioxidant of crude extracts of *C. xanthorrhiza* and *I. aquatica* were determined using DPPH radical scavenging assay and ABTS radical scavenging assay.

3.4.1 DPPH radical scavenging assay

The method described by Brand-Williams with slight modification was employed to determine DPPH radical scavenging activity of *I. aquatica* and *C. xanthorrhiza* extracts as well as standards (Brand-Williams, *et al.*, 1995). Ascorbic acid was diluted in five concentrations of 25, 12.5, 6.25, 3.125 and 1.56 µg/ml and used as standards in the assay to create standard curve. Then in order to compare the scavenging power of the samples, Ascorbic acid BHT, together with silymarin, *C. xanthorrhiza* and *I. aquatica* each was diluted to into two dilutions 25 and 6.25 µg/ml. Each of the prepared dilution was mixed with 195 µl of DPPH (40 X dilution) in triplicate. The decrease in the absorbance due to free radical scavenging activity was recorded at 515 nm for 2 hr with 20 min intervals. The percentage inhibition of DPPH radicals by tested samples was calculated using the following formula and the results were expressed as Mean ± Standard Error Mean (S.E.M.): % of radical scavenging activity = (Abs Blank – Abs Sample) /Abs Blank X100

3.4.2 ABTS radical scavenging assay

ABTS radical scavenging activity of *I. aquatica* and *C. xanthorrhiza* extracts and standards were determined according to the procedure described by Re (Re, *et al.*, 1999). Trolox was used as standard in the assay to create standard curve. Then in order to compare the scavenging power of the samples, Ascorbic acid, BHT, together with silymarin, *C. xanthorrhiza* and *I. aquatica* each was diluted to into two dilutions 100

and 25 µg/ml. The generation of 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical cation take place when is reacted with 7 mM ABTS and 2.45 mM potassium persulfate incubated in the dark for 12–16 hours at room temperature. Fresh ABTS was prepared and diluted with methanol to yield an absorbance of 0.700 ± 0.02 at 734 nm, and the same solution was used for this assay. Then 10 µl of prepared dilutions of sample and standards were mixed with 1 ml ABTS and mixed thoroughly. The reactive mixture was kept at room temperature for 15 min and the absorbance was recorded at 734 nm and the percentage inhibition of ABTS radicals was calculated.

3.5 Cell culture medium

Normal human liver embryo cells WRL-68 obtained from American Type Culture Collection (ATCC, USA) were cultured in RPMI 1640 (Sigma, Aldrich-USA), supplemented with 10% Fetal Bovine Serum (Sigma, Aldrich-USA), 1% Penicillin/Streptomycin (Sigma, Aldrich-USA) and incubated at 37°C in a humidified atmosphere of 5 % CO_2.

3.6 Selection of TAA dose for hepatotoxicity induction in WRL-68 cells

Thioacetamide (TAA) was used to induce oxidative stress and therefore cell damage in the normal human liver embryo cells (WRL-68). The cells were seeded in 200 µl of RPMI containing 10% FBS with density 3000 cells/ well. After 24 hours, cells were treated with 10 µl of different concentrations (0.01, 0.02, 0.04, 0.06, 08 and 0.10g/ml) of freshly prepared TAA (Merck, Darmstadt, Germany) and then again incubated at 37°C in humidified 5 % CO_2 incubator for 2 hours. The WRL-68 growth inhibition as results of TAA toxicity were determined by applying MTT assay described (Mosmann, 1983; Scudiero, *et al.*, 1988). The assay was carried out in three separate experiments. The TAA concentration which demonstrated the cell growth inhibition of approximately

50% was chosen for *in vitro* hepatoprotective activity of the plant extracts and the fractions.

3.7 Hepatoprotective effect of crude extracts against TAA cytotoxicity

Thiacetamide at the concentration of 0.04g/ml was selected to induce approximately 50% cell growth inhibition of normal human liver embryo cells (WRL-68). The cells were seeded in 200 µl of RPMI containing 10% FBS with density 3000 cells/ well and incubated at 37^{o}C and 5% CO_2 for a day. After 24 hours, using 2.5% DMSO, 10 µg/ml media of both plant crude extracts were prepared. The prepared crude extracts were added to the cells as per the designed experiment (Table 3.1) and incubated for 2 hours. The cells were then treated with 10 µl of 0.04g/ml TAA. Normal control cells was kept with media only, toxin control was kept with 0.04 g.ml TAA only and standard drug control (silymarin) was kept with TAA then silymarin at the concentrations 10 µg/ml (Sarkar & Sil, 2007). The cells were incubated for 2 hours and the hepatoprotective effect of plant extracts were determined by cell viability using MTT assay as described by (Mosmann, 1983; Scudiero, *et al.*, 1988). The assay was carried out in three separate experiments.

Table 3.1: The design of in vitro hepatoprotective screening of *C. xanthorrhiza* and *I. aquatica* crude extracts

Group Name	TAA 0.04g/ml	Treatment 10µl/ml
Normal control	No TAA (10µl medium)	DMSO 10µl
TAA control	10µl	DMSO 10µl
Silymarin control	10µl	Silymarin 10µl
C. xanthorrhiza crude	10µl	Crude extract 10µl
I. aquatica crude	10µl	Crude extract 10µl

3.8 Antioxidants of the cell line treated with crude extracts

The crude extracts of both plants were tested for their antioxidant activities. The antioxidant activity was measured using the following kits: superoxide dismutase (SOD) assay kit (Cayman Chemical Company, USA), catalase (CAT) assay kit (Cayman Chemical Company, USA) and molandialdehyde (MDA) assay kit (Cayman Chemical Company according to the manufacturer's instructions. Briefly, to determine the activity of SOD, CAT and lipid peroxidation (MDA), WRL-68 cells were seeded in 200 µl of RPMI containing 10% FBS with density 3000 cells/ well and incubated at 37^{o}C and 5% CO_2 for a day. After 24 hours, using 2.5% DMSO, 10 and 100 µg/ml media of both plant crude extracts were prepared and added to the cells as per the designed experiment (Table 3.2) and incubated for 2 hours. The cells were then treated with 10 µl of 0.04g/ml TAA and reincubated for 2 hours. Normal control cells was kept with media only, toxin control was kept with 0.04 g/ml TAA only and standard drug control (silymarin) was kept with TAA then silymarin at the concentrations 10 and 100 µg/ml. The cells were then washed three times with PBS pH 7.4 and sonicated on ice and the solution was then centrifuged at 13,000 x g for 15 minutes at 4°C. The obtained supernatants were used to determine intracellular antioxidants in a microplate reader at 440 nm for SOD, 540 nm for CAT and 540 nm for MAD. The assay was carried out in three separate experiments.

Table 3.2: The antioxidant and lipid peroxidation experimental design for cell line treated with crude extracts.

Group Name	TAA 0.04g/ml	Treatment 10µl/ml and 100µl/ml
Normal control	No TAA (10µl medium)	DMSO 10µl
TAA control	10µl	DMSO 10µl
Silymarin control	10µl	Silymarin 10µl
Silymarin control	10µl	Silymarin 100µl
C. xanthorrhiza crude	10µl	Crude extract 10µl
C. xanthorrhiza crude	10µl	Crude extract 100µl
I. aquatica crude	10µl	Crude extract 10µl
I. aquatica crude	10µl	Crude extract 100µl

3.9 Acute toxicity test

The procedure for this animal study was reviewed and then approved by Animal Ethics Committee (Ethic No. PM/27/07/2010/MAA). Thirty six Sprague-Dawley rats aged 7-8 weeks (eighteen male and eighteen female) were used for each plant extract in which they were divided into three groups of each gender with six rats each. For *C. xanthorrhiza* the animal groups marked as vehicle, low dose and high dose were orally administered with 5ml/kg of 10% tween 20, 2g/kg and 5g/kg of prepared dose of *C. xanthorrhiza* extract, respectively. In case of acute toxicity study of *I. aquatic*, the animal groups marked as vehicle, low dose and high dose were orally administered with 5ml/kg sterile distilled, 2g/kg and 5g/kg of prepared *I. aquatica* extract, respectively. Prior dosing the rats were kept fasting for overnight and after the dose for another 4 hours. Following the dose the rats were carefully observed for the development of any clinical or toxicological symptoms at 30 minutes and then 2, 4, 8, 24 and 48 hours. On

the 15th day, all the rats were sacrificed and samples for biochemical and histological studies were collected and analyzed as per (Lorke, 1983).

3.10 Hepatoprotective study of the crude extracts

3.10.1 Experimental animals

The study was carried out using male Sprague-Dawley rats (195 - 215 g) obtained from the Animal House Unit, Faculty of Medicine, University of Malaya, Malaysia. The chosen animals were housed in bottomed cages at 25 ± 3°C temperature, 50–60% humidity, and under a 12 hours light-dark cycle for a week before the experiment. The animals were maintained at standard housing conditions and free access to a standard diet and water *ad libitum* during the experiment. The experiment was performed in accordance to the guideline of taking care of animals prepared by the National Academy of Sciences.

3.10.2 Preparation of doses

Thioacetamide (cirrhosis inducer) was dissolved in sterile distilled water and then injected intraperitoneally to the experimental rats at a dosage of 200 mg/kg body weight (Aydın, *et al.*, 2010). Silymarin (a standard drug) was dissolved in sterile distilled water and orally administered to rats in concentrations of 50 mg/kg body weight (Aydin, *et al.*, 2010). Crude dried ethanol extract of *C. xanthorrhiza* rhizome was dissolved in tween 20 (10% w/v) and orally administered to the experimental animals at a dosage of 250 and 500 mg/kg body weight. Crude dried ethanol extract of *I. aquatica* leaves was dissolved in sterile distilled water and orally administered to the experimental animals at a dosage of 250 and 500 mg/kg body weight. For each plant, normal control group of animals received distilled water and 10 % tween 20 for *I. aquatica* and *C. xanthorrhiza* extracts, respectively.

3.10.3 Study design for *Curcuma xanthorrhiza*

Thirty rats were randomly divided into five groups of six animals each. Group 1 (normal control) received intraperitoneal injection of sterile distilled water (1 ml/kg) three times a week for eight weeks and oral administration of tween 20 (10%, 5 ml/kg) daily for eight weeks. Group 2 (cirrhosis control) received intraperitoneal injection of TAA (200 mg/kg) three times a week for eight weeks and oral administration of tween 20 (10%, 5 ml/kg) daily for eight weeks. Group 3 (standard drug control) received intraperitoneal injection of TAA (200 mg/kg) three times a week for eight weeks and oral administration of Silymarin (50 mg/kg) daily for eight weeks. Group 4 (low dose ethanolic extract treated group) received intraperitoneal injection of TAA (200 mg/kg) three times a week for eight weeks and oral administration of *C. xanthorrhiza* (250 mg/kg) daily for eight weeks. Group 5 (high dose ethanol extract treated group) received intraperitoneal injection of TAA (200 mg/kg) three times a week for a period of eight weeks and oral administration of *C. xanthorrhiza* (500 mg/kg) daily for eight weeks.

3.10.4 Study design for *Ipomoea aquatica*

The procedure described for *C. xanthorrhiza* was followed to determine the hepatoprotective activity of *I. aquatica.* Except the group 1 (normal control) received intraperitoneal injection of sterile distilled water (1 ml/kg) three times a week for eight weeks and oral administration of sterile distilled water (5 ml/kg) instead of 10% tween 20 daily for eight weeks.

3.10.5 Analysis of serum biochemical parameters of the liver

Biochemical parameters were analyzed by standard biochemistry automated analyzer at the Central Diagnostic Laboratory of the medical Centre of University Malaya. Blood samples collected from the rats were centrifuged at 3000 rpm for 10 minutes. Then the separated serum samples were used to analyze liver biochemical parameters including

alanine aminotransferase (ALT), alkaline phosphatase (AP), aspartate aminotransferase (AST), total protein, bilirubin and albumin.

3.10.6 Histopathological examination

Histopathological examination of the liver was carried out by means of light microscope. Liver was fixed in 10 % buffered formaldehyde subsequently processed by programmed tissue processing machine and after that embedded in paraffin wax. Following embedding a section of 5 μm thick was prepared and stained with hematoxylin-eosin (HE) and Masson–Trichrome (MT) for photomicroscopic assessment (Al-Bayaty, *et al.*, 2010).

3.11 Alpha smooth muscle actin study

For alpha smooth muscle actin (α-SMA) studies, the sections were mounted on glass slides coated with 0.1% poly-l-lysine. After deparaffination, and subsequent blockage of the endogenous peroxidase activity by incubation in 2.5% methanolic hydrogen peroxide (30 min), the endogenous biotin was blocked by Biotin Blocking. The sections were then washed three times in phosphate-buffered saline (PBS). Mouse monoclonal anti-alpha-SMA antibody diluted 1:40 was used as primary antibody. The sections were incubated for 1 h at room temperature. After three washings in PBS, the sections were incubated for 30 min with the appropriate secondary biotinylated antibody labelled with the Avidin-Biotin Complex. The sections were developed with 3-3_ diaminobenzidine and finally counterstained with haematoxylin. Negative controls were performed using normal mouse antiserum instead of the primary antibody, which uniformly demonstrated no reaction.

3.12 Evaluation of SOD, CAT and MDA contents in liver homogenate

Superoxide dismutase (SOD), catalase (CAT) and Malondialdehyde level (MDA) was analyzed from liver homogenate sample. The Liver sample was rinsed with saline and then homogenized (10 % w/v) in 50 mM cold potassium phosphate buffer (pH 7.4) subsequently centrifuged at 3500 rpm for 10 minutes at 4°C. Then the supernatant was separated and saved for MDA analysis as per thiobarbituric acid procedure (Yasuda, *et al.*, 2003), which is an indicator for lipid peroxidation level. SOD and CAT were analyzed using SOD and CAT assay kits from Cayman Chemical Company, USA.

3.13 Determination of cytokines

Tumor necrosis factor-α (TNF-α) and Transforming growth factor beta 1 (TGFβ1) were analyzed from serum sample whereas, nuclear factor-κB (NF-κB) was analyzed from liver homogenate sample. Collected blood samples from the rats were centrifuged at 3000 rpm for 10 minutes. Then the separated serum samples were used to analyze TNF alpha and TG-Fβ1 by using Quantikine's TNF alpha and TG-Fβ 1 assay kits. Tissue for the assay of NFκ-β was minced in very small pieces and then homogenized in 10 ml of PBS. The resulting suspensions were exposed to two freeze-thaw cycles to completely break the cell membranes. The homogenates were centrifugated for 5 minutes at 5000×g. Then the supernatant was separated and used to analyze NF-κB by using Uscnk assay kit.

3.14 Fractionation of ethanol crude extracts

Five grams of the crude ethanol extract of each plant was dissolved in a minimum amount of acetone solvent. The concentrated crude solution in acetone was mixed with 10 grams of silica gel and followed by drying using rotary evaporator to form powder sample. Each of the obtained powdered samples was subjected to column chromatography (5.0 cm x 30 cm) containing 100 grams silica gel. The amount of silica

gel G60, 70 - 230 mesh (Merck, Darmstadt, Germany) was based on the ratio of 1 g of crude extract to 20 g of silica gel. The fractionation step was based on gradient elution method and the solvent system used was hexane- ethyl acetate eluted stepwise with gradient concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 %. The fractions were collected in clean tubes every 100 ml of eluent. Based on the similarity of spots on thin layer chromatography (TLC), the eluents were pooled to give 10 and 13 major fractions of *curcuma xanthorrhiza* and *ipomoea aquatica*, respectively. The fractions were dried using rotary evaporator, dissolved with minimum amount of acetone and were transferred to test tubes. The samples were dried using Centrivap.

3.15 Hepatoprotective effect of fractions isolated from both plants against TAA cytotoxicity

Hepatoprotective of all fractions isolated from *C. xanthorrhiza* and *I. aquatica* crude extracts were carried out in cell line. Normal human liver embryo cells (WRL-68) were seeded in 200 µl of RPMI containing 10% FBS with density 3000 cells/ well and incubated at 37^{o}C and 5% CO_2 for a day. After 24 hours, using 2.5% DMSO, 10 µg/ml media of both plant isolated fractions were prepared. The prepared fractions were added to the cells as per the designed experiment (Table 3.3and 3.4) and incubated for 2 hours. The cells were then treated with 10 µl of 0.04g/ml TAA. Normal control cells was kept with media only, toxin control was kept with 0.04 g.ml TAA only and standard drug control (silymarin) was kept with TAA then silymarin at the concentrations 10 µg/ml (Sarkar & Sil, 2007). The cells were incubated for 2 hours and the hepatoprotective effect of isolated fractions were determined by cell viability using MTT assay as described by (Mosmann, 1983; Scudiero, *et al.*, 1988). The assay was carried out in three separate experiments.

Table 3.3: The design of *in vitro* hepatoprotective screening of *C. xanthorrhiza* isolated fractions

Group Name	TAA 0.04g/ml	Treatment 10µl/ml
Normal control	No TAA (10µl medium)	DMSO 10µl
TAA control	10µl	DMSO 10µl
Silymarin control	10µl	Silymarin 10µl
C. xanthorrhiza F1	10µl	Fraction 1 10µl
C. xanthorrhiza F2	10µl	Fraction 2 10µl
C. xanthorrhiza F3	10µl	Fraction 3 10µl
C. xanthorrhiza F4	10µl	Fraction 4 10µl
C. xanthorrhiza F5	10µl	Fraction 5 10µl
C. xanthorrhiza F6	10µl	Fraction 6 10µl
C. xanthorrhiza F7	10µl	Fraction 7 10µl
C. xanthorrhiza F8	10µl	Fraction 8 10µl
C. xanthorrhiza F9	10µl	Fraction 9 10µl
C. xanthorrhiza F10	10µl	Fraction 10 10µl

Table 3.4: The design of *in vitro* hepatoprotective screening of *I. aquatica* isolated fractions

Group Name	TAA 0.04g/ml	Treatment 10µl/ml
Normal control	No TAA (10µl medium)	DMSO 10µl
TAA control	10µl	DMSO 10µl
Silymarin control	10µl	Silymarin 10µl
I. aquatica F1	10µl	Fraction 1 10µl
I. aquatica F2	10µl	Fraction 2 10µl
I. aquatica F3	10µl	Fraction 3 10µl
I. aquatica F4	10µl	Fraction 4 10µl
I. aquatica F5	10µl	Fraction 5 10µl
I. aquatica F6	10µl	Fraction 6 10µl
I. aquatica F7	10µl	Fraction 7 10µl
I. aquatica F8	10µl	Fraction 8 10µl
I. aquatica F9	10µl	Fraction 9 10µl
I. aquatica F10	10µl	Fraction 10 10µl
I. aquatica F11	10µl	Fraction 11 10µl
I. aquatica F12	10µl	Fraction 12 10µl
I. aquatica F13	10µl	Fraction 13 10µl

3.16 Antioxidants of the cell line treated with the active fractions

From each plant one isolated fraction which showed best hepatoprotective and prevented the oxidative damage were tested for their antioxidant activities. The antioxidant activity was measured using the following kits: superoxide dismutase (SOD) assay kit (Cayman Chemical Company, USA), catalase (CAT) assay kit

(Cayman Chemical Company, USA) and molandialdehyde (MDA) assay kit (Cayman Chemical Company according to the manufacturer's instructions. Briefly, to determine the activity of SOD, CAT and lipid peroxidation (MDA), WRL-68 cells were seeded in 200 µl of RPMI containing 10% FBS with density 3000 cells/ well and incubated at 37^{o}C and 5% CO_2 for a day. After 24 hours, using 2.5% DMSO, 10 and 100 µg/ml media of fraction which showed the best hepatoprotective results were prepared and added to the cells as per the designed experiment (Table 3.5) and incubated for 2 hours. The cells were then treated with 10 µl of 0.04g/ml TAA and reincubated for 2 hours. Normal control cells was kept with media only, toxin control was kept with 0.04 g/ml TAA only and standard drug control (silymarin) was kept with TAA then silymarin at the concentrations 10 and 100 µg/ml The cells were then washed three times with PBS pH 7.4 and sonicated on ice and the solution was then centrifuged at 13,000 x g for 15 minutes at 4°C. The obtained supernatants were used to determine intracellular antioxidants in a microplate reader at 440 nm for SOD, 540 nm for CAT and 540 nm for MAD. The assay was carried out in three separate experiments.

Table 3.5: The antioxidant and lipid peroxidation experimental design for cell line treated with the active fractions.

Group Name	TAA 0.04g/ml	Treatment 10µl/ml and 100µl/ml
Normal control	No TAA (10µl medium)	DMSO 10µl
TAA control	10µl	DMSO 10µl
Silymarin control	10µl	Silymarin 10µl
Silymarin control	10µl	Silymarin 100µl
C. xanthorrhiza F5	10µl	Fraction 5 10µl
C. xanthorrhiza F5	10µl	Fraction 5 100µl
I. aquatica F11	10µl	Fraction 5 10µl
I. aquatica F11	10µl	Fraction 5 100µl

3.17 Active constituents

The active constituents of the fractions of *C. xanthorrhiza* and *I. aquatica* ethanol extracts which demonstrated hepatoprotective effects in the cell line experiment were identified by using liquid chromatography mass spectrometry (LCMS). The compounds from active fractions were identified by comparing with mass spectrometry library. The protocol was prepared by the technician at BTS.

Sample preparation for LC-MS

Sample concentration used in analysis was 1ug/uL

For both samples, original sample was dissolved in 100% ACN with 0.1% Formic Acid

Instrument

Agilent 1200 Series HPLC system with capillary pump and degasser, micro-well plate sampler with thermostat, and Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source.

LC parameters

Column Used: Agilent Zorbax SB-C18 (0.5 x 150 mm, 5um). Part no: 5064-8256

Flow rate: 18uL/min from Agilent 1200 series capillary pump (micro flow)

Solvents: 0.1% formic acid in water (A); 90% Acetonitrile in water with 0.1% formic acid (B)

Injection volume: 1uL

Sample analysis: Gradient as shown below,

Time (minutes)	B (%)
Initial	60
5	60
30	100
38	100

3.18 Selection of xanthorrhizol and violaxanthin for further study

From the LCMS results of the fractions which showed hepatoprotective, xanthorrhizol from fraction 5 of *Curcuma xanthorrhiza* and violaxanthin from fraction 11 of *Ipomoea aquatic* were selected for further study. The selection of the compounds was based on the available information from the previous studies which could be used to infer that these compounds may be the main contributor in the hepatoprotective activity of the fraction and plant extract as whole. In a recent study violaxanthin isolated from *I. aquatic* has proved to be an excellent free radical scavenger and efficient inhibitor of lipid peroxidation. It has shown to be lipid peroxidation inhibitor, particularly in the isolated mouse liver cells. In another study, violaxanthin revealed to inhibit activation of nuclear factor kappa b (NF-κB). Nuclear factor kappa b activation is known to trigger the overexpression of proinflammatory genes in the nucleus, thereby initiating the inflammatory processes. On the other hand, xanthorrhizol revealed to have potent antioxidant activity and inhibitor of lipid peroxidation. It has also reported that xanthorrhizol reduce proinflammatory mediators such as tumor necrosis factor-alpha and interleukin-6. Moreover, in a short period study, xanthorrhizol abrogated

hepatotoxicity of cisplatin (an anticancer drug which can produce side effects including hepatotoxicity).

3.19 Hepatoprotective effect of xanthorrhizol and violaxanthin against TAA cytotoxicity

The cells were seeded in 200 µl of RPMI containing 10% FBS with density 3000 cells/ well and incubated at 37^{o}C and 5% CO_2 for a day. After 24 hours, using 2.5% DMSO, 10 µg/ml media of xanthorrhizol and violaxanthin were prepared. The prepared compounds were added to the cells as per the designed experiment (Table 3.6) and incubated for 2 hours. The cells were then treated with 10 µl of 0.04g/ml TAA. Normal control cells was kept with media only, toxin control was kept with 0.04 g.ml TAA only and standard drug control (silymarin) was kept with TAA then silymarin at the concentrations 10 µg/ml. The cells were incubated for 2 hours and the hepatoprotective effects of compounds were determined by cell viability using MTT assay. The assay was carried out in three separate experiments.

Table 3.6: The design of *in vitro* hepatoprotective for cell line treated with xanthorhizol and violaxanthin.

Group Name	TAA 0.04g/ml	Treatment 10µl/ml
Normal control	No TAA (10µl medium)	DMSO 10µl
TAA control	10µl	DMSO 10µl
Silymarin control	10µl	Silymarin 10µl
Xanthorrhizol	10µl	Xanthorrhizol 10µl
Violaxanthin	10µl	Violaxanthin 10µl

3.20 Antioxidant of the cell line treated with xanthorrhizol and violaxanthin

The antioxidant activity was measured using the following kits: superoxide dismutase (SOD) assay kit (Cayman Chemical Company, USA), catalase (CAT) assay kit (Cayman Chemical Company, USA) and molandialdehyde (MDA) assay kit (Cayman Chemical Company according to the manufacturer's instructions. Briefly, to determine the activity of SOD, CAT and lipid peroxidation (MDA), WRL-68 cells were seeded in 200 µl of RPMI containing 10% FBS with density 3000 cells/ well and incubated at 37^{o}C and 5% CO_2 for a day. After 24 hours, using 2.5% DMSO, 10 and 100 µg/ml media of xanthorrhizol and violaxanthin added to the cells as per the designed experiment (Table 3.7) and incubated for 2 hours. The cells were then treated with 10 µl of 0.04g/ml TAA and reincubated for 2 hours. Normal control cells was kept with media only, toxin control was kept with 0.04 g.ml TAA only and standard drug control (silymarin) was kept with TAA then silymarin at the concentrations 10 and 100 µg/ml The cells were then washed three times with PBS pH 7.4 and sonicated on ice and the solution was then centrifuged at 13,000 x g for 15 minutes at 4°C. The obtained supernatants were used to determine intracellular antioxidants in a microplate reader at 440 nm for SOD, 540 nm for CAT and 540 nm for MAD. The assay was carried out in three separate experiments.

Table 3.7: The antioxidant and lipid peroxidation experimental design for cell line treated with xanthorhizol and violaxanthin.

Group Name	TAA 0.04g/ml	Treatment 10µl/ml and 100µl/ml
Normal control	No TAA (10µl medium)	DMSO 10µl
TAA control	10µl	DMSO 10µl
Silymarin control	10µl	Silymarin 10µl
Silymarin control	10µl	Silymarin 100µl
Xanthorrhizol	10µl	Xanthorrhizol 10µl
Xanthorrhizol	10µl	Xanthorrhizol 100µl
Violaxanthin	10µl	Violaxanthin 10µl
Violaxanthin	10µl	Violaxanthin 100µl

3.21 Statistical analysis

All of the values are reported as mean ± S.E.M. The statistical significance of variations between groups was analyzed employing one-way ANOVA pursued by Tukey's Post-Hoc test analysis using SPSS version 18 (SPSS Inc. Chicago, IL, USA) with a value of $p< 0.05$ was regarded significant when compared to the control group.

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RESULTS

4.1 DPPH and ABTS radical scavenging capacity of *C. xanthorrhiza* and *I. aquatica* extracts

Free radical scavenging efficacy of *I. aquatica*, *C. xanthorrhiza* ethanolic extracts and the reference standard drug used in the experiment were evaluated by DPPH and ABTS assays and the results are presented in the Figure 4.1 and 4.2. As shown in the Figure 4.1 and 4.2, all the samples exhibited marked free radical scavenging activity expressed in terms percentage. In both the assays, Ascorbic acid exhibited highest radical scavenging capacity value followed by *C. xanthorrhiza* extract, BHT, silymarin and *I. aquatica* extract. The DPPH radical scavenging capacity of Ascorbic acid was approximately 4 and 1.4 times higher than *I. aquatica* and *C. xanthorrhiza* extracts, respectively. Similarly, the ABTS radical scavenging capacity of Ascorbic acid was 3 and 1.2 times higher than *I. aquatica* and *C. xanthorrhiza* extracts, respectively. However, the DPPH radical scavenging activity of *C. xanthorrhiza* extract was about 2 times higher than silymarin the reference standard drug used in liver cirrhosis experiment. Similarly, the ABTS radical scavenging capacity of *C. xanthorrhiza* extract is higher than silymarin.

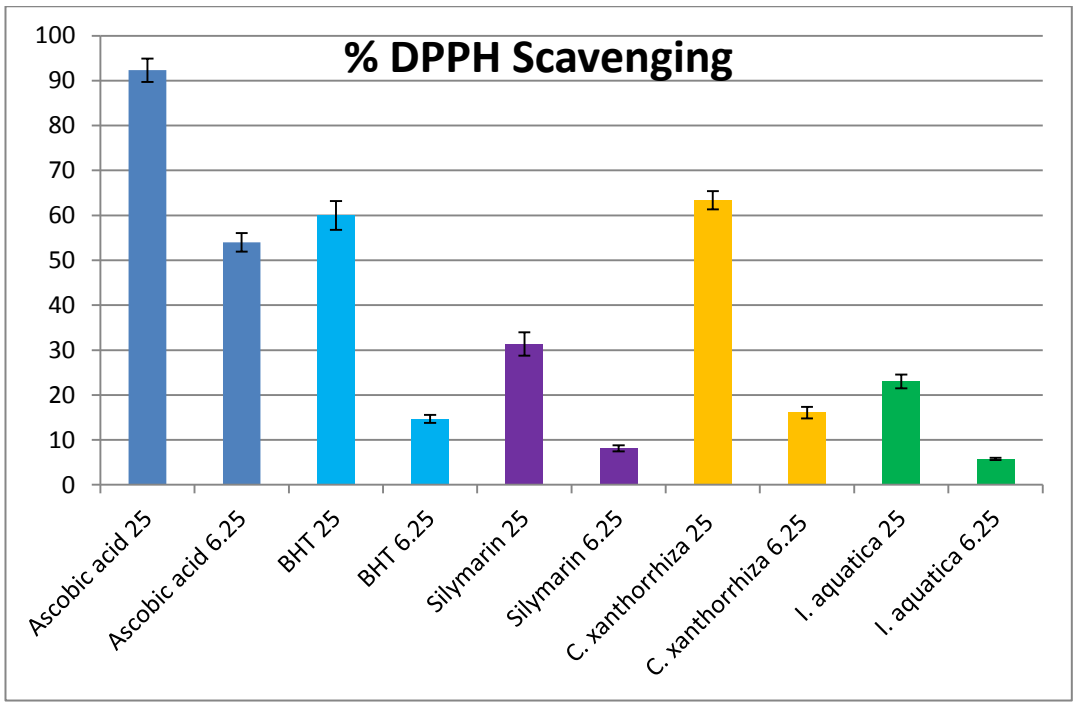

Figure 4.1: DPPH radical scavenging activity of *C. xanthorrhiza* and *I. aqatica* extracts and standards diluted in 25 and 6.25 µg/ml.

