EVALUATION OF HEPATOPROTECTIVE EFFECTS AND BIOACTIVITIES OF *PHYLLANTHUS NIRURI* AND *MELASTOMA MALABATHRICUM*

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ABSTRACT

The liver plays an essential role in the body by regulating several important metabolic functions. Liver injury is associated with distortion of these functions causing many health problems. Pharmaceutical drugs treat liver disorders but carry the risk of causing further damage to the liver. Hence, herbal drugs have been becoming increasingly popular worldwide as both patients and doctors strive to find safer alternatives.

The bioactivities of the crude ethanol extracts of *Phyllnthus niruri L.* (PN) and *Melastoma malabathricum L.* (MM) were investigated in virtue of their *in vitro* antioxidant, immunomodulatory effects and *in vivo* oral toxicity tests. The hepatoprotective activity was evaluated against thioacetamide (TAA) induced liver cirrhosis in rats. Antioxidant activity was evaluated by different assays, including: 2,2-diphenlyl-1-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities and ferric reducing antioxidant power (FRAP). Total phenolic content (TPC) and total flavonoids content (TFC) of the plants extracts were also determined. The immunomodulatory effect was studied on human peripheral mononuclear cells (PBMC) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide **(**MTT) assay. To induce liver injury, male *Sprague Dawley* rats were administered by intraperitoneal injections (i.p.) of thioacetamide (TAA, 200 mg/kg, b.w. thrice weekly) for eight weeks to assess the hepatoprotective effect of the extracts. Daily treatments with plant extracts 100 mg/kg b.w., 200 mg/kg and silymarin (50 mg/kg) administered orally were carried out for eight weeks. At the end of the study, hepatic damage was evaluated by monitoring liver's gross morphology, histopathology, weight changes, biochemical parameters, endogenous antioxidants activities including total antioxidant capacity (TAC), catalase (CAT),

superoxide dismutase (SOD), glutathione peroxidase (GPX) and malondialdehyde (MDA) in the liver homogenates. Gene expressions were also studied by profiling transforming growth factor (TGFβ1), collagen α1 (Collα1), matrix metalloproteinase-2 (MMP2) and tissue inhibitor of metalloproteinase-1 (TIMP1) by Real Time PCR. Besides, different chromatography techniques were used to isolate the active constituents of the plants including column chromatography, thin layer chromatography and ultra performance liquid chromatography (UPLC) with liquid chromatography coupled to mass spectrometry (LC-MS). Our results revealed that treatment with 200 mg/kg of PN and MM significantly reduced the impact of thioacetamide toxicity and they were effectively hepatoprotective, comparable to that of silymarin. The mechanism of the hepatoprotective effects of PN and MM, proposed to be through neutralizing the reactive oxygen species (ROS) and enhancement of endogenous antioxidant activities SOD, GPX and suppression of oxidative stress marker MDA. Additionally, PN and MM treatment normalized the expression of TGFβ, Collα1, MMP2 and TIMP1 genes. The isolated chemical constituents included in *P. niruri* were 4-O-caffeolquinic acid and quercetin 3-O-rhamnoside while in *M. malabathricum* were kaempferol, quercetin and naringenin.

In conclusion, the results of the present study indicate that PN and MM ethanol extracts were hepatoprotective and toxicologically safe when administered orally. We postulated that the hepatoprotective effect of these extracts might be in part due to the active components, antioxidant and immunomodulatory properties.

ABSTRAK

Hati memainkan peranan yang penting di dalam regulasi beberapa fungsi metabolik yang penting. Kerosakan hati dikaitkan dengan perubahan fungsi-fungsi ini yang mengakibatkan banyak masalah kesihatan. Ubat-ubat farmaseutikal yang digunakan untuk merawat penyakit hati menambah pula kerosakan pada hati. Oleh itu ubatan herba semakin popular di seluruh dunia.

Bioaktiviti bagi ekstrak etanol kasar Phyllnthus niruri (PN) dan Melastoma malabathricum (MM) dikaji dari segi antioksidan in vitro, kesan modulasi imun dan ujian ketoksikan oral in vivo. Aktiviti perlindungan ke atas hati dikaji di dalam tikus-tikus yang mengalami sirosis hati akibat thioacetamide (TAA). Aktiviti antioksidan dinilai melalui beberapa asai, iaitu aktiviti memerangkap radikal 2,2-diphenlyl-1-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 'ferric reducing antioxidant power' (FRAP), dan asai jumlah kandungan fenol (TPC) dan jumlah kandungan flavonoid (TFC) manakala aktiviti modulasi imun dikaji dengan asai MTT ke atas sel-sel mononuklear periferi manusia. Di dalam kajian perlindungan hati, tikus-tikus Sprague Dawley jantan diberi suntikan thioacetamide (TAA, 200mg/kg berat badan, tiga kali seminggu) secara intraperitoneal selama lapan minggu. Ekstrak tumbuhan 100 mg/kg berat badan, 200 mg/kg dan silymarin (50mg/kg) diberi secara oral sebagai rawatan harian selama lapan minggu. Pada penghujung tempoh kajian, kerosakan hati dinilai secara: morfologi kasar, histopatologi, perubahan berat badan, parameter-parameter biokimia, aktiviti antioksidan dalaman iaitu jumlah kapasiti antioksidan (TAC), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX) dan pengoksidan lipid (MDA) di dalam homogenat hati. Ekspresi gen

juga dikaji dengan menggunakan RT-PCR untuk memperoleh profil gen expressions transforming growth factor, (TGFβ), collagen α1 (Collα1), matrix metalloproteinase-2 (MMP2) dan tissue inhibitor of matrix metalloproteinase-1 (TIMP1). Beberapa teknik kromatografi turut dilakukan untuk mengasingkan konstituent aktif tumbuhan iaitu kromatografi 'column', kromatografi lapisan nipis dan Ultra Performance Liquid Chromatography (UPLC) dengan Liquid Chromatography yang dipadankan ke Mass Spectrometry (LC-MS). Keputusan yang diperoleh menunjukkan rawatan dengan PN dan MM mengurangkan dengan signifikan ketoksikan hati akibat thioacetamide di mana kedua-dua ekstrak telah melindungi hati, setara dengan perlindungan yang diperoleh daripada silymarin yang merupakan agen pelindung hati yang standard. Cadangan bagi mekanisme PN dan MM di dalam melindungi hati ialah dengan meneutralkan ROS dan meningkathan aktiviti antioksidan dalaman: SOD, GPX dan mengurakan marker stress oksidatif MDA. Tambahan pula rawatan dengan PN dan MM menormalkan ekspresi gen TGFβ, Collα1, MMP2 dan TIMP1. Konstituen kimia yang diasingkan bagi *P. niruri* adalah (4-O-caffeolquinic acid dan quercetin 3-O-rhamnoside) manakala bagi *M. malabathricum* pula ialah (kaempferol, quercetin dan naringenin).

Kesimpulannya, keputusan kajian ini menunjukkan ekstrak etanol PN dan MM adalah selamat dari segi toksikologi apabila diberi secara oral dan mempunyai kesan perlindungan pada hati, antioksidan dan memodulasi sistem imun, hasil daripada konstituen kimia yang diasingkan.

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviation	Description
ABTS	2,2′-azino-bis(3-ethyl benzthiazoline-6-sulfonic acid
ALD	Alcoholic liver disease
ALT	Alanine amino transferase
AST	Aspartate amino transferase
BHA	Butylated hydroxyl anisole
BHT	Butylated hydroxyl toluene
B.W.	Body weight
CAT	Catalase
CCL_4	Carbon tetrachloride
cDNA	Complementary deoxy ribonucleic acid
Coll α	Collagen alpha
Ct	Threshold cycle
Cu-NTA	Cupric nitrilo triacetate
dH_2O	Distilled water
DMSO	Dimethyl sulphoxide
DPPH	2,2-diphenyl-2-picrylhydrazyl
EB	Ethidium bromide
ECM	Extracellular matrix
FBS	Fetal bovine serum
FCC	Flash column chromatography
FMO	Flavin- containg monooxygenase
FRAP	Ferric ion reducing antioxidant power
GAPDH	Glycer aldehyde 3-phosphate dehydrogenase
G	Gram
GPX	Glutathione peroxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HD	High dose
HGF	Hepatocyte growth factor
HPLC	High performance liquid chromatography
Hprt1	Hypoxanthine phosphoribosyl transferase 1
HSC	Hepatic satellite cell
IC_{50}	Inhibitory concentration
IFN-γ	Interferon-gamma
IL-2	Interleukin-2
i.p.	Intraperitoneal
Kg	Kilogram
LC-MS	Liquid chromatography-mass spectrometry
LD	Low dose
LPO	Lipid peroxidation

MDA	Malondialdehyde
Mg	Milligram
Ml	Milliliter
µl	Microliter
MM	*M. malabathricum*
Mmol	Millimole
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide
Ng	Nanogram
NIM	Nimesulide
PAF	Platelet activating factor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PN	*P. niruri*
Ppia	Peptidyl prolylisomerase A
Real Time PCR	Real Time Polymerase Chain Reaction
ROS	Reactive oxygen species
Rpm	Revolution per minute
RPMI-1640	Roswell Park Memorial Institute-1640
RT	Room temperature
SD	*Sprague Dawley*
SGOT	Serum glutamic-oxaloacetic transaminase
SGPT	Serum glutamic-pyruvic transaminase
SOD	Superoxide dismutase
TAA	Thioacetamide
TAC	Total antioxidant capacity
TASO	Thioacetamide-S, oxide
TASO2	Thioacetamide-S,S-dioxide
TBARS	Thiobarbituric acid reactive substances
TBHQ	Tert-butyl hydro quinine
TFC	Total flavonoid content
TGFβ	Transforming growth factor beta
TIMP	Tissue inhibitors of metalloproteinases
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor-α
TP	Total protein
TPC	Total phenolic content
UPLC	Ultra performance liquid chromatography
WHO	World Health Organization
β-actin	Beta actin
γGT	Gamma glutamyl transferase
ºC	Degree Celsius
%	Percentage

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CHAPTER I

INTRODUCTION

1.1 Introduction

The liver is the largest organ in the human body, it is responsible for the metabolism of carbohydrates, proteins and lipids, detoxification and bile secretion, regulating blood clotting, converting food nutrients into essential blood components, storing vitamins and minerals, and maintaining hormone balances. The liver makes factors that help the human immune system and removes bacteria from the blood (Nemeth et al., 2009).

Liver diseases are conditions that involve tissue damage or liver inflammation and affects liver function. Liver diseases can be categorized to causes that include injuries, infections, exposure to drugs, toxic compounds, alcohol and viruses like the hepatitis virus. Exposure to these factors may affect clotting abnormalities, inflammation, obstructions, scarring and liver failure. Liver fibrosis and its end-stage cirrhosis represent a massive universal healthcare problem, according to the National Institutes of Health cirrhosis is the 12th leading cause of death. Liver fibrosis is the last pathological way of many chronic liver injuries and cirrhosis is the last step of hepatic parenchyma fibrosis, leading in the formation of nodules that alter the hepatic function and block the blood flow (Sabir & Rocha, 2008a).

Cirrhosis affects hundreds of millions of patients worldwide. Due to chronic liver disease with fibrosis the overall problem of liver disease continues to expand, as does the associated social and economic costs. Cirrhosis had previously been considered as being the stage at which fibrotic liver disease became irreversible. However nowadays, strong evidences have proven that fibrosis and even cirrhosis is reversible by

elimination of the causes of liver diseases in animal models, which provide vital evidences to the underlying mechanism (Mosmann, 1983).

In spite of medical advances, pharmacological drugs lack efficiency, have unfavorable side effects and relatively high cost (Stickel & Schuppan, 2007). Therefore, alternative drugs are required to prevent and treat these disorders. For thousands of years humankind has traditionally used plants as basis from which to formulate new remedies for the use as medicines. The utilizing of plants in different traditional medicines of various cultures has been comprehensively documented. These plant-based systems continue to play an essential role in health care and the World Health Organization estimates that 80% of the world's inhabitants depend on herbal medicines for their health care (Gurib-Fakim, 2006).

There is a long history regarding the use of traditional medicine in the treatment of liver diseases, starting with the Ayurvedic treatment (the ancient Indian system) and extending to the European, Chinese and other systems of traditional medicine. Many herbs have been deemed to possess liver protecting qualities. Near 160 phyto-constituents from 101 herbs have been deemed to have hepatoprotective activity (Saleem et al., 2010).

In Malaysia, the occurrence of an extraordinary growth in Malaysian herbal medicine has led to the development of many efficacious and safe products for human consumption. Besideswhich, the advances of many chromatography and spectroscopy techniques had a remarkable impact on the analysis of the active constituents of these medicinal plants (Jantan, 2004).

Silybum marianum (milk thistle) is among the well documented hepatoprotective plants, since the silymarin (the seed extract) had been claimed to enclose hepatoprotective activities which has been validated in many *in vivo* and *in vitro*

studies. Also it has been reported to protect the liver from injuries induced by various toxins or chemicals, by performing anti-lipid peroxidative, antioxidative, antifibrotic, anti-inflammatory, liver regenerating and immunomodulatory activities (Cragg & Newman, 2005).

On another hand, the immunomodulatory profile of the medicinal plants has been well defined both *in vitro* and *in vivo*. An old tradition exists worldwide to ingest phytochemicals for improving the human immune system so as to fight infections (Tan & Vanitha, 2004). Classes of medicinal herbs were known to alter the activity of the immune function through the dynamic regulation of certain molecules as cytokines, that in response to injury stimuli, innate inflammatory responses, cell growth and differentiation, angiogenesis, and repair processes (Spelman et al., 2006). For instance, research suggested that in Kampo (Japanese traditional medicine) more than 210 different herbs have been used in Japan to improve and modulate the immune response (Borchers et al., 2000).

Furthermore, the involvement of reactive oxygen species (ROS) and lipid peroxidation products have been clearly reported as the fundamental events of many, if not all, diseases affecting liver tissue through an unbalanced increase of reactive oxygen species (ROS) with overall anti-oxidant defense mechanisms exerted on the body, such as the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) that have been believed to prevent certain degrees of liver damage from being caused. Therefore, in recent years medicinal plants have been investigated for their antioxidant properties and free radical scavenging activities as a natural source of antioxidant with presumed nutritional, safety and therapeutic value to replace the synthetic antioxidants that have been restricted due to their health risks and undesirable toxicities. Plants natural antioxidants are mainly in the form of phenolic

compounds (phenolic acids, flavonoids and tocopherols), ascorbic acid and carotenoids, thus the antioxidant supplements derived from plants were thought to quench free radicals and prevent human disease (Ali et al., 2008).

Phyllanthus niruri, a plant from the Euphorbiaceae family commonly known in Malaysia as dukung anak, has been used in Malay traditional medicines for various purposes and has been investigated extensively worldwide for its medicinal and pharmacological properties (Tang et al., 2010; Murugaiyah & Chan, 2009; Thippeswamy et al., 2011). It has a long history as the medication of chronic liver diseases (Bhattacharjee & Sil, 2006, 2007; Chatterjee et al., 2006; Chatterjee & Sil, 2006).

Melastoma malabathricum, locally known in Malaysia as senduduk, is found in most Asian tropical regions. It has been used in Malay traditional medicine as a drug for treatment of diarrhoea, cholera, fever, leucorrhoea, dysentery, wounds and skin diseases (Sirat et al., 2010). Besideswhich, it has also been reported to exhibit many medicinal properties, such as being an anti-bacterial with wound-healing potential, anticoagulant properties and anti ulcer activities (Choudhury et al., 2011; Sunilson et al., 2008; Manicam et al., 2010; Hussain et al., 2008).

The underlying mechanism of *P. niruri*'s (PN) hepatoprotective effect is still not clearly defined. Therefore, we carried out this project in an attempt to characterize the hepatoprotective effect of PN by combining a systematic methodology with advanced technologies to discover the underlying mechanism involved. Several studies revealed certain medicinal properties of *Melastoma malabathricum,* but to the best of our knowledge, there were no reports pertaining to its hepatoprotective activity, so this study was carried out to discover more pharmacological properties by investigating the basic mechanism of its hepatoprotective action.

1.2 Objectives of the study

1. To evaluate the *in vivo* hepatoprotective activity of *Phyllanthus niruri* and *Melastoma malabathricum* crude ethanol extract on thioacetamide-induced liver cirrhosis in *Sprague Dawley* rats.
2. To investigate the endogenous antioxidant activities of *Phyllanthus niruri* and *Melastoma malabathricum.*
3. To monitor the expression of TGFβ, Collα1, MMP2 and TIMP1genes.
4. To investigate the *in vitro* free radical scavenging properties and phytochemical screening of *Phyllanthus niruri* and *Melastoma malabathricum* crude ethanol extracts.
5. To examine the *in vitro* immunomodulatory role of crude ethanol extract and fractions of *Phyllanthus niruri* and *Melastoma malabathricum*.
6. To identify the active constituents of *Phyllanthus niruri* and *Melastoma malabathricum* from the active fraction by different chromatographic techniques.

CHAPTER II

REVIEW OF LITERATURE

2.1 The liver

2.1.1 General description

The liver is the largest gland, constituting about 2.5% of an adult's body weight. It is located in the upper right side of the abdominal cavity, beneath the diaphragm. The liver weighs about 1.6 kilograms and is dark reddish brown in color. It supports most of the organs in the body and thus is vital for survival. Due to its multidimensional functions and strategic location, the liver is also exposed to many diseases. The liver consists of two large sections, the right and the left lobes. The gallbladder lies under the liver, along with the pancreas and intestines. The liver and these organs together digest, absorb, and process food. Clinical terms referred to the liver mostly start in (hepato) or (hepatic) from the Greek word for liver (hepar) (Tortora & Derrickson, 2008).

2.1.2 Gross and microscopic anatomy of the liver

The liver has a smooth surface and it is connected to the kidney, adrenal glands, inferior vena cava, hepato-duodenal ligament and stomach. As shown in Figure 2.1, it is divided into four lobes, thos being right, left, caudate, and quadrate. The right and left lobes are the largest, while the caudate and quadrate are smaller and located posteriorly. These lobes are further subdivided into small hexagonal lobules (the basic functional units of the liver). The liver tissue includes thousands of lobules; each hexagonal lobule includes

a plate of hepatocytes and portal triads at each corner of the hexagon, cental vein, liver sinusoids, hepatic macrophages (kupffer cells), bile canaliculi and space of disse (Allen, 2002).

Blood is passed to the liver by two large blood vessels, the hepatic artery and the portal vein. The hepatic artery transfers oxygen-rich blood from the aorta while the portal vein transfers blood containing digested food from the small intestine. These blood vessels subdivide into very small capillaries. Each capillary leads to a lobule. The two major functional types of cells are the hepatocytes, which constitutes 80% of the liver's volume and performs the majority of liver functions, and the non-parenchyma cells (like sinusoidal endothelial cells, kupffer cells and hepatic stellate cells) which occupy only 6.5% of the total volume, but have been recognized to regulate many hepatocyte functions under normal and pathological conditions (Kmiec, 2001).

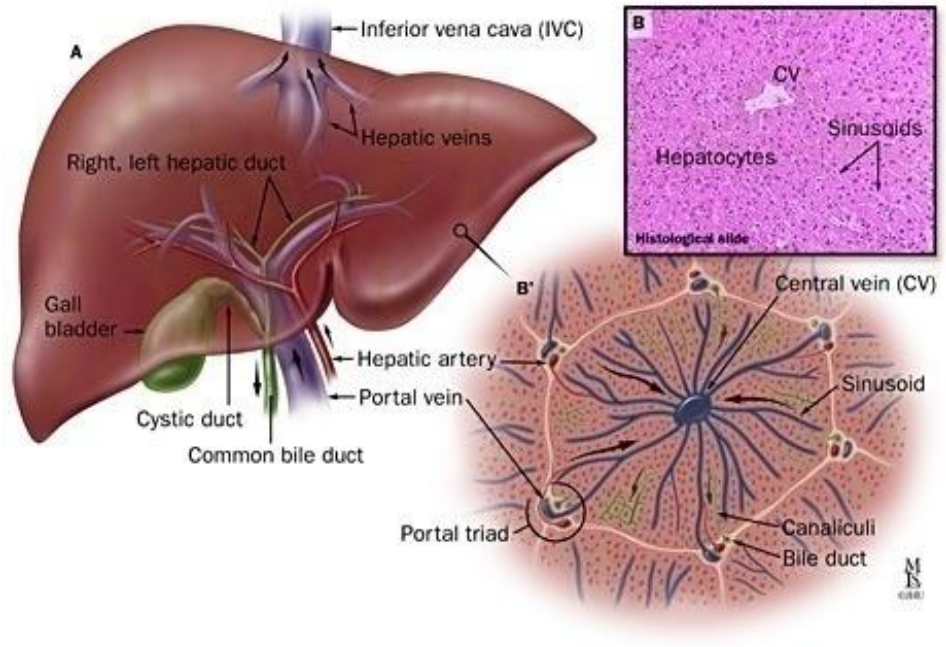


Figure 2.1: Anatomy of the liver: (A) Gross morphology, (B) histology slide and (B*) histological view of the liver. [adapted from (Gurakar et al., 2012)]

2.1.3 Functions of the liver

The liver carries out a large number of critical functions, it regulates chemical levels in the blood and excretes bile that carries out waste products from the liver. The liver processes the blood and breaks down its nutrients and drugs. More than 500 vital functions have been identified like amino acids synthesis and the metabolism of fats, carbohydrates and proteins. In addition, the liver detoxifies chemicals and drugs; and in conjunction with the spleen, it is involved in the filtration of blood, through the destruction of red blood cells and the retrieval of their constituents. It synthesizes and secreets bile proteins important for blood clotting and other functions. (Tso & Mcgill, 2001). It manages the blood glucose level in several ways including glycogenesis (storing glucose as glycogen), glycogenolysis (breaking glycogen to glucose) and (gluconeogenesis) the forming of glucose from non-carbohydrate resources such as amino acids.

2.1.4 Liver diseases

The most common diseases of the liver are acute hepatitis (inflammation), chronic (long duration) hepatitis, alcohol damage, fatty, cirrhosis (scarring),α1 antitrypsin deficiency, progressive familial intrahepatic cholestasis, biliary atresia, alagille syndrome, Langerhans cell histiocytosis and cancer. These diseases accelerate liver's derangement; however, with its ability to regenerate, it only produces symptoms after long-term damage. Liver regeneration is the response to different injuries induced by external stimuli, it involves consequent changes in the growth factor production, gene expression, and morphologic structure (Michalopoulos & Defrances, 1997).

Damage to the liver is mostly accompanied with abnormalities in certain blood biomarker tests, for example, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). In addition, the actual liver injury may cause abnormalities in other liver function tests (LFT) such as bilirubin, albumin, gamma glutamyl transferase (γGT) and prothrombin time. These tests are useful in the evaluation and management of liver dysfunctions in order to detect the presence of hepatic injury, distinguish between different types of liver disorders, determine the level of known liver damage and track the response to different treatments (Giboney, 2005).

Alanine aminotransferase (ALT), previously known as serum glutamic-pyruvic transaminase (SGPT) catalyzes reversible amine group transfer in Krebs cycle and it is a specific indicator of Hepato cellular damage. Aspartate aminotransferase (AST), previously known as serum glutamic-oxaloacetic transaminase (SGOT), catalyzes the conversion of nitrogenous portion of amino acid. It is essential for energy production in Krebs cycle. It is most elevated in acute cellular necrosis. Alkaline phosphatase (ALP) is often associated with cholestasis and biliary obstruction. Gamma glutamyl transferase (γGT) is a liver enzyme involved in the transport of amino acids and peptides into cells, as well as glutathione metabolism. γGT is mainly found in liver cells and is extremely sensitive to alcohol use. Elevated γGT levels may be found in liver disease, alcoholism, obesity, bile-duct obstruction, cholangitis, and drug abuse (Whitfield, 2001).

Total protein (TP) is a measure of the total proteins of blood. It reflects liver disease, nutritional state, kidney disease and others. In general, protein consists of albumin and globulin. Globulin is a building block of antibodies, proteins, and clotting factors. Globulin is made up of about 60 different important proteins. A decreased value of total protein may indicate liver or kidney disease. Albumin is a very common protein

found in the blood with a variety of functions, if its levels are lower than normal it could be suggestive of chronic liver disease. Moreover, bilirubin is a byproduct of the destruction of red blood cells in the liver released as bile in the feces. Elevation of the bilirubin suggests liver dysfunction. (Johnston, 1999; Gopal & Rosen, 2000).

2.2 Cirrhosis of the liver

The word cirrhosis is derived from the Greek word “kirrhos” that means brownish-yellow (Behrns, 2008). It is a worldwide health problem and is a complication of several liver diseases that are described by abnormal structure and functions of the liver. It is the scarring of the liver in response to the development of regenerative nodules surrounded by fibrous septa as consequence of long-term liver injury. The scar tissue blocks the flow of blood through the liver and slows the production of hormones, nutrients, toxins and drugs. It also slows the process of different substances and proteins made by the liver. The spleen then becomes congested which sequentially leads to splenomegaly and ultimately portal hypertension will be responsible for the majority of later complications (Tsukada *et al.*, 2006).

Liver cirrhosis represents a major medical problem with significant morbidity and mortality rates. According to the National Institutes of Health, cirrhosis is the 12th leading cause of death and within the top 15 most important causes of death of 1990 to 2020 as assumed by global burden disease study (Murray & Lopez, 1997).

Liver cirrhosis historically thought to be irreversible process due to the parenchyma collapse and its replacement with collagen, but currently it is considered as a wound-healing model of response to chronic liver damage. Generally, treatment could delay or stop further progression and reduce complications (Mann *et al.*, 2003).

2.2.1 Clinical features of liver cirrhosis

Multi-factorial or single insults to the liver ultimately may lead to cirrhosis. The most common causes are viruses, excessive alcohol consumption, drug-related injury, inherited disorders and exposure to environmental toxins. However, it can also be caused by non-alcoholic steatohepatitis, primary sclerosing cholangitis, hereditary hemochromatosis, wilson's disease, alpha 1-antitrypsin deficiency, galactosemia glycogen storage disease type IV, cystic fibrosis and lysosomal acid lipase deficiency and many other issues. Some patients may have more than one cause of cirrhosis (such as alcohol excess and viral hepatitis), while a large number of patients (up to 20%) do not have an identifiable cause for cirrhosis, which is known as cryptogenic cirrhosis (Schuppan & Afdhal, 2008). In Western countries, chronic hepatitis C and alcohol intake are the main causes of liver cirrhosis, whilst in the Asian Pacific region, chronic hepatitis B is the most common cause. In Malaysia the etiology of liver cirrhosis had peculiar patterns based on the prevalence of hepatitis B and the racial differences in alcohol intake (Qua & Goh, 2011).

Liver cirrhosis is a potentially life-threatening condition that occurs because of scarring-damage to the liver. This scarring replaces healthy tissue and prevents the liver from working normally. Cirrhosis usually develops after years of liver inflammation. Patients with cirrhosis often have few symptoms at first, the signs and symptoms are non-specific or absent at the early stages. As scar tissue replaces healthy tissue and liver function worsens, a variety of liver-related symptoms may develop. Some of the more common symptoms and signs of cirrhosis include fatigue, itching, weakness, loss of appetite, edema (most frequently in the legs) and white nails. The main clinical consequences of liver cirrhosis are spontaneous bacterial peritonitis, spider, ascites, digestive tract bleeding, jaundice, splenomegaly, mental slowing, excess drowsiness,

confusion and slurring of speech, a condition known as hepatic encephalopathy, hepatorenal syndrome, hepatopulmonary syndrome, hypersplenism and liver cancer or hepatocellular carcinoma (Kuo *et al.*, 2010).

The term "cirrhosis" was first introduced in 1826 by Laennec. It derives from the Greek term *scirrhus* and refers to the orange or tawny surface of the liver as seen at autopsy. Macroscopically, the liver enlarges its external surface and causes irregularities in the appearance of its nodularity. It usually becomes shrunken and yellowish, but in some cases became enlarged and yellow such as in alcoholic fatty cirrhosis, or large and green in biliary obstruction and rusty in hemochromatosis. A variation in colors, sizes, shapes and consistency of the nodules helps in the identification of the etiology. Based on the size of the nodules, cirrhosis was classified to micro nodular (nodules $\leq$ 3 mm), macro nodular (nodules >3 mm) and the mixed cirrhosis, which consists of a variety of nodules with different sizes (Wanless, 2004). Histological criteria for cirrhosis (Figure 2.2) is characterized by vascularised fibrotic bands which link the portal tracts with each other and with central veins. The microscopic features include regeneration of hepatocytes nodules, deposition of connective tissue between these nodules and destruction of other normal tissues of the liver, including the sinusoids, the space of Disse and other vascular structures (Schuppan & Afdhal, 2008).

Cirrhosis was previously considered as being irreversible. Nowadays, several studies have demonstrated that progression of liver cirrhosis can be arrested by therapy. That said, the complete regression of cirrhosis has not been clearly documented, instead the anatomic state of the liver has been proposed to be in a balance between two processes, the frequently episodic injury and the slow and continuous repair processes (Wanless *et al.*, 2000).

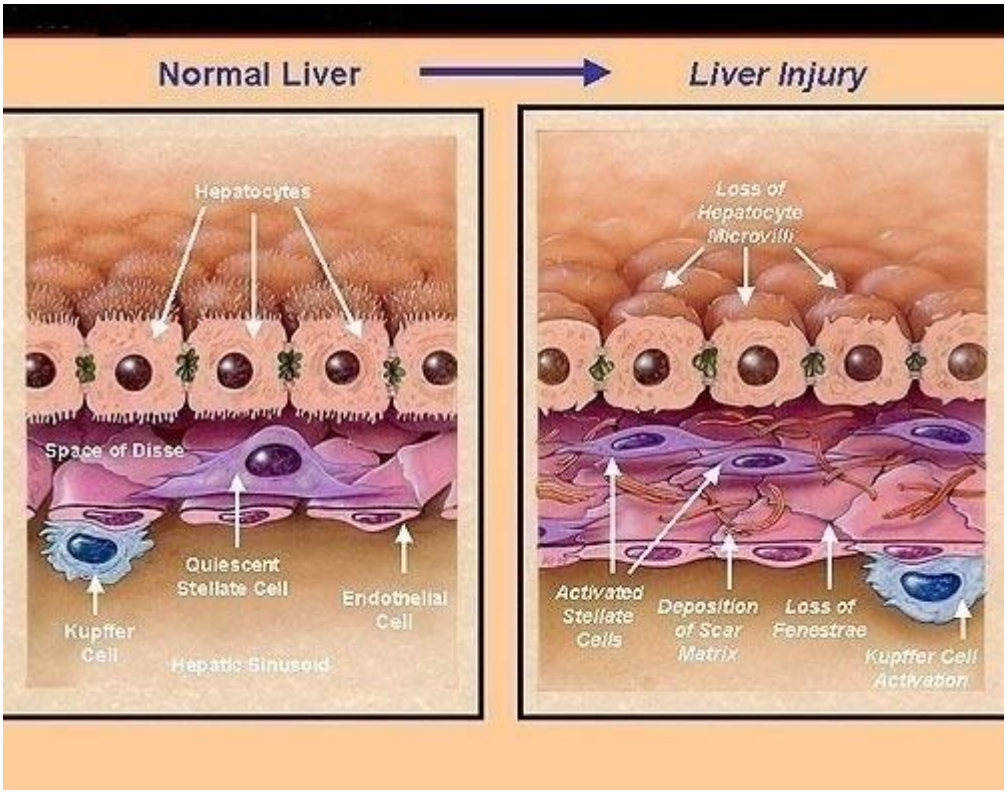


Figure 2.2: Histological criteria of chronic hepatic injury.
[Adapted from (Moreira, 2007)]

2.2.2 Molecular features of liver cirrhosis

The normal liver contains hepatocytes (an epithelium), an endothelial lining, Kupffer cells (tissue macrophages) and hepatic stellate cell (HSC) (mesenchymal cell, previously called lipocyte, Ito cell, peri-sinusoidal cell or fat-storing cell). The cellular components of liver are categorized within the sinusoid with the space of disse separating hepatocytes from the sinusoidal endothelium. This space consists of a matrix, called the extracellular matrix (ECM), that is essential for maintaining the differentiated function of all liver cells (Friedman, 2000). The major components of the ECM in normal liver are collagens I, III, IV, V, VI and various non-collagenous components like laminin, fibronectin, undulin, tenascin, and entactin. In the normal liver, these components are remodeled by matrix degrading enzymes, causing controlled deposition

of matrix components. The matrix metalloproteinases (MMPs) are the most important ECM-degradative enzymes. The activities of these MMPs are regulated through three processes: 1) transcription, 2) zymogen activation and 3) through the action of a family of inhibitory proteins (the tissue inhibitors of metalloproteinases) (TIMPs) (Kossakowska *et al.*, 1998).

A liver cirrhosis case is a wound-healing process as a response to repeated liver injury, associated with alterations in the quantity and composition of ECM, including fibronectin, collagens (I, III, and IV), undulin, laminin, elastin, proteoglycans and hyaluronans (Friedman, 2003). On the other hand, HSC cells are quiescent cells functions in the storing of vitamin A; however, in the response to liver injury these cells undergo an"activation" process which is the key issue affecting the main ECM-producing cells in cases of liver cirrhosis (Reeves & Friedman, 2002).

After HSCs activation it acquires pro-inflammatory, contractile, and fibrogenic properties, accumulating at the sites of tissue repair, secreting large amounts of ECM and regulating ECM degradation. A complex interchange takes place among different liver cell types during cirrhosis process. Hepatocytes release ROS and fibrogenic mediators and induce the white blood cells, then apoptosis of these hepatocytes stimulates the activation of HSCs, which in turn secretes collagen, inflammatory chemokines and modulates the activation of lymphocytes (Figure 2.3). Kupffer cells play’s a role in the liver inflammation process by releasing ROS and cytokines. Therefore, an intense circle is likely to occur (Bataller & Brenner, 2005).

Nevertheless, many genes have been identified as influencing chronic liver diseases. Among these, transforming growth factor beta (TGFβ) is the most potent cytokine that promotes patho-physiological significant liver cirrhosis. It induces the activation of

hepatic stellate cells (HSCs) in necro-inflammatory tissue regions. Upon activation, HSCs differentiate into matrix-producing myofibroblasts producing TGFβ, which in turn, initiate and accelerate the deposition of extracellular matrix components that distort the liver architecture and impair liver function, causing liver cirrhosis (Wang *et al.*, 2008).

Recently it have been demonstrated that oxidative stress accompanying hepatocytes damage was associated with the up-regulation of TGFβ, which enhanced the deposition of extracellular matrix proteins by stimulation of fat-storing cells (Flisiak *et al.*, 2000). ROS up-regulated expression of these genes have been shown to be through activation of a number of critical signal transduction pathways and transcription factors related to fibrogenesis (Mormone *et al.*, 2011).

Moreover, TGFβ has been recognized as a potent inducer of collagen genes. The collagen family is a group of extracellular matrix (ECM) proteins that engage in several activities, like performing structural support of connective tissue and the basement membrane of organs, including the liver. At least 27 different proteins have been recognized from this family; however, of these types I, III, and IV are most associated with liver fibrosis. Increases in expression and deposition of type I collagen are the most-characterized aspect of liver fibrosis. The level of TGFβ mRNA has been found to be correlated with that of collagen α1 mRNA in rat models of cirrhosis induced by thioacetamide (Sato *et al.*, 2000). Furthermore, the main feature of liver fibrosis has been revealed to be the uncontrolled production of collagen α and the molecular basis of its regulation has been shown to be through a complex process involving reactive oxygen species (ROS) as the key mediators (Dikshit et al., 2011).

In addition, the activated HSCs have been shown to increase the expression of matrix metalloproteinases family (MMP) genes, which play an important role in the modulation of ECM accumulation and the endogenous inhibitors of metalloproteinases activity, the tissue inhibitors of metalloproteinases (TIMPs). The interaction between ECM synthesis and its degradation plays a critical role in extracellular homeostasis, and disruption of this balance causes alterations in ECM integrity that can result in deleterious consequences, including the establishment of hepatic fibrosis (Tsukada *et al.*, 2006). TIMP-1 expression has been found to be up-regulated *in vitro* in the HepG2 (human hepatoma) cell line under the influence of certain cytokines such as TGF-β and IL-6, which suggests the contribution of TIMP-1 in the fibrosis process of the liver (Amin et al., 2012).

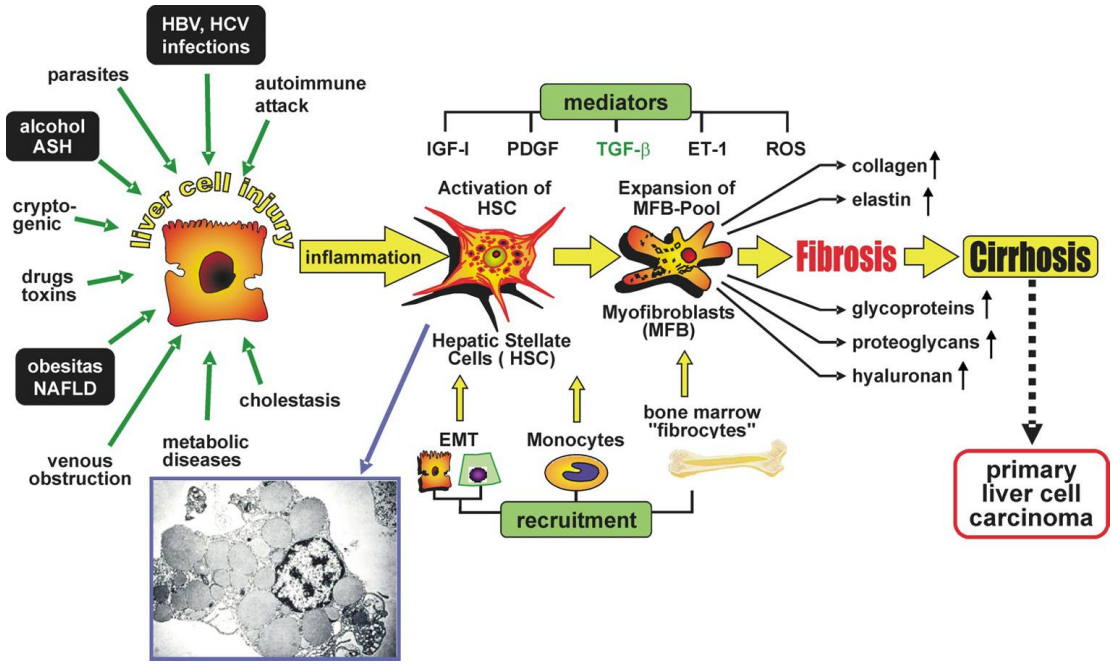


Figure 2.3: Molecular pathogenesis of liver cirrhosis.
[Adapted from (Gressner *et al.*, 2007)]

2.2.3 Experimentally induced cirrhosis

To achieve a better understanding of liver cirrhosis and other hepatic dysfunctions, many models have been established to study the hepatotoxicity of experimental agents, which are adequate models of human cirrhosis.

One of the widely used liver toxicants is carbon tetrachloride (CCL_4), which is employed in animal models for induction of acute and chronic liver diseases and has been reported to induce hepatocellular necrosis, increased lipid peroxidation products and decreased levels of antioxidative enzymes (Kuo *et al.*, 2010). The mechanism of its action has been proposed to be through activation by cytochromes (CYP_2E_1, CYP_2B_1, CYP_2B_2 and CYP_3A) to trichloro methyl radical (CCl_3), that binds to the cellular molecules like nucleic acids, proteins and lipids of the liver thus initiating the damage. CCl_3^- reacts with oxygen to form the trichloro methyl peroxy radical CCl_3OO^- that initiates the reaction of lipid peroxidation which attacks and destroys polyunsaturated fatty acids, resulting in the loss of cellular calcium sequestration and homeostasis, which can contribute heavily to subsequent cell damage (Weber *et al.*, 2003).

Thioacetamide (TAA) is another potent hepatotoxicant that was originally used as a fungicide. Studies have shown that its hepatotoxic effects is due to its metabolite thioacetamide S-oxide (TASO) and further to thioacetamide-S,S-dioxide ($TASO_2$) which is thought to initiate necrosis by covalently binding to liver macromolecules (Chilakapati *et al.*, 2005). Moreover, paracetamol has been used experimentally to induce acute liver failure (Sawant *et al.*, 2006). The liver injury has been postulated to be through mitochondrial oxidant stress and peroxy nitrite formation after its metabolism by P450 system to reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) which bind to cellular proteins and initiates liver damage (Knight *et al.*, 2003).

One more hepatotoxic agent is cupric nitrilo-riacetate (Cu-NTA), which has been used as a model to induce hepatotoxicity. It has been proven to cause damage to the parenchyma cells of the liver when deposited in these cells as an insoluble compound (Toyokuni *et al.*, 1989). Also hepatic necrosis was observed in the livers of rats exposed to Cu-NTA and the levels of 8-hydroxydeoxyguanosine (8-OHdG) have been investigated as being a marker of copper toxicity (Toyokuni & Sagripanti, 1994). Furthermore, liver fibrogenesis was evidenced after the use of an intragastric ethanol infusion model on *Wistar* rats, in which peri-sinusoidal cells were believed to play the key role in fibrogenesis process through increasing DNA synthesis, enhancing gene expression of collagen, transforming growth factor-β1 (Tsukamoto, 1993). Other studies (Tsukamoto *et al.*, 1995) have demonstrated that dietary iron supplementation to intragastric ethanol infusion increased the hepatocyte damage and promoted the fibrogenesis by iron catalyzed oxidant stress.

Nimesulide (NIM) is an analgesic, non-steroidal anti-inflammatory drug (NSAID) that has been reported to cause liver damage, due to the way it is metabolized in the liver and by generating reactive oxygen species (ROS) which in turn have the potential to damage the liver tissues. It has been used in experimental models because it induces significant oxidative stress and thus reduces the levels of antioxidant enzymes like catalase (CAT), superoxide dismutase (SOD) and non protein thiol (GSH) through significant enhancement of lipid peroxidation (LPO) (Chatterjee & Sil, 2006).

2.2.3.1 Thioacetamide

Thioacetamide is a thiono-sulfur compound with the molecular formula of C_2H_5NS (Figure 2.4). Also known as ethane thioamide, it has been used as an organic solvent, fungicide, stabilizer of motor oil and accelerator in the vulcanization of rubber. It is a white monoclinic crystalline solid, which is soluble in water and serves as a source of sulfide ions in the synthesis of organic and inorganic compounds. Thioacetamide was first reported as a hepatotoxic agent by Fitzhugh *et al.* (Fitzhugh & Nelson, 1948). Chronic administration of this toxin can lead to liver cirrhosis and hepatocarcinoma *in vivo* and *in vitro* (Tsukamoto, 1993).

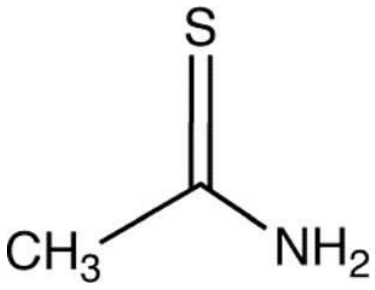


Figure 2.4: Chemical Structure of Thioacetamide.

It undergoes two steps of bioactivation mediated by microsomal CYP_2E_1 and/or flavin-containg monooxygenase (FMO) systems: first to TAA sulfoxide (sulfine), and further to TAA-*S*,*S*-dioxide (sulfene), which is the reactive metabolite, initiates cellular necrosis (Figure 2.5) (Chilakapati *et al.*, 2005). Metabolic activation of TAA then directs to the formation of free radicals, derived from thioacetamide-S,S-oxide and by reactive oxygen species (ROS) (Tsukamoto *et al.*, 1995).

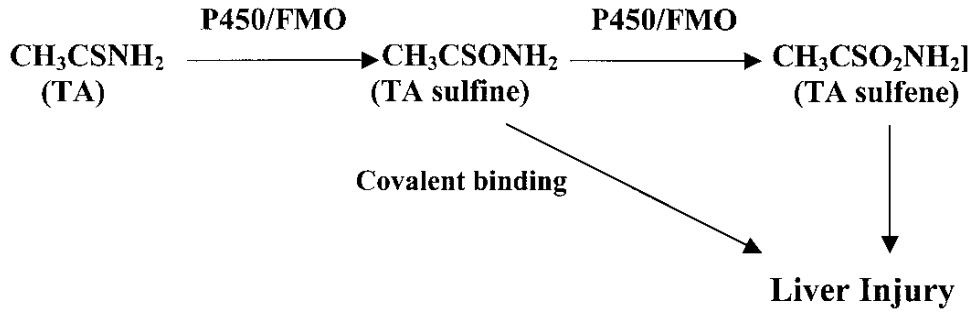


Figure 2.5: Mechanism of TAA induced liver injury.
[adapted from (Ramaiah *et al.*, 2001)]

Thioacetamide induced liver cirrhosis has been extensively studied in rats and in other animal species using different times, doses and routes of administration. The most common types of administration are via drinking water, gastric tube, diet and by subcutaneous or intraperitoneal injections. Overall, the thioacetamide induced experimental liver disorder showed pathology and biochemical changes similar to human liver cirrhosis (Toyokuni & Sagripanti, 1994). Thioacetamide has been revealed to cause severe necrosis of the rat's liver by oxidative stress (Kishioka *et al.*, 2007) and it caused significant increase in the activity of amino transferases (ALT and AST), alkaline phosphatase (ALP), bilirubin and total protein (TP) gamma glutamyl transferase (γGT) and other biochemical markers of the liver (Madani *et al.*, 2008; Kumar *et al.*, 2004; Galisteo *et al.*, 2006b). Long term administration of thioacetamide resulted in distinctive lesions in the rats livers, which resembles the micro nodular cirrhosis after treatment over 3 months (Müller *et al.*, 1988).

Furthermore, thioacetamide has been proven to cause alterations of lipid and lipoproteins metabolism in female *Wistar* rats. It decreased levels of pre-β-lipoproteins, increased levels of β-lipoproteins, decreased concentration of serum triglyceride and decreased hepatic VLDL-TG output into the serum (Zimmermann *et al.*, 1986). Previous studies have described the changes in the collagen deposit after TAA intra

peritoneal injection, accompanied by body weight changes with cellular necrosis and regeneration, pseudo-lobular hepatic fibrosis and the type of liver nodules vary from micro nodular to macro nodular depending on the dosage and the time of TAA exposure (Munoz *et al.*, 1991).

The research of Stankova *et al.* (Stankova *et al.,* 2010) documented the decrease in glutathione (GSH) levels in rat hepatocytes *in vitro* prior to the increase in endogenous ROS production. They suggested that the primary target for TAA metabolites were not the mitochondria, which were affected secondarily due to the stimulation of oxidative stress. Mice treated with thioacetamide have been proven to experience elevated levels of hydroxyl proline and α-smooth muscle actin protein. It also induced increased mRNA levels of the genes collagen alpha1 (collα1) and transforming growth factor beta 1 (TGFβ1) in the liver (Hsieh *et al.,* 2008). TAA treated liver has been found to benefit from reduced mRNA levels of TGF-β and pro-collagen α1 genes, which in turn resulted in pathological recession of liver fibrosis (Sato *et al.,* 2000).

2.3 Herbal medicine

Even today, huge numbers of people on this planet still depend on medicinal plants for their daily healthcare needs. Natural products derived from plants are the source of most active components in modern medications, which in turn play a significant role in the treatment and prevention of human illnesses. Fifty percent of the drugs in the clinical usage of the world have been derived from natural products. Higher plants represent 25% of the total, whilst at least a dozen effective medications were found as derivatives from flowering plants. According to the World Health Organization's (WHO) estimation, 70-95% of the population in developing countries (especially Asia, the

Middle East, Africa and Latin America) depend on traditional medications to manage their primary health care. As a result, tropical plants have been investigated intensively during the last few years in order to evaluate the possibility of developing new, sustainable, natural and affordable cosmetics and drugs (Gurib-Fakim, 2006; Robinson Mm, 2011).

Recent research conducted by Stickel and Schuppan (Stickel & Schuppan, 2007) on medicinal plants signified that new phytochemicals could be developed for many health problems, serving as one of the therapeutic challenges of the new millennium. For example, the vinca alkaloids (vinblastine, vincristine, and vindesine), derived from *Vinca rosea*, *Lochnera rosea* and *Ammocallis rosea* have been utilized for their anticancer properties. Thus, many pharmacological uses have been revealed by studying medicinal plants, such as antipyretics, sedatives, cardioprotectives, analgesics, antiviral, antibacterial, and antiprotozoal (Cragg et al., 2012). Many factors support the rise in requests for herbal medicines in the last few decades, such as herbs being considered more safe to use because they are "natural" and are therefore perceived as being a harmless option, or because no unfavorable side effects exist coupled with easy availability and low cost (Stickel & Schuppan, 2007).

2.3.1 Medicinal plants and their role in treating liver disorders

Medicinal plant extracts play an important role in the management of various liver disorders, as evidenced by herbs having been used in the treatment of liver injuries for over 2,000 years. Up to now, more than 160 plant-derived substances, from 101 plant families, have been recognized regarding their hepatoprotective activity (Negi *et al.*, 2008; Saleem *et al.*, 2010). In spite of many experimental limitations, a number of herbs

showed promising effects, either practically in cell cultures, in animal studies and even in clinical trials (Stickel & Schuppan, 2007). As examples, the hepatoprotective activity of *Leucas ciliata* leaves extract was tested against carbon tetrachloride (CCL_4) induced hepatotoxicity in rat model, the antihepatotoxic activity of *L.ciliata* was documented to be due to its free radical scavenging activity (Pradhan & Girish, 2006). While the aqueous extract of the central stem of *Musa sapientum* showed a hepatoprotective activity against CCL_4 hepatotoxicity, comparable to that of silymarin (a well known hepatoprotective drug) through pathological, antioxidant and biochemical findings (Dikshit et al., 2011).

Aqueous extracts of *Solanum fastigiatum* was used to evaluate the hepatoprotective activity on mice liver damage induced by paracetamol, by monitoring biochemical and antioxidant parameters (Sabir & Rocha, 2008a). Moreover, The results (Galisteo *et al.*, 2006a) revealed that *Rosmarinus tomentosus* ethanol extract administered in the diet affords protection against thioacetamide (TAA) induced liver cirrhosis, preventing the majority of the histological changes and biochemical alterations.

2.3.1.1 Silymarin

Silymarin (Figure 2.6) is the flavono lignan extracted from the dried seeds of milk thistle *Silybum marianum*. It exsits as four flavono lignan isomers, namely isosilybin, silybin, silychristin and silydianin with molecular formula $C_{25}H_{22}O_{10}$ (Pradhan & Girish, 2006).

