

**HEPATOPROTECTIVE, IMMUNOMODULATORY AND  
ANTIBACTERIAL EFFECTS OF SELECTED MALAYSIAN  
MEDICINAL PLANT EXTRACTS**

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## ABSTRACT

*Orthosiphon stamineus* Benth and *Morinda citrifolia* L. are considered an important traditional folk medicine and commonly used in Malaysia for treating many diseases. In this study, the ethanol extracts of *O. stamineus* and *M. citrifolia* were evaluated for their hepatoprotective and antioxidant activities; *in vivo* against thioacetamide-induced liver cirrhosis in rats and *in vitro* against H<sub>2</sub>O<sub>2</sub>-induced hepatotoxicity in WRL-68 liver cell line, as well as to investigate their immunomodulatory and antibacterial effects.

*Orthosiphon stamineus* and *Morinda citrifolia* were evaluated for their antioxidant activities using DPPH, ABTS and FRAP. In addition the phenolic and flavonoids contents were determined. Seven groups of adult SD rats were used in the hepatoprotective experiment. Group 1 as normal control group, while groups 2 to 7 were injected intraperitoneally with 200 mg/kg of thioacetamide (TAA) thrice weekly for two months and orally administered respectively with 10 % Tween 20, 50 mg/kg silymarin, 200 mg/kg and 100 mg/kg ethanol extract of *O. stamineus* and *M. citrifolia* daily for two months. The hepatoprotective activity was evaluated using the following parameters; body and liver weight, serum liver biochemical markers, liver gross morphology and histopathology, as well as endogenous antioxidant markers. Furthermore, the liver fibrosis related genes namely; TGFβ1, MMP2, TIMP1 and Coll α were estimated for the change in gene expression levels using RT-PCR. In addition, the ethanol crude extracts of *O. stamineus* and *M. citrifolia* and their isolated fractions were investigated against H<sub>2</sub>O<sub>2</sub>-induced hepatotoxicity in WRL-68 liver cell line and the percentage of cell viability using MTT assay and the antioxidant level markers were assessed. The immunomodulatory potential of the extracts were investigated by MTT assay against human peripheral blood mononuclear cells (PBMCs), while the antibacterial activity was investigated by disc diffusion and determination of minimum

inhibitory concentration (MIC) against four Gram-positive and Gram-negative bacterial strains. Finally, LC-MS was used for identification of the active constituents of the fractions that proved to have hepatoprotective activity.

*Orthosiphon stamineus* exhibited significant free radical scavenging activity with DPPH (IC<sub>50</sub> 21.4 µg/ml), at the same time, showed high total phenolic and flavonoidal contents. In animal experiments, the hepatotoxic group showed a coarse granulation on the liver surface when compared to the smooth aspect observed on the liver surface of normal and treatment groups. Histopathological study confirmed the result. Moreover, there was a significant ( $P < 0.05$ ) increase in serum liver biochemical parameters (ALT, AST, ALP and bilirubin) and the level of liver lipid peroxidation index malondialdehyde (MDA), accompanied by a significant decrease in the level of total protein, albumin, catalase, superoxide dismutase and glutathione peroxidase in the TAA control group comparing with normal group. The 200 mg/kg treatment groups of both plants significantly restored the elevated liver function enzymes and antioxidant parameters near to normal and significantly down-regulated the expression of the liver fibrosis genes. The oxidative stress by H<sub>2</sub>O<sub>2</sub> resulted in a decrease of cell viability to 41.9 %, while pre-treatment with crude extracts of *O. stamineus* and *M. citrifolia*, as well as with fraction 3 of *O. stamineus* and fraction 2 of *M. citrifolia* were found significantly ( $P < 0.01$ ) increase the cell viability to 81.1 %, 76.4 %, 95.1 % and 86.1 % respectively at concentration 100 µg/ml. The hepatoprotective and antioxidant activity could be claimed to the following flavonoids; ponkanetin, eupatorin, TMF and salvigenin that were identified in *O. stamineus* F3 and scopoletin and *P*- coumaric acid, which were identified in *M. citrifolia* F2. In conclusion, this study showed that *O. stamineus* and *M. citrifolia* exhibit potent antioxidant properties, immunomodulatory activity and could be an effective herbal and efficient remedy for chemical-induced hepatic cirrhosis, thus may be a highly promising candidate drugs.

## ABSTRAK

*Orthosiphon stamineus* dan *Morinda citrifolia* dikenali sebagai ubatan tradisional yang penting dan kerap digunakan untuk mengubati pelbagai penyakit di Malaysia. Bagi kajian ini, ekstrak etanol *O. stamineus* dan *M. citrifolia* diuji untuk menilai aktiviti pelindung hati dan antioksidan masing-masing; secara *in vivo* di dalam tikus yang mengalami sirosis hati akibat thioacetamide dan secara *in vitro* di dalam sel-sel hati WRL-68 yang mengalami ketoksikan akibat H<sub>2</sub>O<sub>2</sub>, serta menyiasat kesan modulasi sistem imun dan antimikrobial.

*O. stamineus* dan *M. citrifolia* dinilai untuk aktiviti antioksidan dengan menggunakan DPPH, ABTS, FRAP, jumlah fenol dan asai kandungan flavanoid. Tujuh kumpulan tikus SD dewasa digunakan untuk eksperimen melindungi hati. Kumpulan 1 adalah kumpulan kawalan normal, manakala kumpulan-kumpulan 2 ke 7 disuntik secara intraperitoneal dengan 200 mg/kg thioacetamide (TAA) tiga kali seminggu selama dua bulan dan diberi 10 % Tween 20, 50 mg/kg silymarin, 200 mg/kg atau 100 mg/kg ekstrak etanol *O. stamineus* and *M. citrifolia* secara oral selama dua bulan. Aktiviti perlindungan hati dinilai melalui parameter-parameter berikut; berat badan dan hati, petanda biokimia hati di dalam serum, morfologi nyata dan histopatologi hati serta petanda antioksidan tisu hati. Di samping itu, gen yang berkait dengan fibrosis hati, yakni; TGFβ1, MMP2, TIMP1 dan Coll α diuji secara RT-PCR untuk mengesan perubahan di dalam ekspresi gen. Ekstrak etanol kasar daripada *O. stamineus* dan *M. citrifolia* serta pecahan kromatografi masing-masing pula diperiksa secara *in vitro* di dalam sel hati WRL-68 yang mengalami ketoksikan akibat H<sub>2</sub>O<sub>2</sub> dan penilaian peratusan viabiliti sel menggunakan asai MTT dan petanda paras antioksidan. Potensi untuk memodulasi sistem imun oleh ekstrak-ekstrak ini dikaji melalui asai MTT ke atas sel-sel mononuklear darah periferi manusia (PBMC). Keberkesanan antibakteria dikaji dengan menggunakan cakera sebar dan kepekatan minimum untuk mencapai perencatan

(MIC) ke atas empat jenis bakteria Gram-positif dan Gram-negatif. LC-MS digunakan untuk mengenal-pasti juzuk aktif yang terbukti mempunyai aktiviti yang mampu melindungi hati.

*O. stamineus* menampakkan aktiviti memerangkap radikal bebas yang signifikan dengan DPPH IC<sub>50</sub> 21.4 µg/ml, dan juga menunjukkan kandungan jumlah fenol dan flavanoid yang tinggi. Eksperimen tikus menunjukkan kumpulan ketoksikan hati mempunyai granulasi kasar di permukaan hati berbanding dengan permukaan licin yang dilihat pada hati-hati daripada kumpulan normal dan kumpulan yang dirawat. Kajian histopatologi mengesahkan keputusan ini; di samping itu terdapat penambahan signifikan ( $P < 0.05$ ) bagi parameter biokimia hati di dalam serum (ALT, AST, ALP dan Bilirubin) dan paras indeks malondialdehyde (MDA) bagi pengoksidan lipid hati, berserta pengurangan signifikan paras jumlah protein, Albumin, katalase, superoxide dismutase dan glutathione peroksida bagi kumpulan kawalan TAA berbanding kumpulan normal. Kumpulan-kumpulan yang dirawat dengan dos 200 mg/kg memulihkan paras tinggi enzim fungsi hati dan parameter antioksidan hampir ke paras normal dan menurunkan dengan signifikan regulasi ekspresi gen fibrosis hati. Tekanan oksidatif H<sub>2</sub>O<sub>2</sub> daripada mengakibatkan viabiliti sel merosot ke 41.9 %, manakala prarawatan dengan ekstrak *O. stamineus*, *M. citrifolia*, pecahan 3 *O. stamineus* dan pecahan 2 *M. citrifolia* berkepekatan 100 100 µg/ml menyebabkan penambahan signifikan ( $P < 0.01$ ) bagi viabiliti sel ke 81.1 %, 76.4 %, 95.1 % dan 86.1 %. Kesimpulan kajian ini ialah *O. stamineus* dan *M. citrifolia* mempunyai kesan antioksidan yang kuat, aktiviti modulasi sistem imun dan berkesan sebagai rawatan herba yang efisien untuk sirosis hati yang diakibatkan oleh bahan kimia, dan mempunyai potensi yang tinggi untuk dijadikan ubat.

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## LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviations	Description
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATCC	American Type Culture Collection
BHT	Butylated hydroxytoluene
CAT	Catalase
CCl <sub>4</sub>	Carbon tetrachloride
CT	Computerized tomography
C <sub>t</sub>	Threshold cycle
CYP2E1	Cytochrome P450 2E1
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DPPH	$\alpha$ , $\alpha$ -diphenyl- $\beta$ -picryl-hydrazyl radical
ECM	Extracellular matrix
FBS	Fetal bovine serum
FC	Flavonoid content
FDA	Food and Drug Administration
FRAP	Ferric reducing antioxidant power
GPx	Glutathione peroxidase
H&E stain	Hematoxylin-eosin stain
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HD	High dose
HPLC	High performance liquid chromatography
HSC	Hepatic stellate cells
I.P	Intraperitoneal
LC-MS	Liquid chromatography-mass spectrometry
LD	Low dose

LD <sub>50</sub>	Lethal dose 50
<i>M. citrifolia</i>	<i>Morinda citrifolia</i>
MBC	Minimum bactericidal concentration
MDA	Malondialdehyde
MIC	Minimum inhibitory concentration
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
NAPBI	N-acetyl-p-benzoquinone imine
NCCLS	National committee for clinical laboratory standards
NK	Natural killer cells
<i>O. stamineus</i>	<i>Orthosiphon stamineus</i>
°C	Degree celcius
<i>P</i> value	Level of significance
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
PT	Prothrombin Time
ROS	Reactive oxygen species
Rpm	Revolution per minute
RT-PCR	Real time polymerase chain reaction
S.C	Subcutaneous
SD	<i>Sprague Dawley</i>
SEM	Standard error of the mean
SOD	Superoxide dismutase
TAA	Thioacetamide
TAC	Total antioxidant capacity
TBARS	Thiobarbituric acid reactive substance
TGF- $\beta$	Transforming growth factor $\beta$
TIMPs	Tissue inhibitors of metalloproteinases
TLC	Thin layer chromatography
TNF- $\alpha$	Tumor necrosis factor- $\alpha$

TP	Total protein
TPC	Total phenolic content
UV	Ultraviolet
WHO	World Health Organization
$\alpha$ -SMA	$\alpha$ -smooth muscle actin

# **CHAPTER I**

## **INTRODUCTION**

### **1.1 Introduction**

The liver is the largest internal organ in the body with four lobes of different size and shape and surrounded by a firm layer of connective tissue called Glisson's capsule encloses the whole liver. It gets the nutrients and oxygen through the main hepatic blood vessels; portal vein and hepatic artery. The lobes of liver are made up of many functional units called lobules. Lobules are the functional units of the liver; each lobule is made up of either parenchymal cells (hepatocytes), which are the basic metabolic cells, or non-parenchymal cells [hepatic stellate cells (HSC), kupffer cells and sinusoidal endothelial cells] (Gressner, 1991).

Liver has a pivotal role in regulation of physiological processes. It is responsible for the synthesis of blood clotting factors, bile secretion, metabolism of lipid, carbohydrate and proteins, elimination of many substances, blood detoxifications, synthesizes, and regulation of essential hormones (Heidelbaugh & Bruderly, 2006). Furthermore, detoxification of several drugs and xenobiotics takes place in liver. Liver diseases have become a worldwide problem and are associated with significant morbidity and mortality. The principal causative factors for the liver diseases in developed countries are excessive alcohol consumption, and viral-induced chronic liver diseases while in the developing countries the most frequent causes are environmental toxins, parasitic disease, hepatitis B and C viruses, and hepatotoxic drugs (certain antibiotics, chemotherapeutic agents, high doses of paracetamol, thioacetamide (TAA), carbon

tetrachloride (CCl<sub>4</sub>), etc) (Schuppan & Afdhal, 2008). Ninety percent of cases with hepatocellular carcinoma are associated with liver cirrhosis (Okazaki *et al.*, 2001).

Liver cirrhosis was introduced for the first time in 1819 by Laennec (Roguin, 2006). It is derived from the Greek word *scirrhous* and is used to describe the yellowish of the liver seen at autopsy. The fatal and irreversible cirrhosis is the end-stage of most liver pathologies of different etiologies and leads to metabolic alterations and chronic liver dysfunction. The high prevalence of these liver disorders worldwide places them among the most serious diseases. Moreover, the cost of cirrhosis on economy such as hospital costs, lost productivity and human suffering is very high. Chronic liver cirrhosis and drug induced liver injury accounting the ninth leading cause of death by disease in western and developing countries (Saleem *et al.*, 2010) . Liver cirrhosis is a disease of liver caused due to the change in structure and function of liver. Liver in patients with cirrhosis characterized by replacement of normal tissue with scar and unhealthy tissue, blocking the blood flow through the organ which in turn leads to accumulation of toxins in the body. These toxins further give rise to other complications, so it is a critical stage of chronic liver diseases that can produce liver failure accompanied with portal hypertension, bleeding tendency, an impaired metabolic function, hepatic encephalopathy and hepatocarcinoma. Liver cirrhosis developing in response to chronic hepatocellular damage characterized by particular cellular reactions that are controlled by a several cytokines and lead finally to the excessive deposition of extracellular matrix proteins (Li & Friedman, 1999). As these processes continue, the liver architecture is change resulting in severe physiological and pathology consequences. Histologically cirrhosis is defined as a widespread hepatic process characterized by the conversion of normal liver architecture into abnormal nodules and fibrosis. An appropriate degenerative development of the sclerosis connective tissue in some

patients, finally leads to cirrhosis, which is the common endpoint of a variety of chronic liver diseases (Poli, 2000).

Oxidative stress plays a key role in cell injury and necrosis induced during liver cirrhosis. To date the pathogenesis of liver fibrosis is not completely clear, but there is no doubt that free radicals including reactive oxygen species (ROS) play an important role in liver pathological changes, particularly in cases of toxic liver diseases and alcoholic intoxication (Poli & Parola, 1997). The free radicals such as ROS, including hydroxyl radicals, superoxide anions, and hydrogen peroxide, play a remarkable role in promoting tissue damage in living organisms. They may lead to cell damage through membrane lipid peroxidation and DNA mutations and as a consequence of that many diseases such as cancer may develop. Lipid peroxidation of unsaturated fatty acids in cell membranes leads to a decrease of membrane fluidity and to a disruption of membrane integrity and function, which is implicated in serious pathological changes (Halliwell, 1987). It is well known that oxidative stress involved in the pathogenesis of hepatic cirrhosis. Consequently, the oxidative stress and free radicals were considered as primary causes of the liver fibrosis. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages in liver. Enhanced lipid peroxidation produced during the liver microsomal metabolism of ethanol may result in hepatitis and cirrhosis. The protective mechanisms of dietary antioxidants may be of a great importance. Therefore, many natural and artificial agents possessing antioxidant properties have been proposed to prevent and treat hepatopathies induced by oxidative stress (Lieber, 1996).

Although fibrosis and cirrhosis are of high incidence worldwide, therapeutic management of these diseases is still insufficiently, as most of them focus mainly on symptoms rather than on blocking fibrosis mechanisms itself (Franklin, 1995). Despite of the tremendous advances made, there is no effective and safe medicine for hepatic



cirrhosis; consequently control of liver diseases has become a major goal of modern medicine. Till date, there is only few numbers of drugs available for the treatment of liver diseases (Chatterjee, 2000). Therefore, different medicinal plant extracts are tested for their potential as antioxidant and hepatoprotective liver injury in experimental animal model.

Long time ago, human started using herbal plants in the treatment of various diseases. Herbal drugs have gained popularity and importance in recent years because of their efficacy, safety, and cost effectiveness. Medicinal plants are widely used all over the world as folk medicine for several purposes. They have been used as antibacterial, antioxidant, antiulcer, anti-inflammatory, antiviral and anticancer agents especially in developing countries where infectious diseases are endemic and health services and hygiene facilities are inadequate. Estimations made by the World Health Organization (WHO) revealed that 80 % of people who live in developed countries generally use traditional medicine (Rahim & Khan, 2006). One of the most important and well-documented use of medicinal plants traditionally is their use as hepatoprotective drugs. Hence, there is an ever increasing need for safe hepatoprotective drugs (Agarwal, 2001). In the absence of reliable hepatoprotective drugs in modern medicine, a large number of herbal preparations have become increasingly popular for the treatment of liver disorders (Chatterjee, 2000). A number of herbals show promising activity, including silymarin for liver cirrhosis, *Phyllanthus amarus* in chronic hepatitis B, glycyrrhizin to treat chronic viral hepatitis, and some herbal combinations from China and Japan that have been scientifically proven for treatment of liver diseases (Stickel & Schuppan, 2007). Silymarin, a flavonolignan from “milk thistle” *Silybum marianum*, is widely used for hepatoprotection. Silymarin showed good protection in different toxic models of induced liver cirrhosis experiments by using laboratory animals (Ghosh *et al.*, 2010).

Liver cell injury caused by various toxicants such as certain chemotherapeutic agents, thioacetamide, CCl<sub>4</sub>, etc., long term alcohol consumption and some viruses is well-studied. Enhanced lipid peroxidation during metabolism of ethanol may result in development of hepatitis leading to cirrhosis. Thioacetamide (TAA) induced hepatotoxicity is an experimental model world widely used for the study of hepatoprotective effects of plant extracts and other kind of drugs. The experimental induction of liver cirrhosis by long exposure of TAA results in histological and biochemical changes resembles human liver cirrhosis (Zimmermann *et al.*, 1987). The TAA model is more reliable and easy for induced liver cirrhosis than the CCl<sub>4</sub> model (Kreft *et al.*, 1999).

Nowadays there is growing focus to evaluate scientific basis for the use of traditional herbal medicines which are claimed to possess hepatoprotective and antioxidant activity. As shown by recent *in vivo* and *in vitro* studies, plant antioxidants, particularly flavonoids and other phenolics, show a magnificent potency to prevent liver cirrhosis of different etiology (Gebhardt, 2002). Since only few number of herbs have been studied so far have just opened a wide horizon, it is worth to screen other plant extracts and natural compounds in appropriate model systems and to identify further compounds combining antioxidant properties with other effectors functions. Such studies may lead to new drugs particularly appropriate and specifically designed to block liver cirrhosis at early steps of disease. This study was carried out to assess the *in vivo* hepatoprotective and antioxidant activities of *Orthosiphon stamineus* Benth and *Morinda citrifolia* L. against thioacetamide-induced hepatotoxicity in rats to prove scientifically the traditional use of both plants against liver disorders as well as, to determine their *in vitro* antioxidant, immunomodulatory and antibacterial properties of both plants.

## **1.2 Objectives**

### **1.2.1 General**

To investigate the *in vivo* and *in vitro* hepatoprotective, antioxidant, immunomodulatory and antibacterial activities of the crude extracts of *O. stamineus* and *M. citrifolia*, as well as to identify the active constituents of both medicinal plants.

### **1.2.2 Specific**

- 1) To assess the hepatoprotective activity of *O. stamineus* and *M. citrifolia* ethanol crude extracts against thioacetamide-induced liver cirrhosis in rats (*in vivo*) and against H<sub>2</sub>O<sub>2</sub>-induced hepatotoxicity in WRL-68 normal liver cell line (*in vitro*).
- 2) To determine the antioxidant properties of the ethanol crude extracts of these two plants (*in vitro*) and in rats liver homogenate (*in vivo*).
- 3) To evaluate the changes in gene expression levels between normal, control and treatment animal groups.
- 4) To evaluate the immunomodulatory activities of the ethanol extracts of both plants against human peripheral blood mononuclear cells (PBMCs).
- 5) To fractionate and identify the active constituents of the plant extracts with hepatoprotective activity.
- 6) To assess the *in vitro* antibacterial activities of both plant extracts.

## **CHAPTER II**

### **LITERATURE REVIEW**

#### **2.1 Liver cirrhosis**

Liver is a vital organ play a major role in metabolism and excretion of drugs and xenobiotics from the body. Liver injury or liver dysfunction is a major health problem that challenges not only health care professionals but also the pharmaceutical industry and drug regulatory agencies (Heidelbaugh & Bruderly, 2006).

Cirrhosis is a complication of many liver diseases that is characterized by abnormal structure and function of the liver. The diseases that lead to cirrhosis do so because they injure and kill liver cells and the inflammation and repair that is associated with the dying liver cells causes scar tissue to form. The liver cells that do not die multiply in an attempt to replace the cells that have died. This results in clusters of newly-formed liver cells (regenerative nodules) within the scar tissue. Septa are connective tissue membranes of various widths. They result from either collapse of pre-existing parenchyma or formation of new connective tissue fibres. Septa are wide when they form after extensive collapse of lobular or nodular parenchyma. They cause functional manifestations when they connect central with portal canals and contain vessels permitting short circuits of blood flow. Cirrhosis is characterized conventionally by progressive hepatocytes injury and a nodular parenchyma followed by regeneration and widespread fibrosis, with hepatocellular necrosis leading to disorganization of lobular architecture (Schuppan & Afdhal, 2008).

Cirrhosis is better described as the result of abnormal reconstruction of the pre-existing lobular architecture. The first problem in cirrhosis, the relationship between blood and liver cells is destroyed. Even though the liver cells that survive or are newly-formed

may be able to produce and remove substances from the blood, they do not have the normal, intimate relationship with the blood, and this interferes with the liver cells' ability to add or remove substances from the blood. In addition, the scarring within the cirrhotic liver obstructs the flow of blood through the liver and to the liver cells. Because of the obstruction to flow and high pressures in the portal vein, blood in the portal vein seeks other veins in which to return to the heart, veins with lower pressures that bypass the liver. Unfortunately, the liver is unable to add or remove substances from blood that bypasses it. It is a combination of reduced numbers of liver cells, loss of the normal contact between blood passing through the liver and the liver cells, and blood bypassing the liver that leads to many of the manifestations of cirrhosis. A second reason for the problems caused by cirrhosis is the disturbed relationship between the liver cells and the channels through which bile flows. Bile is a fluid produced by liver cells that has two important functions: to aid in digestion and to remove and eliminate toxic substances from the body. The bile that is produced by liver cells is secreted into very tiny channels that run between the liver cells that line the sinusoids, called canaliculi. The canaliculi empty into small ducts which then join together to form larger and larger ducts. Ultimately, all of the ducts combine into one duct that enters the small intestine. In this way, bile gets to the intestine where it can help with the digestion of food. At the same time, toxic substances contained in the bile enter the intestine and then are eliminated in the stool. In cirrhosis, the canaliculi are abnormal and the relationship between liver cells and canaliculi is destroyed, just like the relationship between the liver cells and blood in the sinusoids. As a result, the liver is not able to eliminate toxic substances normally, and they can accumulate in the body. To a minor extent, digestion in the intestine also is reduced (Schuppan & Afdhal, 2008).