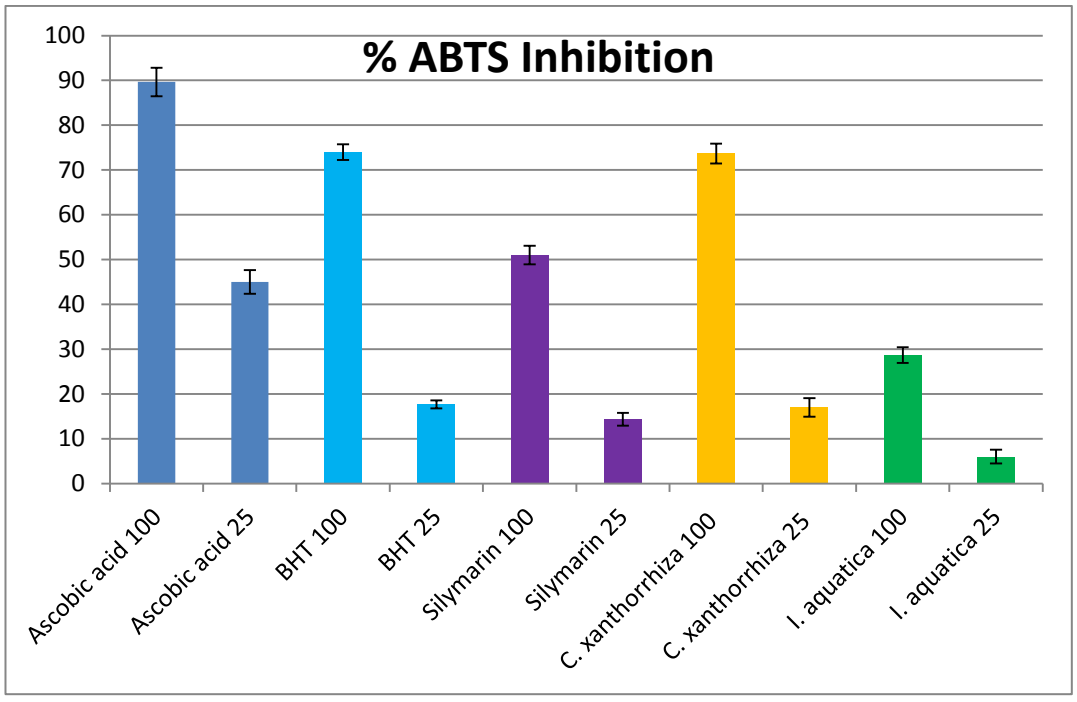

Figure 4.2: ABTS radical scavenging activity of *C. xanthorrhiza* and *I. aqatica* extracts and standards diluted in 100 and 25 µg/ml.

4.2 Selection of TAA dose for hepatotoxicity induction in WRL-68 cells

In order to preselect the suitable concentration of thioacetamide for the hepatotoxicity induction, cytotoxicity assays of thoiacetamide on WRL-68 cells was carried out. A range of TAA concentrations (0.01, 0.02, 0.04, 0.06, 08 and 0.10 g/ml) were tested for WRL-68 cells growth inhibition of approximately fifty percent. Cell growth inhibition quantified by MTT assay was found to be in dose dependent manner. A concentration of 0.04 g/ml was found to inhibit 47.6 percent of WRL-68 cells growth and thus it was selected for the *in vitro* hepatoprotective experiment.

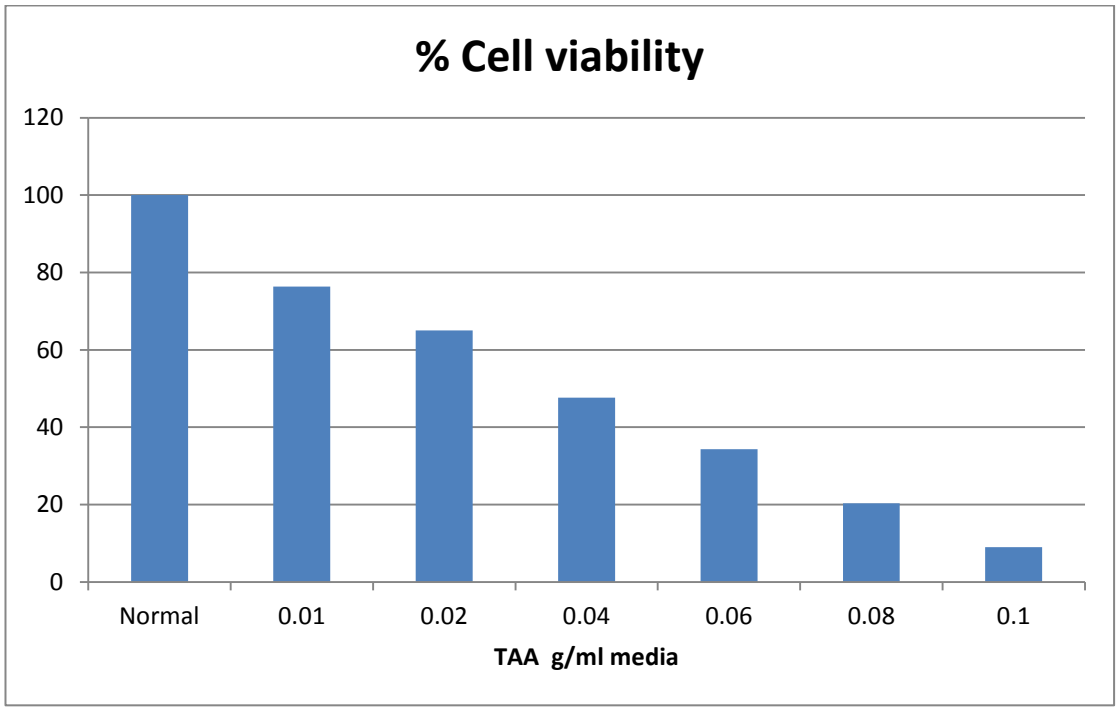

Figure 4.3: Effects TAA on WRL-68 cell viability treated with a range of TAA concentrations.

4.3 *In vitro* hepatoprotective of crude extracts

The result of i*n vitro* hepatoprotective experiment showed that *C. xanthorrhiza* and *I. aquatica* crude extracts have the ability to inhibit WRL-68 cell death which induced by cytotoxicity effects of TAA. Thioacetamide induced 47 percent death in TAA control as compared to normal control. Ethanol crude extract of *C. xanthorrhiza* at the dose of 10

ug/ml significantly increased the cell viability to 81 percent. On the other hand, *I. aquatica* ethanol crude extract showed almost the same results as *C. xanthorrhiza. I. aquatica* ethanol crude extract at the dose of 10 ug/ml significantly increased the cell viability to 80 percent. These results show that *C. xanthorrhiza* and *I. aquatica* ethanol crude extracts at the dose of 10 ug/ml significantly decreased cell death that induced by TAA oxidative stress.

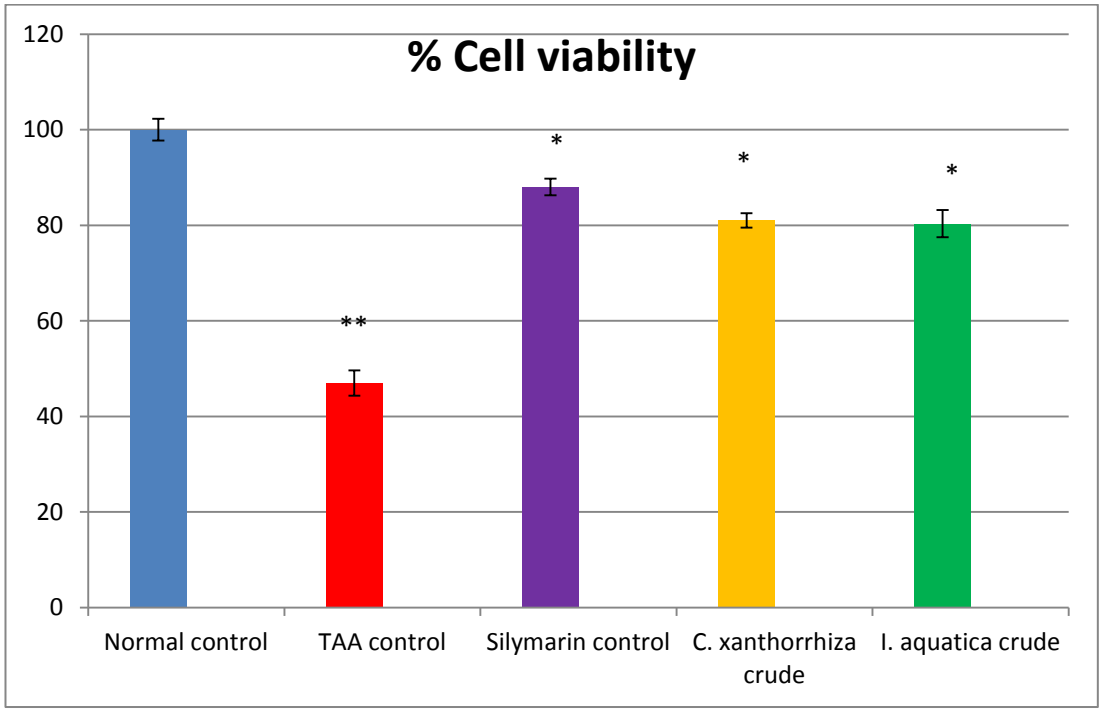

Figure 4.4: Effect of *C. xanthorrhiza* and *I. aquatica* crude extracts on WRL-68 cell viability treated with TAA. Data represent mean ± SEM. *p<0.05, significant compared to TAA control, **p<0.05, significant compared to normal control.

4.4 Antioxidant of WRL-68 cell treated with crude extracts

Antioxidant activities of *C. xanthorrhiza* and *I. aquatica* crude extracts were studeid in WRL-68 normal cell line. The oxidative stress was induced by exposing cells to 0.04g/ml of TAA. The antioxidant activities of *C. xanthorrhiza* and *I. aquatica* crude extracts were found to have a positive correlation with the improvement of the cell viability. It is apparent that TAA bring about the generation of ROS, which reduced the

antioxidant enzymes. Treatment with *C. xanthorrhiza* and *I. aquatica* crude extracts reduced the free radical and improved the antioxidant enzymes levels. The obtained results show that TAA significantly reduced SOD and CAT levels. Treatment with *C. xanthorrhiza* and *I. aquatica* crude extracts significantly improved the levels of these two antioxidant enzymes. At the same time, lipid peroxidation level, determined as MDA generation, significantly increased in TAA control group as compared to normal control group. Treatment with *C. xanthorrhiza* and *I. aquatica* crude extracts significantly prevented lipid peroxidation hence low levels of MDA.

Table 4.1: Effect of *C. xanthorrhiza* and *I. aquatica* crude extracts on SOD, CAT and MDA on WRL-68 cell treated with TAA.

Group Name	SOD U/ml	CAT nmol/min/ml	MDA nmol/ml
Normal control	14.34 ± 0.43	6.03 ± 0.41	13.26 ± 1.03
TAA control	8.048 ± 0.78 **	2.72 ± 0.23 **	35.83 ± 1.72 **
Silymarin control 10	12.39 ± 0.15 *	4.81 ± 0.20 *	18.00 ± 1.00 *
Silymarin control 100	13.80 ± 0.70 *	5.34 ± 0.26 *	16.00 ± 1.15 *
C. xanthorrhiza crude 10	11.91 ± 0.41 *	4.59 ± 0.15 *	23.30 ± 2.12 *
C. xanthorrhiza crude 100	12.23 ± 0.57 *	4.76 ± 0.10. *	20.03 ± 1.76 *
I. aquatica crude 10	12.31 ± 0.42 *	4.42 ± 0.19 *	25.43 ± 0.69 *
I. aquatica crude 100	12.60 ± 0.48 *	4.56 ± 0.20 *	23.40 ± 1.62 *

Data represent mean ± SEM. *$p<0.05$, significant compared to TAA control, **$p<0.05$, significant compared to normal control.

4.5 Acute toxicity study

In the present study, acute oral toxicity of *C. xanthorrhiza* and *I. aquatica* ethanolic extracts were evaluated in male as well as female Sprague-Dawley rats for 14 days. Each group of animal was orally administered with *C. xanthorrhiza* and *I. aquatica* at doses of 2 and 5 g/kg, and were kept under observations for 14 days. The animals did not manifest any significant signs of toxicity and there were no mortality was at any time during the experiment. Histological examination of the kidney and liver tissues showed no difference between extracts treated and control groups (Figure 4.5). Moreover, serum biochemistry data did not reveal any significant differences between extracts treated and control groups (Table 4.2- 4.9).

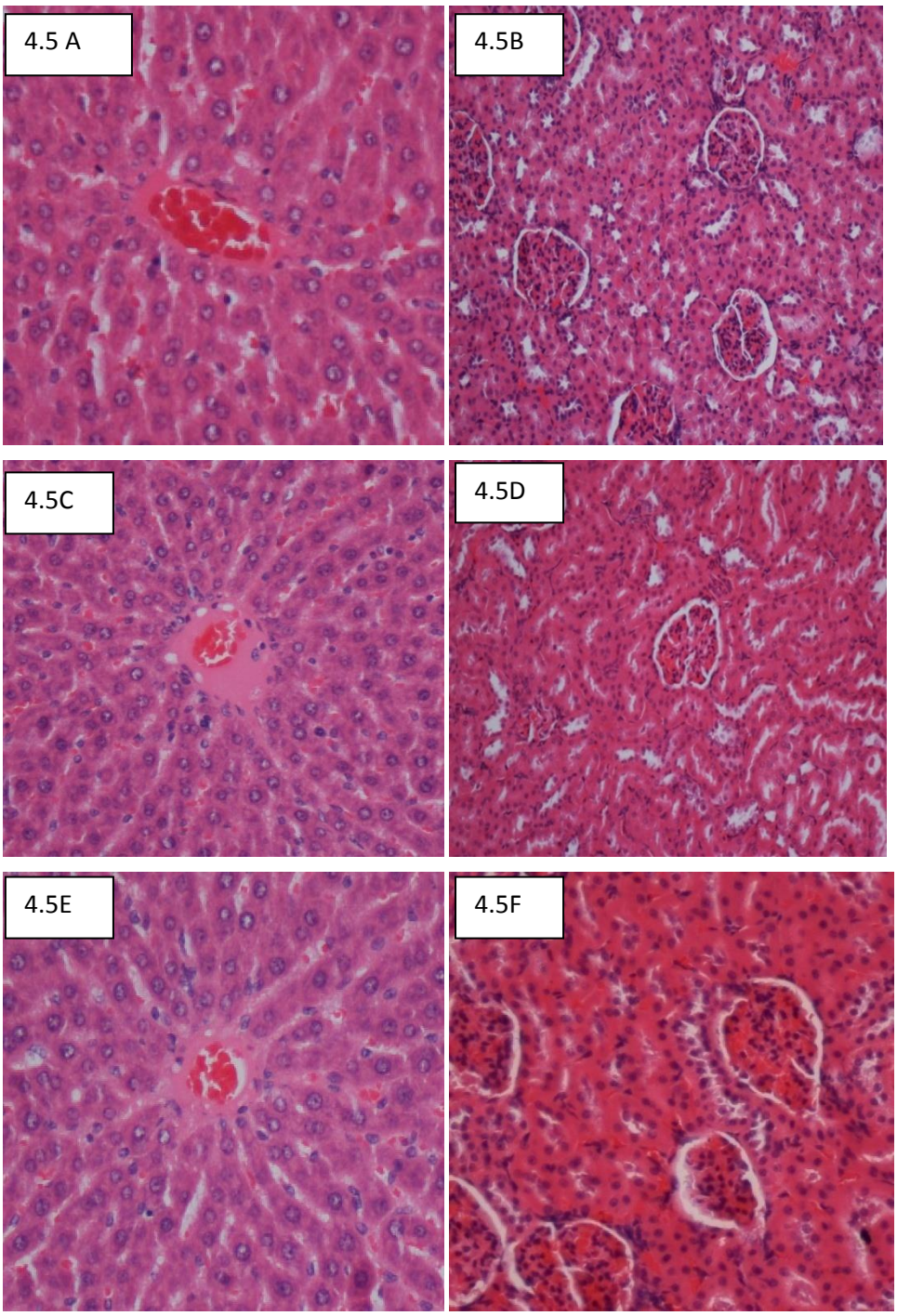

Figure 4.5: Represent acute toxicity histological sections of liver and kidney of different groups. Histological section (4.5A and 4.5B) represent control group (vehicle). Histological section (4.5C and 4.5D) represent animal group received 5g/kg of *C. xanthorrhiza* extract. Histological section (4.5E and 4.5F) represent animal group received 5g/kg of *I. aquatica* extract (H & E stain 20x).

Table 4.2: The results of renal function test of male rats in acute toxicity study of *C. xanthorrhiza* extract.

Dose	**Sodium (mmol/l)**	**Potassium (mmol/l)**	**Chloride (mmol/l)**	**Urea (mmol/l)**	**Creatinine (µmol/l)**
Vehicle (5 ml/kg)	138.80 ± 2.26	4.41 ± 0.20	101.90 ± 2.61	4.61 ± 0.34	50.81 ± 4.61
CXLD (2 g/kg)	137.00 ± 2.69	4.10 ± 0.22	102.36 ± 2.12	4.48 ± 0.33	46.55 ± 5.27
CXHD (5 g/kg)	135.95 ± 3.56	4.23 ± 0.25	104.10 ± 1.91	4.53 ± 0.38	49.46 ± 4.33

Values are expressed as mean ± S.E.M (n=6 rats/group) significant at $p<0.05$. There are no differences between treated groups and control group. CXLD: *C. xanthorrhiza* low dose, CXHD: *C. xanthorrhiza* high dose

Table 4.3: The results of liver function test of male rats in acute toxicity study of *C. xanthorrhiza* extract.

Dose	**Total protein (g/L)**	**Albumin (g/L)**	**TB (mg/dl)**	**AP (IU/L)**	**ALT (IU/L)**	**AST (IU/L)**
Vehicle (5 ml/kg)	69.75 ± 2.97	27.06 ± 1.91	1.39 ± 0.08	77.85 ± 6.85	43.20 ± 3.77	66.11 ± 3.13
CXLD (2 g/kg)	71.36 ± 3.04	28.33 ± 1.81	1.35 ± 0.09	74.31 ± 5.56	42.50 ± 4.04	68.98 ± 4.91
CXHD (5 g/kg)	68.05 ± 2.96	26.66 ± 1.63	1.25 ± 0.06	79.65 ± 8.63	45.00 ± 3.67	64.83 ± 6.26

Values are expressed as mean ± S.E.M (n=6 rats/group) significant at $p<0.05$. There are no differences between treated groups and control group. CXLD: *C. xanthorrhiza* low dose, CXHD: *C. xanthorrhiza* high dose. TB: total protein, AP: alkaline phosphatase, ALT: alanine aminotransferase and AST: aspartate aminotransferase.

Table 4.4: The results of renal function test of female rats in acute toxicity study of *C. xanthorrhiza* extract.

Dose	**Sodium (mmol/l)**	**Potassium (mmol/l)**	**Chloride (mmol/l)**	**Urea (mmol/l)**	**Creatinine (µmol/l)**
Vehicle(5 ml/kg)	136.38 ± 1.70	4.46 ± 0.62	101.56 ± 3.65	4.55 ± 0.44	45.58 ± 5.42
CXLD (2 g/kg)	137.05 ± 2.59	4.73 ± 0.50	104.31 ± 3.53	4.25 ± 0.45	49.81 ± 4.16
CXHD (5 g/kg)	134.63 ± 2.32	4.30 ± 0.58	102.10 ± 2.20	4.66 ± 0.50	47.03 ± 3.22

Values are expressed as mean ± S.E.M (n=6 rats/group) significant at $p<0.05$ There are no differences between treated groups and control group.
CXLD: *C. xanthorrhiza* low dose, CXHD: *C. xanthorrhiza* high dose

Table 4.5: The results of liver function test of female rats in acute toxicity study of *C. xanthorrhiza* extract

Dose	**Total protein (g/L)**	**Albumin (g/L)**	**TB (mg/dl)**	**AP (IU/L)**	**ALT (IU/L)**	**AST (IU/L)**
Vehicle(5 ml/kg)	68.91 ± 6.16	22.71 ± 2.59	1.81 ± 0.17	68.26 ± 5.40	39.25 ± 3.72	60.85 ± 4.25
CXLD (2 g/kg)	64.38 ± 5.37	24.86 ± 3.00	1.66 ± 0.29	63.51 ± 8.34	37.78 ± 4.30	59.65 ± 6.13
CXHD (5 g/kg)	69.08 ± 4.78	21.18 ± 2.26	1.55 ± 0.20	70.23 ± 4.87	35.26 ± 4.90	65.33 ± 6.52

Values are expressed as mean ± S.E.M (n=6 rats/group) significant at $p<0.05$. There are no differences between treated groups and control group. CXLD: *C. xanthorrhiza* low dose, CXHD: *C. xanthorrhiza* high dose. TB: total protein, AP: alkaline phosphatase, ALT: alanine aminotransferase and AST: aspartate aminotransferase.

Table 4.6: The results of renal function test of male rats in acute toxicity study of *I. aquatica* extract.

Dose	**Sodium (mmol/l)**	**Potassium (mmol/l)**	**Chloride (mmol/l)**	**Urea (mmol/l)**	**Creatinine (µmol/l)**
Vehicle (5 ml/kg)	138.53 ± 2.66	4.30 ± 0.20	102.48 ± 2.92	4.63 ± 0.31	48.08 ± 2.93
IALD (2 g/kg)	137.33 ± 3.28	4.18 ± 0.21	101.43 ± 2.19	4.53 ± 0.28	47.81 ± 4.35
IAHD (5 g/kg)	139.66 ± 315	4.40 ± 0.17	104.16 ± 1.95	4.41 ± 0.29	49.66 ± 3.83

Values are expressed as mean ± S.E.M (n=6 rats/group) significant at $p<0.05$ There are no differences between treated groups and control group.
IALD: *I. aquatica* low dose, IAHD: *I. aquatica* high dose

Table 4.7: The results of liver function test of male rats in acute toxicity study of *I. aquatica* extract

Dose	**Total protein (g/L)**	**Albumin (g/L)**	**TB (mg/dl)**	**AP (IU/L)**	**ALT (IU/L)**	**AST (IU/L)**
Vehicle (5 ml/kg)	69.45 ± 2.22	25.26 ±1.09	1.33 ± 0.07	73.15 ±6.88	42.13 ±2.95	64.63 ± 3.38
IALD (2 g/kg)	71.01 ± 3.26	26.16 ±1.32	1.29 ± 0.06	74.03 ±4.42	41.73 ±3.63	65.46 ± 4.86
IAHD (5 g/kg)	68.05 ± 2.65	24.26 ±1.01	1.32 ± 0.05	76.30 ±4.81	43.35 ±3.34	62.33 ± 7.45

Values are expressed as mean ± S.E.M (n=6 rats/group) significant at $p<0.05$. There are no differences between treated groups and control group. IALD: *I. aquatica* low dose, IAHD: *I. aquatica* high dose. TB: total protein, AP: alkaline phosphatase, ALT: alanine aminotransferase and AST: aspartate aminotransferase.

Table 4.8: The results of renal function test of female rats in acute toxicity study of *I. aquatica* extract

Dose	**Sodium (mmol/l)**	**Potassium (mmol/l)**	**Chloride (mmol/l)**	**Urea (mmol/l)**	**Creatinine (µmol/l)**
Vehicle(5 ml/kg)	137.31± 2.58	4.28 ±0.21	101.28 ±2.14	4.61 ± 0.39	47.01 ± 3.53
IALD (2 g/kg)	135.48± 2.33	4.20±0.26	102.70±1.18	4.25 ± 0.38	46.66 ± 4.53
IAHD (5 g/kg)	138.33± 2.29	3.98±0.23	103.05±2.16	4.48 ± 0.42	48.11 ± 5.21

Values are expressed as mean ± S.E.M (n=6 rats/group) significant at $p<0.05$. There are no differences between treated groups and control group. IALD: *I. aquatica* low dose, IAHD: *I. aquatica* high dose.

Table 4.9: The results of liver function test of female rats in acute toxicity study of *I. aquatica* extract

Dose	**Total protein (g/L)**	**Albumin (g/L)**	**TB (mg/dl)**	**AP (IU/L)**	**ALT (IU/L)**	**AST (IU/L)**
Vehicle (5 ml/kg)	69.31 ± 2.77	25.25 ± 1.33	1.25 ± 0.04	75.43 ± 5.32	39.35 ± 3.54	65.63 ± 3.58
IALD (2 g/kg)	70.73 ± 2.43	24.28 ± 1.32	1.20 ± 0.05	74.38 ± 4.83	38.45 ± 2.69	63.73 ± 4.04
IAHD (5 g/kg)	68.68 ± 3.11	23.43 ± 1.02	1.19 ± 0.05	77.01 ± 3.89	41.13 ± 3.84	62.26 ± 6.08

Values are expressed as mean ± S.E.M (n=6 rats/group) significant at $p<0.05$. There are no differences between treated groups and control group. IALD: *I. aquatica* low dose, IAHD: *I. aquatica* high dose. TB: total protein, AP: alkaline phosphatase, ALT: alanine aminotransferase and AST: aspartate aminotransferase.

4.6 Hepatoprotective study of the crude extracts

4.6.1 Liver index measurement

Normal growth of the experimental animal was evaluated by measuring the initial and final body weight, which was also useful to determine liver index [Liver weight (LW)/body weight (BW) %]. Initially prior to experiment, animal weighed between 195 to 215 g of the average body weight. After eight weeks of treatment the body weight of *C. xanthorrhiza* extract low dose group, high dose group, silymarin group and normal control group increased to reach 274, 281, 288 and 321, respectively (Table 4.10). As shown in Table 4.11, the body weight of *I. aquatica* extract low dose group, high dose group, silymarin group and normal control group increased to reach 276, 282, 287 and 308, respectively. However, the cirrhosis control groups showed significantly retardation in the body weight growth (Table 4.10 and 4.11). Liver weight in proportion to the body weight was estimated by calculating the liver index [Liver weight (LW)/body weight (BW) %]. Liver index showed significant differences ($p<0.05$) between the drug treated groups compared to cirrhosis control groups (Table 4.10 and 4.11). TAA induced toxicity altered the normal growth patten (body weight and liver weight) of the animals. Where as, *C. xanthorrhiza* and *I. aquatica* extracts and silymarin treatments improved the the normal animal growth patten.

Table 4.10: Effect of *C. xanthorrhiza* extract on the liver index of experimental rats.

Group	Body weight (gm)	Liver weight (gm)	Liver index (LW/BW %)
Normal control (10% Tween 20)	321 ± 22.17	8.53 ± 0.41	2.68 ± 0.13
Cirrhosis control (TAA)	196 ± 15.12**	9.43 ± 0.15	4.95 ± 0.41**
Silymarin control (50 mg/kg)	288 ± 17.47*	8.44 ± 0.59	2.93 ± 0.14*
C. xanthorrhiza (250 mg/kg)	274 ± 16.54*	9.01 ± 0.48	3.31 ± 0.18*
C. xanthorrhiza (500 mg/kg)	281 ± 19.23*	8.66 ± 0.49	3.12 ± 0.20*

Data represent mean ± SEM. *p<0.05, significant compared to cirrhosis control group, **p<0.05, significant compared to normal control group.

Table 4.11: Effect of *I. aquatica* extract on the liver index of experimental rats.

Group	Body weight (gram)	Liver weight (gram)	Liver index (LW/BW %)
Normal control (Distilled water)	308 ± 21.76	7.85 ± 0.58	2.55 ± 0.11
Cirrhosis control (TAA)	201 ± 16.48**	10.19 ± 0.98	5.04 ± 0.20**
Silymarin control (50 mg/kg)	287 ± 15.85*	8.98 ± 0.64	3.12 ± 0.14*
I. aquatica (250 mg/kg)	276 ± 17.55*	9.83 ± 0.87	3.53 ± 0.15*
I. aquatica (500 mg/kg)	282 ± 17.31*	8.56 ± 0.65	3.02 ± 0.12*

Data represent mean ± SEM. *p<0.05, significant compared to cirrhosis control group, **p<0.05, significant compared to normal control group.

4.6.2 Results of serum biochemical parameters of the liver

The effects of ethanolic extracts of *C. xanthorrhiza* and *I. aquatica* on TAA induced liver cirrhosis in rats was determined by measuring serum levels of specific liver biochemical parameters (Figure 4.6- 4.11). There were significant ($p< 0.05$) increase in serum ALP, AST and ALT in TAA treated rats, which were 3-4 times higher compared to normal control and silymarin and also *C. xanthorrhiza* and *I. aquatica* treatments (Figure 4.6 and 4.7). The observed a significant increase in serum ALP, AST and ALT due to the intoxication by TAA was restored by treatment with *C. xanthorrhiza* as well *I. aquatica* in a dose-dependent manner. As shown in Figure 4.4 and 4.5, *C. xanthorrhiza* and *I. aquatica* extracts at higher dose of 500 mg/kg restored serum levels of ALP, AST and ALT, which were comparable to silymarin control groups (50 mg/kg). Also, the exhibited result was better than their respective lower dose (250 mg/kg).