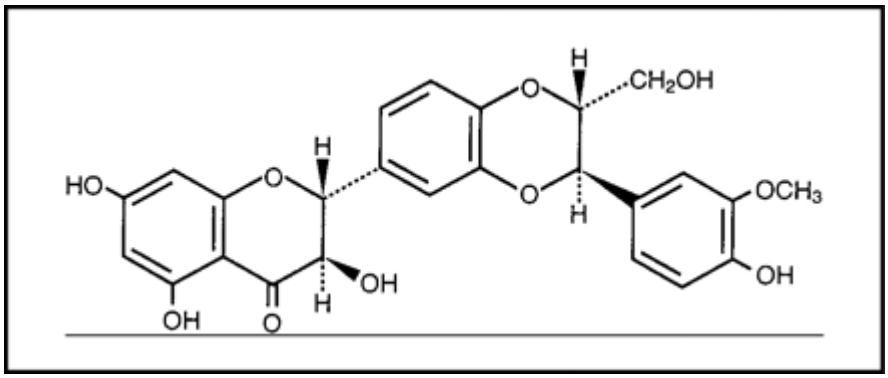


Figure 2.6: Chemical structure of silymarin

[Adapted from (Katiyar *et al.*, 1997)]

The pharmacological properties of silymarin have been well defined and the hepatoprotective profile was reported both *in vitro* and *in vivo*. It has been documented to prevent the liver from different injuries induced by various toxins or chemicals, by performing anti-lipid peroxidative, antioxidative, antifibrotic, membrane stabilizing, anti-inflammatory, liver regenerating and immunomodulatory activities. Its mechanisms of action have been investigated to be through promoting protein synthesis, controlling inflammation, regenerating liver tissue, protecting against glutathione depletion and enhancing glucuronidation (Choudhury et al., 2011; Colegate & Molyneux, 1993; Cragg & Newman, 2005). Silymarin is reported also to enhance the activity of RNA-polymerase I, combined to toxic-free iron and blocks the uptake of toxins such as Amanitaphalloides toxin (Yadav *et al.*, 2008). Furthermore, promising activities of silymarin have been reported in regards its anticancer activity, regulation of apoptosis and the inflammation process. It also has potential as a neuroprotective agent due to its neurotropic activities, which could be used to prevent and treat cardio-pulmonary problems, to treat skin problems and as steroid hormons receptors (Kren & Walterova,

2005). Overall, the mechanisms of the hepatoprotective action of silymarin have been investigated to be through four actions:

1- Free radical scavenging activity, increasing of glutathione cellular content and lipid peroxidation lowering property.
2- Membrane permability regulation and increasing the membrane stability against damage caused by xenobiotics.
3- Regulation of nuclear expression through steroid like effects.
4- Inhibits the formation of myofibroblasts from stellate hepatocytes, which releases collagen and subsequently causes cirrhosis of the liver (Pandy Govind, 2011).

2.3.2. Immunomodulatory roles of medicinal plants

The immunomodulator material modulates the responses of the immune system to produce antibodies to react with the antigens that initiated their production. Examples of immunomodulators are thymosin, corticosteroids, cytotoxic agents, and immunoglobulins. Certain immunomodulators naturally exist in the body and some would be available by pharmacological preparations. Immunomodulation is a therapeutic approach for intervening in auto regulation processes of the defense system; it is one of the three homotoxical treatments alongside detoxification and cellular activation (Spelman *et al.*, 2006). Medicinal plants have been studied widely as a source of botanical immunomodulators in various *in vivo* and *in vitro* studies. Different herbs were found to regulate cytokine secretion, immunoglobulin secretion, histamine release, cellular co-receptor expression, phagocytosis and lymphocyte expression. These herbs might offer an alternative course of therapy to the costly immunotherapeutics (Patwardhan & Gautam, 2005). In a recent study by Bin-Hafeez (Bin-Hafeez *et al.*,

2003) the immunostimulating effects of *Trigonella foenumgraecum* L. was reported at doses of 50 and 100 mg/kg. It revealed that this plant has a stimulatory effect on immune functions in mice body weight, organ weight, as well as causing a delayed type of hypersensitivity response, cellularity of lymphoid organs, phagocytosis, plaque-forming cell assay, quantitative haemolysis of SRBC assay, haemagglutination titre, and lymphoproliferation. While Makare et al (Makare *et al.*, 2001) showed that administration of *Mangifera indica* Linn resulted in a increase in humoral antibodies and delayed type hypersensitivity in mice.

Moreover, results of Yeap *et al.* (Yeap *et al.*, 2007) revealed the proliferation effect of the pre-treatment of peripheral blood mononuclear cells (PBMC) with *Rhaphidophora korthalsii* methanol extract, which stimulated the cytotoxicity toward human hepatocellular carcinoma (HepG2). In addition, (Souza-Fagundes *et al.*, 2002) reported the role of plant extracts as sources of immunomodulators by screening 313 extracts from 136 plant species using the bioassay based on the inhibition of proliferation of PBMC stimulation with phytohemaglutinin A (PHA). Furthermore, *Acoruscalamus* has been reported to exhibit an *in vitro* anti-cellular and immunosuppressive potential, by proliferating the mitogen (phyto-haemagglutinin) and the antigen (purified protein derivative) that stimulate human peripheral blood mononuclear cells (PBMCs), it also inhibits production of interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) (Mehrotra *et al.*, 2003).

2.3.3 Free radicals and oxidative stress

Reactive oxygen species (ROS), such as the free radicals (e.g. hydroxyl, superoxide, nitric acid) and certain non-radicals (e.g. lipid peroxide, hydrogen peroxide) lead to damage of specific molecules by a process called oxidative stress, which consequently leads to the injury of the cells or tissues. High level's of ROS results from inflammation, viral or fungal infection, ageing, pollution, UV radiation, excessive alcohol consumption, cigarette smoking…etc (Mittler, 2002). The ROSs produced *in vivo* in respiratory chains of mitochondria, activated polymorpho-nuclear leukocyte, macrophages and peroxisomes. Exogenous sources of these harmful radicals are items such as tobacco smoke, pollutants, pesticides, ionizing radiation and organic solvents (Kumar, 2011).

Neutralization of ROS is accomplished by antioxidants, which are chemicals or substances that retard the damage caused by these free radicals. Well-known antioxidants include enzymes and other substances which are either endogenous (e.g. superoxide dismutase, catalase, glutathione) or exogenous (e.g. vitamins A, C, E, α-tocopherol, selenium, beta carotene, butylated hydroxyl anisole and butylated hydroxyl toluene) that are capable of counteracting the destructive effects of oxidation. Food products such as vegetable oils and prepared foods are usually supplied with antioxidants to prevent or interrupt their rancidity by the action of air (Halliwell, 1994).

Liver cells, especially hepatocytes, have the excellent ability to metabolize and detoxify ROSs, subsequently repairing damage done by antioxidants. Furthermore, the liver expresses a multi-layered defense system against these ROSs, as it encloses superoxide dismutases (in the cytosol and mitochondria), catalase (in the peroxisomes), glutathione peroxidases (in the cytosol and mitochondria), holds glutathione in its cellular

compartments besides its cell membranes and carries radical chain-breaking antioxidants like vitamin E. Therefore, ROS could cause instability only on cellular homeostasis, it would lead to cell death if not effectively counteracted (Jaeschke & Ramachandran, 2011).

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide ion into oxygen and hydrogen peroxide (H_2O_2). Catalase (CAT) catalyzes the decomposition of hydrogen peroxide to water and oxygen. Glutathione peroxidase (GPX) removes hydrogen peroxide by converting reduced glutathione into oxidized glutathione. These enzymes modulate in several diseases, including multiple sclerosis, alzheimer disease, diabetes, liver cirrhosis and hepatocellular carcinoma (Szymonik-Lesiuk *et al.*, 2003). Previous studies documented a significant decrease in the level of CAT, SOD and GPX enzymes after consequent increase in oxidative stress and free radical levels, resulting in an increase in cellular damage in cirrhotic rats (Abul *et al.*, 2002).

The control of ethanol-induced oxidative stress in alcoholic liver diseases (ALD), hepatic stellate cells (HSC) and inflammatory cells have been shown to initiate the production of hepatocyte growth factor (HGF), which leads to hepatocytes proliferation and repair. HGF has been revealed to decrease ROS production, lipid peroxidation and protein oxidation damage due to ethanol metabolism, with an increase in cell viability. The mechanism involved in the HGF that induced protection against ethanol toxicity revealed that HGF induces the expression of antioxidant enzymes such as CAT, SOD, and GPX (Gómez-Quiroz *et al.*, 2012).

Liver cirrhosis has been reported to be accompanied by excessive oxidation of polyunsaturated membrane lipids (Poli, 2000). Lipid peroxidation induces over

expression of fibrotic cytokines, the key molecules in the fibrosis mechanism, as well as increased synthesis of collagen. Thus the hepatic lipid peroxidation has been extensively studied in *vitro* and *in vivo* experimental models by measuring malondialdehyde (MDA), a major end product of lipid peroxidation (Poli & Parola, 1997). Lipid peroxidation and subsequently fibrosis have been prevented in the liver of animals supplemented with antioxidants like silymarin (Shaker *et al.*, 2010), *Anoectochilus formosanus* (Shih *et al.*, 2005), *Nigella sativa* (Kanter *et al.*, 2003), *Rosmarinus officinalis* (Gutierrez *et al.*, 2010), *Ambrosia maritime* (Ahmed & Khater, 2001) and many other plant extracts.

2.3.3.1 Antioxidant properties of medicinal plants

Many synthetic antioxidants e.g. butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT) or tert-butyl hydro quinine (TBHQ) have been restricted against includion in foods because of their toxicity, therefore, attention has been directed towards the improvement of natural antioxidants from plants materials.

Natural antioxidants could be either phenolic compounds (phenolic acid, flavonoid, and tannin), nitrogen containing compounds (chlorophyll derivative, alkaloid, amino acid, amine and peptide,), ascorbic acid, carotenoids or tocopherols and their derivatives. Thus interest has raisen amongst food manufacturers, scientists and consumers toward's antioxidant constituents of botanical sources in the maintenance of health and protection from different diseases and cancer (Dimitrios, 2006).

There have been various *in vitro* techniques used to determine the effectiveness of plant derived natural antioxidants. These techniques are of two types:

1) Hydrogen atom transfer reactions, such as total radical trapping antioxidant potential (TRAP), oxygen radical absorbance capacity (ORAC) and β- carotene bleaching.
2) Electron transfer reactions such as ferric reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC), α,α-diphenyl-β-picryl-hydrazyl radical scavenging assay (DPPH), hydroxyl radical scavenging assay, superoxide anion radical scavenging assay, total phenolic or flavonoids assays and nitric oxide radical scavenging assay.

However, it is important to apply more than one technique to measure the antioxidant ability of plant extracts because of the complex structure of phytochemicals (Chanda & Dave, 2009).

Medicinal plants have been screened worldwide for their antioxidant effects. In a study by Adewusi and Steenkamp (Adewusi & Steenkamp, 2011) they reported the antioxidant activities of 12 plants traditionally use in South Africa to treat neurological disorders. Their findings support that the antioxidant activities of plants counteract with the ROS and the cholinesterase mechanisms. *In vitro* radical scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) has been detected with *in vivo* superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities enhanced in Chinese hamster lung fibroblasts (V79-4) treated with methanol extracts of nine medicinal plants traditionally used in Chinese medicine (Lee *et al.*, 2003).

Moreover, correlations were found between the antioxidant activities of 42 plants used traditionally in Thailand and their chemical content such as vitamin C, vitamin E, carotenoids, tannin, and totalphenolics (Chanwitheesuk *et al.*, 2005). Also, the antioxidant capacity and the total phenols and flavonoid contents of kiwi fruit (*Actinidia deliciosa*) were determined *in vitro*, by assaying free radical scavenging activities of

1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2′-azino-bis(3-ethyl benzthiazoline-6-sulfonic acid) (ABTS), superoxide anion radical and N,N-dimethyl-p-phenyl enediamine (DMPD), and reducing power of (FRAP) and (CUPRAC) and the metal chelating activities (Bursal & Gulcin, 2011).

The *in vitro* inhibition of lipid peroxidation, DPPH radical scavenging activity and modulation of mutagenicity in *Escherichia coli* induced by ter-butyl hydroperoxide (TBH) have been screened in 45 medicinal plants in Cuba (Ramos *et al.*, 2003). Furthermore, 22 extracts from 14 species of Brazilian medicinal plants were studied for their ability to reduce DPPH free radical and to protect the yeast *Saccharomyces cerevisiae* cells against the lethal oxidative stress caused by tert-butyl hydroperoxide (TBH) (Silva *et al.*, 2005).

2.3.3.2 Plant-derived polyphenolic compounds

Plant-derived polyphenolic compounds are organic compounds of plant origin with more than one phenol group. Polyphenols are classified by their source of origin, chemical structure and biological function into phenolic acids (benzoic acid and cinnamic acid derivatives), flavonoids (anthocyanins, flavanols, flavones and flavanones) and polyphenolic amides, with some highly polymerised compounds like lignans, melanins and hydrolysable tannins (Tsao, 2010).

Polyphenols are widely involved as the active components in many herbal and traditional medicines. More than 5000 plant polyphenols have been identified and are known to possess a wide range of pharmacological properties (Ullah & Khan, 2008). In recent years, plant-derived polyphenolic compounds were shown to possess a wide range of pharmacological properties, such as anti-inflammatory, antioxidant and DNA

repair mechanisms (Nichols & Katiyar, 2010). They are recognized as naturally occurring anti-microbial compounds (Taguri *et al.*, 2004) and have been implicated as cancer chemopreventive agents (Stoner & Mukhtar, 1995).

Furthermore, the hepatoprotective effects of different polyphenolic compounds derived from plant origins were reported by (Adzet T, 1987 ; De Oliveira E Silva Am, 2012) and the mechanisms involved in the hepatoprotective effects have been suggested to be related to the inhibition of iron absorption by polyphenol compounds (Mascitelli *et al.*, 2008).

2.3.4. Modern techniques used for isolation of plants active constituents

The first research on isolating plants active constituents' were scientifically recorded at start of the 19^{th} century. Since then, different organic chemistry techniques were developed and improved upon. These techniques include extraction, separation and identification of the chemical ingredients. Thus, different parameters have been reported to affect the extraction yield, such as the used plants parts, solvent type and extraction time (Ahmad et al., 2009). Various preparation steps like pre-washing plants, drying or freeze drying and grinding are used to gain a homogenous sample. In addition to which, extraction methods such as heating under reflux, sonication, soxhlet extraction and others are also commonly used (Ong, 2004).

Sample preparation is the first critical step of the analysis process, since it is important to extract the preferred active components from the plant extracts for further separations and purifications (Huie, 2002).

Separations have been reported to be through liquid chromatography with gradient elution, by capillary electrophoresis or by using hyphenation procedures. Hyphenated

techniques such as high-performance liquid chromatography (HPLC) coupled to UV spectroscopy (LC/UV), mass spectrometry (LC/MS), gas chromatography coupled to mass spectrometry (GC/MS) and more recently nuclear magnetic resonance (LC/NMR), have all been proven to be highly efficient for the chemical screening of crude plant extracts (Hostettmann *et al.*, 2001).

Testing the biological activities subsequent to the plant fractionation has been performed continuously in the discovery of new metabolites. (Hostettmann *et al.*, 1998). GC/MS have been used mostly for the analysis of volatile and semi-volatile compounds, like essential oils and others, while the LC/MS is the method of choice for many compounds, ranging from small polar molecules to macromolecules, such as proteins, nucleic acids and carbohydrates (Ong, 2004).

Identification of new compounds could be done by comparing their chromatographic retention times and mass-spectral fragmentation outlines to the recognized and expected information available in certain databases (Kopka *et al.*, 2004). Additionally direct comparison with available authentic materials could confirm the identification, but if the authentic compounds were not available, comparison with literature data may suffice for the identification (Harborne, 1998).

2.4 *Phyllanthus niruri Linn*

2.4.1 Botanical classification and characteristics

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Euphorbiales

Family: Euphorbiaceae

Genus: *Phyllanthus*

Species: *niruri*

Phyllanthus niruri Linn. Locally known in Malaysia as dukung anak, is found in most tropical and subtropical regions (Figure 2.7). It is a small herb, growing up to 60 cm in height. Its leaves are small with very short petiole, the flowers vary from white to greenish in color (Murugaiyah & Chan, 2009).

Figure 2.7: *Phyllanthus niruri Linn*.

2.4.2 Traditional uses

Phyllanthus niruri has been used in folk medicine as antipyretic, analgesic, as well as to treat inflammations or similar symptoms that might suggest anti-histamine effects. Also, the decoction of the whole plant has been used as a drug against diarrhoea and as a bath emolient used to treat jaundice. Crushed leaves together with leaves of *Eupatorium odoratum* and lime are applied on boils (Ong & Norzalina, 1999).

Furthermore, it has been used as an antispasmodic, including treatment of stomachaches, abdominal pain, colic, menstrual cramps, dysmenorrhea, relief of spasms, childbirth pains, or similar symptoms (Unander *et al.*, 1995).

2.4.3 Medicinal uses

Previous studies revealed the therapeutic potential of *Phyllanthus niruri* to treat genito-urinary infections, venereal diseases, kidney or bladder stones, as well as acting as a urinary inhibitor of calcium oxalate crystallization and as effective treatments for urolithiasis through its interfere in the growth and aggregation of calcium oxalate crystals (Freitas *et al.*, 2002; Boim *et al.*, 2010; Barros *et al.*, 2006). It has also been shown that the anti-hyperuricemic action might be due to its uricosuric activity through xanthine oxidase inhibitory effect (Murugaiyah & Chan, 2009).

It has been used to treat diabetes through blood glucose lowering properties such as inhibition of glucose absorption and enhancement of glucose storage (Okoli *et al.*, 2011), with the methanol extract of *P. niruri* exhibiting significant anti-hyperglycemic activity in streptozotocin-induced diabetic rats (Mazunder et al., 2005).

In addition, *P. niruri* aqueous extract had cardio-protective effects on doxorubicin-induced myocardial toxicity in rats (Thippeswamy *et al.*, 2011), used as antiplasmodial to treat malaria and to treat skin or throat infections of bacterial origin (Mustofa & Wahyuono, 2007). Khanna *et al.* (Khanna *et al.*, 2002) studied the lipid lowering activity of *Phyllanthus niruri*. They found that this plant caused lowering in the levels of VLDL and LDL when fed to the research animals. In addition, they revealed that this activity was mediated through inhibition of hepatic cholesterol biosynthesis, increased bile acids excretion, whilst also enhancing plasma lecithin and cholesterol acyl transferase activity. Tang *et al.* (Tang *et al.*, 2010) investigated the cytotoxic effects of the aqueous and the methanolic extracts of four *Phyllanthus* species (*P. niruri, P. amarus, P. watsonii* and *P. urinaria)* against prostate cancer cells and skin melanoma. Their results indicated that *Phyllanthus* extracts enclosed the ability to induce apoptosis with no significant cytotoxicity effects on normal cells.

Moreover, *P. niruri* in combination with *Nigella sativa* has proven to treat viral origin acute tonsillo-pharyngitis through anti-inflammatory and immunmodulatory effects (Dirjomuljono *et al.*, 2008).

2.4. 4 Chemical constituents isolated from *Phyllanthus niruri*

The most recent research has characterized a wide variety of phytochemicals and their pharmacological properties from various parts of *P. niruri*. The most known active phytochemicals that have been isolated were flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, coumarins and saponins (Bagalkotkar *et al.*, 2006; Murugaiyah & Chan, 2009; Markom *et al.*, 2007).

The aqueous extract of the intact plant provided an acidic arabinogalactan, which was characterized chemically, along with its effects on peritoneal macrophage activation (Mellinger *et al.*, 2005). Additionally, Benzenoids, phytallates, lipids, sterols, triterpenes have all been identified, and several polyphenolic constituents were isolated from tissue cultures of *P. niruri*, such as gallic acid, catechin, epicatechin, gallocatechin, epigallocatechin, epicatchin-3-O-gallate and epigallocatechin-O-gallate (Calixto *et al.*, 1998).

2.5 *Melastoma malabathricum Linn*

2.5.1 Botanical classification and characteristics

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Myrtales

Family: Melastomataceae

Genus: *Melastoma*

Species: *malabathricum*

Melastoma malabathricum Linn is a plant from the family Melastomaceae with one meter of shrub and plenty of branches. It can grow to a height of bween 3 to 6 meters (Figure 2.8) and the flowers raise 5 to 10 clusters with 5 petals each (Susanti *et al.*, 2007).

Figure 2.8: *Melastoma malabathricum Linn*.

2.5.2 Traditional uses

Melastoma malabathricum (MM) is a plant found in many regions of tropical Asia, where it is known by different local names, such as the Straits Rhododendron (Singapore), Malabar melastome (Australia), Indian-rhododendron or Lutki (India) and Senduduk (Malaysia). According to the tribes of Manipur it is known as a "Wonder drug", since the leaves are medicinally used as an antipyretic drug and commonly recommended to lower fever (Ringmichon et al., 2010).

The Melastomataceae family is used worldwide to treat a range of diseases, such as diarrhea, dysentery, wound-healing and post-partum depression. Leaf extracts from *M. malabathricum* have been used in traditional medicine as a drink to cure diarrhea, while the fruit juice has been used as a lip polish (Ong & Nordiana, 1999).

In Malaysia the Malay population use the leaves and shoots of this plant for the treatment of wounds, post natal health care and to prevent scars of small pox. The root extract's have been used to relieve toothaches and the flowers can be used to brew a tea to treat stomach ache, flowers cuts were used for washing skin and a root decoction was sometimes taken for measles (Rajenderan, 2010).

2.5.3 Medicinal uses

Prior studies on *M. malabathricum* leaves showed that the methanol and acetone extracts had significant antibacterial and wound-healing potential, possessing potent anticoagulant properties compared with heparin (Choudhury *et al.*, 2011; Sunilson *et al.*, 2008; Manicam *et al.*, 2010).

The study of (Hussain *et al.*, 2008) demonstrated that aqueous extracts from *M. malabathricum* leaves had gastro-protective activities toward ethanol-induced gastric ulcers in rats. The phytochemical investigation of (Susanti *et al.*, 2009) isolated a series of flavonoids, triterpenoids and alkaloids by chromatography that scavenged free radicals, prevented lipid peroxidation, and reduced inflammation. Moreover, *M. malabathricum* showed potent antiviral activity against-Herpes simplex virus and Poliovirus and showed cytotoxic activity against human and murine cancer lines (L1210, 3LL, K562, DU145, U251, MCF-7) (Lohezic-Le Devehat *et al.*, 2002). The natural penta cyclic triterpenes and flavonoids from *M. malabathricum* could inhibit platelet activating factor (PAF) and may act as a natural anti-inflammatory agent and may actively scavenge free radicals (Sirat *et al.*, 2010).

2.5.4. Chemical constituents isolated from *Melastoma malabathricum*

Kaempferol 3-0-D-galactopyranoside, kaempferol 3-0-(2",6" -di-O-E-p-coumaroyl-D-galactopyranoside, kaemptero13-O-L-rhamnopyranoside, kaempferol kaempfero13-O-D-glucopyranoside, quercetin and ellagic acid were isolated from the methanol extract of the flowers of *Melastoma malabathricum* (Susanti et al., 2007).

The phytochemical research of (Wong *et al.*, 2011) resulted in the recognition of ursolic acid, asiatic acid, 2-hydroxyursolic acid, sitosterol 3-O-D-glucopyranoside and the glycolipid glycerol 1,2-dilinolenyl-3-O-D-galactopyanoside in the chloroform fraction. Kaempferol, kaempferol 3-O-D-glucopyranoside, kaempferol3-O-L-rhamnopyranoside, kaempferol 3-O-(2, 6-di-O-Ep-coumaryl)-D-galactopyranoside, kaempferol 3-O--D-galactopyranoside, ellagic acid and quercetin were found from the ethyl acetate fraction. Numerous hydrosable tannins have been identified from this plant likedimers named

malabathrins B, C and D oligomers named nobotanin B, monomers named 1,2,4,6-tetra-O-galloyl-b-D-glucoside, 1, 4, 6-tri-O-galloyl-b-D glucoside, strictinin, pedunculagin, casuarictin, nobotanin D, oligomers nobotanins G, H, and J. and pterocarinin. A further research revealed the presence of a-amyrin, b-sitosterol, sitosterol-3-O-b-D-glucopyranoside, uvaol, quercitrin, quercetin, and rutin (Sirat *et al.*, 2010).

CHAPTER III

METHODOLOGY

3.1 Materials

Tween 20 and 95% (v/v) ethanol were purchased from (Sigma-Aldrich, UK). Filter paper (Whatmann No.1, Fitchburg, WI, USA), R-215 rotating evaporator (Buchi, Flawil, Switzerland), freeze-drying machine (LabConco, Kansas City, MO, USA), Genie-2 vortex (Scientific Industries Inc., USA). 2,2 Diphenyl-1-picryl dyhydrazyl (DPPH) reagent, **2, 2-**azinobis 3-ethyl benzothiazoline-6-sulfonic acid (ABTS) reagent, Folin-Ciocalteu reagent, ascorbic acid (product no. A4403), potassium persulfate, methanol, Folin-Ciocalteu reagent, gallic acid, natrium carbonate (Na_2CO_3), butylated hydroxyl toluene (BHT), aluminium chloride, potassium acetate, quercetin, acetate buffer, 2, 4, 6-tripyridyl-s-triazine (TPTZ), HCL, $FeCl_3.6H_2O$. were all purchased either from (Thermo Fisher scientific ,USA) or (Sigma-Aldrich, UK). Power wave X 340 ELISA plate reader from (BIO-TEK instruments, Winooski, VT, USA). Trypan blue, Histopaque-1077, PBS (phosphate buffer saline), Roswell Park Memorial Institute-1640 (RPMI-1640) media supplemented with 2 mM glutamine and $NaHCO_3$ purchased from (Sigma-Aldrich, Gillingham, UK). 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide **(**MTT reagent), FBS (Fetal Bovine Serum), DMSO (dimethyl sulphoxide) purchased from (Merck, Darmstadt, Germany). Neubaur haemocytometer (Weber, Teddington, UK), IR Jacketed incubator (from NUAIRE laboratory equipment supply, Plymouth, MN, USA). Silymarin was purchased from International Laboratory (USA), Thioacetamide, xylazine, ketamine, Formalin, Hematoxylin & Eosin were obtained from (Sigma-Aldrich, Gillingham, UK). Automated tissue processor, Leica Tissue Microtome (Leica, Germany). AM 432 Dino Lite microscope eyepiece camera (Dpro Scientific Bhd. Sdn.) Wise Mix^{TM} HG,15A

homogenizer (DAIHAN Sci., Seoul, Korea), Jouan C312 centrifuge (Santa Fe Springs, CA, USA) and Rotofix 32 refrigerated centrifuge (Hettich Zentrifugen, Germany). Commercially available kits from (Cayman Chemical Co., USA) were used to determine total antioxidant capacity (TAC) (cat. #709001), Malonialdehyde (MDA) (cat. #10009055), Catalase (CAT) (cat. #707002), superoxide dismutase (SOD) (cat. #706002) and glutathione peroxidase (GPX) (cat. #703102) activities. RNA-later solution (Applied Biosystems, Foster City, CA), Qiamp RNA blood mini kit (Qiagen, Germantown, Maryland, USA). RNase- Free DNase set (Qiagen, Germantown, Maryland, USA), Nano Drop ND-2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), agarose gels and Tris-Borate-EDTA (10X TBE) (Applied Biosystems, USA), Ethidium Bromide (EB) and loading dye (Promega, USA). High Capacity RNA-to-cDNA Master Mix, TaqMan Fast Advanced Master Mix, ultrapure DNase free water, Transforming growth factor beta ($TGF\beta_1$), tissue inhibitors of metalloproteinases ($TIMP_1$), matrix metalloproteinase (MMP_2), Collagen alpha (Collα1).glyceraldehyde 3-phosphate dehydrogenase (GAPDH), beta actin (β-actin), hypoxanthine phosphoribosyl transferase 1 (Hprt1) and peptidyl prolylisomerase A (Ppia) assay genes were all purchased from (Applied Biosystems, Foster City, CA), thermal cycler (PTC-100 thermal controller, MJ Research Inc., Basel, Switzerland), Monochrome scientific grade camera and gel documentation system (Vilber Lourmat, Thermo Fisher scientific, USA). Silica gel 60 powder (0.063 - 0.200 mm) and (70 - 230 mesh), silica gel F254 plates (20 x 20 cm, 0.2 mm), HPLC grade n hexane, HPLC grade ethyl acetate, HPLC grade methanol, HPLC grade acetonitrile were purchased from (Merck, Germany). (2 x 30 cm) Kontes column with EYEL- 4 pump (Rikakikai, Tokyo). Milli-Q academic water purification system (Millipore, USA).

3.2 Protocol of the study

Two plants were selected in this study to evaluate their *in vivo* hepatoprotective effects; based on the ethnobotanical information and literature reports, the previously implicated hepatoprotective function of the *Phyllanthus niruri* was further evaluated and the mechanism underlying its hepatoprotective action was investigated using rat model of liver cirrhosis induced chemically by thioacetamide (TAA) administration.

Secondly, the *in vitro* bioactivities were assessed (including the antioxidant activities and the immunomodulatory effects) besides, the isolation of active constituents of both plants by different chromatography techniques.

Then, since no literature was found investigating the activity of *Melastoma malabathricum* as a hepatoprotective agent and the healing properties on liver cirrhosis have not been studied before, this study was set out with the aim of assessing the importance of *M. malabathricum* ethanol extract to prevent thioacetamide induced liver cirrhosis in *Sprague Dawley* rats. The obtained results of both plants were compared with standard hepatoprotective agent “silymarin”.

All of the processes and procedures that employed in our experiments are shown in the Flow Chart of the study in Figure 3.1.

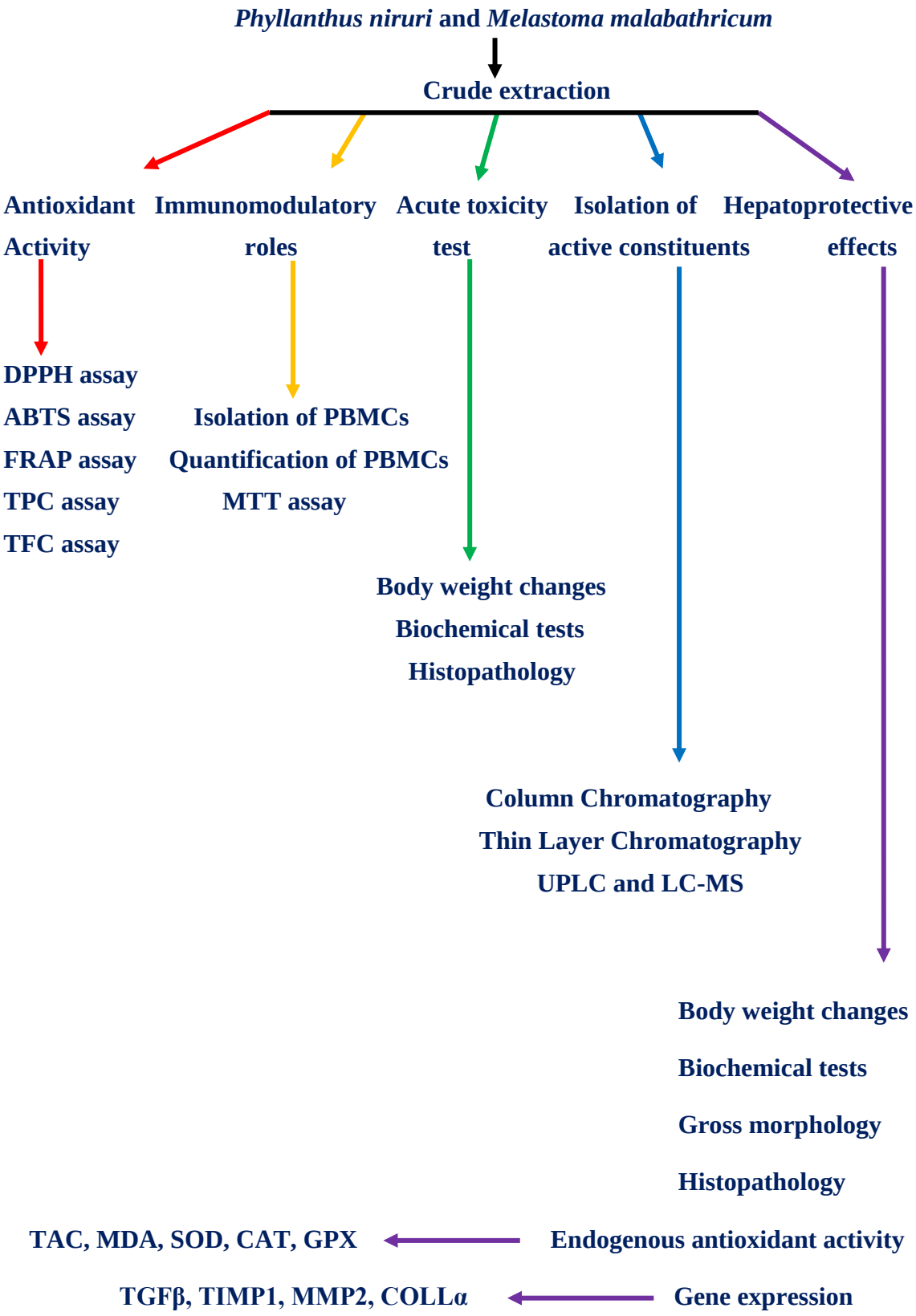


Figure 3.1: Protocol of the study

3.3 Methods

3.3.1 Preparation of plants extracts

Phyllanthus niruri and *Melastoma malabathricum* whole plant parts were obtained from Ethno Resources Sdn Bhd, Selangor Malaysia. For preparation of the ethanol extracts, (100 grams) of the fine powders were soaked in (1000 ml) of (95 % ethanol) for 72 hours. Then the extracts were filtered by filter paper (Whatman No. 1), evaporated under low pressure by using rotary evaporator and finally subjected to lyophilization in freeze dryer to give the crude-dried extract. The dried extracts were stored at -20°C until used.

3.3.2 Antioxidant activity

3.3.2.1 Diphenylpicrylhydrazyl (DPPH) assay

DPPH analysis is one of the tests used to prove the ability of the components of the plants extracts to act as donors of hydrogen atoms. As the Diphenylpicrylhydrazyl (DPPH free radical) molecule has a spare electron over it, gives rise to a deep violet colour, characterized by an absorption band in methanol solution centered at about 515 nm. When a solution of DPPH is mixed with that of a plant extract that can donate a hydrogen atom, then this gives rise to the reduced form diphenylpicrylhydrazine (non radical DPPH) with the loss of its violet colour (to a yellow colour) (Molyneux, 2004). The scavenging activity of stable 2, 2 Diphenyl-1-picryl dyhydrazyl free radical was determined according to the method of (Gorinstein *et al.*, 2005) with slight modifications. (1 mg /1ml) of the plant extracts and the reference standard (ascorbic acid) were prepared as stock solutions then a series dilution with five varying concentrations were tested. 5µl of samples/standards were loaded, and followed by 195

µl of DPPH reagent, the mixtures were then mixed vigorously and incubated in the dark at room temperature for 2 hours, and the absorbance was measured spectrophotometrically at 515nm. The percentage of DPPH free radical scavenging activity was calculated as:

DPPH (%) = [(Abs of blank-Abs of sample)/absorbance of sample] × 100.

The results were expressed as (IC_{50} value) the extract concentration that required to reduce 50% of the hydroxyl radical produced.

3.3.2.2 Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay

The total antioxidant capacity assay was carried out using the improved method, as described by (Re *et al.*, 1999). The radical cation of 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical was generated by oxidation with potassium persulfate to a blue/green ABTS chromophore and further reduced in the presence of the plants extracts (as the hydrogen-donating sources of antioxidants) determined by the decolorization of the ABTS solution and measuring the reduction as the percentage inhibition of absorbance at 734 nm.

Briefly, ABTS radical cation is generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate via incubation at room temperature, in the dark for 12–16 h. The ABTS solution was diluted with methanol to an absorbance of (0.700 ± 0.020) at 734 nm and equilibrated at 30°C while Plant extracts were diluted with distilled water. To one milliter of diluted ABTS, 10 µl of each plant extract solution were added and mixed thoroughly. The reactive mixture was allowed to stand at room temperature for 15 min and the absorbance was recorded immediately at 734 nm. The percentage of ABTS free radical scavenging activity was calculated as:

ABTS (%) = [(Abs of blank-Abs of sample)/absorbance of sample] × 100.

The results were expressed as (IC_{50} value) the extract concentration that required to reduce 50% of the free radical produced.

3.3.2.3 Total phenolic content (TPC) assay

Total phenolic content was determined using Folin-Ciocalteu reagent following the method of (Singleton & Rossi Jr, 1965) using gallic acid as a standard. 10µl of extract solution (1mg /1ml) was added in a test tube followed by 0.5ml of 1:10 Folin-Ciocalteu reagent. The mixture was incubated at room temperature for 5 minutes. After 5 minutes, 0.35 ml of 115mg /ml natrium carbonate (Na_2CO_3) was added and mixed thoroughly. The mixture was then allowed to stand at room temperature for 2 hours. Absorbance readings were taken spectrophotometrically at 765nm and all determinations were done in triplicates. The total phenolic content was expressed as miligrams of gallic acid equivalent to grams dried plant material and butylated hydroxyl toluene (BHT) was used as positive control.

3.3.2.4 Total flavonoid content (TFC) assay

Total flavonoid content of each sample was determined by aluminium chloride colorimetric method by (Chang *et al.*, 2002) and (Harborne & Williams, 2000). Briefly, 0.5ml of extract solutions (1mg /1ml) were added to a separate test tube and mixed with1.5 ml of 95 % ethanol, 0.1ml of 1M of potassium acetate, 0.1 ml of aluminium chloride and 2.8 ml of distilled water. After that, the mixtures were incubated for 30 minutes at room temperature. The absorbance readings were taken

spectrophotometrically at 415nm. Total flavonoid content was expressed as milligrams of quercetin equivalent to grams of dried plant material.

3.3.2.5 Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power assay is presenting a method of measuring antioxidant power by converting ferric tripyridyltriazine complex (Fe III-TPTZ) at low pH to a colored ferrous-tripyridyltriazine complex (Fe II).The ferric reducing activity of the plant extracts were estimated using the method developed by (Benzie & Strain, 1996). The reaction mixture contained 300 mmol / L acetate buffer, 10 mmol/ L TPTZ (2, 4, 6-tripyridyl-s-triazine) in 40 mmol / L of HCL and 20 mmol /L of $FeCl_3.6H_2O$. the working FRAP reagent prepared freshly by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of FeCl3.6H2O. The freshly prepared mixture was incubated at 37°C in water bath for five minutes and then a blank reading was taken spectrophotometrically at 593 nm. After that, 10 µl of extract or standard and 90 µl of distilled water were added to 300 µl of the working FRAP reagent. Absorbance was measured (593nm) at 0 minute immediately upon addition of the working FRAP reagent after vortexing. Thereafter, absorbance reading was taken after four minutes. All results were expressed as mmol ferric reducing activity of the extract per gram of dried weight based on three experiments and butylated hydroxyl toluene (BHT) was used as the positive control.

3.3.3 Immunomodulatory effects

3.3.3.1 Isolation of peripheral blood mononuclear cells (PBMC)

To achieve the immunomodulatory effects of the plant extracts the peripheral blood mononuclear cell (PBMC) proliferation activity was investigated (Boyum, 1968).

Ten milliliters of whole blood was drawn from a healthy donor and diluted with the same volume of Histopaque-1077 (sigma Aldrich). The mixed solution was centrifuged under at 1,000 rpm for 30 min. The mononuclear layer was carefully transferred out and washed, then pelleted down with 30 ml PBS (phosphate buffer saline) and centrifuged at 1,000 rpm for 10 min for thrice and re-suspended with Roswell Park Memorial Institute-1640 (RPMI-1640) media supplemented with 2 mM glutamine and $NaHCO_3$ (Sigma-Aldrich, Gillingham, UK) with addition of 10% FBS (Fetal Bovine Serum).

3.3.3.2 Quantification of peripheral blood mononuclear cell (PBMC)

Haemocytometer grid system was used to determine the concentrations of PMBCs. First of all, 10 µL of the isolated PBMC was mixed with 10 µL of trypan blue (0.4%) and mixed eventually and 10 µL of this suspension was loaded into Neubaur haemocytometer (Weber, Teddington, UK) to find out the PBMC cell number. The PBMCs were visualized under 40X microscope and the numbers were counted following the formula:

PBMC concentration (cells/ml) = Average cell count * dilution factor $*10^4$

Consequently, the cells were mixed with RPMI-1640 media to the required concentration.

3.3.3.3 MTT assay

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide **(**MTT) assay was used to study the effect of the extract on PBMC cells viability (Mosmann, 1983). Briefly, 100 µL of cells suspended in RPMI media (with 10% of FBS) was added into the 96 well plate and incubated at IR Jacketed incubator (from NUAIRE laboratory equipment supply, Plymouth, MN, USA) for 24 h at 37 °C. Then, 10 µL of the extracts (20 mg/ml) were added and further incubated for 24 h.

After the corresponding period 10 µl of MTT reagent (Merck, Darmstadt, Germany) (5 mg/ml PBS) was added into each well and further incubated for 4 h. 100 µl of DMSO were added and shake for 20 min to solubilize and extract the formazan crystal. At the last, the plate was read at 595 nm by using Power wave X 340 ELISA plate reader (from BIO-TEK instruments, Winooski, VT, USA). All extract samples and controls were tested in triplicates in four independent experiments. The percentage of cell viability was calculated by the formula:

% Cell viability = (abs of extract sample – abs of control/abs of control) × 100

3.3.4 Acute oral toxicity test

To determine the safety of the plants extracts when administered orally, the acute toxicity test was performed following OECD-423 guidelines (Oecd, 1998). The experiment design is given in Figure 3.2. which in brief, forty healthy *Sprague Dawley* rats (20 males and 20 females) were randomly assigned equally into 5 groups labeled as vehicle 10% Tween 20 and two large doses 2 g/kg and 5 g/kg for each of *P. niruri* and *M. malabathricum* ethanol extract, respectively. Each rat was made to fast (no food but water) overnight prior to dosing. Food was withheld for another 3 to 4 hours after the dosing. The rats were closely observed for 30 minutes and at 2, 4, 24 and 48 hours after the dosing to detect if there were any acute signs of clinical toxicity, morbidity and mortality twice daily. Comparisons were verified between the individual animals and their respective vehicle treated animals. Detailed behavioral observations include: respiration (dyspenea), salivation, skin piloerection, exopthalmus, convulsion and locomotion changes. After keeping alive for 14 days, on day 15, the rats were sacrificed to measure serum biochemical and (liver and kidney) histological parameters following the standard methods (Abdulla *et al.*, 2010).

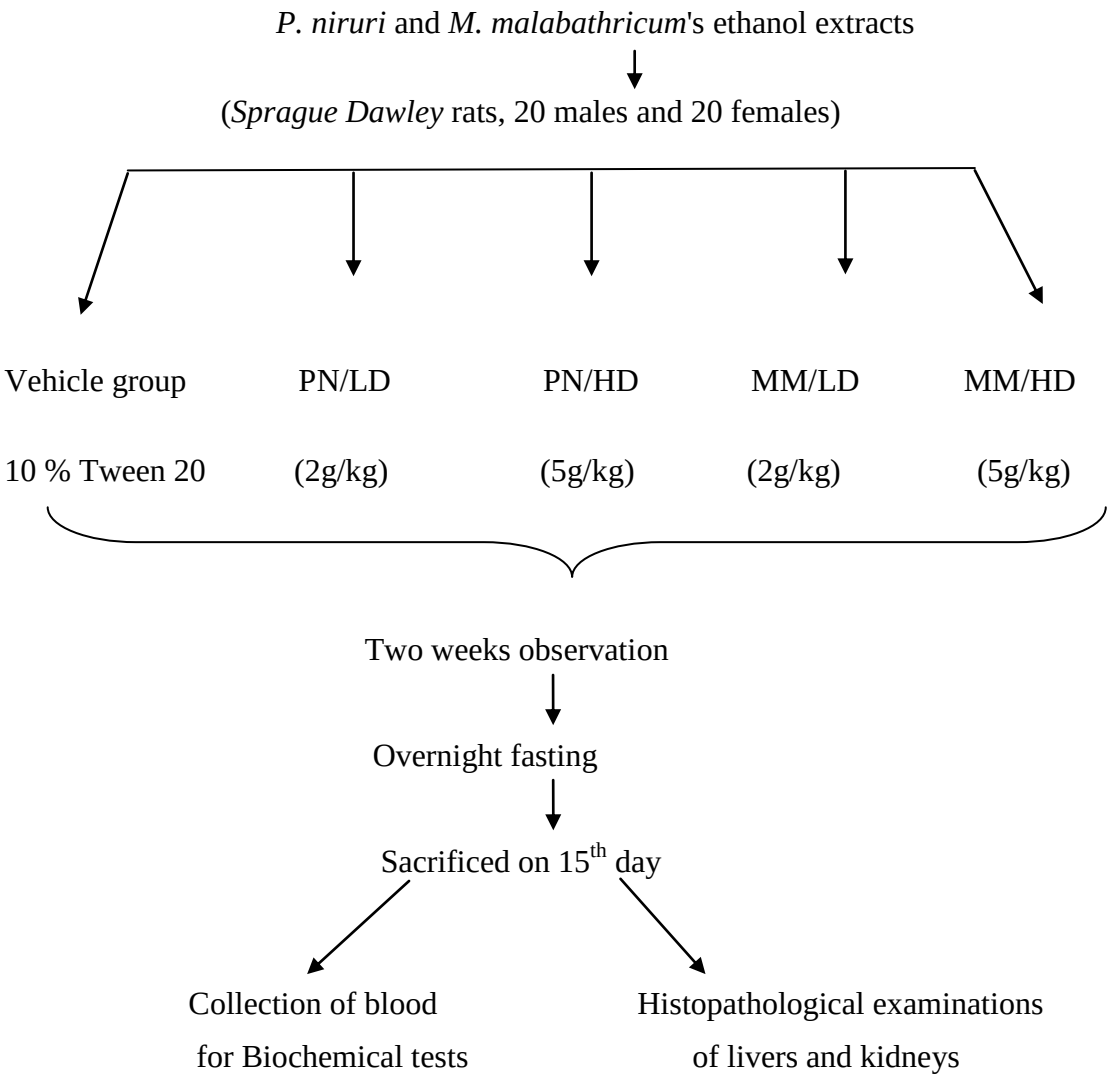


Figure 3.2: Experimental design of acute toxicity test: PN/LD*: P. niruri* low dose group, PN/HD*: P. niruri* high dose group MM/LD: *M. malabathricum* low dose group, MM/HD*: M. malabathricum* high dose group.

3.3.5 Hepatoprotective activity

3.3.5.1 Animals and ethical considerations

This study was approved by the institutional Ethics Committee, University of Malaya, Malaysia with protocol number PM/28/08/2010/MAA. Throughout the experiments, we provided human care to the animals according to the criteria outlined in the "Guide for the Care and Use of laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

Adult healthy *Sprague Dawley* rats weighing between 190 -260 grams were obtained from the Laboratory Animal Centre of the Faculty of Medicine, University of Malaya. The rats were placed individually in a separate cage and maintained on standard pellet diet and water *ad libitum*.

3.3.5.2 Experimental protocol and samples collection

Fifty-six healthy male *Sprague Dawley* rats weighing 190-260 gm were divided into 7 groups randomly, each of eight rats. The plant extract and silymarin were dissolved in 10% Tween 20 and distilled water respectively and (5 ml/kg body weight) whereas thioacetamide was dissolved in distilled water (2 ml/kg body weight) and injected intra-peritoneally to the rats. The plants extracts were administered orally by oral gavages three days prior to TAA injection following the methods of (Bruck et al., 2007; Dorğru-Abbasoğlu et al., 2001) in which the administration of the tested substance together with TAA injection, have shown to diminish the severity of the liver injury. The sample concentrations, durations and the complete experimental design are shown in Table 3.1. **Group 1** (vehicle control group) rats were administered orally with 10% Tween 20 (5 ml/kg) daily and injected ip with sterile distilled water (1 ml/kg) thrice weekly for 8

weeks. **Group 2** (hepatotoxic group) rats were administered orally with 10% Tween 20 (5 ml/kg) and injected intraperitoneally (ip) with Thioacetamide (TAA) (200 mg/kg) thrice weekly for 8 weeks. The injection protocol above was according to the recommendation of Alshawsh *et al.* (Alshawsh *et al.*, 2011) **Group 3** (hepatoprotective group) rats were administered orally with silymarin (50 mg/kg) daily and injected ip with TAA (200 mg/kg) thrice weekly for 8 weeks. **Group 4** and **5** (PN treatment groups) rats were administered orally with the PN extract (100 mg/kg and 200 mg/kg) daily and injected ip with TAA (200 mg/kg) thrice weekly for 8 weeks. **Group 6** and **7** (MM treatment groups) rats were administered orally with the MM extract (100 mg/kg and 200 mg/kg) daily and injected ip with TAA (200 mg/kg) thrice weekly for 8 weeks. The body weights of the animals were taken every week.

After 8 weeks, each rat was made to fast for 24 hours after the last treatment and then perfused under Ketamine and Xylazine (1:10 v/v) anesthesia and quickly sacrificed by exsanguination of jugular vein for blood sample collection. Gross morphology examinations were performed and the absolute weights of livers and spleens were documented.

Table 3.1: *P. niruri* and *M.malabathricum*'s hepatoprotective experimental protocol.

Group number	Group name	Oral feeding (Daily)	Injection of TAA (Thrice weekly)
		5 ml/kg BW	2 ml/kg BW
1	Vehicle control group	10 % Tween 20	D.W.
2	TAA (hepatotoxic) group	10 % Tween 20	200 mg / kg BW
3	Silymarin (hepatoprotective) group	50 mg / kg BW	200 mg / kg BW
4	PN/ LD group	100 mg / kg BW	200 mg / kg BW
5	PN /HD group	200 mg / kg BW	200 mg / kg BW
6	MM/ LD group	100 mg / kg BW	200 mg / kg BW
7	MM /HD group	200 mg / kg BW	200 mg / kg BW

TAA: thioacetamide, *PN: Phyllanthus niruri* MM: *Melastoma malabathricum*, HD: high dose, LD: low dose, D.W.: Distilled Water and BW: body weight.

3.3.5.3 Biochemical tests

The blood was withdrawn and immediately centrifuged at 4000 rpm for 10 minutes (Jouan C312 centrifuge). The serum was separated and subjected to the subsequent biochemical tests at Clinical Diagnostic Laboratory (CDL) division of University of Malaya Medical Center (UMMC) to determine the liver function enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), Bilirubin, albumin, total protein (TP) and gamma glutamyl transferase (γGT).

3.3.5.4 Gross morphology and histopathology

Gross morphology was observed and photographs were taken immediately. Subsequently, the livers and the spleens were carefully collected, weighed, trimmed and kept in 10 % fresh Formalin for 48 hours then, processed by automated tissue processor (Leica, Germany). Molds were prepared by embedding in Paraffin Wax and 5 µm thick sections were sliced by Leica Tissue Microtome. Finally, the slides were kept in oven at 55 °C then stained with Hematoxylin& Eosin.

Histo-pathological observations were documented using Olympus microscope and photographs were captured using (AM 432 Dino Lite) microscope eyepiece camera.

3.3.5.5 Endogenous antioxidant activities

One gram of the livers were kept in 10 ml (10% w/v) PBS (phosphate buffer solution pH 7.4), then homogenized using Wise Mix^{TM} HG 15A homogenizer (DAIHAN Sci., Seoul, Korea) and centrifuged at 4000 rpm for 10 minutes in -4°C using refrigerated centrifuge Rotofix 32 (Hettich Zentrifugen, Germany). The supernatant was separated and kept in -80°C freezer until the assays was performed according to the instruction manual from the manufacturer. Commercially available kits from (Cayman Chemical Co., USA) were used to determine total antioxidant capacity (TAC), Malondialdehyde (MDA), Catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX).

3.3.5.6 Gene expression profile

A small portion of the livers were kept immediately in RNA-later solution (Applied Biosystems, Foster City, CA) for gene expression assay and kept in -80 ºC until the purification experiment was performed. The gene expression assay was done following the Taqman gene expression workflow starting from (RNA isolation and purification), (transcription of RNA to cDNA) and (amplification of cDNA and target genes by Real Time PCR). The assay genes that chosen for this study were: transforming growth factor beta ($TGF\beta_1$) assay number (Rn00572010-m1), tissue inhibitors of metalloproteinases ($TIMP_1$) assay number (Rn00587558-m1), matrix metalloproteinase (MMP_2) assay number (Rn01538167-m1), Collagen alpha (Collα1) assay number (Rn01463848-m1). While glycer aldehyde 3-phosphate dehydrogenase (GAPDH) assay number (Rn0177563-g1), beta actin (β-actin) assay number (Rn00667869-m1), hypoxanthine phosphor ribosyl transferase 1(Hprt1) assay number (Rn01527840-m1) and peptidyl prolylisomerase A (Ppia) assay number (Rn00690933-m1) were used as housekeeping genes.