### **2.1.1 Causes of liver cirrhosis**

Liver cirrhosis is among the most serious diseases. It may be developed as a consequence of chronic viral hepatitis B and/or C, excessive alcohol consumption, biliary obstruction, non-alcoholic fatty liver disease, autoimmune liver diseases, hemochromatosis, alpha-1 antitrypsin deficiency, Wilson's disease, and liver cell injury due to toxic chemicals such as certain antibiotics, chemotherapeutic agents, aflatoxin, thioacetamide, carbon-tetrachloride, chlorinated hydrocarbons, etc.) (Heidelbaugh & Bruderly, 2006). Although less likely, other causes of cirrhosis include parasitic infections and prolonged exposure to environmental toxins. Sometimes more than one cause is present in the same patient. In the western world, chronic alcoholism and hepatitis C are the most common causes. Numerous clinical studies have demonstrated that the patients with hepatitis B virus (HBV) and hepatitis C virus (HCV) infection may progress to liver cirrhosis. More than 60-70 % of individuals infected with HCV develop chronic liver disease with intermittent necrosis and fibrosis. A significant number of patients with HCV chronic hepatitis progress to cirrhosis and even to hepatocellular carcinoma (Davis *et al.*, 1994; Poynard *et al.*, 1997). It has been estimated that approximately 14 - 16 millions of people are infected with this virus in South East Asia region and about 6 % of the total population in that region are carriers of this virus. A vaccine has become available for immunization against hepatitis B virus. Hepatitis C and hepatitis E infections are also common in countries of South East Asia region. However, some liver cirrhosis occurs in patients without evidence of hepatotropic viral infection. Tobacco smoking with alcohol consumption according to (Corrao *et al.*, 1994) plays a role in causing liver cirrhosis. Little is known on modulators of cirrhosis risk, apart from other diseases that cause liver injury (such as the combination of alcoholic liver disease and chronic viral hepatitis, which may act synergistically in leading to cirrhosis). Alcoholic cirrhosis has a worse prognosis than

primary biliary cirrhosis and cirrhosis due to hepatitis. The risk of death due to all causes is increased twelvefold; if one excludes the direct consequences of the liver disease, there is still a fivefold increased risk of death in all disease categories (Sorensen *et al.*, 2003).

### **2.1.2 Pathology**

Hepatic fibrosis is a wound healing process and the pathology of liver cirrhosis is characterized by the abnormal and progressive deposition of large amount of extracellular matrix (ECM) proteins in response to liver injury (Stalnikowitz & Weissbrod, 2003). Liver cirrhosis has been believed to be an irreversible disease which causes serious complications. Cirrhosis is defined as diffuse involvement of the whole liver with slowly progressive necrosis of liver cells (hepatocytes) leading to liver failure. Pathogenesis of cirrhosis is complex. The liver cells are injured by a chronic disease process, which then undergo inflammatory changes leading to cell death (necrosis) and fibrosis. Fibrosis is initiated by activation of stellate cells, which are induced by cytokines, active oxygen intermediates, autocrine signals and paracrine signals implemented by hepatocytes and Kupffer cells. Stellate cells initiate fibrosis by swelling, loosing retinoids and up regulating receptors for proliferative cytokines transforming growth factor  $\beta 1$  (TGF- $\beta 1$ ) and platelet-derived growth factor (PDGF). Stellate cell activation in chronic liver disease also leads to expression of  $\alpha$ -actin, which is a contractile protein. The contractions are induced by endothelin, nitrous oxide and prostaglandins. Normal matrix in spaces of disse in the liver are replaced by collagen 1, 3 and fibronectin. Sub-endothelial fibrosis leads to the loss of endothelial fenestrations resulting in the impairment of liver functions. Tissue inhibitors of metaloproteinases (TIMPs) increase and inhibit matrix metaloproteinases (MMPs) (degrade excessive matrix collagen). The deposition of collagen and its maturation is

enhanced around surfaces with reduced fluid exchange like basement membranes, damaged hepatocytes, and loaded macrophages, as well as by reduced oxygen tension (Popper, 1977). Hepatic stellate cells, which reside in the space of disse in close contact with both sinusoidal endothelial cells and hepatocytes, play multiple roles in the pathophysiology of the liver. When liver injury occurs, they undergo transformation into myofibroblasts that actively proliferate in response to PDGF, produce increased amount of ECM material, show augmented contractility accompanied by the expression of smooth muscle  $\alpha$ -actin, secrete TGF- $\beta$  and monocyte chemotactic protein-1 (MCP-1), lose the retinoid, and exhibit active apoptosis (Friedman, 1997). Liver injury is associated with activation of hepatic stellate cells. Upon liver injury (Figure 2.1), changes occur in the subendothelial space of disse and sinusoid such as alterations in both cellular responses and ECM composition. This contributes to the loss of hepatocyte microvilli and sinusoidal endothelial fenestrae, which result in deterioration of hepatic function. Kupffer cell (macrophage) activation accompanies liver injury and contributes to paracrine activation of stellate cells. HSCs major phenotypic changes after activation include proliferation, contractility, fibrogenesis (synthesis of fibrotic matrix rich in collagen I), matrix degradation, chemotaxis, retinoid loss, and white blood cell chemoattraction. The ECM components in fibrotic liver are similar regardless of the underlying cause (Safadi & Friedman, 2002). The pathological changes seen in cirrhosis are important in the diagnosis of the disease as well as for etiological diagnosis. They include fibrosis involving both central veins and peripheral areas, formation of regenerative nodules due to the hyperplasia of surviving liver cells and distortion of normal architecture of the liver. Macroscopically, the liver is initially enlarged, but with progression of the disease, it becomes smaller. Its surface is irregular, the consistency is firm and the colour is often yellow. Depending on the size of the nodules there are three macroscopic types: micronodular, macronodular and mixed cirrhosis. In micronodular



form (portal cirrhosis) regenerating nodules are less than 3 mm of uniform size. In macronodular cirrhosis (post-necrotic cirrhosis), the nodules are larger than 3 mm. The mixed cirrhosis consists in a variety of nodules with different sizes. Microscopically the nodules are composed of hyperplastic hepatocytes separated by fibrous connective tissue. Excess hepatic connective tissue results from collapse (passive septa) or from formation of new fibres (active septa). Collapse induces additional fibre formation. Connective tissue consists of a matrix of glycoproteins and proteoglycans, many sulfated, and scleroproteins of which collagen is the most important in the liver. The liver cells are enlarged and the vasculature distorted due to the fibrosis involving portal triads. Fistulous communications may be formed between portal veins and hepatic arterioles. The fibrosis in cirrhosis can lead to destruction of other normal tissues in the liver: including the sinusoids, the space of disse, and other vascular structures, which leads to altered resistance to blood flow in the liver and portal hypertension (Heidelbaugh & Bruderly, 2006).

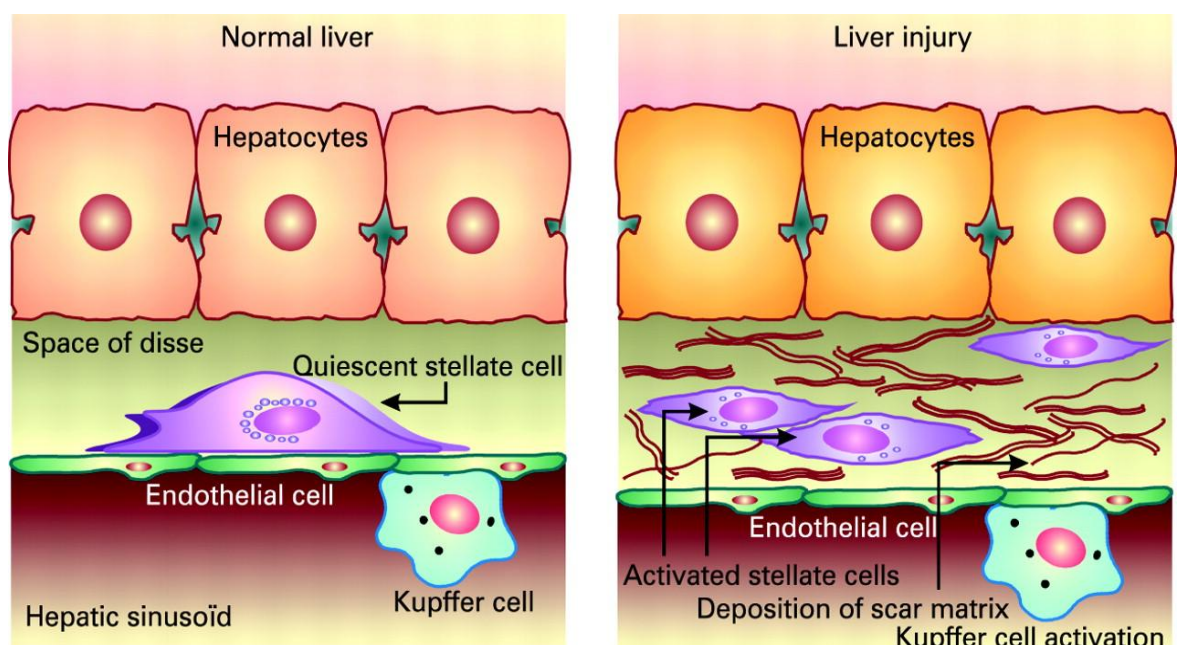


Figure 2.1 Diagram illustrates the four major liver cell types (hepatocytes, Kupffer cells, endothelial cells and stellate cells) in healthy and injured liver. (Adapted from (Asselah *et al.*, 2009)).

### **2.1.3 Immunology (molecular and cellular aspects of liver cirrhosis)**

Complex cellular and molecular mechanisms resulting from chronic activation of tissue repair mechanism following liver tissue injury have been characterized. Liver damage is always associated with cellular necrosis, increase lipid peroxidation and depletion in the tissue glutathione levels. In addition serum level of many biochemical markers like AST, ALT, Alkaline phosphatase and bilirubin are elevated. Thus, cirrhosis is a consequence of chronic liver disease characterized by replacement of liver tissue by scar tissue, fibrosis, regenerative nodules, and blocking the portal flow of blood through the organ leading to loss of liver function. Hepatic fibrosis, or the deposition of ECM, is associated with inflammation and cell death, which accompanies the repair processes, and is a consequence of severe liver damage that occurs in many patients with chronic liver injury of any etiology (Re *et al.*, 1999). Many forms of liver injury are marked by fibrosis. Fibrosis is defined as an excess deposition of the components of ECM (i.e., collagens, glycoproteins, and proteoglycans) within the liver. This response to liver injury potentially is reversible. In contrast, in most patients, cirrhosis is not a reversible process. The fibrotic process recognises the involvement of various cells and different factors in bringing about an excessive fibrogenesis with disruption of intercellular contacts and interactions of extracellular matrix composition. However, Kupffer cells, together with recruited mononuclear cells, and hepatic stellate cells are by far the key-players in liver fibrosis. Their cross-talk is triggered and favoured by a series of chemical mediators, with a prominent role played by the TGF- $\beta$  (Poli, 2000). In liver, the inflammation process gives rise to different pathways of lymphocyte recruitment and migration, probably directly related to type of insult. These pathways involve portal tract, sinusoid and hepatic vein. Composition and distribution of the inflammatory infiltrate may include T lymphocytes (more peripheral) B lymphocytes (mainly central), plasma cells, histiocytes (granuloma), eosinophils, neutrophils, natural killer (NK) cells,

and mast cells, which in turn give rise to secondary changes such as phenotypic differences among different vascular compartments (Gutiérrez-Ruiz *et al.*, 2002). Leukocytes that are recruited to the liver during injury join with Kupffer cells in producing compounds that modulate stellate cell behavior. Monocytes and macrophages are involved in inflammatory actions by producing large amounts of nitric oxide (NO) and inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) which have a direct stimulatory effect on stellate cell collagen synthesis. In recent years, a significant role for pro-inflammatory cytokines such as TNF- $\alpha$ , in the onset of liver disease, has been indicated both by clinical observations of an enhanced circulating level of TNF- $\alpha$  and other cytokines in patients and by results of studies with animal models. Kupffer cells respond to the endotoxin challenge by producing a battery of cytokines and chemokines, including TNF- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6, and prostaglandin E2 (McClain *et al.*, 1999; Thurman *et al.*, 1999). The influx of Kupffer cells coincides with the appearance of stellate cell activation markers. Kupffer cells can stimulate matrix synthesis, cell proliferation, and release of retinoids by stellate cells through the actions of cytokines especially TGF- $\beta$ 1 and reactive oxygen intermediates/lipid peroxides. Kupffer Cells, the resident macrophages in the sinusoids of the liver, have been widely implicated in hepatic injury such as endotoxin-mediated liver injury.

In the injured liver, HSCs are regarded as the primary target cells for inflammatory and per-oxidative stimuli, and are transformed into myofibroblast-like cells. These HSCs are referred to as activated cells, and this activation is accompanied by a loss of cellular retinoid, and the synthesis of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and large quantities of the major components of the ECM, including collagen types I, III, and IV, fibronectin, and laminin (Cassiman *et al.*, 2001). During active hepatofibrogenesis, however, HSCs become the major ECM producing cell type, with a predominant production of collagen type I. Activation of HSC is regulated by several soluble factors, including cytokines

such as TGF- $\beta$ 1, chemokines, growth factors, and products of oxidative stress as well as by extensive changes in composition and organization of extracellular matrix components. There is evidence to show that the products of lipid peroxidation modulate collagen gene expression in HSC (Bedossa *et al.*, 1994). Of all the cytokines and growth factors produced TGF- $\beta$  and IL- 6 are two main fibrogenic cytokines. While HSC activation is taking place in liver and cytokines and signal transduction pathways are being stimulated, TGF- $\beta$ 1 plays a central role in fibrosis, contributing to influx and activation of inflammatory cells as well as activation of HSC. TGF- $\beta$  is produced by Kupffer cells and HSC, it up-regulates the transcription of collagen I genes and induces the expression of TIMP-1, a tissue inhibitor of MMPs involved in collagen degradation. Four members of the family of the TIMPs have been so far described (Arthur, 1998). Interestingly they are able to inhibit all MMPs. IL- 6 which is produced by HSC from normal or cirrhotic livers, it up- regulates the expression of TGF- $\beta$  in HSC from cirrhotic livers. Previous study indicates that reactive oxygen intermediates in general, and H<sub>2</sub>O<sub>2</sub> in particular, are important mediators of TGF- $\beta$  actions in HSC (Friedman, 1997). Following cell activation *in vivo*, HSCs express the genes encoding the key components required for matrix degradation such as MMP-1 and -2. In normal liver, matrix protein degradation is accomplished by a family of enzymes called MMPs. To date, four subclasses of MMPs have been defined on the basis of relative substrate specificity. Stellate cells and Kupffer cells are certainly recognized as source of MMPs (Benyon & Arthur, 2001). However, through the activation of tissue inhibitor of TIMP-1 and -2, activated HSCs also inhibit the activity of interstitial collagenases, which degrade fibrillar collagen. Most of the hepatocellular injury inducer and subsequently hepatic inflammation, finally resulting in HSC activation and collagen deposition (Bataller & Brenner, 2001). Although hepatocytes are the major site for oxygen utilization, non-parenchymal cells also are sources of ROS, and may thereby contribute

to hepatocyte necrosis and/or HSC activation. Kupffer cells are activated by a variety of stimuli to produce ROS. ROS include hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), hydroxyl (OH), superoxide ( $\text{O}_2^-$ ) and nitric oxide (NO) free radicals, highly heterogeneous in terms of reactivity against cellular targets. Inflammatory cells, such as Kupffer cells and invading mononuclear cells, which release cytokines, TGF- $\beta$  1 and PDGF may also contribute to the fibrogenic response to liver injury (Figure 2.2). Furthermore, TGF-  $\beta$  1 is a key fibrogenic mediator that can enhance ECM deposition and inhibit MMP activity (Casini *et al.*, 1993). It is also noteworthy that TGF-  $\beta$  is an inhibitor of the proliferation of hepatocytes, and that, at higher concentrations, TGF-  $\beta$  induces oxidative stress leading to hepatocyte apoptosis. After that, the fibrous tissue (septa) separate hepatocyte nodules, which eventually replace the entire liver architecture, leading to decreased blood flow throughout.

There is extensive evidence of altered immune reactivity in some types of liver disease. Mitochondrial antibody, smooth muscle antibody, and antinuclear factors have been demonstrated in primary biliary cirrhosis and chronic active hepatitis but the pathogenic significance of these auto-antibodies is uncertain. The histology of these diseases shows infiltration with small lymphocytes but specific cell-mediated immune reactivity has been little studied so far (Flier *et al.*, 1993).

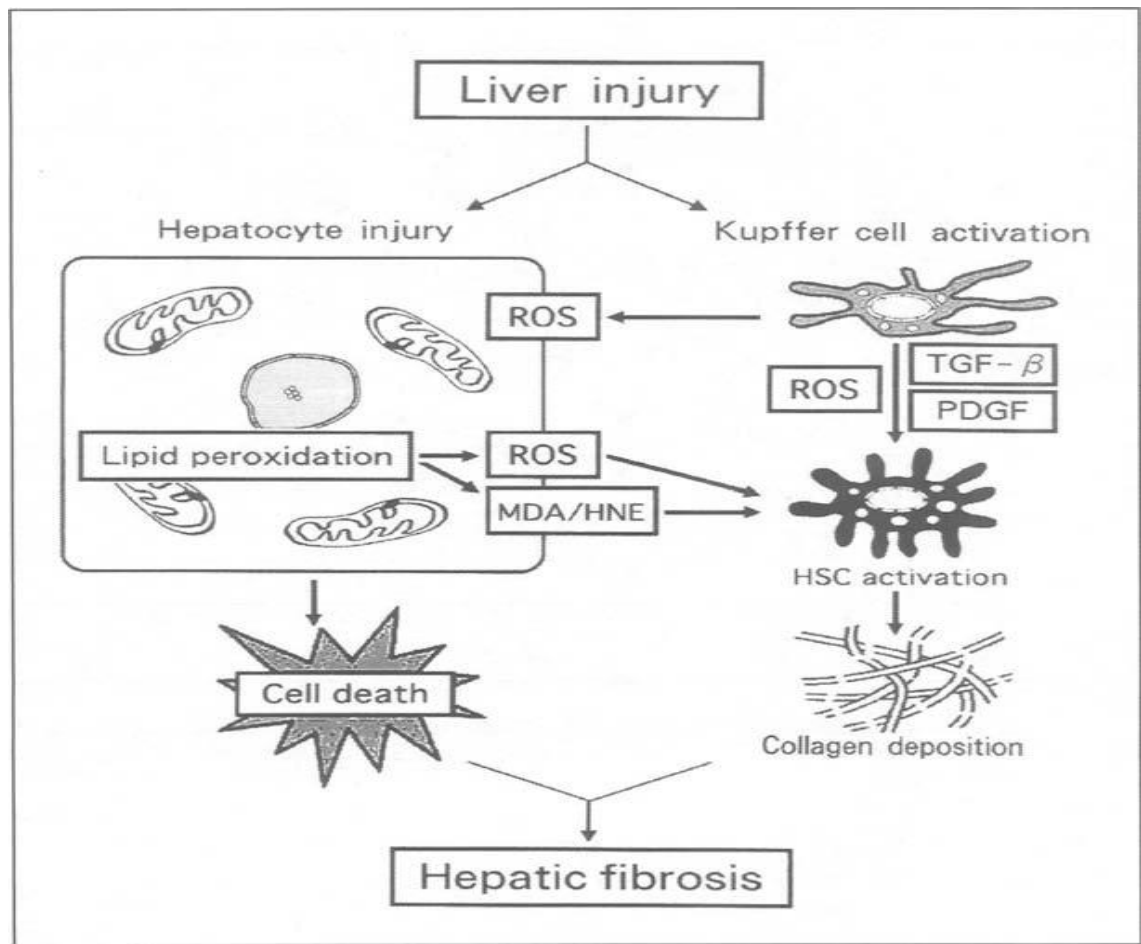


Figure 2.2 Diagram illustrates liver injury-mediated hepatofibrogenesis. MDA: malondialdehyde; HNE: 4-hydroxynonenal; TGF: transforming growth factor; PDGF: platelet-derived growth factor. [Adapted from (Re *et al.*, 1999)].

#### 2.1.4 Symptoms and complication

In most cases with liver cirrhosis have no symptoms in the early stages of the disease. However, as scar tissue replaces healthy cells, liver function starts to fail and a person may experience the following symptoms (Schuppan & Afdhal, 2008):

- Jaundice (Yellowing of the skin and eyes)
- Fatigue and weakness
- Itching
- Loss of appetite and nausea
- Easy bruising from decreased production of blood clotting factors.
- Weight loss

- Nail changes such as Muehrcke's lines and Terry's nails (proximal two-thirds of the nail plate appears white with distal one-third red) due to hypo-albuminemia.
- Hepatomegaly and splenomegaly (increase in size).
- Musty odor in breath as a result of increased dimethyl sulfide.

As the disease progresses, the following complications may develop. In some people, these may be the first signs of the disease:

- Edema and ascites: When the liver loses its ability to make the protein albumin, water accumulates in the leg (edema) and abdomen (ascites).
- Spontaneous bacterial peritonitis
- Esophageal variceal Bleeding
- Hepatic encephalopathy
- Portal hypertension
- Hypersplenism
- Liver cancer (hepatocellular carcinoma)

### **2.1.5 Diagnosis**

Liver cirrhosis may be diagnosed through more than one of the following methods (Schuppan & Afdhal, 2008):

- Physical examination, patient history and symptoms.
- Liver biopsy, which is the key for diagnosis.
- Computerized tomography (CT or CAT) or magnetic resonance imaging (MRI) scans, and ultrasound examinations.
- Laboratory finding:
  1. Abnormal elevation of bilirubin and other liver enzymes in blood (such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST)).

2. Slightly increase in alkaline phosphatase (ALP).
3. Reduced level of albumin in blood.
4. Prothrombin time (PT) increases, thrombocytopenia and reduced blood clotting factors.

#### **2.1.6 Prevention**

There are several ways to reduce the risk of developing liver cirrhosis (Flier *et al.*, 1993):

- Stop abusing alcohol or limiting how much and how often of drink for those who do drink alcohol.
- Avoiding high-risk sexual behaviour such as unprotected sexual contact with multiple partners.
- Wearing protective clothing and a facemask for people who in contact with synthetic chemicals, such as cleaning products and pesticides.
- Vaccination of susceptible patients against hepatitis B.
- Eating a well-balanced, low-fat diet and taking vitamins.