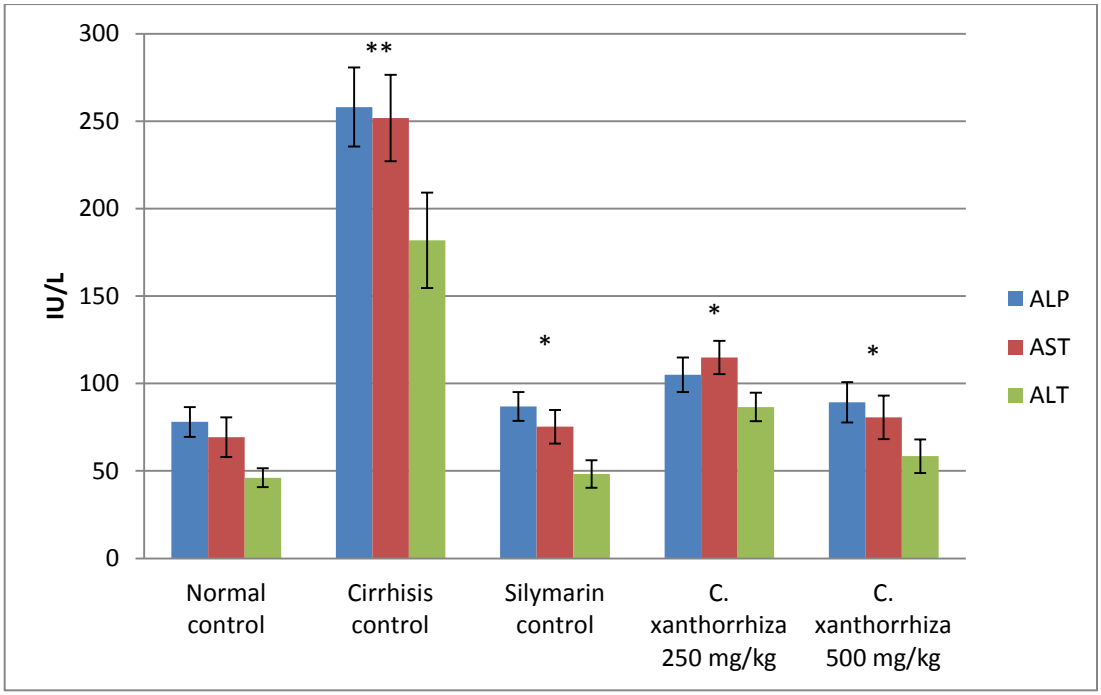

Figure 4.6: Effect of *C. xanthorrhiza* extract on serum liver enzymes of experimental rats. Data represent mean ± SEM. *p<0.05, significant compared to cirrhosis control group, **p<0.05, significant compared to normal control group.
ALP alkaline phosphatase; AST aspartate transferase; ALT alanine transferase.

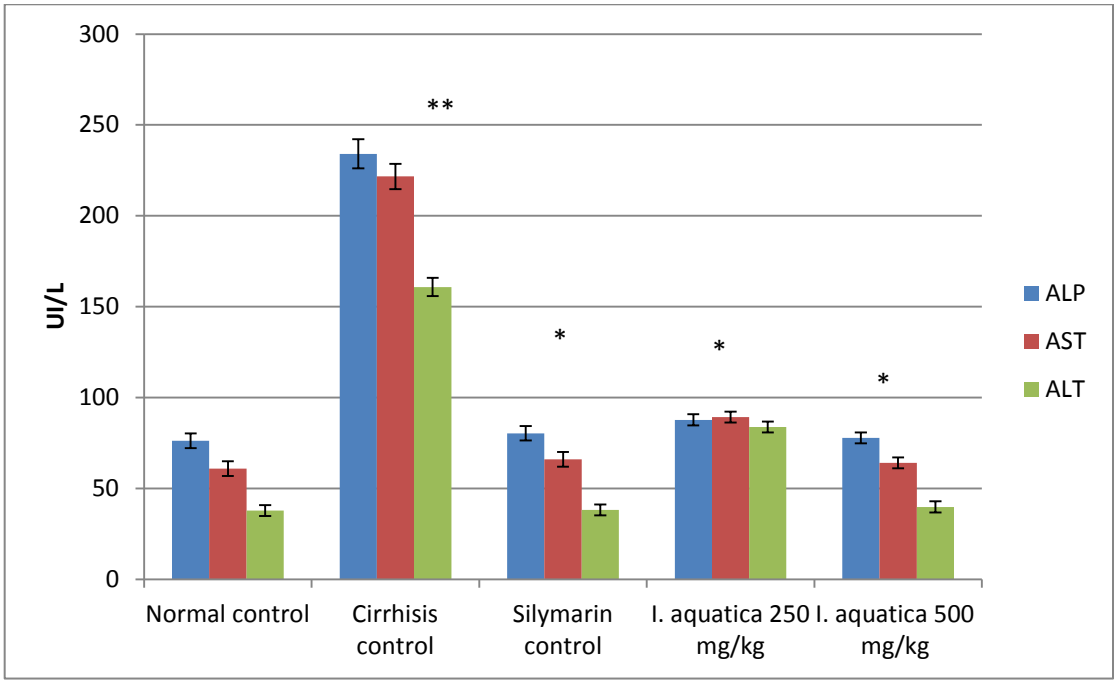

Figure 4.7: Effect of *I. aquatica* extract on serum liver enzymes of experimental rats. Data represent mean ± SEM. *p<0.05, significant compared to cirrhosis control group, **p<0.05, significant compared to normal control group.
ALP alkaline phosphatase; AST aspartate transferase; ALT alanine transferase.

As shown in Figure 4.8 and 4.9, long-term administration of TAA (cirrhosis control group) significantly decreased the total protein and albumin level compared to normal control group, indicating the occurrence of acute liver damage. However, treatment of animals with silymarin, *C. xanthorrhiza* and *I. aquatica* extracts increased the levels of total protein and albumin toward normal control group. Both these plant extracts at a dose of 500 mg/kg significantly restored the altered liver parameters and the effect was more resemble to that of standard drug silymarin.

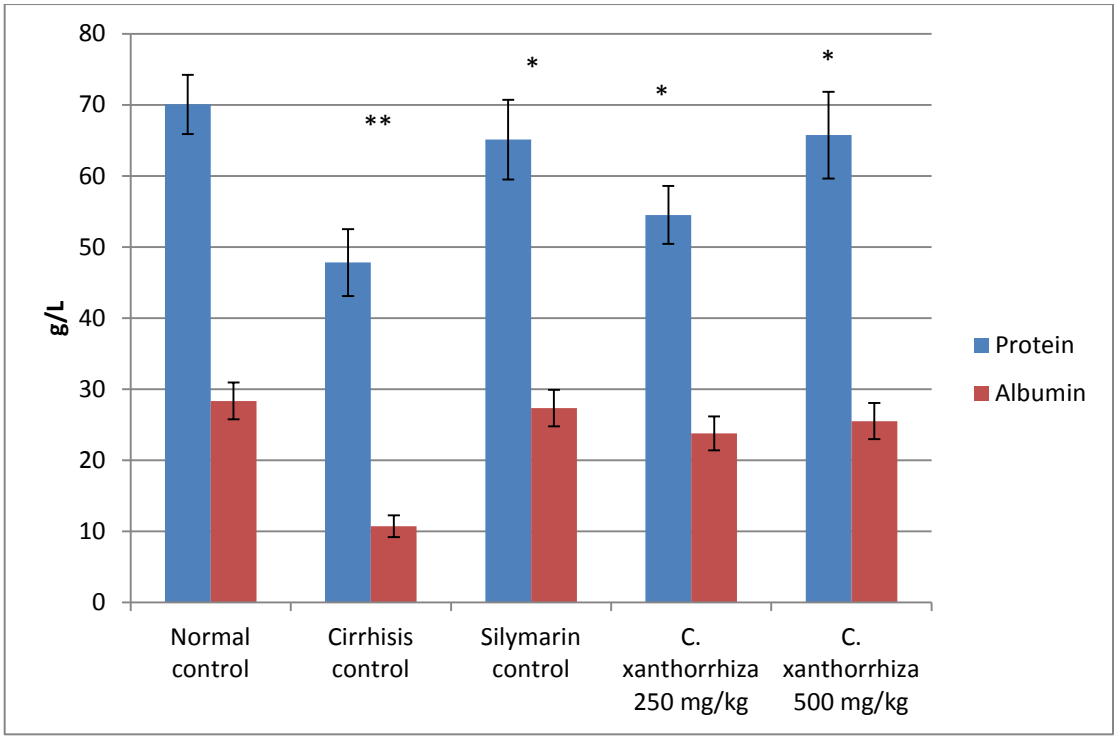

Figure 4.8: Effect of *C. xanthorrhiza* extract on serum liver biomarkers of experimental rats. Data represent mean ± SEM. *p<0.05, significant compared to cirrhosis control group, **p<0.05, significant compared to normal control group.

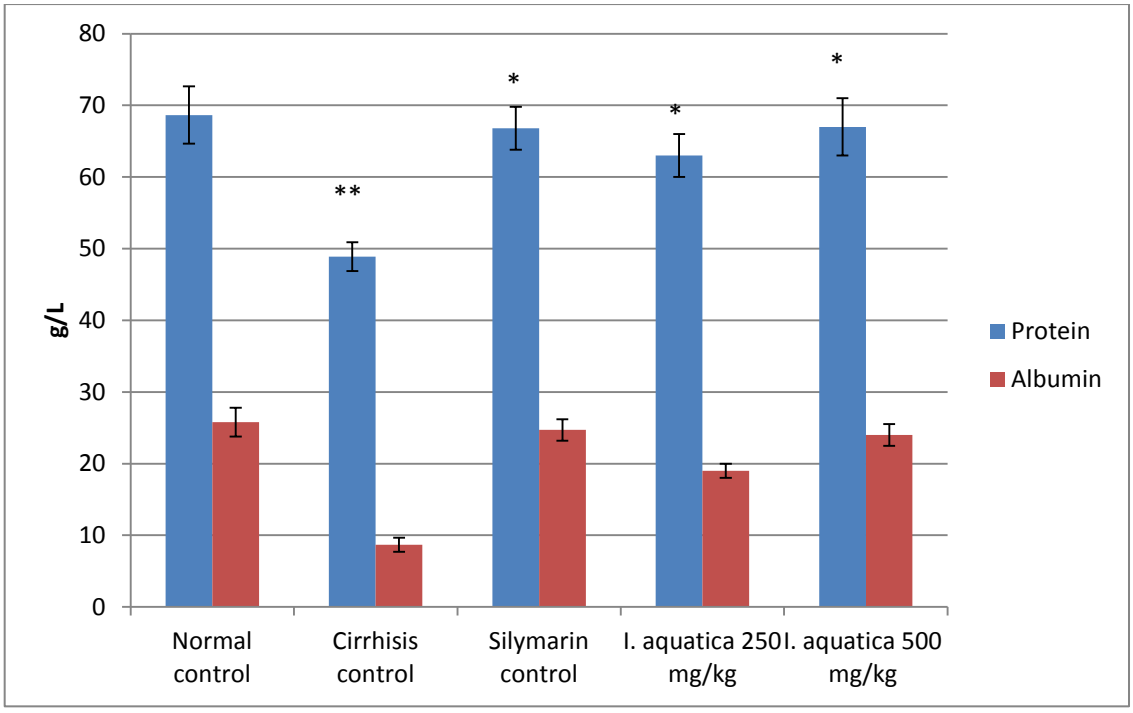

Figure 4.9: Effect of *I. aquatica* extract on serum liver biomarkers of experimental rats. Data represent mean ± SEM. *p<0.05, significant compared to cirrhosis control group, **p<0.05, significant compared to normal control group.

Figure 4.10 and 4.11 represents the effect of silymarin, *C. xanthorrhiza* and *I. aquatica* extracts on bilirubin levels in TAA induced liver damage. It is evident from Figure 4.10 and 4.11 that the elevated levels of bilirubin due TAA intoxication were significantly reduced in the animal groups treated with silymarin, *C. xanthorrhiza* and *I. aquatica* extracts. Both extracts exhibited dose dependent decrease in the bilirubin level with *I. aquatica* extract showing superior effect in comparison with *C. xanthorrhiza* extract and was as effective as the silymarin.

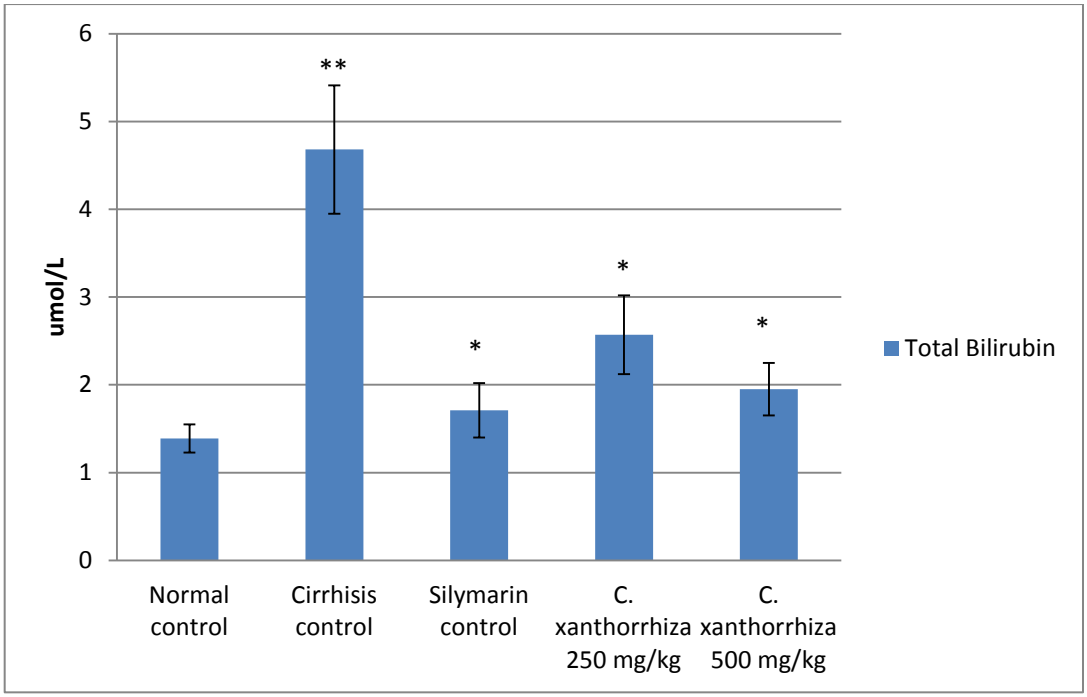

Figure 4.10: Effect of *C. xanthorrhiza* extract on serum total bilirubin of experimental rats. Data represent mean ± SEM. *p<0.05, significant compared to cirrhosis control group, **p<0.05, significant compared to normal control group.

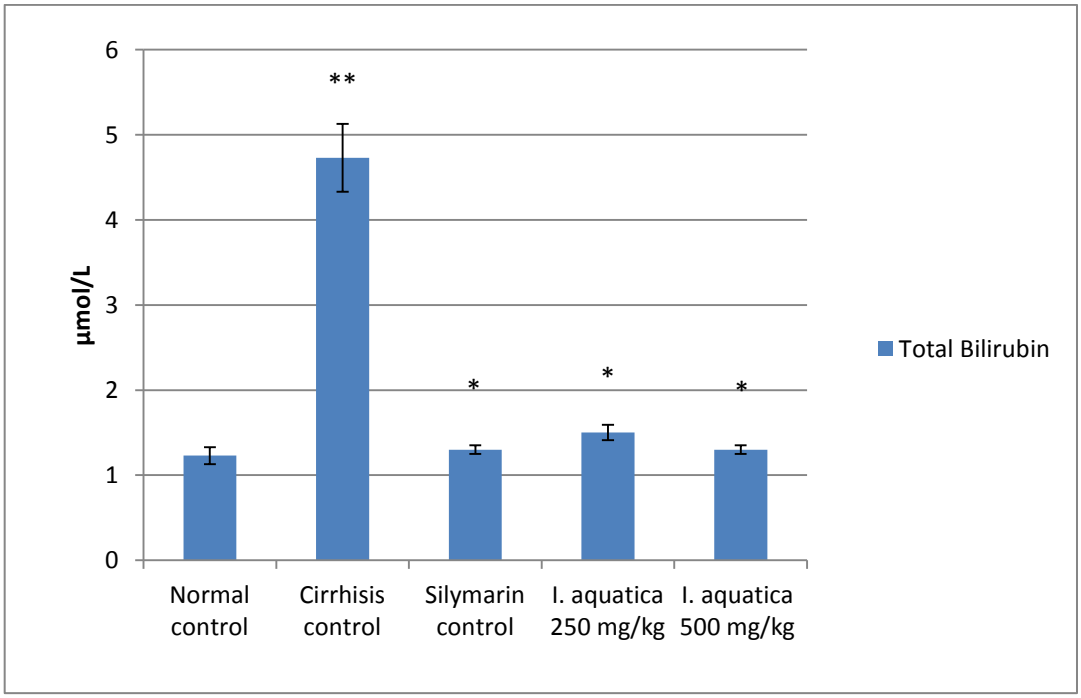

Figure 4.11: Effect of *I. aquatica* extract on serum total bilirubin of experimental rats.

Data represent mean ± SEM. *p<0.05, significant compared to cirrhosis control group, **p<0.05, significant compared to normal control group.

4.6.3 Gross morphology

Macroscopic appearance of rat livers representing different groups of experimental rat model of TAA-induced cirrhosis is presented Figure 4.12 and 4.13. Normal control group (4.12A and 4.13A) shows normal smooth surfaces liver and without any irregularities. The liver of the cirrhosis control group as a result of long-term administration of TAA develops irregular shape and occupied by uniform formations of micronodules and macronodules (4.12B and 4.13B). This kind of nodular transformations is typical signature of the TAA induced liver cirrhosis in the rat which is also seen in human nodular cirrhosis. The treatment of both doses of *C. xanthorrhiza* and *I. aquatica* extract (250 mg/kg and 500 mg/kg) particularly 500 mg/kg (4.12E and 4.13E) and silymarin (4.12C and 4.13C) preserved the anatomical shape and appearance of the liver nearly to normal and prevented the formation of macro- and micronodules.

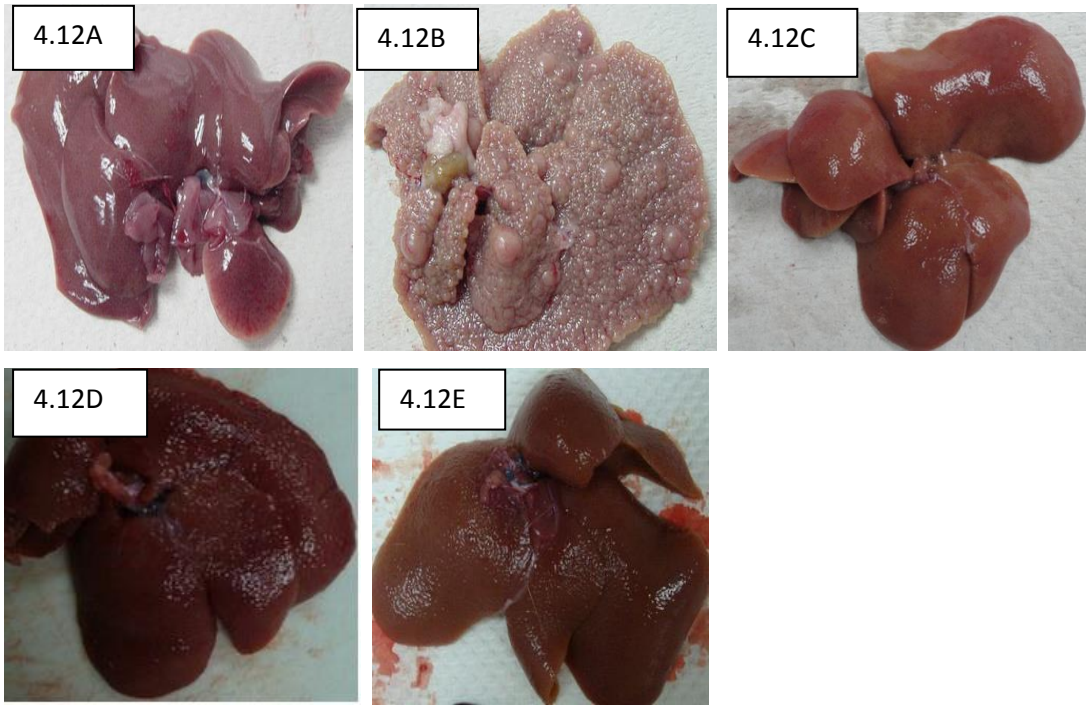

Figure 4.12: Macroscopic appearance of rat livers representing different groups of an experimental model of TAA-induced cirrhosis. The liver of normal control group (4.12A) shows smooth surface of the liver. The liver of cirrhosis control group (4.12B) shows irregular surface and macronodular cirrhosis of the liver. Silymarin contol group (4.12C) shows smooth surface of the liver. (4.12D) The liver of the group 250 mg/kg of *C. xanthorrhiza* extract shows to some extent smooth surface of the liver. (4.12E) The liver of the group received 500 mg/kg of *C. xanthorrhiza* extract shows smooth surface of the liver.

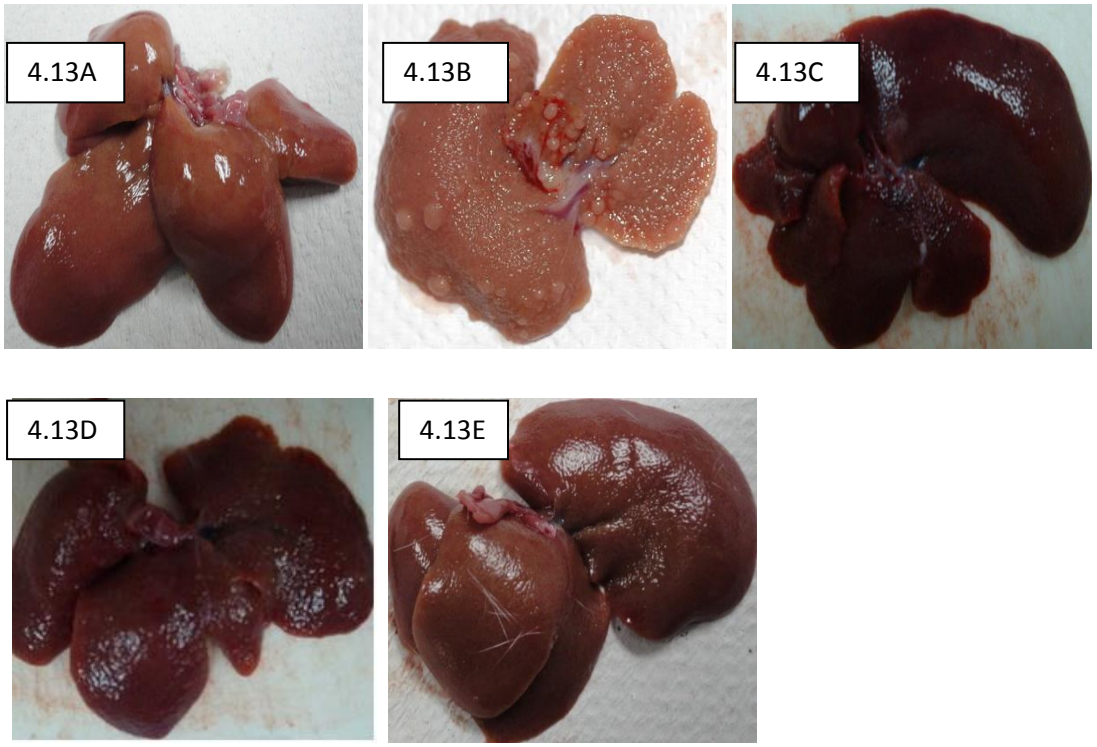

Figure 4.13: Macroscopic appearance of rat livers representing different groups of experimental model of TAA-induced cirrhosis. The liver of normal control group (4.13A) shows smooth surface of the liver. The liver of cirrhosis control group (4.13B) shows irregular surface and macronodular cirrhosis of the liver. Silymarin control group (4.13C) shows smooth surface of the liver. (4.13D). The liver of the group received 250 mg/kg of *I. aquatica* extract shows to some extent smooth surface of the liver. (4.13E) The liver of the group 500 mg/kg of *I. aquatica* extract shows smooth surface of the liver.

4.6.4 Histopathological and special stain examination

Histological studies were carried out to determine the TAA induced liver damage and to determine the hepatoprotective activity of *C. xanthorrhiza* and *I. aquatica* extracts. After eight weeks treatment with TAA alone and in combination with silymarin, lower and higher dose of *C. xanthorrhiza* and *I. aquatica* extracts, liver sections were evaluated for the histological examination using H & E as well as Masson's trichome staining methods. Immunohistochemical staining was used for alpha smooth muscle actin (α-SMA) to determine the extent of hepatic stellate cells activation.

Light micrographs of the rat liver sections representing different groups of experimental rat model of TAA-induced cirrhosis are presented in Figure 14 and 15. The liver section representing normal control group (4.14A and 4.15A) appeared regular with normal cellular architecture and totally free of any kind of pathology. The polygonal hepatic cells display intact cytoplasm, sinusoidal spaces, visible nucleus, nucleolus and central vein. The section of cirrhosis control group (4.14B and 4.15B) confirms liver damage as demonstrated by the presence of inflammation and necrosis. The normal architecture of the liver parenchyma is totally damage with collagen bridges between hepatic triads and the formation of regenerative nodules of hepatocytes or necrosis and fibrotic septa between the nodules. The liver tissue section of silymarin control group (4.14C and 4.15C) shows lesser pathology as compared to the extensive liver damage found in the cirrhosis control group and therefore protected hepatocyte and architecture with smaller section of mild necrosis. (4.14C and 15D) The tissue section of the liver of the group received 250 mg/kg of *C. xanthorrhiza* and *I. aquatica* extract shows partly protected architecture and hepatocyte with smaller section of necrosis and fibrotic septa (4.14E and 15E). The tissue section of the liver of the group received 500 mg/kg of *C. xanthorrhiza* and *I. aquatica* extract shows considerably less pathology when compared with the extensive liver damage found in the cirrhosis control group and therefore protected architecture and hepatocyte with smaller section of mild necrosis (hematoxylin and eosin stain magnification 20x).

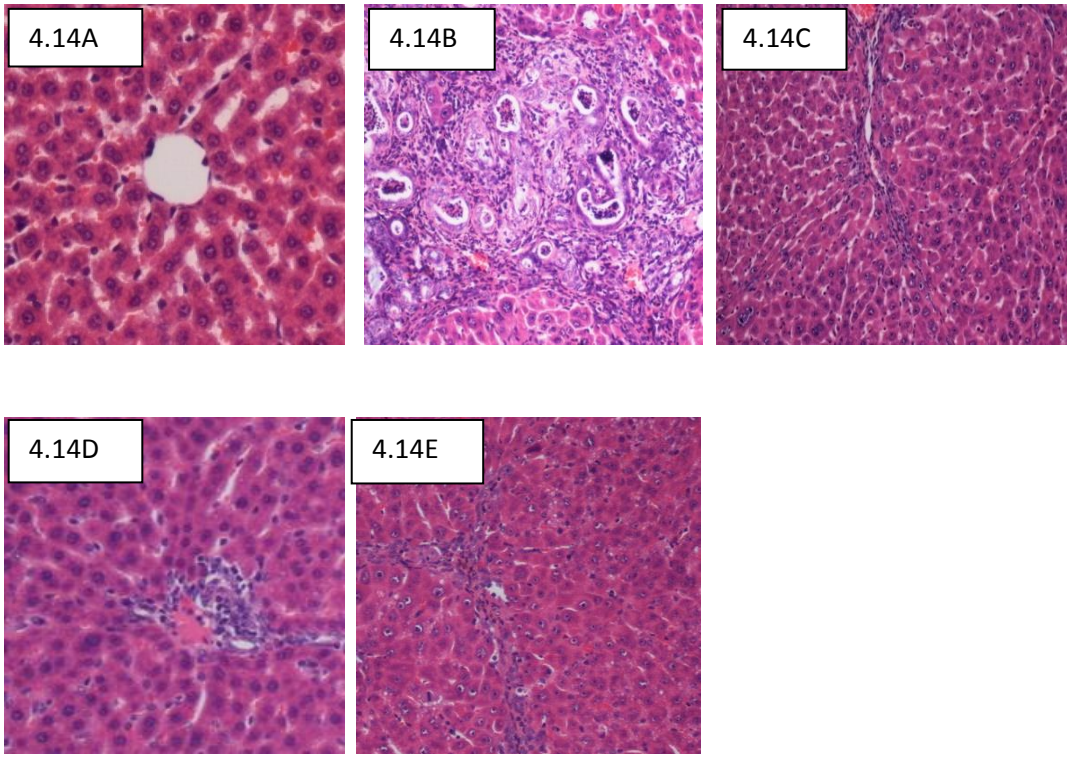

Figure 4.14: Light micrographs of the rat liver sections representing different groups of an experimental model of TAA-induced cirrhosis. The liver tissue section of normal control group (4.14A) shows central vein with cords of normal liver cells radiating from it. The liver tissue section of cirrhosis control group (4.14B) shows severe damage, fibrous bands and nodular liver. The liver tissue section of silymarin control group (4.14C) shows shows partly protected architecture and hepatocyte with smaller section of mild necrosis. (4.14D) The tissue section of the liver of the group received 250 mg/kg of *C. xanthorrhiza* extract shows partly protected architecture and hepatocyte with smaller section of necrosis and fibrotic septa (4.14E). The tissue section of the liver of the group received 500 mg/kg of *C. xanthorrhiza* extract shows partly protected architecture and hepatocyte with smaller section of mild necrosis (hematoxylin and eosin stain magnification 20x).