3.3.5.6.1 RNA isolation and purification

First, total RNA was extracted from the frozen liver using Qiamp RNA blood mini kit following the manufacturer's protocol (Qiagen, Germantown, Maryland, USA). Briefly, 30 mg of frozen *RNAlater* stabilized liver tissue was weighed immediately (without allowing the tissue to thaw), then disrupted and grinded by using a mortar and pestle. The QIA-shredder spin columns were used for homogenization and separation of tissue lysates. Further on column digestion of DNA was performed during RNA purification using RNase- Free DNase set (Qiagen, Germantown, Maryland, USA) as recommended by the manufacturer. Finally, the total RNA was stored at -70 ºC until further use.

RNA purity was quantified using ND-2000 Nano Drop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The absorbance was measured at 230, 260, 280, 320, and 260/280, 260/320 ratios were documented.

While RNA integrity was measured by agarose gels electrophoresis. Electrophoresis buffer Tris-Borate-EDTA (10X TBA) was prepared (Applied Biosystems, USA) to fill the electrophoresis tank and to cast the gel. A 0.5% (w/v) solution of agarose in electrophoresis buffer was prepared and Ethidium Bromide (EB) was added to the molten gel to a final concentration (0.5 μg/ ml) then mixed thoroughly by gentle swirling. A small toothed comb (allows 1μL sample / well) positioned on the plate so a complete well was formed. The 60 ºC warm agarose solution was poured into the mold, and then allowed to set at RT for 30-45 minutes. The gel mounted in the tank and the electrophoresis buffer was added to cover the gel at depth of 1 mm.

1μL of RNA samples were loaded after mixing with the loading dye (Promega, USA). Then the RNAs allowed migrating toward the positive anode. The gel ran for 30 minutes at 95V until the migrated distance is 75% through the gel. The gels were examined under UV light to observe the discrete 18S and 28 S ribosomal RNA and photographs were taken using a Monochrome scientific grade camera in a gel documentation system (Vilber Lourmat, from Fisher Scientific, USA)

3.3.5.6.2 Reverse Transcription of RNA to cDNA

cDNA was synthesized using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA) by loading the reaction plates into thermal cycler (PTC-100 thermal controller, MJ Research Inc., Basel, Switzerland) following the reaction condition of the manufacturer.

3.3.5.6.3 Amplification of cDNA by Real Time PCR

Real Time Polymerase Chain Reaction (Real-time PCR) was performed using StepOnePlus system and TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA). The total reaction volume was 10 μL: 1μL cDNA (20 ng), 5 Ml Taqman Fast Advanced Master Mix, 0.5 μL of each Taqman Gene Expression Assays, and 3.5 μL ultrapure DNase free water. Cycle parameters were as follows: UNG incubation at 50 ºC for 2 min., polymerase activation at 95 ºC for 20 sec., denaturation at 95 ºC for 1 sec. and then annealing and extension at 60 ºC for 20 sec.

3.3.5.6.4 Real Time PCR data normalization

The real-time PCR reaction was performed in triplicate for each sample and the averages of the obtained threshold cycle (Ct) values were processed for further calculations according to the comparative Ct method described in the ABI manual (http://www.appliedbiosystems.com). Two endogenous control genes, hypoxanthine phosphor ribosyl transferase1 (Hprt1) and peptidyl prolylisomerase A (Ppia), were used for the normalization. The comparative CT method was used to calculate relative quantification of gene expression. The following formula was used to calculate the relative amount of the transcripts in all groups and were normalized to the endogenous controls. The final value then was normalized to the housekeeping gene and relative to the control sample values of the studied genes.

$\Delta\Delta CT = \Delta$ CT (sample) - Δ CT (control).

Where ΔCT: is the difference in CT between the target gene and endogenous controls by subtracting the average CT of controls. The fold-change for each treated sample relative to the control sample = $2^{-\Delta\Delta CT}$.

3.3.6 Isolation of the plants active constituents

3.3.6.1 Sample preparation

One gram of each plant fraction was dissolved in 5 ml of methanol then filtered through a 0.45 μm filter and kept in fridge at 4° C until the tests were performed.

3.3.6.2 Flash column chromatography (FCC)

Plants fractionations were done following the method of Fraga *et. Al*. (Fraga *et al.*, 1987). Flash column chromatography was performed on silica gel 60 (0.063 - 0.200 mm) and (70 - 230 mesh) from (Merck, Germany) using a (2 x 30 cm) Kontes column with EYEL- 4 pump (Rikakikai, Tokyo). Elution to plant extract (1gm / 5 ml methanol) starts with the most non-polar solvent (n hexane) then continuous gradient elution with n hexane - ethyl acetate - methanol - acetonitrile and ended with the most polar solvent (dH_2O) which was purified by a Milli-Q academic water purification system (Millipore, USA).

3.3.6.3 Thin layer chromatography (TLC)

The obtained fractions were dissolved in methanol at 10 mg/ml to perform thin layer chromatography (TLC) with silica gel F254 (20 x 20 cm, 0.2 mm) plates. The analyses have been achieved in (n hexane - ethyl acetate), (ethyl acetate - methanol), (methanol - acetonitrile), (acetonitrile - water).

3.3.6.4 UPLC and LC-MS

Waters Synapt HDMS system was used with TOF-mode to provide Ultra Performance Liquid Chromatography (UPLC) and HDMS- mode to provide mass spectrometry, equipped with ACQUITY PDA Detector and an ACQUITY UPLC BEH C18, 1.7 µm, 2.1 x 50 mm Column. The flow rate was (0.5 ml /min) and the injection volume was (3 µL). The analyses were carried out using binary gradients of Milli-Q water (with 0.1 % Formic Acid) (solvent A) and HPLC grade acetonitrile (with 0.1 % Formic Acid) (solvent B). with the following elution profile: from 0 min: 0% (B) in (A); 7 min: 100% (B) in (A); 10 min: 100% (B) in (A); 11 min: 0% (B) in (A).

3.3.6.5 Identification of the isolated components

The identification of the isolated components from the active fractions of *P. niruri* and *M. malabathricum* were performed by comparing ultraviolet (UV) and mass spectrometry (MS) as described by (Halket *et al.*, 2005). The method development and optimization were done at the Cancer Research Initiatives Foundation (CARIF) laboratory. Moreover, special software, namely "The Dictionary of Natural Products on DVD" was used for processing and interpretation of mass spectra by comparison with the data from the commercial library and the available literatures review.

3.4 Data management and statistical analysis

Data obtained from the *in vitro* antioxidant assay were analyzed using Statistical Program for Social Scientists (SPSS program for windows version 19, SPSS Inc. Chicago, IL, USA) using one-way ANOVA followed by Post-Hoc multiple comparisons, Duncan's test. Results presented as (Mean ± S.E.M.) of triplicates for three different experiments. Pearson correlation used to study the correlation between the assays. For the hepatoprotective and immunomodulatory studies, statistical significance differences between the groups were assessed using one-way ANOVA using Bonferroni's test / Post-Hoc multiple comparisons. A value of $P \leq 0.05$ was considered significant as compared to the respective control group.

All graphs were illustrated using Graph Pad Prism program version 5.04 for Windows, (Graph Pad Software, San Diego California USA, www.graphpad.com).

The NormFinder and geNorm algorithm software (MultiD Analyses AB, Göteborg, Sweden) were used to evaluate and calculate the expression stability of four endogenous reference genes: HPRT-1, Ppia, GAPDH and β-actin for normalization in the rat liver samples. Real-time RT-PCR data exported from "StepOnePlus" software to Ms. Excel where all results arranged in one study and finally analyzed by using GenEx program version 5.4. (GenEx software, www.multid.se), fold changes were calculated and T-test was used to examine the differences between groups for all genes and $P \leq 0.05$ was considered as the level of significance.

"The Dictionary of Natural Products on DVD" software Version 20:2 (2011) (CRC Press, Taylor and Francis Group, www.netbeans.org) was used to analyze the obtained data from chromatography profiling of the plants active fractions.

CHAPTER IV

RESULTS

4.1 Extraction yield

As shown in Table 4.1, the percentage yields for the crude ethanol extraction of *Phyllanthus niruri* and *Melastoma malabathricum* were 4.84 % and 10.68 % (w/w) respectively.

4.2 Antioxidant Activity of *P. niruri* and *M. malabathricum*

4.2.1 Scavenging effects on DPPH free radicals

As displayed in Figure 4.1, both plant extracts showed significant effects in inhibiting DPPH, at a comparable extents. The DPPH scavenging activities were observed to be dose dependent and increased gradually with high concentrations.

The ethanol extract of *P. niruri* showed DPPH free radical scavenging activity reaching up to (86.53 %) at concentration 25 µg/ml with IC_{50} value (11.07 ± 1.37 µg/L). while *M. malabathricum* showed DPPH inhibiting activity (80.09 %) at concentration 25 µg/ml and IC_{50} value (11.599 ± 0.84 µg/L) and had a regression coefficients of R^2 = 0.991 as shown in appendix A1. As the lower IC_{50} value indicates a higher free radical quenching activity, so *P. niruri* and *M. malabathricum* shown to be significantly less active than the synthetic reference antioxidant ascorbic acid (Vit. C IC_{50} = 3.346 ± 1.20 µg/L) and significantly better than the synthetic reference antioxidant butylated hydroxytoluene (BHT IC_{50} = 21.1 ± 0.01 µg/L) and no significant differences were found between *P. niruri* and *M. malabathricum* (Table 4.1).

Table 4.1: *In vitro* antioxidant activity of *P. niruri* and *M. malabathricum*.

	Vitamin C	BHT	PN/E	MM/E
Extraction Yield % (w/w)			4.84	10.68
DPPH (IC_{50} µg/mL)	3.35 ± 1.20^{a}	21.30 ± 0.01^{c}	11.07 ± 1.37^{b}	11.60 ± 0.84^{b}
ABTS (IC_{50} µg/mL)	21.37 ± 0.12^{a}	65.7 ± 0.41^{d}	53.34 ± 1.97^{b}	62.66 ± 0.78^{c}
TPC (mg GAE/g)	…	…	270.00 ± 0.03	384.33 ± 0.01
TFC (mg QE/g)	…	…	123.9 ± 0.02	85.8 ± 0.01
FRAP (mmol FeII/g)	101600 ± 0.01^{d}	57300 ± 0.01^{c}	7755 ± 0.02^{a}	33590 ± 0.04^{b}

The data represented as the Mean ± SEM of triplicates; means followed by the same letters are not significantly different at * $P < 0.05$.

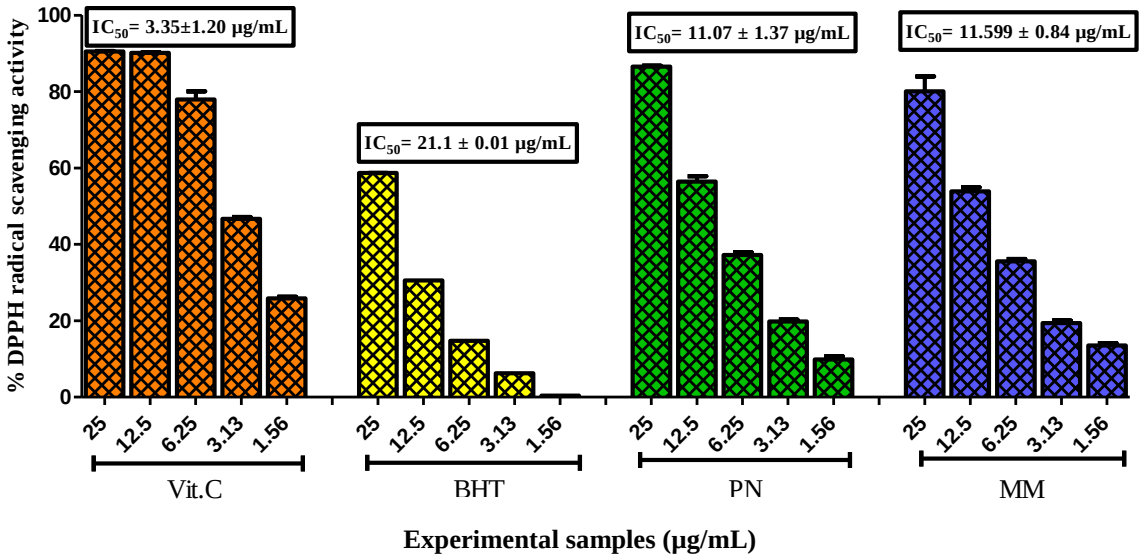


Figure 4.1: DPPH free radical scavenging activities of *P. niruri* and *M. malabathricum*.

4.2.2 Scavenging effects on ABTS free radicals

As shown in Table 4.1 and Figure 4.2, both extracts displayed antioxidant capacities as they were able to scavenge ABTS radical cations in dose dependent manner. The ethanol extract of *P. niruri* showed higher activity to scavenge ABTS free radicals than the ethanol extract of *M. malabathricum* with less IC_{50} values (53.34 ± 1.97µg/mL) and (62.66 ± 0.78 µg/mL) respectively with regression coefficients of R^2 = 0.998 as shown in appendix A2.

The extracts of the present study were shown to exhibit significantly lower antioxidant capacity than the synthetic reference antioxidant ascorbic acid (Vit C IC_{50} = 21.37±0.12µg/mL) but significantly better than the synthetic reference antioxidant butylated hydroxyl toluene (BHT IC_{50} = 65.70 ± 0.41µg/mL) and significant differences were found between *P. niruri* and *M. malabathricum*'s ABTS radical scavenging activities.

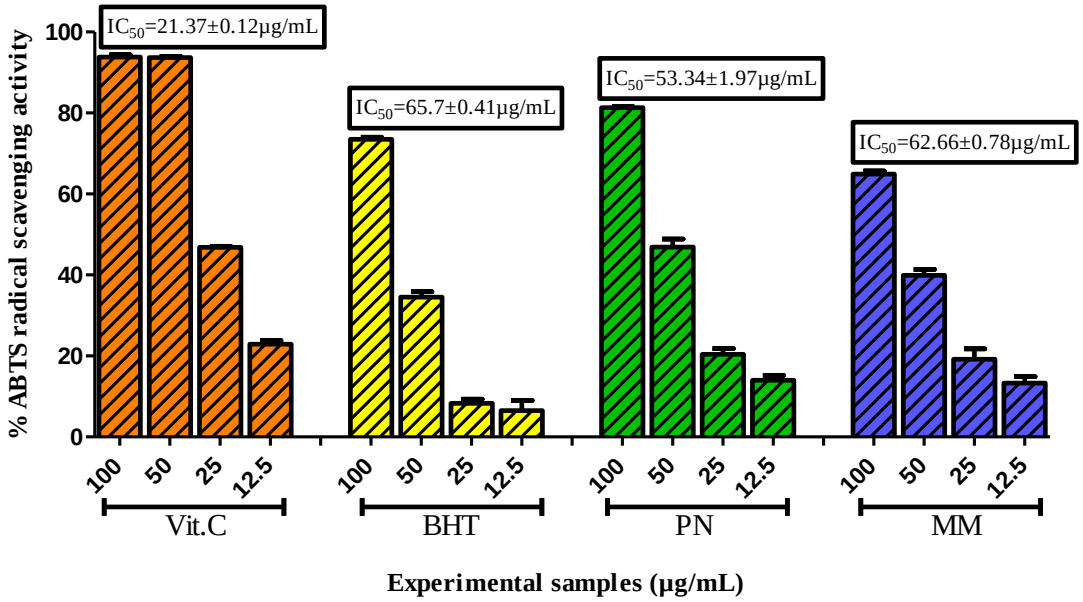


Figure 4.2: ABTS free radical scavenging activities of *P. niruri* and *M. malabathricum*.

4.2.3 Total phenolic and total flavonoids contents (TPC & TFC)

Figure 4.3 and Table 4.1, show that both investigated extracts had appreciable amounts of phenolic compounds as the ethanol extract of *P. niruri* had phenolic and flavonoids contents values (270 ± 0.003 mg GAE/ g extract) and (123.9 ± 0.002mg QE / g extract) respectively, with TFC/TPC ratio of (0.45). While the phenolic and flavonoids contents of the ethanol extract of *M. malabathricum* were (384.33 ± 0.005mg GAE / g extract) and (85.8 ± 0.009 mg QE / g extract) respectively and the ratio TFC/TPC was (0.22).

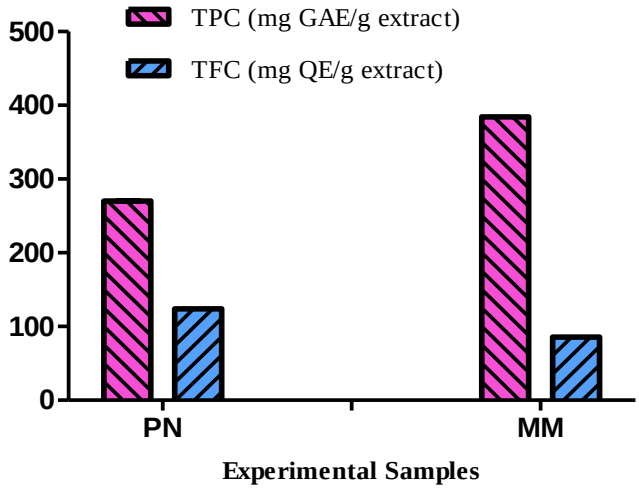


Figure 4.3: Total phenolic (TPC) and flavonoids (TFC) contents of *P. niruri* and *M. malabathricum*.

4.2.4 Ferric reducing antioxidant power (FRAP)

Figure 4.4 shows that *M. malabathricum* had a stronger reducing power as it attained significantly ($P < 0.05$) higher FRAP value (33590 ± 0.038 mmol Fe II/g) as compared to *P. niruri* (7755 ± 0.015 mmol Fe II/g). Furthermore, the FRAP value for the synthetic reference antioxidants ascorbic acid (Vit C) and butylated hydroxyl toluene (BHT) were reported to be (57300 ± 0.01 mmol Fe II/g) and (101600 ± 0.01 mmol Fe II/g) respectively with regression coefficient of $R^2 = 0.982$ as shown in appendix A3.

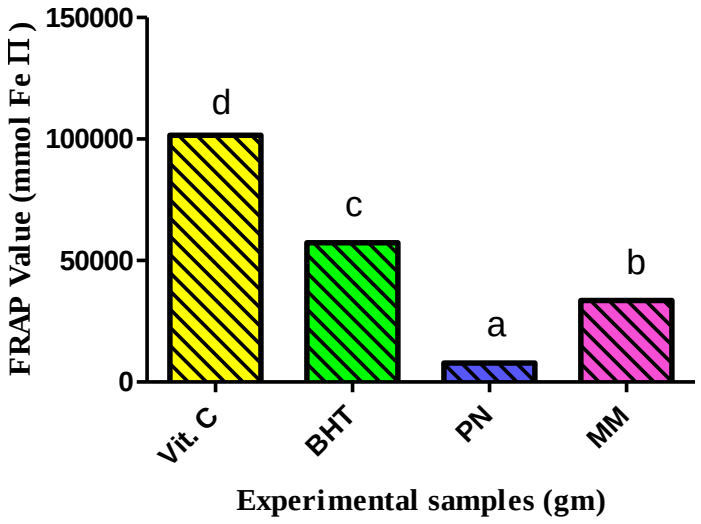


Figure 4.4: FRAP activities of *P. niruri* and *M. malabathricum*. Data expressed as Mean ± SEM, different superscripts indicates significant differences between groups at ($P \leq 0.05$).

Finally, Pearson's correlation coefficient results for both plants showed that the highest positive correlation was between TPC and FRAP ($r = 0.908$) as shown in appendix B.

4.3 Immunomodulatory effects of *P. niruri* and *M. malabathricum* on human peripheral blood mononuclear cells (PBMC) viability

The effects of the ethanol extracts of *P. niruri* and *M. malabathricum* on human peripheral blood mononuclear cells (PBMC) viability show evidence that both investigated plant extracts significantly stimulated the proliferation of PBMCs *in vitro* in a dose-dependent manner, as shown in Figure 4.5 and 4.6.

Results showed that the PBMCs cell viability increased significantly after treatment, which means that the extract was not toxic to the immune cells and it modulated the cellular immune response.

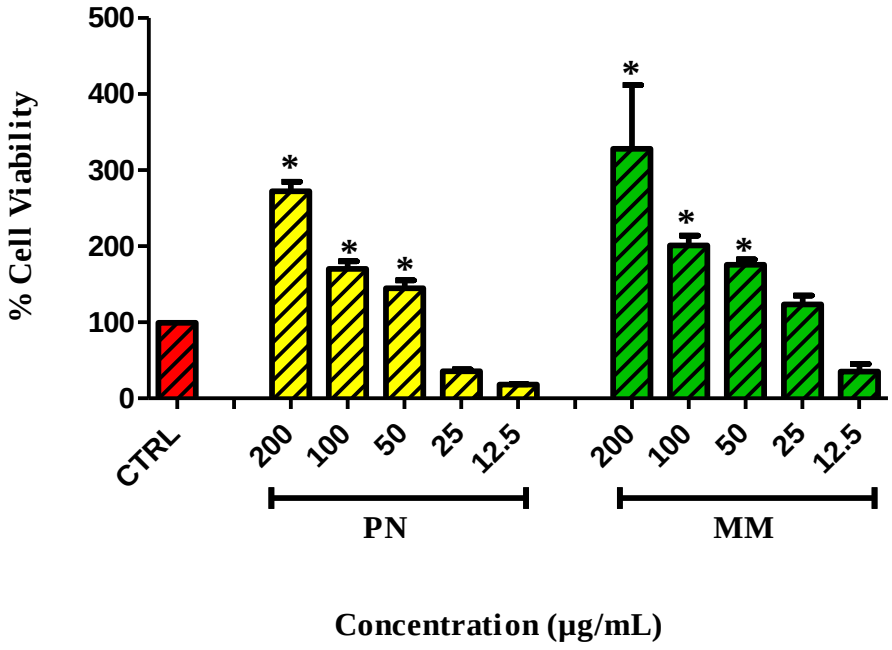


Figure 4.5: Percentage of (PBMC) cell viability after treatment with *P. niruri* and *M. malabathricum*; CTRL= dH_2O; PN= *P. niruri* and MM= *M. malabathricum*. Data expressed as Mean ± SEM, (*) indicates significant differences between groups at ($P \leq 0.05$).

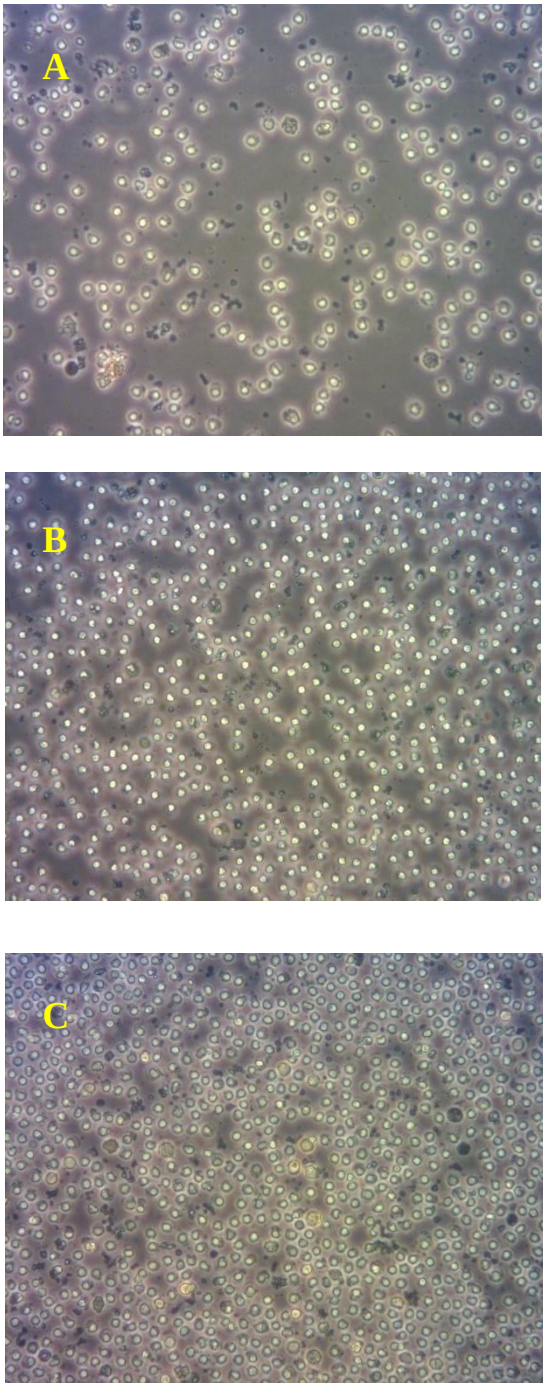


Figure 4.6: Photographs of PBMC cells: (A) normal cells without treatment, (B) cells treated with 200 µg/ml PN, (C) cells treated with 200 µg/ml MM (magnification 10x).

4.4 Acute oral toxicity test

The acute toxicity study at doses of 2 g / kg and 5 g / kg for both plants extracts did not result in any mortality in the treated rats and no toxic effects were observed throughout the 14 days study period. Physical observations indicated no signs of changes in rats' skin and fur, eyes and mucus membrane, behavior patterns, tremors, salivation, diarrhea and sleep. The body weight, clinical observations and biochemical measurements reflected normal status of the kidney and liver functions, and histopathological evaluations of these organs all together revealed that there were no significant differences between the control and the test groups, as shown by the quantitative data in Tables 4.2, 4.3, 4.4, and qualitative data in Figures 4.7 and 4.8. The findings from the applications of the *P. niruri* and *M. malabathricum* extracts to the control rats provided sufficient evidence to conclude that the orally administered extract was safe and presented no extract-related toxicity even at the highest dose of 5 g / kg.

Table 4.2: Effects of 2 g/kg and 5 g/kg of *P. niruri* and *M. malabathricum* extracts on rats body weights.

Groups	BW (At Day 0)	BW (At Day 14)
Tween 20 (10%)	202.69 ± 2.86	206.63 ± 3.06
PN extract (2g/kg)	205.00 ± 2.20	207.88 ± 3.78
PN extract (5g/kg)	210.63 ± 3.96	213.88 ± 3.01
MM extract (2g/kg)	209.25 ± 4.12	205.50 ± 3.62
MM extract (5g/kg)	207.63 ± 2.71	201.44 ± 1.92

Values expressed as mean ± S.E.M. There are no statistically significant differences between the measurements in different groups. The significant value was set at $P < 0.05$.

Table 4.3: Effects of 2 g/kg and 5 g/kg of *P. niruri* and *M. malabathricum* extracts on biochemical parameters.

Groups	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)	CO_2 (mmol/L)	Anion Gap (mmol/L)	Urea (mmol/L)	Creatinine (µmol/L)
Tween 20 (10%)	141.33 ± 1.59	4.33 ± 0.27	102.83 ± 1.05	26.83 ± 1.72	14.33 ± 1.75	4.60 ± 0.70	37.33 ± 0.18
PN (2 g/kg)	140.00 ± 1.77	4.37 ± 0.30	103.17 ± 1.45	26.33 ± 1.59	13.00 ± 1.44	4.57 ± 0.70	37.60 ± 0.19
PN (5 g/kg)	137.17 ± 1.14	4.42 ± 2.39	102.67 ± 0.99	22.17 ± 0.60	17.83 ± 1.45	4.73 ± 0.67	37.52 ± 0.11
MM (2 g/kg)	140.33 ± 1.20	4.54 ± 0.23	105.33 ± 2.08	27.33 ± 1.50	12.80 ± 1.33	4.62 ± 0.65	37.48 ± 0.16
MM (5 g/kg)	136.33 ± 1.50	4.28 ± 2.25	104.60 ± 1.54	25.50 ± 1.15	19.00 ± 0.52	4.56 ± 0.56	36.58 ± 0.82

Values expressed as mean ± S.E.M. There are no statistically significant differences between the measurements in different groups.

Table 4.4: Effects of 2 g/kg and 5 g/kg of *P. niruri* and *M. malabathricum* extracts on biochemical parameters.

Groups	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Total Protein (g/L)	Albumin (g/L)	Bilirubin (µmol/L)	γGT (IU/L)
Tween 20 (10%)	45.00 ± 5.32	27.00 ± 4.16	97.67 ± 13.86	73.00 ± 2.96	44.17 ± 2.59	10.17 ± 2.26	58.17 ± 9.90
PN (2 g/kg)	49.67 ± 4.60	26.33 ± 3.22	92.67 ± 9.76	74.50 ± 2.31	42.83 ± 1.97	8.00 ± 1.06	37.67 ± 6.82
PN (5 g/kg)	45.17 ± 5.30	28.00 ± 3.65	78.33 ± 6.81	74.33 ± 2.72	42.50 ± 2.93	9.50 ± 1.69	55.00 ± 8.83
MM (2 g/kg)	46.50 ± 4.21	26.83 ± 3.84	92.83 ± 11.97	72.67 ± 2.09	45.67 ± 6.73	6.67 ± 1.41	55.00± 10.58
MM (5 g/kg)	44.33 ± 5.94	28.00 ± 3.84	88.17 ± 6.58	74.33 ± 4.62	44.17 ± 4.05	9.50 ± 1.65	58.83 ± 7.15

Values expressed as mean ± S.E.M. There are no statistically significant differences between the measurements in different groups.

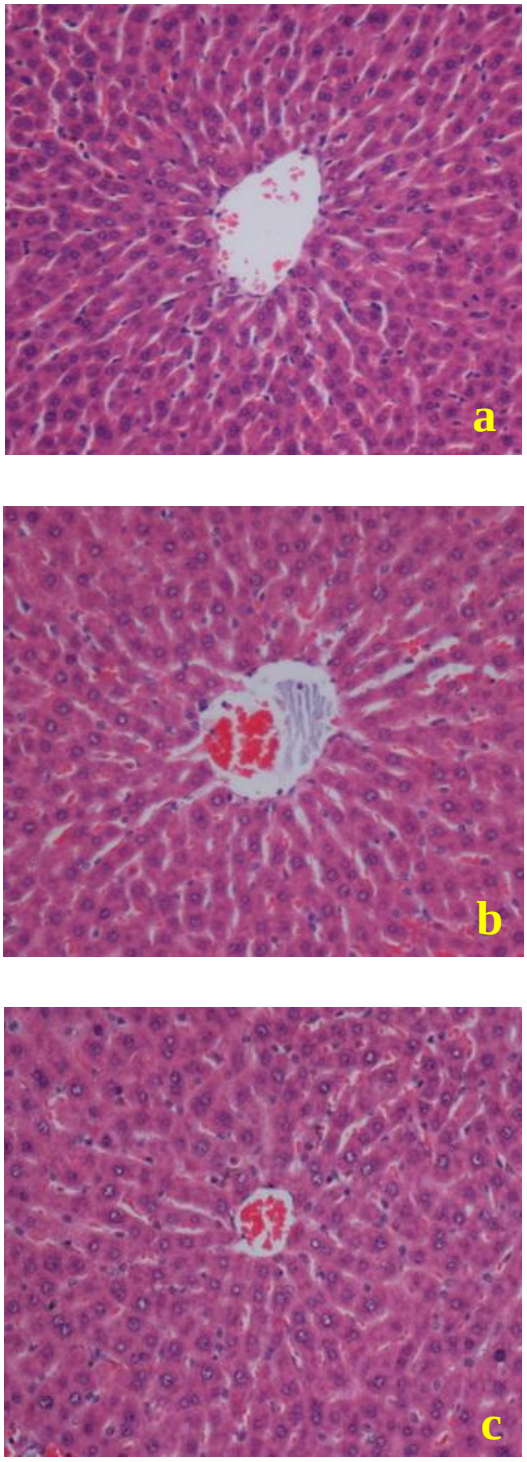


Figure 4.7: Histological sections of livers from the acute toxicity test. (a) Control treated with 5 ml/kg vehicle (10% Tween20); (b and c) PN and MM extracts (5 g/kg) respectively. No structural differences were seen between the plants treated groups and the control group. (H & E stain; 20x magnification).

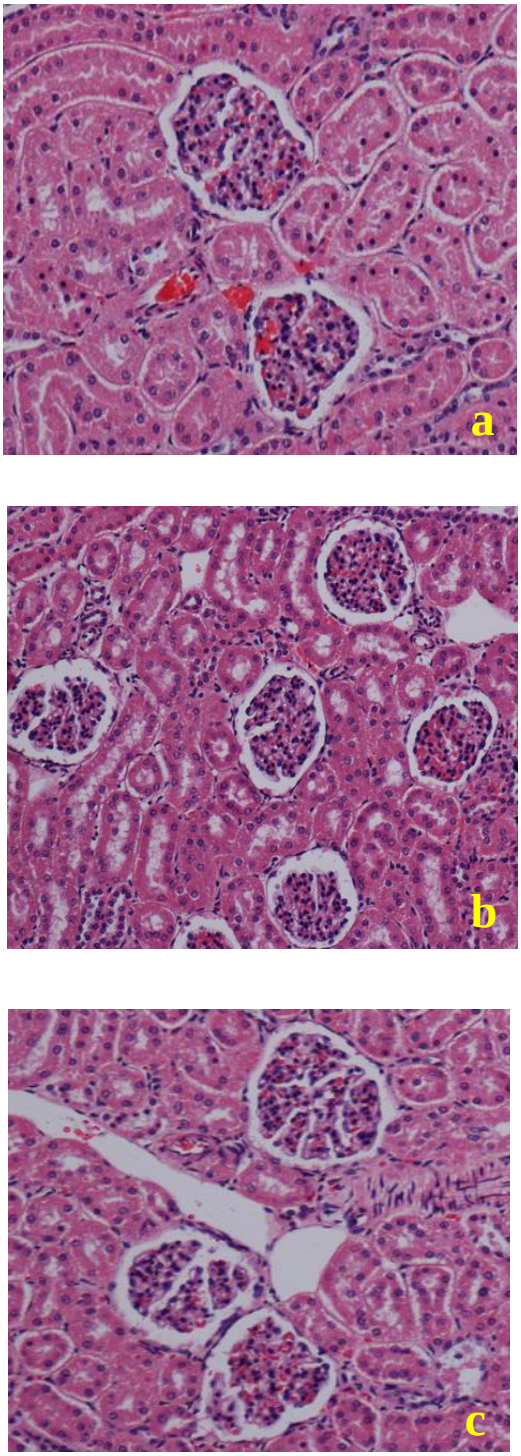


Figure 4.8: Histological sections of kidneys from the acute toxicity test. (a) Control treated with 5 ml/kg vehicle (10% Tween20); (b and c) PN and MM extracts (5 g/kg) respectively. No structural differences were seen between the plants treated groups and the control group. (H & E stain; 20x magnification).

4.5 Hepatoprotective effects of *P. niruri* and *M. malabathricum*

4.5.1 Body, liver and spleen weights

The measurements of the body, liver and spleen weights of the rats by the end of the study in all groups were listed in Table 4.5. Through out the eight weeks of study (Figure 4.9), the hepatotoxic rats weighted significantly less, but their livers and spleens weighted significantly more compared with the control rats. The rats subjected to the treatment with either silymarin or PN or MM extracts exhibited gains in the body weight and no increase in the liver and spleen masses were observed.

Table 4.5: Body, Liver and Spleen weight changes of the rats in the different treatment groups.

Groups	BW (gm)	LW (gm)	LW/BW (%)	SW (gm)	SW/BW (%)
Normal	237.00 ± 17.24	6.20 ± 0.49	2.63 ± 0.13	0.36± 0.02	0.16 ± 0.02
TAA	195.40 ± 18.35	9.40 ± 0.98	4.81 ± 0.23[a]	0.51 ± 0.10	0.25 ± 0.04[a]
S	222.00 ± 12.00	9.20 ± 0.97	4.10 ± 0.22[a]	0.53 ± 0.04	0.24 ± 0.01
PN/LD (100mg/kg)	258.60 ± 14.02	8.20 ± 1.02	3.22 ± 0.41[c]	0.46 ± 0.04	0.18 ± 0.02
PN/HD (200mg/kg)	253.40 ± 16.41	7.80 ± 0.58	3.18 ± 0.24[c]	0.40 ± 0.04	0.16 ± 0.01[b]
MM/LD (100mg/kg)	249.40 ± 14.70	10.80 ± 0.58[a]	4.35 ± 0.15[a]	0.46 ± 0.02	0.19 ± 0.01
MM/HD (200mg/kg)	231.20 ± 13.85	9.20 ± 0.37	4.05 ± 0.34[a]	0.41 ± 0.01	0.18 ± 0.01

The data were stated as mean ± S.E.M.; the mean difference is significant at [a] *P <0.01* against Normal group, [b] *P <0.05* against TAA group and at [c] $P < 0.01$ against TAA group. BW: Body Weight, LW: Liver Weight, SW: Spleen Weight.

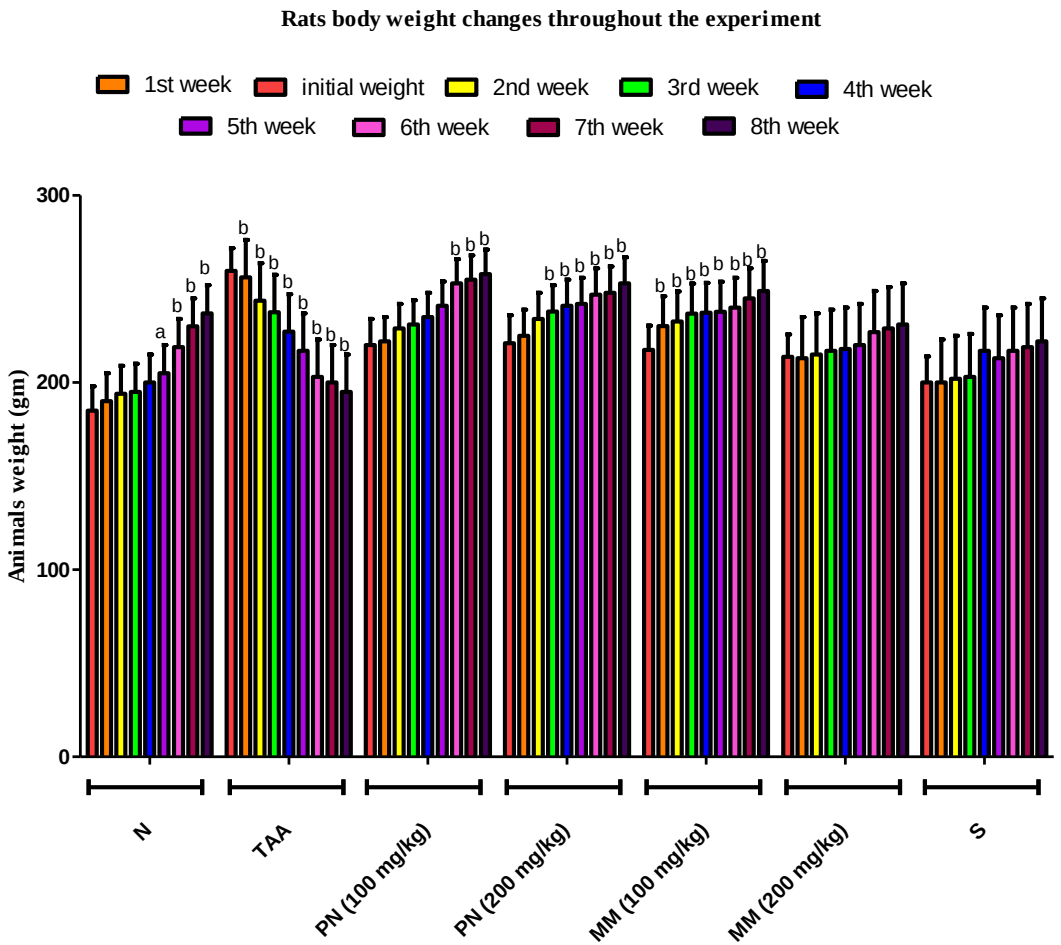


Figure 4.9: Changes of rats body weights in different experimental groups throughout the eight weeks of the study. The data were stated as Mean ± S.E.M.; the mean difference is significant from initial weight at [a] *P <0.05* and [b] *P <0.01.*

4.5.2 Gross morphology

As depicted in Figure 4.10, the control rats had livers with smooth surfaces. In the hepatotoxic group, exposure to TAA made the liver attain irregular shape and at the same time be occupied with uniform formations of micro nodules and macro nodules (whitish granules), some of which were localized close to the surface. The nodular transformation in liver parenchyma is also a typical characteristic of human cirrhosis. The treatment with either silymarin or the PN and MM extracts (Figures 4.11 and 4.12) prevented the development of the macro- and micro nodules and made the liver preserve its nearly normal anatomical shape and appearance in rats. Although based on visual evaluations, these results clearly demonstrated that the PN and MM extracts effectively prevented the development of the nodules and hence protected the liver from further deterioration of its structure and function.

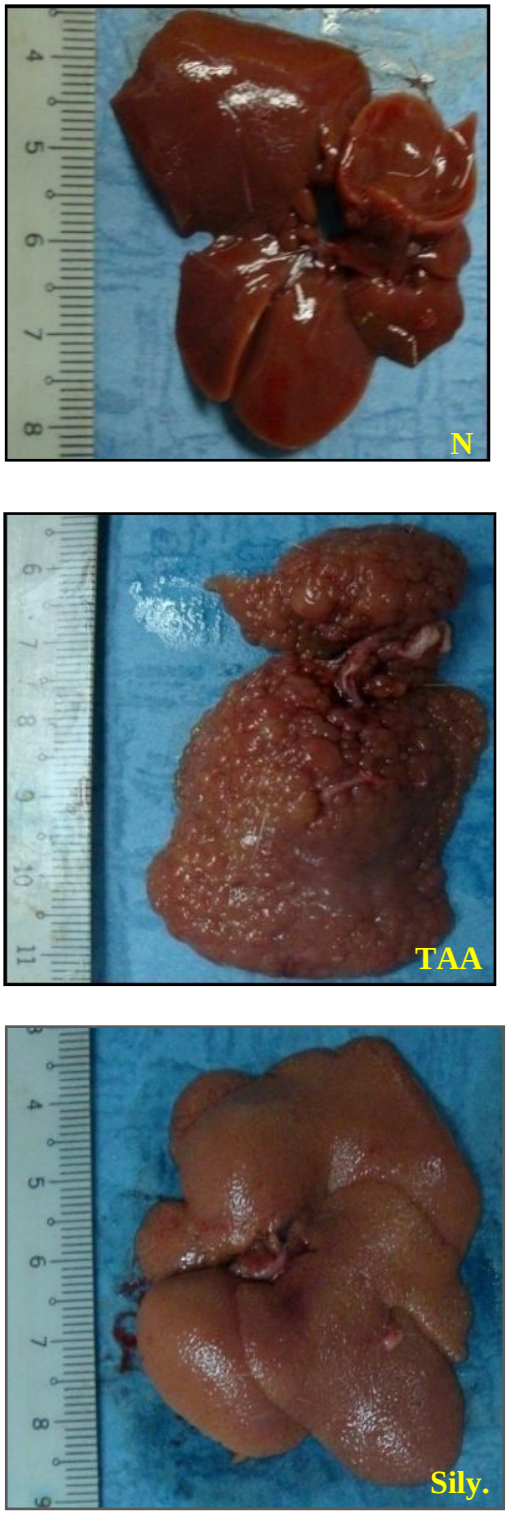


Figure 4.10: Images showing the macroscopic appearances of livers from N, TAA and Sily groups. (N) control group rats liver with regular smooth surface. (TAA) thioacetamide rats liver showing iirregular whitish micro- and macronodules and a large area of ductular cholangiocellular proliferation embedded within fibrosis. (Sily) silymarin treated rats liver with smooth surface.

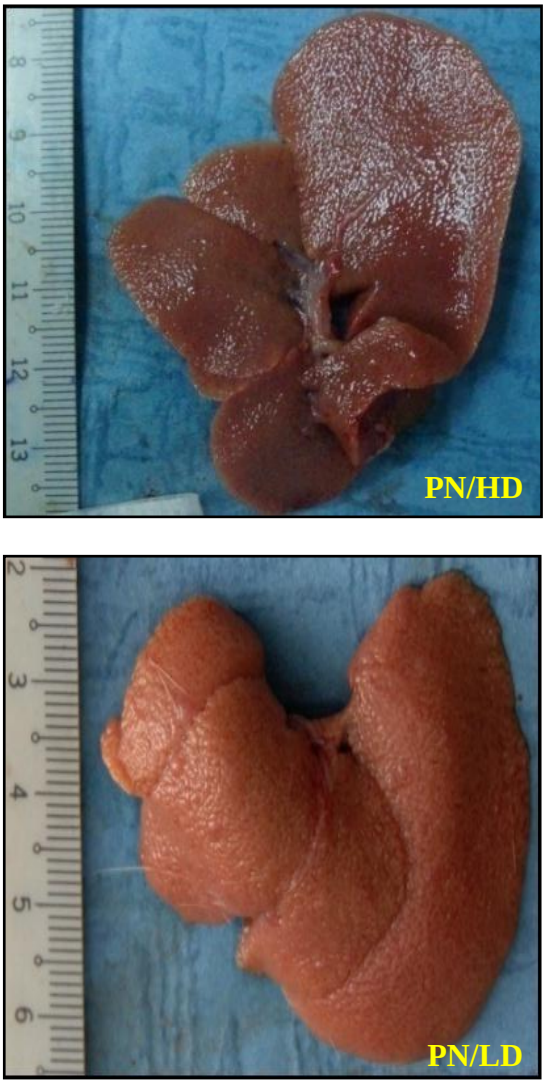


Figure 4.11: Images showing the macroscopic appearances of livers from *P. nirui* treated groups. (PN/LD 100 mg/kg) *P. niruri's* low dose treatment group showing slight micrnodules on the liver surface. (PN/HD 200 mg/kg) *P. niruri s* high dose treatment group showing nearly smooth surface.

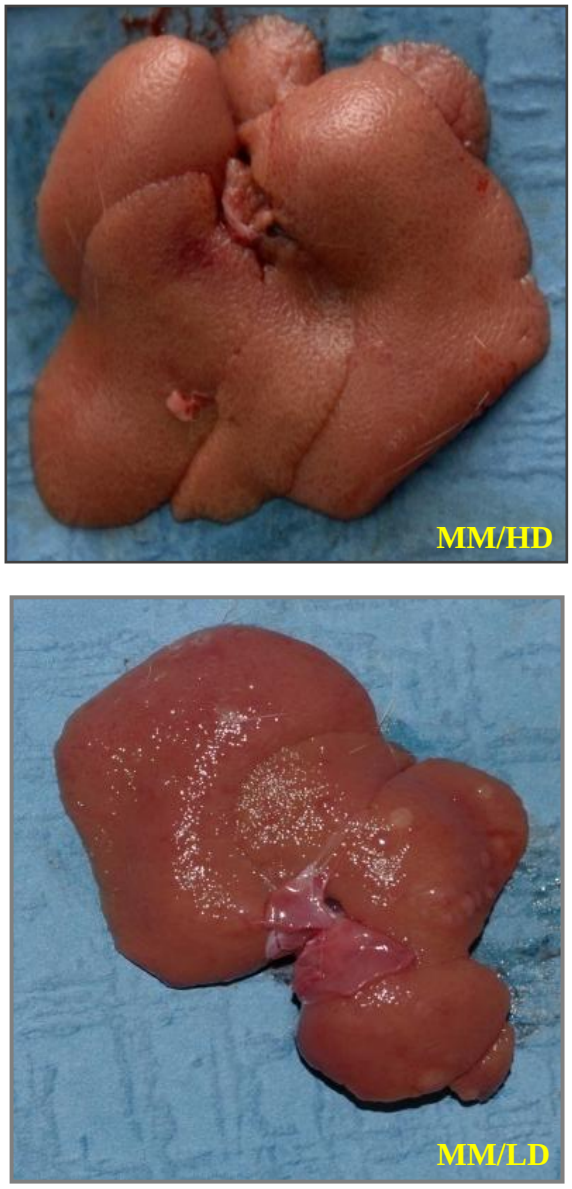


Figure 4.12: Images showing the macroscopic appearances of livers from *M. malabathricum* treated groups. (MM/LD 100 mg/kg) *M. malabathricum's* low dose treatment group showing slight micrnodules on the liver surface. (MM/HD 200 mg/kg) *M. malabathricum's* high dose treatment group showing nearly smooth surface.

4.5.3 Histopathology of the liver

Histological examination of Haematoxylin and Eosin-stained sections was performed to determine the extent of liver injury and fibrosis for each section. Example slides obtained from the histopathological examinations under the light microscope were given in Figure 4.13, 4.14 and 4.15. In the control rats, there were no pathological abnormalities, the liver sections looked normal with regular cellular architecture and were clear of any pathology. Polygonal hepatic cells had intact cytoplasm, sinusoidal spaces, prominent nucleus, nucleolus, and central vein. In the hepatotoxic rats, the histology confirmed liver damage as evidenced by the presence of inflammation and necrosis in the respective liver sections. Histological examination showed loss of normal architecture of the liver parenchyma with fibrous septa forming collagen bridges between hepatic triads delineating small and large regenerative nodules of hepatocytes or necrosis. The nodules were surrounded by thick fibrous septa, which divided the liver into pseudo lobules. The histology also delineated cytoplasmic vacuolization, megalocytosis, bile duct proliferation, hepatocyte degeneration, necrosis, infiltration of inflammatory cells, and centrilobular necrosis with portal triaditis, expanded portal tracts and collagen deposition. Proliferation of spindle cells and the hepatic cells surrounding the central vein showed various degenerative changes like cloudy swelling, hydropic degeneration, loss of nucleus and nucleolus and necrosis (Cassiman *et al.*, 2002). The rats in silymarin treated group exhibited significantly lesser pathology as compared to the extensive liver damage found in the hepatotoxic group. Silymarin administration prevented the incidences of liver lesions including hepatic cells cloudy swelling, lymphocytes infiltration, hepatic necrosis, and fibrous connective tissue proliferation induced by TAA. Consequently, the liver tissue preserved its nearly normal hepatic lobular architecture with central veins and radiating hepatic cords. These results

reconfirmed the preventive and protective functions of silymarin against the TAA-induced liver damage. The histopathological examination of the liver sections from the rats treated with the PN and MM extracts revealed reduced degree of fibrosis induced by TAA, but small size necrotic zones with nodules and centrolobular veins still occupied the parenchyma. The slides showed appreciable level of normal parenchymal architecture with thinner fibrous septa. The livers of the treated rats experienced lesser amount of cytoplasmic vacuolization, megalocytosis, bile duct proliferation, and nuclei injury. These results provided microscopic evidence again demonstrating the significant hepatopreservation and hepatoprotection functions of the PN and MM extract to counter balance the negative effects of TAA in the liver tissue.

Masson's trichrome staining of liver sections was used to determine the extent of hepatic fibrosis induced by TAA treatment in the presence and absence of PN and MM (Figures 4.16, 4.17 and 4.18). No fibrosis was detected in the liver sections of the untreated control animals. In contrast, extensive fibrosis was observed in the liver sections from TAA-treated animals (Figure 4.16). Sections from the PN (Figure 4.17) or MM (Figure 4.18) treated animals showed mild fibrosis; however, the degree of fibrosis was substantially less than the cirrhosis control animals. Thus, TAA treatment for 8-week induced hepatic fibrosis (blue color), but treatment with PN or MM reduced collagen accumulation in the liver.