#### **2.1.7 Treatment**

Treatment options for liver diseases such as cirrhosis, fatty liver, and chronic hepatitis are problematic. Although there is no cure for cirrhosis of the liver, but treatment can stop or delay further progression, minimize the damage to liver cells and reduce complications. The effectiveness of treatments such as interferon, colchicine, penicillamine, and corticosteroids are associated with the risk of relapse and the incidence of side-effects profound (Mavier & Mallat, 1995). So to date, no treatment has revealed efficient to prevent or to avoid the progress of this pathology and, in most



instances, patients receive treatment for the symptoms of cirrhosis (Mavier & Mallat, 1995). Mostly the treatment aims the following:

- Preventing further damage to the liver
- Treating the complications of cirrhosis.
- Preventing liver cancer or detecting it early.

When complications cannot be controlled or when the liver becomes so damaged from scarring that it completely stops functioning, a liver transplant is necessary. Physicians and patients are in need of effective therapeutic agents with low incidence of side-effects.

#### **2.1.8 Liver cirrhosis and medicinal plants**

Medicinal plants have formed the basis for treatment of diseases in traditional medicine for thousands of years and continue to play a major role in the primary health. World Health Organization (WHO) reported that 80 % of the world population rely on the use of traditional medicine which is predominantly based on plant materials (WHO, 1993). According to Newman *et al.* (2003) all drugs approved by the US Food and Drug Administration (FDA), finding that 42 % of the 1031 drugs approved between 1981 and 2002 are associated to natural products. The available synthetic drugs to treat liver disorders in this condition also cause further damage to the liver. Hence, herbal drugs have become increasingly popular and their use is wide-spread. The use of natural remedies for the treatment of liver diseases has a long history, starting with the Ayurvedic treatment, and extending to the Chinese, European and other systems of traditional medicines. The 21<sup>st</sup> century has seen a paradigm shift towards therapeutic evaluation of herbal products in liver disease models by carefully synergizing the strengths of the traditional systems of medicine with that of the modern concept of

evidence-based medicinal evaluation, standardization and randomized placebo controlled clinical trials to support clinical efficacy (Thyagarajan *et al.*, 2002). Different herbal plants as natural sources have been evaluated for the treatment of hepatocellular damage in experimental animal models (Luper, 1998). In recent years many researchers have examined the effects of plants used traditionally by indigenous healers and herbalists to support liver function and treat diseases of the liver. In most cases, research has confirmed traditional experience and wisdom by discovering the mechanisms and modes of action of these plants as well as reaffirming the therapeutic effectiveness of certain plants or plant extracts in clinical studies. Several hundred plants have been examined for use in a wide variety of liver disorders. Just a handful has been fairly well researched. The latter category of plants include: *Silybum marianum* (milk thistle), *Picrorhiza kurroa* (kutkin), *Curcuma longa* (turmeric), *Camellia sinensis* (green tea), *Chelidonium majus* (greater celandine), *Glycyrrhiza glabra* (licorice), and *Allium sativa* (garlic) (Luper, 1999). The constituents of plant extracts have immune stimulating, liver protective and anti-inflammatory actions. The herbs stimulate hepatocytes production and for repairing and regeneration hepatocytes. Accumulative evidence for the effectiveness against fibrosis is now available for several plant-derived antioxidants. The most successful liver protective natural product is silymarin, a flavonolignan from the seeds of milk thistle *Silimum marianum* L. This extract is used exclusively for liver protection. Silymarin proved to be antifibrogenic in a rat fibrosis model (Jia *et al.*, 2001), where it led to a reduction of hepatic collagen accumulation by more than 35 %. It is well known that silymarin and its component silibinin have potent antioxidant activity (Pietrangelo *et al.*, 1995). Medicinal plants are an important source of antioxidants (Rice-Evans, 2004). The secondary metabolites like phenolics and flavonoids from plants have been reported to be potent free radical scavengers. They are

found in all parts of plants such as leaves, fruits, seeds, roots and bark (Mathew & Abraham, 2006).

## **2.2 Approaches to study hepatoprotective activity and screening models**

Hepatoprotective agents are those compounds, which prevent the liver injury caused by hepatotoxic agents (Lee *et al.*, 2000). There are several different approaches for the assessment of hepatoprotective activity such as the experimental animal (*in vivo*) or using *in vitro* model systems like rat isolated hepatocytes. Although the efficacy of hepatoprotective agents can best be demonstrated by *in vivo* studies, mechanistic details of this activity that can be derived from these studies, however, are sparse. Therefore, many *in vitro* studies with plant extracts, less complex fractions or isolated compounds give more information.

### **2.2.1 *In vivo* models**

*In vivo* studies are the primary tools used to study liver damage and resolution of liver injury. For this purpose, animals are treated or pretreated with plant extracts, and liver injury is induced chemically. By induced hepatotoxicity using toxic chemical dose or repeated dose of hepatotoxin in rats and mice as they virtually mimic any form of naturally occurring liver disease. Hepatoprotective effects of herbal drugs and plant extraction are studied *in vivo* against chemicals (thioacetamide (TAA), Carbon tetrachloride (CCl<sub>4</sub>), alcohol, beta galactosamine,) and drugs (paracetamol, isoniazid, and rifampicin) (Sumanth, 2007). The hepatoprotective tested plant is usually administrated prior or along with the toxin, and if it's effective it will prevent or reduce the liver injury. Morphological parameters such as liver volume, visible signs of necrosis and cirrhosis, biochemical parameters are evaluated to assess the damage. Among regular clinical analyses, the most useful are serum bilirubin, serum albumin,

serum alkaline phosphatase, serum ALT, AST, prothrombin time, gamma-glutamyl transpeptidase, and lactate dehydrogenase tests (Rahman *et al.*, 2001; Schuppan *et al.*, 1999).

Mode of hepatotoxicity of CCl<sub>4</sub> is through membrane damage of hepatocyte, while TAA leads to centrilobular necrosis. The TAA model is easier to perform and more reliable in liver cirrhosis induction than the CCl<sub>4</sub> models (Kreft *et al.*, 1999). Since the TAA, CCl<sub>4</sub> and Paracetamol induced liver cirrhosis are the most widely used models, the mechanism of liver injury by these chemicals are explained below.

#### **2.2.1.1 Thioacetamide induced hepatotoxicity model**

Thioacetamide (Figure 2.3), originally used as a fungicide, is potent hepatotoxin bio-activated by cytochrome P450 to sulfine (sulfoxide) and sulfene (sulfone) metabolites; it is known to induce liver cirrhosis in rats, which is caused by free radical-mediated lipid peroxidation. Thioacetamide administration leads to liver damage in rats marked by increase ALT, AST in serum and malondialdehyde (MDA) in liver, also centrilobular necrosis in hepatic architecture (Ahmad *et al.*, 1999). Thioacetamide interferes with the movement of RNA from the nucleus to cytoplasm which may cause membrane injury. A metabolite of thioacetamide is responsible for hepatic injury. Thioacetamide reduce the number of viable hepatocytes as well as rate of oxygen consumption. Usually TAA dosage is 100 to 300 mg/kg, administrated subcutaneous (S.C) or intraperitoneal (I.P) (Ahmad *et al.*, 2002; Aydin *et al.*, 2009; Madani *et al.*, 2008). Long term administration and/or high doses of TAA results in a biochemical changes, histological and characteristic lesion in rat liver, which corresponds to cirrhosis-like patterns of micro nodular liver cirrhosis in humans and associated protein-energy malnutrition. Investigation on therapeutic principles should be done during thioacetamide

administration (prophylactic agents) or within 2 months after withdrawal of toxic agents (therapeutics) (Muller *et al.*, 1988; Zimmermann *et al.*, 1987).

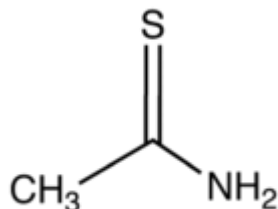


Figure 2.3 Thioacetamide chemical structure

#### **2.2.1.2 Carbon tetrachloride induced hepatotoxicity model**

Carbon tetrachloride is a strong hepatotoxin producing hepatic necrosis. Liver injury due to CCl<sub>4</sub> in rats has been successfully used by many investigators (Ha *et al.*, 2005). Carbon tetrachloride is metabolized by cytochrome P450 in endoplasmic reticulum and mitochondria with the formation of a highly reactive trichloromethyl and trichloromethyl peroxy free radicals, which initiate lipid peroxidation and finally cell necrosis (Vlacheva-Kuzmanova *et al.*, 2004). Administration of a single dose of CCl<sub>4</sub> to a rat produces, within 24 hrs, a centrilobular necrosis and fatty changes. The development of necrosis is associated with the leakage of hepatic enzymes into serum. Dose of CCl<sub>4</sub> is 0.1 to 3 ml/kg administrated intraperitoneally (Handa & Sharma, 1990; Parola *et al.*, 1992a).

#### **2.2.1.3 Paracetamol induced hepatotoxicity model**

Paracetamol, a widely used analgesic and antipyretic drug, produces acute liver damage in high doses (Bhanwra *et al.*, 2000). Paracetamol administration causes necrosis of the centrilobular hepatocytes characterized by nuclear pyknosis and eosinophilic cytoplasm

followed by large excessive hepatic lesion. The covalent binding of N-acetyl-P-benzoquinoneimine, an oxidative product of paracetamol to sulphydryl groups of protein, result in degradation and lipid peroxidation of glutathione level and thereby, produces cell necrosis in the liver. Dose of Paracetamol is 1 to 2 gm/kg administrated orally (Bhanwra *et al.*, 2000; Maheswari *et al.*, 2008).

### **2.2.2 *In vitro* methods**

*In vitro* assays using cultured liver cell line is more convenient, because it allows more samples be tested, is less costly regarding animals, and gives more reproducible results. Identification of active compounds of plants with known hepatoprotective properties requires screening of large numbers of samples obtained during fractionation and purification processes. Because of the expense and the time involved, the use of animal models or freshly isolated hepatocytes is not convenient for this large-scale screening of material separated from crude plant extracts. Thabrew *et al.* (1997) introduced a reproducible microplate-screening assay based on protection of liver cells line against toxic damage that can be used for rapid identification of active fractions of plant extract. Cell lines offer the unique possibility to elucidate interactions with vital cellular functions such as metabolism, cell growth, and death that were formerly difficult to address (Gebhardt, 2000). Either isolated rat hepatocyte cells or primary cultured hepatocytes are used to study the hepatoprotective effect of drugs. Most investigators obtain their established cell lines from cell banks such as American Type Culture Collection (ATCC). These cell lines often retain many features of the primary cell lines, are usually well documented and well characterized for specific experimental purposes. The cells treated with the hepatotoxin such as H<sub>2</sub>O<sub>2</sub> and the effect of plant extract is evaluated. Parameters such as cell viability, morphology, antioxidant defence imbalance and oxidative stress are determined (Bladier *et al.*, 1997; Huang *et al.*, 1999).

### **2.2.3 Markers for hepatoprotective activity evaluation**

Several parameters are used to evaluate the effect of drug on liver:

1. Biochemical blood analysis of liver markers: ALT, AST, ALP and bilirubin.  
When the liver cell is injured or dies, ALT, AST, ALP can leak through the liver cell membrane into the circulation and serum levels will rise. Bilirubin level rises in diseases of hepatocytes, obstruction to biliary excretion into duodenum, in hemolysis and defects of hepatic uptake and conjugation of bilirubin treatment.
2. Gross liver examination: for macroscopic abnormal morphology.
3. Histopathology examination of the liver: for microscopic abnormalities and changes.
4. Serum protein: liver cells synthesize total proteins, albumin and alpha fetoproteins. The blood levels of these plasma proteins are decreased in extensive liver damage.
5. Coagulation factors: fibrinogen, prothrombin, and other coagulation factors reduced in liver damage, which they are measured direct or through prothrombin time.
6. Relative liver weight: (liver weight/body weight) percentage.
7. Level of the antioxidant enzymes: superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase to evaluate the antioxidant defense status, using liver homogenates.
8. Level of oxidative stress and lipid peroxidation: by measuring the MDA.
9. Gene expression: to study the mechanism of action for hepatoprotective activity on the genetic level changes.

#### 2.2.4 Silymarin

Silymarin (Figure 2.4) is a chemical constituent isolated from the seeds of milk thistle (*Silybum marianum*) exhibits protective effects against induced hepatotoxicity in rats. The flavonolignan mixture can protect from liver cirrhosis. It is a mixture of mainly 3 flavonolignans isomers, i.e. silybin, silydianin and silychristine, with silybin being the most active component and is largely responsible for the claimed benefit of the silymarin (Kvasnička *et al.*, 2003). Silymarin has been used to treat toxic liver disease and for the supportive treatment of chronic active hepatitis and hepatic cirrhosis (Flora *et al.*, 1998). Silymarin has drawn increasing attention because of its antifibrogenic properties; it reduces collagen accumulation by 30 % in secondary biliary fibrosis in rats. Due to its antioxidant activity it decreases hepatic injury by both cytoprotection and inhibition of indicated a slight survival advantage of treated compared with untreated controls (Li & Friedman, 1999). Its mechanisms of action include inhibition of hepatotoxin binding to receptor sites on the hepatocytes membrane, reduction of glutathione oxidation to enhance its level in the liver and intestine, antioxidant activity, free radical scavenging properties, anti-inflammatory, anti-fibrotic effects and stimulation of ribosomal RNA polymerase and subsequent protein synthesis leading to enhanced hepatocyte regeneration. It is orally absorbed, but has very poor bioavailability due to its poor water solubility (Kshirsagar *et al.*, 2009). Hepatoprotective activity of silymarin has been demonstrated by various researchers against toxic models in experimental animals by using TAA, paracetamol, CCl<sub>4</sub> and ethanol (Dixit *et al.*, 2007; Ghosh *et al.*, 2010; Pradhan & Girish, 2006).



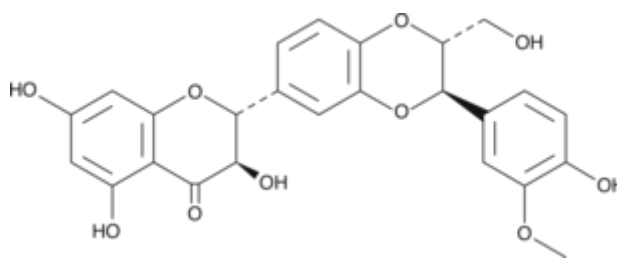


Figure 2.4 Chemical structure of the main isomer of silymarin (silybin)

## 2.3 Antioxidant activity

### 2.3.1 Free radicals and reactive oxygen species

Free radicals are chemical compounds which contain unpaired electrons in their outer electron orbit. The free radicals are energetic and highly unstable, in order to gain stability, they always seek other electrons to pair with, they attack and steal electrons from other molecules such as lipids, proteins, DNA and carbohydrates. Even they can damage DNA and lead to mutation and chromosomal damage. The attacked molecule loses its electron and becomes free radicals itself, this initiates an uncontrolled chain reaction that can damage the natural function of the living cell, resulting in various diseases (Valko *et al.*, 2006). The free radicals can be classified as reactive oxygen species (ROS) or reactive nitrogen species. ROS are the most important free radicals in our body and it refers to any free radical involving oxygen-centered free radical containing two unpaired electrons in outer shell. There are two sources of free radicals namely endogenous and exogenous sources. Endogenous sources include free radicals produced during nutrient metabolism and energy production in the mitochondria (Bergendi *et al.*, 1999). Another endogenous source of ROS, especially in the liver, is a group of enzymes called the cytochrome P450 mixed-function oxidases. The biochemical reactions catalyzed by the cytochrome P450 molecules use molecular oxygen, and during these reactions small amounts of ROS are generated. The extent of

ROS generation may vary considerably depending on the compound to be degraded and on the cytochrome P450 molecule involved. One type of cytochrome molecule that is especially active in producing ROS is known as cytochrome P450 2E1 (CYP2E1) (Lieber, 1997). The exogenous sources come from the environmental contaminants such as smoking, toxic chemicals, radiation, air pollution, organic solvents and pesticides (Büyükkokuroğlua *et al.*, 2001). Free radicals are involved in many physiological processes and human diseases such as cancer, arteriosclerosis, aging, arthritis, Parkinson syndrome, ischaemia, toxin induced reaction, alcoholism and liver injury (Willcox *et al.*, 2004). The damage to hepatic parenchymal cells, leading to hepatic injury, is due to oxidative stress within the cells caused by partially reduced free oxygen species such as superoxide anions, hydroxyl radicals, hydrogen peroxide and singlet oxygen (Halliwell, 1995). The elevation of free radical levels seen during the liver damage is due to enhanced production of free radicals and decreased scavenging potential of the cells. Natarajan *et al* suggested that evidence of oxygen free radicals is also found early in the development of fibrosis and cirrhosis (Natarajan *et al.*, 2006).

### **2.3.2 Oxidative stress in liver cirrhosis**

Oxidative stress is an imbalance between the excessive formation of ROS and endogenous antioxidants, which may lead to cell injury. While large levels of ROS are attained, which may induce severe oxidation of biomolecules and dysregulation of signal transduction and gene expression, leading to cell death through necrotic and/or apoptotic mechanisms (Dröge, 2002). Undoubtedly ROS production and excessive oxidative stress in liver cells contributes to the progression and pathological findings of liver cirrhosis and finally to hepatocellular carcinoma, regardless of the etiology (viral infection, alcohol consumption, and drug overload) and serves as a link between hepatic injury and fibrosis (Halliwell, 1999; Zhu *et al.*, 2012). Liver fibrosis due to chronic

ethanol intoxication is always accompanied by indices of excessive oxidation of polyunsaturated membrane lipids. The pro-oxidant effect of ethanol has been clearly demonstrated to be dependent upon alcohol metabolism, and in particular to the induction of a defined isoform of the cytochrome P450 family, namely the CYP2E1 (Ekstrom & Ingelman-Sundberg, 1989). On the other hand, oxidative stress seems to be responsible for the progression of the activation of HSC, TGF- $\beta$  induction and collagen synthesis during chemical-induced fibrogenesis (Albano, 2000; Kim *et al.*, 2000). Under the conditions of excessive oxidative stress, the aldehydes formed from membrane lipid peroxidation (e. g., malondialdehyde and 4-hydroxynonenal) attack various cellular and extracellular proteins in the hepatocytes and stimulate the progression of collagen deposition in the inflamed tissue (Albano, 2006). They seem to affect gene expression in adjacent HSC as demonstrated by the induction of matrix components (collagen type I, fibronectin), matrix metalloproteinases and other factors (Svegliati-Baroni *et al.*, 2001).

### **2.3.3 Antioxidant and liver cirrhosis**

Antioxidants are compounds that dispose, scavenge and suppress the formation of free radicals, or oppose their actions. Generally, antioxidants scavenge free radicals through four mechanisms. The first mechanism involves termination of free radicals production by electron donation. In the second mechanism, ROS initiator is removed by antioxidant. The third mechanism is by reducing the potency of ROS. Antioxidant serves as transition metal catalysts chelating agent in the fourth mechanism. Antioxidants protect the human body against free radical attacks that may cause pathological conditions such as liver cirrhosis (Hasani-Ranjbar *et al.*, 2009). Antioxidants, particularly those of plant origin, have emerged as potent antifibrotic and hepatoprotective agents. Moreover, lipid peroxidation was significantly increased along

with significant decrease in antioxidant levels in patients with alcoholic liver disease (Shinde & Ganu, 2009). In recent years there has been increasing interest in the presence and availability of compounds in plant materials that may possess bioactive properties, in particular, antioxidant activity. Plant antioxidants are composed of a broad variety of different substances like polyphenolic compounds, tocopherols or terpenoids. Most antioxidants isolated from higher plants are phenolic compounds (e.g. phenolic acids, tannins, coumarins, anthraquinones, flavonoids) (Middleton Jr & Kandaswami, 1994). The antioxidant activity of phenolic compounds was found to be mainly due to their scavenging and redox properties, through neutralizing and quenching free radicals (Galato *et al.*, 2001). Several anti-inflammatory, anti-necrotic, and hepatoprotective drugs have recently been shown to have an antioxidant and/or anti-radical scavenging mechanism as part of their activity. Cells are equipped with different kinds of cellular enzymes and compounds that may stand for to fight against ROS and to maintain the redox homeostasis of cell. For example, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) play important roles in scavenging the free radicals and preventing cell injury. Molecules such as vitamin C and E inhibit lipid peroxidation in cell. When the mechanism of antioxidant protection becomes unbalanced in human body, antioxidant supplement may be used to help reduce oxidative damage (Bergendi *et al.*, 1999).

#### **2.3.4 Evaluation of antioxidant activity**

*In vitro* and *in vivo* antioxidant models were used to assess the antioxidant potential of the herbal extract. A great number of *in vitro* methods have been developed to measure the efficiency of natural antioxidants either as pure compounds or as plant extracts. Electron transfer reactions are the most famous *In vitro* methods, like trolox equivalent antioxidant capacity, ferric reducing antioxidant power (FRAP),  $\alpha$ ,  $\alpha$ -diphenyl- $\beta$ -picryl-

hydrazyl radical scavenging assay (DPPH), hydroxyl radical scavenging assay, nitric oxide radical scavenging assay and total phenol content. These methods are popular due to their high speed and sensitivity. However, it is essential to use more than one method to evaluate antioxidant capacity of plant materials because of the complex nature of phytochemicals (Chanda & Dave, 2009). In addition to the *in vitro* assay that can be carried out on plant extracts, there are many *in vivo* cellular enzymes and compounds may achieve on experimental animal tissue homogenate such as SOD, CAT, GPx and MDA, which is indicate the lipid peroxidation status (Pietrangelo *et al.*, 1995).

#### **2.4 Immunomodulatory activity**

An immunomodulator is a substance used for its effect on the immune system, which can suppress, stimulate or modulate the immune system. The immunostimulants are investigated to enhance body's immunity against infection, allergy, autoimmunity and cancer. In healthy individuals the immunostimulants are expected to serve as prophylactic by enhancing the basal levels of immune response, while in individuals with impairment of immune response as immunotherapeutic agent (Agarwal & Singh, 1999). Some medicinal plants and their active components such as Ginseng, Echinacea and Astragalus possess immunomodulatory properties and show potential against malignant diseases and infections (Block & Mead, 2003). Blood mononuclear cells including natural killer (NK) cells have an important function in the defence against bacterial infections, virus-infected cells and malignant cells. High frequency of infections and cancer development are always related to the reduction of NK cell number or cytolytic activity (Lotzová, 1991). The *in vitro* methods used to study immunomodulation are principally based on the assessment of the proliferation, the metabolic activity and the activation of immune cells (measurement of cytokine

production). The most common method used to investigate the immunomodulatory properties of new compounds is the one using the human peripheral blood mononuclear cells (PBMCs) or mice splenocytes as target cells (Ajaya Kumar *et al.*, 2004; Swamy & Tan, 2000). The activation of PBMCs proliferation is always related to the immunomodulating potential of the extract. A few methods are being widely used to evaluate the immunomodulating activity based on the assessment of the proliferation, metabolic activity [such as 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay] and cell number quantitation (direct cell counting using a hemocytometer) (Durrieu *et al.*, 2005).