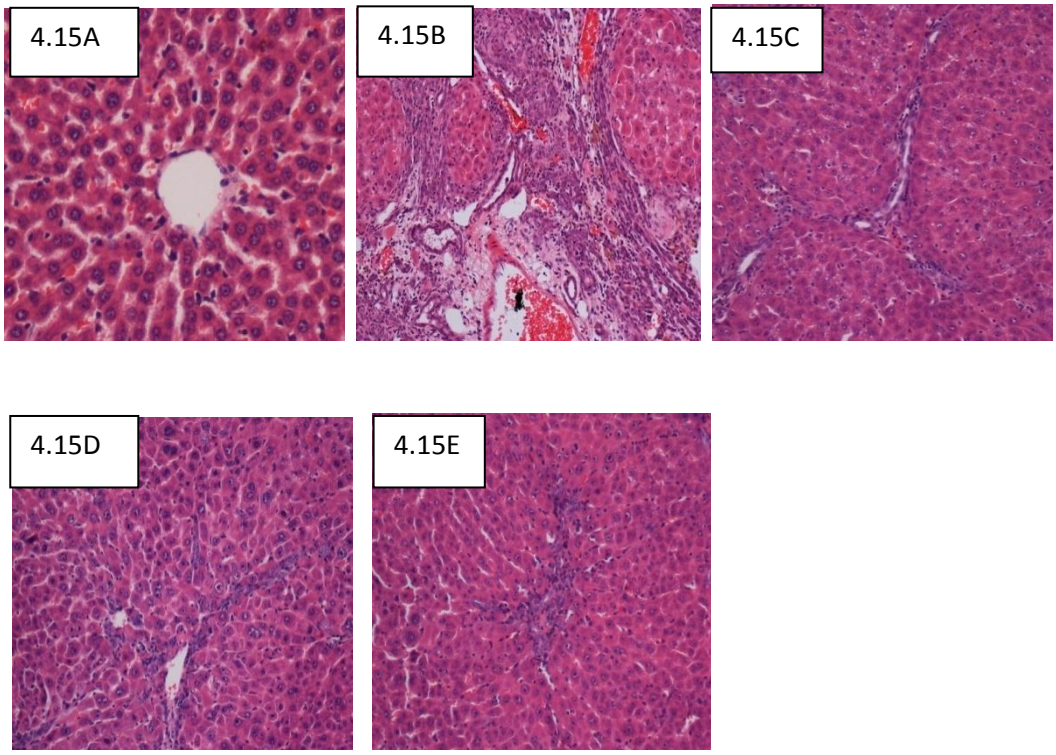

Figure 4.15: Light micrographs of the rat liver sections representing different groups of an experimental model of TAA-induced cirrhosis. The liver tissue section of normal control group (4.15A) shows central vein with cords of normal liver cells radiating from it. The liver tissue section of cirrhosis control group (4.15B) shows severe damage, fibrous bands and nodular liver. The liver tissue section of silymarin control group (4.15C) shows partly protected architecture and hepatocyte with smaller section of mild necrosis. (4.15D) The tissue section of the liver of the group received 250 mg/kg of *I. aquatica* extract shows partly protected architecture and hepatocyte with smaller section of necrosis and fibrotic septa . (4.15E). The tissue section of the liver of the group treated with 500 mg/kg of *I. aquatica* extract shows partly protected architecture and hepatocyte with smaller section of mild necrosis (hematoxylin and eosin stain magnification 20x).

Masson's trichome staining of liver sections was employed to evaluate the deposition of collagen induced by TAA treatment. This staining method normally detects the collagen distribution in the fibrous liver. Figure 4.16 and 4.17 represents the influence of silymarin, lower and higher dose of *C. xanthorrhiza* and *I. aquatica* extracts. Liver sections of normal control group show normal hepatocytes without any collagen deposition (4.16A and 4.17A). Liver section of rats intoxicated with TAA shown

severe liver damage with the apparent collagen fibrous band deposition (4.16B and 4.17B). The hepatohistological changes induced by TAA were dramatically ameliorated by treatment with silymarin, *C. xanthorrhiza* and *I. aquatica* extracts (500 mg/kg) which shown mild collagen deposition. However, plant extracts at the lower dose of 250 mg/kg displayed moderate collagen deposition in the liver Masson's trichome staining method.

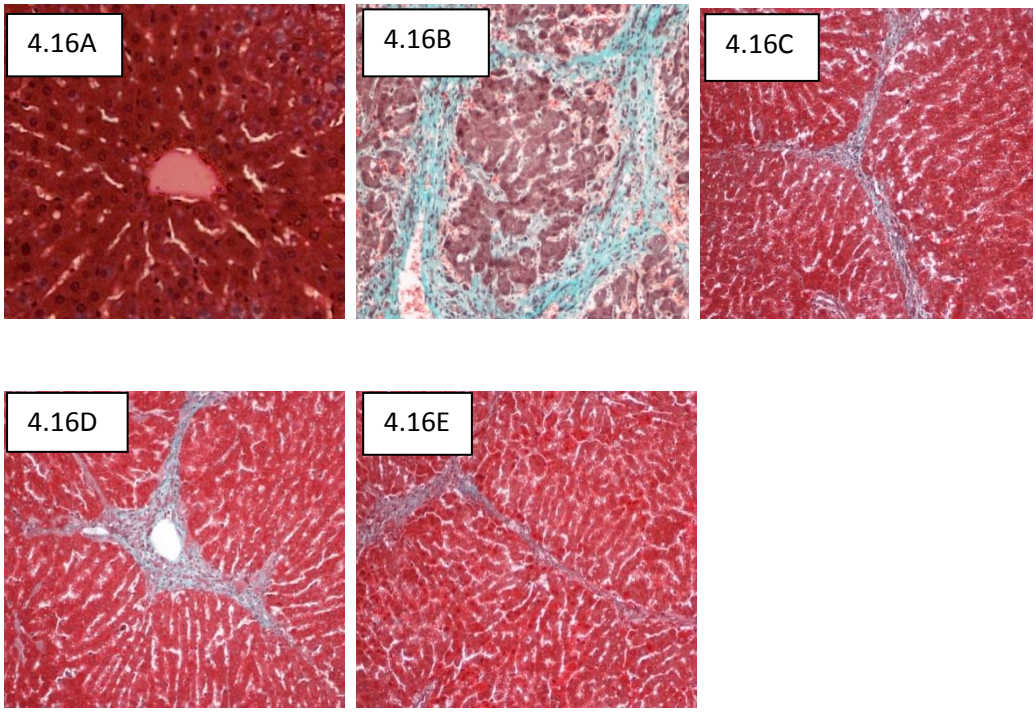

Figure 4.16: Light micrographs of the rat liver sections representing masson's trichrome staining of collagen deposition from different groups of an experimental model of TAA-induced cirrhosis. The liver tissue section of normal control group (4.16A) shows normal hepatocyte and architecture of the liver without any collagen deposition. The liver tissue section of cirrhosis control group (4.16B) shows severe damage and green blue collagen fibrous bands. The liver tissue section of silymarin control group (4.16C) shows mild collagen fibrotic septa deposition. (4.16D) The tissue section of the liver of the group treated with 250 mg/kg of *C. xanthorrhiza* extract shows moderate collagen fibrotic septa. (4.16E) The tissue section of the liver of the group received 500 mg/kg of *C. xanthorrhiza* extract shows mild collagen fibrotic septa deposition (Masson's trichrome stain magnification 20x).

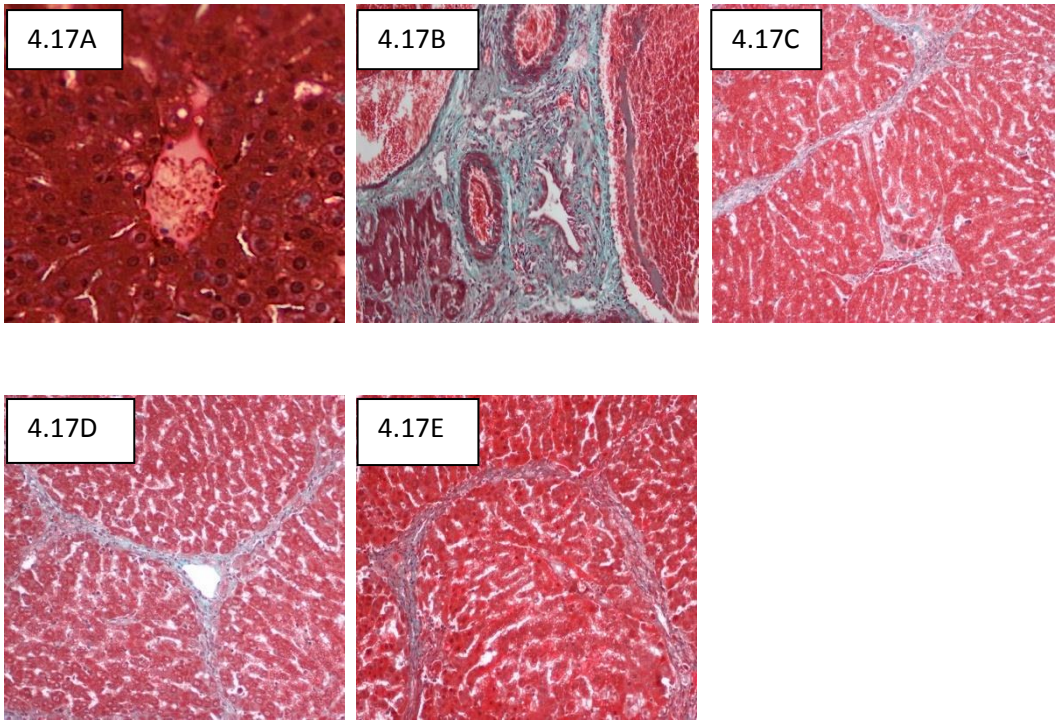

Figure 4.17: Light micrographs of the rat liver sections representing masson's trichrome staining of collagen deposition from different groups of an experimental model of TAA-induced cirrhosis. The liver tissue section of normal control group (4.17A) shows normal hepatocyte and architecture of the liver without any collagen deposition. The liver tissue section of cirrhosis control group (4.17B) shows severe damage and green blue collagen fibrous bands. The liver tissue section of silymarin control group (4.17C) shows mild collagen fibrotic septa deposition. (4.17D) The tissue section of the liver of the group received 250 mg/kg of *I. aquatica* extract shows moderate collagen fibrotic septa. (4.17E) The tissue section of the liver of the group received 500 mg/kg of *I. aquatica* extract shows mild collagen fibrotic septa deposition (Masson's trichrome stain magnification 20x).

To evaluate the effectiveness of *C. xanthorrhiza* and *I. aquatica* on hepatic stellate cell (HSC) indirectly activation, liver tissues of the animals were subjected for immunofluorescence staining (Figure 4.18 and 4.19). Alpha smooth muscle actin (α-SMA) is considered as a marker of activated HSC. As can be seen in Figure 4.16A and 4.17A, the normal control group shows negative staining of α-SMA. Whereas the number of positively stained α-SMA cells was increased around fibrotic septa in the liver sections of cirrhosis control (Figure 4.18 B and 4.19 B). This reveals that TAA

intoxication stimulates the HSC in the liver of the rat models. Concurrent treatments with a lower dose (250 mg/kg) of *C. xanthorrhiza* and *I. aquatica* for eight weeks displayed moderate number of positive stains for α-SMA (Figure 4.18D and 4.19D). The number of positively α-SMA cells was dramatically reduced in the liver tissues of the animals treated with silymarin (4.18C and 4.19C), *C. xanthorrhiza* and *I. aquatic* in a dose dependent manner, possibly by improving antioxidant status and suppressing proinflammatory mediators therefore reduce the number of activated HSC.

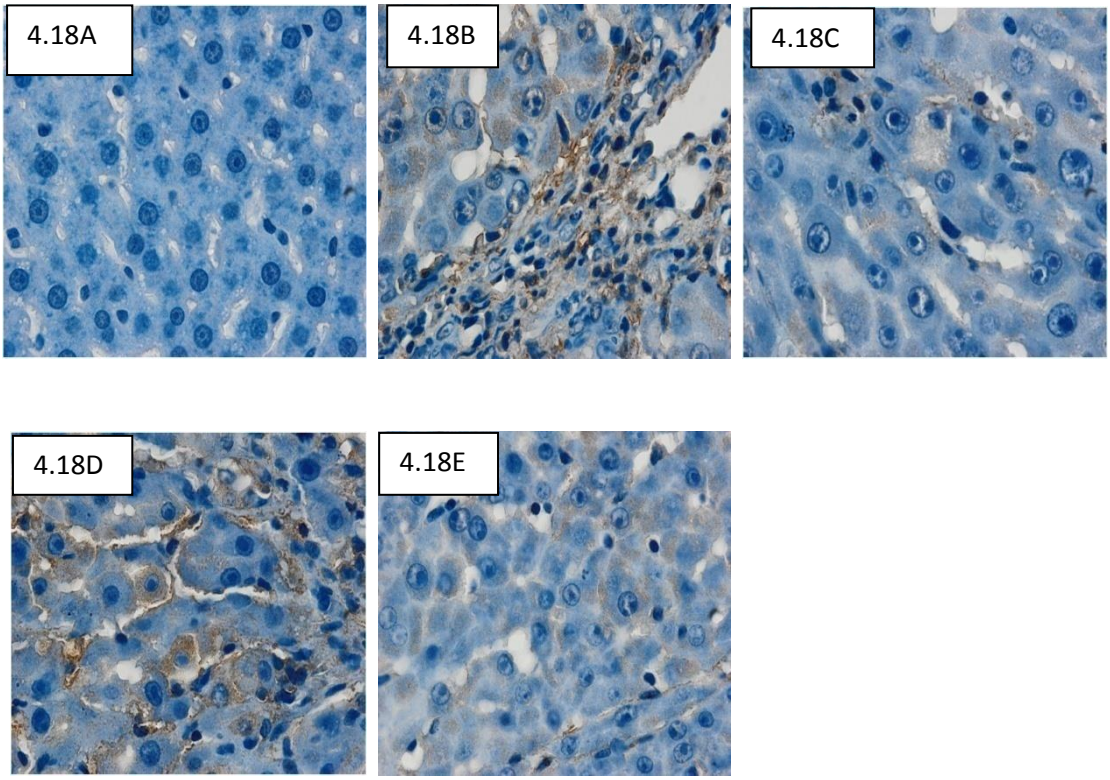

Figure 4.18: Light micrographs of the rat liver sections representing immunohistochemical staining of α-SMA from different groups of an experimental model of TAA-induced cirrhosis. The liver tissue section of normal control group (4.18A) shows negative staining of α-SMA. The liver tissue section of cirrhosis control group (4.18B) shows strong positivity for α-SMA. The liver tissue section of silymarin control group (4.18C) shows mild positive stain for α-SMA. (4.18D) The tissue section of the liver of the group received 250 mg/kg of *C. xanthorrhiza* extract shows moderate positivity for α-smooth muscle actin. (4.18E) The tissue section of the liver of the group received 500 mg/kg of *C. xanthorrhiza* extract shows mild positive stain for α-SMA (α-SMA stain magnification 100x).

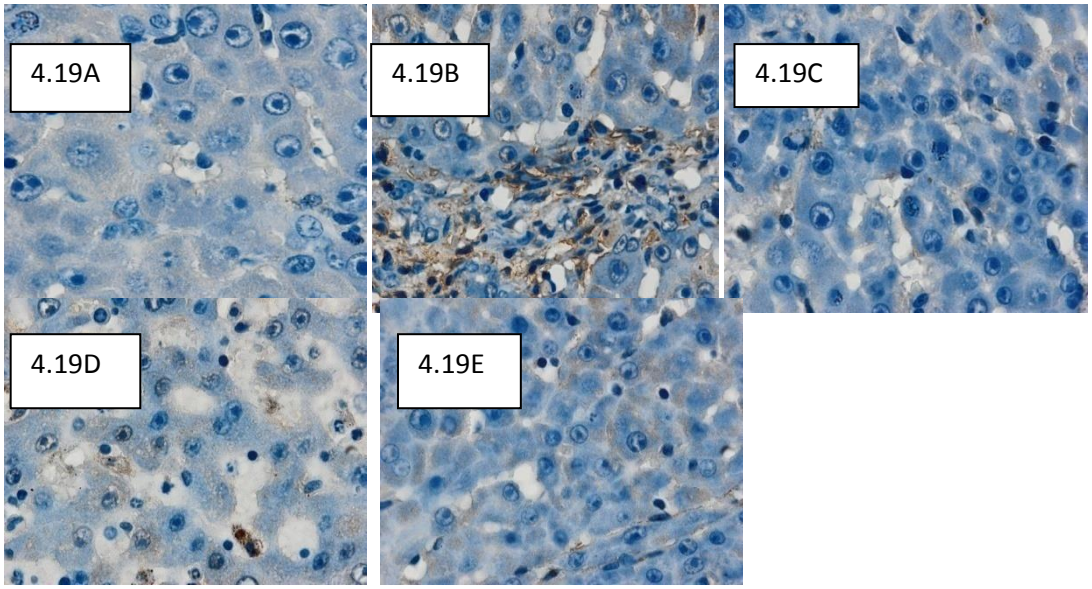

Figure 4.19: Light micrographs of the rat liver sections representing immunohistochemical staining of α-SMA from different groups of an experimental model of TAA-induced cirrhosis. The liver tissue section of normal control group (4.19A) shows negative staining of α-SMA. The liver tissue section of cirrhosis control group (4.19B) shows strong positivity for α-smooth muscle actin. The liver tissue section of silymarin control group (4.19C) shows mild positive stain for α-SMA. (4.19D) The tissue section of the liver of the group received 250 mg/kg of *I. aquatica* extract shows moderate positivity for α-SMA. (4.19E) The tissue section of the liver of the group received 500 mg/kg of *I. aquatica* extract shows mild positive stain for α-SMA (α-SMA stain magnification 100x).

4.7 SOD, CAT and MDA contents in liver homogenates

Antioxidant enzyme status and oxidative stress induced by the TAA in the liver of experimental rats were determined by measuring the amount of superoxide dismutase (SOD), catalase (CAT) and also malondialdehyde (MDA). Table 4.12 and 4.13 clearly reveals the influence of *C. xanthorrhiza* and *I. aquatica* extracts on SOD, CAT and MDA contents in liver homogenates of experimental rats, respectively. In the liver tissues of cirrhosis control group, the antioxidant enzymes SOD and CAT were significantly (p<0.05) reduced in comparison with the normal control group. Conversely, SOD and CAT were increased significantly (p<0.05) in the liver tissues of animals treated with *C. xanthorrhiza*, *I. aquatica* extract and silymarin when

compared with cirrhosis control group (Table 4.12 and 4.13). At the tested doses (250 and 500 mg/kg. p.o), *C. xanthorrhiza* and *I. aquatica* extracts exhibited the dose dependent increase in the antioxidant enzyme level toward normal control group.

Table 4.12: Effect of *C. xanthorrhiza* extract on SOD, CAT and MDA contents in liver homogenates of experimental rats.

Group	**SOD U/mg protein**	**CAT U/mg protein**	**MDA nmol/mg protein**
Normal control	19.07 ± 1.24	39.89 ± 3.35	1.32 ± 0.10
Cirrhosis control	7.17 ± 0.44**	17.55 ± 0.91**	4.64 ± 0.32**
Silymarin control	14.66 ± 0.48*	35.45 ± 2.67*	1.62 ± 0.15*
C. xanthorrhiza (250 mg/kg)	13.62 ± 1.02*	26.89 ± 2.07*	2.41 ± 0.27*
C. xanthorrhiza (500 mg/kg)	15.79 ± 1.20*	37.20 ± 3.18*	1.70 ± 0.16*

Data represent mean ± SEM. *$p<0.05$, significant compared to cirrhosis control group, **$p<0.05$, significant compared to normal control group.

Table 4.13: Effect of *I. aquatica* extract on SOD, CAT and MDA contents in liver homogenates of experimental rats.

Group	**SOD U/mg protein**	**CAT U/mg protein**	**MDA nmol/mg protein**
Normal control	18.03 ± 0.24	38.76 ± 0.32	1.19 ± 0.04
Cirrhosis control	8.63 ± 0.32**	18.73 ± 0.32**	4.80 ± 0.06**
Silymarin control	14.68 ± 0.21*	37.13 ± 0.40*	1.7 ± 0.04*
I. aquatica (250 mg/kg)	12.41 ± 0.27*	26.46 ± 0.64*	2.33 ± 0.0*
I. aquatica (500 mg/kg)	14.56 ± 0.29*	37.95 ± 0.24*	1.40 ± 0.09*

Data represent mean ± SEM. *p<0.05, significant compared to cirrhosis control group, **p<0.05, significant compared to normal control group.

4.8 Effect of the plant extracts on TNF-α, TG-Fβ1 and NF-κB

Table 4.14 and 4.15 represents the effect of *C. xanthorrhiza* and *I. aquatica* extracts on the inflammatory cytokines such as TNF-α, TG-Fβ1 and NF-κB. As shown in Table 4.14 and 4.15, animals treated with TAA exhibited a significant increase of the amount of TNF -α, TG-Fβ1 and NF-κB and these values were almost 2, 4 and 3 times higher than the normal control groups, respectively. However, the amount of TNF-α, TG-Fβ1 and NF-κB was significantly decreased in the livers of the TAA and extracts cotreated animals. The treatment of *C. xanthorrhiza* and *I. aquatica* at the dose of 500 mg/kg reduced the TNF-α, TG-Fβ1 and NF-κB to the levels similar to that of the silymarin control group which represents that these plant extracts have the ability to inhibit the expression of cytokines biomarkers in TAA induced liver cirrhosis.

Table 4.14: Effect of *C. xanthorrhiza* extract on serum level of TNF alpha, TG-Fβ1 and liver tissue (homogenate) NF-κB of experintal rats.

Group	TNF α pg/ml	TGFβ1 ng/ml	NF-κB ng/ml
Normal control	16.23 ± 0.68	0.97 ± 0.05	1.23 ± 0.11
Cirrhosis control	30.73 ± 1.85**	4.31 ± 0.30**	3.23 ± 0.23**
Silymarin control	17.35 ± 1.49*	1.52 ± 0.13*	1.37 ± 0.17*
C. xanthorrhiza (250 mg/kg)	23.34 ± 2.42*	1.75 ± 0.16*	1.73 ± 0.14*
C. xanthorrhiza (500 mg/kg)	18.48 ± 1.78*	1.33 ± 0.14*	1.50 ± 0.15*

Data represent mean ± SEM. *p<0.05, significant compared to cirrhosis control group, **p<0.05, significant compared to normal control group.

Table 4.15: Effect of *I. aquatica* extract on serum level of TNF alpha, TG-Fβ1 and liver tissue (homogenate) NF-κB of experintal rats.

Group	TNF α pg/ml	TGFβ1 ng/ml	NF-kB ng/ml
Normal control	15.64 ± 0.66	1.11 ± 0.07	1.16 ± 0.10
Cirrhosis control	32.42± 2.18**	4.05 ± 0.47**	3.06 ± 0.19**
Silymarin control	16.85± 1.10*	1.62 ± 0.10*	1.33 ± 0.09*
I.aquatica (250 mg/kg)	19.67± 1.37*	1.81 ± 0.14*	1.59 ± 0.11*
I. aquatica (500 mg/kg)	17.27± 1.17*	1.53 ± 0.17*	1.32 ± 0.13*

Data represent mean ± SEM. *p<0.05, significant compared to cirrhosis control group, **p<0.05, significant compared to normal control group.

4.9 *In vitro* hepatoprotective of isolated fractions

The result of *in vitro* hepatoprotective screening showed that the fractions isolated from *C. xanthorrhiza* and *I. aquatic*, to different degree, have the ability to inhibit WRL-68 cell death which induced by cytotoxicity effects of TAA. Thioacetamide induced 47 percent death in TAA control as compared to normal control. The results of the 10 fractions of *C. xanthorrhiza* showed that fraction number 5 (*C. xanthorrhiza* F5) was the most effective fraction which at the dose of 10 ug/ml significantly increased the cell viability to 85 percent. On the other hand, isolated fractions from *I. aquatica* showed almost the same results as the ones isolated from *C. xanthorrhiza.* Among the 13 fractions of *I. aquatica*, fraction number 11 (. *I. aquatica* F11) was the most effective which at the dose of 10 ug/ml significantly increased the cell viability to 86.7 percent. These results show that fraction number 5 from *C. xanthorrhiza* and fraction number 11 from *I. aquatica* were more effective than the crudes in preventing TAA oxidative stress and therefore cell death.

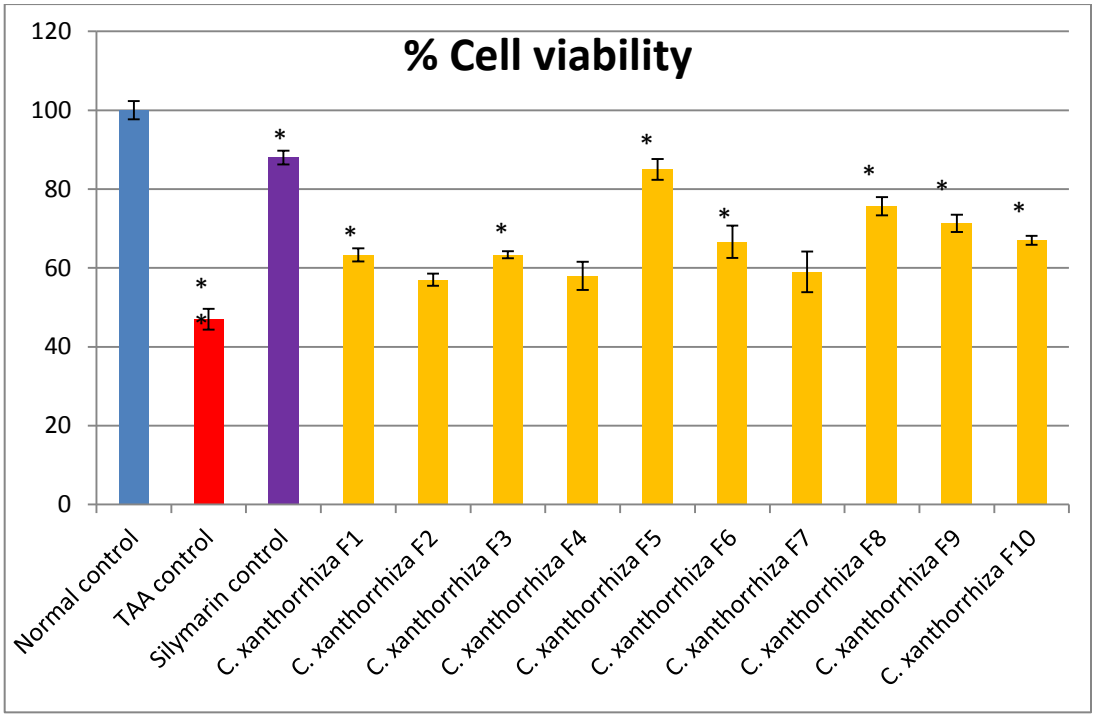

Figure 4.20: Effect of *C. xanthorrhiza* isolated fractions (F1 – F10) on WRL-68 cell viability treated with TAA. Data represent mean ± SEM. **p*<0.05, significant compared to TAA control, ***p*<0.05, significant compared to normal control.

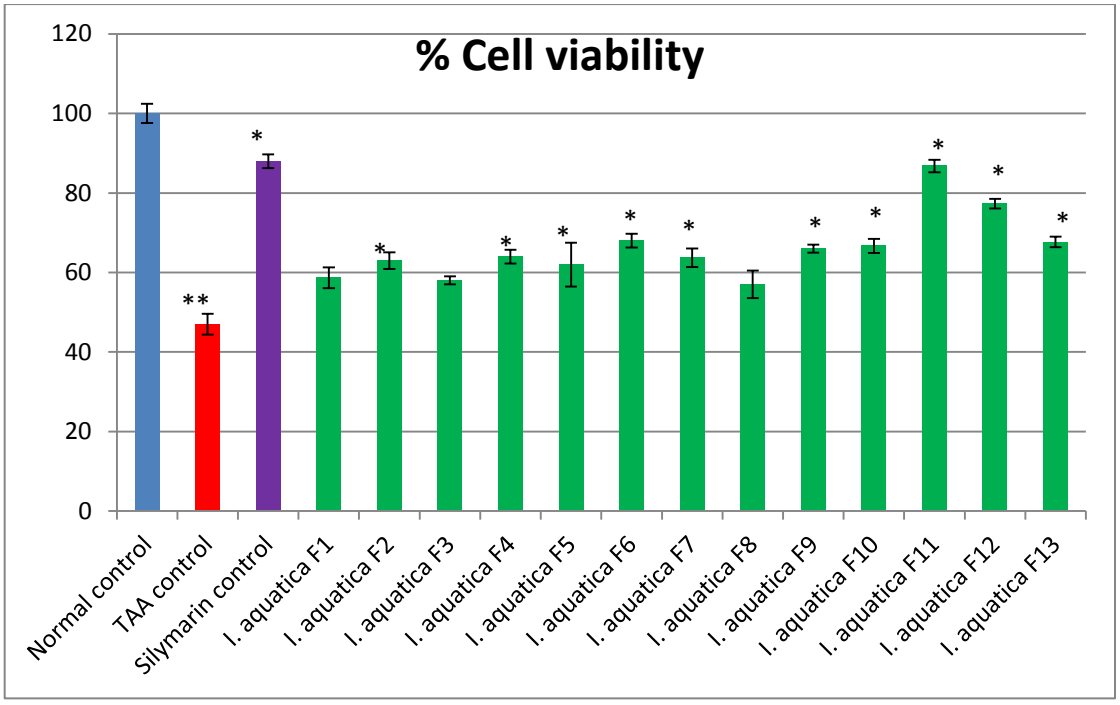

Figure 4.21: Effect of *I. aquatica* isolated fractions (F1-13) on WRL-68 cell viability treated with TAA. Data represent mean ± SEM. **p*<0.05, significant compared to TAA control, ***p*<0.05, significant compared to normal control.

4.10 Antioxidant of WRL-68 cell treated with active fractions

Antioxidant results of isolated fractions from *C. xanthorrhiza* and *I. aquatica* crude extracts which showed best hepatoprotective and prevented cell death in WRL-68 cell. The oxidative stress was induced by exposing cells to 0.04g/ml of TAA. The antioxidant activities of fraction 5 and 11 from *C. xanthorrhiza* and *I. aquatic*, respectively, were found to have a positive correlation with the improvement of the cell viability. It is apparent that TAA bring about the generation of ROS, which reduced the antioxidant enzymes. Treatment with these fractions reduced the free radical and improved the antioxidant enzymes levels. The obtained results show that TAA significantly reduced SOD and CAT levels. Treatment with active fractions significantly improved the levels of these two antioxidant enzymes. At the same time, lipid peroxidation level, determined as MDA generation, significantly increased in TAA control group as compared to normal control group. Treatment with active fractions significantly prevented lipid peroxidation hence low levels of MDA.

Table 4.16**:** Effect of *C. xanthorrhiza* isolated fraction 5 (F5) and *I. aquatica* isolated fraction 11 (11) on SOD, CAT and MDA on WRL-68 cell treated with TAA.