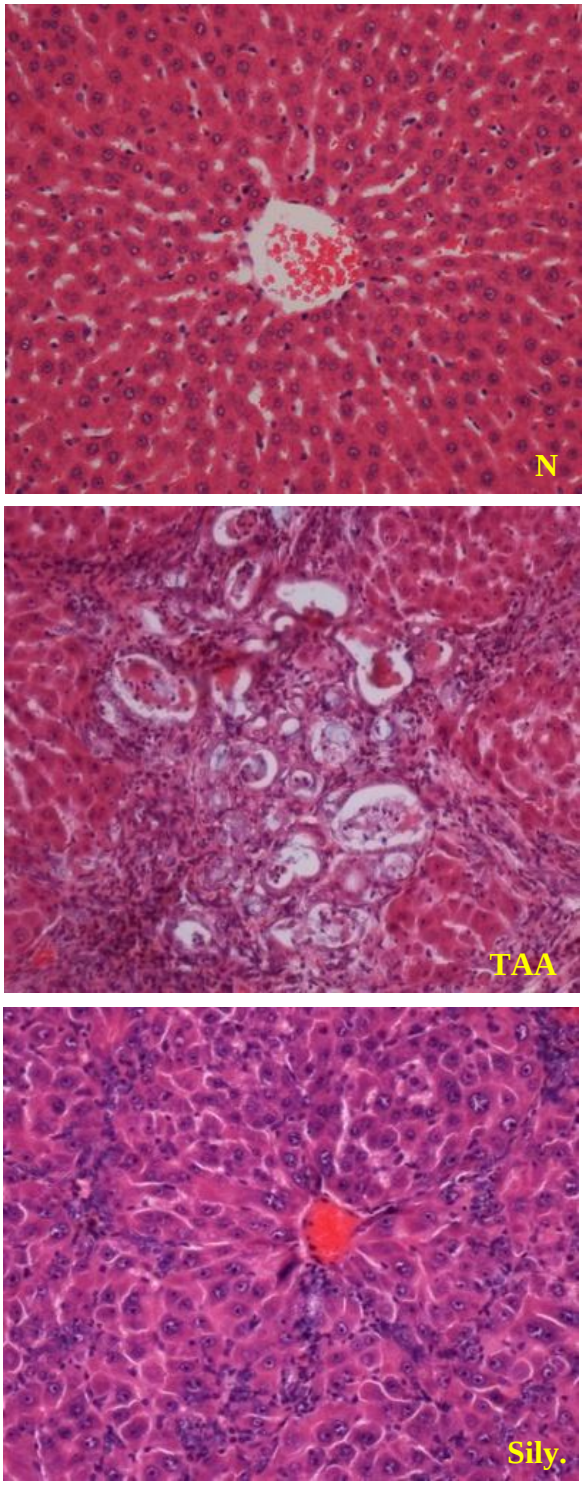


Figure 4.13: Histopathological changes of livers from N, TAA and Sily groups. (N) control rats liver section shows normal histological structure and architecture. (TAA) Severe structural damage, formation of pseudolobules with thick fibrotic septa and necrotic areas were present in the liver of the hepatotoxic rat. (Sily) Mild inflammation but no fibrotic septa was depicted in the liver of the rats treated with silymarin. . (H & E stain Original magnification 20x).

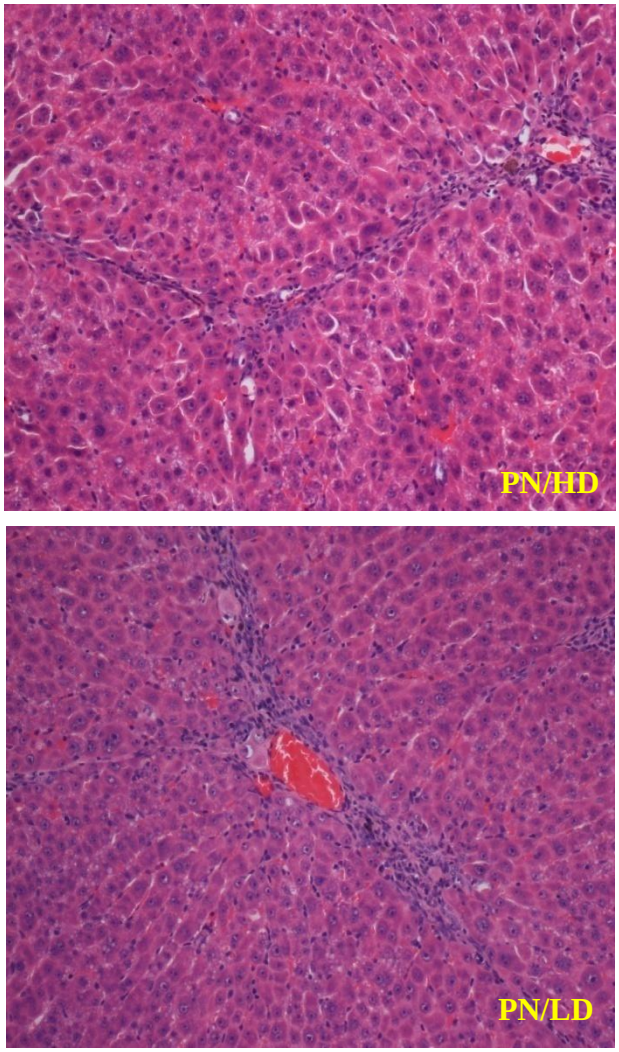


Figure 4.14: Histopathological changes of livers from *P. niruri* treated groups: (PN/LD 100 mg/kg) and (PN/HD 200 mg/kg). Preserved hepatocyte and architecture with small areas of mild necrosis were observed in the liver of the rat treated with 200 mg/kg of the PN. (H & E stain Original magnification 20x).

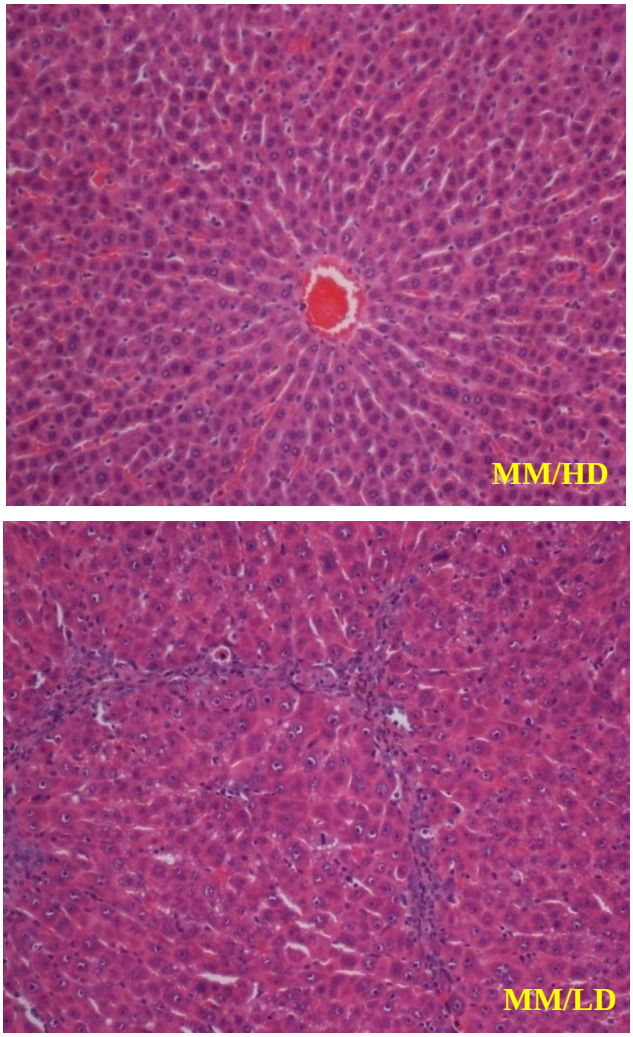


Figure 4.15: Histopathological changes of livers from *M. malabathricum* treated groups: (MM/LD 100mg/kg) and (MM/HD 200 mg/kg). Preserved hepatocyte and architecture with small areas of mild necrosis were observed in the liver of the rat treated with 200 mg/kg of the MM extract. (H & E stain Original magnification 20x).

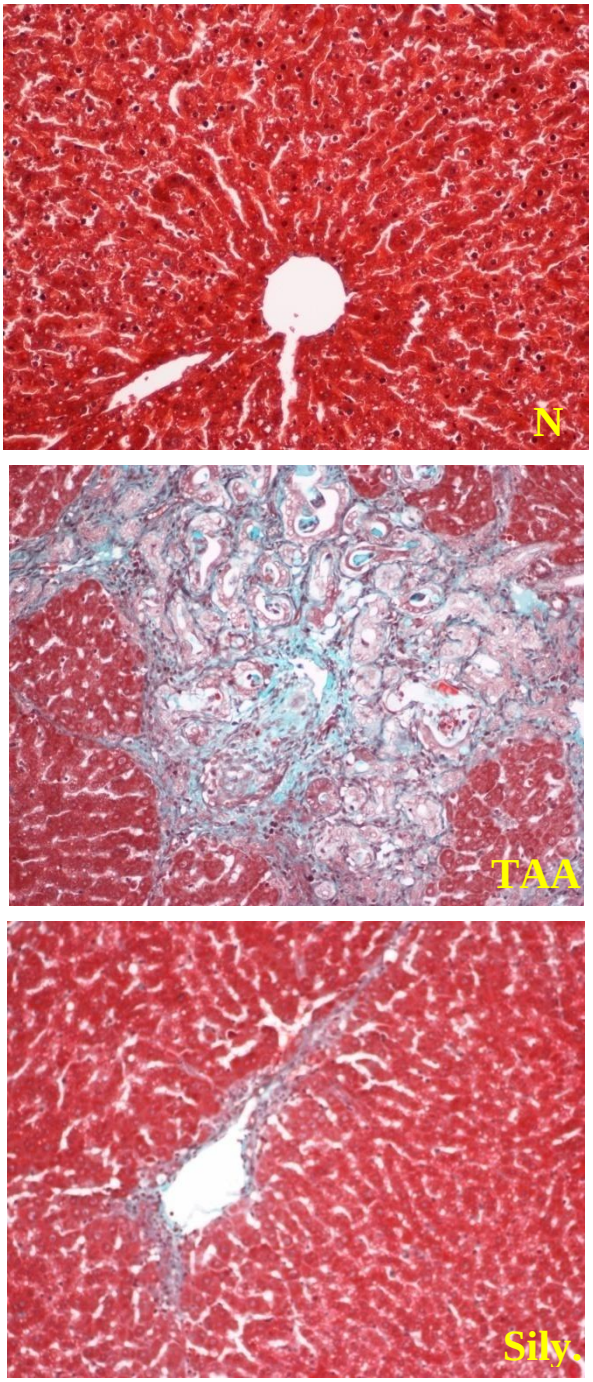


Figure 4.16: Histological section of N, TAA and Sily. Groups liver parenchyma stained with Masson's trichrome stain. N: normal rat's liver section showing normal liver architecture. TAA: Extensive hepatic fibrosis (blue color) with proliferating bile ducts was observed in TAA-treated liver sections. Sily.: silymarin treated rats showed mild hepatic fibrosis (blue color) in liver section.

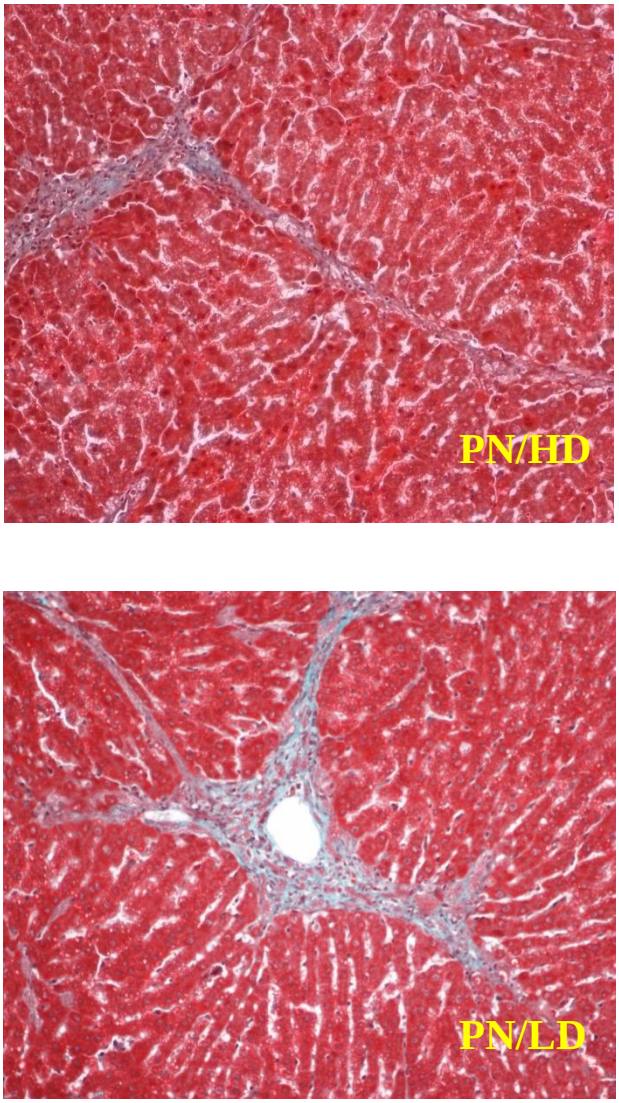


Figure 4.17: Effects of *P. niruri* on TAA-induced liver cirrhosis in rats. (PN/LD 100 mg/kg): *P. niruri* low dose treated rats showed mild hepatic fibrosis (blue color) in liver section. (PN/HD 200 mg/kg): *P. niruri* high dose treated rats showed less hepatic fibrosis (blue color) in liver section. (Masson's trichrome stain, Original magnification 20x).

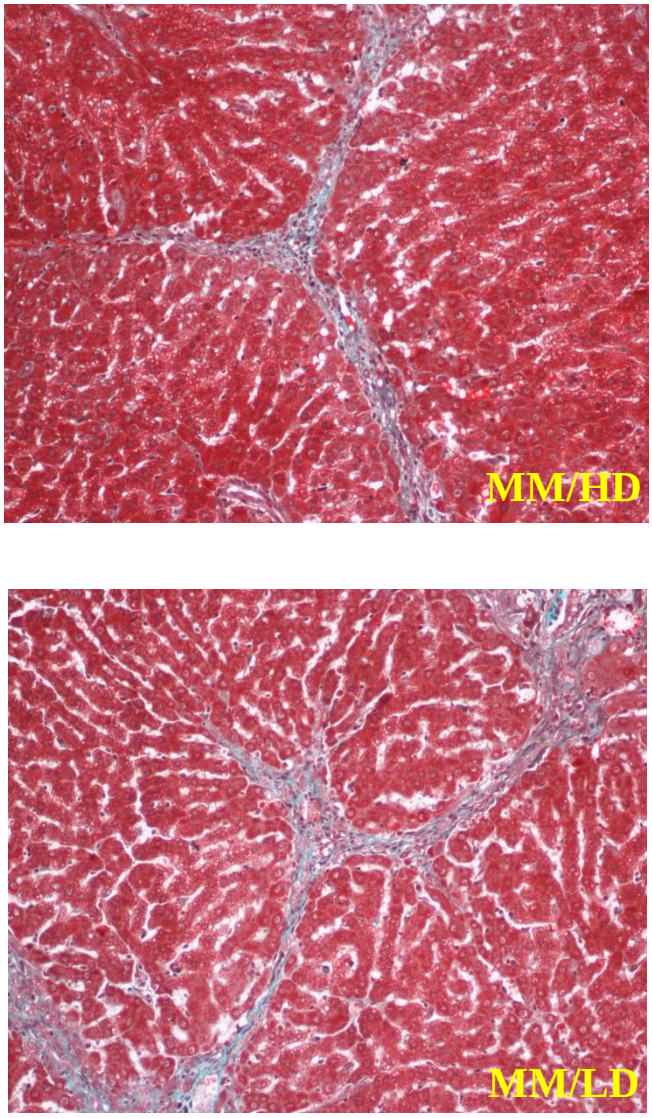


Figure 4.18: Effects of *M. malabathricum* on TAA-induced liver cirrhosis in rats. (MM/LD 100 mg/kg): *M. malabathricum* low dose treated rats showed mild hepatic fibrosis (blue color) in liver section. (MM/HD 200 mg/kg): *M. malabathricum* high dose treated rats showed less hepatic fibrosis (blue color) in liver section. (Masson's trichrome stain, Original magnification 20x).

4.5.4 Hepatic biochemical parameters

At the end of the experimental duration and as compared to normal group rats, TAA rats had elevated serum activities of liver function enzymes: ALT, AST and ALP. Similarly, there were elevated levels of Bilirubin and γGT and subsequently reduced levels of albumin. The raised parameters monitored in TAA group were reduced markedly by feeding with (100 and 200 mg) PN and MM extracts per kg body weight of rats, as shown in Figure 4.19 and Figure 4.20.

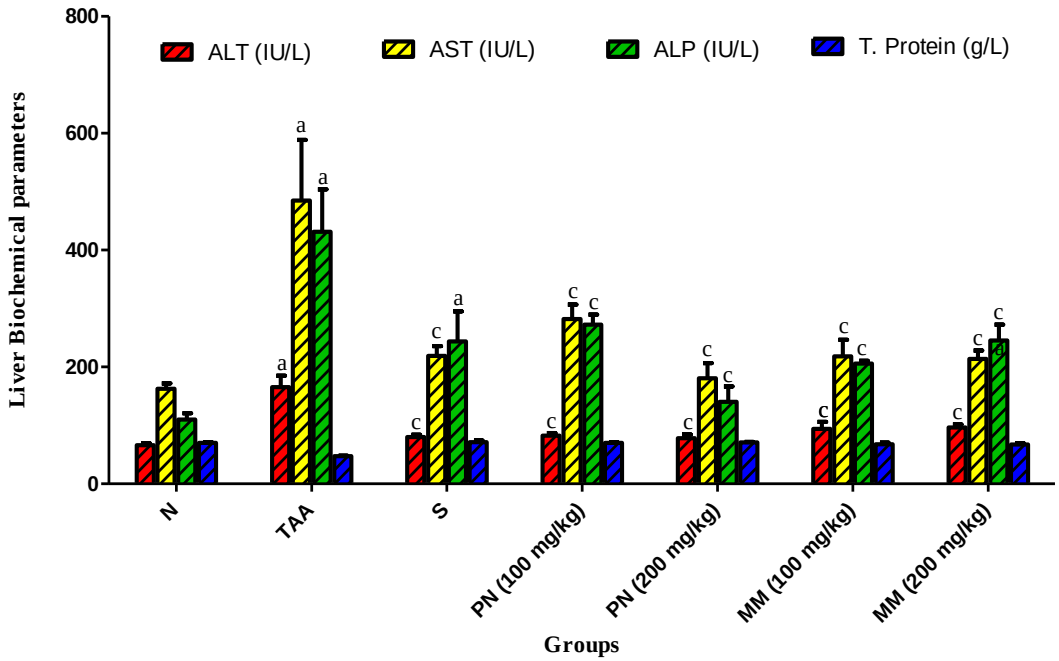


Figure 4.19: Effect of Experimental samples on Alanine Amino Transferase (ALT), Aspartate Amino Transferase (AST), Alkaline Phosphatase (ALP) and Total Protein levels. Data expressed as Mean ± SEM, (a) indicates significance versus normal group at $P \leq 0.01$; (b) indicates significance versus thioacetamide group at $P \leq 0.05$ and; (c) indicates significance versus thioacetamide group at $P \leq 0.01$

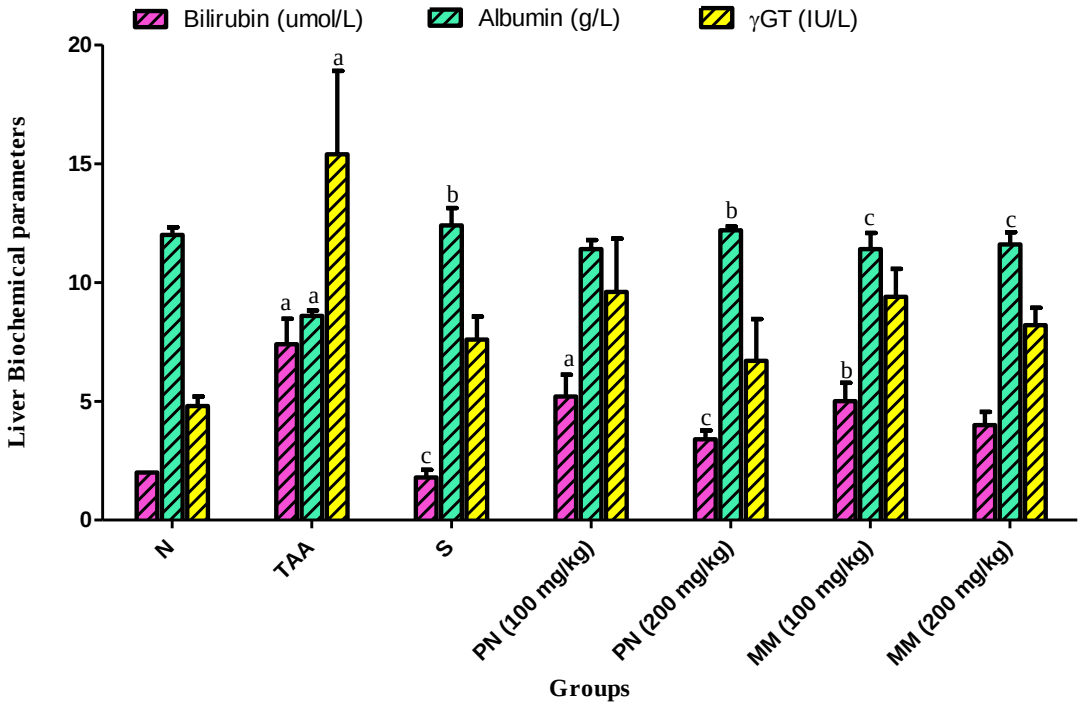


Figure 4.20: Effect of Experimental samples on Bilirubin, Albumin and Gamma Glutamyl transferase (γGT) levels. Data expressed as Mean ± SEM, (a) indicates significance versus normal group at $P \leq 0.01$; (b) indicates significance versus thioacetamide group at $P \leq 0.05$ and; (c) indicates significance versus thioacetamide group at $P \leq 0.01$

4.5.5 Endogenous antioxidant activities

Figure 4.21 shows *in vivo* activities of oxidative enzymes involved in the hepatic antioxidant defense system. SOD and GPX enzyme levels were decreased significantly in TAA groups when compared with the normal control group and similarly their levels were lower in the low dose plant group when compared with the high dose group. However, the decrease was non-significant for CAT levels. Furthermore, under the reaction conditions used, the capacity of the total antioxidants in both plants to prevent ABTS oxidation was proportional to their concentration in the livers of the animals where the values were substantially increased in the treatment groups in comparison to their HD and LD groups. However, Figure 4.21 indicates that treatment with MM or PN or Silyamrin significantly decreased the MDA values as compared to TAA group.

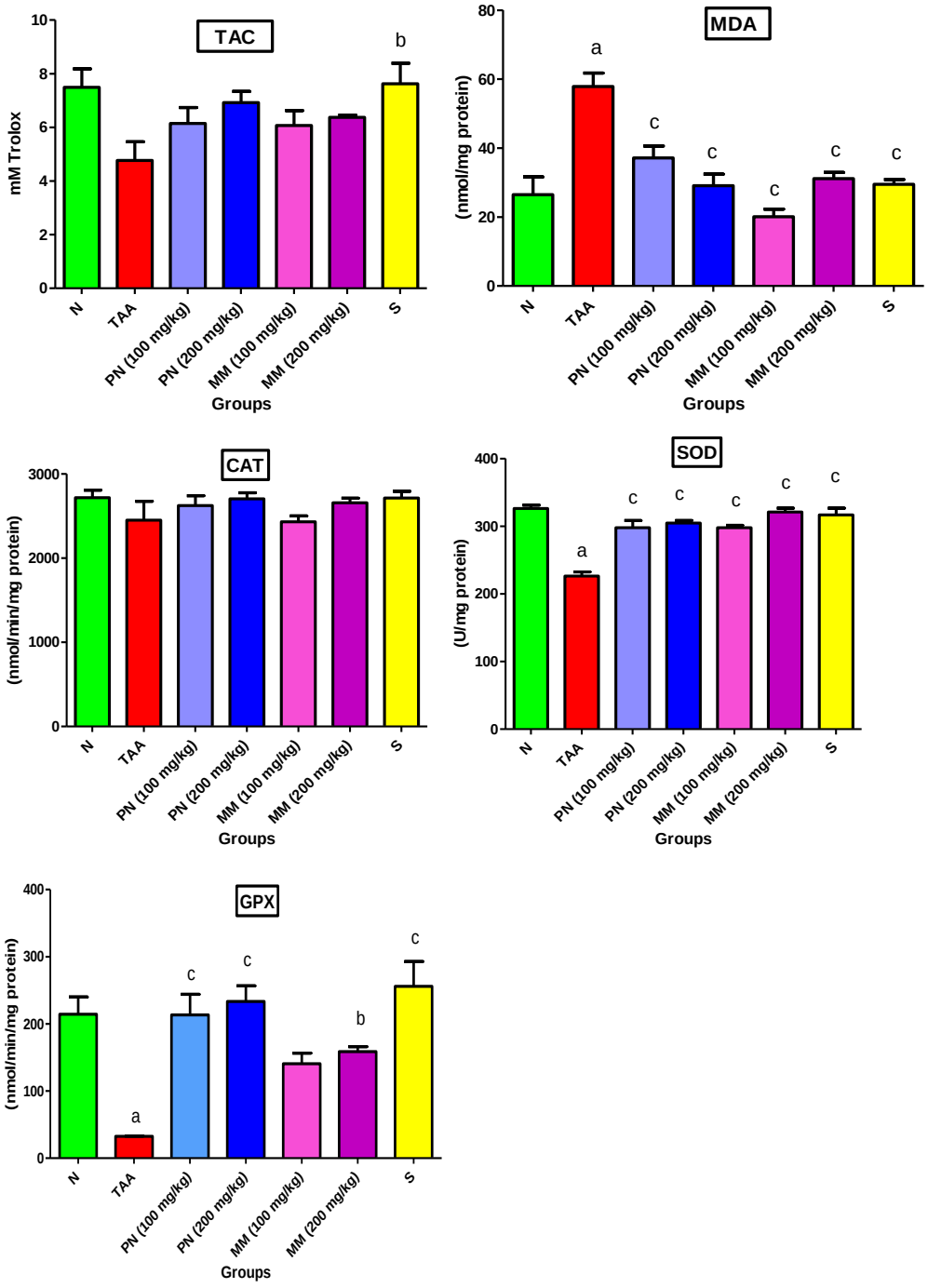


Figure 4.21: *P. niruri* and *M. malabathricum*'s *in vivo* antioxidant activities. Data expressed as Mean ± SEM, **(**a) indicates significant differences at $P < 0.01$ versus Normal control group, (b) at $P < 0.05$ versus TAA group and (c) at $P < 0.01$ versus TAA group.

4.5.6 Gene expression profile

4.5.6.1 Integrity of RNA

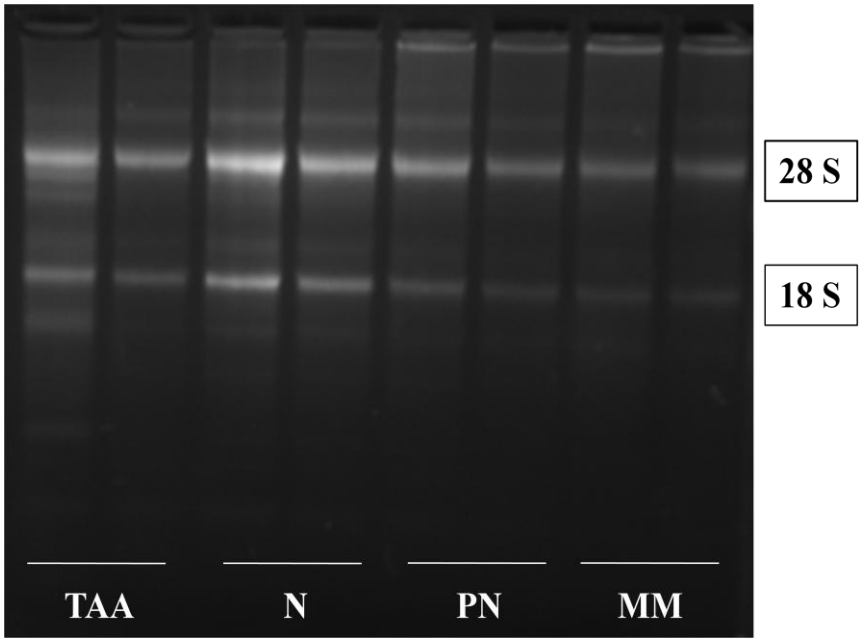
Total RNA was extracted from the liver tissues and quantity of RNA was determined by reading the absorbance at 260nm spectrophotometrically with ND-2000 Nano Drop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The ratio of absorbance reading at 260nm and 280 nm was used to indicate the quality of the RNA. The 260/280 ratio for our RNA preparation ranged from 1.6-2.1 in most cases (Table 4.6), which suggested good quality RNA.

The integrity of RNA was checked by agarose gel electrophoresis. Discrete 28S and 18S ribosomal RNA bands were obtained in each case, with the 28S band about twice the 18S band, indicating that the RNA extracted was intact and could be applied for RT-PCR. (Figure 4.22) shows a typical ethidium bromide stained-agarose RNA gel.

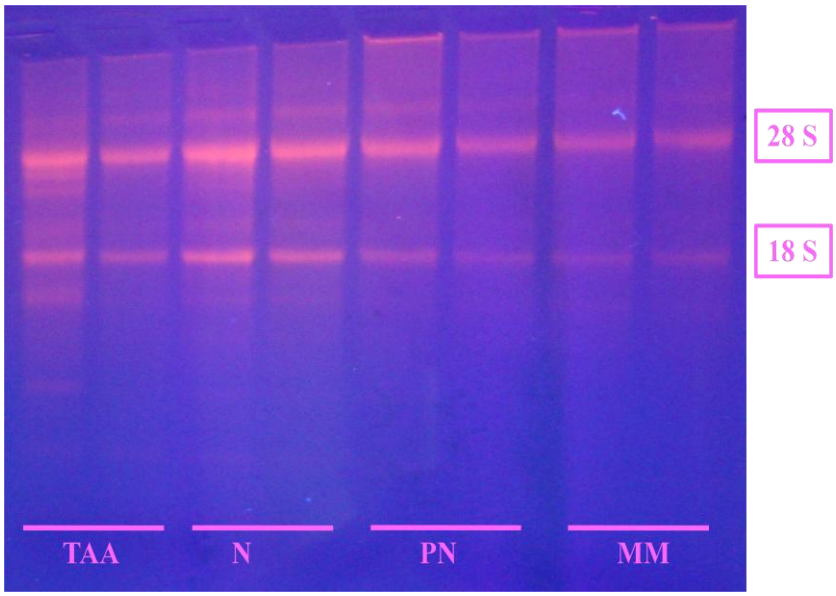
Table 4.6: The quality of the RNA as measured by Nano Drop spectrophotometer at 230, 260, 280, 320, 260/280 and 260/320 nm absorbances.

Sample name	Concentration ng/µl	260/280	260/230	230nm	260nm	280nm	320nm
N1	603	1.993	1.813	1.283	1.959	1.208	0.452
N2	496	2.091	2.191	0.372	1.046	0.339	0.194
N3	625	2.08	1.818	0.668	1.371	0.56	0.191
N4	600	2.059	2.062	0.529	1.301	0.53	0.198
N5	764	1.833	1.99	1.335	2	1.417	0.375
TAA1	145	2.017	1.276	0.8	0.95	0.825	0.584
TAA2	567	2.052	2.026	0.7	0.414	0.691	0.004
TAA3	272	2.072	1.616	0.443	0.717	0.346	0
TAA4	416	1.964	1.512	1.093	1.445	0.935	0.406
TAA5	690	1.977	2.029	1.077	1.952	1.116	0.227
PN1	243	2.082	1.906	0.32	0.609	0.293	0.001
PN2	224	2.102	0.809	0.63	0.562	0.269	0.003
PN3	105	1.13	0.25	1.13	0.251	0.113	0.011
PN4	494	2.057	1.836	1.726	1.288	0.654	0.054
PN5	308	2.059	2.059	0.38	0.776	0.38	0.006
MM1	130	2	1.5	0.23	0.33	0.168	0.011
MM2	102	2.1	1.81	0.2	0.35	0.21	0.098
MM3	417	2	2	0.5	1.05	0.5	0.01
MM4	262	2	2	1.01	1.35	1.018	0.69
MM5	385	2	1.9	0.5	0.96	0.47	0.007

N: normal control group, TAA: thioacetamide treated group, PN: *P. niruri* treated group and MM: *M. malabathricum* treated group.



(A)



(B)

Figure 4.22: A typical ethidium bromide-stained agarose gel showing the integrity of the extracted RNA; (A) visualized under Vilber Lourmat gel documentation system (B) visualized under UV light.

3.5.6.2 Real Time PCR analysis

Between the four endogenous reference genes used, the lowest variabilities have been shown by HPRT-1 and Ppia in comparison to GAPDH and β-actin, according to the analysis of NormFinder and geNorm algorithm softwares (Figure 4.23). Therefore, HPRT-1 and Ppia were chosen for further analyses and normalization of the target genes in animal liver tissue samples.

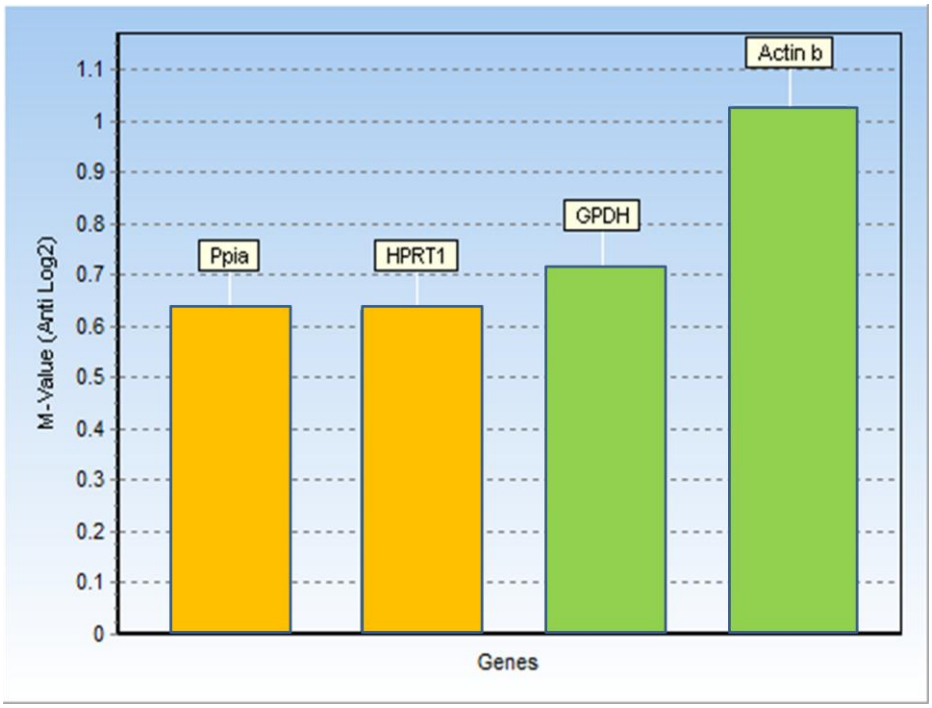


Figure 4.23: The best genes with less variabilities were Ppia and HPRT-1 among the four endogenous genes used (Analyzed by geNorm software).

Prior to running the quantitative Real Time PCR, standardization processes have been done for all investigated target and endogenous genes as shown in Table 4.7. All obtained data were within the reference range to perform the Real Time PCR.

Table 4.7: The standard curve properties of all investigated genes

Genes	Efficiency (%)	Slope	Y-intercept	R^2 value
HPRT1	97.78	-3.376	28.026	0.995
Ppia	92.679	-3.511	27.154	0.998
TGFβ1	98.056	-3.369	28.85	0.993
Coll α	95.351	-3.439	32.567	0.990
MMP2	108.376	-3.136	31.042	0.991
TIMP1	99.408	-3.336	34.01	0.989
Reference Range	(90-110)	(-3.1)-(-3.5)	….	(≥ 0.98)

Ct values of Real Time PCR data were calculated by GenEX software and normalized to the reference genes HPRT-1 and Ppia. The analysis showed significant differences in mRNA levels of investigated genes expression between controls, TAA, PN and MM treated rats.

In the control rats the mRNA levels of transforming growth factor β (TGFβ) gene, collagen α type I (collα1) gene, metalloproteinase- 2 (MMP2) gene or tissue inhibitor of metalloproteinase-1 (TIMP1) gene did not change suggesting that the hepatic satellite cells

(HSCs) were in their quiescent state (Figure 4.24). When treated with thioacetamide (200 mg/kg b.w), the livers expressed up-regulations of all investigated genes. The up-regulation was significant at ($P < 0.01$) in TAA group in comparison to N group for the genes TGFβ (1.677 ± 0.120 fold increase), Coll α1 (47.062 ± 7.716 fold increase) and MMP2 (14.500 3.528 fold increase). However, the difference was non-significant for TIMP1 gene (1.738 ± 0.486 fold increase).

Oral administration of PN and MM (200 mg/kg b.w.) before cirrhosis induction prevented and resolved the activation of HSCs and the remaining cells expressed decreased levels of TGFβ, Collα1 and MMP2 in comparison to TAA group as shown in Figure 4.24 and Table 4.8. For TGFβ1 gene; PN treated group did not show significant difference with TAA group (1.644 ± 0.158 fold increase) while in contrast, MM group showed significant difference at ($P < 0.05$) (1.113 ± 0.180 fold increase).

For Collα1 and MMP2 genes; in comparison to TAA group, mRNA expression decreased significantly in PN and MM groups than TAA group at ($P < 0.01$) (9.948 ± 8.021 and 8.555 ± 7.762 fold increases) for Collα1 gene and at ($P < 0.05$) (4.167 ± 3.557 and 3.436 ± 3.601 fold increases) for MMP2 gene respectively.

The mRNA expression of TIMP1 gene in the TAA treated group showed decreased levels than PN and MM treated groups (2.706 ± 0.703 and 1.912 ± 0.441 fold changes) respectively as shown in Figure 4.24 and Table 4.8.

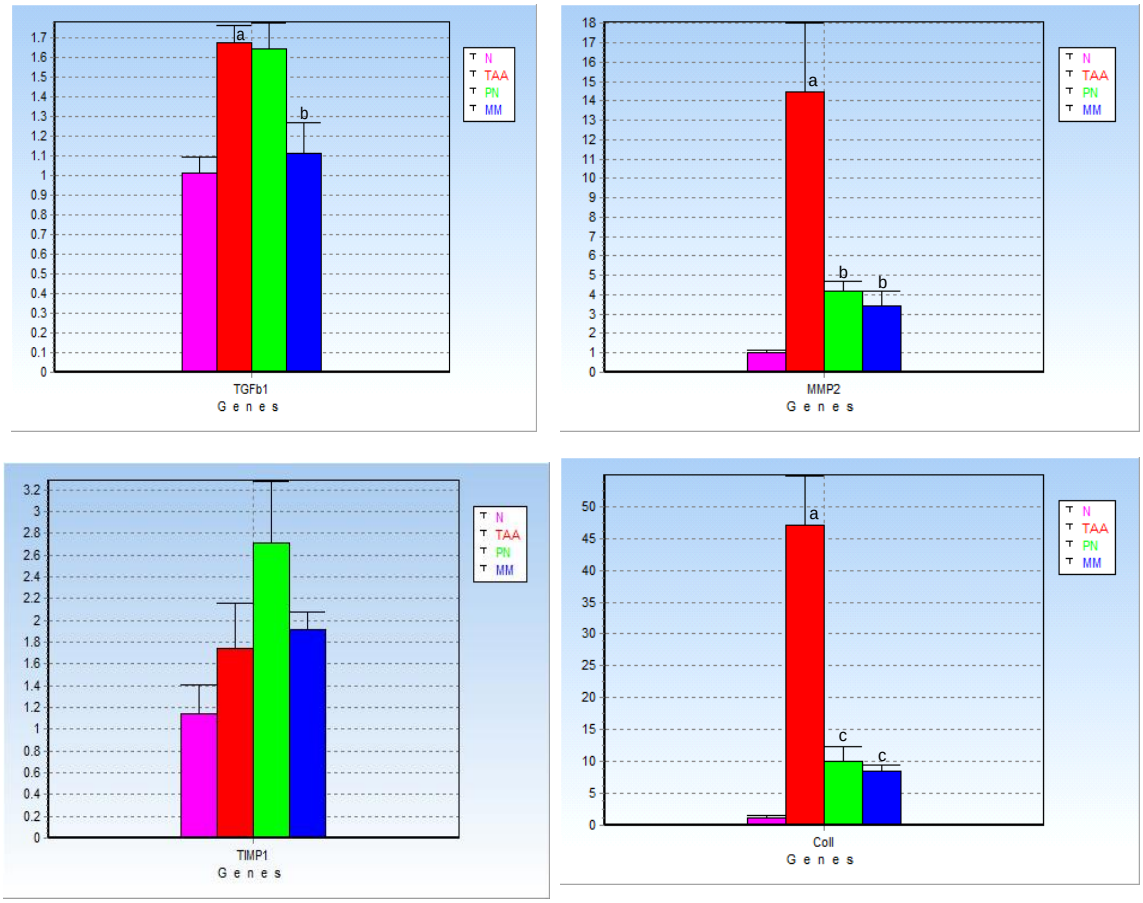


Figure 4.24: Real Time PCR analysis showing relative fold changes of transforming growth factor beta (TGFb), matrix metalloproteinase 2 (MMP2),tissue inhibitors of metalloproteinases 1 (TIMP1) and collagen alpha (Coll) genes between all experimental groups. Values expressed as Mean ± S.E.M. with different superscripts are significantly different. (a) Indicates significance versus Normal group at $P \leq 0.05$; (b) indicates significance versus TAA group at $P \leq 0.05$ and (c) indicates significance versus TAA group at $P \leq 0.01$.

Table 4.8: TAA, PN and MM effects on mRNA expressions of investigated genes.

Group	TGFβ1	TIMP1	MMP2	Collα1
N	1	1	1	1
TAA	1.677 ± 0.120 [a]	1.738 ± 0.486	14.500 ± 3.528 [a]	47.062 ± 7.716 [a]
PN (200 mg/kg)	1.644 ± 0.158	2.706 ± 0.703	4.167 ± 3.557 [b]	9.948 ± 8.021 [c]
MM (200 mg/kg)	1.113 ± 0.180 [b]	1.912 ± 0.441	3.436 ± 3.601 [b]	8.55482 ± 7.762 [c]

Values expressed as Mean ± S.E.M. with different superscripts are significantly different. (a) Indicates significance versus Normal group at $P \leq 0.05$; (b) indicates significance versus TAA group at $P \leq 0.05$ and (c) indicates significance versus TAA group at $P \leq 0.01$.

3.6 Chromatography profile

After crude extraction of *P. niruri* and *M. malabathricum,* the ethanol extracts were objected to flash column chromatography to separate the constituents of the extracts according to molecular size, molecular mass and polarity. Thus, (12) fractions were obtained from *P. niruri* and (9) fractions were obtained from *M. malabathricum*; and by performing thin layer chromatography the subsequent fractions with the same Rf (retention factor) and the spot colors after visualizing under UV light at 240 nm and 360 nm, were combined to give five fractions for *P. niruri* (PNF1, PNF2, PNF3, PNF4 and PNF5) and four fractions for *M. malabathricum* (MMF1, MMF2, MMF3 and MMF4). The best resolutions of plates were given by acetonitrile- water.

Subsequently the immunomodulatory activity for all fractions was tested to examine their abilities to proliferate human peripheral mononuclear cells (PBMC). As shown in Figures 4.25 and 4.26, both plants fractions showed high activities to proliferate PMBCs percentages of viability but best fractions were fraction number one for *P. niruri* (PNF1) and *M. malabathricum* (MMF1).

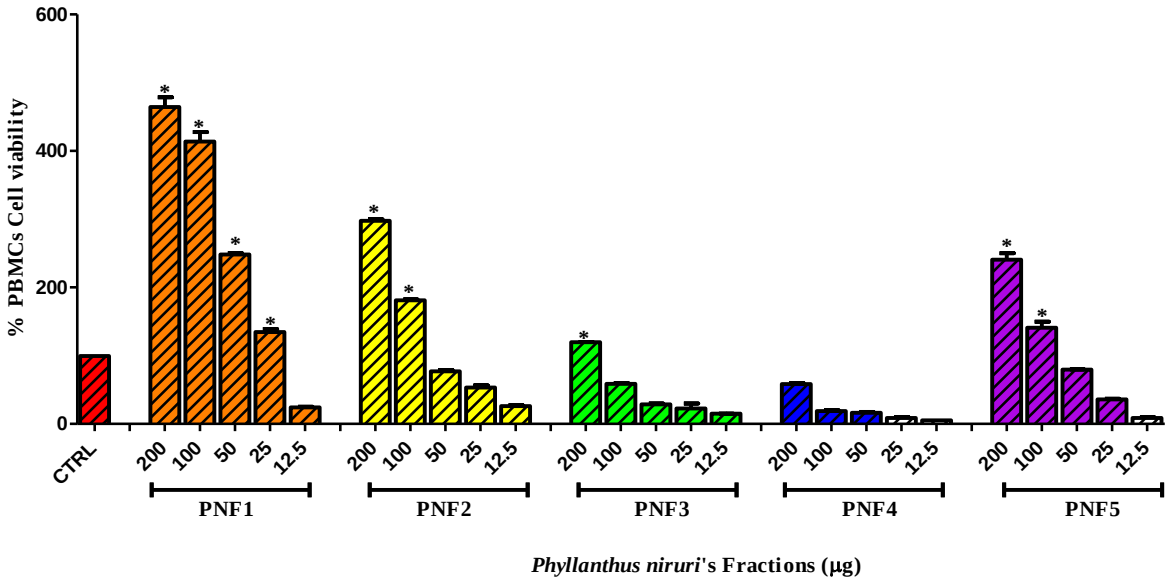


Figure 4.25: The effects of *P. niruri* fractions on human peripheral mononuclear cells (PBMC) proliferation. Data expressed as Mean ± SEM for triplicates; (*) indicates significance versus control group (CTRL= dH_2O) at $P \leq 0.05$

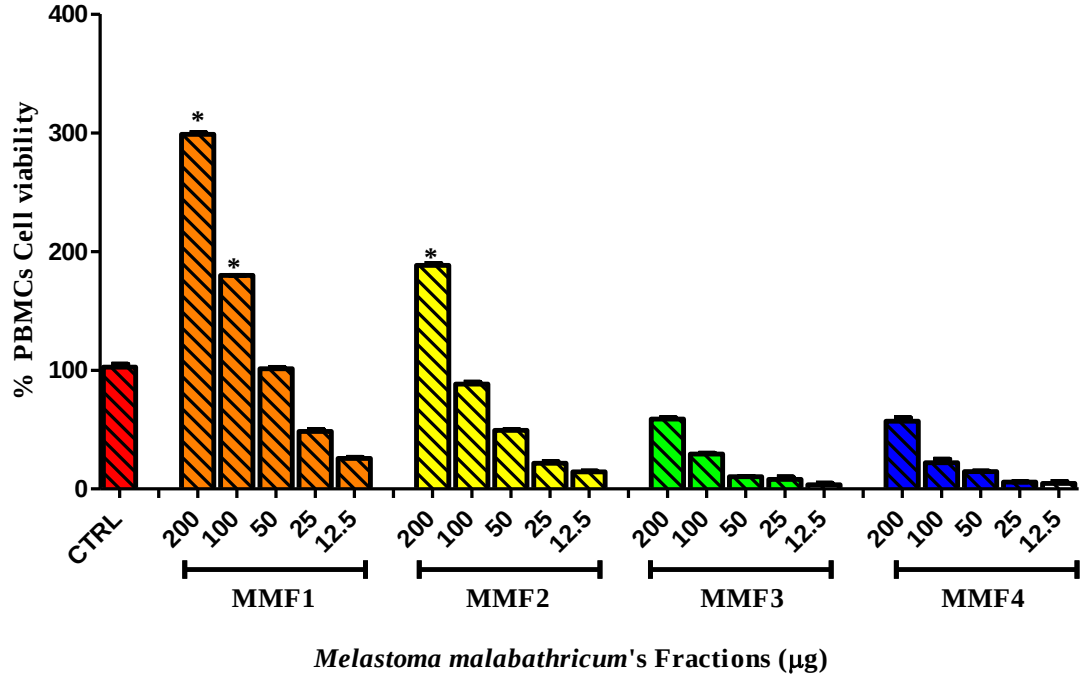


Figure 4.26: The effects of *M. malabathricum* fractions on human peripheral mononuclear cells (PBMC) proliferation. Data expressed as Mean ± SEM for triplicates; (*) indicates significance versus control group (CTRL= dH_2O) at $P \leq 0.05$.

LC-MS was performed to the fractions (PNF1 and MMF1) that showed higher activities to proliferate the PBMC cells. The concept of the combination of LC partition with UV spectrophotometric detection and MS enables the recognition of identified compounds in the extract by comparing their LC retention time and UV and MS spectra with those obtained from authentic compounds. Afterward, In LC-MS/MS by using the positive ionization mode four peaks were observed from PNF1 (Figures 4.27, 4.28, 4.29 and Table 4.9). However, only peaks number 2 and 4 were identified. the peak number 2 at (RT= 4.454 min, λ=221 and 280 nm, MW=355) (Figure 4.27) had $[M + H]^+$ at *m*/*z* 356 identified as Caffeolquinic acid (an isomer of chlorogenic acid) with fragments at *m*/*z* 340 (loss of CH_3) and the predominant fragments at *m*/*z* 191,165,151 and 147 respectively (Tang *et al.*, 2010). The peak number 4 (Figure 4.28) at (RT= 7.96 min , λ= 225 nm, MW= 448) had $[M + H\text{-}H_2O]^+$ at *m*/*z* 430 identified as Quercetin 3-O-rhamnoside with loss of H_2O and with loss the rhamnoside the ion appeared at *m*/*z* 303 identified as quercetin with other fragments 219, 205 and 165, respectively (Bravo *et al.*, 2007; Seeram *et al.*, 2006).

At the same time, four peaks were recognized from MMF1, (Figure 4.30). Among them, only the first peak was not identified. The second peak at (RT= 2.31 min, λ= 210,263 and 313 nm, MW= 286) as shown in Figure 4.31 was identified as kaempferol with MS/MS fragmention at 147, 241 and 317. The third peak appeared at (RT 2.61 min, λ= 203, 254 and 368 nm, MW= 302) at *m*/*z* 303 identified as quercetin (Figure 4.32) with the predominant fragments at *m*/*z* 121, 153, 165, 245 and 257 respectively. The forth peak at *m/z* 273 (RT= 3.27 min, λ= 219,267 and 312 nm, MW= 272) with daughter peaks at 119, 147 and 181, identified as naringenin as shown in Figure 4.33. All the chemical and molecular informations of the identified chemicals were given in Table 4.9.