## **2.5 Antibacterial activity**

Nowadays bacterial infections have increased all over the world and antibiotics resistance has emerged as a challenging therapeutic problem, therefore screening of medicinal plants to discover new antibacterial agents has gained priority (Austin *et al.*, 1999). The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency (Nascimento *et al.*, 2000). Resistance to antimicrobial agents has resulted in morbidity and mortality from treatment failures and increased health care costs. The association between increased rates of antimicrobial use and resistance has been documented for nosocomial infections as well as for resistant community acquired infections. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, actions must be taken to reduce this problem, for example, to control the use of antibiotic, develop research to exact understand the genetic mechanisms of resistance, and to continue studies to develop new drugs, either synthetic or natural. The ultimate goal is to offer appropriate and efficient antimicrobial

drugs to the patient (Nascimento *et al.*, 2000). Natural products of higher plants represent a rich source of antimicrobial agents with possibly novel mechanisms of action. Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases (Iwu *et al.*, 1999). The most widely used screening methods to measure the antibacterial efficacy of a compound or medicinal plant and their constituents are both disc diffusion and minimum inhibitory concentration (MIC) against bacterial strains (NCCLS, 2000). In disk diffusion test, dried- filter paper impregnated with a specified amount of drug are applied to the surface of an agar medium inoculated with the microorganism. The compound in the disk diffuses through the agar creating inhibition zone around the disk, the larger the zone diameter, the lower the MIC. Moreover, the strength of the antimicrobial activity can be determined by dilution of compound in agar or broth. In dilution susceptibility test, serial dilution of the extract in a number of test tubes contain media broth followed by addition of test microorganism to determine the MIC for the microorganism using the turbidity as indication of growth. The determination of the lowest concentration of an antimicrobial agent needed to inhibit the growth of microorganism being tested called MIC, where is no visible growth in a nutrient medium. However, the minimum bactericidal concentration (MBC) is the lowest concentration of an antimicrobial agent where the culture has been completely sterilized after subculture onto antibiotic-free media. While agar diffusion method is excellent for screening compounds, the MIC is better for determination the exact inhibitory concentration. The broth micro-dilution method was used to determine the MIC according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 2003).

## 2.6 Investigated medicinal plants

### 2.6.1 *Orthosiphon stamineus*

*Orthosiphon stamineus* Benth (Family: Lamiaceae), also known as Misai Kucing in Malaysia, kumis kucing in Indonesia, and Java tea in Europe, this is native plant to South East Asia (Indubala & Ng, 2000).

#### 2.6.1.1 Description

*O. stamineus* (Figure 2.5) is a herbaceous perennial that bears 3-6 long flower spikes from summer to fall, which grows to a height of 1.5 m. The flowers are hermaphrodite in nature, white and airy with long white stamens that look like cat whiskers, hence the common name. Cat whiskers can reach 18-24 in height and double that in width and is a great asset to attract wildlife into the garden. *O. stamineus* will do great in full to partially sunny areas as long as it does not dry out. In the wild, it can be seen growing along the forest edges, roadsides and wastelands. The leaves are arranged in opposite pairs. They are simple, green, and glabrous with a lanceolate leaf blade and a serrate margin. The leaf apice is acuminate with an acute leaf base. The petiole is relatively short, about 0.3 cm in length and reddish purple in color. The stem is quadrangle, reddish in color, erect and with profuse branching. Although it looks similar to peppermint, the plant has a dry, salty, bitter taste. The plant is found in an area extending from tropical Asia, China and Thailand through Malaysia to tropical Australia, and is a 40 to 80 cm high herb. The medicinal parts are the leaves and stem tips collected during the flowering season. Various herbal preparations (notably aqueous and ethanolic extracts) are used in traditional medicines (Wiart, 2002).





Figure 2.5 *Orthosiphon stamineus* Benth

#### 2.6.1.2 Uses, pharmacological properties and safety

*O. stamineus* has been widely used in Malaysia as herbal tea , as diuretic, for treating kidney problems, fever, hypertension, abdominal pain, edema, gout, to treat rheumatism, diabetes, hepatitis, and jaundice (Basheer & Majid, 2010; Chin *et al.*, 2008b; Wiart, 2002). *O. stamineus* is listed in the French, Indonesia, Dutch, and Swiss pharmacopoeias for conditions related to renal cleansing and function, and related disorders that include nephritis, cystitis, and urethritis. In Europe, people use the leaves of *O. stamineus* extract as a tonic for kidney and bladder stones, liver and gallbladder problems and urinary tract infections. It is also used to reduce cholesterol and blood pressure. Researchers have found it to be mildly antiseptic as well. *O. stamineus* has been proven using animal models to treat diabetes mellitus and improving lipid profile in diabetic rats (Sriplang *et al.*, 2007), kidney problem diuretic and hypouricemic effects in rats (Arafat *et al.*, 2008), as anti-inflammatory (Yam *et al.*, 2008), for the treatment of hypertension (Ohashi *et al.*, 2000), and antipyretic activity (Yam *et al.*, 2009). Moreover, the plant's strong antioxidant action is what makes many researchers to explore the potential pharmacological properties of this plant. It has been scientifically proven that *O. stamineus* exhibits a range of pharmacological properties such as

antioxidant, antibacterial, cytotoxic, diuretic, antihypertensive and anti-angiogenesis properties (Basheer & Majid, 2010; Ho *et al.*, 2010; Sahib *et al.*, 2009). *O. stamineus* has been extensively studied in rodents with no signs of toxicity. In a study, researchers administered the botanical orally to rats for 14 days and compared it to a control group receiving distilled water. The four test groups were treated with 0.5 g/kg, 1 g/kg, 3 g/kg and 5 g/kg body weight of *O. Stamineus* respectively. No lethality or adverse toxic signs were seen during the experimental period. The study concluded that *O. stamineus* within these range and treatment duration would not cause any severe toxic effects and organ damage in rats. Individuals in Malaysia, Vietnam and Japan have consumed *O. stamineus* for centuries, further supporting its safety. Furthermore, recently the herb has been shown to be exceptionally safe with no toxicity *in vitro* and *in vivo* (Chin *et al.*, 2008b; Mohamed *et al.*, 2011).

### **2.6.1.3 Phytochemistry**

The literature review shows that this plant contains phenolic compounds and flavonoids. More than twenty phenolic compounds were isolated from *O. stamineus*, the most important constituents are nine lipophilic flavones, two flavonol glycosides, and nine caffeic acid derivatives (Sumaryono *et al.*, 1991). The well-known chemical constituents of *O. stamineus* (Figure 2.6) are caffeic acid, cirrchoric acid, diterpenes, orthosiphols, monoterpenes, triterpenes, saponins, hexoses, organic acids, rosmarinic acids, sinensetin, eupatorin, and 3-hydroxyl-5,6,7,4-tetramethoxyflavone (TMF) (Akowuah *et al.*, 2005a; Olah *et al.*, 2003; Tezuka *et al.*, 2000). The plant contains high amount of flavones, polyphenols, bioactive proteins, glycosides, a volatile oil, and vast quantities of potassium. Earlier studies reported bioactive pentacyclic triterpenes

betulinic acid, oleanolic acid, ursolic acid and sterols from the leaves of this plant (Tezuka *et al.*, 2000).

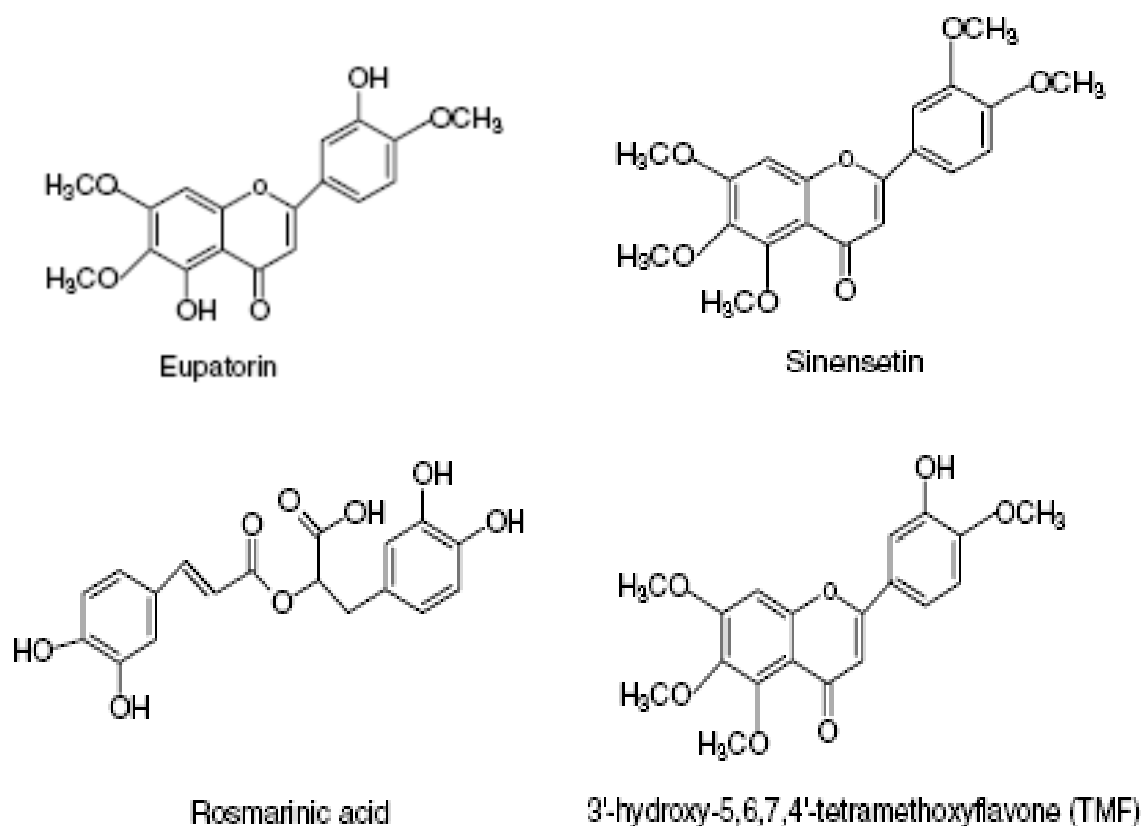


Figure 2.6 Chemical structures of the main phenolic compounds in *O. Stamineus*

### 2.6.2 *Morinda citrifolia*

The genus *Morinda* (Family: Rubiaceae), including the species *Morinda citrifolia* L., is made up of around 80 species. *M. citrifolia* commercially known as noni, is indigenous to tropical countries and is considered as an important traditional folk medicine. Apart from this appellation, there are many local names that are also widely used in their respective countries namely, Mengkudu (Malaysia), Noni Apple, Polynesia Fruit, Indian Mulberry (India), Bumbo (Africa), Cheeserut (Australia), Painkiller Tree (Caribbean Islands), Nhau (Southeast Asia), Morinda (Vietnam), and Hai Ba Ji (China) (Chan-Blanco *et al.*, 2006).

### 2.6.2.1 Description

*M. citrifolia* (Figure 2.7) is a shrub which grows in sandy areas along many tropical coastal regions at sea level and in forest areas of up to about 1300 feet above sea level. *M. citrifolia* is a bush or small evergreen tree, 3–10 m tall, with abundant wide elliptical leaves (5–17 cm length, 10–40 cm width). It can be identify by its straight trunk, large, bright green and elliptical leaves, white tubular flowers, and its distinctive, ovoid, “grenade-like” yellow fruit. The small tubular white flowers are grouped together and inserted on the peduncle. The petioles leave ring-like marks on the stalks and the corolla is greenish-white. The noni fruit (3–10 cm length, 3–6 cm width) is oval and fleshy with an embossed appearance. It is slightly wrinkly, semi-translucent, and ranges in colour from green to yellow, to almost white at the time of picking. It is covered with small reddish-brown buds containing the seeds. The pulp is juicy and bitter, light dull yellow or whitish, gelatinous when the fruit is ripe; numerous hard triangular reddish-brown pits are found, each containing four seeds (3.5 mm). The mature fruit has a foul taste and odour. *M. citrifolia* grows in shady forests, as well as on open rocky or sandy shores. It reaches maturity in about 18 months, then yields between 4 and 8 kg of fruit every month throughout the year. It is tolerant of saline soils, drought conditions, and secondary soils. It is therefore found in a wide variety of habitats: volcanic terrains, lava-strewn coasts, and clearings or limestone outcrops (Chan-Blanco *et al.*, 2006).



Figure 2.7 *Morinda citrifolia* plant with fruit

#### **2.6.2.2 Uses, pharmacological properties and safety**

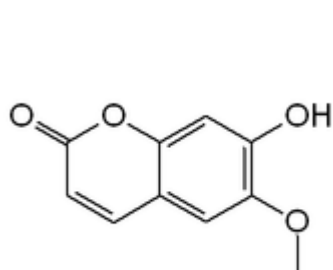
All parts of the plant have traditional and/or modern uses, including roots and bark (dyes, medicine), trunks (firewood, tools), leaves and fruits (food, medicine). The medicinal applications, both traditional and modern, span a vast array of conditions and illnesses, although most of these have yet to be scientifically supported (Chan-Blanco *et al.*, 2006). The tree has attained significant economic importance worldwide in recent years through a variety of health and cosmetic products made from its leaves and fruits. These include fruit juices as well as powders made from the fruit or leaves. Fruit extracts have been traditionally used as a sedative, a remedy for diarrhoea, a topical disinfectant, and as a gargle to relieve sore-throat. *M. citrifolia* has been used in folk remedies by Polynesians for over 2000 years, and is reported to have a broad range of therapeutic effects, including antibacterial, antiviral, antifungal, anticancer, analgesic, hypotensive, anti-inflammatory, and immune enhancing effects (Akihisa *et al.*, 2007; Aneupankul *et al.*, 2007; Chan-Blanco *et al.*, 2006; Wang *et al.*, 2002). The fruit juice is in high demand as an alternative medicine for different kinds of illnesses such as jaundice, to alleviate menstrual cramps, arthritis, diabetes, gastric ulcers, sprains, poor digestion, problems associated with high blood pressure, muscle aches and pains,

headaches, heart disease, mental depression, senility and drug addiction. Scientific evidence on the benefits of the noni fruit juice is limited but there is some anecdotal evidence for successful treatment of colds and influenza (Wang *et al.*, 2002). Extracts of *M. citrifolia* have been shown to possess several pharmacological properties, *e.g.* analgesic, anti-inflammatory, antioxidant, chemoprotective, antimicrobial, and immunomodulatory properties (Hirazumi *et al.*, 1996; Wang *et al.*, 2002). An acute toxicity studies were conducted for the *M. citrifolia* extract showed that the plant is safe up to 4 g/kg body weight. All animals survived with no mortality and no any sign of toxicity were noted. No signs of gross toxicity were seen in the organs after necropsy (Nayak *et al.*, 2009; Wang *et al.*, 2002).

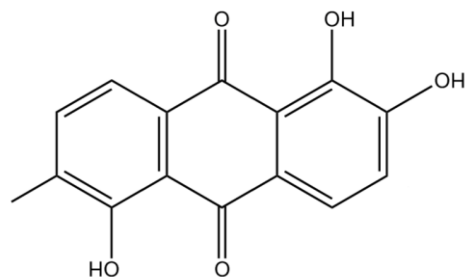
#### **2.6.2.3 Phytochemistry**

The fruit contains 90 % of water and the main components of the dry matter appear to be soluble solids, dietary fibers and proteins. The fruit protein content is surprisingly high, representing 11.3 % of the juice dry matter, and the main amino acids are aspartic acid, glutamic acid and isoleucine (Chunhieng, 2003). About 160 phytochemical compounds have been already identified in *M. citrifolia*, and the major micronutrients are phenolic compounds, organic acids and alkaloids. Of the phenolic compounds, the most important reported are anthraquinones (damnacanthal, morindone, morindin, etc.), and also aucubin, asperuloside, and scopoletin (Figure 2.8). The main organic acids are caproic and caprylic acids, while the principal reported alkaloid is xeronine (Wang & Su, 2001). The other major components have been identified in *M. citrifolia* include octanoic acid, potassium, vitamin C, terpenoids, alkaloids, anthraquinones (such as nordamnacanthal, morindone, rubiadin, and rubiadin-1-methyl ether, anthraquinone glycoside),  $\beta$ -sitosterol, carotene, vitamin A, flavone glycosides, linoleic acid, alizarin, amino acids, aucubin, L-asperuloside, caproic acid, caprylic acid, ursolic acid, rutin, and

a putative proxeronine, while the fruit juice contains a polysaccharide rich compounds (Farine *et al.*, 1996; Hirazumi *et al.*, 1994; Jayaraman *et al.*, 2008; Levand & Larson, 1979).



Scopoletin



Morindone

Figure 2.8 Chemical structures of the main phenolic compounds in *M. citrifolia* fruits

## CHAPTER III

### METHODOLOGY

#### 3.1 Plant materials and chemicals

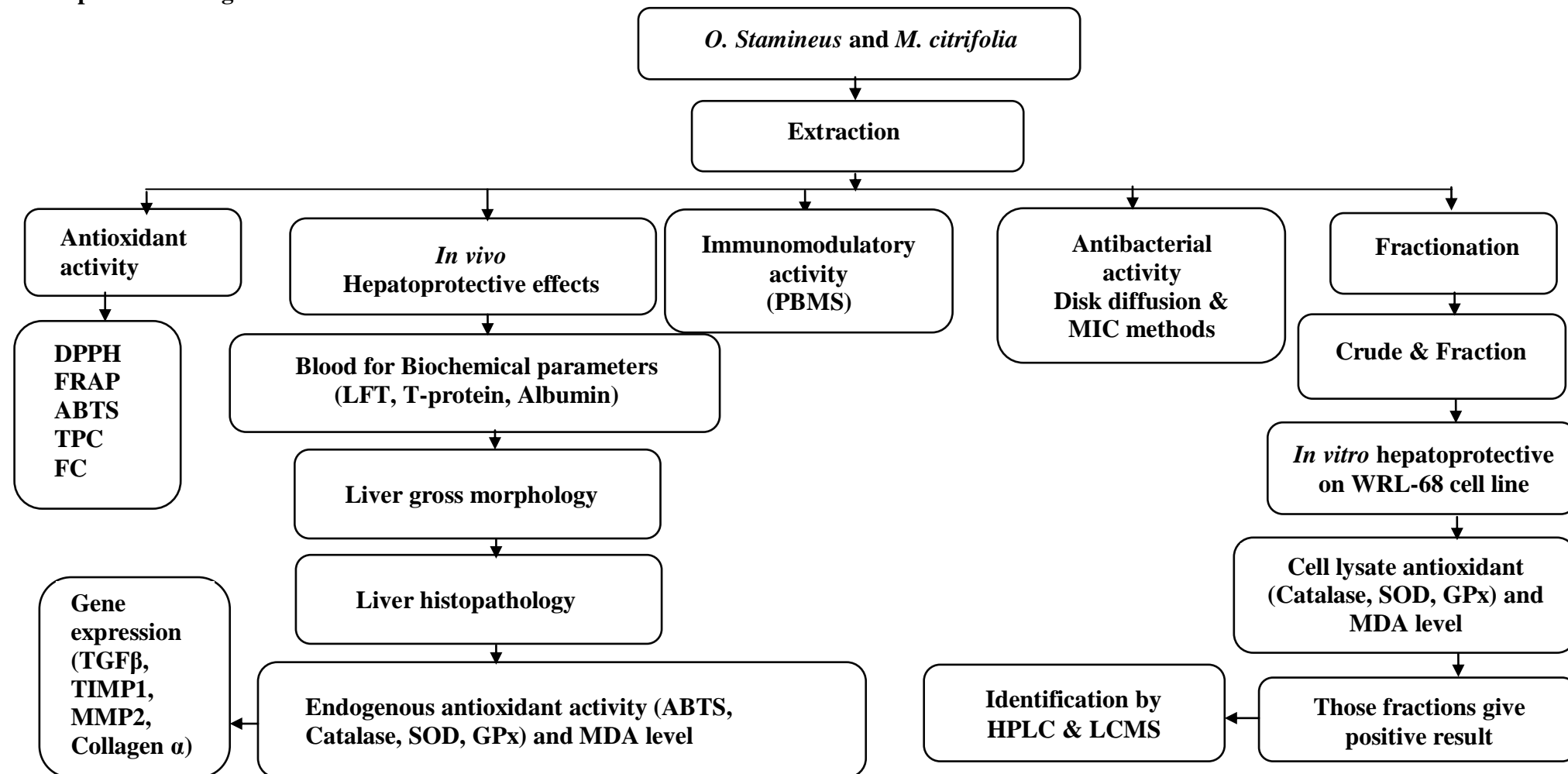
*O. stamineus* plant leaves and *M. citrifolia* plant fruits (Table 3.1) were obtained from the Ethno Resource Sdn Bhd Selangor, Malaysia. The plants were identified, and voucher specimens were kept in our laboratory for future references. Totally 100 gm of the dried and fine powdered of plant were extracted with 900 ml of 95 % ethanol for 48 h, after that the residue was extracted two times again with 95 % ethanol. The ethanol extracts were filtered by filter paper (Whatman No. 1) and evaporated under low pressure by using rotary evaporator (Buchi, type R-215, Rotavapor, Switzerland) and finally subjected to lyophilization in freeze dryer (LabConco, Kansas City, MO, USA) to give the crude-dried extract. The percentage yields of ethanol extracts for *O. stamineus* and *M. citrifolia* were found to be 8.1 % and 16.0 % (w/w) respectively. The dried extracts were stored at -20°C until used (Alshawsh *et al.*, 2011). Thioacetamide from (Sigma-Aldrich, Switzerland), silymarin from (International Laboratory, USA) and all other chemicals used were of analytical grade and purchased mostly from Sigma-Aldrich, UK and Fisher Scientific, UK.

Table 3.1 Investigated Medicinal plants

Botanical name	Local name	Family	Part tested	Traditional uses
<i>Orthosiphon stamineus</i> Benth	Misai kuching	Lamiaceae	Leaves	Hepatitis and jaundice, kidney problems, fever, hypertension, gout, diabetes
<i>Morinda citrifolia</i> L.	Mengkudu	Rubiaceae	fruits	Tonic, skin wounds and abscesses, Antipyretic, gum and throat problems, constipation



### 3.2 Experiment design



### 3.3 Antioxidant activity

The investigated plants examined to determine their antioxidant activity using the following assays; DPPH, FRAP, total phenolic content (TPC) and flavonoid content (FC).