Group Name	SOD U/ml	CAT nmol/min/ml	MDA nmol/ml
Normal control	14.34 ± 0.43	6.03 ± 0.41	13.26 ± 1.03
TAA control	8.048 ± 0.78 **	2.72 ± 0.23 **	35.83 ± 1.72 **
Silymarin control 10	12.39 ± 0.15 *	4.81 ± 0.20 *	18.00 ± 1.00 *
Silymarin control 100	13.80 ± 0.70 *	5.34 ± 0.26 *	16.00 ± 1.15 *
C. xanthorrhiza F5 10	11.97 ± 0.26 *	4.76 ± 0.15 *	21.00 ± 1.32 *
C. xanthorrhiza F5 100	12.73 ± 0.84 *	4.99 ± 0.16 *	18.00 ± 0.75 *
I. aquatica F11 10	12.35 ± 0 39 *	4.75 ± 0.18 *	19.43 ± 2.05 *
I. aquatica F11 100	13.16 ± 0.63 *	5.13 ± 0.34 *	17.66 ± 1.01 *

Data represent mean ± SEM. *p<0.05, significant compared to TAA control, **p<0.05, significant compared to normal control.

4.11 LCMS for the identification of active constituents of the active fractions

In order to identify the active constituents of the *Curcuma xanthorrhiza* fraction 5 (F5) and *Ipomoea aquatica* fraction 11 (F11), LCMS was applied using negative and positive mode ionization. All identified peaks are presented in negative and positive mode ionization in Figure 4.22 to 4.25. Table 4.17 to 4.20 present tentative compounds of the identified peaks detected with their retention time, observed *m/z*, mass and formula. Among the active constituents are xanthorrhizol from *Curcuma xanthorrhiza* fraction 5 and violaxanthin from the *Ipomoea aquatica* fraction 11. In the LCMS positive mode peak 7 (cpd 7) represents xanthorrhizol of the *Curcuma xanthorrhiza* fraction 5 (Figure 4.23). Whereas the LCMS negative mode peak 3 (cpd 3) represents violaxanthin of the *Ipomoea aquatica* fraction 11 (Figure 4.24).

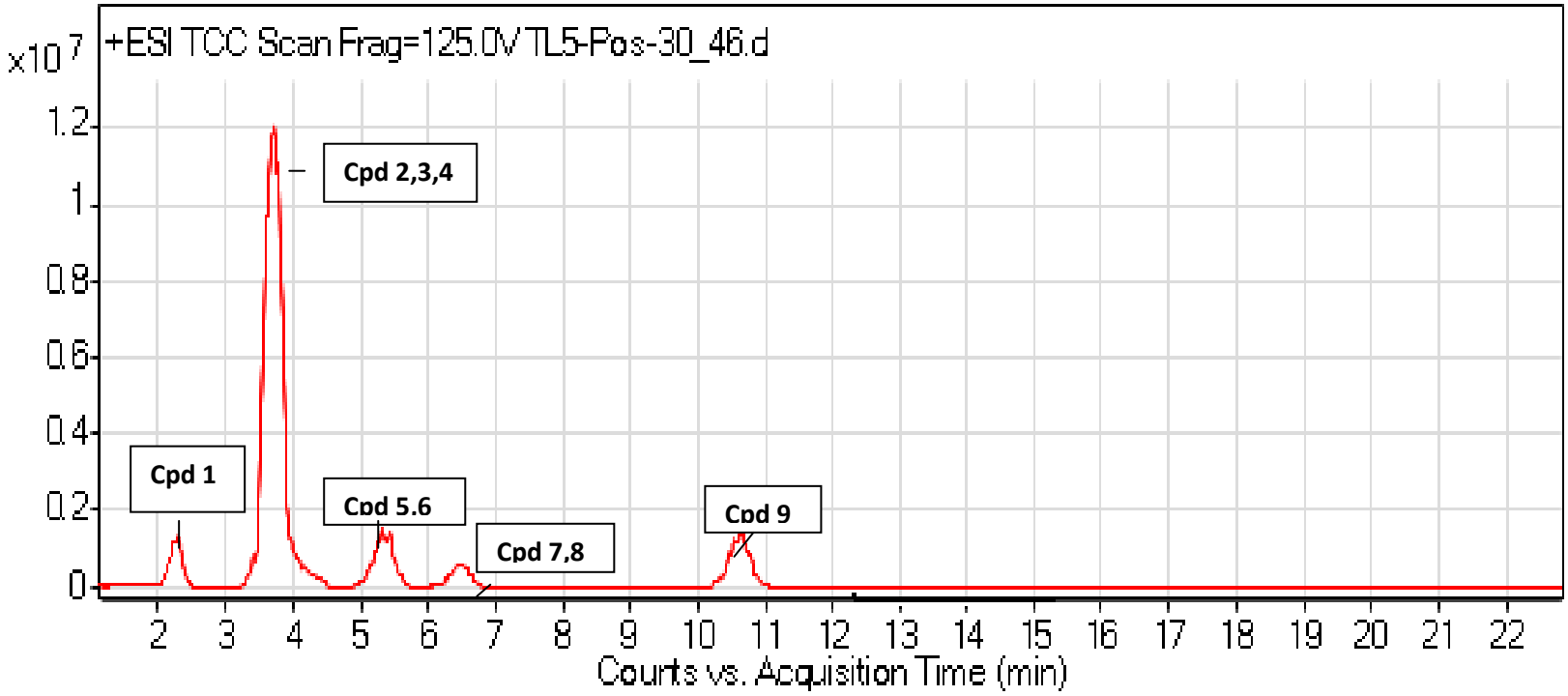

Figure 4.23: LCMS of *Curcuma xanthorrhiza* fraction 5 (F5) peaks in negative mode ionization. The details of the identified compounds are presented in Table 4.17. cpd: Compound

Table 4.17: Present the result of negative mode ionization LCMS of *Curcuma xanthorrhiza* fraction 5 (F5).

Peak	Label	Formula	Score	Mass	m/z	RT
cpd 1	Loxoprofen Metabolite (Benzeneacetic acid, 4-[(2-hydroxycyclopentyl)methyl]-a-methyl-, [1S-[1a(R*),2	C_{15} H_{20} O_3	97.46	248.14	249.15	2.26
cpd 2	Loxoprofen Metabolite (Benzeneacetic acid, 4-[(2-hydroxycyclopentyl)methyl]-a-methyl-, [1S-[1a(R*),2	C_{15} H_{20} O_3	94.44	248.14	249.15	3.46
cpd 3	Loxoprofen	C_{15} H_{18} O_3	98.41	246.13	247.13	3.47
cpd 4	3alpha-Hydroxy-4,4-Bisnor-8,11,13-Podocarpatriene	C_{15} H_{20} O	92.95	216.15	217.16	3.73
cpd 5	Punctaporin B	C_{15} H_{24} O_3	99.17	252.17	253.18	5.24
cpd 6	Helenine	C_{15} H_{20} O_2	99.81	232.15	233.15	5.38
cpd 7	Nabumetone	C_{15} H_{16} O_2	99.97	228.12	229.12	6.46
cpd 8	helenine	C_{15} H_{20} O_2	99.15	232.15	233.15	6.52
cpd 9	Trichodiene	C_{15} H_{24}	99.99	204.19	205.2	10.63

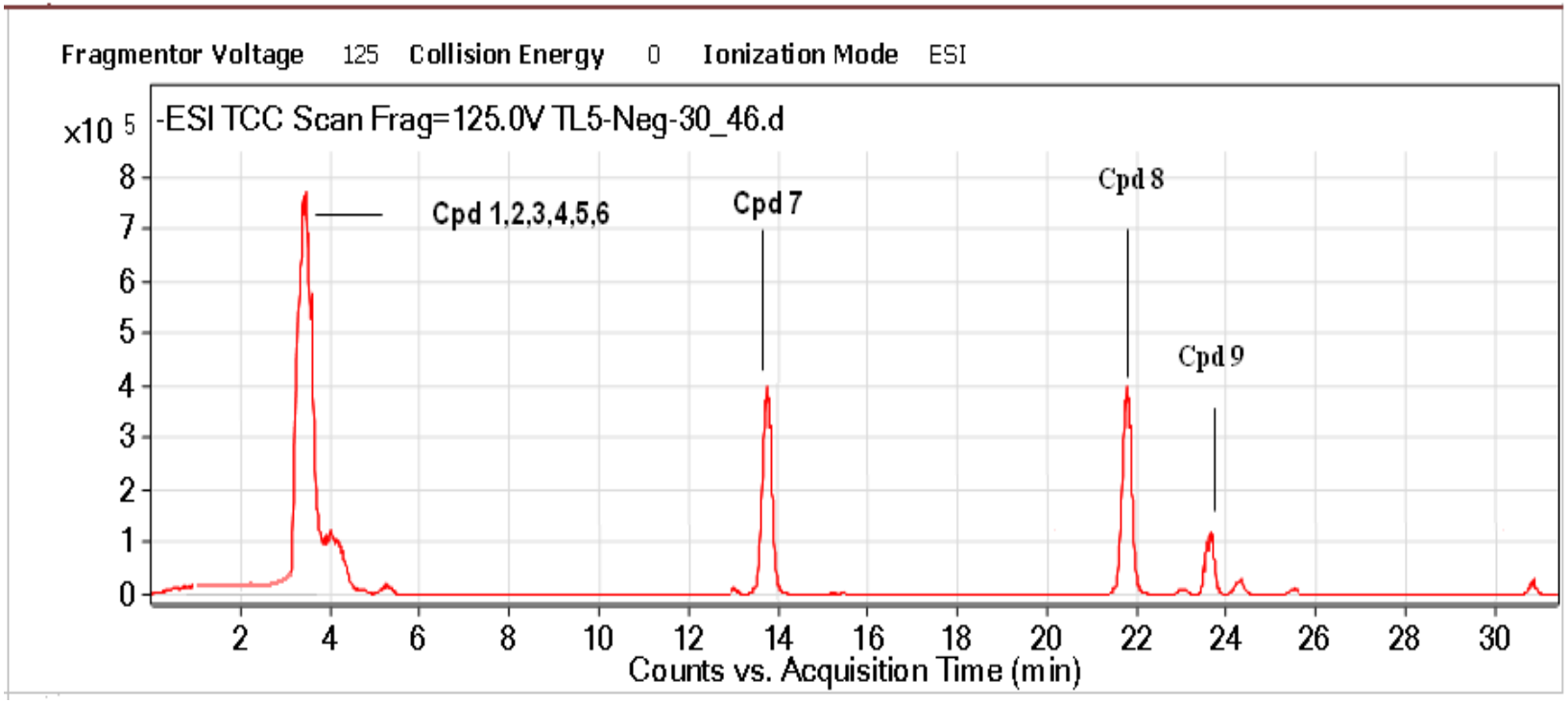

Figure 4.24: LCMS of *Curcuma xanthorrhiza* fraction 5 (F5) peaks in positive mode ionization. The details of the identified compounds are presented in Table 4.18. cpd: Compound

Table 4.18: Present the result of positive mode ionization LCMS of *Curcuma xanthorrhiza* fraction 5 (F5).

Compound	Label	Formula	Score	Mass	m/z	RT
cpd 1	Gemfibrozil	$C_{15}H_{22}O_3$	98.56	250.16	249.15	3.38
cpd 2	Lactone of PGF-MUM	$C_{16}H_{24}O_5$	97.35	296.16	295.16	3.41
cpd 3	Cucurbitacin S	$C_{30}H_{42}O_6$	99.36	498.3	497.29	3.52
cpd 4	2,4-Pentadienoic acid, 3-methyl-5-(2,6,6-trimethyl-3-oxo-1-cyclohexen-1-yl)-	$C_{15}H_{20}O_3$	99.7	248.14	247.13	3.55
cpd 5	Helenine	$C_{15}H_{20}O_2$	99.77	232.15	231.14	3.65
cpd 6	Artemether	$C_{16}H_{26}O_5$	99.68	298.18	297.17	3.83
cpd 7	Xanthorizol	$C_{15}H_{22}O$	99.54	218.17	217.16	13.56
cpd 8	20-Dihydrodexamethasone	$C_{22}H_{31}FO_5$	98.35	394.22	393.21	21.79
cpd 9	Milbemycin A3	$C_{31}H_{44}O_7$	38.34	528.31	527.3	23.04

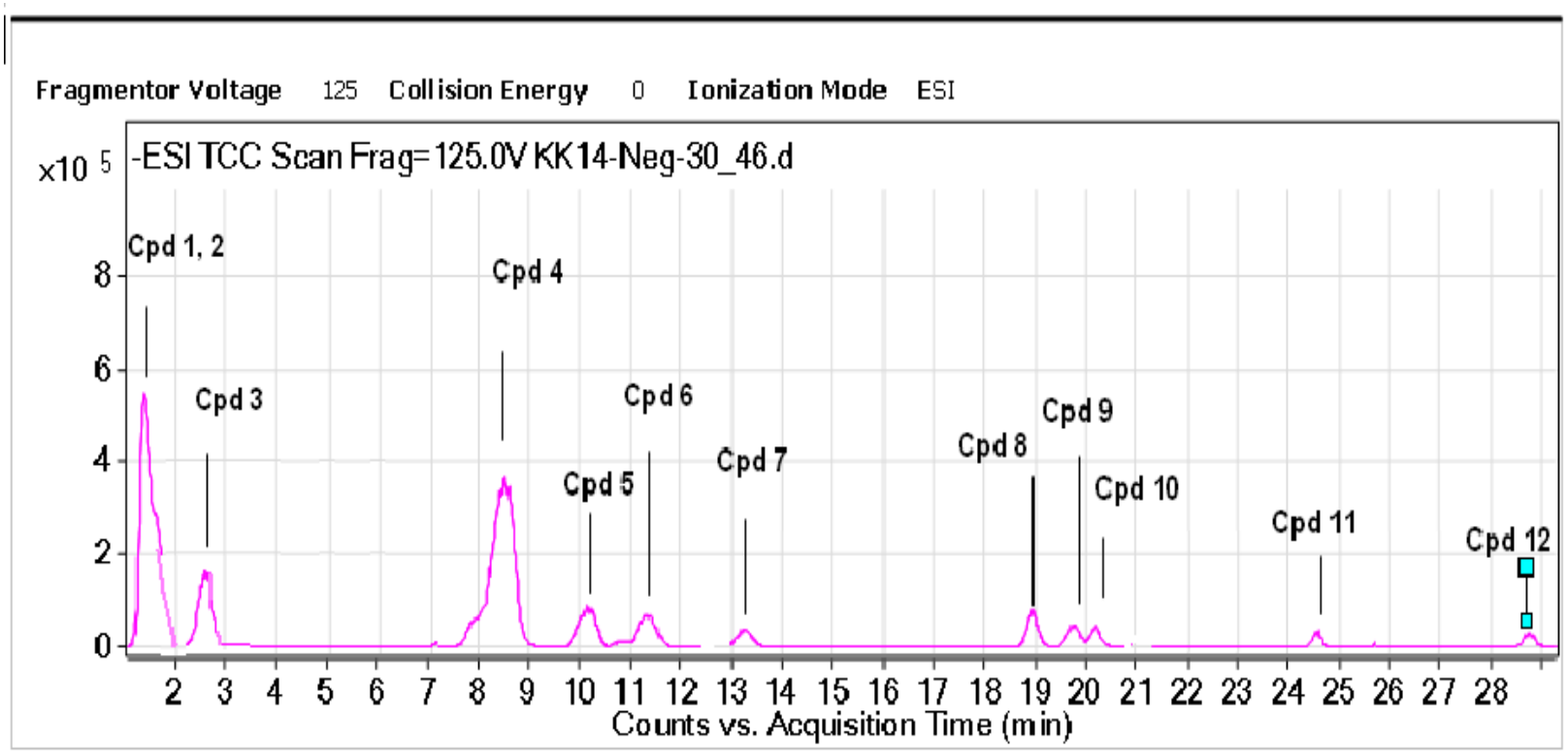

Figure 4.25: LCMS of *Ipomoea aquatica* fraction 11 (F11) peaks in negative mode ionization. The details of the identified compounds are presented in Table 4.19. cpd: Compound

Table 4.19: Present the result of negative mode ionization LCMS of *Ipomoea aquatica* fraction 11 (F11).

Compound	Label	Formula	Score	Mass	m/z	RT
cpd 1	Umbelliferone	$C_9 H_6 O_3$	97.14	162.03	163.04	1.49
cpd 2	Methyl jasmonate	$C_{13} H_{20} O_3$	96.68	224.14	225.15	1.51
cpd 3	Violaxanthin	$C_{40}H_{56}O_4$	92.62	600.85	601.4	2.64
cpd 4	Tetranor Iloprost	$C_{18} H_{26} O_4$	99.8	306.18	307.19	2.68
cpd 5	9-deoxy-9-methylene-PGE2	$C_{21} H_{34} O_4$	99.2	350.25	351.25	3.52
cpd 6	gamma-Linolenic acid	$C_{18} H_{30} O_2$	99.97	278.22	279.23	8.3
cpd 7	13(S)-HOTrE(gamma)	$C_{18} H_{30} O_3$	99.72	294.22	295.23	9.86
cpd 8	3alpha-Hydroxy-4,4-Bisnor-8,11,13-Podocarpatriene	$C_{15} H_{20} O$	98.94	216.15	217.16	10.62
cpd 9	1-hexadecanoyl-sn-glycerol	$C_{19} H_{38} O_4$	99.95	330.28	331.28	21.88
cpd 10	Harderoporphyrin	$C_{35} H_{36} N_4 O_6$	99.41	608.26	609.27	22.23
cpd 11	Oleamide	$C_{18} H_{35} N O$	99.89	281.27	282.28	24.47
cpd 12	1-octadecanoyl-rac-glycerol	$C_{21} H_{42} O_4$	99.67	358.31	359.32	28.41

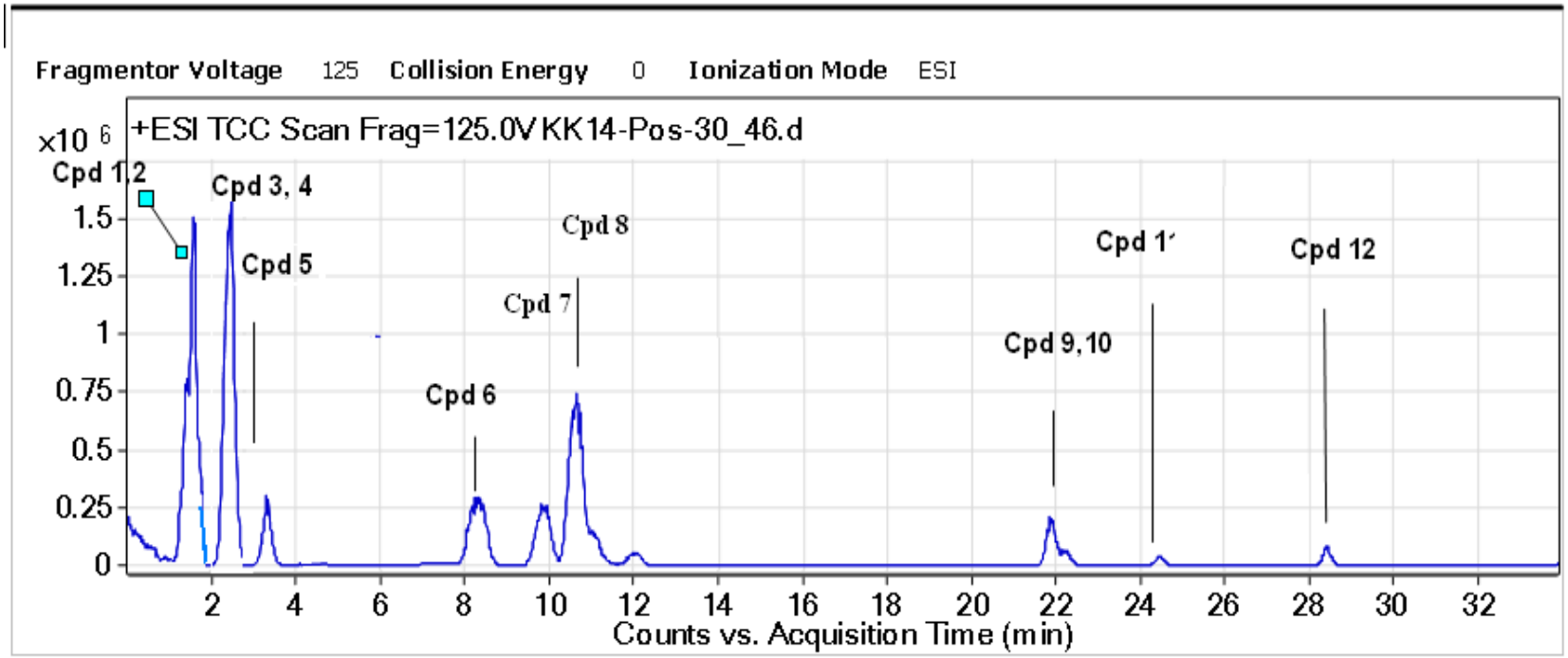

Figure 4.26: LCMS of *Ipomoea aquatica* fraction 11 (F11) peaks in positive mode ionization. The details of the identified compounds are presented in Table 4.20. cpd: Compound

Table 4.20: Present the result of positive mode ionization LCMS of *Ipomoea aquatica* fraction 11 (F11).

Compound	Label	Formula	Score	Mass	m/z	RT
cpd 1	Isoquinoline N-oxide	$C_9 H_7 N O$	99.85	145.05	144.05	1.61
cpd 2	Traumatic acid	$C_{12} H_{20} O_4$	99.9	228.14	227.13	1.73
cpd 3	8,13-dihydroxy-9,11-octadecadienoic acid	$C_{18} H_{32} O_4$	99.61	312.23	311.22	2.78
cpd 4	13S-hydroxy-9E,11Z-octadecadienoic acid	$C_{18} H_{32} O_3$	97.05	296.24	295.23	8.51
cpd 5	12-oxo-9-octadecynoic acid	$C_{18} H_{30} O_3$	99.24	294.22	293.21	10.16
cpd 6	12-oxo-9-octadecynoic acid	$C_{18} H_{30} O_3$	99.51	294.22	293.21	11.35
cpd 7	13(S)-HOTrE(gamma)	$C_{18} H_{30} O_3$	99.72	294.22	295.23	9.86
cpd 8	2-acetoxy-butanedioic acid bis-(2-ethyl-hexyl) ester	$C_{22} H_{40} O_6$	49.83	400.28	399.28	18.95
cpd 9	Unknown	$C_{22} H_{44} O_7$	49.73	420.31	419.3	19.73
cpd 10	D-Glucopyranoside	$C_{20} H_{40} O_6$	49.52	376.28	375.28	22.15
cpd 11	7-palmitoleic acid	$C_{16} H_{30} O_2$	99.96	254.22	253.22	24.56
cpd 12	Sorbitan monooleate	$C_{22} H_{44} O_6$	49.59	404.31	403.31	28.76

4.12 *In vitro* hepatoprotective of xanthorrhizol and violaxanthin

The result of i*n vitro* hepatoprotective experiment showed that xanthorrhizol and violaxanthin have the ability to inhibit WRL-68 cell death which induced by cytotoxicity effects of TAA. Thioacetamide induced nearly 50 percent death in TAA control as compared to normal control. Xanthorrhizol and violaxanthin at the dose of 10 ug/ml significantly increased the cell viability to 89 and 91 percent, respectively.

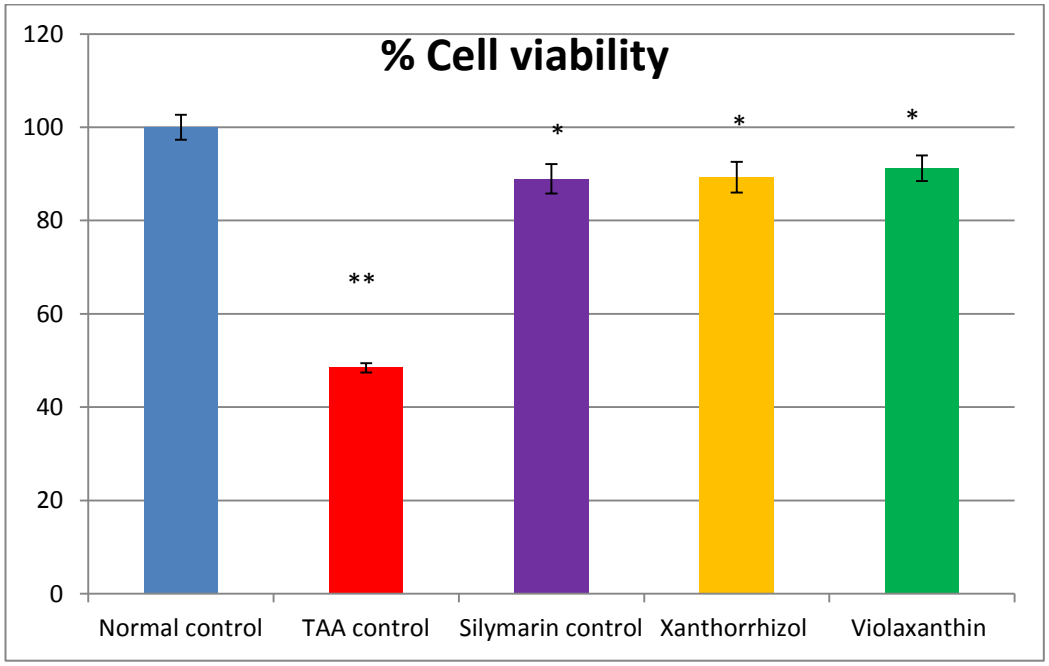

Figure 4.27: Effect of xanthorrhizol and violaxanthin on WRL-68 cell viability treated with TAA. Data represent mean ± SEM. *p<0.05, significant compared to TAA control, **p<0.05, significant compared to normal control.

4.13 Antioxidant of WRL-68 cell treated with xanthorrhizol and violaxanthin

Antioxidant activities of xanthorrhizol and violaxanthin were found to have a positive correlation with the improvement of the cell viability in WRL-68 cell. It is apparent that TAA bring about the generation of ROS, which reduced the antioxidant enzymes. Treatment with xanthorrhizol and violaxanthin reduced the free radical and improved

the antioxidant enzymes levels. The obtained results show that TAA significantly reduced SOD and CAT levels. Treatment with xanthorrhizol and violaxanthin significantly improved the levels of these two antioxidant enzymes. At the same time, lipid peroxidation level, determined as MDA generation, significantly increased in TAA control group as compared to normal control group. Treatment with xanthorrhizol and violaxanthin significantly prevented lipid peroxidation hence low levels of MDA.

Table 4.21: Effect of xanthorrhizol and violaxanthin on SOD, CAT and MDA on WRL-68 cell treated with TAA.

Group Name	SOD U/ml	CAT nmol/min/ml	MDA nmol/ml
Normal control	14.34 ± 0.43	6.03 ± 0.41	13.26 ± 1.03
TAA control	8.048 ± 0.78 **	2.72 ± 0.23 **	35.83 ± 1.72 **
Silymarin control 10	12.39 ± 0.15 *	4.81 ± 0.20 *	18.00 ± 1.00 *
Silymarin control 100	13.80 ± 0.70 *	5.34 ± 0.26 *	16.00 ± 1.15 *
Xanthorrhizol 10	12.52 ± 0.36 *	4.90 ± 0.13 *	18.94 ± 1.00 *
Xanthorrhizol 100	13.31 ± 0.582 *	5.02 ± 0.170 *	17.28 ± 1.45 *
Violaxanthin 10	13.34 ± 0.34 *	5.10 ± 0.12 *	17.63 ± 1.15 *
Violaxanthin 100	13.91 ± 0.18 *	5.30 ± 0.19 *	16.20 ± 0.98 *

Data represent mean ± SEM. *p<0.05, significant compared to TAA control, **p<0.05, significant compared to normal control.

CHAPTER V

DISCUSION AND CONCLUSION

5.1 Mechanism of liver cirrhosis and the potential of medicinal plants on hepatoprotective

Liver cirrhosis regarded as the ending stage of chronic liver disease in which chronic fibrosis leads to the accumulation of scar tissue and therefore structural and functional alteration. Fibrosis is a reversible wound healing process which takes place virtually in all chronic liver damage. Despite the fact that the wound healing response generally starts with damage to the hepatocyte, the total response extends far beyond this event (Baldo, *et al.*, 2008). Liver is the critical organ of the body for maintaining overall health and detoxification and therefore more than all other organ is exposed to toxic injury. Drug-induced liver injury as a result of widely used has grown to be a really serious health issue. Thereby studies on the mechanism of drug-induced liver damage can be very useful in prevention of drug-induced liver injury and therapy (Xu & Qu, 2008). The damage to the liver generates a cascade of events (Holstege, *et al.*, 2005; Svegliati Baroni, *et al.*, 2003). Molecular and cellular mechanisms of hepatic fibrotic degradation as a result of chronic hepatic tissue damage have been described. Fibrosis is a physiologic mechanism, which happens to be beneficial in the beginning, but then develops into pathological in the event that chronic hepatic injury persist (Jarnagin, *et al.*, 1994; Meyer, *et al.*, 2005; Minagawa, *et al.*, 2004). Fibrosis process triggered upon hepatic tissue damage, followed by inflammatory response with activation of macrophage of the liver, Kupffer cells, and the effector cells, stellate cells, which in turn bringing about increased expression of pro-fibrotic and pro-inflammatory cytokines. The HSC's generally activated by cytokines that includes tumor necrosis factor alpha (TNFα), tumor growth factor beta (TGFβ) and platelet derived growth factor (PDGF)

as well as excessive alterations in composition and arrangement of ECM components and products of oxidative stress. Once activated, stellate cells recruit and activate fibroblast which is responsible for increased production of ECM proteins (Flier, *et al.*, 1993). In addition to the increase in the production of ECM, activated HSCs also increase release of tissue inhibitors of metalloproteinases (TIMPs) which prevent matrix metaloproteinases (MMPs) to degrade excessive matrix collagen. The process is driven by signalling pathways mediated by proinflammatory cytokines including TNFα and also transforming growth factor TGFβ1 (Wang, *et al.*, 2006). The most accumulated ECM involved in fibrosis are the interstitial collagens, types I, and III (Schuppan, 1990). In all different chronic liver disease, the progression of fibrosis is a step by step and gradually process starting from minimal fibrosis limited to in and within the portal tracts causing periportal stellate fibrous accumulates and extension (periportal or zone one fibrosis)(Marcellin, *et al.*, 2006). As the process gets progressively worse over time, fibrous tissues increase to the nearby mesenchymal arrangements with the development of fibrous septa. Then it progressively grows into the lobules towards the central veins (zone three) with septa development. Finally, when almost all of the central veins or portal tracts interconnected, bridge fibrosis generated, and annular fibrosis surrounding nodules of the liver cells, cirrhosis occur (Z.D. Goodman & K.G. Ishak, 1995; Scheuer, *et al.*, 2005).