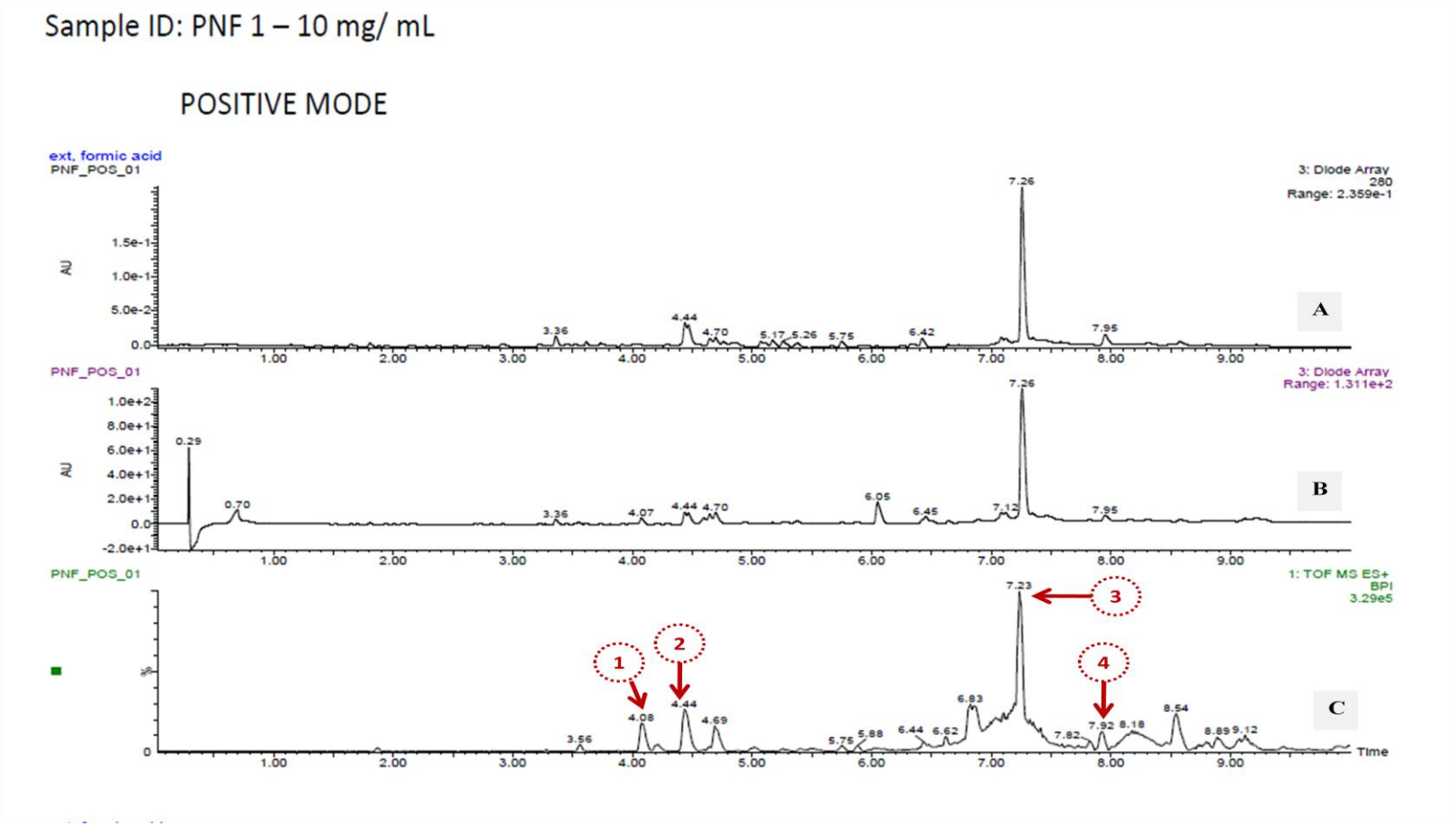


Figure 4.27: HPLC-TOF/MS and UV diode array chromatograms of *P. niruri* fraction 1 (PNF1); (A) Diode array detection UV spectra at 280 nm (B) Diode array detection UV spectra at range between 190 and 800 nm (C) TOF MS peaks in positive mode ionization

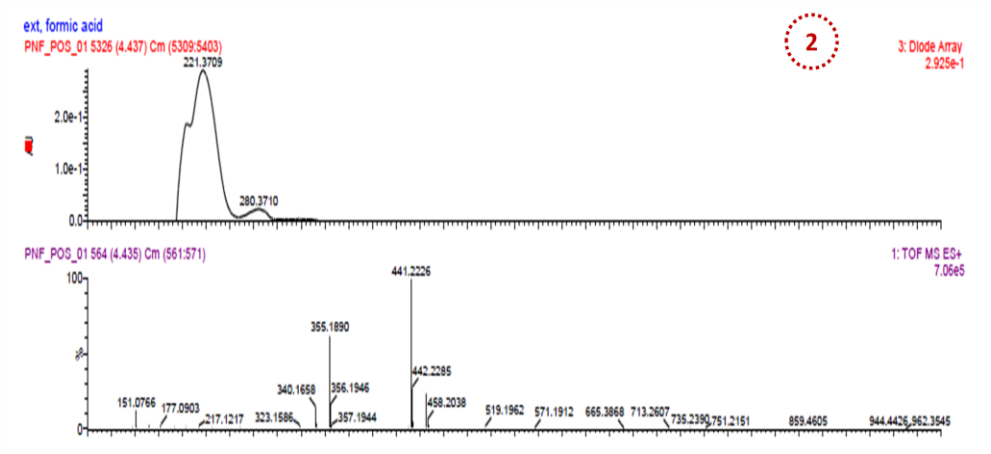


Figure 4.28: UV max spectra and Mass Spectrum (TOF MS ES+) of peak no. 2 in *P. niruri* F1 (identified as 4-O-Caffeolquinic acid).

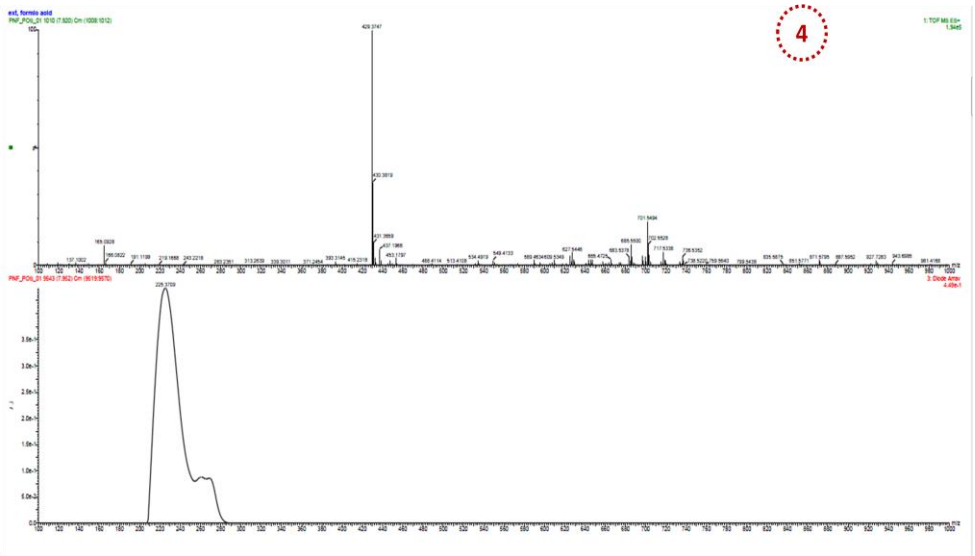


Figure 4.29: Mass Spectrum (TOF MS ES+) and UV max spectra of peak no. 4 in *P. niruri* F1 (identified as Quercetin 3-O-rhamnoside).

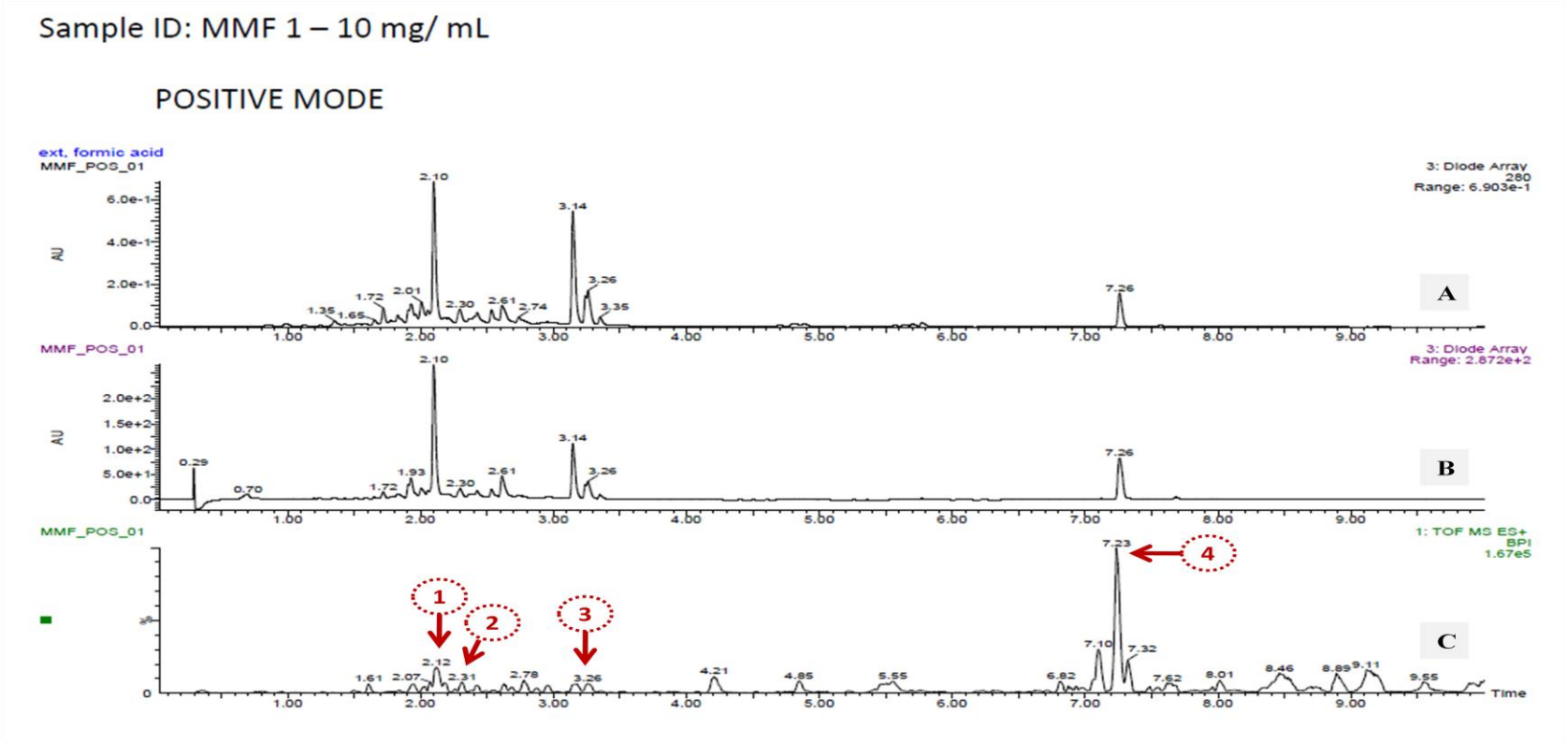


Figure 4.30: HPLC-TOF/MS and UV diode array chromatograms of *M. malabathricum* fraction 1 (MMF1); (A) Diode array detection UV spectra at 280 nm (B) Diode array detection UV spectra at range between 190 and 800 nm (C) TOF MS peaks in positive mode ionization.

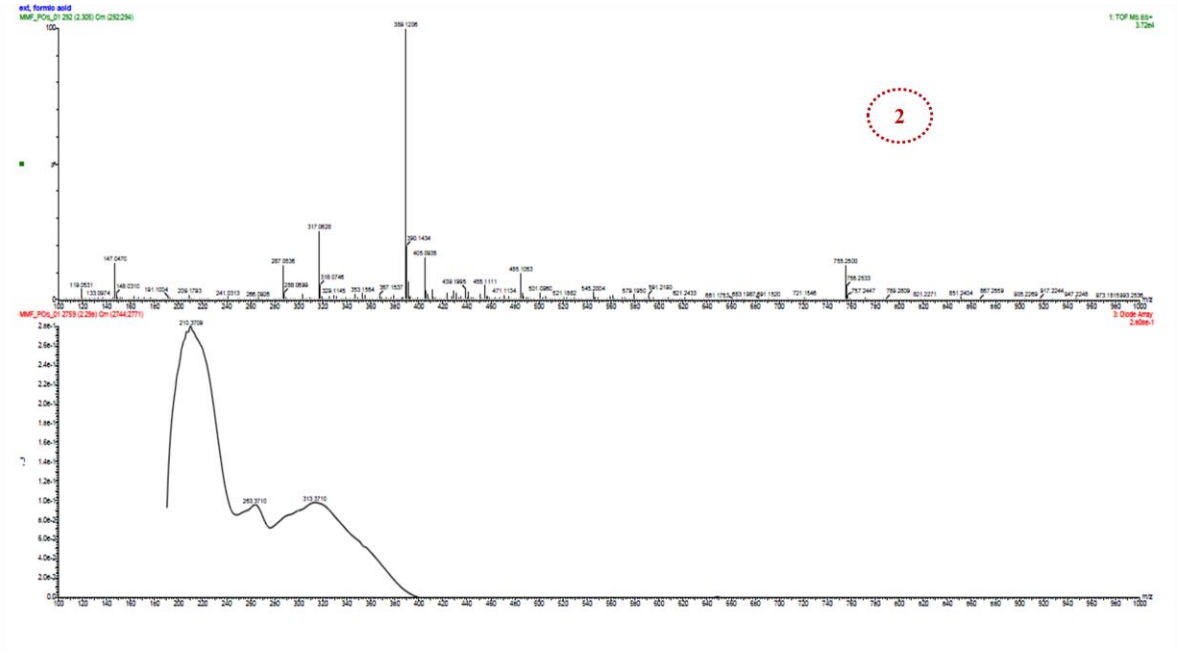


Figure 4.31: Mass Spectrum (TOF MS ES+) and UV max spectra of peak no. 2 in *M. malabathricum* F1 (identified as Kaempferol).

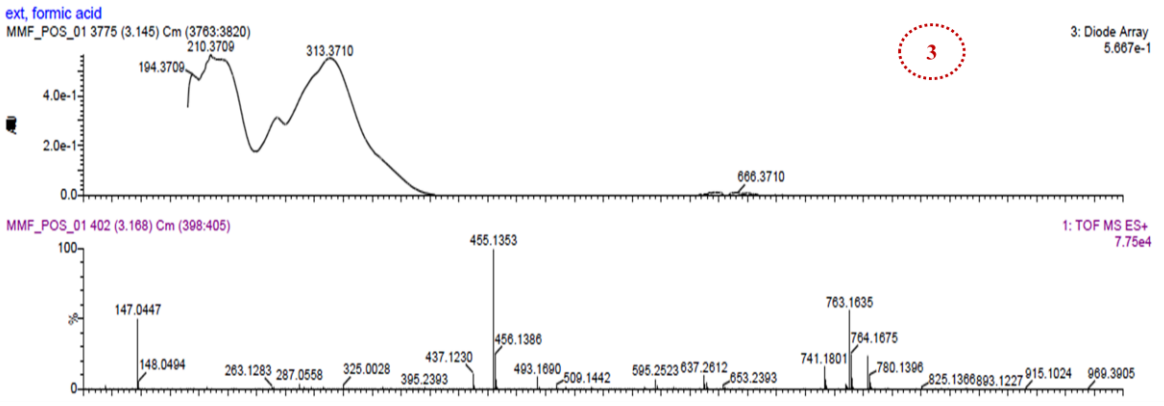


Figure 4.32: UV max spectra and Mass Spectrum (TOF MS ES+) of peak no. 3 in *M. malabathricum* F1 (identified as Quercetin).

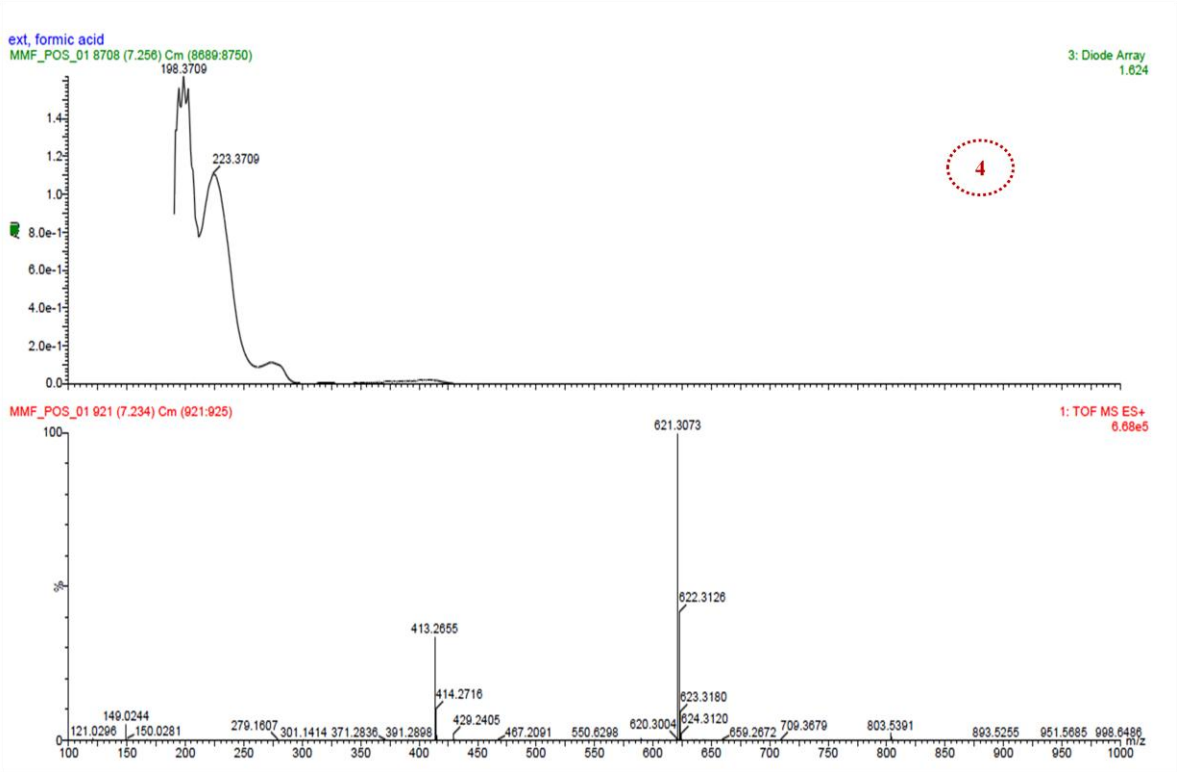


Figure 4.33: UV max spectra and Mass Spectrum (TOF MS ES+) of peak no. 4 in *M. malabathricum* F1 (identified as Naringenin).

Table 4.9: Putative identification of main components of *P. niruri* fraction (PNF1) and *Melastoma malabathricum* fraction (MMF1).

Plant Fraction	Rt.Time (min)	λ (nm)	Molecular Weight	[M+H] *m/z*	MS/MS Fragmentation	Tentative identification	Molecular Formula
PNF1	4.437	221, 280	355	356	340, 191, 165 , 151, 147	4-O-Caffeolquinic acid	$C_{16}H_{18}O_9$
	7.92	225	448	430	303, 219, 205, 165	Quercetin3-O-rhamnoside	$C_{21}H_{20}O_{11}$
MMF1	2.31	210, 263, 313	286	287	147, 241, 317	Kaempferol	$C_{15}H_{10}O_6$
	2.61	203, 254, 368	302	303	121, 153, 165, 245, 257	Quercetin	$C_{15}H_{10}O_7$
	3.27	219, 267, 312	272	273	119, 147, 181	Naringenin	$C_{15}H_{12}O_5$

CHAPTER V

DISCUSSION AND CONCLUSION

5.1 Discussion

5.1.1 Antioxidant Activities of *P. niruri* and *M. malabathricum*

Reactive oxygen species (ROS) are prospective carcinogenic substances due to generating free radicals including hydroxyl, superoxide, peroxyl, hydroperoxyl and alkoxyl radicals, which participate in tumor promotion, mutagenesis and progression. If there is no effective regulation, the excess ROS would damage proteins, lipids or DNA and in turn inhibit the normal function and modulation of gene expression, cell cycle, cell metabolism, cell adhesion and cell death (Ha *et al.*, 2010).

Medicinal plants have been presumed to be rich resources of natural antioxidants that counterbalance these ROSs, remove the free radicals and minimize the damage to the tissues. In this investigation, we have tested the crude ethanol extract of *Phyllanthus niruri* and *Melastoma malabathricum* for their radical scavenging potentials.

The data of the present study demonstrated that DPPH and ABTS free radicals were reduced in the presence of the plant extracts following the trend Vit C > *P. niruri* > *M. malabathricum* > BHT. In addition, both plants showed a high ability for reducing the ferric ions. The antioxidant activities of *Phyllanthus niruri* and *Melastoma malabathricum* ethanol extracts may be attributed to their phenolic and flavonoid contents, which are powerful *in vitro* antioxidant molecules (Rice-Evans, 2001) and predictable free radical scavengers due to their reducing properties as hydrogen or electron-donating agents (Rice-

Evans, 1995). The mechanism of these plant derived polyphenolic compounds may be through free radical scavenging, complexion of pro-oxidant metals and quenching of the formation of singlet oxygen (Pratt, 1992).

These findings seems to be in agreement with the recent study of Mollataghi et al. and Arya et al. (Mollataghi et al., 2012; Arya et al., 2012), who determined the DPPH scavenging activity of ascorbic acid and found the IC_{50} to be less than 4 µg / mL.

Poh-Hwa *et al.* (Poh-Hwa, 2011) proposed that over 95% of the antioxidant capacity in the *Phyllanthus* species was due to the involvement of polyphenolic compounds. In addition, it is encouraging to compare such findings with those found by Harish *et al.* (Harish & Shivanandappa, 2006), who discovered that methanol and aqueous extracts of the leaves and fruits of *P. niruri* showed scavenging of 1,1-diphenyl-2picrylhydrazyl (DPPH) radical and inhibition of reactive oxygen species (ROS) *in vitro*.

Furthermore, many recent studies reported the *in vitro* antioxidant and free radical potentials of *M. malabathricum*'s methanol and aqueous extracts, suggesting that the high phenol component explains the high antioxidant activity (Faravani, 2009).

5.1.2 Immunomodulatory Roles of *P. niruri* and *M. malabathricum*

The immune system is one of the body's means for detecting different pathogens, by producing immediate responses through the activation of immune cells, chemokines, cytokines and the release of inflammatory mediators, which in turn modulate and adapt the immune system (Singh Virendra Kumar, 2011).

Extracts of many medicinal plants have been shown to possess significant roles as immunomodulators and antitumor agents (Wong *et al.*, 1994). The naturally derived

immunomodulators have great prospectives for future development of new pharmaceutical products, serving as the initial point from which we may discover modern medicines (Ganju *et al.*, 2003) .

The ethanol extracts of *P. niruri* and *M. malabathricum* have shown to stimulate the PBMCs proliferation to more than double or triple the amount of the initial PBMCs control quantity, which may be of value in combination with other therapies in the treatment of immunodeficiency, cancer, infections and even autoimmune disorders.

Several attempts have been made to report on the immunomodulatory roles of *Phyllanthus* species, as the aqueous and the aceton extracts of *Phyllanthus tenellus* stimulates the macrophages activation through nitric oxide production (Ignácio *et al.*, 2001) and *Phyllanthus emblica* was named as " an immunomodulator" that enhanced natural killer cells activities and antibody dependent cellular cytotoxicity in BALB/c mice when administered orally (Suresh & Vasudevan, 1994).

Moreover, the study of Nworu *et al.* (Nworu *et al.*, 2010) suggested that *P. niruri*'s stimulation of the immune system was through an expression of surface activation maker (CD69) and proliferation of B and T lymphocytes. These activities were proposed to be achieved by lysosomal enzymes activity, phagocytosis and TNF-α release.

Additionally, the Melastomaceae family has been reported to include a wide variety of immune modulator species, such as *Osbeckia aspera* that has proven to induce *in vitro* phagocytosis by peripheral blood mononuclear cells (PBMC) and enclosed inhibitory patterns on mitogen/antigen stimulation with PBMC from patients with chronic hepatitis C viral infections (Nicholl *et al.*, 2001). In fact the anticancer activity of several isolated

compounds from *M. malabathricum* was reported by Susanti *et al.* (Susanti *et al.*, 2009) to involve cell proliferation and changes in the cell morphology.

Perhaps, the immunomodulatory potentials of *P. niruri* and *M. malabathricum* are due to their high antioxidant and free radical scavenging activities, which have been identified to innate pro-inflammatory immune responses and acts as therapeutic pathways for controlling inflammatory-mediated diseases (Tse *et al.*, 2004).

5.1.3 Acute Oral Toxicity Tests of *P. niruri* and *M. malabathricum*

Many clinical and toxicological studies have been carried out on medicinal plant extracts to ensure the secure and safe use and of such plants. In the 'Global Harmonized System of Classification and Labeling of Chemicals' and the Environmental Protection Agency 'Health Effects Test Guidelines', an LD_{50} value higher than 5000 mg/kg shown by plant extracts are considered to be practically safe (Ghs, 2005).

Therefore, the investigated plant extracts are toxicologically safe by oral administration, since they did not show any signs of body weight loss, no behavioral abnormalities were observed and no significant differences were found in the PN and MM treated groups in comparison to the control group regarding liver and renal function tests. Thus, our findings supports the logical usage of these plants in Malaysian folk medicine.

5.1.4 Hepatoprotective Activities of *P. niruri* and *M. malabathricum*

The experimental model of liver cirrhosis induced by thioacetamide in rats has been shown to reveal many alterations similar to human cirrhosis (Fontana *et al.*, 1996). The present study aimed to evaluate the protective role of *P. niruri* and *M. malabathricum* against thioacetamide induced liver cirrhosis on *Sprague Dawley* rats. The livers of rats that were administered TAA showed anatomical macroscopic and microscopic symptoms of cirrhosis, alteration of hepatic biochemical markers, significant loss of rat's body weights accompanied with significant increase in the liver and spleen masses.

On the contrary, the administration of the plant extracts prior to TAA injection has been proven to reduce the incidence of cirrhosis process and to decrease the necrosis, calcium levels and leakage of enzymes (Bruck et al., 2007; Balkan et al., 2001). Thus, the administration of crude ethanol extracts of *P. niruri* and *M. malabathricum* revealed significant hepatoprotective activity comparable to silymarin, based on normal body weight gaining, normal liver and spleen masses, preservation of the liver's normal morphologic shape and appearance, normal microscopic liver architecture and regular levels of hepatic biochemical tests.

These results support earlier studies by Bhattacharjee & Sil (Bhattacharjee & Sil, 2006, 2007) who mentioned that *P. niruri* exhibited hepatoprotective effects due to its antioxidant contents, which could protect liver from oxidative stress induced by different chemicals *in vivo* (Chatterjee *et al.*, 2006; Chatterjee & Sil, 2006) and *in vitro* (Erra Rajeshwar *et al.*, 2008; Sarkar & Sil, 2007).

Much literature can be found to verify the protective activity of *Phyllanthus niruri* against different drug and toxin induced hepatic disorders. Earlier studies (Harish &

Shivanandappa, 2006) have shown that the aqueous and methanol extracts of *P. niruri* demonstrated hepatoprotective action against carbon tetrachloride (CCL_4) induced formation of lipid perioxidation in the livers of rats, judged by the raised serum enzymes levels. While (Manjrekar *et al.*, 2008) studied the effects of the aqueous extracts of *P. niruri* against carbon tetrachloride (CCL_4) induced liver, kidney and testes injuries, and they conducted that the hepatoprotective and the antioxidant activity of this plant was associated with a deleterious effects on kidney and testes. In the study of (Bhattacharjee & Sil, 2007) the hepatoprotective potential of the protein isolated from *P. niruri* against carbon tetrachloride (CCL_4) induced liver damage was investigated. The results suggested that this protein protected the liver against oxidative stress and stimulated liver repair mechanisms.

A study was conducted by Chatterjee *et al.* (Chatterjee *et al.*, 2006; Chatterjee & Sil, 2006) that evaluated the preventive and curative effects of a protein fraction of *P. niruri* against nimesulide-induced oxidative stress *in vivo*, using a murine model through promotion of antioxidant defense. Moreover, the aqueous extracts of leaves of *P. niruri* exhibited distinctive antioxidant and hepatoprotective effects against paracetamol-induced liver injury in mice, which might be due to the high free radical scavenging activity, lipid peroxidation and inhibition of reactive oxygen species (Sabir & Rocha, 2008b) and pre-treatment by n-hexane extract of *P. niruri* all of which may have improved paracetamol-induced injury of the rat liver (Iqbal *et al.*, 2008).

Phyllanthin, known as "the principle active constituent" of *P. niruri*, has been demonstrated as being a hepatoprotective agent against ethanol-induced oxidative stress in rat liver cells (Chirdchupunseree & Pramyothin, 2010). On the other hand, in the *in vitro* study of Sarkar

and Sil (Sarkar & Sil, 2007) the hepatoprotective effect of the protein isolate of *P. niruri* was evaluated against thioacetamide (TAA) induced cytotoxicity in mice hepatocytes. Their data suggested that the protein isolate protected mice hepatocytes by its radical scavenging and antioxidative properties.

However, to the best of our knowledge, this is the first time that *Melastoma malabathricum* has been evaluated for hepatoprotective activity. Thus, we suggest a strong association between the *in vitro* free radical scavenging activities and *in vivo* hepatoprotective effects of *Melastoma malabathricum* crude ethanol extract, since thioacetamide induced hepatotoxicity is through its bioactivation to thioacetamide-S-oxide, which covalently binds to the macromolecules and induces oxidative stress (Pallottini *et al.*, 2006).

The hepatoprotective effects of the investigated plants are not only due to *in vitro* antioxidant activities. Previous studies have proved that plant extracts possess protective effects against toxic chemical caused liver injury through the endogenous oxidative enzyme systems involved in the hepatic defense system, like catalase (CAT), superoxide dismutase (SOD) and glutathione perioxidase (GPX), which counter balance the oxidative stress induced by thioacetamide (Kuo *et al.*, 2010; Huang *et al.*, 2011).

TAA injection has been reported to produce ROSs that followed lipid peroxidation (Sun *et al.*, 2000), glutathione depletion and reduction in the SH-thiol groups (Zaragoza *et al.*, 2000). The present study's finding demonstrated that TAA administration caused severe damage to rats endogenous antioxidant system, represented by down- regulation of the total antioxidant capacity (TAC) of the rats, alongside lipid peroxidation that was characterized by up-regulation in MDA levels and disturbance of oxidative stress enzymes (CAT, SOD and GPX) activities which led to a decrease in their levels. These results are in agreement with the previous research of Balkan *et al.* (Balkan *et al.*, 2001), who reported that

treatment of experimental rats with thioacetamide caused an increase in the MDA levels and decrease in the GPX levels. Moreover, Yogalakshmi *et al.* (Yogalakshmi *et al.*, 2010) displayed that TAA administration to rats resulted in significant decrease in the activities of SOD, CAT, GPX.

P. niruri and *M. malabathricum* crude ethanol extracts enhanced the levels of oxidative stress enzymes: SOD, GPX and reduced the lipid peroxidation (MDA) levels back to their normal levels after damage induced by thioacetamide.

Nevertheless, peripheral blood mononuclear cells (PBMCs) are critical cells of the immune system that fight different types of infections. PBMCs comprise the circulating mononuclear cells, including monocytes, T-cells, B-cells, and natural killer (NK) cells. These cells have been verified recently as specific markers of several diseases including inflammatory (e.g. rheumatoid arthritis, preeclampsia and chronic pancreatitis) and malignant (renal cell carcinoma and chronic lymphocytic leukemia) diseases (Baine et al., 2011). Strong relations found between plants immunomodulatroy activities and hepatoprotective roles since the PBMCs have high expression of the gene encoding TGF-β (Navikas et al., 1994; Reinhold & Ansorge, 2007; Turner et al., 1990) which is an essential regulator of chronic liver disease including initial liver injury, inflammation, fibrosis, cirrhosis and hepatocellular carcinoma (Dooley & Ten Dijke, 2012). On the other hand, as mentioned earlier (section 2.2.2) liver cirrhosis activates the hepatic satellite cells (HSC), which in turn increases the extracellular matrix (ECM) synthesis and causes a relative imbalance between production and degradation of matrix metalloproteins. In addition, the activated HSCs constitute various collagenases and tissue inhibitors of metalloproteinases to remodel the ECM (Bruck *et al.*, 2001).

In the present study results, the mRNA levels of transforming growth factor β (TGF-β1), collagen αI (Coll-α1), metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-1 (TIMP-1) genes did not change in the control group. This supports the idea that the hepatic satellite cells (HSCs) were still in their quiescent state. However, those HSCs activated by the presence of TAA led to the high production of ECM and consequently high expressions of TGFβ, Collα1, MMP2 and the TIMP1 gene. PN and MM treatment successfully prevented the high synthesis of ECM and reduced the mRNA expression of TGFβ, Collα1, MMP2 genes in comparison to TAA group.

Most studies of human liver diseases and animal models of progressive fibrosis have showed that TIMP-1 mRNA expression was up-regulated at early stages of fibrosis (Arthur *et al.,* 2006) since the TIMP-1 functions not only in reduction of the MMP activity (Tsukada *et al.,* 2006), but also acts on suppression of the apoptosis process of HSCs (Yoshiji *et al.,* 2002). So in our findings, hepatic reduction in TIMP-1 mRNA expression in the TAA group can be explained as a consequence of the increased HSC apoptosis. (Arthur, 2000).

Figure 5.1 shows the putative mechanism of alteration of mRNA levels of the investigated genes in TAA treated rats. First of all TAA converted to the free radical thioacetameide-S-oxide and other ROSs (Chilakapati *et al.,* 2005) causes lipid peroxidation (elevation levels of MDA), alters the antioxidant defense system of (CAT, SOD and GPX) (Yogalakshmi *et al.,* 2010) and activates the HSCs, which in turn, releases more ECM and subsequently increase the TGFβ gene expression that affects the release of collagen α gene, MMP1 gene and the TIMP1 gene. Thus, scar tissue develops and the liver loses its normal functions, anatomical shape and architecture (Wang *et al.,* 2008; Mormone *et al.,* 2011).

Treatment with PN and MM significantly reduced the impact of thioacetamide toxicity. Firstly through removing the causative stimuli of TAA, neutralizing the ROSs by their high antioxidant content and attenuation of endogenous antioxidant enzymes to their normal levels. Secondly, by maintaining the HSCs in their quiescent state. Thirdly, through increasing the TIMP1 release to counter balance the MMP2 and complete remodeling of the hepatocyte cellular system that preserves or sustains the normal liver function, shape and appearance.

These findings confirm the previous findings of Wills & Asha (Wills & Asha, 2007), who suggested the hepatoprotective role of *Lygodium flexuosum* plant extract is to reduce the mRNA levels of growth factors, pro inflammatory cytokines and other signaling molecules, which are involved in hepatic fibrosis including TGF-β1, procollagen-I, and TIMP-1. Also Chen *et al.* (Chen *et al.*, 2012) demonstrated the hepatoprotective effects of silymarin against TAA-induced liver damage to be due to down-regulation of hepatic MMP-2, TIMP-1,TGF-β1, COL-α1 and other genes in the mouse model of chronic liver fibrosis. While the antifibrotic and hepatoprotective properties of silybin–phosphatidylcholine- Vitamin E complex in the rat model of hepatic fibrosis induced by bile duct ligation and dimethylnitrosamine administration was postulated to attribute reduced mRNA levels of procollagen type I, TGFβ1, TIMP-1 and MMP2 genes expression. Furthermore, the administration of the complex has been reported to reduce hepatic stellate cell activation and proliferation with collagen deposition. (A.Di Sario, 2005).

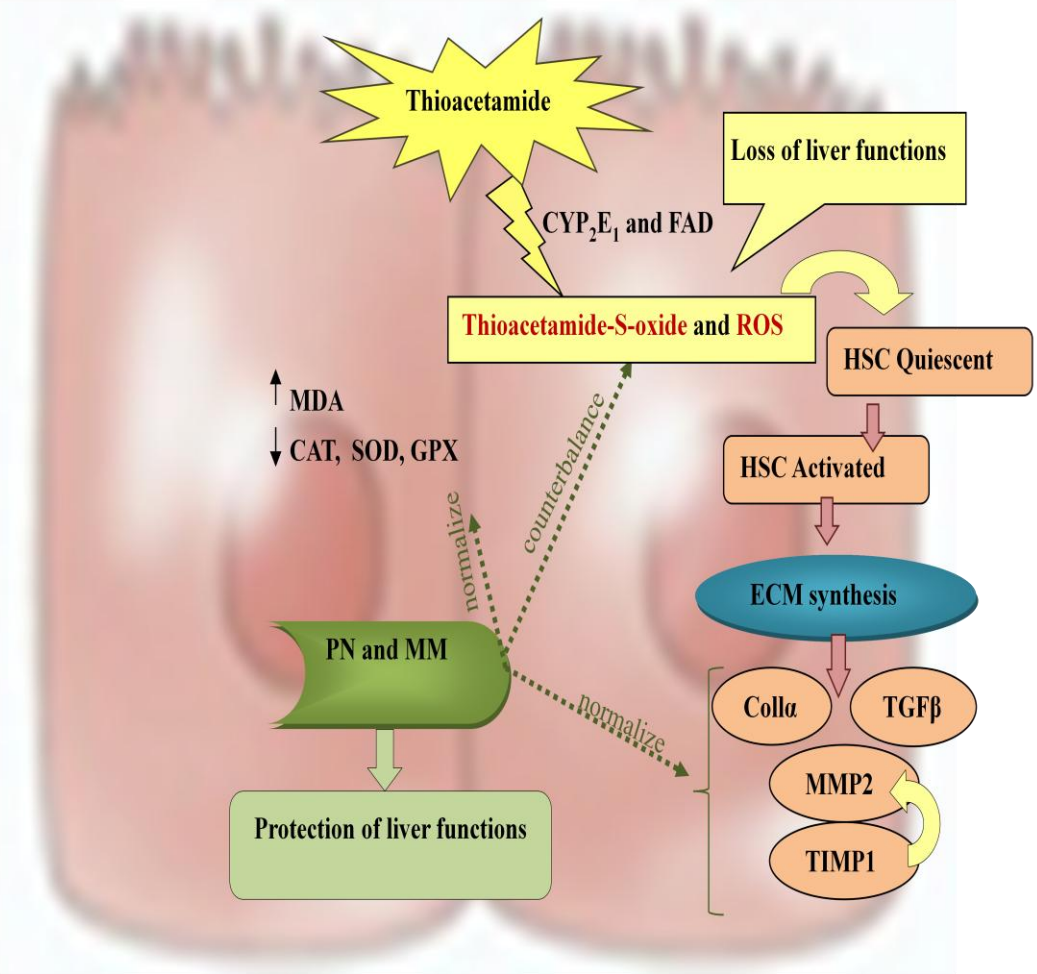


Figure 5.1: Putative mechanism of *Phyllanthus niruri* and *Melastoma malabathricum*'s hepatoprotective effects as a respond to TAA induced hepatotoxicty.

5.1.5 Active constituents of *P. niruri* and *M. malabathricum*

The identified chemical constituents included in *P. niruri* (4-O-caffeolquinic acid and quercetin 3-O-rhamnoside) and *M. malabathricum* (kaempferol, quercetin and naringenin) can further interpret the above-mentioned medicinal activities.

4-O-caffeolquinic acid classified as tannin has been isolated previously from *P. niruri* (Tang *et al.*, 2010) and proven to posses antioxidant, immunomodulatory and hepatoprotective effects in several *in vivo* and *in vitro* assays (Chun *et al.*, 2003) (Silva *et al.*, 2004; Adzet *et al.*, 1987; Kapil *et al.*, 1995). Additionally, all of quercetin 3-O-rhamnoside, kaempferol, quercetin and naringenin belong to flavonoid groups of compounds that are considered as having a wide range of pharmacological benefits including: antimicrobial, antiviral, antioxidant, gastroprotective, hepatoprotective, anti-infalmmatory and chemopreventive effects (Kähkönen *et al.*, 1999; Zayachkivska *et al.*, 2005; Ozcelik *et al.*, 2006; Oh *et al.*, 2004). Furthermore, a potentially valuable working mechanism of flavonoids during injuries and tissue damage has been suggested to occur through interfere with ≥3 different free radical producing systems, whilst also increasing the functions of the endogenous antioxidants CAT, SOD and GPX (Nijveldt *et al.*, 2001). The findings of the present study seem to confirm the findings of Joffry *et al.* (Joffry *et al.*, 2012) who reviewed the chemical constituents of *Melastoma malabathricum* and demonstrated the presence of kaempferol, quercetin and naringenin in this plant. Otherwise, quercetin 3-O-rhamnoside was confirmed to be present in *Phyllanthus* species and other species of Euphorbiaceae family (Zhang *et al.*, 2002; Calixto *et al.*, 1998; Kuti & Konuru, 2004).

Finally, all isolated chemical compounds from *P. nrirui* and *Melastoma malabathircum* (tannins and flavonoids) are classified as “polyphenols”, which are one of the most frequent

groups of plant metabolites and has an important role in human and animal diets (Bravo, 1998). Recent studies have shown that plant-derived polyphenols are promising nutraceuticals for the control of various disorders such as cardiovascular, neurological and neoplastic diseases (Ullah & Khan, 2008). In addition, the use of plant derived polyphenolic compounds were explored for use as hepatoprotective agents against different types of liver damage inducers like CCL_4 (Shimoda *et al.*, 2008), paracetamol (Chen *et al.*, 2009) and thioacetamide (Madani *et al.*, 2008), which explains the high interest of many researchers interested in studying the biological activity and bioavailabilities of the polyphenolic compounds.

5.2 Conclusion

1. *Phyllanthus niruri* and *Melastoma malabathricum* have significant amounts of phenolic and flavonoid contents and exhibited potent antioxidant activity *in vitro* by virtue of DPPH, ABTS radical scavenging activities and ferric ion reducing power.
2. *P. niruri* and *M. malabathricum* possessed significant *in vitro* immunomodulatory roles on human peripheral mononuclear cells (PBMC).
3. *P. niruri* and *M. malabathricum* ethanol extracts were toxicologically safe *in vivo* by oral administration.
4. 200 mg/kg of *P. niruri* and *M. malabathricum* extracts were significantly effective *in vivo* to prevent liver cirrhosis induced by thioacetamide based on biochemical parameters, gross morphology and histopathology and accordingly they might have promising hepatoprotective activities. The mechanism underlied the hepatoprotective effects of *Phyllanthus niruri* and *Melastoma malabathricum* were suggested to be through maintaining the endogenous antioxidant system of the liver and monitoring the expression of TGFβ, Collα1, MMP2 and TIMP1genes.
5. The antioxidant, immunmodulatory and hepatoprotective activities were probably due to the identified chemical compounds (4-O-caffeolquinic acid, quercetin 3-O-rhamnoside) and (kaempferol, quercetin and naringenin) of *Phyllanthus niruri* and *Melastoma malabathricum* respectively.
6. From this study promising potential has been identified that can be harnessed towards the development of new therapies, which must focus on gene expression for the treatment of liver cirrhosis.

5.3 Future Work

1. Purification, identification and structure elucidation using 1D- and 2D-NMR spectroscopic processes should be done for the unidentified chemical components.
2. Future studies should focus on how to effectively transition herbal remedies from the *in vivo* animal models, to the *in vitro* cell line models which can establish the hepatoprotective efficiency of the plants.
3. The fractionations of the plant extracts might be repeated several times so as to isolate sufficient quantities of the active fractions in order to test their bioactivities in the experimental animals.

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APPENDICES

Appendix A: Analytical techniques and preparations

A1. DPPH free radical scavenging assay

Reagents

2, 2-diphenyl-1-picrylehydrazyl ($C_{18}H_{12}N_5O_0$) solution

Ascorbic acid (Vit. C)

Preparation of reagent

0.001761 g/ml Vit. C was prepared as a standard.

The DPPH solution was prepared by dissolving 0.001972 g of DPPH in 50 ml of absolute ethanol then stirred until the DPPH was completely dissolved.

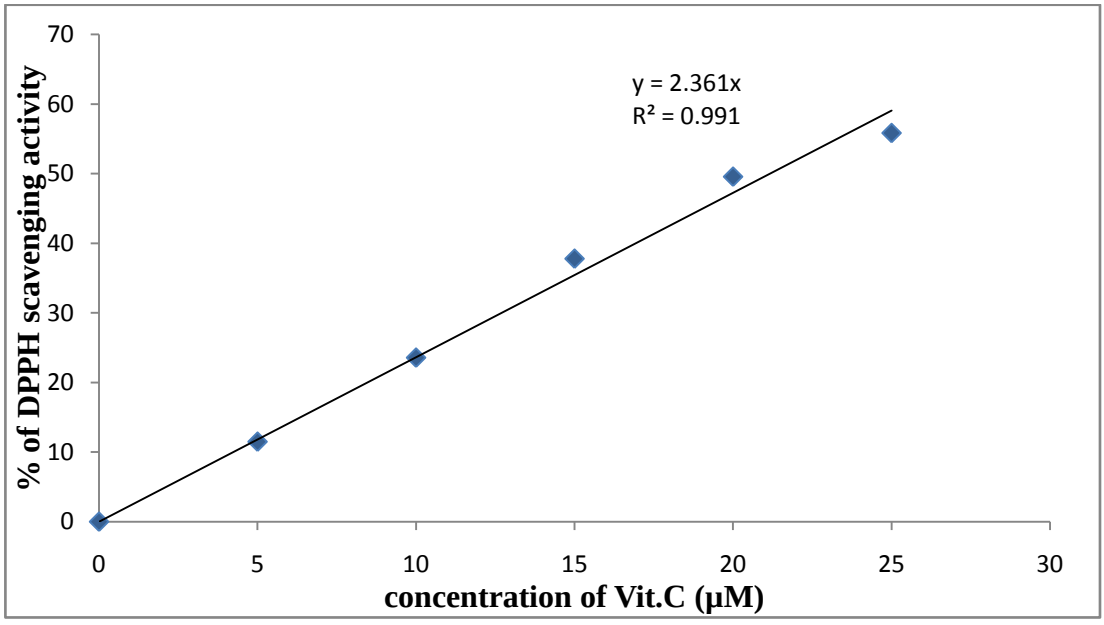


Figure A.1: DPPH Standard curve

A2. ABTS free radical scavenging assay

Reagents

2.5 mM Trolox (Co-hydroxy-2,5,7,8-tetramethyl chroman-2- carboxylic acid) (Standard)

7 mM ABTS ($C_{18}H_{24}N_6O_6S_4$)

2.45 mM potassium persulfate ($K_2S_2O_8$)

Preparation of reagent

1- The trolox standard was prepared by dissolving 0.00625g of trolox in 10 ml of absolute methanol. It was then stirred until completely dissolved.

2- To prepare the ABTS solution, 0.0384 g was dissolved in 10 ml dH_2O and 0.033 g of potassium persulfate dissolved in 5 ml dH_2O then both were mixed and incubated for (12-16 hrs) in a dark place at room temperature. Then the prepared ABTS stock was diluted with methanol to an absorbance of (0.700 ± 0.020) at 734 nm.

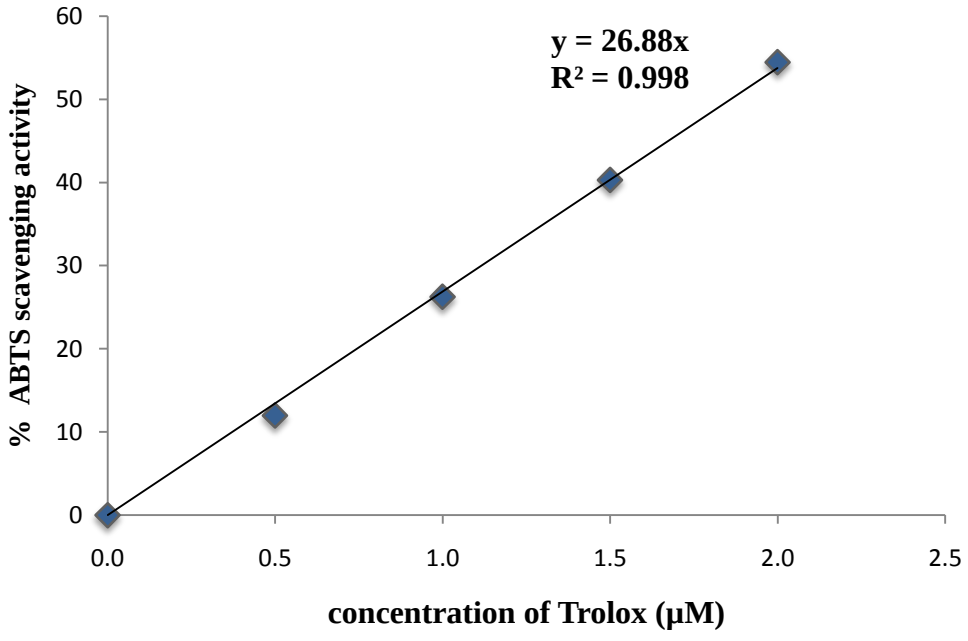


Figure A.2: ABTS Standard curve

A3. Total Phenolic Content (TPC)

Reagents

Commercial Folin-Ciocalteu solution

Sodium carbonate

Gallic acid

Preparation of reagent

1- A ratio of (1:10) of Folin-Ciocalteu solution was prepared by diluting 10 ml of it with 90 ml dH_2O in a dark place.
2- 5.75 g of sodium carbonate was dissolved in 50 ml of dH_2O to give a final concentration of 0.115 mg/ml.
3- 0.2 mg/ml of gallic acid solution was prepared and used as the standard solution.

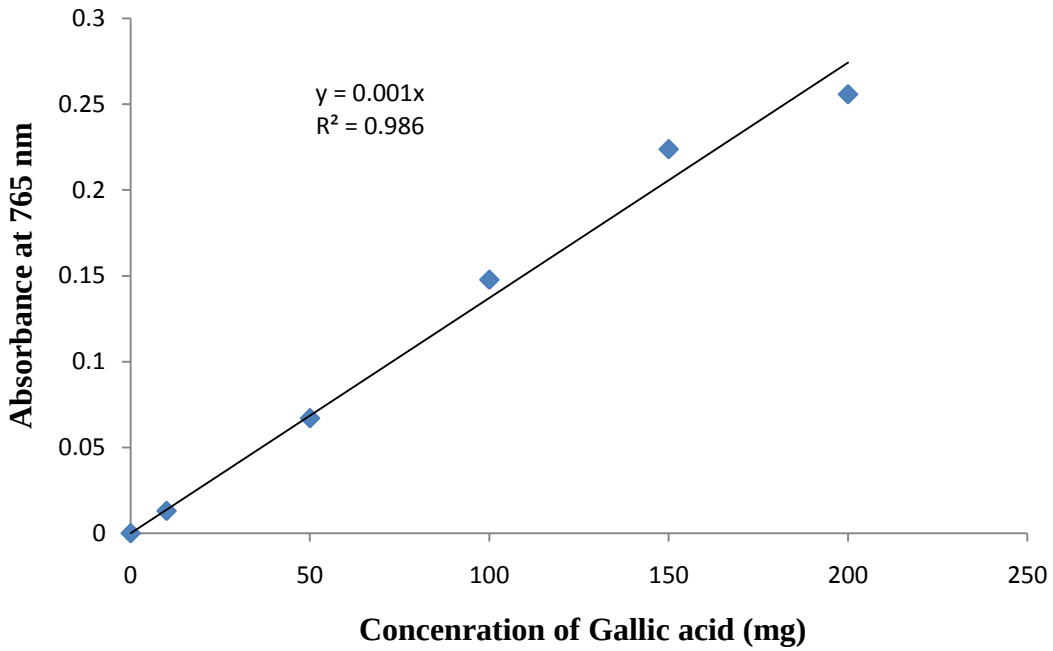


Figure A.3: Gallic acid calibration plot

A4. Total flavonoid content (TFC)

Reagents

Aluminium trichloride (Al Cl_3)

Quercetin (Standard)

Potassium acetate (CH_3COOK)

Preparation of reagents

One gram of Al Cl_3 was dissolved in 10 ml dH_2O.