#### 3.3.1 Scavenging activity of DPPH

The antioxidant activities of the crude extracts were determined using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. DPPH is a molecule containing a stable free radical. In the presence of an antioxidant which can donate an electron to DPPH, the purple color which is typical for free DPPH radical decays and the change in absorbency at 515 nm can be read. This assay measures the free radical scavenging capacity of the investigated extracts. The assay was carried out as described by Brand *et al* (Brand-Williams *et al.*, 1995) with minor modification. Briefly 1 mg from ethanolic and aqueous extracts dissolved in 1 ml solvent then diluted to got five different concentrations (25, 12.5, 6.25, 3.125 and 1.56 µg/ml) and Ascorbic acid used as antioxidant standards. A quantity of (5 µl) from each plant extract and standard were mixed with 195 µl of DPPH (40 X dilution) in triplicate. The decrease in absorbance value was measured at 515 nm for 2 hr with 20 min intervals. The radical scavenging activity was calculated from the following equation and the results were expressed as mean ± standard error mean (SEM):

$$\% \text{ of radical scavenging activity} = (\text{Abs Blank} - \text{Abs Sample}) / \text{Abs Blank} \times 100$$

#### 3.3.2 Ferric reducing antioxidant power (FRAP) assay

The ferric reducing activity of the plant extracts were estimated using with some modification 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 acid and 20 mmol/l of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ . The working FRAP reagent prepared freshly by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ . The freshly prepared mixture was incubated at  $37^\circ\text{C}$  in water bath for five minutes and then a blank reading was taken spectrophotometrically at 593 nm. After that, 10  $\mu\text{l}$  of extract or standard were added to 300  $\mu\text{l}$  of the working FRAP reagent. Absorbance is measured at 0 minute immediately upon addition of the working FRAP reagent after mixing. Thereafter, absorbance reading was taken after four minutes. The standard curve was plotted using  $\text{FeSO}_4$  solution absorbance as standard, and the results were expressed as  $\mu\text{mol Fe(II)}/\text{g}$  dry weight of crude extract (appendix IV).

### 3.3.3 ABTS assay

The total antioxidant capacity assay was carried out using the improved method, as described by (Re *et al.*, 1999). Briefly, 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid (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 reach to the absorbance of  $0.700 \pm 0.020$  at 734 nm and equilibrated at  $30^\circ\text{C}$  while plant extracts were diluted with distilled water. To 1 ml of diluted ABTS, 10  $\mu\text{l}$  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.

### **3.3.4 Total phenolic content (TPC) and flavonoids determination**

The *O. stamineus* and *M. citrifolia* extracts were evaluated for their total phenolic content by using Folin-Ciocalteu reagent and were calculated as gallic acid equivalents in mg (GAE)/g of extract according to Folin-Denis colorimetric method (AOAC, 1995). However, the total flavonoids were determined by using the aluminium chloride colorimetric method, and expressed as quercetin equivalents in mg (QE)/g of extract as described by Dowd (Dowd, 1959). Both assays were carried out in triplicate.

### **3.4 Acute toxicity**

Three groups of 10 adult Sprague–Dawley (SD) rats (5 males and 5 females) were given single oral doses of 5 g/kg body weight of the crude extract, 2 g/kg body weight of the crude extract and the control group received single dose of vehicle at the same volume. Observations for any abnormal behavioural pattern, clinical signs and mortality were made systematically for 48 h after doses administration and at the end of this period mortality was recorded for each group. The number of survivors was then maintained for a further 14 days. The last day of the experiment, all surviving animals were sacrificed then liver and kidney organs were collected, gross necropsy and histopathology examined (Lorke, 1983; OECD, 2001). The acute toxicity protocol was approved by Animal Ethics Committee; with an ethic No. (PM /07/05/2008/1111/ MAA (a) (R).

### **3.5 *In vivo* hepatoprotective activity of plant extracts**

#### **3.5.1 Animals**

Adult male healthy SD rats weighing 200-250 gm were obtained from Animal House Unit, Faculty of Medicine, University of Malaya, Malaysia. They were kept in wire bottomed cages at  $25 \pm 3^{\circ}\text{C}$  temperature, 50-60 % humidity, and a 12 h light–dark cycle

for at least a week before the experiment. They were maintained at standard housing conditions and free access to standard diet and water ad libitum during the experiment. The experimental protocol was approved by Animal Ethics Committee; with an ethic No. (PM 28/08/2009/ MAA (R) (appendix I). Throughout the experiments, all criteria of taking care of animals prepared by the National Academy of Sciences and outlined in the “guide for the care and use of laboratory animals” were applied (Clark *et al.*, 1997).

### **3.5.2 *In vivo* hepatoprotective activity experimental design**

The dry extract was dissolved in Tween 20 (10 % w/v) and administered orally to rats in concentrations of 200 mg/kg high dose (HD) and 100 mg/kg low dose (LD) body weight. The TAA chemical was dissolved in sterile distilled water and injected intraperitoneally to the rats in concentrations of 200 mg/kg body weight (Aydin *et al.*, 2009). Silymarin as a standard drug was dissolved in Tween 20 (10 % w/v) and orally administered to rats in concentrations of 50 mg/kg body weight (Ahmad *et al.*, 2002). For each plant the animals were randomly divided into five groups of eight rats each and treated for 2 months the doses administered showed in (Table 3.2). Body weights of all animals were measured every week. All rats were sacrificed 24 hours after last treatment and overnight fasting under anesthesia. Blood samples were collected; serum was separated for assay of the liver biomarkers. The liver and spleen were harvested, washed in normal saline, blotted with filter paper and weighed. Gross examination was conducted to examine of any abnormalities developed in the organs. The liver of all animals was subsequently subjected to histopathological examination in a blinded fashion.

Table 3.2 *In vivo* hepatoprotective activity of plant extracts experimental design

Group	Name of group	Intraperitoneal injection	Oral administration
1	Normal Control group	1ml/kg Distilled water/thrice weekly	5ml/kg 10 % Tween 20/daily
2	Thioacetamide Hepatotoxic (TAA) Control group	200mg/kg TAA/thrice weekly	5ml/kg 10 % Tween 20/daily
3	Treatment (High dose) group	200mg/kg TAA/thrice weekly	200mg/kg Extract suspended in 10% Tween 20/daily
4	Treatment (Low dose) group	200mg/kg TAA/thrice weekly	100mg/kg Extract suspended in 10% Tween 20/daily
5	Hepatoprotective group (Standard drug)	200mg/kg TAA/thrice weekly	50 mg/kg Silymarin/daily

### 3.5.3 Biochemical and histopathological examination

The collected blood samples were separated at 2500 rpm for 15 minutes after been completely become clotted. Serum for assay of the liver biomarkers such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, total protein (TP), and albumin was assayed spectrophotometrically by standard automated techniques according to the procedures described by the manufacturers in Central Diagnostic Laboratory, University of Malaya Medical Centre (appendix IV). The Liver was sliced and pieces and placed in tissue cassettes using a scalpel and forceps. Each tissue cassette labeled and fixed for 4 h in 10 % buffered formalin solution (appendix II) for histological study. The fixed tissues were dehydrated, cleared and infiltrated in automated tissue processing machine (Leica Microsystems, Nussloch, Germany) using standard technique (appendix II). Tissues were embedded in paraffin wax by conventional methods. Sections of 5  $\mu$ m in thickness were cut using the microtome (Leica Microsystems, Nussloch, Germany) and then stained with hematoxylin-eosin (H&E) staining according to the standard protocol (appendix II). Then the stained sections were observed under the light microscope

(Olympus, Tokyo, Japan) for histopathological changes, and their photomicrographs were captured with a Nikon microscope digital camera (Nikon, Tokyo, Japan).

### **3.6 *In vivo* antioxidant activity in liver tissue**

Liver samples were washed immediately with ice cold saline to remove the blood. Liver homogenates (10 % w/v) were prepared in a cold 50 mM potassium phosphate buffer saline (PBS) (pH 7.4) using homogenizer in ice (Wise Mix HG-15, Daihan Scientific, Seoul, Korea). The cell debris was removed by centrifugation at 4500 rpm for 15 min at 4°C using refrigerated centrifuge Rotofix 32 (Hettich Zentrifugen, Germany). The supernatant was used for the estimation of the following *in vivo* antioxidant using commercially available kits from (Cayman Chemical Company, USA): total antioxidant capacity (TAC) (Item No. 709001), malondialdehyde (MDA) or thiobarbituric acid reactive substance (TBARS) (Item No. 10009055), catalase (CAT) (Item No. 707002), superoxide dismutase (SOD) (Item No. 706002) and glutathione peroxidase (GPx) (Item No. 703102) activities. All assays performed according to the instruction manual of the manufacturer (appendix III). Protein concentration was determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

### **3.7 Gene expression assay using real time PCR (RT-PCR)**

Liver samples after sacrificing the rats were kept immediately in the preservative RNA *later* Soln. (Ambion, Austin, Texas, USA) and kept at 4°C overnight, after that stored in -80°C until used. The selected genes for the expression assay were; TGF- $\beta$ , TIMP-1, MMP-2 and collagen1 $\alpha$ 1.

### **3.7.1 RNA isolation and purification**

Total RNA was extracted from liver tissue with QIAamp RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol (appendix III). A DNase digestion step was included to eliminate genomic DNA contamination using the RNase-Free DNase Set (cat. no. 79254) (Qiagen, Hilden, Germany) (appendix III). The RNA concentration was measured spectrophotometrically with a Nano Drop ND-2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The ratio of the readings at 260 nm and 280 nm ( $A_{260}/A_{280}$ ) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. Pure RNA has an  $A_{260}/A_{280}$  ratio of 1.7–2.1 (appendix IV). The integrity and size distribution of total RNA purified with QIAamp RNA Mini Kits was checked by denaturing agarose gel electrophoresis and ethidium bromide staining (appendix II) using agarose gel electrophoresis apparatus (Bio-Rad, Richmond, CA, USA). The apparent ratio of 28S RNA to 18S RNA should be approximately 2:1 (Figure 3.1). The extracted RNA was stored at  $-80^{\circ}\text{C}$  until used.



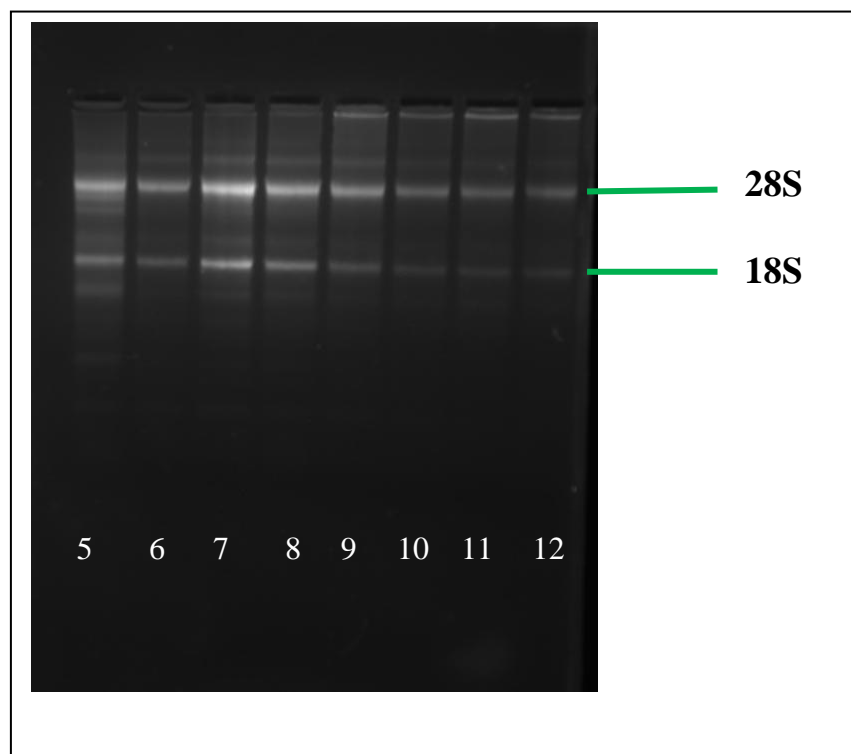
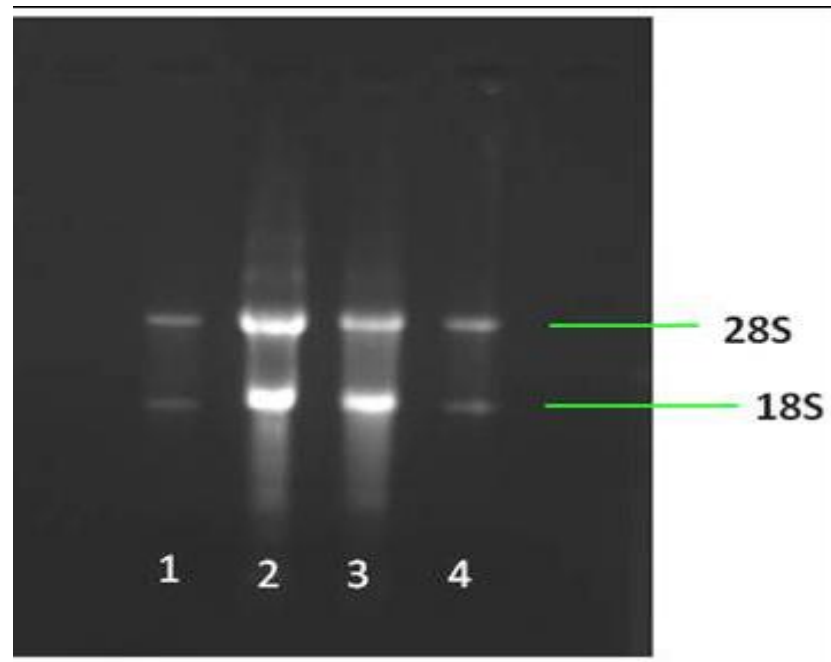


Figure 3.1 RNA profile using ethidium bromide-stained agarose gel

### 3.7.2 Reverse transcription and cDNA synthesis

One microgram of total RNA was reverse transcribed to cDNA using High capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA) according to

the manufacturer's protocol (appendix III). The following total volumes of components were prepared to obtain total volume of 20  $\mu$ l of cDNA per reaction as in (Table 3.3):

Master Mix	4.0 $\mu$ l
RNA template	Variable (up to 16 $\mu$ l), calculated according to RNA concentration in order to obtain 1 $\mu$ g cDNA per reaction (Table 3.3)
Nuclease-free H <sub>2</sub> O	sufficient to 20 $\mu$ l

All components were mixed well and loaded in a thermal cycler (Major Science, CA, USA) under the following conditions: 5 minutes at 25°C, then 30 minutes at 42°C, in step three 5 minutes at 85°C, finally holding at 4°C. The cDNA was stored at -20°C until used.

Table 3.3 Reverse transcription components volume calculation

Sample	RNA Concentration ng/μl	RNA volume μl	Nuclease-free H <sub>2</sub> O μl
Calibrator 1	647	1.6	14.4
Calibrator 2	329	3.0	13.0
Calibrator 3	503	2.0	14.0
Calibrator 4	464	2.2	13.8
Calibrator 5	641	1.6	14.4
Calibrator 6	516	1.9	14.1
Calibrator 7	879	1.1	14.9
TAA 1	225	4.4	11.6
TAA 2	577	1.7	14.3
TAA 3	281	3.6	12.4
TAA 4	522	1.9	14.1
TAA 5	884	1.1	14.9
TAA 6	724	1.4	14.6
TAA 7	284	3.5	12.5
<i>O. stamineus</i> 1	76.9	13.0	3.0
<i>O. stamineus</i> 2	130	7.7	8.3
<i>O. stamineus</i> 3	276	3.6	12.4
<i>O. stamineus</i> 4	279	3.6	12.4
<i>O. stamineus</i> 5	482	2.1	13.9
<i>O. stamineus</i> 6	364	2.8	13.2
<i>O. stamineus</i> 7	248	4.0	12.0
<i>M. citrifolia</i> 1	554	1.8	14.2
<i>M. citrifolia</i> 2	551	1.8	14.2
<i>M. citrifolia</i> 3	556	1.8	14.2
<i>M. citrifolia</i> 4	575	1.7	14.3
<i>M. citrifolia</i> 5	316	3.2	12.8
<i>M. citrifolia</i> 6	629	1.6	14.4
<i>M. citrifolia</i> 7	541	1.9	14.1

### 3.7.3 Real time PCR amplification

The following TaqMan rat assay genes from (Applied Biosystems, Foster City, CA, USA) were used for RT-PCR: TGFβ1 (Rn00572010\_m1), TIMP-1 (Rn00587558\_m1), MMP-2 (Rn01538167\_m1) and Col 1a1 (Rn01463848\_m1) (Table 3.4). The NormFinder and geNorm algorithm (MultiD Analyses AB, Göteborg, Sweden) were used to evaluate and calculate the expression stability of four endogenous reference genes – HPRT-1 (Rn01527840\_m1), Ppia (Rn00690933\_m1), GAPDH

(Rn0177563\_g1) and Act b (Rn00667869\_m1) – for normalisation of rat's liver samples (Chen *et al.*, 2006a). According to the NormFinder algorithm, the gene that showed the lowest variability was HPRT-1, while geNorm showed that the HPRT-1 and Ppia are the best endogenous reference genes combination (appendix IV). The transcriptional levels of the target genes were normalized using both HPRT-1 and Ppia endogenous reference genes. These two genes were consistently expressed in rat liver tissue and experiments showed a stable expression of both endogenous reference genes in all groups. The cDNA was diluted with nuclease-free water (Applied Biosystems, Foster City, CA, USA) to a concentration of 20 ng/μl, after that 1 μl was applied for each RT-PCR. The PCR was carried out using TaqMan fast advanced Master Mix (Applied Biosystems, Foster City, CA, USA) (appendix III). The volume and concentration of each component in the RT-PCR reaction mix was adjusted according to (Table 3.5). All components were added to 1.5 ml optical tube with cap (Applied Biosystems, Foster City, CA, USA). The amplification reaction was performed using the step one plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to TaqMan gene expression assay protocol (appendix III) with the following cycling conditions: 2 min at 50°C, 20 sec at 95°C, followed by 40 cycles of 1 sec at 95°C and 20 sec at 60°C. For each sample, the real-time PCR reaction was performed in triplicate, and the averages of the obtained threshold cycle ( $C_t$ ) values were processed for further calculations according to the comparative  $C_t$  method. Gene expression values were calculated with the  $2^{-\Delta\Delta C_t}$  method (Livak & Schmittgen, 2001). The  $\Delta C_t$  value of each sample was determined by subtracting the average  $C_t$  value of the endogenous reference gene from the average  $C_t$  value of the target gene. The  $\Delta\Delta C_t$  value was then calculated by subtracting the  $\Delta C_t$  value of the treated sample from the control (untreated)  $\Delta C_t$  value. Finally, the gene expression level was calculated as  $2^{-\Delta\Delta C_t}$  giving the final value that is normalized to the reference genes and relative to the control sample values of the

studied genes. GenEx Enterprise software for quantitative real-time PCR (qRT-PCR) expression profiling (MultiD Analyses AB, Göteborg, Sweden) was used to analyze and normalize the qRT-PCR data (Kubista *et al.*, 2006). This software allows the correction of PCR efficiencies and the normalization with more than one endogenous reference genes. Statistical analysis of differences was performed by a two-tailed unpaired Student's t test. A *p* value of <0.05 was considered indicative of a statistically significant difference.

Table 3.4 Investigated genes and reference genes with their Amplicon length, GenBank accession numbers and TaqMan gene expression assay numbers

Gene Symbol	Gene Name	TaqMan gene expression assay number	GenBank accession number	Amplicon length
TGFβ1	Transforming growth factor, beta 1	Rn00572010_m1	NM_021578.2	65
TIMP-1	Tissue inhibitors of metalloproteinases-1 (metallopeptidase inhibitor 1)	Rn00587558_m1	NM_053819.1	91
MMP-2	Matrix metalloproteinases-2 (matrix metallopeptidase 2)	Rn01538167_m1	NM_031054.2	72
Col 1a1	collagen, type I, alpha 1	Rn01463848_m1	NM_053304.1	115
HPRT-1	Hypoxanthine phosphoribosyl transferase 1 (reference gene)	Rn01527840_m1	NM_012583.2	64
Ppia	Peptidylprolyl isomerase A (cyclophilin A) (reference gene)	Rn00690933_m1	NM_017101.1	149
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase (reference gene)	Rn0177563_g1	NM_017008.3	175
Act b	Actin, beta (reference gene)	Rn00667869_m1	NM_031144.2	91

Table 3.5 Components volume and concentration used in the RT-PCR reaction mix (total volume 10 µl)

Component	Volume (µl) for 1 reaction	Final concentration
TaqMan fast advanced Master Mix	5.0 µl	1X
TaqMan Gene expression assay	0.5 µl	1X
cDNA template	1.0 µl	20 ng
Nuclease-free water	3.5 µl	-
Total volume per reaction	10.0 µl	

### 3.8 Immunomodulatory activity

#### 3.8.1 Peripheral blood mononuclear cell (PBMCs) isolation and cell culture

Fresh blood was collected from healthy adult volunteer using heparinised tubes. Peripheral blood mononuclear cells (PBMCs) were separated by density-gradient centrifugation over Histopaque 1077 (Sigma-Aldrich, UK) (appendix III). The remnant erythrocytes in the recovered PBMCs layer were eliminated using lysis buffer to lysis the RBCs and washed three times in sterile PBS. The PBMC were re-suspended in 1 ml RPMI 1640 media (Sigma-Aldrich, UK). The mononuclear cells were counted and adjusted to an appropriate concentration in complete RPMI 1640 supplemented with 2 mM glutamine, 1 % (v/v) Penicillin-Streptomycin antibiotic (Sigma-Aldrich, UK) and containing 10 % (v/v) fetal bovine serum (FBS) (J R Scientific, Inc, USA) for further assays (Gayathri *et al.*, 2007). Their viability was determined by trypan blue exclusion test (Moldeus *et al.*, 1978) and only cell viability greater than 95 %, as assessed by the trypan blue undergoes the MTT assay. The cells were then seeded onto 96-well flat bottom sterile tissue culture plates (Jet Biofil, China) at a density of  $5 \times 10^4$  cells/ml. All cell cultures were maintained at 37 °C in a humidified IR water-jacketed incubator (NuAire, Plymouth, MN, USA) with 5 % CO<sub>2</sub> condition.