At the same time, enhanced oxidative stress and significant decrease of antioxidant defence have been reported in all types of liver damage (Loguercio & Federico, 2003). Reactive oxygen species (ROS) appear to have a key role in the induction and in the progression of liver disease (Choi & Ou, 2006). ROS are well-recognized to have important role in establishment and maintaining of liver fibrosis in viral hepatitis infection, alcohol intoxication and metabolic disorders. Excessive production of reactive oxygen species and depletion of antioxidant enzymes such as catalase (CAT) and

superoxide dismutase (SOD) play an important role in lipid peroxidation leading to hepatic tissue damage and necrosis. To a large extent oxidative stress is the result of a necrotic activity, but nevertheless, ROS generated by activated macrophages (Kupffer cells) in addition to reactive aldehydes resulting from membrane lipid peroxidation can certainly stimulate the expansion of collagen accumulation in the inflamed tissue. It is well established that ROS may well be underlying cause and also the results of the cellular injury. Several hepato-toxins, as an example, result in increased levels of ROS which overcome the normal antioxidant system of the cells (Marí, *et al.*, 2001). Increased ROS levels lead to lipid peroxidation resulting in increased in highly reactive aldehydic end products. Consequently, alteration of signal transduction, regulation of gene expression, rearrangement of the redox condition which include deficit of glutathione concentrations, and promotion of apoptosis and necrosis (Dalton, *et al.*, 1999).

In accordance with molecular and cellular mechanisms of hepatic fibrogenesis, a number of strategies may be applied for hepatoprotective. These strategies may include controlling oxidative stress by scavenging of free radicals or enhancing cellular antioxidant system, reducing inflammation and cell dying, and promoting excess ECM breaking down. These strategies should indirectly down regulate the activation of HSC which is responsible for the increased production of ECM, however, a strategy of reducing HSC can be directly by stimulating apoptosis. There are already good candidatures of hepatoprotective agents which are known to reduce oxidative stress, and blocking HSC activation. A good example of agent that reduces oxidative stress is silymarin, which was used as a reference drug in this our study (Li & Friedman, 2002).

In view of absence effective therapy in the modern medicine, and serious side effects as a results of synthetic agents, a good number of medicinal plants increasingly used to treat liver disorders (Dhiman, *et al.*, 2012). These recommended medicinal plants for the

treatment of liver disorder have been in use for long time and claimed to produce significant results (Sanmugapriya & Venkataraman, 2006). Some of traditional medicinal plants which have been scientifically studied for various liver disorders have exhibited promising results. For example, Silymarin, a well known hepatoprotective agent with antioxidant and antinflammatory, derived from *Silybum marianum* has demonstrated potential in the prevention of cirrhosis (Stickel & Schuppan, 2007). There is a number of other medicinal plants that have proved to have hepatoprotective properties including *Camellia sinensis, Glycyrrhiza glabra and Curcuma longa* (Luper, 1999). Therefore it is obviously beneficial to conduct studies in medicinal plants searching for safe and reliable liver-protective therapy. Hepatoprotective studies, particularly, of the medicinal plants possess antioxidants and antinflammatory properties may lead to a discovery of new drug, which can prevent or even treat the cirrhosis by blocking the progression of fibrosis process.

5.2 Antioxidant activities of *Curcuma xanthorrhiza* and *Ipomoea aquatica* extracts

Curcuma xanthorrhiza ethanolic extract exhibited high efficacy in free radical scavenging property, whereas its counterpart *I. aquatica* extract exhibited lesser efficacy in free radical scavenging property evaluated by DPPH and ABTS assays. Along with *C. xanthorrhiza, I. aquatica* ethanol extracts and Silymarin the reference standard drug used in our liver cirrhosis experiment, all the samples exhibited marked free radical scavenging activity expressed in terms percentage. In both the assays, Ascorbic acid exhibited highest radical scavenging capacity value followed by *C. xanthorrhiza* extract, BHT, silymarin and *I. aquatica* extract. The DPPH radical scavenging capacity of Ascorbic acid was approximately 4 and 1.4 times higher than *I. aquatica* and *C. xanthorrhiza* extracts, respectively. Similarly, the ABTS radical scavenging capacity of Ascorbic acid was 3 and 1.2 times higher than *I. aquatica* and *C. xanthorrhiza* extracts,

respectively. However, the DPPH radical scavenging activity of *C. xanthorrhiza* extract was about 2 times higher than silymarin the reference standard drug used in liver cirrhosis experiment. Similarly, the ABTS radical scavenging capacity of *C. xanthorrhiza* extract is higher than silymarin.

In the oral toxicity of *C. xanthorrhiza* and *I. aquatica* ethanol extracts at higher dose of 5 g/kg did not exhibit any mortality, behavioral changes or alter serum chemistry. The oral LD_{50} value of both these extracts was predicted to be higher than 5mg/kg. Hence, they were considered to be practically safe in Sprague-Dawley rats. For further animal experimental studies, $1/10^{th}$ and $1/20^{th}$ of the highest dose of *C. xanthorrhiza* and *I. aquatica* ethanolic extracts were employed.

5.3 *In vitro* hepatoprotective and antioxidant properties of crude extracts

The usage of cell line as an *in vitro* hepatic model to screen the effectiveness of crude extracts before moving to the animal experiment is the most suitable when considering the time involved together with expenses. Normal human embryonic liver cells (WRL 68 cells) are regarded as the convenient *in vitro* model for toxicological and pharmaceutical studies (Wilkening, Stahl & Bader, 2003). For the hepatoprotective studies, liver cell damage is induced by the use of hepatotoxin to which the effectiveness of the treatment can be evaluated. The effectiveness of the treatment can be evaluated by means of parameters which include cell viability and oxidative stress (Bladier, *et al.*, 1997; Huang, *et al.*, 1999). Hence *in vitro* hepatoprotective experiment was conducted in the normal liver cell line WRL-68 treated with TAA to study the effects of *C. xanthorrhiza* and *I. aquatica* ethanol extracts and their fractions. TAA produces oxidative stress, equally by both increased generation of ROS and also by reduction of cellular oxidative defences system. It is well established that mitochondria has an essential role in the apoptotic response, therefore functional alteration in

mitochondrial following TAA treatment lead to cell death through necrosis or apoptosis (Hoek & Pastorino, 2002). Studies have also revealed that liver cells intoxicated with TAA tend to be more susceptible to the cytotoxic effects of TNF-α along with other cytokines.

It has been established that TAA induce damage in the cell line by suppressing antioxidant defence system. Antioxidant defence system which include SOD and catalase act together to eliminate ROS and therefore play key role in the prevention of cellular lipids, proteins and DNA from oxidative damage. Reduction in antioxidant defence system result in the release of ROS from the mitochondrial matrix into the cytosol. Study in the cell line treated with TAA has also revealed the correlation between ROS production and increased lipid peroxidation. Imbalance between the production of ROS and the level on antioxidant defence system result in oxidative stress damage and therefore cell death.

Curcuma xanthorrhiza and *Ipomoea aquatica* crude extracts were screened for their *in vitro* hepatoprotective activity against TAA-induced damage in WRL-68 cell. In this study, *C. xanthorrhiza* and *I. aquatica* crude extracts inhibited WRL-68 cell death which induced by TAA oxidative stress. Both plant crude extracts prevented TAA oxidative stress damage and enhanced the WRL-68 cell viability. In the antioxidant study of the WRL-68 induced-TAA oxidative stress, the results supported the hepatoprotective findings in terms of improving antioxidant defence system which prevent cell death from TAA-induced oxidative stress. *C. xanthorrhiza* and *I. aquatica* crude extracts significantly increased the cellular SOD and CAT as compare to the TAA control group. The levels of MDA which represent the lipid peroxidation was also significantly reduced by the treatment with *C. xanthorrhiza* and *I. aquatica* crude extracts. This reprodiuceble *in vitro* hepatoprotective results justified the *in vivo* experiment.

5.4 *In vivo* hepatoprotective, antioxidant and antinflammatory properties of *Curcuma xanthorrhiza* and *Ipomoea aquatica* crude extracts

Thioacetamide is a selective hepatotoxin and widely used to induce acute and chronic liver injury in rats and other experimental animals depending on the dose and duration of administration (Hung, *et al.*, 2005; Wang, *et al.*, 2004). TAA induces rat liver cirrhosis with biochemical and morphological observations, resemble that of human liver cirrhosis and therefore serves as a suitable animal model for studying human liver fibrosis and cirrhosis (Fan & Weng, 2005).Oxidative stress and inflammation have been reported to contribute to the pathogenesis of TAA liver damage in animal model (Lin, *et al.*, 2009). The appropriate dose of TAA to induce liver damage in experimetal animals through intraperitoneal route of administration is reported to be 200 mg/kg (Chen, *et al.*, 2012). Its long-term administration induces hepatic fibrosis in rats, and causes the development of cirrhosis associated with an increased extent of lipid peroxidation (Kadir, *et al.*, 2011). Following administration, TAA is metabolised by the mixed function of oxidase system to acetamide and TAA-S-oxide ((Ahmed, *et al.*, 2008; Bastway Ahmed, *et al.*, 2010). Then TAA-S-oxide is metabolised, at least in part, by cytochrome P-450 monoxygenases to, a very highly reactive compound, TAA-S-dioxide (Wang, *et al.*, 2006). The binding of TAA-S-dioxide to tissue macromolecules induce hepatic necrosis and oxidative stress (Túnez, *et al.*, 2005). Besides, TAA produces oxidative stress, equally by both increased generation of ROS and also by reduction of cellular oxidative defences system. Studies have also revealed that liver cells intoxicated with TAA tend to be more susceptible to the cytotoxic effects of TNF-α along with other cytokines. It is well established that mitochondria has an essential role in the apoptotic response, therefore functional alteration in mitochondrial following TAA treatment lead to cell death through necrosis or apoptosis (Hoek & Pastorino, 2002).

TAA treatment (cirrhosis control) resulted in significant ($p<0.05$) body weight reduction as the animals suffered growth retardation and increase in liver weight and therefore significantly increased liver index. The increased liver index might be attributed to swelling of the liver by the TAA induced chronic inflammatory response. In accordance with our study, a study has also reported the decrease in the body weight and larger liver size in Sprague-Dawley rats treated with TAA in comparison with the untreated rats (Ljubuncic, *et al.*, 2005). Eight weeks treatment with ethanolic extracts of *C. xanthorrhiza*, *I. aquatica* extract and silymarin allowed natural growth pettern (body weight gain and liver index) towords normal.

Liver function biomarkers such as aspartate amino transferase (AST) and alanine aminotransferae (ALT) are cytosolic enzymes present in liver cells. TAA treatment causes the hepatocellular membrane damages through lipid peroxidation which leads to the alteration of the membrane permeability and leakage of liver enzymes in to the circulation system (Poli, 2000; Rees & Spector, 1961; Túnez, *et al.*, 2005). The elevated level of AST and ALT indicates the cellular leakage, loss of functional integrity of hepatic cell membrane and thus hepatotoxicity. (Drotman & Lawhorn, 1978; Fontana, *et al.*, 1996). Hence, in the present study experimental animals treated with TAA exhibited significant increase in the serum levels of AST and ALT (Figure 4.4 and 4.5), which ensure hepatotoxic effect of TAA. Treatment with *C. xanthorrhiza* and *I. aquatica* extracts at doses of 250 and 500 mg/kg, attenuated the elevated AST and ALT and brought them toward the level of normal control group and these results were in comparable with the standard drug silymarin. These results suggest that ethanolic extracts of *C. xanthorrhiza* and *I. aquatica* might protect the hepatocellular membrane structural integrity or enhance the regeneration of damaged hepatocytes.

Increased biliary pressure during the liver damage increases the alkaline phosphatase (ALP) and bilirubin synthesis and thus the increased serum level of them (Muriel, *et al.*,

1992). In the present study TAA induced the elevation of serum level of ALP and bilirubin which were reduced toward normal control level with the treatment of *C. xanthorrhiza* and *I. aquatica* extracts. These results suggest the ability of the selected plant extracts for the stability of biliary dysfunction in rats with TAA induced liver damage.

It is well known that following the hepatocellular damage the capacity of liver cells to synthesize proteins is reduced and as the extent of damage increases, the levels of these proteins in the plasma will tend to decrease. Figure 4.6 and 4.7 shows the similar observations in our studies, where the total protein and albumin levels were decreased markedly. However, silymarin as well *C. xanthorrhiza* and *I. aquatica* extract treated groups restored these levels of protein toward normal.

Mammalian cells contain various endogenous antioxidant enzymes such as superoxide dismutase(SOD), catalase (CAT), glutathione peroxidase and reduced glutathione which scavenge the reactive oxygen species (ROS) generated and maintains the balance (Saeed, *et al.*, 2005). These enzymes serve as biomarkers of hepatocellular injury as they easily inactivated by lipid peroxides or ROS formed by the use of alcohol and drug (Chattopadhyay & Bandyopadhyay, 2005; Recknagel, *et al.*, 1989). However, during the pathological conditions an imbalance occur between the endogenous antioxidative system and ROS leading to oxidative stress (Karan, *et al.*, 1999; Khan & Sultana, 2009). SOD is clinically important endogenous antioxidative enzymes that scavenge the highly reactive and potentially toxic superoxide anion to form hydrogen peroxide. Decrease in the level of this enzyme activity is considered as a sensitive index of liver cell damage (Curtis, *et al.*, 1972; Reiter, *et al.*, 2000). Catalase is another endogenous antioxidative enzyme which widely distributed in all animal tissues but highest activity is found the red blood cells and liver. CAT helps in eliminating highly reactive hydrogen peroxide radical and protects the tissue from the oxidative injury. Hence, reduction in the activity

of SOD and CAT may results in a number of deleterious effects due to accumulation of highly reactive superoxide anion and hydrogen peroxide radicals.

Lipid peroxidation refers to the oxidative degradation of lipids, is a multifarious and natural harmful process (Tribble, *et al.*, 2005). Malondialdehyde is a major breakdown output of lipid peroxidation and useful indicative for lipid peroxidation and free radical activity (Kurata, *et al.*, 1993). Increase in the lipid peroxidation causes changes in the hepatocellular metabolism which leads to cell deformity and death (Halliwell, *et al.*, 1992). Thiobarbituric acid reactive substances of liver are increased during lipid peroxidation which was measured to estimate the amount of Malondialdehyde.

Results of the present study revealed that TAA intoxication caused the imbalance between free radical production and the antioxidant defence which resulted in oxidative stress in the liver with significant reduction in the SOD and CAT and marked increase in the MDA. This suggests that TAA induce the hepatocellular damage through the oxidative stress. However, pretreatment with silymarin, *C. xanthorrhiza* and *I. aquatica* ethanolic extracts significantly accelerated the return of altered activities of SOD and CAT to the values of normal control and markedly reduced the amount of MDA. These evidences suggest that both *C. xanthorrhiza* and *I. aquatica* particularly at higher dose (500 mg/kg) exhibit hepatoprotective activity, at least in part, by improving endogenous antioxidant enzymes status and therefore inhibiting lipid peroxidation.

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine found in the fibrous liver tissues. Kupffer cells are the main source of TNF-α and are produced in higher amount when these cells are exposed to lipopolysaccaride, viruses, alcohol or to TAA (Reeves & Friedman, 2002; Schultze, *et al.*, 1999). Even HSC and sinusoidal endothelial cells upon activation secrete this inflammatory cytokine (Kurosaka, *et al.*, 2001). Elevated level of TNF-α is responsible for the inflammation of the liver and also for the necrosis as well as fat accumulation. Results of the present study shown that,

TAA significantly elevated the level of TNF-α in the serum of the cirrhotic control animal group as compared to the corresponding normal control animal group. This increase in TNF-α level might be due to the liver cirrhosis induced by TAA (Marinos, *et al.*, 1995). However, treatment with silymarin, *C. xanthorrhiza* and *I. aquatica* significantly reduced the serum level of TNF-α toward the level of normal control in dose dependent manner. Possessing antinflammatory properties, both plants, at least in part, might have inhibited fibrosis progression through the down regulation of proinflammatory cytokines in which TNF-α is the most important.

Transforming growth factor beta 1 (TGFβ1) is the most potent fibrogenic cytokine produced by the nonparenchymal cells such as Kupffer cells, HSC and endothelial cells during the tissue damage and oxidative stress (Bissell, *et al.*, 2003). Once TGFβ1 expressed in higher level it activates the HSC which in turn releases higher amount of platelet derived growth factors, collagen and also TGFβ1 (Freedman, *et al.*, 1992). This leads to the increased extracellular matrix production and finally ends up with the liver fibrosis (Kimura, *et al.*, 1999). The abnormal expression of TGFβ1 serves as a useful serologic marker in detecting the hepatocellular carcinoma. Since TGFβ1 have a low molecular weight it is released easily into the blood and leads to the presence of higher concentration (Song, *et al.*, 2001). Hence, the down regulation of TGFβ1 is very important in the effective treatment of liver fibrosis (Prosser, *et al.*, 2006).

In our study, animal's intoxicatied with TAA exhibited significant amount level of serum TGFβ1 compared to normal control group. Concurrent administration of *C. xanthorrhiza* and *I. aquatica* extracts as well as silymarin dramatically reduced the increased levels of TGFβ1. Hence, antinflammatory property of these plant extracts through inhibition of inflammation (which include inactivation of kupffer cell) might have indirectly reduced the expression of TGFβ1 from liver cells.

Nuclear factor-κB (NF-κB) is a transcriptional factor which is normally retained in the cytoplasm in an inactive form and play a pivotal role in the regulation of inflammation (Inoue, *et al.*, 1995). NF-κB is activated by several factors including interleukin-1, lipopolysccharide, TNF-α and oxidative stress. NF-κB normally induces the expression of genes involved in the proliferation, apoptosis and carcinogenesis of cells (Akira & Kishimoto, 1997). Once it is activated it induces the acute and chronic liver injury. Results obtained from this study reveals that treatment of experimental animals with TAA induced the elevation of NF-κB in a significant amount in comparison with the normal control group. In contrast, eight weeks treatment with *C. xanthorrhiza* and *I. aquatica* extracts significantly reduced the amount of NF-κB toword the level of normal control group. Hence, these plant samples might show their hepatoprotective effect by suppressing inflammation and fibrogenesis through NF-κB which regulate inflammatory in which TNF-α is the most impotent.

The hepatoprotective effect of *C. xanthorriza* and *I. aquatica* extract was confirmed by the histopathological findings. TAA administration leads to severe damage, congested central veins, sinusoid and multifocal area of necrosis, fatty changes and inflammatory cell with granular swelling in cirrhosis control group. Treatment *C. Xanthorriza, I. aquatic* extract and silymarin to some extent prevented the liver from these effects and preserved the liver architecture nearly to normal control group. Small necrosed area and also decrease in the infiltration of the inflammatory cells in the liver lobules confirm the efficacy of *C. xanthorriza* and *I. aquatic* in hepatoprotective. In addition, the Masson's trichome stain clearly demonstrated that fiber extension and collagen accumulation in cirrhosis control group. The deposition of collagen was markedly reduced in dose dependent manner by *C. xanthorriza* and *I. aquatic* extract and silymarin treatment.

Alpha smooth muscle actin (α-SMA) staining has been widely employed to detetct the extent of hepatic stellate cell (HSC) activation as the activation of HSCs leads to the

generation of α-SMA. The expession of α-SMA widely used as marker of activated HSC and also considered to be responsible for the liver fibrosis (Magness, *et al.*, 2004; Mann & Smart, 2002). Since HSC is activated by increased expression of TGFβ1 and TNF-α, down regulation of these cytokines by the plant extracts and silymarin might have reduced the activation of HSC and therefore expression of α-SMA.

5.5 *In vitro* hepatoprotective and antioxidant properties of isolated active fractions, xanthorrhizol and violaxanthin

In addition to time and expense, there are other benefits in *in vitro* hepatoprotective studies using human liver cells model in comparison with standard animal model. Particularly, one importent benefit is the fact that the cell line will not needed large quantity of the examined sample as in the case of animal model study and therefore suits the study of isolated fraction or compound, which generally yeiled in small amount. Furthermore usefulness of working with human cell models is to eliminate the issue of extrapolating animal data to humans (Olivares, *et al.*, 1997).

To find out the most effective fraction of *Curcuma xanthorrhiza* and *Ipomoea aquatica* crude extracts, all isolated fractions from both extracts were screened for their i*n vitro* hepatoprotective activity against TAA-induced damage in WRL-68 cell. The results showed that isolated fractions number 5 from *C. xanthorrhiza* and fraction number 11 from *I. aquatic* are the most. Both fractions produced the best results in term of normalization of the oxidative stress generated by TAA induction and therefore improving WRL-68 cell viability. All other fractions were less effective than their crude extracts. In the antioxidant study of the WRL-68 induced-TAA oxidative stress, the results supported the hepatoprotective findings in terms of improving antioxidant defence system which prevent cell death from TAA-induced oxidative stress. Similarly and even better than their crude extracts, fractions 5 from *C. xanthorrhiza* and fraction

11 from *I. aquatica* exhibited strong overall antioxidant properties by increasing SOD and CAT levels, and reducing MDA levels.

The LCMS results showed about 15 compounds in the fraction 5 of *C. xanthorrhiza* and about 19 compounds in the fraction 11 of *I. aquatic*. Among the compound found in these active fractions were xanthorrhizol and violaxanthin from *C. xanthorrhiza* and *I. aquatic*, respectively. Xanthorrhizol and violaxanthin were selected for further study based on the available information from the previous studies which could be used to infer that these compounds may be the main contributor in the hepatoprotective activity of the fraction and plant extract as whole. In a recent study violaxanthin isolated from *I. aquatic* has proved to be an excellent free radical scavenger and efficient inhibitor of lipid peroxidation (Fu, *et al.*, 2011). It has shown to be lipid peroxidation inhibitor, particularly in the isolated mouse liver cells. In another study, violaxanthin revealed to inhibit activation of nuclear factor kappa b (NF-κB). Nuclear factor kappa b activation is known to trigger the overexpression of proinflammatory genes in the nucleus, thereby initiating the inflammatory processes (Soontornchaiboon, *et al.*, 2012). On the other hand, xanthorrhizol revealed to have potent antioxidant activity and inhibitor of lipid peroxidation (Lim, *et al.*, 2005). It has also been reported that xanthorrhizol reduce proinflammatory mediators such as tumor necrosis factor-alpha (TNFα) and interleukin-6 (IL-6). Moreover, in a short period study, xanthorrhizol abrogated hepatotoxicity of cisplatin (an anticancer drug which can produce side effects including hepatotoxicity) (Hwan Kim, *et al.*, 2004).

The results of our *in vitro* study exhibited that both violaxanthin and xanthorrhizol are highly hepatoprotective against thioacetamide induced cell damage in WRL-68 normal liver cell line. Both compounds were more efficient than crude extracts and fractions in preventing TAA oxidative stress damage and enhancing the WRL-68 cell viability. It is probably that the efficiency of these compounds in preventing TAA oxidative stress

damage and enhancing the WRL-68 cell viability is antioxidant activity which was found to be stronger in of improving antioxidant defense system than that found in fraction of crude extracts. Violaxanthin and xanthorrhizol significantly increased the cellular SOD and CAT and reduced lipid peroxidation levels in the cell line. In fact, violaxanthin, the compound from *Ipomoea aquatic*, was more effective in hepatoprotective and improving antioxidant status than the silymarin, the standard drug used as control in this study.

5.6 Conclusion

The results obtained from this study demonstrated that ethanol crude extracts of *Curcuma xanthorrhiza* and *Ipomoea aquatica* possess hepatoprotective activities *in vivo* against thioacetamide-induced liver cirrhosis in rats which was proven in biochemical and histopathological findings. In the preliminary *in vitro* screening of free radical scavenging of both plants, *Curcuma xanthorrhiza* showed higher free radical scavenging activity than silymarin, however, the free radical scavenging of *Ipomoea aquatica* was moderate and less than silymarin. Nevertheless, both plant extracts significantly improved the overall oxidant status by increasing antioxidant enzymes and reducing lipid peroxidation. These evidences suggest that both *Curcuma xanthorrhiza* and *Ipomoea aquatic* particularly at higher dose (500 mg/kg) exhibit hepatoprotective activity, at least in part, by improving endogenous antioxidant enzymes status and therefore inhibiting lipid peroxidation. In addition to that, both plant extracts exhibited antinflammatory properties to which, at least in part, might have inhibited fibrosis progression through the down regulation of proinflammatory and profibrotic cytokines which lead to the decrease in hepatic stellate cell activation and therefore less extracellular matrix deposition. This was proven by liver tissue immunohistochemical staining of α-SMA and Masson’s trichome staining.

The hepatoprotective activities of both plant crude extracts were confirmed *in vitro* against thioacetamide induced cell damage in WRL-68 normal liver cell line. Furthermore, in hepatoprotective screening study of the fractions from both plant extracts, separated fraction 5 from *Curcuma xanthorrhiza* and fraction 11 from *Ipomoea aquatica* exhibited the highest hepatoprotective activities against thioacetamide induced cell damage in WRL-68 normal liver cell line. From the LCMS results of the fractions which exhibited best hepatoprotective results, violaxanthin from *Ipomoea aquatica* and xanthorrhizol from *Curcuma xanthorrhiza* were selected for further study. The selection of these compounds was based on the available information from the previous studies which could be used to infer that these compounds may be the main contributor in the hepatoprotective activity of the fraction and plant extract as whole. Both compounds were highly hepatoprotective against thioacetamide induced cell damage in WRL-68 normal liver cell line. Similar to the animal study, both crude extracts, their fractions and compounds improved antioxidant enzymes, inhibited lipid peroxidation. Both compounds were more effective than their crude extracts in protecting cell damage, improving antioxidant status. In fact, violaxanthin, the compound from *Ipomoea aquatic*, was more effective than the silymarin, the standard drug used as control in this study. These results propose that the possible mechanism of action of both plant extracts in hepatoprotective is likely to be through improving antioxidant enzymes, inhibiting lipid peroxidation and decreasing proinflammatory and profibrotic cytokines. Moreover, in addition to the fact that they are known to have antinflammatory activities, the extent of effectiveness of violaxanthin and xanthorrhiza in preventing cell death, improving antioxidant levels and inhibiting lipid peroxidation, suggest that they are likely to be the active compounds for the hepatoprotective activities of their plant extracts.

5.7 Future work

Violaxanthin and xanthorrhizol are quite expensive, however, they worth attention. Further studies are needed on pharmacological evaluation of these compounds in animal experiment in order to determine their pharmacodynamics, bioavailability and pharmacokinetics.

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APPENDIX A Preparation of reagents

A1. Reagent of hematoxylin

1. Pot or aluminium alum	6 gm
2. Mercuric oxide	1.25 gm
3. Hematoxylin	1.5 gm
4. Absolute alcohol	25 ml
5. Glacial Acetic acid	1 ml

Hematoxylin was dissolved in absolute alcohol, pot or aluminum alum was heated in 500 ml of distilled water until dissolved. Hematoxylin was added to mixture until boiled, then removed from heater and mercuric oxide was added. After it cooled, glacial acetic acid was added then filtered before use.

A2. Reagent of ethyl eosin

1.	Ethyl eosin	1 gm
2.	Absolute alcohol	95 ml
3.	Acetic acid	2 ml
4.	Distilled water	5 ml

Mixed well and filtered before use.

A3. Reagent of Masson's Trichromes stain

1. Lugol's Iodine

1. Iodine	1 g
2. Potassium iodide	2 g
3. Distilled water	100 ml

2. 5% Sodium thiosulphate

20 g of sodium thiosulphate were dissolved in 400 ml distilled water

3. Weigert iron hematoxylin

A. 10 g hematoxylin dissolved in 100 ml Alcohol

B. 15 g Ferric chloride dissolved in 100 ml distilled water

C. i) 10 ml from stock A mixed in 90 ml 95% alcohol

ii) 4 ml from stock B mixed in 95 ml distilled water and 1 ml HCl Equal part of solution (i) and (ii) were taken at time of use.

4. 1% acid alcohol

99 ml 70% OH mixed with 1 ml HC1 pure

5. 1% Ponceau fuschin

4 g Ponceau fuschin dissolved in 400 ml of 1% acetic acid

6. 1% Phosphomolybdic acid

4 g phosphomolybdic acid dissolved in 400 ml distilled water

7. 2% Light green

8 g Light green dissolved in 400 ml 2% acetic acid

8. 1% Acetic acid

1 ml acetic acid dissolved in 99 ml distilled water

A4. Preparation of thioacetamide

Thioacetamide stock solution of 5 mg L^{-1} was prepared by diluting the pure thioacetamide which is in the crystal form with distilled water and stirred well until all the crystals were dissolved.

E. Preparation of 10% Tween 20

100 ml of the stock solution was prepared by dissolving 10ml of 10% Tween-20 in 90 ml of distilled water.

A5. Preparation of 0.9% normal saline

9 g of NaCl was added to 1 L of distilled water.

A6. Preparation of phosphate buffer saline (PBS) PH 7.3+ 0.2

1 Liter PBS was prepared as follows: sodium chloride 8.0 g L^{-1}, potassium chloride 0.2 g L^{-1}, di-sodium phosphate 1.15 g L^{-1} and potassium di-hydrogen phosphate 0.2 g L^{-1} were dissolved in distilled water at 25°C.

A7. Preparation of 10% fresh formalin (Buffered formalin)

1 L of fresh formalin was prepared by dissolving 6.5 g di-sodium hydrogen phosphate with 4 g sodium di-hydrogen phosphate monohydrate and 100 ml concentrated formalin (38 - 40%) in 900 ml of phosphate buffer saline (PBS).

APPENDIX B Procedures and kits instructions

B1 Techniques for processing the tissues

Fixation

The tissue processing started with fixation by using 10% buffered formalin. The purpose of fixation is to preserve tissues permanently in life - like state as possible after removal of the tissues. The tissues were excised into small pieces of about 1 cm in size and then put in cassettes containing fresh formalin of 10:1 ratio of fixative to tissue for 48 hr.

a) Tissue processing

The technique of getting fixed tissue into paraffin is called tissue processing. The main steps in this process were:

i) Dehydration

Water from the tissues was removed by a procedure called dehydration in series of alcohol.

ii) Clearing

The removal of dehydrant was done with toluene that is miscible with the embedding medium, paraffin. Toluene is more tolerant of small amounts of water left in the tissues and less toxic than xylene.

iii) Infiltration and impregnation

Finally, the tissues were infiltrated with embedding agent, usually with the paraffin in oven less than 60 °C.