0.9815 g of potassium acetate was dissolved in 10 ml dH_2O.

And 0.02g of quercetin was dissolved in 4 ml (95%) methanol.

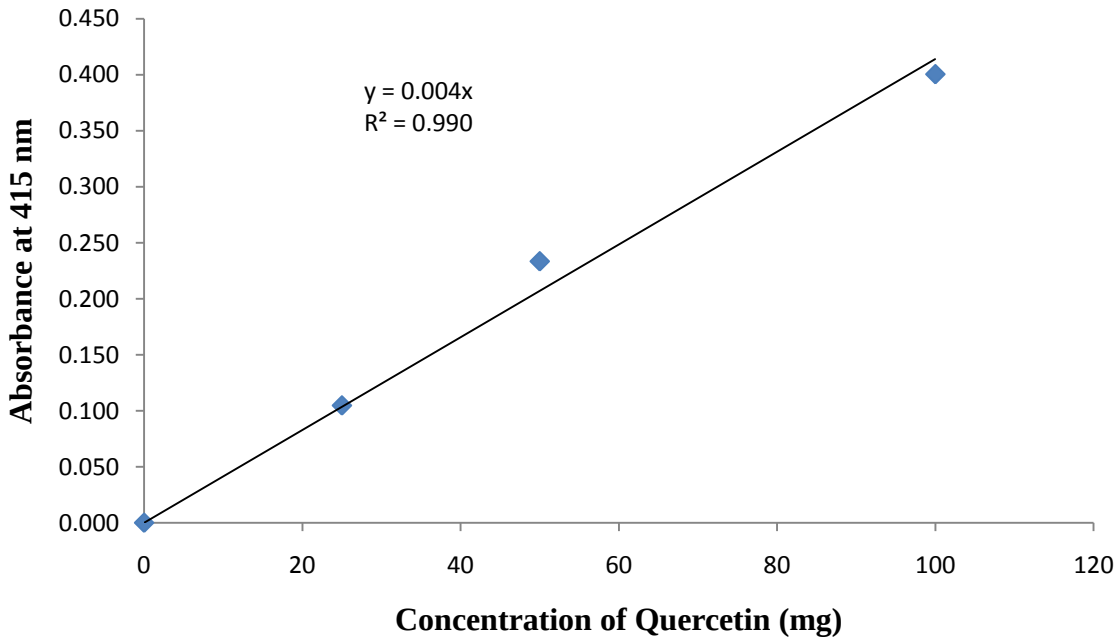


Figure A.4: Quercetin calibration plot

A5. Ferric reducing antioxidant power (FRAP) assay

Reagents

Iron (II) sulfate hepatohydrate ($FeSO_4$. $7H_2O$)

Sodium acetate trihydrate buffer ($C_2H_3O_2N_9$. $3H_2O$)

2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) 98%

$FeCl_3$. $6H_2O$

Preparation of reagents

0.0278 g of $FeSO_4.7H_2O$ was dissolved in 100 ml dH_2O.

0.0775 g of acetate buffer was dissolved in 25 ml dH_2O mixed previously with 0.4 ml glacial acetic acid.

0.00781 g of TPTZ was dissolved in 2.5 ml dH_2O mixed previously with 0.1 ml (1M HCL).

0.0135g $FeCl_3$. $6H_2O$ was dissolved in 2.5 ml dH_2O.

The freshly prepared: acetate buffer, TPTZ and $FeCl_3$. $6H_2O$ solutions were mixed and vortexed to obtain the ready to use FRAP reagent.

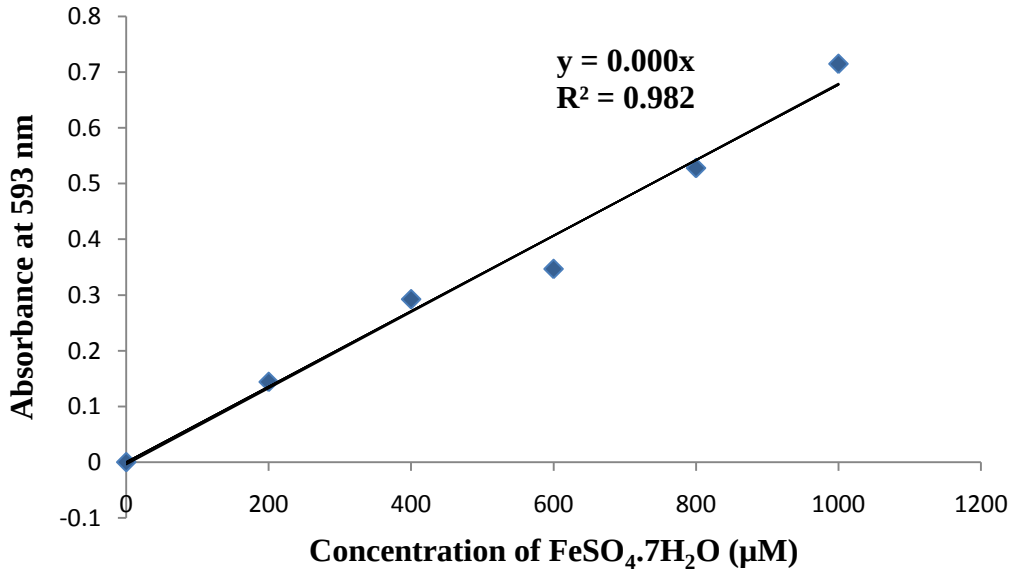


Figure A.5: $FeSO_4.7H_2O$ calibration plot

A6. (MTT) assay

Reagents

3-(4, 5-Dimethyl-2-thiazolyl)-2,3-diphenyl-2H-tetrazolium bromide (MTT) reagent

RPMI media

Preparation of reagents

1- 5 mg / ml MTT reagent prepared in PBS and filtered through sterilized filter 0.22 µm.
2- RPMI media reconstitute with 10% (v/v) Fetal bovine serum (FBS).

A7. Total Antioxidant Capacity (TAC) assay Catalog no.709001

Precautions

Please read these instructions carefully before beginning this assay.

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

It is recommended to take appropriate precautions when using the kit reagents (*i.e.*, lab coat, gloves, eye goggles, etc.) as some of them can be harmful.

Hydrogen Peroxide is corrosive and is harmful if swallowed. Contact with skin may cause burns. In case of contact with skin or eyes, rinse immediately with plenty of water for 15 minutes. Keep away from combustible materials.

If You Have Problems

Technical Service Contact Information

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

Fax: 734-971-3641

E-Mail: 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 4°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 absorbance at 750 nm or 405 nm. *NOTE: The absorbance may be read at 405 nm; however, there is less interference at 750 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

Reactive oxygen species (ROS) are produced as a consequence of normal aerobic metabolism. Unstable free radical species attack cellular components causing damage to lipids, proteins, and DNA which can initiate a chain of events resulting in the onset of a variety of diseases.[1] Living organisms have developed complex antioxidant systems to counteract ROS and to reduce their damage. These antioxidant systems include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase; macromolecules such as albumin, ceruloplasmin, and ferritin; and an array of small molecules, including ascorbic acid, α-tocopherol, β-carotene, reduced glutathione, uric acid, and bilirubin. The sum of endogenous and food-derived antioxidants represents the total antioxidant activity of the system. The cooperation among different antioxidants provides greater protection against attack by reactive oxygen or nitrogen species, than any single compound alone. Thus, the overall antioxidant capacity may provide more relevant biological information compared to that obtained by the measurement of individual components, as it considers the cumulative effect of all antioxidants present in plasma and body fluids.

About This Assay

Cayman's Antioxidant Assay can be used to measure the total antioxidant capacity of plasma, serum, urine, saliva, or cell lysates. Aqueous- and lipid-soluble antioxidants are not separated in this protocol, thus the combined antioxidant activities of all its constituents including vitamins, proteins, lipids, glutathione, uric acid, etc. are assessed (see Figure 1 on page 6). The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of $ABTS^{\bullet}$ (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^{\bullet+}$ by metmyoglobin. The amount of $ABTS^{\bullet+}$ produced can be monitored by reading the absorbance at 750 nm or 405 nm. Under the reaction conditions used, the antioxidants in the sample cause suppression of the absorbance at 750 nm or 405 nm to a degree which is proportional to their concentration.[2-5] The capacity of the antioxidants in the sample to prevent $ABTS^{\bullet}$ oxidation is compared with that of Trolox, a water-soluble tocopherol analogue, and is quantified as millimolar Trolox equivalents.

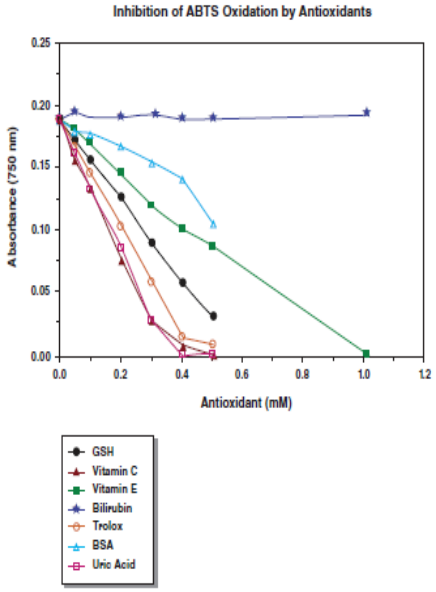


Figure 1. Inhibition of ABTS Oxidation by Antioxidants

PRE-ASSAY PREPARATION

Reagent Preparation

Some of the kit components are in lyophilized (under vacuum) or concentrated form and need to be reconstituted or diluted prior to use. Follow the directions carefully to ensure proper volumes of water or Assay Buffer are used. For lyophilized components, stoppers must be removed slowly to allow air to enter the vials gradually, thereby preventing loss of material.

1. **Antioxidant Assay Buffer (10X) - (Catalog No. 10004872)**

   Dilute 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water. This diluted Assay Buffer (5 mM potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose) should be used to reconstitute the metmyoglobin. *NOTE: The use of 'Assay Buffer' in the remainder of the instructions refers to the diluted buffer.* When stored at 4°C, this Assay Buffer is stable for at least six months.

2. **Antioxidant Assay Chromogen - (Catalog No. 10004873)**

   These vials contain a lyophilized powder of ABTS®. Reconstitute the Chromogen by adding 6 ml of HPLC-grade water to the vial and vortex well. One reconstituted vial will be sufficient for 40 wells. Reconstitute only the number of vials needed to assay the standards and samples. The reconstituted reagent is stable for 24 hours at 4°C.

3. **Antioxidant Assay Metmyoglobin - (Catalog No. 10004875)**

   These vials contain a lyophilized powder of metmyoglobin. Reconstitute the metmyoglobin by adding 600 µl of Assay Buffer to the vial and vortex well. One reconstituted vial will be sufficient for 60 wells. The reconstituted reagent is stable for at least one month if stored at -20°C.

4. **Antioxidant Assay Trolox - (Catalog No. 10004876)**

   These vials contain a lyophilized powder of Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Reconstitute the Trolox vial by adding 1 ml of HPLC-grade water and vortexing well. The reconstituted reagent is used to prepare the Trolox standard curve. The reconstituted reagent is stable for 24 hours at 4°C.

5. **Antioxidant Assay Hydrogen Peroxide - (Catalog No. 10004877)**

   This vial contains an 8.82 M solution of hydrogen peroxide. Dilute 10 µl of hydrogen peroxide with 990 µl of HPLC-grade water. Further dilute by removing 20 µl and diluting with 3.98 ml of HPLC-grade water to yield a 441 µM working solution. The hydrogen peroxide working solution is stable for four hours at room temperature.

Sample Preparation

Plasma

Typically human plasma has an antioxidant capacity of 0.5-2 mM.[2,5-7]

1. Collect blood using an anticoagulant such as heparin or citrate. Do not use 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. Plasma should be diluted 1:20 or 1:30 with Assay Buffer before assaying.

Serum

Typically human serum has an antioxidant capacity of 0.5-2 mM.[8,9]

1. Collect blood without using an anticoagulant such as heparin or citrate. 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 1:20 or 1:30 with Assay Buffer before assaying.

Urine

Typically human urine has an antioxidant capacity of 0.2-3 mM.[9]

1. Collect urine in a clean beaker or flask and store on ice. If not assaying on the same day, freeze the sample at -80°C.
2. Urine should be diluted 1:10 or 1:20 with Assay Buffer before assaying.

Saliva

Typically human saliva has an antioxidant capacity of 0.3-1 mM.[9]

1. Collect saliva in a clean beaker or flask and store on ice. If not assaying on the same day, freeze the sample at -80°C.
2. Saliva should be diluted 1:2 with Assay Buffer before assaying.

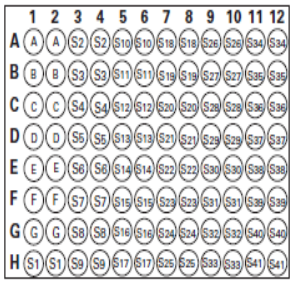
Cell Lysate

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. The cell pellet can be homogenized or sonicated on ice in 1-2 ml of cold buffer (*i.e.*, 5 mM potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose).
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.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of Trolox 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 19).



A-G = Standards
S1-S41 = 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

- The final volume is 210 µl in all of 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 antioxidant level 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 Trolox standards be assayed at least in duplicate (triplicate recommended).
- Monitor the absorbance at 750 nm or 405 nm using a plate reader.

Performing the Assay

1. Preparation of the Trolox standards: Take seven clean glass test tubes and mark them A-G. Add the amount of reconstituted Trolox and Assay Buffer to each tube as described in Table 1.

Tube	Reconstituted Trolox (µl)	Assay Buffer (µl)	Final Concentration (mM Trolox)
A	0	1,000	0
B	30	970	0.045
C	60	940	0.090
D	90	910	0.135
E	120	880	0.18
F	150	850	0.225
G	220	780	0.330

Table 1. Trolox standard preparation

2. **Trolox Standard Wells** - add 10 µl of Trolox standard (tubes A-G), 10 µl of Metmyoglobin, and 150 µl of Chromogen per well in the designated wells on the plate (see sample plate format, Figure 2, page 10).
3. **Sample Wells** - add 10 µl of sample, 10 µl of Metmyoglobin, and 150 µl of Chromogen to two wells. To obtain reproducible results, antioxidant levels of the sample should fall within the standard curve. When necessary, samples can be diluted with Assay Buffer to bring antioxidants to this level.
4. Initiate the reactions by adding 40 µl of hydrogen peroxide working solution to all the wells being used. Add the hydrogen peroxide as quickly as possible (within one minute is recommended).
5. Cover the plate with the plate cover and incubate on a shaker for five minutes at room temperature. Remove the cover and read the absorbance at 750 nm or 405 nm using a plate reader.

ANALYSIS

Calculations

1. Calculate the average absorbance of each standard and sample.
2. Plot the average absorbance of standards as a function of the final Trolox concentration (mM) from Table 1. A typical standard curve is shown below.

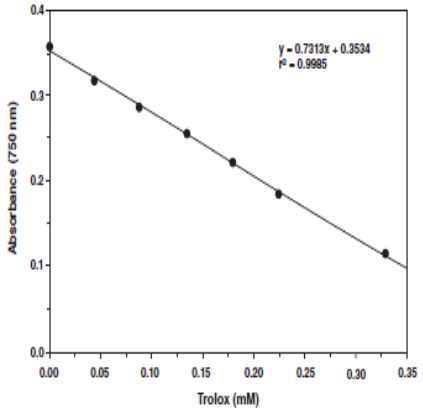


Figure 3. Trolox standard curve

3. Calculate the antioxidant concentration of the samples using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the equation.

$$\text{Antioxidant (mM)} = \frac{\text{Sample average absorbance - (y-intercept)}}{\text{Slope}} \times \text{Dilution}$$

Performance Characteristics

Precision:

Inter-assay coefficient of variation = 3% (n = 20). Intra-assay coefficient of variation = 3.4% (n = 84).

Assay Range:

Samples containing antioxidants between 0.044-0.330 mM can be assayed without further dilution or concentration.

Interferences

The following reagents were tested for interference in the assay.

	Reagent	Will Interfere (Yes or No)
Buffers:	Tris	Yes
	Borate	Yes
	HEPES (≤10 mM)	No
	Phosphate(≤10 mM)	No
Detergents:	Tween 20 (≤1%)	No
	Triton X-100 (≤1%)	No
Protease Inhibitors/ Chelators:	Antipain (≤10 µg/ml)	No
	PMSF (≤200 µM)	No
	Leupeptin (≤10 µg/ml)	No
	Pepstatin (≤0.1 mg/ml)	No
	Chymostatin (≤5 µg/ml)	No
	EGTA (1 mM)	Yes
	EDTA (1 mM)	Yes
Others:	Glycerol (≤5%)	No
	Bovine serum albumin (≤0.1%)	No

Ethanol or dimethylsulfoxide (10 µl) can be used in the assay. However, the solvent (10 µl) needs to be added to the Trolox standard wells to compensate for the decrease in absorbance exerted by the solvent. Methanol interferes with the chromogen and can not be added to the assay.

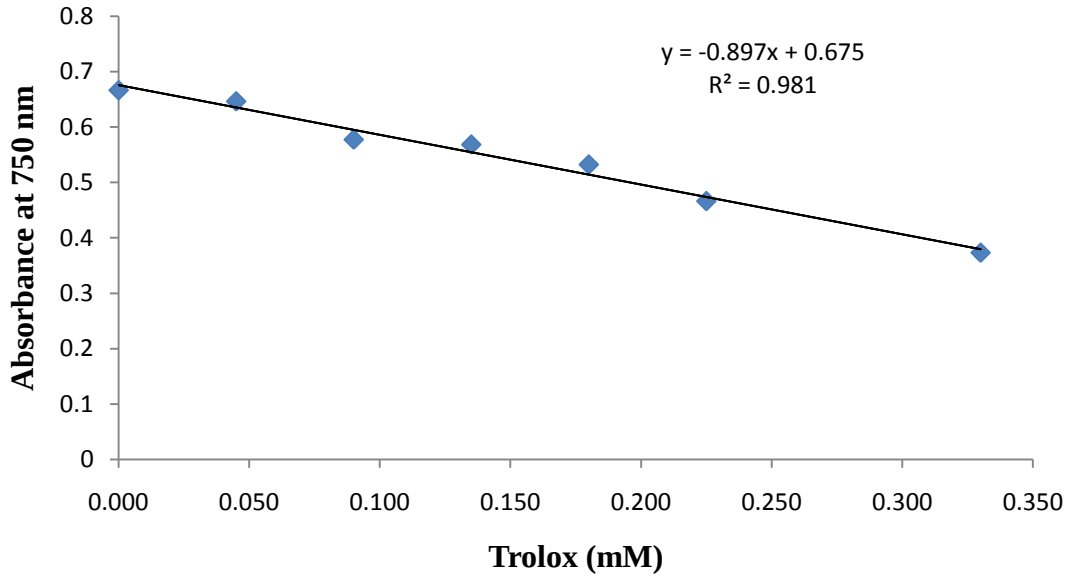


Figure A.6: Trolox standard curve

A8.Catalase (CAT) assay Catalog 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 catalase are found in liver, kidney, and erythrocytes, where it is believed to account for the majority of hydrogen peroxide 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 Catalog No. 700416 or you can supply your own.

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

   Each vial contains 5 ml of Assay Buffer. Dilute 2 ml of 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. **Sample Buffer (10X) – (Catalog No. 707012)**

   Each vial contains 10 ml of Sample Buffer. Dilute 5 ml of 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, CAT (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. **Formaldehyde Standard - (Catalog No. 707014)**

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

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

   Each vial contains a lyophilized powder of bovine liver CAT and is used as a positive control. Reconstitute the CAT by adding 2 ml of Sample Buffer (dilute) to the vial and vortex well. Take 100 µl of the reconstituted enzyme and dilute with 1.9 ml of Sample Buffer (dilute). 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 CAT is stable for one month at -20°C.

5. **Potassium Hydroxide – (Catalog 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 potassium hydroxide pellets are dissolved in water. The KOH solution is stable for at least three months if stored at 4°C.*

6. **Hydrogen Peroxide – (Catalog No. 707011)**

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

7. **Purpald (Chromagen) – (Catalog 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. **Potassium Periodate – (Catalog 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 Assay Buffer (dilute) 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 formaldehyde standard (vial #3) with 9.99 ml of Sample Buffer (dilute) 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 Sample Buffer (dilute) 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 Assay Buffer (dilute), 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 Assay Buffer (dilute), 30 µl of methanol, and 20 µl of diluted CAT (control) to two wells.
3. **Sample Wells** - Add 100 µl of Assay Buffer (dilute), 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 0.25-4 nmol/min/ml. When necessary, samples should be diluted with Sample Buffer (dilute) 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 hydrogen peroxide (dilute) to all the wells being used. Make sure to note the precise time the reaction is initiated and add the 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 potassium hydroxide to each well to terminate the reaction and then add 30 µl of Purpald (chromogen) 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 potassium periodate 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.

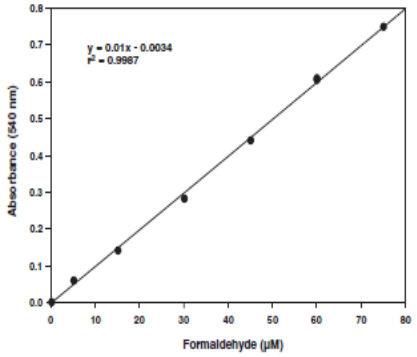


Figure 2. Formaldehyde standard curve

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 Catalase 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.

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

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 2-34 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%.

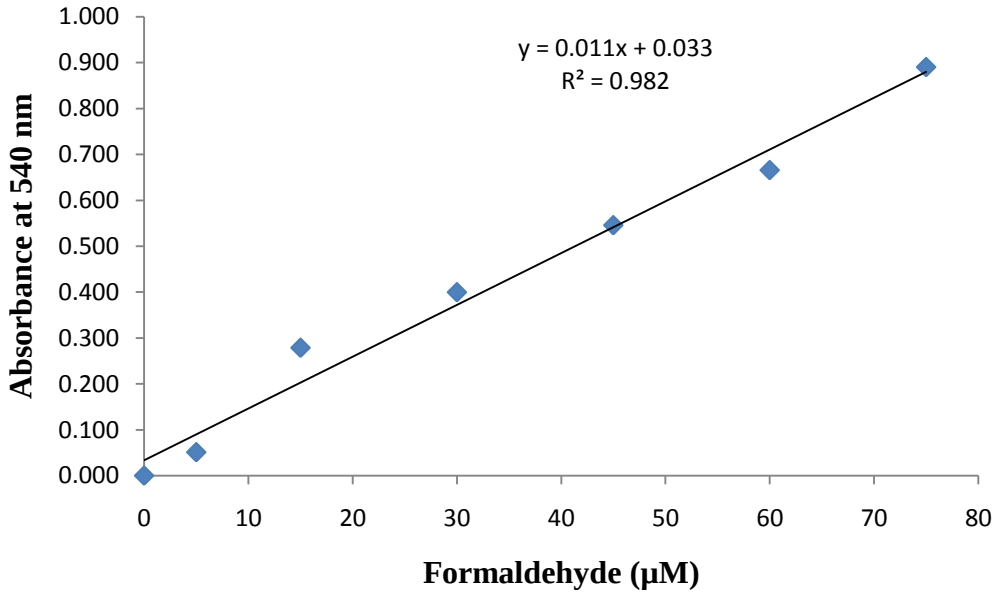


Figure A.7: Formaldehyde standard curve

A9. Superoxide Dismutase (SOD) assay Catalog no.70600

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
E-Mail: 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).

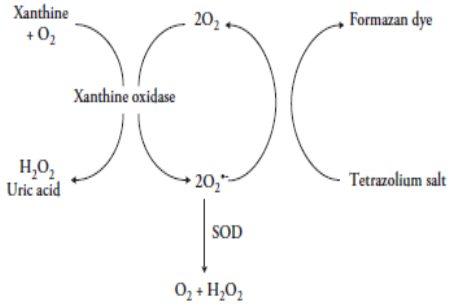


Figure 1. Scheme of the Superoxide Dismutase Assay

PRE-ASSAY PREPARATION

Reagent Preparation

1. Assay Buffer (10X) - (Catalog 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) - (Catalog 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 - (Catalog 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.

4. SOD Standard - (Catalog No. 706005)

The vials contain 100 µl of bovine erythrocyte SOD (Cu/Zn). The enzyme is ready to use as supplied. Store the thawed enzyme on ice.

5. Xanthine Oxidase - (Catalog 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. Do not refreeze the thawed enzyme. This is enough Xanthine Oxidase for 96 wells. Prepare additional Xanthine Oxidase as needed.

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 ssettings:
- 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 19).

	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

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 230 µl in all the wells.
- It is not necessary to use all the wells on the plate at one time.
- The assay temperature is 25°C.
- All reagents except samples and xanthine oxidase must be equilibrated to room temperature before beginning the assay.
- It is recommended that the samples and SOD standards be assayed at least in duplicate.
- Monitor the absorbance at 440-460 nm using a plate reader.

Standard Preparation

Dilute 20 µl of the SOD Standard (Catalog No. 706005) with 1.98 ml of Sample Buffer (dilute) to obtain the SOD stock solution. Take seven clean glass test tubes and mark them A-G. Add the amount of SOD stock and Sample Buffer (dilute) to each tube as described in Table 1 on page 12.

Tube	SOD Stock (µl)	Sample Buffer (µl)	Final SOD Activity (U/ml)
A	0	1,000	0
B	20	980	0.025
C	40	960	0.05
D	80	920	0.1
E	120	880	0.15
F	160	840	0.2
G	200	800	0.25

Table 1. Superoxide Dismutase standards

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 10).
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 amounts 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 14) 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.

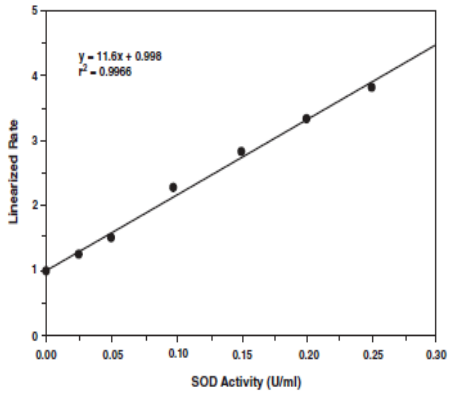


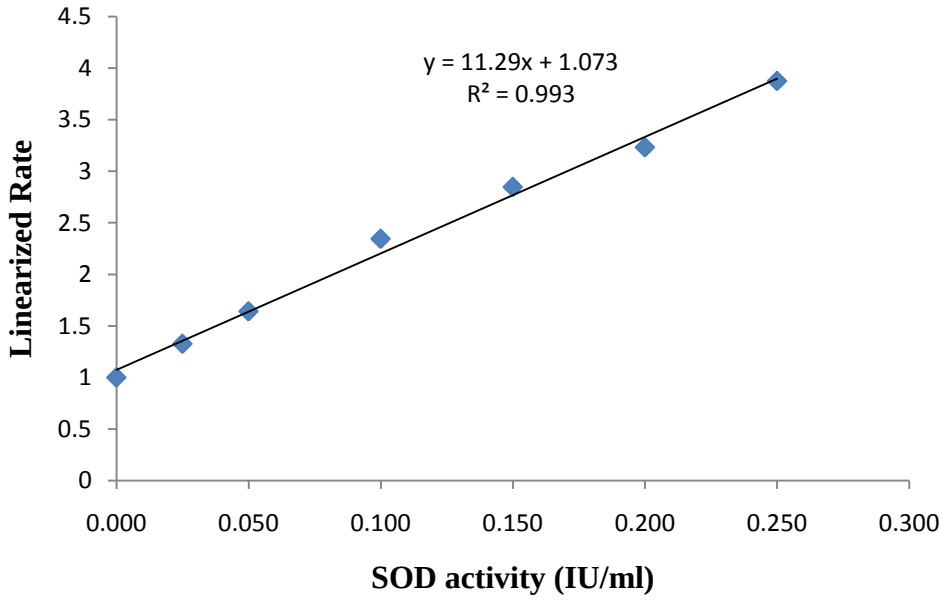
Figure 3. Superoxide Dismutase standard curve

RESOURCES

Interferences

The following reagents were tested for interference in the assay.

	Reagent	Will Interfere (Yes or No)
Buffers:	Tris	No
	Borate	Yes
	HEPES	No
	Phosphate	No
Detergents:	SDS (0.1%)	Yes
	Triton X-100 (≤0.1%)	No
	Tween 20 (0.1%)	Yes
	CHAPS (0.1%)	Yes
Proteases/Inhibitors/ Chelators:	Antipain (0.1 mg/ml)	Yes
	PMSF (1 mM)	Yes
	Leupeptin (1 mg/ml)	Yes
	Trypsin (0.1 mg/ml)	Yes
	Chymostatin (0.1 mg/ml)	Yes
	EGTA (≤1 mM)	No
	EDTA (≤1 mM)	No
Solvents:	Ethanol (10 µl)	No
	Methanol (10 µl)	Yes
	Dimethyl Sulfoxide (10 µl)	Yes
Others:	Glutathione (≤1.5 mM)	No
	Glycerol (≤1%)	No
	BSA (≤1%)	No
	Dithiothreitol (3 mM)	Yes



sod

Figure A.8: Superoxide Dismutase standard curve

A10. TBARS assay Catalog 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.[2] 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).

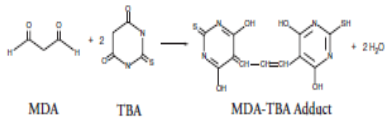


Figure 1.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Thiobarbituric Acid - (Catalog 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 - (Catalog 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) - (Catalog No. 10009201)**

   The vial contains a solution of sodium hydroxide (NaOH). Dilute 20 ml of TBA Sodium Hydroxide 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 - (Catalog 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 - (Catalog 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 (Catalog 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 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 (Catalog 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 (Catalog 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.

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

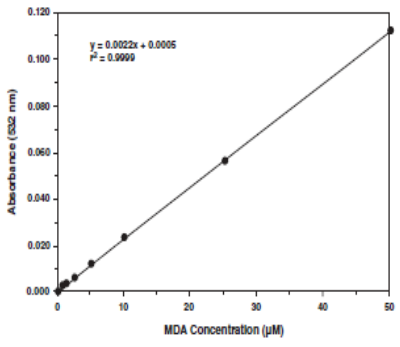


Figure 3. MDA colorimetric standard curve

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.

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

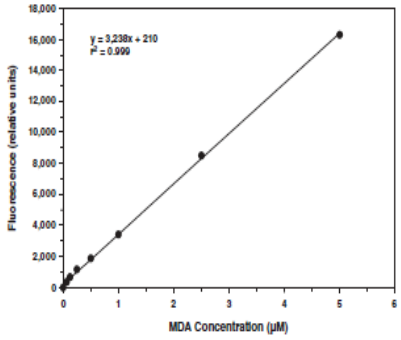


Figure 4. MDA fluorometric standard curve

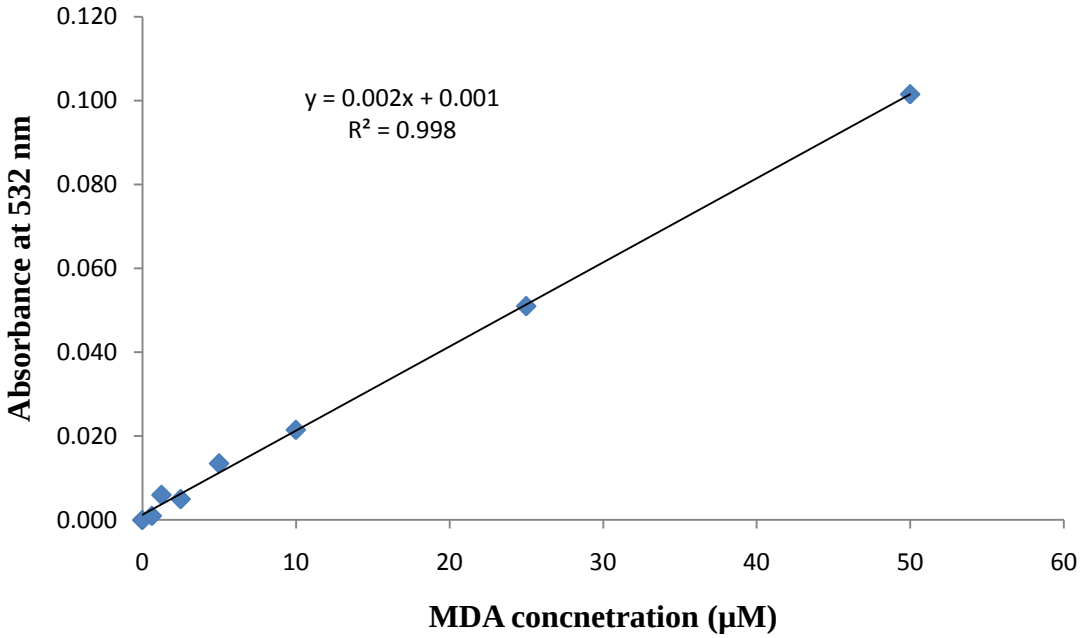


Figure A.9: MDA standard curve

A11. Glutathione peroxidase (GPX) assay Catalog no.703102

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

E-Mail: 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 absorbance at 340 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

Glutathione peroxidase (GPx) catalyzes the reduction of hydroperoxides, including hydrogen peroxide, by reduced glutathione and functions to protect the cell from oxidative damage. With the exception of phospholipid-hydroperoxide GPx, a monomer, all of the GPx enzymes are tetramers of four identical subunits.[1,2] Each subunit contains a selenocysteine in the active site which participates directly in the two-electron reduction of the peroxide substrate.[1,2] The enzyme uses glutathione as the ultimate electron donor to regenerate the reduced form of the selenocysteine.[1,2]

About This Assay

Cayman's GPx Assay measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH:

$$R\text{-}O\text{-}O\text{-}H + 2GSH \xrightarrow{GPx} R\text{-}O\text{-}H + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample.[3] The Cayman GPx Assay Kit can be used to measure all of the glutathione-dependent peroxidases in plasma, erythrocyte lysates, tissue homogenates, and cell lysates.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **GPx Assay Buffer (10X) - (Catalog No. 703110)**

   Each vial contains 3 ml of Assay Buffer. Dilute the contents of the vial with 27 ml of HPLC-grade water. This final Assay Buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA) should be used in the assay. When stored at 4°C, this diluted Assay Buffer is stable for at least six months. Prepare the additional vial as needed.

2. **GPx Sample Buffer (10X) - (Catalog No. 703112)**

   Dilute 2 ml of Sample Buffer concentrate with 18 ml of HPLC-grade water. This final Sample Buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA and 1 mg/ml BSA) should be used to dilute the GPx control and the GPx samples prior to assaying. When stored at 4°C, this diluted Sample Buffer is stable for at least one month.

3. **Glutathione Peroxidase (Control) - (Catalog No. 703114)**

   This vial contains 50 µl of bovine erythrocyte GPx. To avoid repeated freezing and thawing, the GPx should be aliquoted into several small vials and stored at -20°C. Prior to use, transfer 10 µl of the supplied enzyme to another vial and dilute with 490 µl of diluted Sample Buffer and keep on ice. The diluted enzyme is stable for four hours on ice. A 20 µl aliquot of this diluted enzyme per well causes a decrease of approximately 0.051 absorbance unit/minute under the standard assay conditions described in Performing the Assay (see page 11).

4. **GPx Co-Substrate Mixture - (Catalog No. 703116)**

   The 96-well kit contains three 40 well size of Co-Substrate Mixture. These vials contain a lyophilized powder of NADPH, glutathione, and glutathione reductase. Each reconstituted vial will be enough reagent for 40 wells. Reconstitute the number of vials that you will need by adding 2 ml of HPLC-grade water to each vial and vortex well. The 480-well kit contains five 96 well size of Co-Substrate Mixture. Reconstitute the number of vials that you will need by adding 6 ml of HPLC-grade water to each vial and vortex well. Each reconstituted vial will be enough reagent for 96 wells. The reconstituted reagent should be kept at 25°C while assaying and then stored at 4°C. If stored at 4°C, the reconstituted reagent is stable for two days. ***NOTE: Do not freeze the reconstituted reagent.***

5. **GPx Cumene Hydroperoxide - (Catalog No. 703118)**

   The 96-well kit contains one 2.5 ml vial of cumene hydroperoxide. The 480-well kit contains one 12 ml vial of cumene hydroperoxide. The vials should be stored at -20°C when not being used. The reagent is ready to use as supplied.

Sample Preparation

Tissue Homogenate

1. Prior to dissection, perfuse or rinse tissue with a PBS (phosphate buffered saline) solution, pH 7.4, to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold buffer (*i.e.*, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT) 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 cell pellet in cold buffer (*i.e.*, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT).
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. Pipet 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 4 volumes 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.

NOTE: It has been reported that heme peroxidase activity of hemoglobin can lead to falsely elevated GPx activity in erythrocyte lysates. There was no significant effect in the GPx activity when assayed with cumene hydroperoxide as the substrate. Therefore, it is not necessary to treat the sample with Drabkin's Reagent (potassium ferricyanide/potassium cyanide) to convert hemoglobin to cyanmethemoglobin before assaying.

Tissue Homogenization using the Precellys 24 Homogenizer

- Freeze organs immediately upon collection and then store at -80°C. Snap-freezing of tissues in liquid nitrogen is preferred.
- Add 1 ml of homogenization buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA and 1 mM DTT) per 100 milligrams of tissue.
- Homogenize the sample using the Precellys 24 according to appropriate settings:

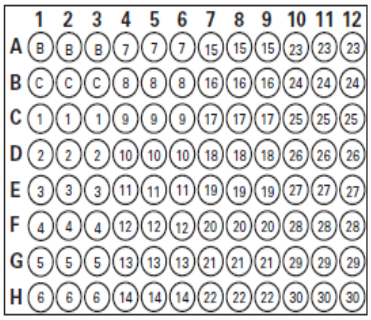
Organ	Speed (rpm)	Cycle Length (seconds)	Cycle Break (seconds)	Number of Cycles	Beads
Heart (aorta)	5,000	30	30	3	CK28 Large Cetamic

- 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. However, it is necessary to have three wells designated as non-enzymatic or background wells. The absorbance rate of these wells must be subtracted from the absorbance rate measured in the GPx sample and control wells. We suggest that there be at least three wells designated as positive controls and that you record the contents of each well on the template sheet provided on page 19.



B - Background Wells
C - Positive Control Wells
1-30 - Sample Wells

Figure 2. Sample plate format

ANALYSIS

Calculations

1. Determine the change in absorbance (ΔA_{340}) per minute by:
   a. Plotting the absorbance values as a function of time to obtain the slope (rate) of the linear portion of the curve (a graph is shown on page 13 using bovine erythrocyte GPx) *-or-*
   b. Select two points on the linear portion of the curve and determine the change in absorbance during that time using the following equation:

$$\Delta A_{340}/\text{min.} = \frac{A_{340}\ (\text{Time 2}) - A_{340}\ (\text{Time 1})}{\text{Time 2 (min.)} - \text{Time 1 (min.)}}$$

2. Determine the rate of ΔA_{340}/min. for the background or non-enzymatic wells and subtract this rate from that of the sample wells.
3. Use the following formula to calculate the GPx activity. The reaction rate at 340 nm can be determined using the NADPH extinction coefficient of 0.00373 μM^{-1}*. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to NADP* per minute at 25°C.

$$\text{GPx activity} = \frac{\Delta A_{340}/\text{min.}}{0.00373\ M^{-1}} \times \frac{0.19\ \text{ml}}{0.02\ \text{ml}} \times \text{Sample dilution} = \text{nmol/min/ml}$$

*The actual extinction coefficient for NADPH at 340 nm is 0.00622 $\mu M^{-1}cm^{-1}$. This value has been adjusted for the pathlength of the solution in the well (0.6 cm).

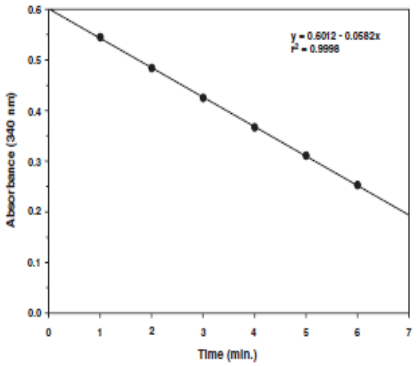


Figure 1. Activity of bovine erythrocyte GPx

Performance Characteristics

Precision:

When a series of seventy-seven GPx measurements were performed on the same day, the intra-assay coefficient of variation was 5.7%. When a series of seventy-seven GPx measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 7.2%.

Assay Range:

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 GPx activity between 50-344 nmol/min/ml can be assayed without further dilution or concentration. This GPx activity is equivalent to an absorbance decrease of 0.02 to 0.135 per minute.

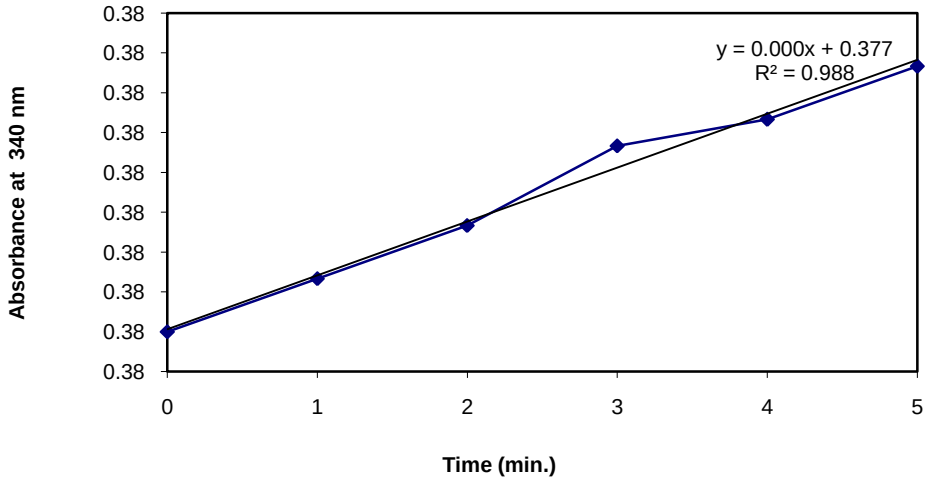


Figure A.10: Activity of GPX in TAA treated rat liver.

A12. Histopathology Techniques

1) Routine slide preparation

a- Tissue trimming and fixation

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

b- Processing

The technique of getting fixed tissue into paraffin is called tissue processing. This process have been done by using automatic tissue processor. The main steps in this process were: dehydration, clearing and infiltration in a programmed sequence.

c- 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.

d- 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 less than 50°C temperature.

e- 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 stains. 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.

f- Mounting

Finally, to protect the stained sections from damage, the stained sections were dehydrated in series of alcohol, cleared in xylene and mounted with the mounting media DPX and the use of coverslip in 45º angle then bubbles were removed carefully and left to dry overnight at room temperature.

2) Haematoxylin and Eosin (H&E) stain

Reagents required:

1- Harris haematoxylin working solution
2- Eosin working solution
3- 0.5 % acid alcohol
4- 2 % soidium acetate
5- 80 % alcohol
6- 95 % alcohol
7- Absolute alcohol

Procedure:

1-	Bring section to water	
2-	Stain in Harris haematoxylin	10 min.
3-	Wash in running water until excess blue color goes off	
4-	Differentiation: Dip 2 to 3X in 0.5% acid alcohol and wash in running tab water	
5-	Wash well in running tab water	2-3 min.
6-	Blue section with 2% sodium acetate	2 sec.
7-	Wash again in running tab water	2-3 min.
8-	Rinse in 80% alcohol	
9-	Stain in eosin solution	5 min.
10-	Dehydration: 95 % alcohol I	5 sec
	95 % alcohol II	2 min.
	Absolute alcohol I	2 min.
	Absolute alcohol II	2 min.
11-	Clear in xyline	2 min. x3
12-	Mount with DPX	
13-	Wipe slide to remove excess xyline	
14-	Label slide appropriately.	

Results:

Nuclei blue
Cytoplasm various shades of pink and red.

3) Masson's trichrome stain

Reagents required:

1- Bouin's fixative
2- Celestine blue stain
3- Harris haemtoxylin
4- 0.5 % acid alcohol
5- BSAF stain (Biebrich scarlet acid fuchsin)
6- 5 % aqueous phosphotungstic acid
7- Light green stain (2 % light green in 1 % acetic acid)

Procedure:

1- Bring section to water
2- Stain in Celestine blue 6 min.
3- Brief rinse in water
4- Stain in Harris haematoxylin 6 min
5- Wash in running tap water until excess blue color goes off
6- Differentiate with acid alcohol until background is colorless
7- Wash well in running water
8- Stain with BSAF 5 min.
9- Brief rinse in water
10- Differentiate in phosphotungstic acid 10 min.
11- Brief rinse in water
12- Stain in light green solution 10 min.
13- Drain off excess light green then dehydrate quickly
14- Clear and mount

Results:

Nuclei black
Cytoplasm red
Collagen blue

A13. **Gene expression techniques:**

1- RNA Purification by QIAamp® RNA Blood Mini kit catalog no. 52304

Tissues

Protocol: Purification of Total RNA from Tissues

Important points before starting

- Use an appropriate amount of tissue (see page 13).
- When using QIAamp RNA Blood Mini Kits for the first time, please read "Important Notes", page 11.
- When preparing RNA for the first time, please read "General Remarks for Handling RNA" (Appendix A, page 35).
- Some tissues, including heart, spleen, and brain, are difficult to homogenize. The volume of lysis buffer may have to be increased to facilitate complete homogenization and to avoid reduced yields. See protocol for amounts.
- For best results, stabilize tissues immediately in RNA*later* RNA Stabilization Reagent. Tissues can be stored in RNA*later* TissueProtect Tubes for up to 1 day at 37°C, 7 days at 18–25°C, 4 weeks at 2–8°C, or for archival storage at –20°C or –80°C. See the *RNAlater Handbook* for more information about RNA*later* RNA Stabilization Reagent and about stabilizing RNA in tissues.
- Fresh, frozen, or RNA*later* stabilized tissue can be used. To freeze tissue for long-term storage, flash-freeze in liquid nitrogen,* and immediately transfer to –70°C. Tissue can be stored for several months at –70°C. To process, do not allow tissue to thaw during weighing or handling before disruption in Buffer RLT. Homogenized tissue lysates (in Buffer RLT, step 1) can also be stored at –70°C for several months. To process frozen lysates, thaw samples at room temperature (15–25°C) or at 37°C in a water bath until they are completely thawed and salts in the lysis buffer are dissolved. Avoid extended treatment at 37°C, which can cause chemical degradation of the RNA. Continue with step 2.
- All steps of this protocol (including centrifugation) should be performed at room temperature (15–25°C). During the procedure, work quickly.

Things to do before starting

- Buffer RLT may form a precipitate upon storage. If necessary, warm to redissolve.
- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. Add 10 µl β-ME per 1 ml of Buffer RLT. This solution is stable for 1 month at room temperature (15–25°C).
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) to obtain a working solution.
- If performing optional on-column DNase digestion, prepare DNase I stock solution as deseribed in Appendix D (page 41).

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Procedure

1. **Disrupt tissue and homogenize lysate according to step 1a, 1b, or 1c.**

   See "Lysis and homogenization", pages 14–16, for more details on disruption and homogenization.

   Note: Incomplete disruption and homogenization will lead to significantly reduced yields, and can cause clogging of the QIAamp spin column. Homogenization with the TissueLyser, TissueRuptor, or equivalent rotor–stator homogenizers generally results in higher RNA yields than with other methods.

   Note: Ensure that β-ME is added to Buffer RLT before use (see "Things to do before starting").

   After storage in RNA*later* RNA Stabilization Reagent, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization using standard methods is usually not a problem. For easier disruption and homogenization, we recommend using 600 µl Buffer RLT.

1a. Disruption and homogenization using the QIAGEN TissueRuptor or equivalent rotor–stator homogenizer:

Place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in a suitably sized vessel for the homogenizer. Add the appropriate volume of Buffer RLT (see Table 3). Immediately disrupt and homogenize the tissue using the TissueRuptor or equivalent rotor–stator homogenizer until it is uniformly homogeneous (usually 20–40 s). Centrifuge the lysate for 3 min at maximum speed and use only the supernatant. Proceed to step 2.

Following centrifugation, for some samples, very small amounts of insoluble material may be present, making the pellet invisible.

Table 3. Volumes of Buffer RLT Used for Sample Lysis

Amount of starting material	Volume of Buffer RLT
Up to 20 mg	350 µl
20 to 30 mg, if tissue is difficult to lyse	600 µl

As a guide, 3 mm³ of most tissues weighs 30–35 mg.

1b. Disruption and homogenization using the TissueLyser:

Place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in a 2 ml microcentrifuge tube. Add the appropriate volume of Buffer RLT (see Table 3), and add one stainless steel bead (5 mm diameter). Homogenize the lysate on the TissueLyser for 2 min at 20 Hz. Rotate the TissueLyser rack, and homogenize for another 2 min at 20 Hz. Centrifuge the lysate for 3 min at maximum speed and use only the supernatant. Proceed to step 2.

Note: The instructions in step 1b are only guidelines. They may need to be changed depending on the sample being processed or if a different bead mill is used. See the TissueLyser Handbook for more details.

1c. **Disruption using a mortar and pestle followed by homogenization using a QIAshredder homogenizer:**

Immediately place the weighed (fresh, frozen, or RNAlater stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle, keeping the sample immersed in liquid nitrogen. Transfer the tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled 2 ml microcentrifuge tube. Allow the liquid nitrogen to evaporate, but do not let allow the tissue to thaw.

Add 600 µl Buffer RLT. Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge at maximum speed for 2 min to homogenize the sample. Discard the QIAshredder spin column, and use only the supernatant. Proceed to step 2.

Note: This method may result in lower yields than those obtained when using the TissueLyser, TissueRuptor, or equivalent rotor–stator homogenizer (see above).

2. **Add 1 volume (usually 350 µl or 600 µl) of 70% ethanol to the cleared lysate and mix well by pipetting. Do not centrifuge.**

If some lysate is lost during homogenization, reduce volume of ethanol accordingly. A precipitate may form after the addition of ethanol but this will not affect the QIAamp procedure.

3. **Carefully pipet 700 µl of the sample, including any precipitate that may have formed, into a QIAamp spin column in a 2 ml collection tube (provided) without moistening the rim. Centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). If sample volume exceeds 700 µl, load aliquots successively onto the QIAamp spin column and centrifuge as above.**

Discard flow-through* and collection tube.

Optional: If performing optional on-column DNase digestion (see "DNase treatment", page 41), follow steps D1–D4 (page 42) after performing this step.

4. **Transfer QIAamp spin column to a new 2 ml collection tube (provided). Pipet 700 µl Buffer RW1 onto the QIAamp spin column and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash.**

Discard flow-through* and collection tube.

5. **Transfer QIAamp spin column to a new 2 ml collection tube (provided). Pipet 500 µl of Buffer RPE onto the QIAamp spin column and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash.**

Discard flow-through* and collection tube.

* Flow-through contains Buffer RW1 or RLT and is therefore incompatible with bleach. See page 6 for safety information.

Note: Ensure ethanol is added to Buffer RPE before use (see "Things to do before starting").

6. **Carefully open the QIAamp spin column and add 500 µl of Buffer RPE. Close the cap and centrifuge at full speed (20,000 x *g*, 14,000 rpm) for 3 min.**

   Note: Some centrifuge rotors may distort slightly upon deceleration, resulting in flow-through, containing Buffer RPE, contacting the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp spin column.

7. **Recommended: Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

   This step helps to eliminate the chance of possible Buffer RPE carryover.