### 3.8.2 MTT cell viability assay

The effect of the extracts on cell viability of human PBMCs were determined by using MTT assay as described by Mosmann and Scudiero using MTT reagent (5 mg/ml) (Mosmann, 1983; Scudiero *et al.*, 1988). Briefly, 100 µl of the isolated PBMCs suspension were cultured in 96-well flat bottom micro titer plates at  $5 \times 10^4$  cells/ml in RPMI medium containing 10 % (v/v) FBS and incubated at 37°C, 5 % CO<sub>2</sub> and 90 % humidity incubator for 48 hr. The second day, the cells were treated with 20 µl of two fold serial dilution with final concentration (200, 100, 50, 25 and 12.5 µg/ml) of crude extracts and re-incubated at 37°C in humidified 5 % CO<sub>2</sub> incubator for 24 hr. After 48 hr, 10 µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) (Merck, Germany) was added into each well in the plates and again re-incubated for four hr at 37°C in humidified 5 % CO<sub>2</sub> incubator. The yellow MTT is reduced to purple formazan in the mitochondria of living cells (Mosmann, 1983). Approximately 80 µl of medium with MTT was removed from all wells and 100 µl of dimethyl sulfoxide (DMSO) (Fisher Scientific, UK) was added to each well to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution measured at 595 nm using power wave x340 ELISA Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The assay performed in triplicates in three independent experiments. The percentage of cell viability was calculated by the following formula and the results were expressed as mean  $\pm$  SEM:

$$\% \text{ Cell viability} = (\text{Abs sample} - \text{Abs blank}) / \text{Abs blank} \times 100$$

### 3.9 Profiling and Fractionation of crude extracts

The ethanol crude extract of both plants (10 gm) were subjected to column chromatography fractionation using a 3.0 x 50 cm glass columns (Kontes Scientific

Glassware, Vineland, NJ, USA) packed with silica gel G60, 70 - 230 mesh (Merck, Darmstadt, Germany) and connected with an EYEL-L1 type pump (Tokyo Rikakikai, Tokyo, Japan). The crude extracts were eluted stepwise with gradient (25 ml each time of five different concentrations; 20, 40, 60, 80 and 100 %) of different solvents in the order of increasing polarity, started by less polarity toward the higher polarity. The solvents used were hexane, ethyl acetate, methanol, acetone, acetonitrile and water, all fractions collected in clean tubes and all eluents were then pooled to give six major fractions (Fraction 1–6) based on similarity of spots on thin layer chromatography (TLC) using aluminium foils pre-coated with silica gel 60 F254 plate, 20x20 cm, 0.2 mm (Merck, Darmstadt, Germany). The solvents were evaporated under reduced pressure in centrifuge evaporator and freeze dryer (Awaad *et al.*, 2008). The isolation of the compounds was monitored by TLC using mixtures of ethyl acetate and methanol. All fractions and the crude extract subjected to *in vitro* hepatoprotective cell line assay and those given positive result identified by high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) to determine the active constituents.

### **3.10 *In vitro* hepatoprotective activity of plant crude extracts and isolated fractions**

#### **3.10.1 Cell line and culture conditions**

The normal hepatic human cell line (WRL-68) obtained from American Type Culture Collection (ATCC) (appendix III). WRL-68 cells were cultured in Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich, UK) supplemented with 10 % (v/v) FBS (J R Scientific, Inc, USA) and 1 % (v/v) penicillin-streptomycin solution (Sigma-Aldrich, UK). WRL-68 cell lines were cultured at 37° C in humidified 5 % CO<sub>2</sub> incubator, and



cells were split twice weekly using trypsin / EDTA (Sigma-Aldrich, UK). Cells from passage 20-25 were used for the experiment.

### **3.10.2 Hydrogen peroxide treatment evaluation**

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was added to WRL-68 cell line to induce cell oxidative damage *in vitro*. Approximately 100 µl of WRL-68 cell line suspension was seeded onto 96-well flat bottom sterile tissue culture plates at a density of  $5 \times 10^4$  cells/ml and incubated at 37°C, 5 % CO<sub>2</sub> and 90 % humidity incubator for 24 hr. The second day, the cells were exposed to 10 µl of different freshly prepared H<sub>2</sub>O<sub>2</sub> dilutions (Merck, Darmstadt, Germany) with final concentration (100, 200, 400, 600, 800 and 1000 µM) and re-incubated at 37°C in humidified 5 % CO<sub>2</sub> incubator for 2 hr (Spitz *et al.*, 1987). The effect of the H<sub>2</sub>O<sub>2</sub> on cell viability was determined by MTT assay as described previously (Mosmann, 1983; Scudiero *et al.*, 1988). The H<sub>2</sub>O<sub>2</sub> concentration that can inhibit the WRL-68 cell viability to 40-50 % comparing with untreated cells was chosen for *in vitro* hepatoprotective activity experiment.

### **3.10.3 *In vitro* hepatoprotective activity and cell viability test**

The MTT assay was used to assess cell damage by the H<sub>2</sub>O<sub>2</sub> and cell viability protection by the crude extracts and isolated fractions as described by (Chen *et al.*, 2006b; Weecharangsan *et al.*, 2006) with some modification. Briefly, 100 µl of the WRL-68 cell line suspension were seeded in 96-well flat bottom micro titer plates at  $5 \times 10^4$  cells/ml in DMEM medium containing 10 % (v/v) FBS and allowed to attach overnight. The second day, the cells were treated with 100 µg of various treatment doses according to (Table 3.6) and incubated at 37°C with 5 % CO<sub>2</sub> for 2 hr. The treated cells were induced by 10 µl of freshly prepared 1000 µM H<sub>2</sub>O<sub>2</sub> (this concentration obtained from

H<sub>2</sub>O<sub>2</sub> treatment evaluation experiment) and re-incubated at 37°C for 2 hr. The hepatoprotective effect of plant extracts and isolated fractions were determined by cell viability using MTT assay as described previously (Mosmann, 1983; Scudiero *et al.*, 1988). Cell viability was calculated as a percentage compared to the untreated control and the H<sub>2</sub>O<sub>2</sub> groups. The assay performed in triplicates and the percentage of cell viability was calculated as means  $\pm$  SEM.

Table 3.6 *In vitro* hepatoprotective activity of plant extracts and isolated fractions experimental design

Group Name	Oxidant agent (1000 $\mu$ M H <sub>2</sub> O <sub>2</sub> )	Treatment (100 $\mu$ g/ml)
Control (Normal cell group)	No oxidant agent (10 $\mu$ l medium)	No treatment (10 $\mu$ l solvent)
H <sub>2</sub> O <sub>2</sub> (Oxidative damage group)	10 $\mu$ l	No treatment (10 $\mu$ l solvent)
<i>O. stamineus</i>	10 $\mu$ l	10 $\mu$ l <i>O. Stamineus</i> ethanol extract
<i>M. citrifolia</i>	10 $\mu$ l	10 $\mu$ l <i>M. citrifolia</i> ethanol extract
<i>O. stamineus</i> F1	10 $\mu$ l	10 $\mu$ l <i>O. Stamineus</i> fraction No. 1
<i>O. stamineus</i> F2	10 $\mu$ l	10 $\mu$ l <i>O. Stamineus</i> fraction No. 2
<i>O. stamineus</i> F3	10 $\mu$ l	10 $\mu$ l <i>O. Stamineus</i> fraction No. 3
<i>O. stamineus</i> F4	10 $\mu$ l	10 $\mu$ l <i>O. Stamineus</i> fraction No. 4
<i>O. stamineus</i> F5	10 $\mu$ l	10 $\mu$ l <i>O. Stamineus</i> fraction No. 5
<i>O. stamineus</i> F6	10 $\mu$ l	10 $\mu$ l <i>O. Stamineus</i> fraction No. 6
<i>M. citrifolia</i> F1	10 $\mu$ l	10 $\mu$ l <i>M. citrifolia</i> fraction No. 1
<i>M. citrifolia</i> F2	10 $\mu$ l	10 $\mu$ l <i>M. citrifolia</i> fraction No. 2
<i>M. citrifolia</i> F3	10 $\mu$ l	10 $\mu$ l <i>M. citrifolia</i> fraction No. 3
<i>M. citrifolia</i> F4	10 $\mu$ l	10 $\mu$ l <i>M. citrifolia</i> fraction No. 4
<i>M. citrifolia</i> F5	10 $\mu$ l	10 $\mu$ l <i>M. citrifolia</i> fraction No. 5
<i>M. citrifolia</i> F6	10 $\mu$ l	10 $\mu$ l <i>M. citrifolia</i> fraction No. 6

### 3.11 *In vitro* antioxidant for cell line experiment

The crude extracts of both *O. stamineus* and *M. citrifolia* and the fractions that prevent the oxidative damage in the *in vitro* hepatoprotective activity experiment (*O. stamineus* F3 and *M. citrifolia* F2) were used for antioxidant assay experiment. Approximately, 1000  $\mu$ l of the WRL-68 cell line suspension were seeded in 12-well flat bottom micro titer plates at  $2 \times 10^6$  cells/ml in DMEM medium containing 10 % (v/v) FBS and allowed to attach overnight. The second day, the cells were treated with 100  $\mu$ g of various treatment doses in triplicate according to (Table 3.7) and incubated at 37°C with 5 % CO<sub>2</sub> for 2 hr. The treated cells were induced by 100  $\mu$ l of freshly prepared 1000  $\mu$ M H<sub>2</sub>O<sub>2</sub> and re-incubated at for 2 hr. The H<sub>2</sub>O<sub>2</sub>-treated and -untreated cells after removing the medium, were harvested, washed twice with PBS and lysed in lysis buffer (25 mmol/l Tris-HCl). WRL-68 cell lysates were prepared in a 0.5 ml cold phosphate buffer saline (PBS) (pH 7.4). All The cell debris was removed by centrifugation at 100 rpm for 10 min at 4°C using refrigerated centrifuge Rotofix 32 (Hettich Zentrifugen, Germany). All samples were sonicated for 5 min with 10 sec rest after each min. The samples were kept at -20°C until used. The supernatant was used for the estimation of the following antioxidant using commercially available kits from (Cayman Chemical Company, USA): malondialdehyde (MDA) (Item No. 10009055), Catalase (Item No. 707002), superoxide dismutase (Item No. 706002) and glutathione peroxidase (Item No. 703102) activities. All assays performed according to the instruction manual of the manufacturer (appendix III). Protein concentration was determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

Table 3.7 *In vitro* antioxidant for cell line experimental design

Group Name	Oxidant agent (1000 $\mu$ M H <sub>2</sub> O <sub>2</sub> )	Treatment (100 $\mu$ g/ml)
Control (Normal cell group)	No oxidant agent (100 $\mu$ l medium)	No treatment (100 $\mu$ l solvent)
H <sub>2</sub> O <sub>2</sub> (Oxidative damage group)	100 $\mu$ l	No treatment (100 $\mu$ l solvent)
<i>O. stamineus</i>	100 $\mu$ l	100 $\mu$ l <i>O. Stamineus</i> ethanol extract
<i>M. citrifolia</i>	100 $\mu$ l	100 $\mu$ l <i>M. citrifolia</i> ethanol extract
<i>O. stamineus</i> F3	100 $\mu$ l	100 $\mu$ l <i>O. Stamineus</i> fraction No. 1
<i>M. citrifolia</i> F2	100 $\mu$ l	100 $\mu$ l <i>M. citrifolia</i> fraction No. 2
Gallic acid (Standard drug)	100 $\mu$ l	100 $\mu$ l <i>M. citrifolia</i> fraction No. 3

### 3.12 Identification of active constituents

In this study liquid chromatography-mass spectrometry (LC-MS) was used for identification of phenolics and other active constituents in the fractions that give positive result with *in vitro* hepatoprotective activity assay i.e., *O. stamineus* fraction 3 (F3) and *M. citrifolia* fraction 2 (F2). The identification and characterization of isolated compounds from active fractions were performed by comparing ultraviolet (UV) and mass spectrometry (MS) as described by (Lin *et al.*, 2008; Masuda *et al.*, 1992). The components of fractions such as phenolics give positive or negative ion mass spectra containing intense  $[M+H]^+$ ,  $[M-H]^-$  ions as well as fragment ions created after the cleavage of compound bonds. The method development and optimization were done at Cancer Research Initiatives Foundation (CARIF) laboratory (appendix I).

### 3.12.1 Sample preparation

Samples of fraction (5 mg each) were dissolved in 5 ml of methanol, and sonicated for 10 - 15 min. The contents filtered through a 0.45 µm syringe filter. The stock solutions were used for further dilutions and kept in fridge at 4°C prior to analysis.

### 3.12.2 Instrumentation and parameter used

Instrument details: Waters Ultra Performance Liquid Chromatography (Waters UPLC)

Software used: Waters MassLynx 4.1

Column specification: ACQUITY UPLC BEH C18 1.7µm, 2.1 x 50mm Column

Detector: ACQUITY PDA Detector (ACQ-PDA) Version 1.40.1932

The fractions were monitored at 280 nm, and UV spectra from 190 to 800 nm were recorded for peak characterization. Full scan mass spectra were measured between  $m/z$  150 and 1000 u in both positive and negative ion mode.

Waters Synapt HDMS

Electron spray ionization (ESI)

Source (same for +ve and –ve mode):

- a) Capillary voltage: 2.7 kV
- b) Sampling cone: 40
- c) Extraction cone: 4.0
- d) Source temperature: 100° C
- e) Desolvation temperature: 350° C
- f) Cone gas flow: 30 L/h
- g) Desolvation gas flow: 700 L/h

Company name: Cancer Research Initiatives Foundation (CARIF)

Type & length of column: Reverse phase C-18; 2.1 x 50 mm; 1.7 µm particle size

Injection volume: 3  $\mu$ L

Flow rate: 0.5 ml/ min

Positive and negative ions are analyzed in mass spectrometers.

Solvent system: H<sub>2</sub>O + 0.1 % Formic acid/ acetonitrile (ACN) + 0.1 % Formic acid according to the gradient condition mentioned in (Table 3.8).

Table 3.8 UPLC and LC-MS gradient conditions

Time (min)	H <sub>2</sub> O + 0.1 % F.A (%)	ACN + 0.1 % F.A (%)
0.00	100	0
7.00	0	100
10.00	0	100
11.00	100	0
15.00	100	0

### 3.13 Antibacterial assay

#### 3.13.1 Microorganisms

The following bacteria strains were used as test organisms: *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (laboratory isolate supplied from the Clinical Microbiology Laboratory / University of Malaya), *Escherichia coli* (ATCC 25922) and *Klebsilla pneumonia* (ATCC 700603).

#### 3.13.2 Disk diffusion method

Antimicrobial susceptibility test of the isolated organisms was done by disc diffusion method using the Kirby-Bauer technique (Bauer *et al.*, 1966) and according to the standards of the National Committee for Clinical technique Laboratory Standards (NCCLS, 2003). All tests were performed on Mueller-Hinton agar, the surface of media

was lightly and uniformly inoculated by cotton swab. Prior to inoculation, the swab stick was dipped into bacterial suspension having visually equivalent turbidity to 0.5 McFarland standards (approximately  $1.5 \times 10^6$  CFU/ml). The swab stick was then took out and squeezed on the wall of the test tube to discard extra suspension. Sterile 6.0 mm diameter blank discs (Oxoid, Basingstokc, England) were used to load 50 µl of the plant extracts (equivalent to 5 mg/disc) dissolved in 5 % sterile DMSO for ethanol extracts and in sterile distilled water for aqueous extracts, then discs were allowed to dry. Amoxicillin 2 µg/disc, Gentamicin 30 µg/disc and Vancomycin 5 µg/disc were obtained from Oxoid Ltd (Oxoid, Basingstokc, England) were used as a positive control, whereas solvent loaded discs as negative control. The extracts, 5 % sterile DMSO, sterile distilled water impregnated discs and the standard drug antibiotic discs were placed on Muller-Hinton agar and incubated at 35°C for 18-20 hours. On the next day, the diameters of inhibition zones in mm were recorded, the experiment was performed in triplicates and average diameter of zone of inhibition was calculated.

### **3.13.3 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)**

The Broth micro-dilution method was used to determine the MIC according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 2003), this assay was done for the extracts that exhibited considerable activity against bacteria in the disk diffusion method (inhibition zone  $\geq 8$  mm). Using sterile round-bottom 96-well plates, twofold serial dilutions of extracts were prepared in the appropriate broth containing 5 % (v/v) DMSO. Briefly, each plant extract was issued to a serial dilution by using sterile Nutrient broth. The concentrations of plant extracts were 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 mg/ml. Approximately 10 µl of a bacterial cell suspension prepared in Nutrient broth, corresponding to 0.5 McFarland standards, was added to all

wells except those served as negative control. A generally recommended initial cell concentration is  $5 \times 10^5$  CFU/tube. Controls for bacterial growth without plant extracts were also included on each plate; plate was then incubated at 37°C for 18-20 h. The higher dilution of the plant's extracts [i.e., lowest concentration] that produced no visible growth of the bacteria (no turbidity) when compared with the control wells were considered as the MIC of the extract. After that, the contents of all wells that showed no visible growth were cultured on nutrient agar and incubated further at 37°C overnight, the next day, the lowest concentration that showed no single bacterial colony on agar considered as the MBC.

### **3.14 Statistical analysis**

The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Bonferroni's or LSD multiple comparison test. All values were expressed as mean  $\pm$  SEM and a value of  $p < 0.05$  was considered significant as compared to the respective control group using SPSS programme for windows version 18 (SPSS Inc. Chicago, IL, USA).



## CHAPTER IV

### RESULTS

#### 4.1 Antioxidant activity

*O. stamineus* exhibited high free radical scavenging activity towards DPPH and ABTS radicals (Figure 4.1 and Figure 4.2). DPPH data in (Table 4.1) showed that there is no significant difference between the IC<sub>50</sub> of the ethanol extract of *O. stamineus* ( $21.4 \pm 0.104$  µg/ml), and the IC<sub>50</sub> of the synthetic antioxidant standard BHT ( $21.1 \pm 0.031$  µg/ml). On the other hand, ethanolic extract of *M. citrifolia* does not have significant free radical scavenging activity with IC<sub>50</sub> >25 µg/ml (Table 4.1). Moreover, *O. stamineus* exhibited high antioxidant activity as proven by FRAP. Data in (Table 4.1) showed that the FRAP result of *O. stamineus* is comparable to that of standard ascorbic acid as there is no statistically significant difference between them, while the *M. citrifolia* has very low FRAP activity. On the other hand, the total phenolic contents (TPC) in ethanolic extract of *O. stamineus* were  $294.3 \pm 0.051$  mg (Gallic acid equivalents) per g of extracts (standard curve equation:  $y = 0.0013x + 0.0032$ ,  $R^2 = 0.987$ ). At the same time, flavonoids were  $171.4 \pm 0.016$  mg (Quercetin equivalents) per g of extracts (standard curve equation:  $y = 0.0040x + 0.0085$ ,  $R^2 = 0.991$ ), and a ratio flavonoids/phenolic of 0.58 (Figure 4.3 and Table 4.1). The correlation between the TPC results and antioxidant activity represented by DPPH, FRAP and ABTS are 0.996, 0.983 and 0.997 respectively (Figure 4.4). Thus, phenolic compounds were the predominant antioxidant components in *O. stamineus* ethanol extracts, which lead to more potent radical scavenging effect and antioxidant activity.

Table 4.1 Crude extracts antioxidant activity, total phenolic and flavonoids of the investigated plants

Plant extracts and standards	DPPH IC <sub>50</sub> (µg/ml)	FRAP (µmol Fe (II)/g)	ABTS IC <sub>50</sub> (µg/ml)	TPC value mg Gallic acid/g of extract	Flavonoids mg Quercetin/g of extract	Flavonoids/ Phenolic Ratio
<i>O. stamineus</i> Ethanol Extract	21.4± 0.104 <sup>b</sup>	1692.8± 85.09 <sup>c</sup>	83.5± 0.285 <sup>c</sup>	294.3± 0.051 <sup>b</sup>	171.4± 0.016 <sup>b</sup>	0.58
<i>M. citrifolia</i> Ethanol Extract	>25	280.6± 41.76 <sup>a</sup>	>100	46.0 ± 0.013 <sup>a</sup>	31.8± 0.023 <sup>a</sup>	0.69
BHT	21.1±0.031 <sup>b</sup>	988.8± 24.83 <sup>b</sup>	69.7±0.470 <sup>b</sup>	-----	-----	-----
Ascorbic acid	4.6±0.006 <sup>a</sup>	1987.4± 34.98 <sup>c</sup>	24.5± 0.384 <sup>a</sup>	-----	-----	-----

All results are represented the mean ± SEM of triplicates; <sup>a-c</sup> Means followed by the same letters are not significantly different, \*  $P < 0.05$ . Both ascorbic acid and (BHT) butylated hydroxytoluene used as standards.

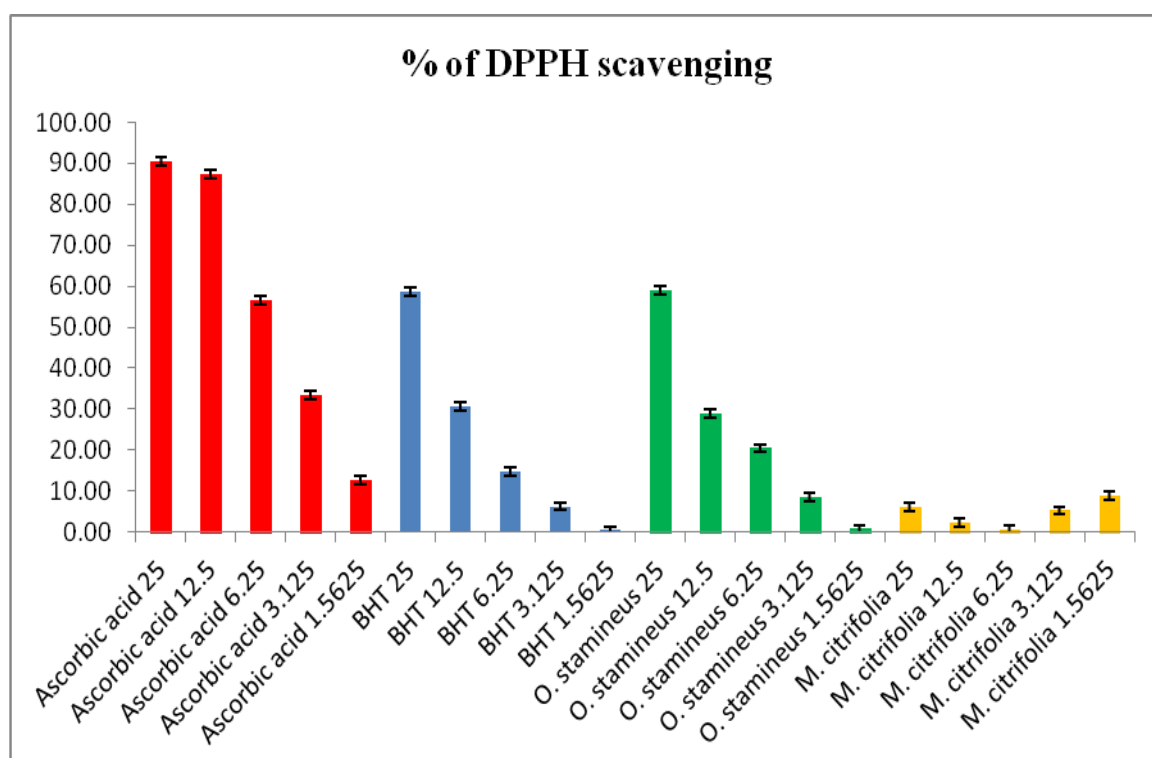


Figure 4.1 Percentage of DPPH scavenging activity of *O. Stamineus*, *M. citrifolia* and standards, all serial dilution in  $\mu\text{g/ml}$ .