All the above processes were done b**y** using automatic tissue processor, which performed fixation, dehydration**,** clearing and paraffin impregnation of tissues in a programmed sequence. The protocols are provided in appendixes section.

iv) Tissue embedding

After the above processes, the tissues were manually transferred from the cassettes and put into the blocks with molten paraffin over them, with proper orientation of tissue in the block of paraffin.

c) Sectioning

Once the melted paraffin was cooled and hardened, the blocks were trimmed into an appropriately sized block and put into freezer under -4°C for 1 hr before sectioning.

Each block was then mounted in a specially designed slicing machine, a microtome. They were cut with steel knife into sections of 5 µm thickness. These sections were floated in a 40 °C warm water bath to avoid wrinkling, then they were picked up on a labeled glass microscopic slides. All these slides were then dried under 50 °C temperature.

b) Staining

Before staining, all the slides were deparaffinized by running them through xylenes I, II for 5 min each, in order to remove the paraffin wax out of the tissues and allow water soluble dyes to penetrate the sections.

The stains which were used in our experiment were H and E and special stains and Masson's Trichrome stain for detecting of collagen and elastic fibrous tissues.

i) Staining technique of hematoxylin and eosin

Thick paraffin sections (5 µm) of liver were de-waxed in xylene, dehydrated in series of alcohol to water then immersed in hematoxylin for 15 min. Sections were then differentiated with 1% acid alcohol and washed in tap water, followed by staining with eosin for 5 min. Finally, sections were dehydrated in series of alcohol, cleared in xylene, mounted with DPX.

ii) Staining technique of Masson's Trichrome

Sections were brought to water, stained with lugol's iodine for 5 min, then decolorized with 5% sodium thiosulphate and washed with tap water until clear and before staining with weigert's iron hematoxylin for 20 min. Section were decolorized with 1% acid alcohol and tap water, then stained with 1% ponceau- fuchsin for 5 min, mordant in 1% phosphomolybdic acid for 5 min and stained with 2% light green, then differentiated with 1% acetic acid. Finally, sections were dehydrated in series of alcohol, cleared in xylene, mounted with DPX .

B2 Catalase assay kit (Cayman)

Catalase Assay Kit

Item No. 707002

INTRODUCTION

Background

Catalase (EC 1.11.1.6; $2H_2O_2$ oxidoreductase) is an ubiquitous antioxidant enzyme that is present in most aerobic cells. Catalase (CAT) is involved in the detoxification of hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), which is a toxic product of both normal aerobic metabolism and pathogenic ROS production. This enzyme catalyzes the conversion of two molecules of H_2O_2 to molecular oxygen and two molecules of water (catalytic activity). CAT also demonstrates peroxidatic activity, in which low molecular weight alcohols can serve as electron donors. While aliphatic alcohols serve as specific substrates for CAT, other enzymes with peroxidatic activity do not utilize these substrates.

(Catalytic Activity) $2H_2O_2 \xrightarrow{\text{Catalase}} O_2 + 2H_2O$

(Peroxidatic Activity) $H_2O_2 + AH_2 \xrightarrow{\text{Catalase}} A + 2H_2O$

In humans, the highest levels of CAT are found in liver, kidney, and erythrocytes, where it is believed to account for the majority of H_2O_2 decomposition.

About This Assay

Cayman's Catalase Assay Kit utilizes the peroxidatic function of CAT for determination of enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen.[1,2] Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple color.[1,2] The assay can be used to measure CAT activity in plasma, serum, erythrocyte lysates, tissue homogenates, and cell lysates.

PRE-ASSAY PREPARATION

Reagent Preparation

NOTE: Methanol is no longer supplied in this kit. It can be purchased separately under Item No. 700016 or you can supply your own.

1. **Catalase Assay Buffer (10X) – (Item No. 707010)**

   Each vial contains 5 ml of Assay Buffer. Dilute 2 ml of Catalase Assay Buffer concentrate with 18 ml of HPLC-grade water. This final Assay Buffer (100 mM potassium phosphate, pH 7.0) should be used in the assay. When stored at 4°C, this diluted Assay Buffer is stable for at least two months. Prepare the additional vial as needed.

2. **Catalase Sample Buffer (10X) – (Item No. 707012)**

   Each vial contains 10 ml of Sample Buffer. Dilute 5 ml of Catalase Sample Buffer concentrate with 45 ml of HPLC-grade water. This final Sample Buffer (25 mM potassium phosphate, pH 7.5, containing 1 mM EDTA and 0.1% BSA) should be used to dilute the formaldehyde standards, Catalase (Control), and CAT samples prior to assaying. When stored at 4°C, this diluted Sample Buffer is stable for at least two months. Prepare the additional vial as needed.

3. **Catalase Formaldehyde Standard - (Item No. 707014)**

   The vial contains 4.25 M formaldehyde. The reagent is ready to use as supplied.

4. **Catalase (Control) – (Item No. 707013)**

   Each vial contains a lyophilized powder of bovine liver CAT and is used as a positive control. Reconstitute the Catalase (Control) by adding 2 ml of diluted Sample Buffer to the vial and vortex well. Take 100 µl of the reconstituted enzyme and dilute with 1.9 ml of diluted Sample Buffer. A 20 µl aliquot of this diluted enzyme per well causes an absorbance of approximately 0.29 after subtracting the background absorbance. The diluted enzyme is stable for 30 minutes. The reconstituted Catalase (Control) is stable for one month at -20°C.

5. **Catalase Potassium Hydroxide – (Item No. 707015)**

   Each vial contains potassium hydroxide (KOH) pellets. Place the vial on ice, add 4 ml of cold HPLC-grade water, and vortex to yield a 10 M solution. *CAUTION: Heat is generated when Catalase Potassium Hydroxide pellets are dissolved in water. The diluted Potassium Hydroxide solution is stable for at least three months if stored at 4°C.*

6. **Catalase Hydrogen Peroxide – (Item No. 707011)**

   The vial contains an 8.82 M solution of H_2O_2. Dilute 40 µl of Catalase Hydrogen Peroxide with 9.96 ml of HPLC-grade water. The diluted Hydrogen Peroxide solution is stable for two hours.

7. **Catalase Purpald (Chromagen) – (Item No. 707017)**

   Each vial contains 4 ml of 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) in 0.5 M hydrochloric acid. The reagent is ready to use as supplied.

8. **Catalase Potassium Periodate – (Item No. 707018)**

   Each vial contains 1.5 ml of potassium periodate in 0.5 M potassium hydroxide. The reagent is ready to use as supplied.

Sample Preparation

Overheating can inactivate catalase. The enzyme should be kept cold during sample preparation and assaying. In general, catalase is very unstable at high dilution. It is recommended to store samples concentrated and assay within 30 minutes after dilution.

Tissue Homogenate

1. Prior to dissection, either perfuse tissue or rinse tissue with a phosphate buffered saline (PBS) solution, pH 7.4, to remove any red blood cells and clots.
2. Homogenize the tissue on ice in 5-10 ml of cold buffer (*i.e.*, 50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA) per gram tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Cell Lysate

1. Collect cells by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. Homogenize or sonicate the cell pellet on ice in 1-2 ml of cold buffer (*i.e.*, 50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Plasma and Erythrocyte Lysate

1. Collect blood using an anticoagulant such as heparin, citrate, or EDTA.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample will be stable for at least one month.
3. Remove the white buffy layer (leukocytes) and discard.
4. Lyse the erythrocytes (red blood cells) in four times its volume of ice-cold HPLC-grade water.
5. Centrifuge at 10,000 x g for 15 minutes at 4°C.
6. Collect the supernatant (erythrocyte lysate) for assaying and store on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month.

Serum

1. Collect blood without using an anticoagulant. Allow blood to clot for 30 minutes at 25°C.
2. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month.

Tissue Homogenization using the Precellys 24 Homogenizer

- Prior to dissection, either perfuse or rinse tissue with phosphate buffered saline (PBS), pH 7.4, to remove any red blood cells and clots.
- Freeze organs immediately upon collection and then store at -80°C. Snap-freezing of tissues in liquid nitrogen is preferred.
- Add cold 50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA.
- Homogenize the tissue sample using the Precellys 24 according to appropriate settings.
- Spin the tissue homogenates at 10,000 x g for 15 minutes at 4°C.
- Collect supernatant and assay samples according to the kit booklet protocol. Samples may need to be diluted appropriately for assay and should be normalized using a protein assay.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. We suggest that there be at least two wells designated as positive controls. A typical layout of formaldehyde standards and samples to be measured in duplicate is shown in Figure 1. We suggest you record the contents of each well on the template sheet provided on page 23.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	B	B	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	C	C	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	D	D	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	E	E	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	F	F	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	G	G	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	+	+	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

A-G = Standards
+ = Positive Controls
S1-S40 = Sample Wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 240 µl in all the wells.
- All reagents except samples must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- If the expected CAT activity of the sample is not known or if it is expected to be beyond the range of the standard curve, it is prudent to assay the sample at several dilutions.
- It is recommended that the samples and formaldehyde standards be assayed at least in duplicate.
- Use the diluted Assay Buffer in the assay.
- Monitor the absorbance at 540 nm using a plate reader.

Standard Preparation

1. Preparation of the Formaldehyde Standards - Dilute 10 µl of Catalase Formaldehyde Standard (Item No. 707014) with 9.99 ml of diluted Sample Buffer to obtain a 4.25 mM formaldehyde stock solution. Take seven clean glass test tubes and mark them A-G. Add the amount of formaldehyde stock and diluted Sample Buffer to each tube as described in Table 1 (below).

Tube	Formaldehyde (µl)	Sample Buffer (µl)	Final Concentration (µM formaldehyde)*
A	0	1,000	0
B	10	990	5
C	30	970	15
D	60	940	30
E	90	910	45
F	120	880	60
G	150	850	75

Table 1

*Final formaldehyde concentration in the 170 µl reaction.

Performing the Assay

1. **Formaldehyde Standard Wells** - Add 100 µl of diluted Assay Buffer, 30 µl of methanol, and 20 µl of standard (tubes A-G) per well in the designated wells on the plate (see sample plate format, Figure 1, page 12).
2. **Positive Control Wells (bovine liver CAT)** - Add 100 µl of diluted Assay Buffer, 30 µl of methanol, and 20 µl of diluted Catalase (Control) to two wells.
3. **Sample Wells** - Add 100 µl of diluted Assay Buffer, 30 µl of methanol, and 20 µl of sample to two wells. To obtain reproducible results, the amount of CAT added to the well should result in an activity between 2-35 nmol/min/ml. When necessary, samples should be diluted with diluted Sample Buffer or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 100,000 to bring the enzymatic activity to this level.
4. Initiate the reactions by adding 20 µl of diluted Hydrogen Peroxide to all the wells being used. Make sure to note the precise time the reaction is initiated and add the diluted Hydrogen Peroxide as quickly as possible.
5. Cover the plate with the plate cover and incubate on a shaker for 20 minutes at room temperature.
6. Add 30 µl of diluted Potassium Hydroxide to each well to terminate the reaction and then add 30 µl of Catalase Purpald (Chromagen) (Item No. 707017) to each well.
7. Cover the plate with the plate cover and incubate for 10 minutes at room temperature on the shaker.
8. Add 10 µl of Catalase Potassium Periodate (Item No. 707018) to each well. Cover with plate cover and incubate five minutes at room temperature on a shaker.
9. Read the absorbance at 540 nm using a plate reader.

ANALYSIS

Calculations

Determination of the Reaction Rate

1. Calculate the average absorbances of each standard and sample.
2. Subtract the average absorbance of standard A from itself and all other standards and samples.
3. Plot the corrected absorbance of standards (from step 2 above) as a function of final formaldehyde concentration (µM) from Table 1. See Figure 2 for a typical standard curve.

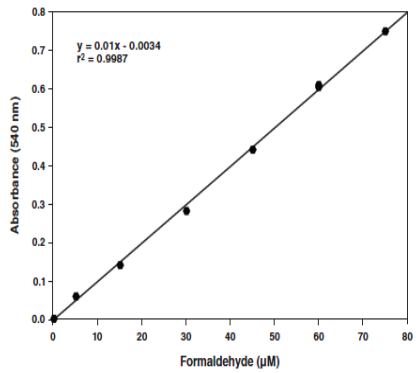


Figure 2. Formaldehyde standard curve

4. Calculate the formaldehyde concentration of the samples using the equation obtained from the linear regression of the standard curve substituting corrected absorbance values for each sample.

$$\text{Formaldehyde (µM)} = \left[\frac{\text{sample absorbance - (y-intercept)}}{\text{slope}}\right] \times \frac{0.17\ \text{ml}}{0.02\ \text{ml}}$$

5. Calculate the CAT activity of the sample using the following equation. One unit is defined as the amount of enzyme that will cause the formation of 1.0 nmol of formaldehyde per minute at 25°C.

$$\text{CAT Activity} = \frac{\text{µM of sample}}{20\ \text{min.}} \times \text{Sample dilution} = \text{nmol/min/ml}$$

Performance Characteristics

Sensitivity:

The dynamic range of the assay is limited only by the accuracy of the absorbance measurement. Most plate readers are linear to an absorbance of 1.2. Samples containing CAT activity between 2-35 nmol/min/ml can be assayed without further dilution or concentration.

Precision:

When a series of 45 CAT measurements were performed on the same day, the intra-assay coefficient of variation was 3.8%. When a series of 45 CAT measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 9.9%.

B3 Superoxide dismultase assay kit (Cayman)

Superoxide Dismutase Assay Kit

Item No. 706002

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring an absorbance at 440-460 nm
2. Adjustable pipettes and a repeat pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Superoxide dismutases (SODs) are metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide and thus form a crucial part of the cellular antioxidant defense mechanism.[1]

$$2O_2^{\bullet-} + 2H^+ + SOD \rightarrow H_2O_2 + O_2$$

Three types of SODs have been characterized according to their metal content: copper/zinc (Cu/Zn), manganese (Mn), and iron (Fe). SOD is widely distributed in both plants and animals. It occurs in high concentrations in brain, liver, heart, erythrocytes, and kidney. In humans, there are three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial MnSOD, and extracellular SOD.[2] Extracellular SOD is found in the interstitial spaces of tissues and also in extracellular fluids, accounting for the majority of the SOD activity in plasma, lymph, and synovial fluid.[3,4]

The amount of SOD present in cellular and extracellular environments is crucial for the prevention of diseases linked to oxidative stress. Mutations in SOD account for approximately 20% of familial amyotrophic lateral sclerosis (ALS) cases. SOD also appears to be important in the prevention of other neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases.[5,6] The reaction catalyzed by SOD is extremely fast, having a turnover of 2×10^9 $M^{-1}sec^{-1}$ and the presence of sufficient amounts of the enzyme in cells and tissues typically keeps the concentration of superoxide (O_2^-) very low.[1] However, in a competing reaction, nitric oxide (NO) reacts with O_2^- with a rate constant of 6.7×10^9 $M^{-1}sec^{-1}$ to form the powerful oxidizing and nitrating agent, peroxynitrite.[7] Under conditions in which SOD activity is low or absent (*i.e.*, SOD mutation) or which favor the synthesis of µM concentrations of NO (*i.e.*, ischemia/reperfusion, iNOS upregulation, etc.), NO out-competes SOD for superoxide, resulting in the formation of peroxynitrite. The presence of nitrotyrosine as a "footprint" for peroxynitrite, and hence the prior co-existence of both O_2^- and NO, has been observed in a variety of medical conditions, including atherosclerosis, sepsis, and ALS.[7]

About This Assay

Cayman's Superoxide Dismutase Assay Kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine (see scheme 1, below). One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The SOD assay measures all three types of SOD (Cu/Zn, Mn, and FeSOD). The assay provides a simple, reproducible, and fast tool for assaying SOD activity in plasma, serum, erythrocyte lysates, tissue homogenates, and cell lysates. Mitochondrial MnSOD can be assayed separately following the procedure outlined under sample preparation (see page 8).

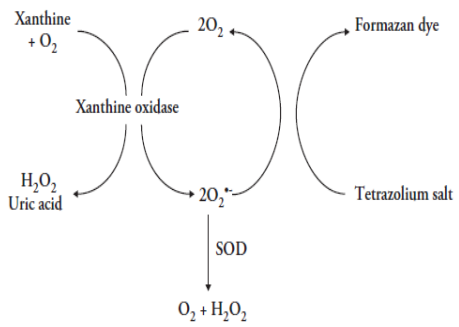


Figure 1. Scheme of the Superoxide Dismutase Assay

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Assay Buffer (10X) - (Item No. 706001)**

   Dilute 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water for assaying 96 wells. Prepare additional Assay Buffer as needed. This final Assay Buffer (50 mM Tris-HCl, pH 8.0, containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and 0.1 mM hypoxanthine) should be used to dilute the radical detector. When stored at 4°C, this diluted Assay Buffer is stable for at least two months.

2. **Sample Buffer (10X) - (Item No. 706003)**

   Dilute 2 ml of Sample Buffer concentrate with 18 ml of HPLC-grade water for assaying 96 wells. Prepare additional Sample Buffer as needed. This final Sample Buffer (50 mM Tris-HCl, pH 8.0) should be used to prepare the SOD standards and dilute the xanthine oxidase and SOD samples prior to assaying. When stored at 4°C, this diluted Sample Buffer is stable for at least six months.

3. **Radical Detector - (Item No. 706004)**

   The vials contain 250 µl of a tetrazolium salt solution. Prior to use, transfer 50 µl of the supplied solution to another vial and dilute with 19.95 ml of diluted Assay Buffer. Cover with tin foil. The diluted Radical Detector is stable for two hours. This is enough Radical Detector for 96 wells. Prepare additional detector as needed. Store unused Radical Detector at -20°C.

4. **SOD Standard - (Item No. 706005)**

   The vials contain 100 µl of bovine erythrocyte SOD (Cu/Zn). Store the thawed enzyme on ice and see **Standard Preparation** on page 13 for preparing the standard curve. Store unused enzyme at -20°C. The enzyme is stable for at least two freeze/thaw cycles.

5. Xanthine Oxidase - (Item No. 706006)

These vials contain 150 µl of Xanthine Oxidase. Prior to use, thaw one vial and transfer 50 µl of the supplied enzyme to another vial and dilute with 1.95 ml of Sample Buffer (dilute). Store the thawed and diluted xanthine oxidase on ice. The diluted enzyme is stable for one hour. This is enough Xanthine Oxidase for 96 wells. Prepare additional Xanthine Oxidase as needed. Do not refreeze the thawed enzyme. Any unused enzyme should be thrown away.

Sample Preparation

The procedures listed below for tissue homogenates and cell lysates will result in assaying total SOD activity (cytosolic and mitochondrial). To separate the two enzymes, centrifuge the 1,500 x g supernatant at 10,000 x g for 15 minutes at 4°C. The resulting 10,000 x g supernatant will contain cytosolic SOD and the pellet will contain mitochondrial SOD.[8] Homogenize the mitochondrial pellet in cold buffer (*i.e.*, 20 mM Hepes, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose). If not assaying on the same day, freeze the samples at -80°C. The samples will be stable for at least one month.

The addition of 1-3 mM potassium cyanide to the assay will inhibit both Cu/Zn-SOD and extracellular SOD, resulting in the detection of only Mn-SOD activity.[3,9]

Samples can be assayed in the absence of Xanthine Oxidase to generate a sample background. This sample background absorbance should be subtracted from the sample absorbance generated in the presence of Xanthine Oxidase thus correcting for non-SOD generated absorbance.

Tissue Homogenate

1. Prior to dissection, either perfuse or rinse tissue with phosphate buffered saline (PBS), pH 7.4, to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose per gram tissue.
3. Centrifuge at 1,500 x g for five minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Cell Lysate

1. Collect cells by centrifugation at 1,000-2,000 x g for 10 minutes at 4°C. For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. Homogenize or sonicate the cell pellet in cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose.
3. Centrifuge at 1,500 x g for five minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Plasma and Erythrocyte Lysate

1. Collect blood using an anticoagulant such as heparin, citrate, or EDTA.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample will be stable for at least one month. Plasma should be diluted 1:5 with Sample Buffer before assaying for SOD activity.
3. Remove the white buffy layer (leukocytes) and discard.
4. Lyse the erythrocytes (red blood cells) in four times its volume of ice-cold HPLC-grade water.
5. Centrifuge at 10,000 x g for 15 minutes at 4°C.
6. Collect the supernatant (erythrocyte lysate) for assaying and store on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month. The erythrocyte lysate should be diluted 1:100 with Sample Buffer before assaying for SOD activity.

Serum

1. Collect blood without using an anticoagulant such as heparin, citrate, or EDTA. Allow blood to clot for 30 minutes at 25°C.
2. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month.
3. Serum should be diluted 1:5 with Sample Buffer before assaying for SOD activity.

Tissue Homogenization using the Precellys 24 Homogenizer

- Prior to dissection, either perfuse or rinse tissue with phosphate buffered saline (PBS), pH 7.4, to remove any red blood cells and clots.
- Freeze organs immediately upon collection and then store at -80°C. Snap-freezing of issues in liquid nitrogen is preferred.
- Add cold 20 mM HEPES buffer, pH 7.2, containing 1mM EGTA, 210 mM mannitol, and 70mM sucrose.
- Homogenize the tissue sample using the Precellys 24 according to appropriate settings:
- Spin the tissue homogenates at 10,000 x g for 15 minutes at 4°C.
- Collect supernatant and assay samples according to the kit booklet protocol. Samples may need to be diluted appropriately for assay and should be normalized using a protein assay.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of SOD standards and samples to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 23).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
B	B	B	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
C	C	C	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
D	D	D	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
E	E	E	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
F	F	F	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
G	G	G	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
H	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41

A-G = Standards
S1-S41 = Sample Wells

Figure 2. Sample plate format

Performing the Assay

1. **SOD Standard Wells** - add 200 µl of the diluted Radical Detector and 10 µl of Standard (tubes A-G) per well in the designated wells on the plate (see sample plate format, Figure 2, page 11).
2. **Sample Wells** - add 200 µl of the diluted Radical Detector and 10 µl of sample to the wells. *NOTE: If using an inhibitor, add 190 µl of the diluted Radical Detector, 10 µl of inhibitor, and 10 µl of sample to the wells. The amount of sample added to the well should always be 10 µl. Samples should be diluted with Sample Buffer (dilute) or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 10,000 to bring the enzymatic activity to fall within the standard curve range.*
3. Initiate the reactions by adding 20 µl of diluted Xanthine Oxidase to all the wells you are using. Make sure to note the precise time you started and add the Xanthine Oxidase as quickly as possible. *NOTE: If assaying sample backgrounds, add 20 µl of Sample Buffer instead of Xanthine Oxidase.*
4. Carefully shake the 96-well plate for a few seconds to mix. Cover with the plate cover.
5. Incubate the plate on a shaker for 20 minutes at room temperature. Read the absorbance at 440-460 nm using a plate reader.

ANALYSIS

Calculations

1. Calculate the average absorbance of each standard and sample. If assayed, subtract sample background absorbance from the sample.
2. Divide standard A's absorbance by itself and divide standard A's absorbance by all the other standards and samples absorbances to yield the linearized rate (LR) (*i.e.*, LR for Std A = Abs Std A/Abs Std A; LR for Std B = Abs Std A/Abs Std B).
3. Plot the linearized SOD standard rate (LR) (from step 2 above) as a function of final SOD Activity (U/ml) from Table 1. See Figure 3 (on page 17) for a typical standard curve.
4. Calculate the SOD activity of the samples using the equation obtained from the linear regression of the standard curve substituting the linearized rate (LR) for each sample. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. SOD activity is standardized using the cytochrome c and xanthine oxidase coupled assay

$$\text{SOD (U/ml)} = \left[\left(\frac{\text{sample LR - y-intercept}}{\text{slope}}\right) \times \frac{0.23\ \text{ml}}{0.01\ \text{ml}}\right] \times \text{sample dilution}$$

Performance Characteristics

Precision:

When a series of 60 SOD standard measurements were performed on the same day, the intra-assay coefficient of variation was 3.2%. When a series of 60 SOD standard measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 3.7%.

Assay Range:

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0.025-0.25 units/ml SOD.

Representative Superoxide Dismutase Standard Curve

The standard curve presented here is an example of the data typically provided with this kit; however, your results will not be identical to these. You must run a new standard curve - do not use this one to determine the values of your samples.

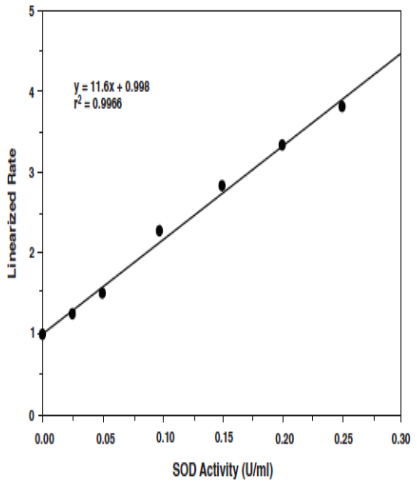


Figure 3. Superoxide Dismutase standard curve

B4 TBARS (MDA) assay kit (Cayman)

TBARS Assay Kit

Item No. 10009055

INTRODUCTION

Background

Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation. Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals and is used as an indicator of oxidative stress in cells and tissues.[1,2] Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds, such as MDA. In human platelets, thromboxane synthase also catalyzes the conversion of PGH_2 to thromboxane A_2, 12(S)-HHTrE, and MDA in a ratio of 1:1:1.[3]

The measurement of Thiobarbituric Acid Reactive Substances (TBARS) is a well-established method for screening and monitoring lipid peroxidation.[1,2] Modifications of the TBARS assay by many researchers have been used to evaluate several types of samples including human and animal tissues and fluids, drugs, and foods.[4-8] Even though there remains a controversy cited in literature regarding the specificity of TBARS toward compounds other than MDA, it still remains the most widely employed assay used to determine lipid peroxidation.[2] If lipoprotein fractions are first acid precipitated from the sample, interfering soluble TBARS are minimized, and the test becomes quite specific for lipid peroxidation.[5] Lipids with greater unsaturation will yield higher TBARS values. It is recommended that if high TBARS values are obtained, a more specific assay such as HPLC should be performed.

About This Assay

Cayman's TBARS Assay Kit provides a simple, reproducible, and standardized tool for assaying lipid peroxidation in plasma, serum, urine, tissue homogenates, and cell lysates. The MDA-TBA adduct formed by the reaction of MDA and TBA under high temperature (90-100°C) and acidic conditions is measured colorimetrically at 530-540 nm or fluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 550 nm. Although this reaction has a much higher sensitivity when measured fluorometrically, protocols for both methods are provided (see Figure 1 below).

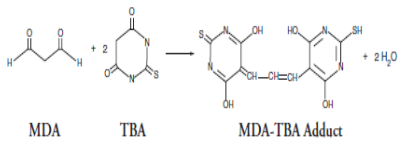


Figure 1.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Thiobarbituric Acid - (Item No. 10009199)**

   The vial contains 2 g of thiobarbituric acid (TBA). It is ready to use to prepare the Color Reagent.

2. **TBA Acetic Acid - (Item No. 10009200)**

   Each vial contains 20 ml of concentrated acetic acid. Slowly add both vials (40 ml) of TBA Acetic Acid to 160 ml of HPLC-grade water. This diluted Acetic Acid Solution is used in preparing the Color Reagent. The diluted Acetic Acid Solution is stable for at least three months at room temperature.

3. **TBA Sodium Hydroxide (10X) - (Item No. 10009201)**

   The vial contains a solution of sodium hydroxide (NaOH). Dilute 20 ml of TBA NaOH with 180 ml of HPLC-grade water. This diluted NaOH Solution is used in preparing the Color Reagent. The diluted NaOH Solution is stable for at least three months at room temperature. Store the diluted NaOH Solution in a plastic container suitable for corrosive materials.

4. **TBA Malondialdehyde Standard - (Item No. 10009202)**

   The vial contains 500 µM Malondialdehyde (MDA) in water. It is ready to use to prepare the standard curve.

5. **TBA SDS Solution - (Item No. 10009203)**

   The vial contains a solution of sodium dodecyl sulfate (SDS). The solution is ready to use as supplied.

6. **To prepare the Color Reagent:**

   The following amount of Color Reagent is sufficient to evaluate 24 samples. Adjust the volumes accordingly if more or less samples are going to be assayed. Weigh 530 mg of TBA (Item No. 10009199) and add to ≥150 ml beaker containing 50 ml of diluted TBA Acetic Acid Solution. Add 50 ml of diluted TBA Sodium Hydroxide and mix until the TBA is completely dissolved. The solution is stable for 24 hours.

Sample Preparation

Plasma

Typically, normal human plasma has a lipid peroxide level (expressed in terms of MDA) of 1.86-3.94 µM.[1,8]

1. Collect blood using an anticoagulant such as heparin, EDTA, or citrate.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice. If not assaying the same day, freeze at -80°C. The plasma sample will be stable for one month while stored at -80°C.
3. Plasma does not need to be diluted before assaying.

Serum

Typically, normal human serum has a lipid peroxide level (expressed in terms of MDA) of 1.86-3.94 µM.[1]

1. Collect blood without using an anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The serum sample will be stable for one month while stored at -80°C.
4. Serum does not need to be diluted before assaying.

Urine

Typically, normal human urine has a lipid peroxide level (expressed in terms of MDA) of 0.8-2 µmol/g creatinine.[9,10]

1. Urine does not require any special treatments. If not assaying the same day, freeze at -80°C.

Tissue Homogenates

1. Weigh out approximately 25 mg of tissue into a 1.5 ml centrifuge tube.
2. Add 250 µl of RIPA Buffer (Item No. 10010263) with protease inhibitors of choice (see **Interferences** section on page 19).
3. Sonicate for 15 seconds at 40V over ice.
4. Centrifuge the tube at 1,600 x g for 10 minutes at 4°C. Use the supernatant for analysis. Store supernatant on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for one month.
5. Tissue homogenates do not need to be diluted before assaying.