8. **Transfer QIAamp spin column into a 1.5 ml microcentrifuge tube (provided) and pipet 30–50 µl of RNase-free water (provided) directly onto the QIAamp membrane. Centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute. Repeat if the expected RNA yield is >30 µg.**

   If a second elution step is performed, elute into the same tube using another 30–50 µl RNase-free water.

Appendix E: DNase Digestion of RNA before RNA Cleanup

This protocol describes how to use the RNase-Free DNase Set (cat. no. 79254) to digest contaminating DNA in RNA solutions prior to RNA cleanup. DNase digestion can alternatively be carried out during RNA cleanup (see Appendix D, page 41). For samples highly contaminated with DNA, we recommend DNase digestion in solution, as it is more efficient than on-column DNase digestion.

Important points before starting

- Generally, DNase digestion is not required with the QIAamp RNA Blood Mini Kit since the silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., real-time RT-PCR analysis with a low-abundance target).
- **Do not vortex the reconstituted DNase I.** DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.

Things to do before starting

- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the vial. Do not vortex.
- For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

Procedure

1. **Mix the following in a microcentrifuge tube:**
   - **≤87.5 µl RNA solution (contaminated with genomic DNA)**
   - **10 µl Buffer RDD**
   - **2.5 µl DNase I stock solution**

   Make the volume up to 100 µl with RNase-free water.

   The reaction volumes can be doubled if necessary (to 200 µl final volume).
2. **Incubate on the benchtop (20–25°C) for 10 min.**
3. **Clean up the RNA according to "Protocol: RNA Cleanup" on page 30.**

Protocol: RNA Cleanup

Important point before starting

- Do not exceed the binding capacity (100 µg) of the QIAamp spin column.

Things to do before starting

- Buffer RLT may form a precipitate upon storage. If necessary, warm to redissolve.
- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. Add 10 µl of β-ME per 1 ml of Buffer RLT. Buffer RLT is stable for 1 month after addition of β-ME.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) to obtain a working solution.
- If performing optional on–column DNase digestion, prepare DNase I stock solution as deseribed in Appendix D (page 41).

Procedure

1. **Adjust sample volume to 100 µl with RNase-free water (provided), add 350 µl Buffer RLT to the sample, and mix thoroughly.**

   Note: β-ME must be added to Buffer RLT before use (see "Things to do before starting").

2. **Add 250 µl ethanol (96–100%) to the lysate and mix by pipetting. Do not centrifuge.**

3. **Pipet sample (700 µl) into a QIAamp spin column in a 2 ml collection tube (provided) without moistening the rim, and centrifuge for 15 s at ≥8,000 x *g* (≥10,000 rpm).**

   Discard flow-through* and collection tube.

   Optional: If performing optional on-column DNase digestion (see "DNase treatment", page 41), follow steps D1–D4 (page 42) after performing this step.

4. **Place the QIAamp spin column into a new 2 ml collection tube (provided), add 500 µl Buffer RPE, and centrifuge for 15 s at ≥8,000 x *g* (≥10,000 rpm).**

   Discard flow-through and collection tube.

   Note: Ensure ethanol is added to Buffer RPE (see "Things to do before starting").

5. **Carefully open the QIAamp spin column and add 500 µl of Buffer RPE. Close the cap and centrifuge at full speed (20,000 x *g*, 14,000 rpm) for 3 min.**

* Flow-through contains Buffer RW1 and is therefore incompatible with bleach. See page 6 for safety information.

Note: Some centrifuge rotors may distort slightly upon deceleration, resulting in flow-through, containing Buffer RPE, contacting the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp spin column.

6. **Recommended: Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

   This step helps to eliminate the chance of possible Buffer RPE carryover.

7. **Transfer the QIAamp spin column to a new 1.5 ml collection tube (provided) and pipet 30–50 µl of RNase-free water (provided) directly onto the QIAamp membrane. Centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute. Repeat if the expected RNA yield is >30 µg.**

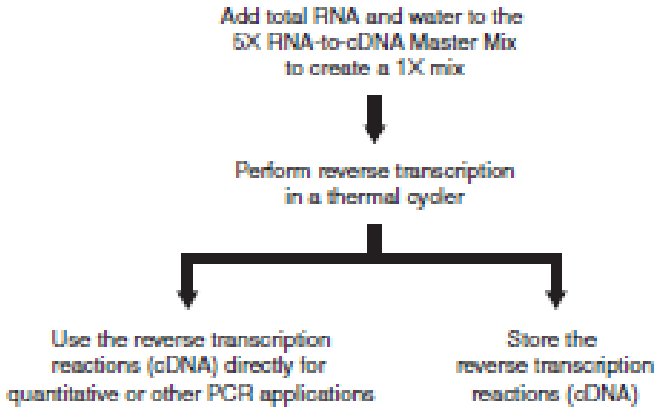
   A second elution step into the same collection tube with a further 30–50 µl RNase-free water is recommended when the expected RNA yield is >30 µg.

2- **Transcription of RNA to cDNA by High Capacity RNA-to-cDNA Master Mix**

Using the High Capacity RNA-to-cDNA Master Mix

Overview Diagram

To synthesize single-stranded cDNA from RNA using the High Capacity RNA-to-cDNA Master Mix, refer to the diagram below.



Optimizing the RNA Template

For optimal performance of the Master Mix, Applied Biosystems recommends using RNA that is:

- Free of inhibitors of reverse transcription and PCR
- Dissolved in PCR-compatible buffer or water
- Free of RNase activity

Amount of RNA

Use 1 pg to 1µg of RNA per 20-µL reaction.

Preparing the Reverse Transcription Reactions with High Capacity RNA-to-cDNA Master Mix

Prepare the Master Mix (or No-RT Control) before preparing the reaction plate.

Note: Follow the same procedure to prepare No-RT controls.

To prepare the reverse transcription reactions (20 µL per reaction):

1. Place the Master Mix (or No-RT Control) components on ice, then mix and briefly centrifuge them.

2. Calculate the total volume of components needed to prepare the required number of reactions. Use the table below.

Component	Volume per Reaction	Final Concentration
Master Mix (or No-RT Control)	4.0 µL	1X
RNA template	up to 16 µL	1 pg - 1 µg
Nuclease-free H_2O	sufficient to 20 µL	—
Total	20.0 µL	—

IMPORTANT! Include additional reactions in the calculations to provide excess volume for the loss that occurs during reagent transfers.

3. Prepare the reaction mix according to your calculations in step 2.

WARNING **CHEMICAL HAZARD. High Capacity RNA-to-cDNA Master Mix** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate eyewear, clothing, and gloves.

IMPORTANT! Prepare the reaction mix on ice.

4. Seal the plate or tubes.

5. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate air bubbles.

To prepare the reverse transcription reactions (20 µL per reaction): *(continued)*

6.	Place the plate or tubes on ice until you are ready to load the thermal cycler.

Performing Reverse Transcription with High Capacity RNA-to-cDNA Master Mix

To perform reverse transcription:

1.	Program the thermal cycler conditions as shown below, using one of the thermal cyclers listed in Table 4 on page 16. **IMPORTANT!** These conditions are optimized for the High Capacity RNA-to-cDNA Master Mix.
2.	Set the reaction volume to 20 µL.
3.	Load the reaction plates or tubes into the thermal cycler.
4.	Start the reverse transcription run. Perform PCR amplification with one-tenth of the first-strand reaction.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	42	85	4
Time	5 min	30 min	5 min	∞

3- **Gene Expression by Real time PCR**

applied biosystems

For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

TaqMan® Fast Advanced Master Mix Product Insert

Insert PN 4444602 Rev. C

Product part numbers and storage conditions

Product	Quantity/part number						Storage conditions
	1 × 1 mL	1 × 5 mL	2 × 5 mL	5 × 5 mL	10 × 5 mL	1 × 50 mL	
TaqMan® Fast Advanced Master Mix	4444556	4444557	4444963	4444964	4444965	4444553	Store at -15 to -25 °C until first use, then store at 4 °C.

Protocol for TaqMan® and Custom TaqMan® Gene Expression Assays

Note:

This Product Insert briefly describes how to perform gene expression experiments using the TaqMan® Fast Advanced Master Mix with TaqMan® and Custom TaqMan® Gene Expression Assays. For more detailed procedures, or for procedures on performing gene expression experiments with TaqMan® MicroRNA Assays or TaqMan® Array Micro Fluidic Cards, refer to the *TaqMan® Fast Advanced Master Mix Protocol* (PN 4444605).

Prepare the PCR reaction mix

1. Thoroughly mix the TaqMan® Fast Advanced Master Mix.
2. Thaw frozen samples and frozen TaqMan® assays on ice. Resuspend by vortexing, then briefly centrifuge.
3. Calculate the total volume required for each component: *volume for 1 reaction* × *the total number of reactions*.

Component	Volume (µL) for 1 reaction		Final concentration
	384-well plate	96-well and 48-well plates (both Standard and Fast)	
TaqMan® Fast Advanced Master Mix (2X)	5.0	10.0	1X
TaqMan® Gene Expression Assay (20X) or Custom TaqMan® Gene Expression Assay (20X)	0.5	1.0	1X
cDNA template	1.0	2.0	100 ng to 1 pg
Nuclease-free water	3.5	7.0	
Total volume per reaction	**10.0**	**20.0**	–

4. Add all components to a 1.5-mL microcentrifuge tube, cap the tube, then vortex briefly to mix.
5. Centrifuge the tube briefly to spin down the contents and eliminate air bubbles.

Prepare and run the PCR reaction plate

1. Transfer the appropriate volume of PCR reaction mix to each well of an optical reaction plate.
2. Cover the reaction plate, then centrifuge the plate briefly to spin down the contents and eliminate air bubbles.
3. Run the PCR reaction plate using the parameters below.
   - Run mode:

Applied Biosystems Real-Time PCR System	Mode
7900HT, 7900HT Fast (384-Well Block Module and Standard 96-Well Block Module), 7500, and 7300 systems	Standard
ViiA™ 7, StepOne™, StepOnePlus™, 7900HT Fast (Fast 96-Well Block Module), and 7500 Fast systems	Fast

- Thermal cycling conditions:

Applied Biosystems Real-Time PCR System	Thermal-cycling profile				
	Parameter	UNG incubation†	Polymerase activation‡	PCR (40 cycles)	
		Hold	Hold	Denature	Anneal/ extend
	Temp. (°C)	50	95	95	60
7900HT system	Time (mm:ss)	02:00	00:20	00:01	00:20
7900HT Fast system (Fast 96-Well, Standard 96-Well, or 384-Well Block Modules)					
ViiA™ 7 system					
StepOne™ system					
StepOnePlus™ system					
7500 Fast system	Time (mm:ss)	02:00	00:20	00:03	00:30
7500 system					
7300 system					

† Required for optimal UNG activity.
‡ Required to activate the AmpliTaq® Fast DNA Polymerase.

Analyze the results

Data analysis varies, depending on the instrument. For further information, refer to the *TaqMan® Fast Advanced Master Mix Protocol* (PN 4444605) and the appropriate documentation for your instrument.

Safety information

IMPORTANT!
For safety and biohazard guidelines, refer to the "Safety" section in the *TaqMan® Fast Advanced Master Mix Protocol* (PN 4444605).

Obtaining SDSs

The Safety Data Sheet (SDS) for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain SDSs:

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **SDS**.
2. In the Keyword Search field, enter the chemical name, product name, SDS part number, or other information that appears in the SDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose

Note:
For the SDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Part Number 4444602 Rev. C 06/2010

4- Genes efficiencies and Standard curves

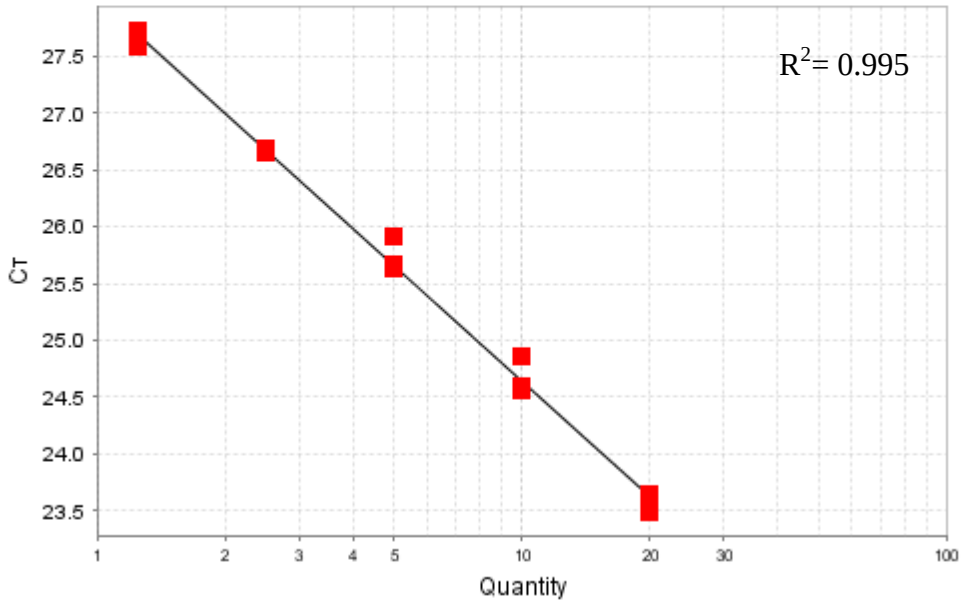


Figure: Hprt1 standard curve

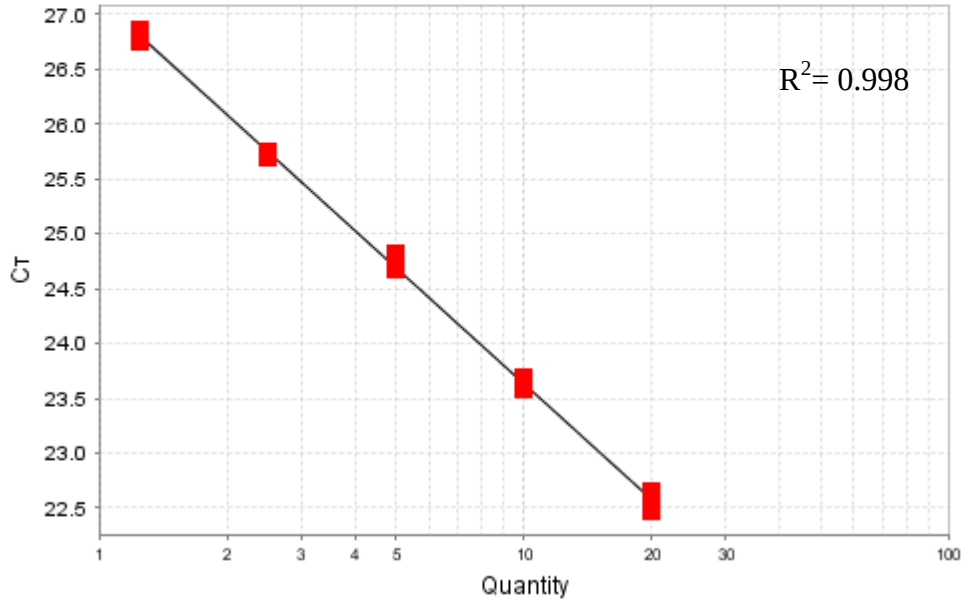


Figure: Ppia standard curve

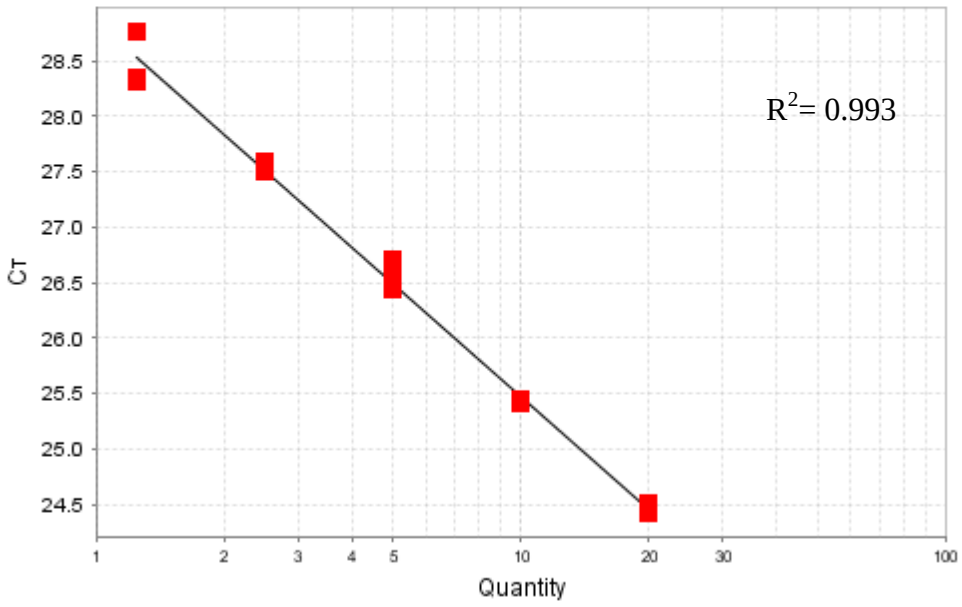


Figure: TGFβ standard curve

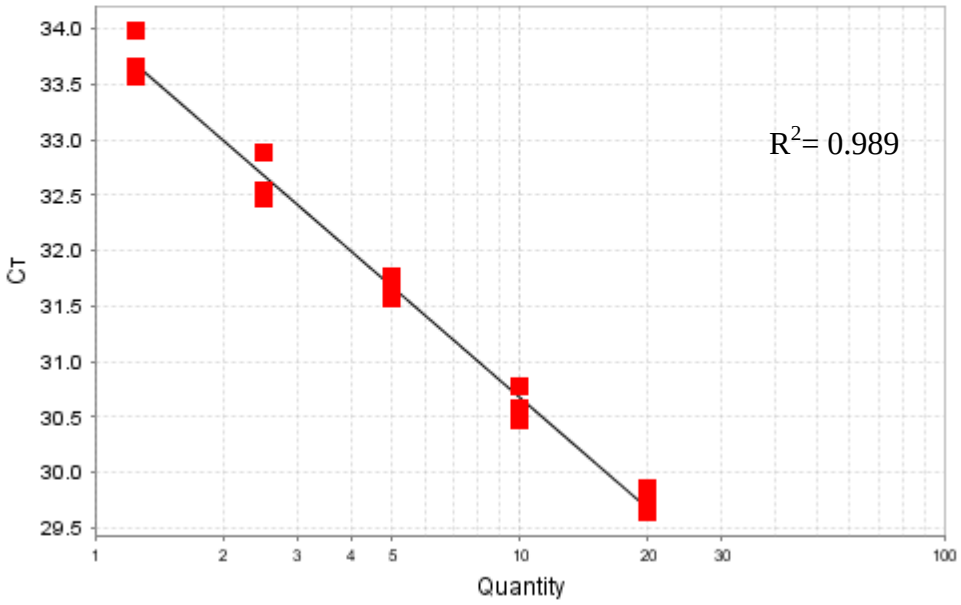


Figure: TIMP1 standard curve

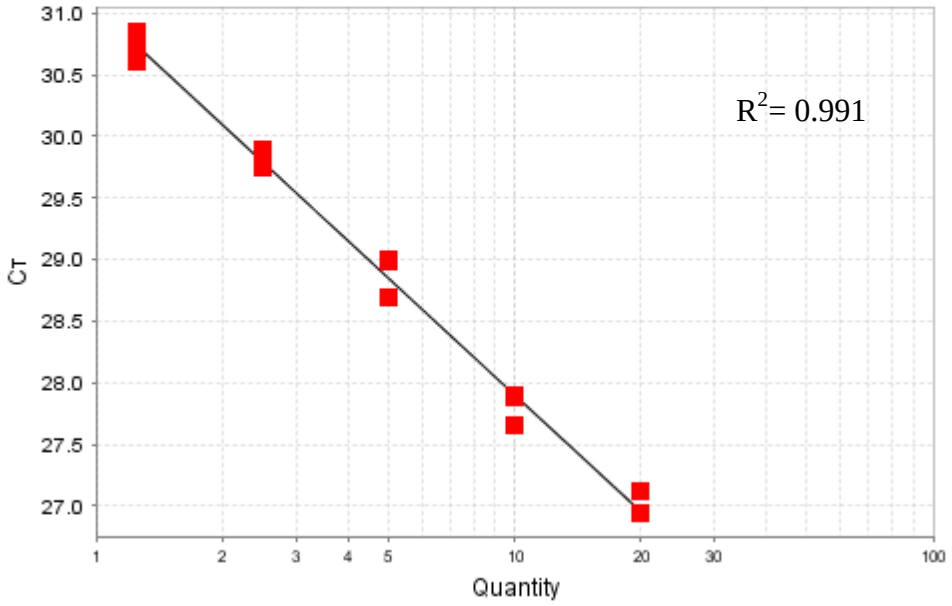


Figure: MMP2 standard curve

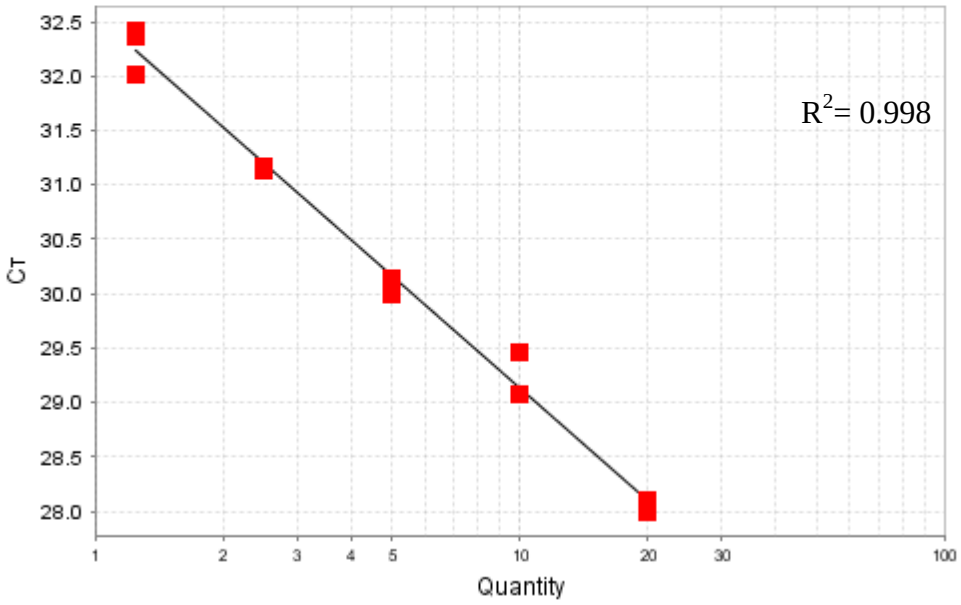


Figure: Collα standard curve

Appendix B: Data analysis and statistics

B1: *In vitro* antioxidant analysis

1- DPPH analysis

DPPH

Duncan test

GROUPS	N	Subset for alpha = 0.05		
		A	b	C
VITC	3	3.3587		
PN	3		11.0233	
MM	3		11.2000	
BHT	3			21.1000
Sig.		1.000	.775	1.000

Means for groups in homogeneous subsets are displayed.

2- ABTS analysis

ABTS

Duncan test

GROUPS	N	Subset for alpha = 0.05			
		A	b	c	d
VITC	3	21.3227			
PN	3		53.1133		
MM	3			62.2200	
BHT	3				65.2333
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

3- FRAP analysis

FRAP

Duncan test

GROUPS	N	Subset for alpha = 0.05			
		A	b	C	d
PN	3	7755.0000			
MM	3		33589.6667		
BHT	3			57303.3333	
VITC	3				101603.3333
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

4- Correlation between *in vitro* antioxidant tests

		ABTS	FRAP	TPC	TFC
DPPH	Pearson Correlation	.631*	.383	-.355-	.276
	Sig. (2-tailed)	.012	.158	.257	.385
	N	15	15	12	12
ABTS	Pearson Correlation		.404	-.173-	.174
	Sig. (2-tailed)		.135	.591	.589
	N		15	12	12
FRAP	Pearson Correlation			.908**	-.805-**
	Sig. (2-tailed)			.000	.002
	N			12	12
TPC	Pearson Correlation				-.766-**
	Sig. (2-tailed)				.004
	N				12

B2: <u>Hepatoprotective Test Statistics</u>

1- Body, Liver and Spleen weights

Multiple Comparisons

BW

Bonferroni

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
N	TAA	41.60000	21.72411	1.000	-30.9672-	114.1672
	S	15.00000	21.72411	1.000	-57.5672-	87.5672
	PN/LD	-21.60000-	21.72411	1.000	-94.1672-	50.9672
	PN/HD	-16.40000-	21.72411	1.000	-88.9672-	56.1672
	MM/LD	-12.40000-	21.72411	1.000	-84.9672-	60.1672
	MM/HD	5.80000	21.72411	1.000	-66.7672-	78.3672
TAA	N	-41.60000-	21.72411	1.000	-114.1672-	30.9672
	S	-26.60000-	21.72411	1.000	-99.1672-	45.9672
	PN/LD	-63.20000-	21.72411	.147	-135.7672-	9.3672
	PN/HD	-58.00000-	21.72411	.262	-130.5672-	14.5672
	MM/LD	-54.00000-	21.72411	.403	-126.5672-	18.5672
	MM/HD	-35.80000-	21.72411	1.000	-108.3672-	36.7672
S	N	-15.00000-	21.72411	1.000	-87.5672-	57.5672
	TAA	26.60000	21.72411	1.000	-45.9672-	99.1672
	PN/LD	-36.60000-	21.72411	1.000	-109.1672-	35.9672
	PN/HD	-31.40000-	21.72411	1.000	-103.9672-	41.1672
	MM/LD	-27.40000-	21.72411	1.000	-99.9672-	45.1672

	MM/HD	-9.20000-	21.72411	1.000	-81.7672-	63.3672
PN/LD	N	21.60000	21.72411	1.000	-50.9672-	94.1672
	TAA	63.20000	21.72411	.147	-9.3672-	135.7672
	S	36.60000	21.72411	1.000	-35.9672-	109.1672
	PN/HD	5.20000	21.72411	1.000	-67.3672-	77.7672
	MM/LD	9.20000	21.72411	1.000	-63.3672-	81.7672
	MM/HD	27.40000	21.72411	1.000	-45.1672-	99.9672
PN/HD	N	16.40000	21.72411	1.000	-56.1672-	88.9672
	TAA	58.00000	21.72411	.262	-14.5672-	130.5672
	S	31.40000	21.72411	1.000	-41.1672-	103.9672
	PN/LD	-5.20000-	21.72411	1.000	-77.7672-	67.3672
	MM/LD	4.00000	21.72411	1.000	-68.5672-	76.5672
	MM/HD	22.20000	21.72411	1.000	-50.3672-	94.7672
MM/LD	N	12.40000	21.72411	1.000	-60.1672-	84.9672
	TAA	54.00000	21.72411	.403	-18.5672-	126.5672
	S	27.40000	21.72411	1.000	-45.1672-	99.9672
	PN/LD	-9.20000-	21.72411	1.000	-81.7672-	63.3672
	PN/HD	-4.00000-	21.72411	1.000	-76.5672-	68.5672
	MM/HD	18.20000	21.72411	1.000	-54.3672-	90.7672
MM/HD	N	-5.80000-	21.72411	1.000	-78.3672-	66.7672
	TAA	35.80000	21.72411	1.000	-36.7672-	108.3672
	S	9.20000	21.72411	1.000	-63.3672-	81.7672
	PN/LD	-27.40000-	21.72411	1.000	-99.9672-	45.1672

	PN/HD	-22.20000-	21.72411	1.000	-94.7672-	50.3672
	MM/LD	-18.20000-	21.72411	1.000	-90.7672-	54.3672

Multiple Comparisons

LW

Bonferroni

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
N	TAA	-3.20000-	1.06904	.120	-6.7710-	.3710
	S	-3.00000-	1.06904	.189	-6.5710-	.5710
	PN/LD	-2.00000-	1.06904	1.000	-5.5710-	1.5710
	PN/HD	-1.60000-	1.06904	1.000	-5.1710-	1.9710
	MM/LD	-4.60000-*	1.06904	.004	-8.1710-	-1.0290-
	MM/HD	-3.00000-	1.06904	.189	-6.5710-	.5710
TAA	N	3.20000	1.06904	.120	-.3710-	6.7710
	S	.20000	1.06904	1.000	-3.3710-	3.7710
	PN/LD	1.20000	1.06904	1.000	-2.3710-	4.7710
	PN/HD	1.60000	1.06904	1.000	-1.9710-	5.1710
	MM/LD	-1.40000-	1.06904	1.000	-4.9710-	2.1710
	MM/HD	.20000	1.06904	1.000	-3.3710-	3.7710
S	N	3.00000	1.06904	.189	-.5710-	6.5710
	TAA	-.20000-	1.06904	1.000	-3.7710-	3.3710
	PN/LD	1.00000	1.06904	1.000	-2.5710-	4.5710
	PN/HD	1.40000	1.06904	1.000	-2.1710-	4.9710

	MM/LD	-1.60000-	1.06904	1.000	-5.1710-	1.9710
	MM/HD	.00000	1.06904	1.000	-3.5710-	3.5710
PN/LD	N	2.00000	1.06904	1.000	-1.5710-	5.5710
	TAA	-1.20000-	1.06904	1.000	-4.7710-	2.3710
	S	-1.00000-	1.06904	1.000	-4.5710-	2.5710
	PN/HD	.40000	1.06904	1.000	-3.1710-	3.9710
	MM/LD	-2.60000-	1.06904	.455	-6.1710-	.9710
	MM/HD	-1.00000-	1.06904	1.000	-4.5710-	2.5710
PN/HD	N	1.60000	1.06904	1.000	-1.9710-	5.1710
	TAA	-1.60000-	1.06904	1.000	-5.1710-	1.9710
	S	-1.40000-	1.06904	1.000	-4.9710-	2.1710
	PN/LD	-.40000-	1.06904	1.000	-3.9710-	3.1710
	MM/LD	-3.00000-	1.06904	.189	-6.5710-	.5710
	MM/HD	-1.40000-	1.06904	1.000	-4.9710-	2.1710
MM/LD	N	4.60000*	1.06904	.004	1.0290	8.1710
	TAA	1.40000	1.06904	1.000	-2.1710-	4.9710
	S	1.60000	1.06904	1.000	-1.9710-	5.1710
	PN/LD	2.60000	1.06904	.455	-.9710-	6.1710
	PN/HD	3.00000	1.06904	.189	-.5710-	6.5710
	MM/HD	1.60000	1.06904	1.000	-1.9710-	5.1710
MM/HD	N	3.00000	1.06904	.189	-.5710-	6.5710
	TAA	-.20000-	1.06904	1.000	-3.7710-	3.3710
	S	.00000	1.06904	1.000	-3.5710-	3.5710

	PN/LD	1.00000	1.06904	1.000	-2.5710-	4.5710
	PN/HD	1.40000	1.06904	1.000	-2.1710-	4.9710
	MM/LD	-1.60000-	1.06904	1.000	-5.1710-	1.9710

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

SW

Bonferroni

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
N	TAA	-.14800-	.06599	.693	-.3684-	.0724
	S	-.16600-	.06599	.376	-.3864-	.0544
	PN/LD	-.10000-	.06599	1.000	-.3204-	.1204
	PN/HD	-.04000-	.06599	1.000	-.2604-	.1804
	MM/LD	-.09800-	.06599	1.000	-.3184-	.1224
	MM/HD	-.04200-	.06599	1.000	-.2624-	.1784
TAA	N	.14800	.06599	.693	-.0724-	.3684
	S	-.01800-	.06599	1.000	-.2384-	.2024
	PN/LD	.04800	.06599	1.000	-.1724-	.2684
	PN/HD	.10800	.06599	1.000	-.1124-	.3284
	MM/LD	.05000	.06599	1.000	-.1704-	.2704
	MM/HD	.10600	.06599	1.000	-.1144-	.3264
S	N	.16600	.06599	.376	-.0544-	.3864
	TAA	.01800	.06599	1.000	-.2024-	.2384

	PN/LD	.06600	.06599	1.000	-.1544-	.2864
	PN/HD	.12600	.06599	1.000	-.0944-	.3464
	MM/LD	.06800	.06599	1.000	-.1524-	.2884
	MM/HD	.12400	.06599	1.000	-.0964-	.3444
PN/LD	N	.10000	.06599	1.000	-.1204-	.3204
	TAA	-.04800-	.06599	1.000	-.2684-	.1724
	S	-.06600-	.06599	1.000	-.2864-	.1544
	PN/HD	.06000	.06599	1.000	-.1604-	.2804
	MM/LD	.00200	.06599	1.000	-.2184-	.2224
	MM/HD	.05800	.06599	1.000	-.1624-	.2784
PN/HD	N	.04000	.06599	1.000	-.1804-	.2604
	TAA	-.10800-	.06599	1.000	-.3284-	.1124
	S	-.12600-	.06599	1.000	-.3464-	.0944
	PN/LD	-.06000-	.06599	1.000	-.2804-	.1604
	MM/LD	-.05800-	.06599	1.000	-.2784-	.1624
	MM/HD	-.00200-	.06599	1.000	-.2224-	.2184
MM/LD	N	.09800	.06599	1.000	-.1224-	.3184
	TAA	-.05000-	.06599	1.000	-.2704-	.1704
	S	-.06800-	.06599	1.000	-.2884-	.1524
	PN/LD	-.00200-	.06599	1.000	-.2224-	.2184
	PN/HD	.05800	.06599	1.000	-.1624-	.2784
	MM/HD	.05600	.06599	1.000	-.1644-	.2764
MM/H	N	.04200	.06599	1.000	-.1784-	.2624

D	TAA	-.10600-	.06599	1.000	-.3264-	.1144
	S	-.12400-	.06599	1.000	-.3444-	.0964
	PN/LD	-.05800-	.06599	1.000	-.2784-	.1624
	PN/HD	.00200	.06599	1.000	-.2184-	.2224
	MM/LD	-.05600-	.06599	1.000	-.2764-	.1644

Multiple Comparisons

LWBW

Bonferroni

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
N	TAA	-2.18340-*	.37298	.000	-3.4293-	-.9375-
	S	-1.47520-*	.37298	.010	-2.7211-	-.2293-
	PN/LD	-.59120-	.37298	1.000	-1.8371-	.6547
	PN/HD	-.48920-	.37298	1.000	-1.7351-	.7567
	MM/LD	-1.72100-*	.37298	.002	-2.9669-	-.4751-
	MM/HD	-1.42540-*	.37298	.014	-2.6713-	-.1795-
TAA	N	2.18340*	.37298	.000	.9375	3.4293
	S	.70820	.37298	1.000	-.5377-	1.9541
	PN/LD	1.59220*	.37298	.004	.3463	2.8381
	PN/HD	1.69420*	.37298	.002	.4483	2.9401
	MM/LD	.46240	.37298	1.000	-.7835-	1.7083
	MM/HD	.75800	.37298	1.000	-.4879-	2.0039
S	N	1.47520*	.37298	.010	.2293	2.7211

	TAA	-.70820-	.37298	1.000	-1.9541-	.5377
	PN/LD	.88400	.37298	.523	-.3619-	2.1299
	PN/HD	.98600	.37298	.279	-.2599-	2.2319
	MM/LD	-.24580-	.37298	1.000	-1.4917-	1.0001
	MM/HD	.04980	.37298	1.000	-1.1961-	1.2957
PN/LD	N	.59120	.37298	1.000	-.6547-	1.8371
	TAA	-1.59220-*	.37298	.004	-2.8381-	-.3463-
	S	-.88400-	.37298	.523	-2.1299-	.3619
	PN/HD	.10200	.37298	1.000	-1.1439-	1.3479
	MM/LD	-1.12980-	.37298	.110	-2.3757-	.1161
	MM/HD	-.83420-	.37298	.703	-2.0801-	.4117
PN/HD	N	.48920	.37298	1.000	-.7567-	1.7351
	TAA	-1.69420-*	.37298	.002	-2.9401-	-.4483-
	S	-.98600-	.37298	.279	-2.2319-	.2599
	PN/LD	-.10200-	.37298	1.000	-1.3479-	1.1439
	MM/LD	-1.23180-	.37298	.055	-2.4777-	.0141
	MM/HD	-.93620-	.37298	.381	-2.1821-	.3097
MM/LD	N	1.72100*	.37298	.002	.4751	2.9669
	TAA	-.46240-	.37298	1.000	-1.7083-	.7835
	S	.24580	.37298	1.000	-1.0001-	1.4917
	PN/LD	1.12980	.37298	.110	-.1161-	2.3757
	PN/HD	1.23180	.37298	.055	-.0141-	2.4777
	MM/HD	.29560	.37298	1.000	-.9503-	1.5415

MM/HD	N	1.42540*	.37298	.014	.1795	2.6713
	TAA	-.75800-	.37298	1.000	-2.0039-	.4879
	S	-.04980-	.37298	1.000	-1.2957-	1.1961
	PN/LD	.83420	.37298	.703	-.4117-	2.0801
	PN/HD	.93620	.37298	.381	-.3097-	2.1821
	MM/LD	-.29560-	.37298	1.000	-1.5415-	.9503

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

SWBW

Bonferroni

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
N	TAA	-.09700-*	.02623	.020	-.1846-	-.0094-
	S	-.08120-	.02623	.093	-.1688-	.0064
	PN/LD	-.02520-	.02623	1.000	-.1128-	.0624
	PN/HD	-.00320-	.02623	1.000	-.0908-	.0844
	MM/LD	-.03000-	.02623	1.000	-.1176-	.0576
	MM/HD	-.02140-	.02623	1.000	-.1090-	.0662
TAA	N	.09700*	.02623	.020	.0094	.1846
	S	.01580	.02623	1.000	-.0718-	.1034
	PN/LD	.07180	.02623	.223	-.0158-	.1594
	PN/HD	.09380*	.02623	.027	.0062	.1814
	MM/LD	.06700	.02623	.344	-.0206-	.1546

	MM/HD	.07560	.02623	.158	-.0120-	.1632
S	N	.08120	.02623	.093	-.0064-	.1688
	TAA	-.01580-	.02623	1.000	-.1034-	.0718
	PN/LD	.05600	.02623	.875	-.0316-	.1436
	PN/HD	.07800	.02623	.126	-.0096-	.1656
	MM/LD	.05120	.02623	1.000	-.0364-	.1388
	MM/HD	.05980	.02623	.639	-.0278-	.1474
PN/LD	N	.02520	.02623	1.000	-.0624-	.1128
	TAA	-.07180-	.02623	.223	-.1594-	.0158
	S	-.05600-	.02623	.875	-.1436-	.0316
	PN/HD	.02200	.02623	1.000	-.0656-	.1096
	MM/LD	-.00480-	.02623	1.000	-.0924-	.0828
	MM/HD	.00380	.02623	1.000	-.0838-	.0914
PN/HD	N	.00320	.02623	1.000	-.0844-	.0908
	TAA	-.09380-*	.02623	.027	-.1814-	-.0062-
	S	-.07800-	.02623	.126	-.1656-	.0096
	PN/LD	-.02200-	.02623	1.000	-.1096-	.0656
	MM/LD	-.02680-	.02623	1.000	-.1144-	.0608
	MM/HD	-.01820-	.02623	1.000	-.1058-	.0694
MM/LD	N	.03000	.02623	1.000	-.0576-	.1176
	TAA	-.06700-	.02623	.344	-.1546-	.0206
	S	-.05120-	.02623	1.000	-.1388-	.0364
	PN/LD	.00480	.02623	1.000	-.0828-	.0924

	PN/HD	.02680	.02623	1.000	-.0608-	.1144
	MM/HD	.00860	.02623	1.000	-.0790-	.0962
MM/HD	N	.02140	.02623	1.000	-.0662-	.1090
	TAA	-.07560-	.02623	.158	-.1632-	.0120
	S	-.05980-	.02623	.639	-.1474-	.0278
	PN/LD	-.00380-	.02623	1.000	-.0914-	.0838
	PN/HD	.01820	.02623	1.000	-.0694-	.1058
	MM/LD	-.00860-	.02623	1.000	-.0962-	.0790

*. The mean difference is significant at the 0.05 level.

2- Hepatic Biochemical parameters

Multiple Comparisons

ALT

Bonferroni

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
N	TAA	-99.60000-*	13.54841	.000	-144.8571-	-54.3429-
	S	-41.25000-	13.54841	.106	-86.5071-	4.0071
	PN/LD	-16.60000-	13.54841	1.000	-61.8571-	28.6571
	PN/HD	-12.20000-	13.54841	1.000	-57.4571-	33.0571
	MM/LD	-30.60000-	13.54841	.670	-75.8571-	14.6571
	MM/HD	-28.00000-	13.54841	1.000	-73.2571-	17.2571
TAA	N	99.60000*	13.54841	.000	54.3429	144.8571
	S	58.35000*	13.54841	.004	13.0929	103.6071

	PN/LD	83.00000*	13.54841	.000	37.7429	128.2571
	PN/HD	87.40000*	13.54841	.000	42.1429	132.6571
	MM/LD	69.00000*	13.54841	.000	23.7429	114.2571
	MM/HD	71.60000*	13.54841	.000	26.3429	116.8571
S	N	41.25000	13.54841	.106	-4.0071-	86.5071
	TAA	-58.35000-*	13.54841	.004	-103.6071-	-13.0929-
	PN/LD	24.65000	13.54841	1.000	-20.6071-	69.9071
	PN/HD	29.05000	13.54841	.858	-16.2071-	74.3071
	MM/LD	10.65000	13.54841	1.000	-34.6071-	55.9071
	MM/HD	13.25000	13.54841	1.000	-32.0071-	58.5071
PN/LD	N	16.60000	13.54841	1.000	-28.6571-	61.8571
	TAA	-83.00000-*	13.54841	.000	-128.2571-	-37.7429-
	S	-24.65000-	13.54841	1.000	-69.9071-	20.6071
	PN/HD	4.40000	13.54841	1.000	-40.8571-	49.6571
	MM/LD	-14.00000-	13.54841	1.000	-59.2571-	31.2571
	MM/HD	-11.40000-	13.54841	1.000	-56.6571-	33.8571
PN/HD	N	12.20000	13.54841	1.000	-33.0571-	57.4571
	TAA	-87.40000-*	13.54841	.000	-132.6571-	-42.1429-
	S	-29.05000-	13.54841	.858	-74.3071-	16.2071
	PN/LD	-4.40000-	13.54841	1.000	-49.6571-	40.8571
	MM/LD	-18.40000-	13.54841	1.000	-63.6571-	26.8571
	MM/HD	-15.80000-	13.54841	1.000	-61.0571-	29.4571
MM/LD	N	30.60000	13.54841	.670	-14.6571-	75.8571

	TAA	-69.00000-*	13.54841	.000	-114.2571-	-23.7429-
	S	-10.65000-	13.54841	1.000	-55.9071-	34.6071
	PN/LD	14.00000	13.54841	1.000	-31.2571-	59.2571
	PN/HD	18.40000	13.54841	1.000	-26.8571-	63.6571
	MM/HD	2.60000	13.54841	1.000	-42.6571-	47.8571
MM/HD	N	28.00000	13.54841	1.000	-17.2571-	73.2571
	TAA	-71.60000-*	13.54841	.000	-116.8571-	-26.3429-
	S	-13.25000-	13.54841	1.000	-58.5071-	32.0071
	PN/LD	11.40000	13.54841	1.000	-33.8571-	56.6571
	PN/HD	15.80000	13.54841	1.000	-29.4571-	61.0571
	MM/LD	-2.60000-	13.54841	1.000	-47.8571-	42.6571

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

AST

Bonferroni

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
N	TAA	-322.20000-*	61.97843	.000	-529.2327-	-115.1673-
	S	-56.40000-	61.97843	1.000	-263.4327-	150.6327
	PN/LD	-39.60000-	61.97843	1.000	-246.6327-	167.4327
	PN/HD	-28.00000-	61.97843	1.000	-235.0327-	179.0327
	MM/LD	-51.20000-	61.97843	1.000	-258.2327-	155.8327
	MM/HD	-55.40000-	61.97843	1.000	-262.4327-	151.6327

TAA	N	322.20000*	61.97843	.000	115.1673	529.2327
	S	265.80000*	61.97843	.004	58.7673	472.8327
	PN/LD	282.60000*	61.97843	.002	75.5673	489.6327
	PN/HD	294.20000*	61.97843	.001	87.1673	501.2327
	MM/LD	271.00000*	61.97843	.003	63.9673	478.0327
	MM/HD	266.80000*	61.97843	.004	59.7673	473.8327
S	N	56.40000	61.97843	1.000	-150.6327-	263.4327
	TAA	-265.80000-*	61.97843	.004	-472.8327-	-58.7673-
	PN/LD	16.80000	61.97843	1.000	-190.2327-	223.8327
	PN/HD	28.40000	61.97843	1.000	-178.6327-	235.4327
	MM/LD	5.20000	61.97843	1.000	-201.8327-	212.2327
	MM/HD	1.00000	61.97843	1.000	-206.0327-	208.0327
PN/LD	N	39.60000	61.97843	1.000	-167.4327-	246.6327
	TAA	-282.60000-*	61.97843	.002	-489.6327-	-75.5673-
	S	-16.80000-	61.97843	1.000	-223.8327-	190.2327
	PN/HD	11.60000	61.97843	1.000	-195.4327-	218.6327
	MM/LD	-11.60000-	61.97843	1.000	-218.6327-	195.4327
	MM/HD	-15.80000-	61.97843	1.000	-222.8327-	191.2327
PN/HD	N	28.00000	61.97843	1.000	-179.0327-	235.0327
	TAA	-294.20000-*	61.97843	.001	-501.2327-	-87.1673-
	S	-28.40000-	61.97843	1.000	-235.4327-	178.6327
	PN/LD	-11.60000-	61.97843	1.000	-218.6327-	195.4327
	MM/LD	-23.20000-	61.97843	1.000	-230.2327-	183.8327

	MM/HD	-27.40000-	61.97843	1.000	-234.4327-	179.6327
MM/LD	N	51.20000	61.97843	1.000	-155.8327-	258.2327
	TAA	-271.00000-*	61.97843	.003	-478.0327-	-63.9673-
	S	-5.20000-	61.97843	1.000	-212.2327-	201.8327
	PN/LD	11.60000	61.97843	1.000	-195.4327-	218.6327
	PN/HD	23.20000	61.97843	1.000	-183.8327-	230.2327
	MM/HD	-4.20000-	61.97843	1.000	-211.2327-	202.8327
MM/HD	N	55.40000	61.97843	1.000	-151.6327-	262.4327
	TAA	-266.80000-*	61.97843	.004	-473.8327-	-59.7673-
	S	-1.00000-	61.97843	1.000	-208.0327-	206.0327
	PN/LD	15.80000	61.97843	1.000	-191.2327-	222.8327
	PN/HD	27.40000	61.97843	1.000	-179.6327-	234.4327
	MM/LD	4.20000	61.97843	1.000	-202.8327-	211.2327

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

ALP

Bonferroni

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
N	TAA	-321.40000-*	53.08026	.000	-498.7093-	-144.0907-
	S	-333.95000-*	53.08026	.000	-511.2593-	-156.6407-
	PN/LD	-103.60000-	53.08026	1.000	-280.9093-	73.7093
	PN/HD	-96.40000-	53.08026	1.000	-273.7093-	80.9093

	MM/LD	-96.00000-	53.08026	1.000	-273.3093-	81.3093
	MM/HD	-135.40000-	53.08026	.346	-312.7093-	41.9093
TAA	N	321.40000*	53.08026	.000	144.0907	498.7093
	S	-12.55000-	53.08026	1.000	-189.8593-	164.7593
	PN/LD	217.80000*	53.08026	.007	40.4907	395.1093
	PN/HD	225.00000*	53.08026	.005	47.6907	402.3093
	MM/LD	225.40000*	53.08026	.005	48.0907	402.7093
	MM/HD	186.00000*	53.08026	.033	8.6907	363.3093
S	N	333.95000*	53.08026	.000	156.6407	511.2593
	TAA	12.55000	53.08026	1.000	-164.7593-	189.8593
	PN/LD	230.35000*	53.08026	.004	53.0407	407.6593
	PN/HD	237.55000*	53.08026	.002	60.2407	414.8593
	MM/LD	237.95000*	53.08026	.002	60.6407	415.2593
	MM/HD	198.55000*	53.08026	.018	21.2407	375.8593
PN/LD	N	103.60000	53.08026	1.000	-73.7093-	280.9093
	TAA	-217.80000-*	53.08026	.007	-395.1093-	-40.4907-
	S	-230.35000-*	53.08026	.004	-407.6593-	-53.0407-
	PN/HD	7.20000	53.08026	1.000	-170.1093-	184.5093
	MM/LD	7.60000	53.08026	1.000	-169.7093-	184.9093
	MM/HD	-31.80000-	53.08026	1.000	-209.1093-	145.5093
PN/HD	N	96.40000	53.08026	1.000	-80.9093-	273.7093
	TAA	-225.00000-*	53.08026	.005	-402.3093-	-47.6907-
	S	-237.55000-*	53.08026	.002	-414.8593-	-60.2407-

	PN/LD	-7.20000-	53.08026	1.000	-184.5093-	170.1093
	MM/LD	.40000	53.08026	1.000	-176.9093-	177.7093
	MM/HD	-39.00000-	53.08026	1.000	-216.3093-	138.3093
MM/LD	N	96.00000	53.08026	1.000	-81.3093-	273.3093
	TAA	-225.40000-*	53.08026	.005	-402.7093-	-48.0907-
	S	-237.95000-*	53.08026	.002	-415.2593-	-60.6407-
	PN/LD	-7.60000-	53.08026	1.000	-184.9093-	169.7093
	PN/HD	-.40000-	53.08026	1.000	-177.7093-	176.9093
	MM/HD	-39.40000-	53.08026	1.000	-216.7093-	137.9093
MM/HD	N	135.40000	53.08026	.346	-41.9093-	312.7093
	TAA	-186.00000-*	53.08026	.033	-363.3093-	-8.6907-
	S	-198.55000-*	53.08026	.018	-375.8593-	-21.2407-
	PN/LD	31.80000	53.08026	1.000	-145.5093-	209.1093
	PN/HD	39.00000	53.08026	1.000	-138.3093-	216.3093
	MM/LD	39.40000	53.08026	1.000	-137.9093-	216.7093

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

TPROTEIN

Bonferroni

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
N	TAA	13.40000*	2.54320	.000	4.9047	21.8953
	S	5.00000	2.54320	1.000	-3.4953-	13.4953

	PN/LD	2.20000	2.54320	1.000	-6.2953-	10.6953
	PN/HD	.95000	2.54320	1.000	-7.5453-	9.4453
	MM/LD	4.40000	2.54320	1.000	-4.0953-	12.8953
	MM/HD	5.00000	2.54320	1.000	-3.4953-	13.4953
TAA	N	-13.40000-*	2.54320	.000	-21.8953-	-4.9047-
	S	-8.40000-	2.54320	.055	-16.8953-	.0953
	PN/LD	-11.20000-*	2.54320	.003	-19.6953-	-2.7047-
	PN/HD	-12.45000-*	2.54320	.001	-20.9453-	-3.9547-
	MM/LD	-9.00000-*	2.54320	.030	-17.4953-	-.5047-
	MM/HD	-8.40000-	2.54320	.055	-16.8953-	.0953
S	N	-5.00000-	2.54320	1.000	-13.4953-	3.4953
	TAA	8.40000	2.54320	.055	-.0953-	16.8953
	PN/LD	-2.80000-	2.54320	1.000	-11.2953-	5.6953
	PN/HD	-4.05000-	2.54320	1.000	-12.5453-	4.4453
	MM/LD	-.60000-	2.54320	1.000	-9.0953-	7.8953
	MM/HD	.00000	2.54320	1.000	-8.4953-	8.4953
PN/LD	N	-2.20000-	2.54320	1.000	-10.6953-	6.2953
	TAA	11.20000*	2.54320	.003	2.7047	19.6953
	S	2.80000	2.54320	1.000	-5.6953-	11.2953
	PN/HD	-1.25000-	2.54320	1.000	-9.7453-	7.2453
	MM/LD	2.20000	2.54320	1.000	-6.2953-	10.6953
	MM/HD	2.80000	2.54320	1.000	-5.6953-	11.2953