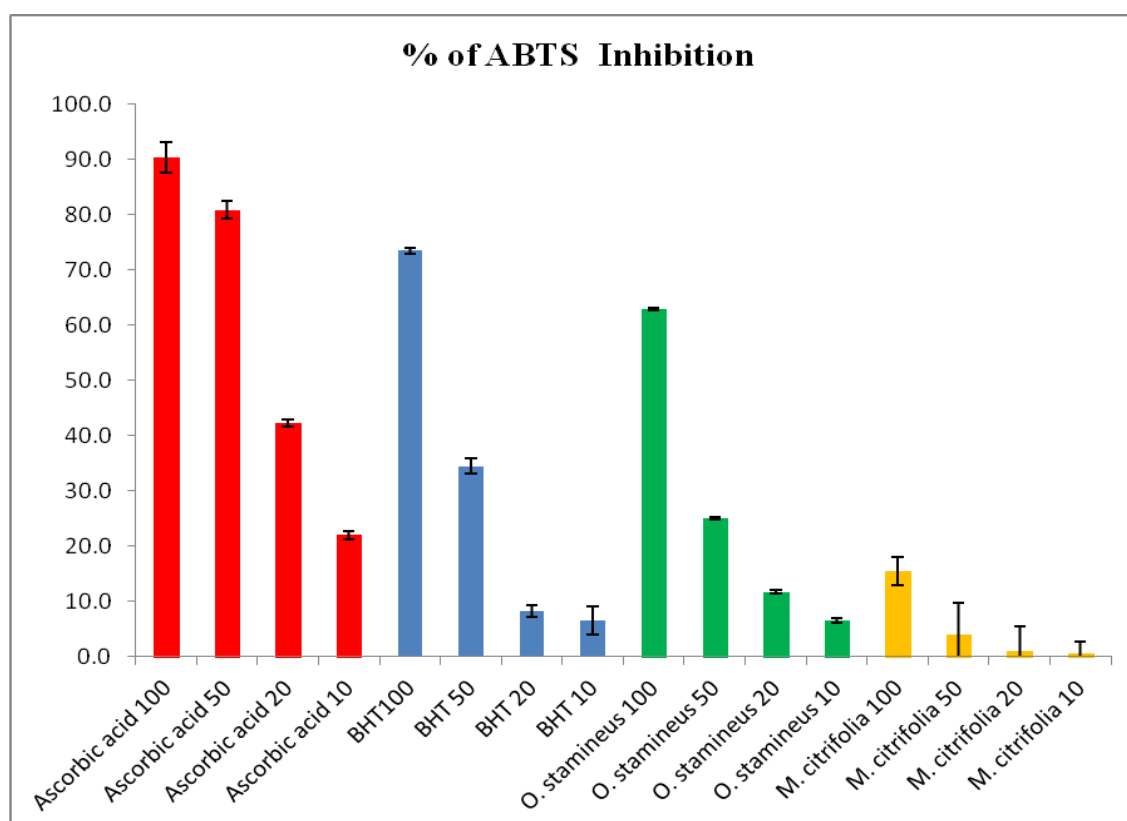


Figure 4.2 Percentage of ABTS inhibitions of *O. Stamineus*, *M. citrifolia* and standards, all serial dilution in  $\mu\text{g/ml}$ .

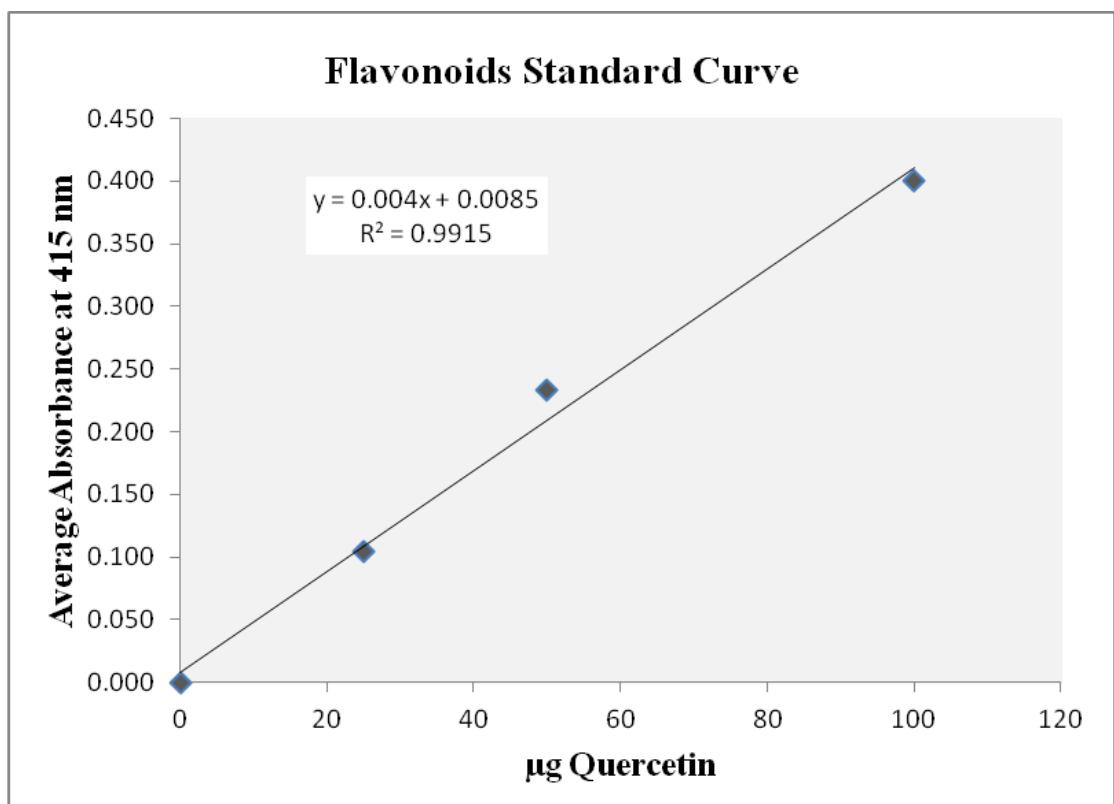
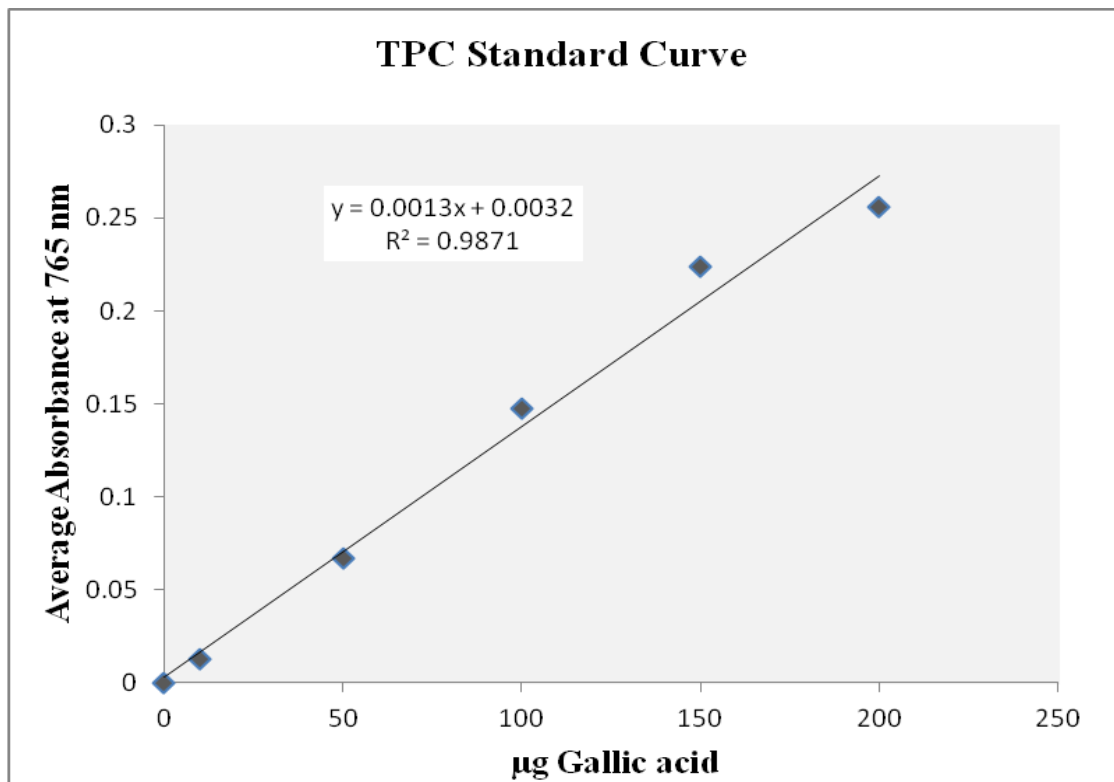


Figure 4.3 Standard curve equations of TPC and flavonoids.

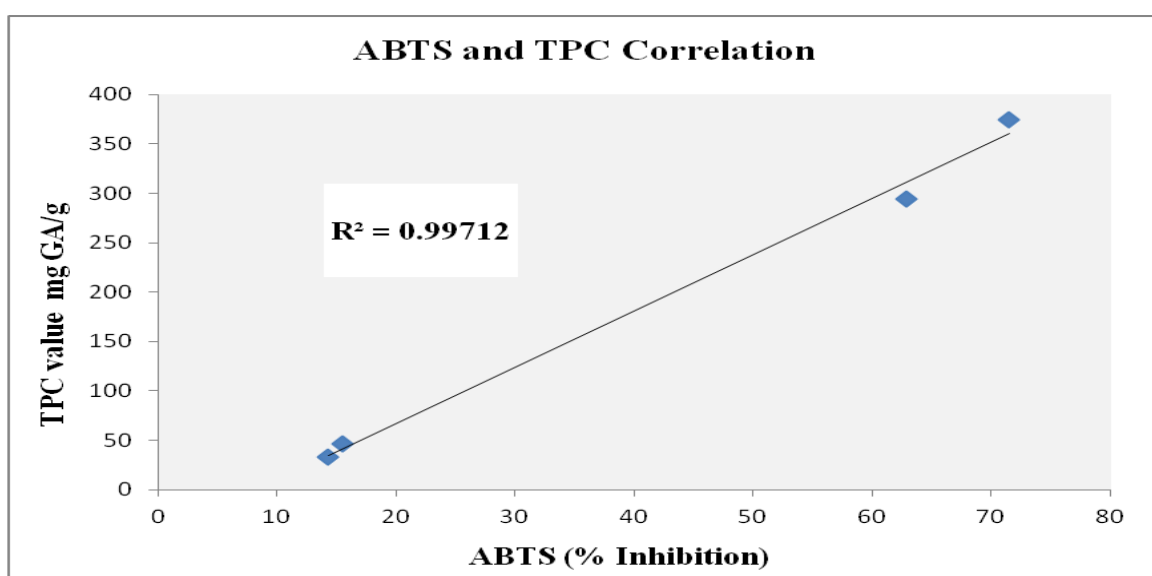
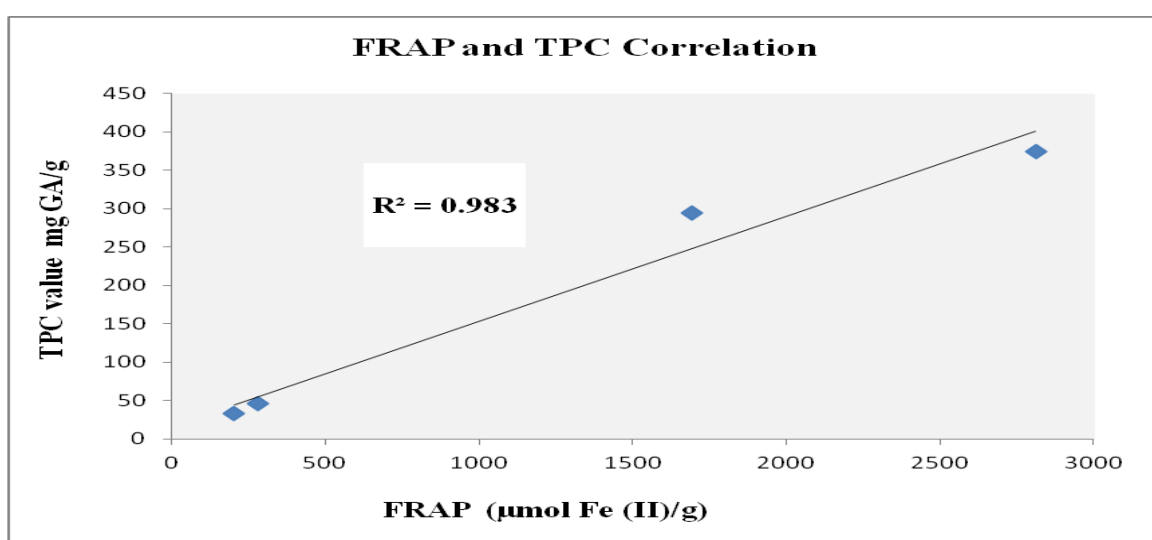
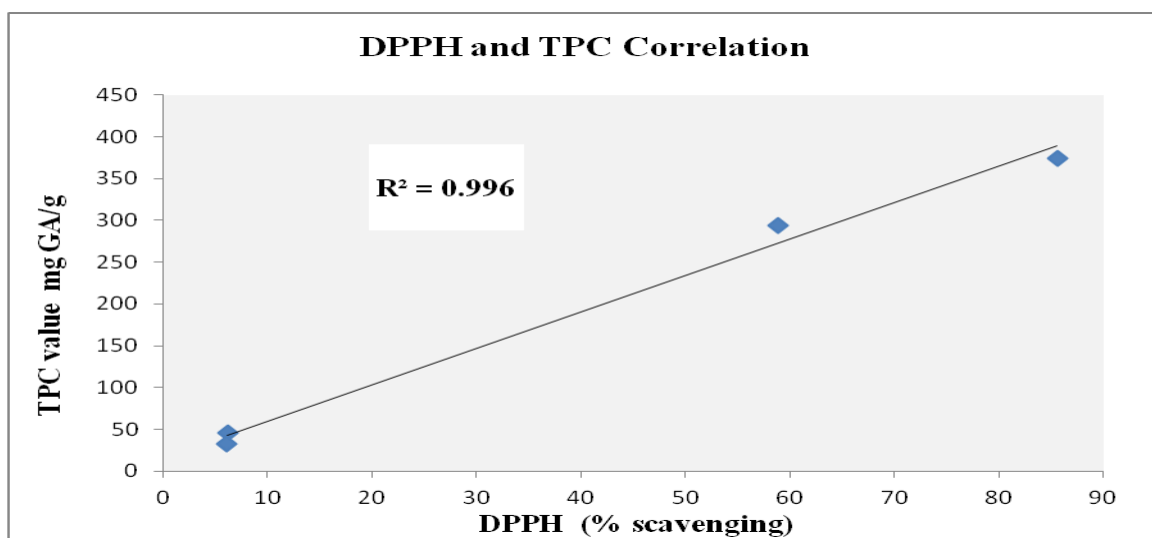


Figure 4.4 Correlation between TPC and antioxidant activity represented by DPPH, FRAP and ABTS

## 4.2 Acute toxicity

All animals showed no mortality and did not manifest any significant signs of toxicity. There were no abnormal signs, behavioural changes, or body weight changes at any time of observation. There was no mortality up to 5 g/kg dose of both plants at the end of 14 days of observation. Histological examination of liver and kidney, revealed no significant changes between the different groups (Figure 4.5). Consequently, it is concluded that the crude extracts of *O. stamineus* and *M. citrifolia* are safe, even at these higher doses and have no toxicity and the oral lethal dose (LD<sub>50</sub>) is greater than 5 g/kg body weight.

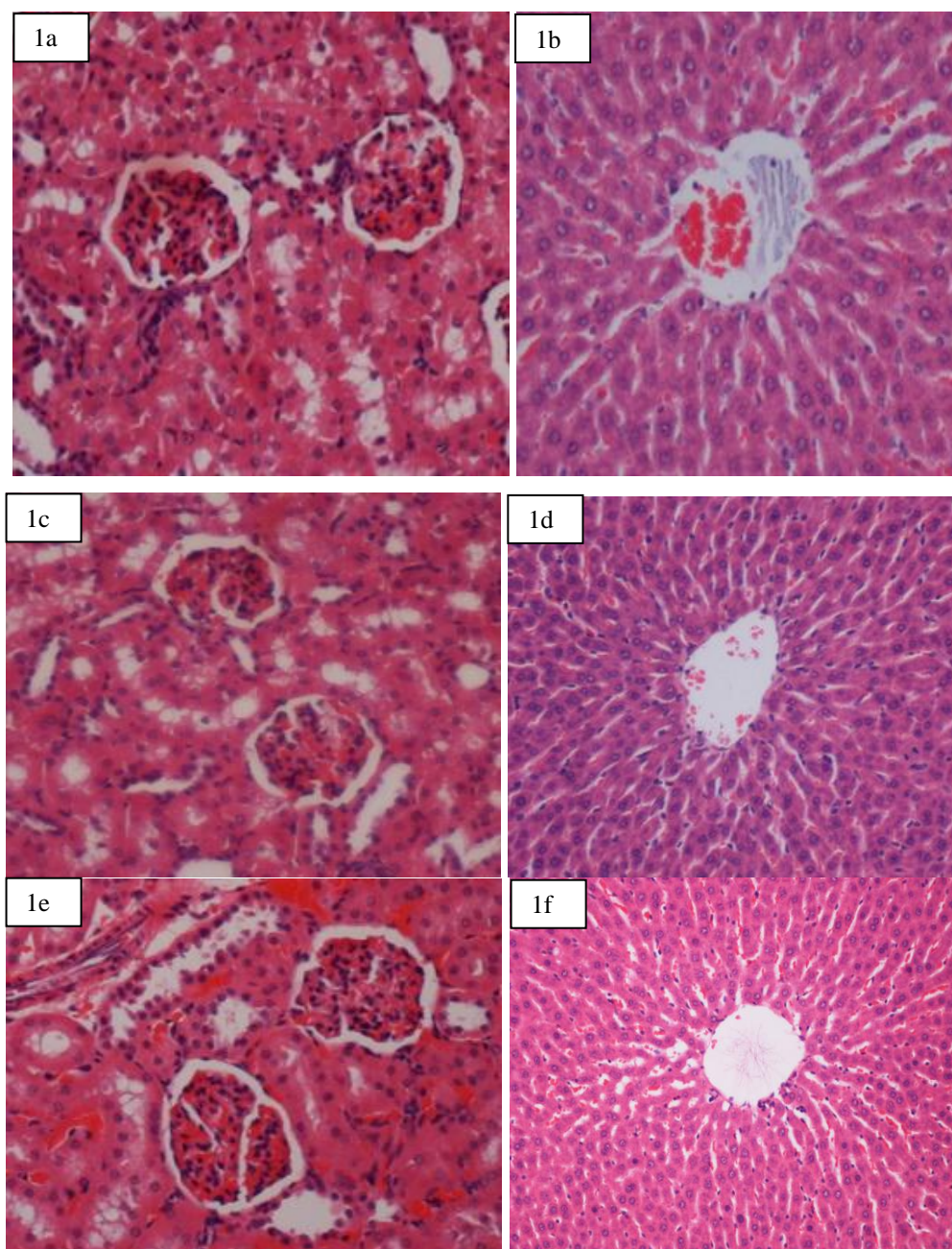


Figure 4.5 Histological sections of liver and kidney in acute toxicity test. Rats (1a and 1b) treated with 5 ml/kg vehicle as control group. Rats (1c and 1d) treated with (5 g/kg (5 ml/kg) *O. stamineus* extract. Rats (1e and 1f) treated with 5 g/kg (5 ml/kg) *M. citrifolia* (H & E stain 20x).

### 4.3 *In vivo* hepatoprotective activity of plant extracts

#### 4.3.1 Body, liver and spleen weight

Before starting of treatment the rats weighed 200–250 gm and after two months animals of normal, *O. stamineus* HD, *O. stamineus* LD, *M. citrifolia* HD, *M. citrifolia* LD and Silymarin groups reached average body weights of 254.9, 232.7, 263.3, 244.5, 235.7

and 257.0 gm respectively. However, TAA positive control group the average body weight was decreased to 202.0 gm but without a significant difference compared to the normal control group. There was no significant difference between the groups but long term taken of TAA led to significant increase of the liver weight compared to normal rats. Values of mean relative liver weight (LW/BW) percent, showed a significant difference between treated groups compared to TAA group (Table 4.2).

#### **4.3.2 Biochemical parameters**

Long term administration of TAA led to significant increase of biochemical markers ALT, AST, ALP, and Bilirubin level, while significantly decreased total protein and albumin compared to the normal control group, indicating acute hepatocytes damage. Treatment of animals with *O. Stamineus*, *M. citrifolia* extracts and Silymarin significantly reduced the level of liver function biomarker (ALT, AST, ALP and bilirubin), in addition significantly increased in total protein and albumin comparing with the TAA group. The toxic effect of TAA was controlled in the rats treated with 200 mg/kg ethanolic extracts of both plants and that's approved by restored of the levels of the liver biomarker. At a dose of 100 mg/kg, in *O. stamineus* the effect was only marginal, whereas there was no significant difference for *M. citrifolia* (Table 4.3). The ethanol extracts of *O. stamineus* significantly restored the altered liver parameters and made it more resemble to that of standard drug Silymarin (50 mg/kg). Moreover, *O. stamineus* extract at 200 mg/kg ( $P<0.01$ ) demonstrated the most potent effect in protecting rats against TAA-induced liver damage, as evidenced by the reduced in all enzyme levels of AST, ALT and ALP and increased in total protein and albumin levels compared to the control.



Table 4.2 Effects of TAA, Silymarin *O. stamineus* and *M. citrifolia* ethanolic extracts intake on the body, liver and spleen weight of rats

Animal Group	Body weight (gm) (BW)	Liver weight (gm) (LW)	LW/BW (%)	Spleen weight (g) (SW)	SW/BW (%)
Normal Control	254.9± 28.69	6.71± 0.64	2.71± 0.18	0.47± 0.08	0.18± 0.02
TAA Control (hepatotoxic group)	202.0± 19.10	11.00 ± 1.11 <sup>*a</sup>	5.43± 0.17 <sup>*a</sup>	0.52± 0.07	0.26± 0.03
<i>O. stamineus</i> HD 200mg/kg	232.7± 16.12	10.43 ± 0.69	4.50± 0.19 <sup>*b</sup>	0.54± 0.04	0.23± 0.01
<i>O. stamineus</i> LD 100mg/kg	263.3± 8.53	10.43 ± 0.72	3.94± 0.16 <sup>*c</sup>	0.55± 0.03	0.21± 0.01
<i>M. citrifolia</i> HD 200mg/kg	244.5 ± 15.84	10.67 ± 0.71	4.45 ± 0.37	0.47 ± 0.05	0.22 ± 0.02
<i>M. citrifolia</i> LD 100mg/kg	235.7 ± 17.52	10.00 ± 0.87	4.27 ± 0.26 <sup>*b</sup>	0.48 ± 0.05	0.23 ± 0.01
Silymarin 50mg/kg (hepatoprotective group)	257.0± 21.97	7.71 ± 1.08	2.94± 0.13 <sup>*c</sup>	0.53± 0.07	0.20± 0.01

All values are expressed as mean ± S.E.M. Means with different superscripts are significantly different. <sup>a</sup>  $P < 0.05$  versus Normal control group, <sup>b</sup>  $P < 0.05$  versus TAA control group, and <sup>c</sup>  $P < 0.01$  versus TAA control group.