Cell Lysates

1. Collect 2 x 10^7 cells in 1 ml of cell culture medium or buffer of choice, such as PBS.
2. Sonicate 3X for five second intervals at 40V setting over ice.
3. Use the whole homogenate in the assay, being sure to use the culture medium as a sample blank.
4. Cell lysates do not need to be diluted before assaying.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of standards and samples to be measured in duplicate is shown below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 23).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	B	B	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	C	C	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	D	D	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	E	E	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	F	F	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	G	G	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	H	H	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

A-H = Standards
S1-S40 = Sample Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- All reagents except samples must be equilibrated to room temperature before beginning the assay. The SDS Solution will take at least one hour to equilibrate to room temperature if stored at 2-8°C. Briefly heating the SDS Solution at 37°C will re-dissolve the precipitated SDS. The SDS Solution can then be stored at room temperature.
- The final volume of the assay is 150 µl in all wells.
- The assay is performed at room temperature.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples and standards be assayed at least in duplicate.
- It is recommended that the samples and standards be kept at 4°C after preparation to increase sensitivity and reproducibility.
- Monitor the absorbance at 530-540 nm or read fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 550 nm.

Colorimetric Standard Preparation

Dilute 250 µl of the MDA Standard (Item No. 10009202) with 750 µl of water to obtain a stock solution of 125 µM. Take eight clean glass test tubes and label them A-H. Add the amount of 125 µM MDA stock solution and water to each tube as described in Table 1.

Tube	MDA (µl)	Water (µl)	MDA Concentration (µM)
A	0	1,000	0
B	5	995	0.625
C	10	990	1.25
D	20	980	2.5
E	40	960	5
F	80	920	10
G	200	800	25
H	400	600	50

Table 1. MDA colorimetric standards

Fluorometric Standard Preparation

Dilute 25 µl of the MDA Standard (Item No. 10009202) with 975 µl of water to obtain a stock solution of 12.5 µM. Take eight clean glass test tubes and label them A-H. Add the amount of 12.5 µM MDA stock solution and water to each tube as described in Table 2.

Tube	MDA (µl)	Water (µl)	MDA Concentration (µM)
A	0	1,000	0
B	5	995	0.0625
C	10	990	0.125
D	20	980	0.25
E	40	960	0.5
F	80	920	1
G	200	800	2.5
H	400	600	5

Table 2. MDA fluorometric standards

Performing the Assay

1. Label vial caps with standard number or sample identification number.
2. Add 100 µl of sample or standard to appropriately labeled 5 ml vial.
3. Add 100 µl of SDS Solution to vial and swirl to mix.
4. Add 4 ml of the Color Reagent forcefully down side of each vial.
5. Cap vials and place vials in foam or some other holder to keep the tubes upright during boiling.
6. Add vials to vigorously boiling water. Boil vials for one hour.
7. After one hour, immediately remove the vials and place in ice bath to stop reaction. Incubate on ice for 10 minutes.
8. After 10 minutes, centrifuge the vials for 10 minutes at 1,600 x g at 4°C. Vials may appear clear or cloudy. Cloudiness will clear upon warming to room temperature.
9. Vials are stable at room temperature for 30 minutes.
10. Load 150 µl (in duplicate) from each vial to either the clear plate (colorimetric version) or to the black plate (fluorometric version).
11. Read the absorbance at 530-540 nm or read fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 550 nm.

ANALYSIS

Colorimetric Calculations

1. Calculate the average absorbance of each standard and sample.
2. Subtract the absorbance value of the standard A (0 µM) from itself and all other values (both standards and samples). This is the corrected absorbance.
3. Plot the corrected absorbance values (from step 2 above) of each standard as a function of MDA concentration (see Table 1, on page 13).
4. Calculate the values of MDA for each sample from the standard curve. An example of the MDA standard curve is shown below in Figure 3.

$$\text{MDA (µM)} = \left[\frac{\text{(Corrected absorbance) - (y-intercept)}}{\text{Slope}}\right]$$

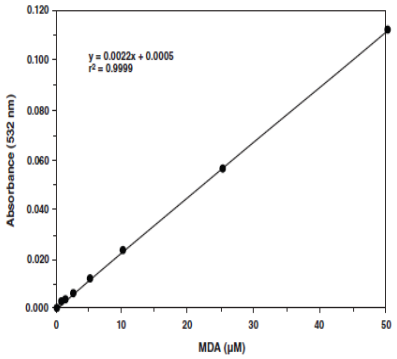


Figure 3. MDA colorimetric standard curve

Fluorometric Calculations

1. Calculate the average fluorescence of each standard and sample.
2. Subtract the fluorescence value of the standard A (0 µM) from itself and all other values (both standards and samples). This is the corrected fluorescence.
3. Plot the corrected fluorescence values (from step 2 above) of each standard as a function of MDA concentration (see Table 2, on page 14).
4. Calculate the values of MDA for each sample from the standard curve. An example of the MDA standard curve is shown below in Figure 4.

$$\text{MDA (µM)} = \left[\frac{\text{(Corrected fluorescence) - (y-intercept)}}{\text{Slope}}\right]$$

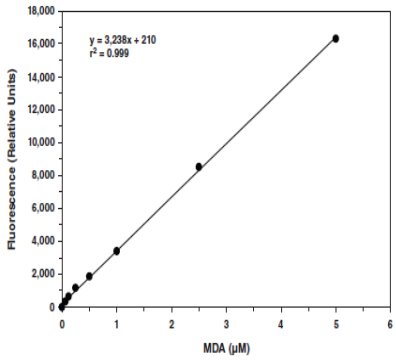


Figure 4. MDA fluorometric standard curve

B5 TNF-α assay kit (Quantikine)

Rat TNF-α Immunoassay

Catalog Number RTA00
SRTA00
PRTA00

For the quantitative determination of rat tumor necrosis factor alpha (TNF-α) concentrations in cell culture supernates, rat serum, and plasma.

INTRODUCTION

Tumor necrosis factor alpha (TNF-α), also known as cachectin; and tumor necrosis factor beta (TNF-β), also known as lymphotoxin, are two closely related proteins (approximately 34% amino acid sequence identity) that bind to the same cell surface receptors and show many common biological functions. TNF-α and -β play critical roles in normal host resistance to infection and to the growth of malignant tumors, serving as immunostimulants and as mediators of the inflammatory response. Over-production of TNFs, however, has been implicated as playing a role in a number of pathological conditions, including cachexia, septic shock, and autoimmune disorders. TNF-α is produced by activated macrophages and other cell types including T and B cells, NK cells, LAK cells, astrocytes, endothelial cells, smooth muscle cells and some tumor cells (1 - 4).

Rat TNF-α cDNA encodes a 235 amino acid (aa) residue type II membrane protein (5). The 156 aa residue soluble TNF-α is released from the C-terminus of the membrane-anchored TNF-α by TNF-α-converting enzyme (TACE), a matrix metalloprotease (6, 7). The membrane-anchored form of TNF-α has been shown to have lytic activity and may also play an important role in intercellular communication (8). The biologically active TNF-α has been shown to exist as a trimer (9, 10).

Two distinct TNF receptors, referred to as type I (or type B or p55) and type II (or type A or p75), that specifically bind TNF-α and TNF-β with equal affinity have been identified (11, 12). The two TNF receptors transduce signals independently of one another. The amino acid sequence of the extracellular domains of the two receptors are homologous and both receptors are members of the TNF receptor family which also include the NGF receptor, fas antigen, CD27, CD30, and CD40. The intracellular domains of the two receptors are apparently unrelated, suggesting that the two receptors employ different signal transduction pathways. Soluble forms of both types of receptors have been found in human serum and urine (13 - 15). These soluble receptors are capable of neutralizing the biological activities of the TNFs and may serve to modulate the activities of TNF.

The Quantikine Rat TNF-α Immunoassay is a 4.5 hour solid phase ELISA designed to measure rat TNF-α levels in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant rat TNF-α and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant rat TNF-α accurately. Results obtained using natural rat TNF-α showed dose response curves that were parallel to the standard curves obtained using the recombinant kit standards. These results indicate that the Quantikine Rat TNF-α Immunoassay kit can be used to determine relative mass values for natural rat TNF-α.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Note: *Grossly hemolyzed or lipemic samples may not be suitable for measurement of rat TNF-α with this assay.*

SAMPLE PREPARATION

Rat serum and plasma samples require a 2-fold dilution into Calibrator Diluent RD5-17 prior to assay. A suggested 2-fold dilution is 75 µL sample + 75 µL Calibrator Diluent RD5-17. Mix well.

Rat cell culture supernate samples require a 3-fold dilution into Calibrator Diluent RD5-17 prior to assay. A suggested 3-fold dilution is 50 µL sample + 100 µL Calibrator Diluent RD5-17. Mix well.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Rat TNF-α Kit Control - Reconstitute the Kit Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL Wash Buffer Concentrate into deionized or distilled water to prepare 625 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

Rat TNF-α Standard - Reconstitute the rat TNF-α Standard with 2.0 mL of Calibrator Diluent RD5-17. Do not substitute other diluents. This reconstitution produces a stock solution of 800 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 200 µL of Calibrator Diluent RD5-17 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted rat TNF-α Standard serves as the high standard (800 pg/mL). Calibrator Diluent RD5-17 serves as the zero standard (0 pg/mL).

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and control be assayed in duplicate.

1. Prepare reagents, working standards, control, and samples as directed in the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 50 µL of Assay Diluent RD1-41 to each well.

4. Add 50 µL of Standard, Control, or sample* to each well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.

6. Add 100 µL of Rat TNF-α Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**

9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

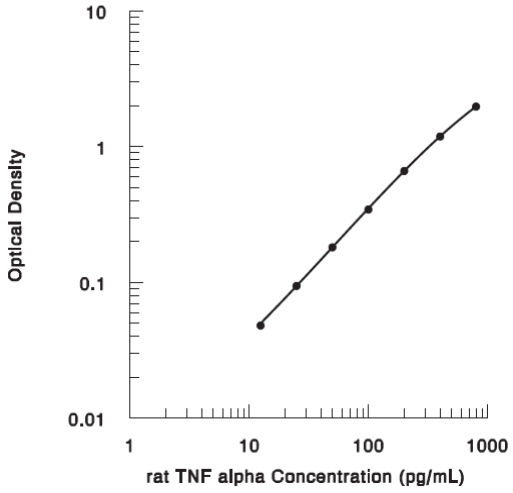
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the rat TNF-α concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

Because samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.034 0.034	0.034	—
12.5	0.085 0.080	0.082	0.048
25	0.128 0.127	0.128	0.094
50	0.214 0.216	0.215	0.181
100	0.383 0.372	0.378	0.344
200	0.692 0.698	0.695	0.661
400	1.218 1.222	1.220	1.186
800	2.023 1.988	2.006	1.972

B6 TFGB assay kit (Quantikine)

INTRODUCTION

Transforming growth factor beta (TGF-β) proteins (including the three closely related mammalian isoforms TGF-β1, -2 and -3) are pleiotropic cytokines that regulate extracellular matrix production, wound healing, immune functions, cell proliferation and differentiation. They belong to the large TGF-β superfamily, which also includes the activins/inhibins/MIS, bone morphogenetic proteins (BMPs), growth/differentiation factors (GDFs), Lefty1 and 2, and the distantly related GDNF family of neurotrophic factors. All family members show a characteristic cysteine-knot that is formed from multiple intrachain disulfide bonds (1 - 3).

The mouse, rat, porcine and canine TGF-β1 cDNAs encode a 390 amino acid (aa) residue precursor that contains a 29 aa signal peptide and a 361 aa pro-protein. The pro-protein for each species is proteolytically processed via a furin-like convertase to generate an N-terminal 249 aa latency-associated peptide (LAP), and a C-terminal 112 aa mature TGF-β1 (4 - 9). Both LAP and mature TGF-β1 exist as disulfide-linked homodimers. After proteolytic cleavage and secretion, the two homodimers remain non-covalently associated as the small latent TGF-β1 complex. In most cell types, this complex is also covalently linked via LAP to a latent TGF-β binding protein (LTBP). This creates a secreted, large latent complex. The TGF-β1 present in either the small or the large latent complex is not available for TGF-β receptor binding and activation, and is, therefore, latent. Whereas LAP is both necessary and sufficient to confer latency to TGF-β1, LTBP facilitates the proper folding and secretion of the small latent complex. LTBP is also a structural component of the extracellular matrix and directs the localization of the latent complex to the extracellular matrix (10 - 15). To date, four LTBPs that share multiple EGF-like, LTBP- and fibrillin-specific domains have been cloned. Three of the four LTBPs (#1, 3, and 4) have been shown to bind the small latent complexes of all TGF-β isoforms (16). Activation of the latent TGF-β complex is an important step that regulates TGF-β function *in vivo*. Multiple activation mechanisms have been identified. These involve protease-dependent (plasmin and matrix metalloprotease) and protease-independent (binding of LAP to thrombospondin 1 or a subset of integrins) pathways (5, 15, 17 - 21). Mature mouse TGF-β1 shows 100% aa sequence identity to mature rat TGF-β1 (6, 7), and both rat and mouse TGF-β1 show 99% aa sequence identity with mature porcine, human, and canine TGF-β1 (4, 9, 22). Mature porcine TGF-β1 shows absolute identity to both mature human and canine TGF-β1.

The signalling high-affinity receptor for TGF-β1 is a heteromeric complex consisting of a type I (TGF-β RI) and a type II (TGF-β RII) transmembrane serine/threonine kinase receptor. TGF-β RII is a constitutively active kinase. Upon binding TGF-β1, it phosphorylates and activates the TGF-β RI. In turn, TGF-β RI phosphorylates and activates Smad proteins that regulate transcription. TGF-β RI, alternatively named activin receptor-like kinase (ALK-5), is present in almost all cell types. Two other type I receptors, ALK-1 and ALK-2 have also been implicated as alternative partners for TGF-β RII in the TGF-β signalling receptor complex. Besides the type I and type II receptors, accessory receptors including the type III receptor, TGF-β RIII (also known as betaglycan), and endoglin, which modulate TGF-β responses, have been identified (2, 23 - 26).

The Quantikine TGF-β1 Immunoassay is a 4.5 hour solid phase ELISA designed to measure TGF-β1 in cell culture supernates, serum, and plasma. It contains recombinant TGF-β1 expressed by CHO cells and has been shown to quantitate the recombinant factor accurately. Results obtained using natural TGF-β1 showed linear curves that were parallel to the standard curves obtained using the recombinant kit standards. These results indicate that the Quantikine immunoassay will provide accurate quantitation for both recombinant and natural TGF-β1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TGF-β1 has been pre-coated onto a microplate. Standards, control and samples are pipetted into the wells and any TGF-β1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-β1 is added to the wells to sandwich the TGF-β1 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TGF-β1 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- It is important that the Assay Diluent selected be consistent with the samples being assayed.
- If samples generate values higher than the highest standard, further dilute the samples after activation and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in this immunoassay, the possibility of interference cannot be excluded.

SAMPLE COLLECTION AND STORAGE

Activated samples must be assayed immediately. Do not freeze activated samples.

Cell Culture Supernates - Remove particulates by centrifugation and assay (see activation procedure) immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Note: *Animal serum used in the preparation of cell culture media may contain high levels of latent TGF-β1. For best results, do not use animal serum for growth of cell cultures when assaying for TGF-β1 production. If animal serum is used as a supplement in the media, precautions should be taken to prepare the appropriate control and run the control in the immunoassay to determine the baseline concentration of TGF-β1.*

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay (see activation procedure) immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Plasma* - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2 - 8° C is recommended for complete platelet removal. Assay (see activation procedure) immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

*TGF-β1 is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of TGF-β1, platelet-poor plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the National Committee for Clinical Laboratory Standards (NCCLS), result in incomplete removal of platelets from blood. This will cause variable and irreproducible results for assays of factors contained in platelets and released by platelet activation. The recommended plasma collection protocol is designed to minimize platelet degranulation. However, since even the best methods for plasma collection may result in some platelet degranulation on occasion, it is recommended that markers for platelet degranulation be determined in samples containing elevated TGF-β1 levels.

ACTIVATION REAGENT PREPARATION

To activate latent TGF-β1 to the immunoreactive form, prepare the following solutions for acid activation and neutralization. The solutions may be stored in polypropylene bottles at room temperature for up to one month.

Caution: *Wear protective clothing and safety glasses during preparation or use of these reagents.*

1 N HCl (100 mL) - To 91.67 mL of deionized water, slowly add 8.33 mL of 12 N HCl. Mix well.

1.2 N NaOH/0.5 M HEPES (100 mL) - To 75 mL of deionized water, slowly add 12 mL of 10 N NaOH. Mix well. Add 11.9 g of HEPES. Mix well. Bring final volume to 100 mL with deionized water.

For each new lot of acidification and neutralization reagents, measure the pH of several representative samples after neutralization to ensure that it is within pH 7.2 - 7.6. Adjust the volume and corresponding dilution factor of the neutralization reagent as needed.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

TGF-β1 Control - Reconstitute the Kit Control with 1.0 mL deionized or distilled water. Assay the Control undiluted. Do not activate.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 625 mL of Wash Buffer.

Calibrator Diluent RD5-53 (1X) - Dilute 20 mL of Calibrator Diluent RD5-53 Concentrate into 60 mL of deionized or distilled water to prepare 80 mL of Calibrator Diluent RD5-53 (1X).

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

TGF-β1 Standard - Reconstitute the TGF-β1 Standard with 2.0 mL of Calibrator Diluent RD5-53 (1X). Do not substitute other diluents. This reconstitution produces a stock solution of 2000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 200 µL of Calibrator Diluent RD5-53 (1X) into each tube. Use the standard stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted TGF-β1 Standard serves as the high standard (2000 pg/mL). Calibrator Diluent RD5-53 (1X) serves as the zero standard (0 pg/mL).

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and control be assayed in duplicate.

1. Prepare all reagents, standard dilutions, and activated samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1-21 (*for cell culture supernate samples*) or Assay Diluent RD1-73 (*for serum/plasma samples*) to each well.
4. Add 50 µL of Standard, Control, or activated sample* per well. Tap the plate gently for one minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 100 µL of TGF-β1 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

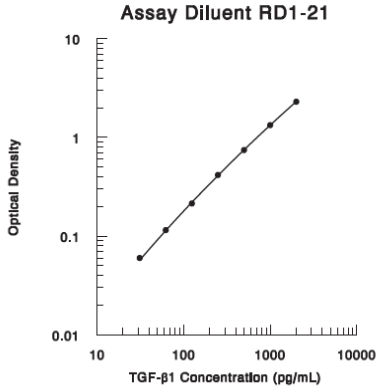
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the TGF-β1 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

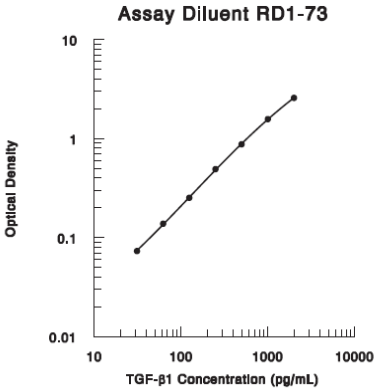
Because samples have been diluted in the activation step prior to the assay, the measured concentrations must be multiplied by the final dilution factor.

TYPICAL DATA

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.



pg/mL	O.D.	Average	Corrected
0	0.050 0.052	0.051	—
31.2	0.107 0.114	0.111	0.060
62.5	0.165 0.167	0.166	0.115
125	0.262 0.267	0.265	0.214
250	0.474 0.459	0.467	0.416
500	0.781 0.809	0.795	0.744
1000	1.372 1.390	1.381	1.330
2000	2.322 2.368	2.345	2.294



pg/mL	O.D.	Average	Corrected
0	0.053 0.053	0.053	—
31.2	0.124 0.128	0.126	0.073
62.5	0.189 0.193	0.191	0.138
125	0.303 0.307	0.305	0.252
250	0.542 0.545	0.544	0.491
500	0.924 0.934	0.929	0.876
1000	1.611 1.638	1.625	1.572
2000	2.624 2.630	2.627	2.574

B7 NFKB assay kit (*Uscn*)

E91824Ra 96 Tests
Enzyme-linked Immunosorbent Assay Kit
For Nuclear Factor Kappa B (NFkB)
Organism: Rattus norvegicus (Rat)
Instruction manual

FOR IN VITRO USE AND RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC OR THERAPEUTIC PROCEDURES

7th Edition (Revised in November, 2011)

[INTENDED USE]

The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of NFkB in rat tissue homogenates, cell lysates and other biological fluids.

[ASSAY PROCEDURE]

1. Determine wells for diluted standard, blank and sample. Prepare 7 wells for standard, 1 well for blank. Add 100μL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 2 hours at 37°C.
2. Remove the liquid of each well, don't wash.
3. Add 100μL of **Detection Reagent A** working solution to each well. Incubate for 1 hour at 37°C after covering it with the Plate sealer.
4. Aspirate the solution and wash with 350μL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1~2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
5. Add 100μL of **Detection Reagent B** working solution to each well. Incubate for 30 minutes at 37°C after covering it with the Plate sealer.
6. Repeat the aspiration/wash process for total 5 times as conducted in step 4.
7. Add 90μL of **Substrate Solution** to each well. Cover with a new Plate sealer. Incubate for 15 - 25 minutes at 37°C (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.
8. Add 50μL of **Stop Solution** to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450nm immediately.

Note:

1. **Assay preparation:** Keep appropriate numbers of wells for 1 experiment and remove extra wells from microplate. Rest wells should be resealed and stored at -20°C.
2. **Samples or reagents addition：Please use the freshly prepared Standard.** Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of standards, samples, and reagents. Also, use separated reservoirs for each reagent.
3. **Incubation:** To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents are added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be controlled.
4. **Washing:** The wash procedure is critical. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and false elevated absorbance reading.
5. **Controlling of reaction time:** Observe the change of color after adding **TMB Substrate** (e.g. observation once every 10 minutes), if the color is too deep, add **Stop Solution** in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. **TMB Substrate** is easily contaminated. Please protect it from light.
7. The environment humidity which is less than 60% might have some effects on the final performance, therefore, a humidifier is recommended to be used at that condition.

[TEST PRINCIPLE]

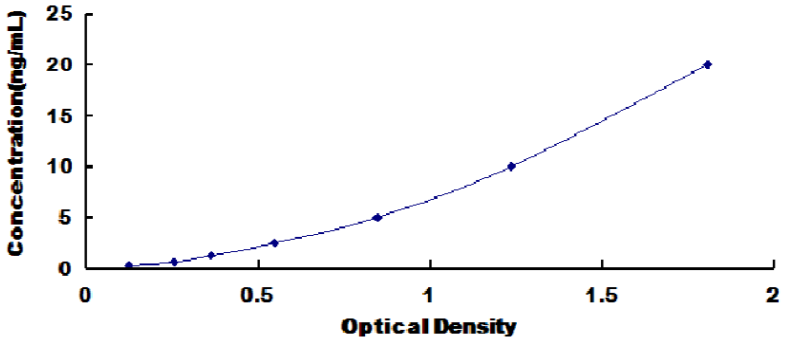
The microtiter plate provided in this kit has been pre-coated with an antibody specific to NFkB. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for NFkB. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain NFkB, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of NFkB in the samples is then determined by comparing the O.D. of the samples to the standard curve.

[CALCULATION OF RESULTS]

Average the duplicate readings for each standard, control, and samples and subtract the average zero standard optical density. Create a standard curve on log-log graph paper, with NFkB concentration on the y-axis and absorbance on the x-axis. Draw the best fit straight line through the standard points and it can be determined by regression analysis. Using some plot software, for instance, curve expert 1.30, is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

[TYPICAL DATA]

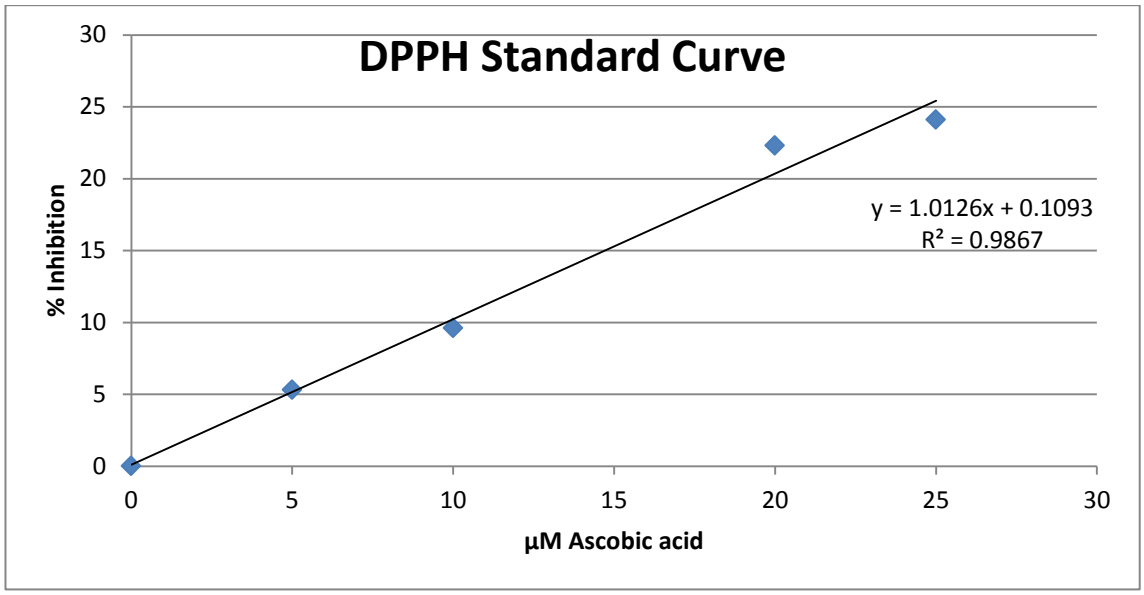
In order to make the calculation easier, we plot the O.D. value of the standard (X-axis) against the known concentration of the standard (Y-axis), although concentration is the independent variable and O.D. value is the dependent variable. However, the O.D. values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), plotting log of the data to establish standard curve for each test is recommended. Typical standard curve below is provided for reference only.

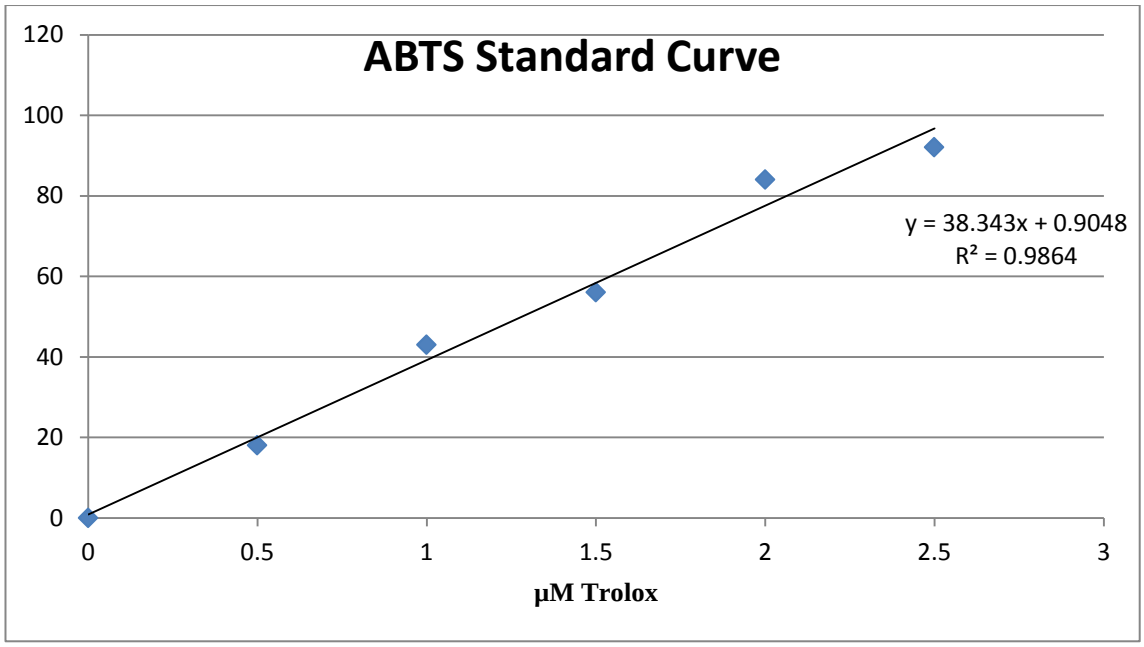


Typical Standard Curve for Rat NFkB ELISA.

APENDIX C Standard curves and cytotoxicity

C1. DPPH and ABTS standard curves





C2. Cytotoxicity

Screening crudes and compounds for cytotoxicity after 24 hrs							
Sample	Conc.	Rep 1	Rep 2	Mean	Inhibition %	SD	SEM
DMSO	5%	0.919	0.842	0.881	0	0.054	0.031
Siimarin	1 µg	0.629	0.792	0.711	19.307	0.115	0.066
Siimarin	1 µg	1.244	1.308	1.276	-44.918	0.045	0.026
Siimarin	10 µg	1.305	1.398	1.352	-53.492	0.066	0.038
C. xanthorrhiza	1 µg	0.967	0.999	0.983	-11.641	0.023	0.013
C. xanthorrhiza	10 µg	1.001	1.004	1.003	-13.856	0.002	0.001
C. xanthorrhiza	100 µg	0.929	1.027	0.978	-11.073	0.069	0.040
I. aquatica	1 µg	0.895	0.943	0.919	-4.373	0.034	0.020
I. aquatica	10 µg	1.106	1.091	1.099	-24.759	0.011	0.006
I. aquatica	100 µg	0.987	1.023	1.005	-14.140	0.025	0.015
Xanthorrhizol	1 µg	1.277	1.482	1.380	-56.672	0.145	0.084
Xanthorrhizol	10 µg	1.372	1.381	1.377	-56.332	0.006	0.004
Xanthorrhizol	100 µg	1.304	1.44	1.372	-55.821	0.096	0.055
Violaxathin	1 µg	1.437	1.569	1.503	-70.698	0.093	0.054
Violaxathin	10 µg	1.439	1.554	1.532	-73.992	0.081	0.047
Violaxathin	100 µg	1.343	1.565	1.52	-72.629	0.157	0.090

APENDIX D Grants and publication

D1. Grants

The work was funded by University of Malaya Grant No. PVO42-2011A.

D2. Publication

Published

Ipomoea aquatica leaves extract shows protective action against thioacetamide induced hepatotoxicity (Molecules)

Submitted

Hepatoprotective effect of *Curcuma xanthorrhiza* against thioacetamide induced hepatotoxicity

Under review

Hepatoprotective activities of violaxanthin and xanthorrhizol