PN/HD	N	-.95000-	2.54320	1.000	-9.4453-	7.5453
	TAA	12.45000*	2.54320	.001	3.9547	20.9453
	S	4.05000	2.54320	1.000	-4.4453-	12.5453
	PN/LD	1.25000	2.54320	1.000	-7.2453-	9.7453
	MM/LD	3.45000	2.54320	1.000	-5.0453-	11.9453
	MM/HD	4.05000	2.54320	1.000	-4.4453-	12.5453
MM/LD	N	-4.40000-	2.54320	1.000	-12.8953-	4.0953
	TAA	9.00000*	2.54320	.030	.5047	17.4953
	S	.60000	2.54320	1.000	-7.8953-	9.0953
	PN/LD	-2.20000-	2.54320	1.000	-10.6953-	6.2953
	PN/HD	-3.45000-	2.54320	1.000	-11.9453-	5.0453
	MM/HD	.60000	2.54320	1.000	-7.8953-	9.0953
MM/HD	N	-5.00000-	2.54320	1.000	-13.4953-	3.4953
	TAA	8.40000	2.54320	.055	-.0953-	16.8953
	S	.00000	2.54320	1.000	-8.4953-	8.4953
	PN/LD	-2.80000-	2.54320	1.000	-11.2953-	5.6953
	PN/HD	-4.05000-	2.54320	1.000	-12.5453-	4.4453
	MM/LD	-.60000-	2.54320	1.000	-9.0953-	7.8953

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

BILIRUBIN

Bonferroni

(I)	(J)	Mean	Std. Error	Sig.	95% Confidence Interval

GROUPS	GROUPS	Difference (I-J)			Lower Bound	Upper Bound
N	TAA	-5.40000-*	.94718	.000	-8.5639-	-2.2361-
	S	-1.00000-	.94718	1.000	-4.1639-	2.1639
	PN/LD	-4.80000-*	.94718	.000	-7.9639-	-1.6361-
	PN/HD	-1.80000-	.94718	1.000	-4.9639-	1.3639
	MM/LD	-3.00000-	.94718	.078	-6.1639-	.1639
	MM/HD	-3.00000-	.94718	.078	-6.1639-	.1639
TAA	N	5.40000*	.94718	.000	2.2361	8.5639
	S	4.40000*	.94718	.002	1.2361	7.5639
	PN/LD	.60000	.94718	1.000	-2.5639-	3.7639
	PN/HD	3.60000*	.94718	.015	.4361	6.7639
	MM/LD	2.40000	.94718	.360	-.7639-	5.5639
	MM/HD	2.40000	.94718	.360	-.7639-	5.5639
S	N	1.00000	.94718	1.000	-2.1639-	4.1639
	TAA	-4.40000-*	.94718	.002	-7.5639-	-1.2361-
	PN/LD	-3.80000-*	.94718	.009	-6.9639-	-.6361-
	PN/HD	-.80000-	.94718	1.000	-3.9639-	2.3639
	MM/LD	-2.00000-	.94718	.919	-5.1639-	1.1639
	MM/HD	-2.00000-	.94718	.919	-5.1639-	1.1639
PN/LD	N	4.80000*	.94718	.000	1.6361	7.9639
	TAA	-.60000-	.94718	1.000	-3.7639-	2.5639
	S	3.80000*	.94718	.009	.6361	6.9639
	PN/HD	3.00000	.94718	.078	-.1639-	6.1639

	MM/LD	1.80000	.94718	1.000	-1.3639-	4.9639
	MM/HD	1.80000	.94718	1.000	-1.3639-	4.9639
PN/HD	N	1.80000	.94718	1.000	-1.3639-	4.9639
	TAA	-3.60000-*	.94718	.015	-6.7639-	-.4361-
	S	.80000	.94718	1.000	-2.3639-	3.9639
	PN/LD	-3.00000-	.94718	.078	-6.1639-	.1639
	MM/LD	-1.20000-	.94718	1.000	-4.3639-	1.9639
	MM/HD	-1.20000-	.94718	1.000	-4.3639-	1.9639
MM/LD	N	3.00000	.94718	.078	-.1639-	6.1639
	TAA	-2.40000-	.94718	.360	-5.5639-	.7639
	S	2.00000	.94718	.919	-1.1639-	5.1639
	PN/LD	-1.80000-	.94718	1.000	-4.9639-	1.3639
	PN/HD	1.20000	.94718	1.000	-1.9639-	4.3639
	MM/HD	.00000	.94718	1.000	-3.1639-	3.1639
MM/HD	N	3.00000	.94718	.078	-.1639-	6.1639
	TAA	-2.40000-	.94718	.360	-5.5639-	.7639
	S	2.00000	.94718	.919	-1.1639-	5.1639
	PN/LD	-1.80000-	.94718	1.000	-4.9639-	1.3639
	PN/HD	1.20000	.94718	1.000	-1.9639-	4.3639
	MM/LD	.00000	.94718	1.000	-3.1639-	3.1639

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

ALBUMIN

Bonferroni

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
N	TAA	2.58000*	.67140	.013	.3373	4.8227
	S	.25000	.67140	1.000	-1.9927-	2.4927
	PN/LD	.58000	.67140	1.000	-1.6627-	2.8227
	PN/HD	.28000	.67140	1.000	-1.9627-	2.5227
	MM/LD	-.40000-	.67140	1.000	-2.6427-	1.8427
	MM/HD	-.60000-	.67140	1.000	-2.8427-	1.6427
TAA	N	-2.58000-*	.67140	.013	-4.8227-	-.3373-
	S	-2.33000-*	.67140	.036	-4.5727-	-.0873-
	PN/LD	-2.00000-	.67140	.124	-4.2427-	.2427
	PN/HD	-2.30000-*	.67140	.040	-4.5427-	-.0573-
	MM/LD	-2.98000-*	.67140	.003	-5.2227-	-.7373-
	MM/HD	-3.18000-*	.67140	.001	-5.4227-	-.9373-
S	N	-.25000-	.67140	1.000	-2.4927-	1.9927
	TAA	2.33000*	.67140	.036	.0873	4.5727
	PN/LD	.33000	.67140	1.000	-1.9127-	2.5727
	PN/HD	.03000	.67140	1.000	-2.2127-	2.2727
	MM/LD	-.65000-	.67140	1.000	-2.8927-	1.5927
	MM/HD	-.85000-	.67140	1.000	-3.0927-	1.3927

PN/LD	N	-.58000-	.67140	1.000	-2.8227-	1.6627
	TAA	2.00000	.67140	.124	-.2427-	4.2427
	S	-.33000-	.67140	1.000	-2.5727-	1.9127
	PN/HD	-.30000-	.67140	1.000	-2.5427-	1.9427
	MM/LD	-.98000-	.67140	1.000	-3.2227-	1.2627
	MM/HD	-1.18000-	.67140	1.000	-3.4227-	1.0627
PN/HD	N	-.28000-	.67140	1.000	-2.5227-	1.9627
	TAA	2.30000*	.67140	.040	.0573	4.5427
	S	-.03000-	.67140	1.000	-2.2727-	2.2127
	PN/LD	.30000	.67140	1.000	-1.9427-	2.5427
	MM/LD	-.68000-	.67140	1.000	-2.9227-	1.5627
	MM/HD	-.88000-	.67140	1.000	-3.1227-	1.3627
MM/LD	N	.40000	.67140	1.000	-1.8427-	2.6427
	TAA	2.98000*	.67140	.003	.7373	5.2227
	S	.65000	.67140	1.000	-1.5927-	2.8927
	PN/LD	.98000	.67140	1.000	-1.2627-	3.2227
	PN/HD	.68000	.67140	1.000	-1.5627-	2.9227
	MM/HD	-.20000-	.67140	1.000	-2.4427-	2.0427
MM/HD	N	.60000	.67140	1.000	-1.6427-	2.8427
	TAA	3.18000*	.67140	.001	.9373	5.4227
	S	.85000	.67140	1.000	-1.3927-	3.0927
	PN/LD	1.18000	.67140	1.000	-1.0627-	3.4227
	PN/HD	.88000	.67140	1.000	-1.3627-	3.1227

	MM/LD	.20000	.67140	1.000	-2.0427-	2.4427

*. The mean difference is significant at the 0.05 level.

Multiple Comparisons

GGT

Bonferroni

(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
N	TAA	-10.00000-*	2.58664	.013	-18.6404-	-1.3596-
	S	-4.85000-	2.58664	1.000	-13.4904-	3.7904
	PN/LD	-7.20000-	2.58664	.200	-15.8404-	1.4404
	PN/HD	-2.60000-	2.58664	1.000	-11.2404-	6.0404
	MM/LD	-1.00000-	2.58664	1.000	-9.6404-	7.6404
	MM/HD	-1.80000-	2.58664	1.000	-10.4404-	6.8404
TAA	N	10.00000*	2.58664	.013	1.3596	18.6404
	S	5.15000	2.58664	1.000	-3.4904-	13.7904
	PN/LD	2.80000	2.58664	1.000	-5.8404-	11.4404
	PN/HD	7.40000	2.58664	.166	-1.2404-	16.0404
	MM/LD	9.00000*	2.58664	.035	.3596	17.6404
	MM/HD	8.20000	2.58664	.077	-.4404-	16.8404
S	N	4.85000	2.58664	1.000	-3.7904-	13.4904
	TAA	-5.15000-	2.58664	1.000	-13.7904-	3.4904
	PN/LD	-2.35000-	2.58664	1.000	-10.9904-	6.2904
	PN/HD	2.25000	2.58664	1.000	-6.3904-	10.8904

	MM/LD	3.85000	2.58664	1.000	-4.7904-	12.4904
	MM/HD	3.05000	2.58664	1.000	-5.5904-	11.6904
PN/LD	N	7.20000	2.58664	.200	-1.4404-	15.8404
	TAA	-2.80000-	2.58664	1.000	-11.4404-	5.8404
	S	2.35000	2.58664	1.000	-6.2904-	10.9904
	PN/HD	4.60000	2.58664	1.000	-4.0404-	13.2404
	MM/LD	6.20000	2.58664	.492	-2.4404-	14.8404
	MM/HD	5.40000	2.58664	.967	-3.2404-	14.0404
PN/HD	N	2.60000	2.58664	1.000	-6.0404-	11.2404
	TAA	-7.40000-	2.58664	.166	-16.0404-	1.2404
	S	-2.25000-	2.58664	1.000	-10.8904-	6.3904
	PN/LD	-4.60000-	2.58664	1.000	-13.2404-	4.0404
	MM/LD	1.60000	2.58664	1.000	-7.0404-	10.2404
	MM/HD	.80000	2.58664	1.000	-7.8404-	9.4404
MM/LD	N	1.00000	2.58664	1.000	-7.6404-	9.6404
	TAA	-9.00000-*	2.58664	.035	-17.6404-	-.3596-
	S	-3.85000-	2.58664	1.000	-12.4904-	4.7904
	PN/LD	-6.20000-	2.58664	.492	-14.8404-	2.4404
	PN/HD	-1.60000-	2.58664	1.000	-10.2404-	7.0404
	MM/HD	-.80000-	2.58664	1.000	-9.4404-	7.8404
MM/HD	N	1.80000	2.58664	1.000	-6.8404-	10.4404
	TAA	-8.20000-	2.58664	.077	-16.8404-	.4404
	S	-3.05000-	2.58664	1.000	-11.6904-	5.5904

	PN/LD	-5.40000-	2.58664	.967	-14.0404-	3.2404
	PN/HD	-.80000-	2.58664	1.000	-9.4404-	7.8404
	MM/LD	.80000	2.58664	1.000	-7.8404-	9.4404

*. The mean difference is significant at the 0.05 level.

3- Endogenous antioxidant analysis

Multiple Comparisons

Bonferroni

Dependent Variable	(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
TAC	N	TAA	3.35567*	.80428	.020	.3804	6.3309
		SILY	-.53467	.80428	1.000	-3.5099	2.4406
		PNLD	1.10033	.80428	1.000	-1.8749	4.0756
		PNHD	1.11133	.80428	1.000	-1.8639	4.0866
		MMLD	2.10700	.80428	.424	-.8682	5.0822
		MMHD	1.50500	.80428	1.000	-1.4702	4.4802
	TAA	N	-3.35567*	.80428	.020	-6.3309	-.3804
		SILY	-3.89033*	.80428	.006	-6.8656	-.9151
		PNLD	-2.25533	.80428	.295	-5.2306	.7199
		PNHD	-2.24433	.80428	.303	-5.2196	.7309
		MMLD	-1.24867	.80428	1.000	-4.2239	1.7266
		MMHD	-1.85067	.80428	.783	-4.8259	1.1246
	SILY	N	.53467	.80428	1.000	-2.4406	3.5099
		TAA	3.89033*	.80428	.006	.9151	6.8656
		PNLD	1.63500	.80428	1.000	-1.3402	4.6102

	PNHD	1.64600	.80428	1.000	-1.3292	4.6212
	MMLD	2.64167	.80428	.114	-.3336	5.6169
	MMHD	2.03967	.80428	.499	-.9356	5.0149
PNLD	N	-1.10033	.80428	1.000	-4.0756	1.8749
	TAA	2.25533	.80428	.295	-.7199	5.2306
	SILY	-1.63500	.80428	1.000	-4.6102	1.3402
	PNHD	.01100	.80428	1.000	-2.9642	2.9862
	MMLD	1.00667	.80428	1.000	-1.9686	3.9819
	MMHD	.40467	.80428	1.000	-2.5706	3.3799
PNHD	N	-1.11133	.80428	1.000	-4.0866	1.8639
	TAA	2.24433	.80428	.303	-.7309	5.2196
	SILY	-1.64600	.80428	1.000	-4.6212	1.3292
	PNLD	-.01100	.80428	1.000	-2.9862	2.9642
	MMLD	.99567	.80428	1.000	-1.9796	3.9709
	MMHD	.39367	.80428	1.000	-2.5816	3.3689
MMLD	N	-2.10700	.80428	.424	-5.0822	.8682
	TAA	1.24867	.80428	1.000	-1.7266	4.2239
	SILY	-2.64167	.80428	.114	-5.6169	.3336
	PNLD	-1.00667	.80428	1.000	-3.9819	1.9686
	PNHD	-.99567	.80428	1.000	-3.9709	1.9796
	MMHD	-.60200	.80428	1.000	-3.5772	2.3732
MMHD	N	-1.50500	.80428	1.000	-4.4802	1.4702
	TAA	1.85067	.80428	.783	-1.1246	4.8259
	SILY	-2.03967	.80428	.499	-5.0149	.9356

		PNLD	-.40467	.80428	1.000	-3.3799	2.5706
		PNHD	-.39367	.80428	1.000	-3.3689	2.5816
		MMLD	.60200	.80428	1.000	-2.3732	3.5772
TBARS	N	TAA	-29.66667*	5.48718	.002	-49.9650	-9.3683
		SILY	-.33333	5.48718	1.000	-20.6317	19.9650
		PNLD	-7.66667	5.48718	1.000	-27.9650	12.6317
		PNHD	1.00000	5.48718	1.000	-19.2983	21.2983
		MMLD	7.83333	5.48718	1.000	-12.4650	28.1317
		MMHD	-.75000	5.48718	1.000	-21.0483	19.5483
	TAA	N	29.66667*	5.48718	.002	9.3683	49.9650
		SILY	29.33333*	5.48718	.002	9.0350	49.6317
		PNLD	22.00000*	5.48718	.027	1.7017	42.2983
		PNHD	30.66667*	5.48718	.001	10.3683	50.9650
		MMLD	37.50000*	5.48718	.000	17.2017	57.7983
		MMHD	28.91667*	5.48718	.002	8.6183	49.2150
	SILY	N	.33333	5.48718	1.000	-19.9650	20.6317
		TAA	-29.33333*	5.48718	.002	-49.6317	-9.0350
		PNLD	-7.33333	5.48718	1.000	-27.6317	12.9650
		PNHD	1.33333	5.48718	1.000	-18.9650	21.6317
		MMLD	8.16667	5.48718	1.000	-12.1317	28.4650
		MMHD	-.41667	5.48718	1.000	-20.7150	19.8817
	PNLD	N	7.66667	5.48718	1.000	-12.6317	27.9650
		TAA	-22.00000*	5.48718	.027	-42.2983	-1.7017
		SILY	7.33333	5.48718	1.000	-12.9650	27.6317
		PNHD	8.66667	5.48718	1.000	-11.6317	28.9650

		MMLD	15.50000	5.48718	.284	-4.7983	35.7983
		MMHD	6.91667	5.48718	1.000	-13.3817	27.2150
	PNHD	N	-1.00000	5.48718	1.000	-21.2983	19.2983
		TAA	-30.66667*	5.48718	.001	-50.9650	-10.3683
		SILY	-1.33333	5.48718	1.000	-21.6317	18.9650
		PNLD	-8.66667	5.48718	1.000	-28.9650	11.6317
		MMLD	6.83333	5.48718	1.000	-13.4650	27.1317
		MMHD	-1.75000	5.48718	1.000	-22.0483	18.5483
	MMLD	N	-7.83333	5.48718	1.000	-28.1317	12.4650
		TAA	-37.50000*	5.48718	.000	-57.7983	-17.2017
		SILY	-8.16667	5.48718	1.000	-28.4650	12.1317
		PNLD	-15.50000	5.48718	.284	-35.7983	4.7983
		PNHD	-6.83333	5.48718	1.000	-27.1317	13.4650
		MMHD	-8.58333	5.48718	1.000	-28.8817	11.7150
	MMHD	N	.75000	5.48718	1.000	-19.5483	21.0483
		TAA	-28.91667*	5.48718	.002	-49.2150	-8.6183
		SILY	.41667	5.48718	1.000	-19.8817	20.7150
		PNLD	-6.91667	5.48718	1.000	-27.2150	13.3817
		PNHD	1.75000	5.48718	1.000	-18.5483	22.0483
		MMLD	8.58333	5.48718	1.000	-11.7150	28.8817
SOD	N	TAA	99.93548*	13.83137	.000	48.7701	151.1009
		SILY	9.52021	13.83137	1.000	-41.6452	60.6856
		PNLD	28.47414	13.83137	1.000	-22.6913	79.6396
		PNHD	21.48081	13.83137	1.000	-29.6846	72.6462
		MMLD	28.45548	13.83137	1.000	-22.7099	79.6209

	MMHD	5.19114	13.83137	1.000	-45.9743	56.3566
TAA	N	-99.93548*	13.83137	.000	-151.1009	-48.7701
	SILY	-90.41527*	13.83137	.000	-141.5807	-39.2498
	PNLD	-71.46133*	13.83137	.003	-122.6268	-20.2959
	PNHD	-78.45467*	13.83137	.001	-129.6201	-27.2892
	MMLD	-71.48000*	13.83137	.003	-122.6454	-20.3146
	MMHD	-94.74433*	13.83137	.000	-145.9098	-43.5789
SILY	N	-9.52021	13.83137	1.000	-60.6856	41.6452
	TAA	90.41527*	13.83137	.000	39.2498	141.5807
	PNLD	18.95393	13.83137	1.000	-32.2115	70.1194
	PNHD	11.96060	13.83137	1.000	-39.2048	63.1260
	MMLD	18.93527	13.83137	1.000	-32.2302	70.1007
	MMHD	-4.32907	13.83137	1.000	-55.4945	46.8364
PNLD	N	-28.47414	13.83137	1.000	-79.6396	22.6913
	TAA	71.46133*	13.83137	.003	20.2959	122.6268
	SILY	-18.95393	13.83137	1.000	-70.1194	32.2115
	PNHD	-6.99333	13.83137	1.000	-58.1588	44.1721
	MMLD	-.01867	13.83137	1.000	-51.1841	51.1468
	MMHD	-23.28300	13.83137	1.000	-74.4484	27.8824
PNHD	N	-21.48081	13.83137	1.000	-72.6462	29.6846
	TAA	78.45467*	13.83137	.001	27.2892	129.6201
	SILY	-11.96060	13.83137	1.000	-63.1260	39.2048
	PNLD	6.99333	13.83137	1.000	-44.1721	58.1588
	MMLD	6.97467	13.83137	1.000	-44.1908	58.1401
	MMHD	-16.28967	13.83137	1.000	-67.4551	34.8758

	MMLD	N	-28.45548	13.83137	1.000	-79.6209	22.7099
		TAA	71.48000*	13.83137	.003	20.3146	122.6454
		SILY	-18.93527	13.83137	1.000	-70.1007	32.2302
		PNLD	.01867	13.83137	1.000	-51.1468	51.1841
		PNHD	-6.97467	13.83137	1.000	-58.1401	44.1908
		MMHD	-23.26433	13.83137	1.000	-74.4298	27.9011
	MMHD	N	-5.19114	13.83137	1.000	-56.3566	45.9743
		TAA	94.74433*	13.83137	.000	43.5789	145.9098
		SILY	4.32907	13.83137	1.000	-46.8364	55.4945
		PNLD	23.28300	13.83137	1.000	-27.8824	74.4484
		PNHD	16.28967	13.83137	1.000	-34.8758	67.4551
		MMLD	23.26433	13.83137	1.000	-27.9011	74.4298
CAT	N	TAA	281.79123*	70.83483	.029	19.7570	543.8255
		SILY	123.72587	70.83483	1.000	-138.3084	385.7601
		PNLD	35.20223	70.83483	1.000	-226.8320	297.2365
		PNHD	30.91243	70.83483	1.000	-231.1218	292.9467
		MMLD	117.37303	70.83483	1.000	-144.6612	379.4073
		MMHD	52.03363	70.83483	1.000	-210.0006	314.0679
	TAA	N	-281.79123*	70.83483	.029	-543.8255	-19.7570
		SILY	-158.06537	70.83483	.893	-420.0996	103.9689
		PNLD	-246.58900	70.83483	.077	-508.6233	15.4453
		PNHD	-250.87880	70.83483	.068	-512.9131	11.1555
		MMLD	-164.41820	70.83483	.753	-426.4525	97.6161
		MMHD	-229.75760	70.83483	.124	-491.7919	32.2767
	SILY	N	-123.72587	70.83483	1.000	-385.7601	138.3084

	TAA	158.06537	70.83483	.893	-103.9689	420.0996
	PNLD	-88.52363	70.83483	1.000	-350.5579	173.5106
	PNHD	-92.81343	70.83483	1.000	-354.8477	169.2208
	MMLD	-6.35283	70.83483	1.000	-268.3871	255.6814
	MMHD	-71.69223	70.83483	1.000	-333.7265	190.3420
PNLD	N	-35.20223	70.83483	1.000	-297.2365	226.8320
	TAA	246.58900	70.83483	.077	-15.4453	508.6233
	SILY	88.52363	70.83483	1.000	-173.5106	350.5579
	PNHD	-4.28980	70.83483	1.000	-266.3241	257.7445
	MMLD	82.17080	70.83483	1.000	-179.8635	344.2051
	MMHD	16.83140	70.83483	1.000	-245.2029	278.8657
PNHD	N	-30.91243	70.83483	1.000	-292.9467	231.1218
	TAA	250.87880	70.83483	.068	-11.1555	512.9131
	SILY	92.81343	70.83483	1.000	-169.2208	354.8477
	PNLD	4.28980	70.83483	1.000	-257.7445	266.3241
	MMLD	86.46060	70.83483	1.000	-175.5737	348.4949
	MMHD	21.12120	70.83483	1.000	-240.9131	283.1555
MMLD	N	-117.37303	70.83483	1.000	-379.4073	144.6612
	TAA	164.41820	70.83483	.753	-97.6161	426.4525
	SILY	6.35283	70.83483	1.000	-255.6814	268.3871
	PNLD	-82.17080	70.83483	1.000	-344.2051	179.8635
	PNHD	-86.46060	70.83483	1.000	-348.4949	175.5737
	MMHD	-65.33940	70.83483	1.000	-327.3737	196.6949
MMHD	N	-52.03363	70.83483	1.000	-314.0679	210.0006
	TAA	229.75760	70.83483	.124	-32.2767	491.7919

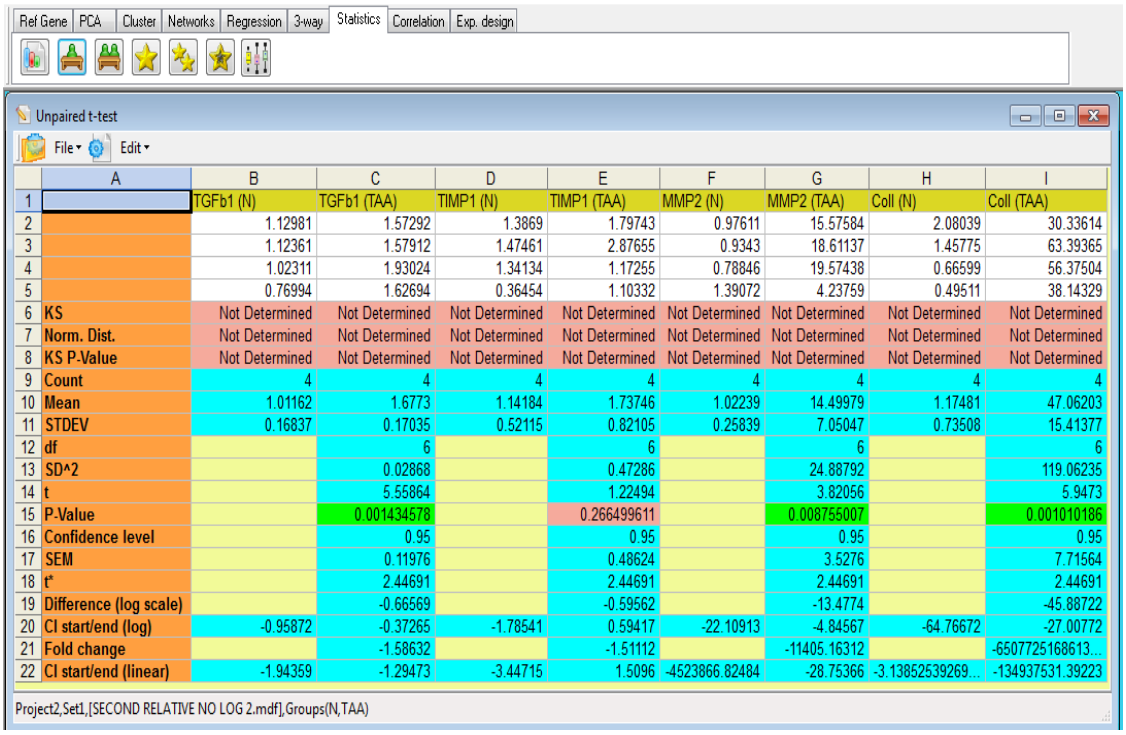
		SILY	71.69223	70.83483	1.000	-190.3420	333.7265
		PNLD	-16.83140	70.83483	1.000	-278.8657	245.2029
		PNHD	-21.12120	70.83483	1.000	-283.1555	240.9131
		MMLD	65.33940	70.83483	1.000	-196.6949	327.3737
GPX	N	TAA	20.12164	13.43171	1.000	-29.5653	69.8086
		SILY	-8.78836	13.43171	1.000	-58.4753	40.8986
		PNLD	9.55164	13.43171	1.000	-40.1353	59.2386
		PNHD	-2.80170	13.43171	1.000	-52.4887	46.8853
		MMLD	-.50836	13.43171	1.000	-50.1953	49.1786
		MMHD	-10.44170	13.43171	1.000	-60.1287	39.2453
	TAA	N	-20.12164	13.43171	1.000	-69.8086	29.5653
		SILY	-28.91000	13.43171	1.000	-78.5970	20.7770
		PNLD	-10.57000	13.43171	1.000	-60.2570	39.1170
		PNHD	-22.92333	13.43171	1.000	-72.6103	26.7636
		MMLD	-20.63000	13.43171	1.000	-70.3170	29.0570
		MMHD	-30.56333	13.43171	.822	-80.2503	19.1236
	SILY	N	8.78836	13.43171	1.000	-40.8986	58.4753
		TAA	28.91000	13.43171	1.000	-20.7770	78.5970
		PNLD	18.34000	13.43171	1.000	-31.3470	68.0270
		PNHD	5.98667	13.43171	1.000	-43.7003	55.6736
		MMLD	8.28000	13.43171	1.000	-41.4070	57.9670
		MMHD	-1.65333	13.43171	1.000	-51.3403	48.0336
	PNLD	N	-9.55164	13.43171	1.000	-59.2386	40.1353
		TAA	10.57000	13.43171	1.000	-39.1170	60.2570
		SILY	-18.34000	13.43171	1.000	-68.0270	31.3470

		PNHD	-12.35333	13.43171	1.000	-62.0403	37.3336
		MMLD	-10.06000	13.43171	1.000	-59.7470	39.6270
		MMHD	-19.99333	13.43171	1.000	-69.6803	29.6936
	PNHD	N	2.80170	13.43171	1.000	-46.8853	52.4887
		TAA	22.92333	13.43171	1.000	-26.7636	72.6103
		SILY	-5.98667	13.43171	1.000	-55.6736	43.7003
		PNLD	12.35333	13.43171	1.000	-37.3336	62.0403
		MMLD	2.29333	13.43171	1.000	-47.3936	51.9803
		MMHD	-7.64000	13.43171	1.000	-57.3270	42.0470
	MMLD	N	.50836	13.43171	1.000	-49.1786	50.1953
		TAA	20.63000	13.43171	1.000	-29.0570	70.3170
		SILY	-8.28000	13.43171	1.000	-57.9670	41.4070
		PNLD	10.06000	13.43171	1.000	-39.6270	59.7470
		PNHD	-2.29333	13.43171	1.000	-51.9803	47.3936
		MMHD	-9.93333	13.43171	1.000	-59.6203	39.7536
	MMHD	N	10.44170	13.43171	1.000	-39.2453	60.1287
		TAA	30.56333	13.43171	.822	-19.1236	80.2503
		SILY	1.65333	13.43171	1.000	-48.0336	51.3403
		PNLD	19.99333	13.43171	1.000	-29.6936	69.6803
		PNHD	7.64000	13.43171	1.000	-42.0470	57.3270
		MMLD	9.93333	13.43171	1.000	-39.7536	59.6203

*. The mean difference is significant at the 0.05 level.

B3 / Gene expression analysis using Unpaired T-test

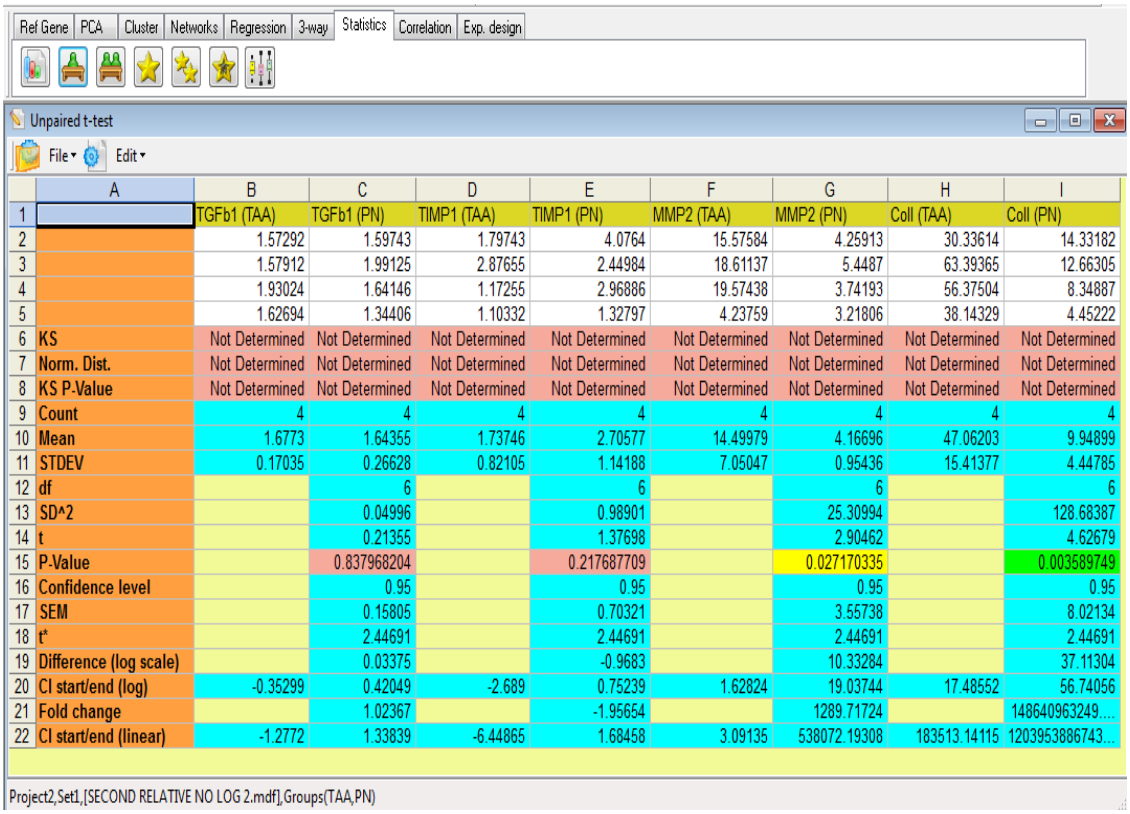
1- Comparison between N and TAA



	A	B	C	D	E	F	G	H	I
1		TGFb1 (N)	TGFb1 (TAA)	TIMP1 (N)	TIMP1 (TAA)	MMP2 (N)	MMP2 (TAA)	Coll (N)	Coll (TAA)
2		1.12981	1.57292	1.3869	1.79743	0.97611	15.57584	2.08039	30.33614
3		1.12361	1.57912	1.47461	2.87655	0.9343	18.61137	1.45775	63.39365
4		1.02311	1.93024	1.34134	1.17255	0.78846	19.57438	0.66599	56.37504
5		0.76994	1.62694	0.36454	1.10332	1.39072	4.23759	0.49511	38.14329
6	KS	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined
7	Norm. Dist.	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined
8	KS P-Value	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined
9	Count	4	4	4	4	4	4	4	4
10	Mean	1.01162	1.6773	1.14184	1.73746	1.02239	14.49979	1.17481	47.06203
11	STDEV	0.16837	0.17035	0.52115	0.82105	0.25839	7.05047	0.73508	15.41377
12	df		6		6		6		6
13	SD^2		0.02868		0.47286		24.88792		119.06235
14	t		5.55864		1.22494		3.82056		5.9473
15	P-Value		0.001434578		0.266499611		0.008755007		0.001010186
16	Confidence level		0.95		0.95		0.95		0.95
17	SEM		0.11976		0.48624		3.5276		7.71564
18	t*		2.44691		2.44691		2.44691		2.44691
19	Difference (log scale)		-0.66569		-0.59562		-13.4774		-45.88722
20	CI start/end (log)	-0.95872	-0.37265	-1.78541	0.59417	-22.10913	-4.84567	-64.76672	-27.00772
21	Fold change		-1.58632		-1.51112		-11405.16312		-6507725168613...
22	CI start/end (linear)	-1.94359	-1.29473	-3.44715	1.5096	-4523866.82484	-28.75366	-3.13852539269...	-134937531.39223

Project2,Set1,[SECOND RELATIVE NO LOG 2.mdf],Groups(N,TAA)

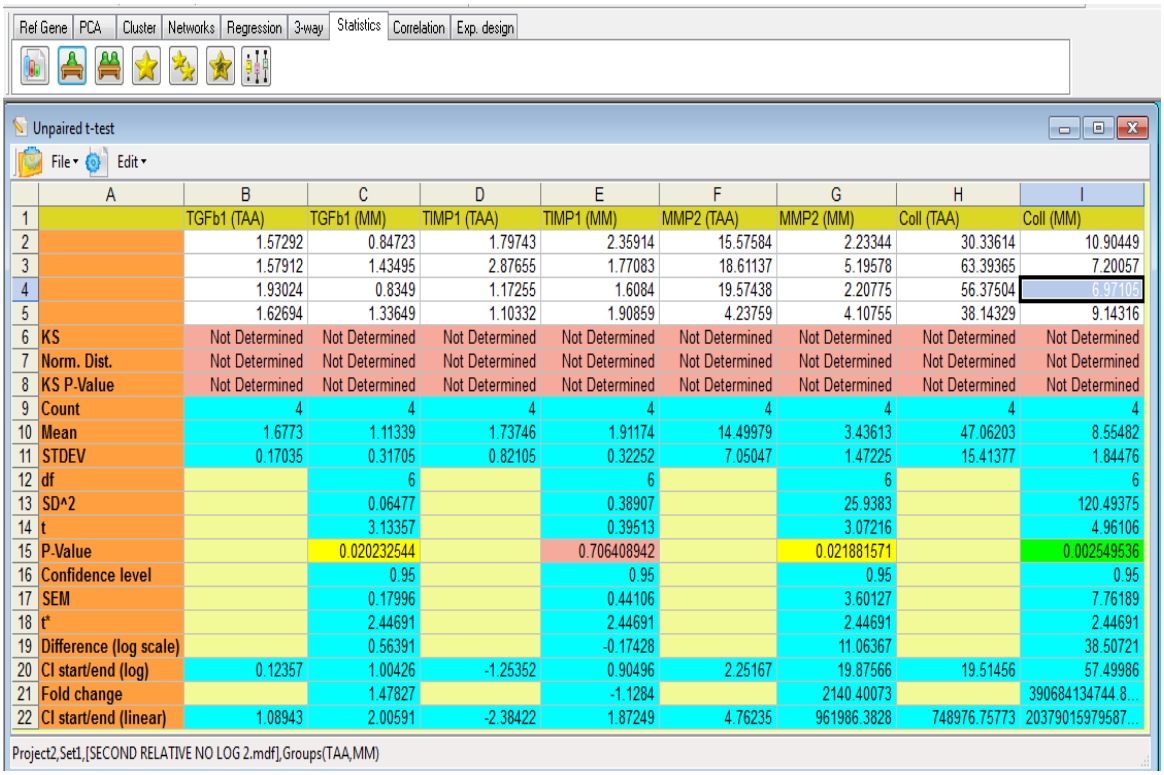
2- Comparison between PN and TAA



	A	B	C	D	E	F	G	H	I
1		TGFb1 (TAA)	TGFb1 (PN)	TIMP1 (TAA)	TIMP1 (PN)	MMP2 (TAA)	MMP2 (PN)	Coll (TAA)	Coll (PN)
2		1.57292	1.59743	1.79743	4.0764	15.57584	4.25913	30.33614	14.33182
3		1.57912	1.99125	2.87655	2.44984	18.61137	5.4487	63.39365	12.66305
4		1.93024	1.64146	1.17255	2.96886	19.57438	3.74193	56.37504	8.34887
5		1.62694	1.34406	1.10332	1.32797	4.23759	3.21806	38.14329	4.45222
6	KS	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined
7	Norm. Dist.	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined
8	KS P-Value	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined
9	Count	4	4	4	4	4	4	4	4
10	Mean	1.6773	1.64355	1.73746	2.70577	14.49979	4.16696	47.06203	9.94899
11	STDEV	0.17035	0.26628	0.82105	1.14188	7.05047	0.95436	15.41377	4.44785
12	df		6		6		6		6
13	SD^2		0.04996		0.98901		25.30994		128.68387
14	t		0.21355		1.37698		2.90462		4.62679
15	P-Value		0.837968204		0.217687709		0.027170335		0.003589749
16	Confidence level		0.95		0.95		0.95		0.95
17	SEM		0.15805		0.70321		3.55738		8.02134
18	t*		2.44691		2.44691		2.44691		2.44691
19	Difference (log scale)		0.03375		-0.9683		10.33284		37.11304
20	CI start/end (log)	-0.35299	0.42049	-2.689	0.75239	1.62824	19.03744	17.48552	56.74056
21	Fold change		1.02367		-1.95654		1289.71724		148640963249....
22	CI start/end (linear)	-1.2772	1.33839	-6.44865	1.68458	3.09135	538072.19308	183513.14115	1203953886743...

Project2,Set1,[SECOND RELATIVE NO LOG 2.mdf],Groups(TAA,PN)

3- Comparison between MM and TAA



	A	B	C	D	E	F	G	H	I
1		TGFb1 (TAA)	TGFb1 (MM)	TIMP1 (TAA)	TIMP1 (MM)	MMP2 (TAA)	MMP2 (MM)	Coll (TAA)	Coll (MM)
2		1.57292	0.84723	1.79743	2.35914	15.57584	2.23344	30.33614	10.90449
3		1.57912	1.43495	2.87655	1.77083	18.61137	5.19578	63.39365	7.20057
4		1.93024	0.8349	1.17255	1.6084	19.57438	2.20775	56.37504	6.97105
5		1.62694	1.33649	1.10332	1.90859	4.23759	4.10755	38.14329	9.14316
6	KS	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined
7	Norm. Dist.	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined
8	KS P-Value	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined	Not Determined
9	Count	4	4	4	4	4	4	4	4
10	Mean	1.6773	1.11339	1.73746	1.91174	14.49979	3.43613	47.06203	8.55482
11	STDEV	0.17035	0.31705	0.82105	0.32252	7.05047	1.47225	15.41377	1.84476
12	df		6		6		6		6
13	SD^2		0.06477		0.38907		25.9383		120.49375
14	t		3.13357		0.39513		3.07216		4.96106
15	P-Value		0.020232544		0.706408942		0.021881571		0.002549536
16	Confidence level		0.95		0.95		0.95		0.95
17	SEM		0.17996		0.44106		3.60127		7.76189
18	t*		2.44691		2.44691		2.44691		2.44691
19	Difference (log scale)		0.56391		-0.17428		11.06367		38.50721
20	CI start/end (log)	0.12357	1.00426	-1.25352	0.90496	2.25167	19.87566	19.51456	57.49986
21	Fold change		1.47827		-1.1284		2140.40073		390684134744.8...
22	CI start/end (linear)	1.08943	2.00591	-2.38422	1.87249	4.76235	961986.3828	748976.75773	20379015979587...

Project2,Set1,[SECOND RELATIVE NO LOG 2.mdf],Groups(TAA,MM)

Appendix C: Ph.D project selected photographs

Figure C1: Animal house experiment

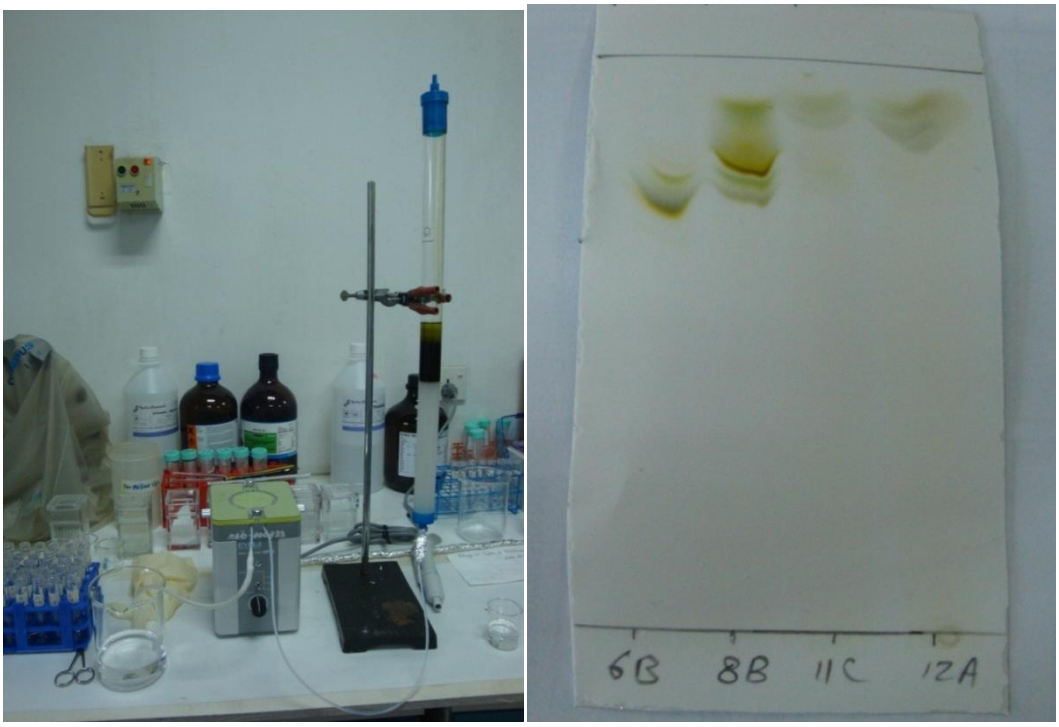


Figure C2: Flash Column Chromatography and Thin layer Chromatography plate.

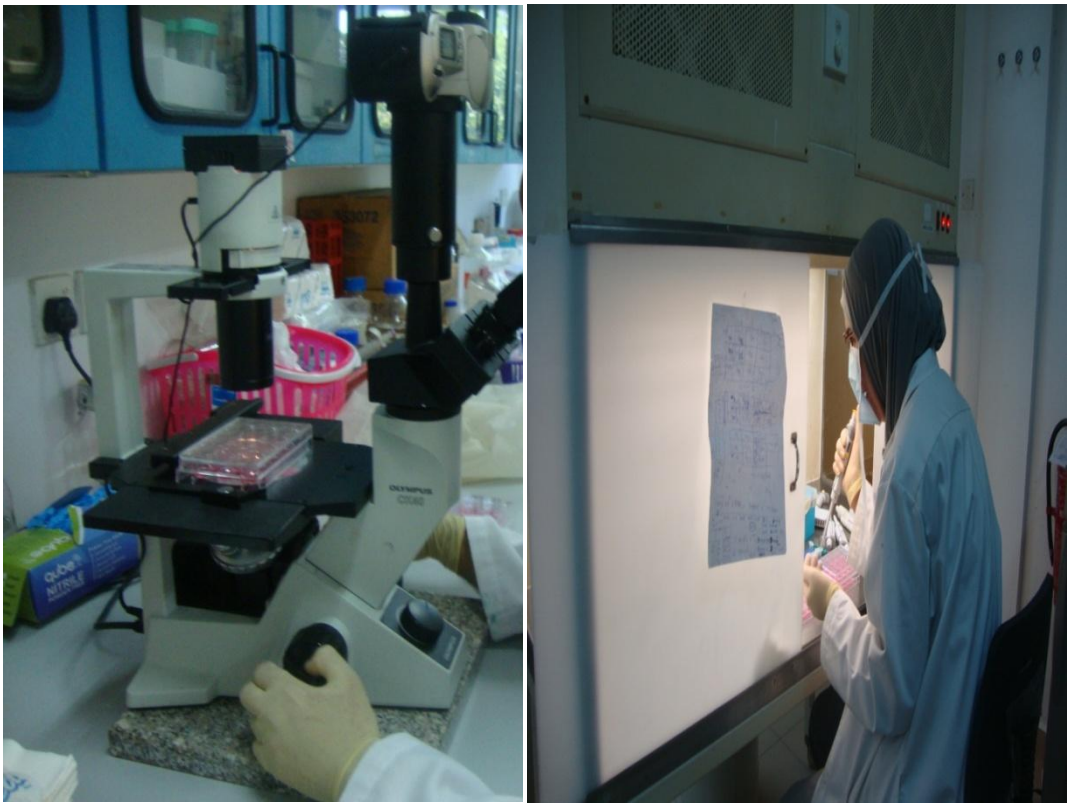


Figure C3: Immunomodulatory test photographs.

Figure C4: Histopathology slide preparation

Appendix D: Additional outputs of the Ph.D project

1. PUBLICATIONS

A/ Published articles

1) Zahra A. Amin, Mehmet Bilgen, Mohammed A. Alshawsh, Hapipah M. Ali, A. Hamid A. Hadi, and Mahmood A. Abdulla (2012). Protective Role of *Phyllanthus niruri* Extract against Thioacetamide-Induced Liver Cirrhosis in Rat Model. Evidence-Based Complementary and Alternative Medicine. doi:10.1155/2012/241583. (ISI cited journal, IF: 4.774, ranking: Q1)

2) Alnajar, Zahra A. Amin; Abdulla, Mahmood A.; Ali, Hapipah M.; Alshawsh, Mohammed A.; Hadi, A. Hamid A. 2012. "Acute Toxicity Evaluation, Antibacterial, Antioxidant and Immunomodulatory Effects of *Melastoma malabathricum*." *Molecules* 17, no. 3: 3547-3559. (ISI cited journal, IF: 2.386, ranking: Q2)

3) Amin, Z. A., Abdulla, M. A., Ali, H. M., Alshawsh, M. A. and Qadir, S. W. (2012), Assessment of *In vitro* antioxidant, antibacterial and immune activation potentials of aqueous and ethanol extracts of *Phyllanthus niruri*. J. Sci. Food Agric. doi: 10.1002/jsfa.5554. (ISI cited journal, IF: 1.36, ranking: Q1)

B/ Submitted manuscripts

1) Amin, Z. A., Abdulla, M. A., Ali, H. M., Alshawsh, M. A. Gene expression profiling reveals the underlying molecular mechanism of the hepatoprotective effect of *Phyllanthus niruri* on thioacetamide induced hepatotoxicity on *Spargue Dawely* rats. Phytomedicine, Ms. Ref. No.: PHYMED-D-12-01106. (ISI cited journal, IF: 2.66, ranking: Q1)

2) Amin, Z. A., Abdulla, M. A., Ali, H. M., Alshawsh, M. A. "Hepatoprotective efficacy of *Melastoma malabathricum* against thioacetamide induced hepatotoxicity in Sprague Dawley rats and profiling of TGFβ, Collα, MMP2 and TIMP1 genes. PLOS ONE, manuscript number: PONE-D-12-38718. (ISI cited journal, IF: 4.3, ranking: Q1)

2. ORAL PRESENTATIONS

1) Oral presentation in the 4th Kurdistan Conference on Biological Sciences held from 8th – 10th May 2012, Duhok, Iraq.

2) Oral presentation in the 3rd International Graduate Conference on Engineering, Sciences and Humanities (IGCESH) 2010 held from 2nd -4th Nov. 2010, at Universiti Teknologi Malaysia, Johor Bahru, Malaysia.

3. POSTER PRESENTATIONS

1) Poster presentation in the 15th Biological Sciences Graduate Congress 2010 held from 15th -17th Dec 2010 at Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.

2) Poster presentation in the Malaysia Technology Expo 2010 (Malaysian Association of Research Scientists) held from 4th - 6th Feb 2010, PWTC, Kuala Lumpur, Malaysia.

3) Poster presentation in the 21st International Invention, Innovation & Technology Exhibition ITEX 2010, held from 14th - 16th May 2010 Kuala Lumpur, Malaysia.

4) Poster presentation in the BioMalaysia 2010 Conference & Exhibition held from 1st -3rd Nov 2010, at Convention Centre, Kuala Lumpur, Malaysia.

5) Poster presentation in the University of Malaya Innovation and Creativity Expo 2010 held from 1st -3rd April 2010, at Dewan Tunku Canselor (DTC) University of Malaya, Kuala Lumpur, Malaysia.

4. AWARDS

1) Silver Medal, Preventive Effect of Ethanolic Extract of *Phyllanthus niruri* on Thioacetamide-induced Liver Cirrhosis in Rats. INVENTION & INNOVATION Award, 21st International Invention, Innovation & Technology Exhibition ITEX 2010 held from 14th - 16th May 2010 Kuala Lumpur, Malaysia.

2) Bronze Medal, Hepatoprotective Activity, Prophylactic and Therapeutic of Skin Diorders by Phyllannthaceae Extract. Bio Inno Awards Bio Malaysia 2010 Conference & Exhibition held from 1st – 3rd Nov 2010 at Convention Centre, Kuala Lumpur, Malaysia.