Table 4.3 Effect of TAA, Silymarin, *O. stamineus* and *M. citrifolia* ethanolic extracts intake on serum hepatic biomarker in TAA-induced liver cirrhosis in rats

Animal Group	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Bilirubin (mg/dl)	Total Protein (g/l)	Albumin (g/l)
Normal Control	64.9± 4.19	164.4± 10.74	109.6± 9.80	1.86± 0.14	74.3± 1.15	12.1± 0.51
TAA Control (hepatotoxic group)	213.3± 25.98 <sup>a</sup>	372.6± 29.98 <sup>a</sup>	435.8 ± 29.78 <sup>a</sup>	8.7± 0.57 <sup>a</sup>	60.7± 0.97 <sup>a</sup>	8.3± 0.57 <sup>a</sup>
<i>O. stamineus</i> HD 200mg/kg	95.7± 9.35 <sup>c</sup>	228.6± 14.10 <sup>c</sup>	289.0 ± 14.23 <sup>c</sup>	4.8± 0.59 <sup>c</sup>	68.0± 2.06 <sup>c</sup>	11.1± 0.63 <sup>b</sup>
<i>O. stamineus</i> LD 100mg/kg	108.0± 11.15 <sup>c</sup>	253.4± 18.67 <sup>c</sup>	383.6± 20.89	6.4± 0.72 <sup>b</sup>	64.6± 1.29	9.3± 0.36
<i>M. citrifolia</i> HD 200mg/kg	100.8± 10.09 <sup>c</sup>	248.8± 24.66 <sup>b</sup>	301.2 ± 9.81 <sup>c</sup>	4.7± 0.49 <sup>c</sup>	69.5± 1.23 <sup>c</sup>	11.3± 0.49 <sup>c</sup>
<i>M. citrifolia</i> LD 100mg/kg	143.0± 17.95 <sup>b</sup>	273.1± 31.88 <sup>b</sup>	387.9± 25.20	6.7± 0.99	66.1± 2.58	10.4± 0.53
Silymarin 50mg/kg (hepatoprotective group)	70.4± 5.60 <sup>c</sup>	171.6± 10.19 <sup>c</sup>	139.4 ± 9.54 <sup>c</sup>	3.0± 0.31 <sup>c</sup>	70.9± 0.91 <sup>c</sup>	11.7± 0.68 <sup>c</sup>

All values are expressed as mean ± S.E.M. of eight rats in each group. Means with different superscripts are significantly different. <sup>a</sup>  $P < 0.05$  versus Normal control group, <sup>b</sup>  $P < 0.05$  versus TAA control group, and <sup>c</sup>  $P < 0.01$  versus TAA control group. ALT: alanine aminotransferase, AST: aspartate aminotransferase, and ALP: alkaline phosphatase.

### 4.3.3 Histopathology examination

Histopathological examination of liver sections of the normal group showed regular cellular architecture with distinct hepatic cells, sinusoidal spaces and a central vein. The hepatocytes are polygonal cells with well preserved cytoplasm, nucleus with prominent nuclei. On the other hand, in the hepatotoxic positive control group histological examination showed loss of architecture, inflammation and congestion with cytoplasmic vacuolation, fatty change, sinusoidal dilatation, centrilobular necrosis, displayed bundles of collagen surrounding the lobules, which resulted in huge fibrous septa and distorted tissue architecture. In *O. stamineus* and *M. citrifolia* treated animals liver sections showed mild inflammation and mild necrosis of hepatocytes with mild cytoplasmic vacuolation, and mostly no visible changes observed. Histopathological examination also showed good recovery of thioacetamide-induced necrosis by ethanolic extracts as compared to Silymarin. Animals treated with the low dose showed regeneration of hepatocytes surrounded by septa of fibrous tissue with a significant increase in bile ductules, fat storing cells, and Kupffer cells. Animals treated with the higher dose of both plant extracts showed remarkable histological regeneration compared to those of the LD groups. They showed nearly ordinary patterns with an increase normal hepatocytes parenchyma and a reduced development of fibrous septa and lymphocyte infiltration. Results of the gross and histopathological examination are shown in the figures (Figure 4.6 and Figure 4.7).

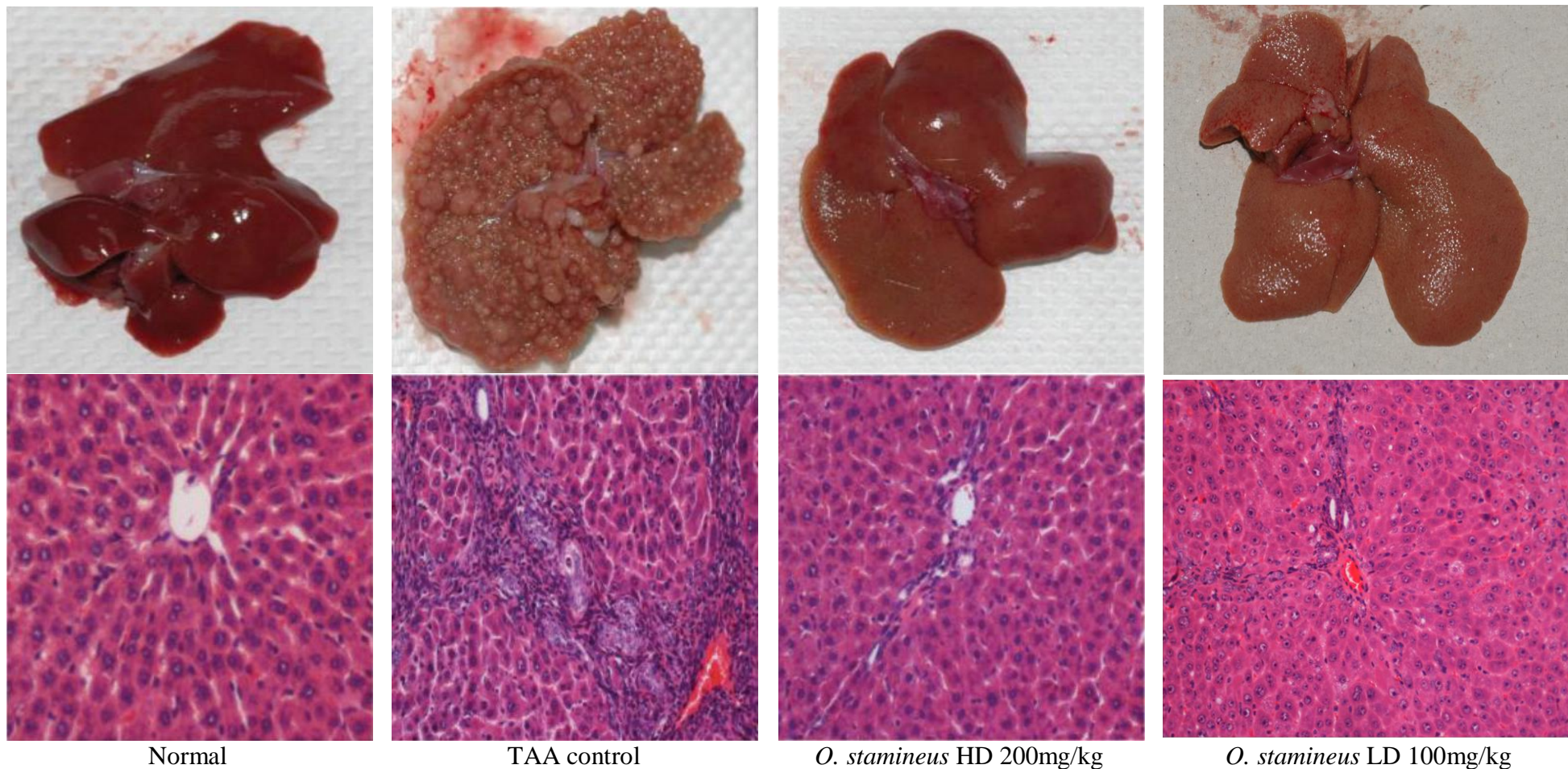


Figure 4.6 Effect of TAA and *O. Stamineus* ethanolic extracts on liver gross and histology in TAA -induced liver cirrhosis rats after two months treatments, (H & E stain 20x).



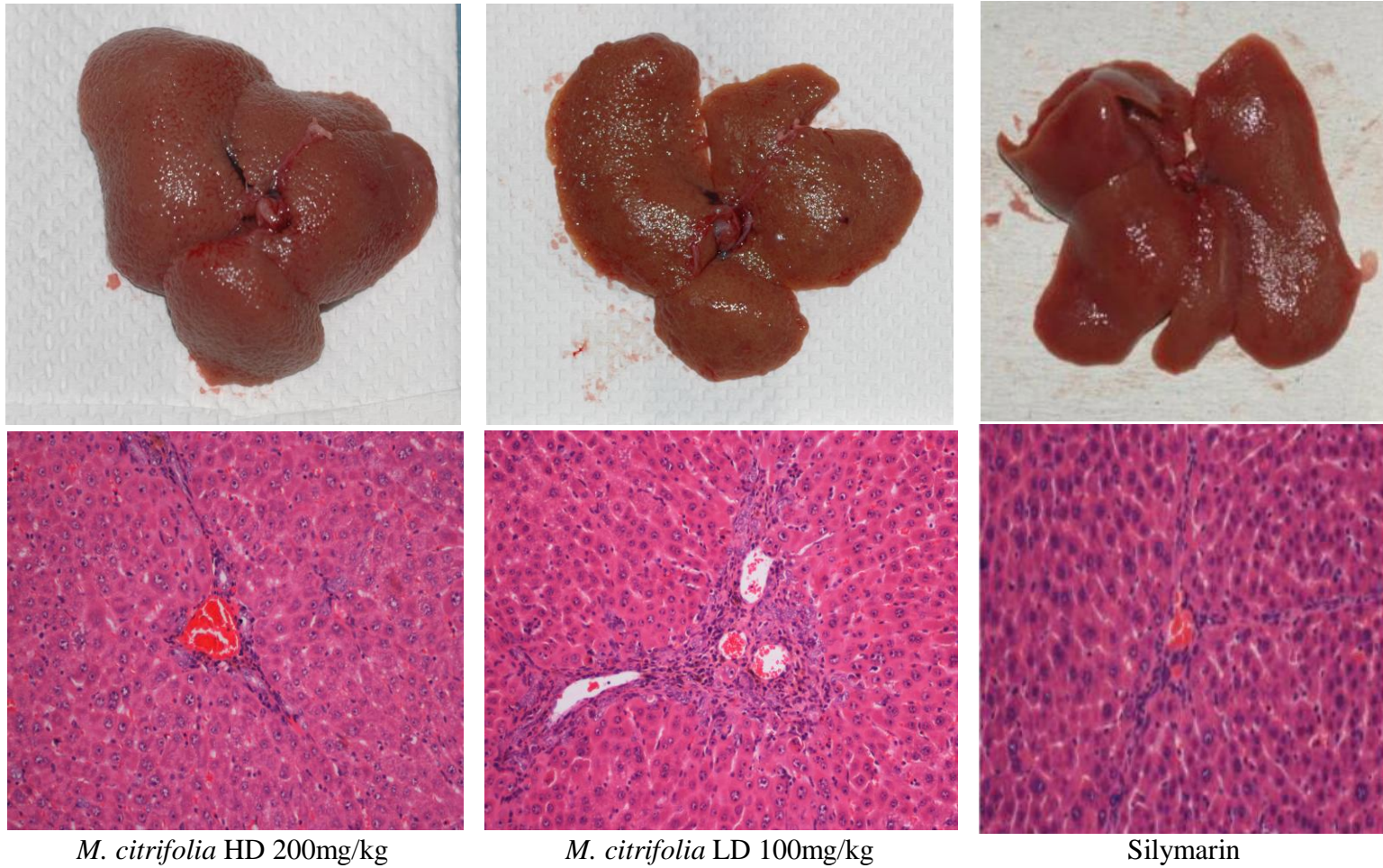


Figure 4.7 Effect of Silymarin and *M. citrifolia* ethanolic extracts on liver gross and histology in TAA -induced liver cirrhosis rats after two months treatments, (H & E stain 20x).

#### **4.4 *In vivo* antioxidant activity in liver tissue**

The ABTS which represents the total antioxidant capacity significantly increased in high dose treatment groups of both plants and silymarin, while decreased in TAA induced groups due to long term excretion of free radicals. In addition, catalase, SOD, and GPx are some of the components of intrinsic antioxidant defence system; it is responsible for dissemination of free radicals such as superoxide radicals. During oxidative stress the body uses its defence mechanism to minimize the process of lipid peroxidation by using these antioxidant enzymes, thus, the activity of those enzymes become higher in early stages of TAA induction, but when the insult continue for long period, the enzymes become depleted and unable to fight against free radicals, which means that in advance stages of peroxidation due to TAA the activity of catalase, SOD, and GPx declined as shown in (Table 4.4). While the levels of these enzymes persist high in treatment groups, because the antioxidant properties of plant extracts against TAA induced free radicals. On the other hand, long term administration of TAA led to significant increase of MDA level compared to the normal control group, indicating acute hepatocytes damage. Treatment of animals with *O. stamineus*, *M. citrifolia* extracts and silymarin significantly reduced the level of MDA and lipid peroxidation.

Table 4.4 Effect of TAA, Silymarin, *O. stamineus* and *M. citrifolia* ethanolic extracts intake on some *in vivo* antioxidant parameters in TAA-induced liver cirrhosis in rats

Group	ABTS (mM Trolox)	CAT (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)	MDA (nmol/ mg protein)
Normal Control	5.5 ± 0.60	2785.24 ± 34.32	326.23 ± 7.14	144.53 ± 0.63	38.74 ± 2.61
TAA Control	3.23 ± 0.28	2123.57 ± 244.87 <sup>*a</sup>	226.3 ± 8.58 <sup>*a</sup>	81.44 ± 0.58 <sup>*a</sup>	107.14 ± 3.71 <sup>*a</sup>
<i>O. stamineus</i> HD 200mg/kg	7.87 ± 0.21 <sup>*c</sup>	2682.56 ± 40.78	298.77 ± 1.17 <sup>*c</sup>	125.51 ± 1.07 <sup>*c</sup>	45.34 ± 3.52 <sup>*c</sup>
<i>O. stamineus</i> LD 100mg/kg	6.52 ± 0.56 <sup>*b</sup>	2492.06 ± 105.29	285.8 ± 6.82 <sup>*c</sup>	117.43 ± 0.58 <sup>*c</sup>	72.6 ± 3.94 <sup>*c</sup>
<i>M. citrifolia</i> HD 200mg/kg	7.19 ± 1.14 <sup>*c</sup>	2580.26 ± 56.01	270.47 ± 3.44 <sup>*c</sup>	120.95 ± 0.41 <sup>*c</sup>	54.35 ± 1.73 <sup>*c</sup>
<i>M. citrifolia</i> LD 100mg/kg	6.59 ± 0.98 <sup>*b</sup>	2479.75 ± 105.09	264.73 ± 0.93 <sup>*b</sup>	109.84 ± 1.13 <sup>*c</sup>	78.85 ± 2.26 <sup>*c</sup>
Silymarin 50mg/kg	8.53 ± 0.13 <sup>*c</sup>	2684.16 ± 75.89	309.9 ± 9.50 <sup>*c</sup>	126.01 ± 0.65 <sup>*c</sup>	40.34 ± 2.8 <sup>*c</sup>

All values are expressed as mean ± S.E.M. Means with different superscripts are significantly different. <sup>a</sup> *P* < 0.05 versus Normal control group, <sup>b</sup> *P* < 0.05 versus TAA control group, and <sup>c</sup> *P* < 0.01 versus TAA control group.

#### 4.5 Genes expression

According to the NormFinder and geNorm algorithm, the endogenous reference genes that showed the lowest variability were HPRT-1 and Ppia. However, GAPDH and Act b were shown differentially expressed, therefore excluded from further analyses (appendix IV). The gene expression levels of the target genes in animal liver tissue samples were normalized using both HPRT-1 and Ppia endogenous reference genes. The expression levels of TGF $\beta$ 1, TIMP1, MMP2, Coll  $\alpha$  and those two endogenous reference genes were subsequently validated by RT-PCR measurements. According to standard curve (appendix IV) all genes showed that the efficiency were between 90-110 % and the slope between (-3.1) and (-3.5) which are within the reference criteria to run the quantitative RT-PCR (Table 4.5). After data analysis of  $C_t$  values by Gen EX software and normalized to the reference genes HPRT-1 and Ppia, all measured mRNAs showed a significantly different expression between calibrator group (normal liver tissue) and TAA group (cirrhosis liver tissue). All four target genes were up-regulated, three of them (TGF $\beta$ 1, MMP2 and Coll  $\alpha$ ) were significantly over expressed in comparison to the calibrator. In contrast, we have demonstrated down-regulation of those three target genes in the *O. stamineus* and *M. citrifolia* treated groups, furthermore the down-regulations were highly significant difference ( $P < 0.01$ ); in *O. stamineus* treated group, while non significant in *M. citrifolia* group. Moreover, observed no significant changes in TIMP-1 gene expression between TAA-induced group, *O. stamineus* and *M. citrifolia* treatment groups, but significant up-regulation in TAA group in comparison to the calibrator (Figure 4.8). The expression of TGF $\beta$ 1 in TAA group increased  $1.45 \pm 0.21$ , ( $P = 0.02647$ ) fold compared with calibrator (normal rats), whereas in *O. stamineus* and *M. citrifolia* treated groups expression was  $0.72 \pm 0.27$ , ( $P = 0.00291$ ) and  $1.22 \pm 0.33$  fold lower compared with the expression in TAA group.



However, mRNA levels of MMP2 were over expressed in TAA group to  $10.78 \pm 0.54$ , ( $P = 0.000035$ ) fold higher compared to the calibrator and expressed  $2.61 \pm 0.57$ , ( $P = 0.00355$ ) and  $4.18 \pm 0.56$ , ( $P = 0.03084$ ) in *O. stamineus* and *M. citrifolia* treated groups respectively. The fold induction of Coll  $\alpha$ , in TAA group compared with calibrator was  $16.10 \pm 0.92$ , ( $P = 0.00092$ ) fold, whereas  $3.36 \pm 0.49$ , ( $P = 0.00062$ ) and  $8.70 \pm 0.56$  folds in *O. stamineus* and *M. citrifolia* treated rats respectively (Table 4.6).

Table 4.5 Measured efficiency slope and  $R^2$  of target and endogenous reference genes using the Ct slope method with 5 concentrations points

Genes	TGF $\beta$ 1	TIMP1	MMP2	Coll $\alpha$	HPRT1	Ppia	Reference criteria
<b>Efficiency (%)</b>	98.056	99.408	108.376	95.351	97.78	92.679	90 – 110
<b>Slope</b>	-3.369	-3.336	-3.136	-3.439	-3.376	-3.511	(-3.1) - (-3.5)
<b>Y-inter</b>	28.85	34.01	31.042	32.567	28.026	27.154	-----
<b>R<sup>2</sup></b>	0.993	0.989	0.991	0.99	0.995	0.998	Not less than 0.98

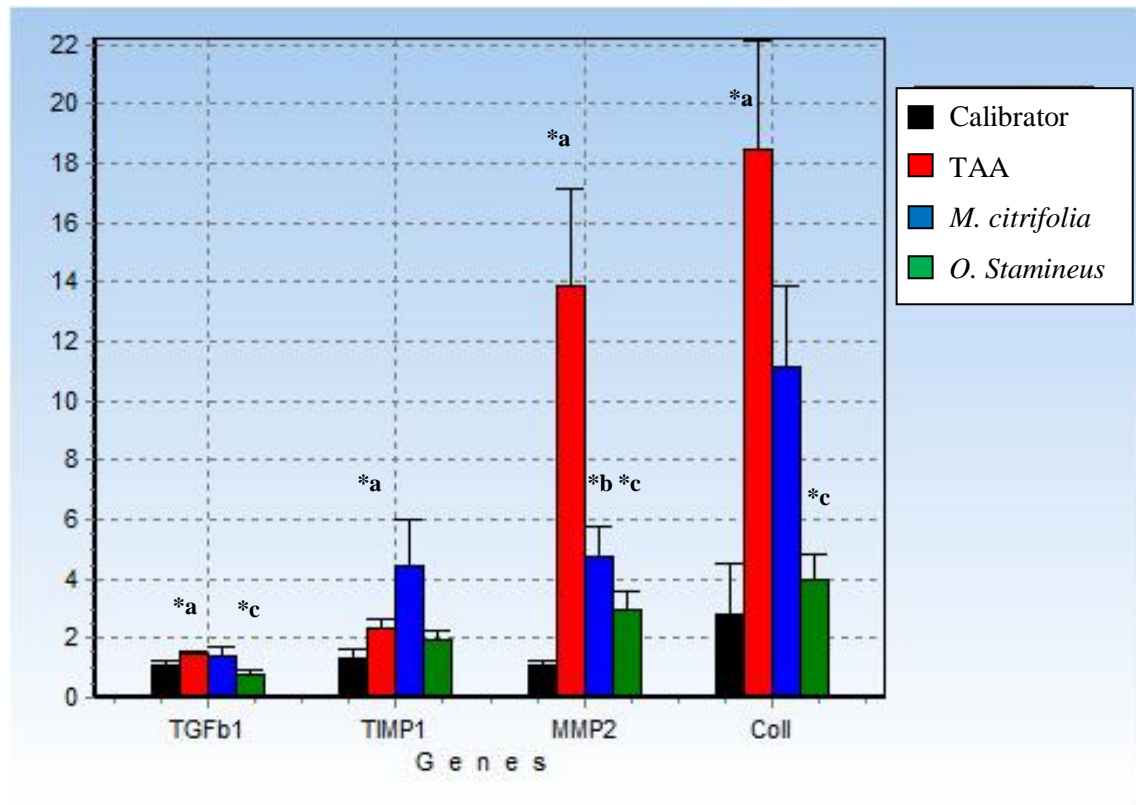


Figure 4.8 Relative gene expression levels (Fold change) of TAA, *O. stamineus* and *M. citrifolia* ethanolic extracts treatment in comparison to the calibrator in TAA-induced rat liver cirrhosis tissue. All values are expressed as mean  $\pm$  SEM. Means with different superscripts are significantly different. <sup>a</sup> $P < 0.05$  versus Calibrator, <sup>b</sup> $P < 0.05$  versus TAA group, and <sup>c</sup> $P < 0.01$  versus TAA group.

Table 4.6 Effect of TAA, *O. stamineus* and *M. citrifolia* ethanolic extracts intake on mRNA gene expression levels in TAA-induced rat liver cirrhosis tissue

Group	TGFβ1	TIMP1	MMP2	Coll α
Calibrator	1	1	1	1
TAA	1.45 ± 0.2106 <sup>*a</sup>	2.23 ± 0.4991 <sup>*a</sup>	10.78 ± 0.5378 <sup>*a</sup>	16.10 ± 0.9189 <sup>*a</sup>
<i>M. citrifolia</i>	1.22 ± 0.3335	2.98 ± 0.5639	4.18 ± 0.5588 <sup>*b</sup>	8.70 ± 0.5589
<i>O. Stamineus</i>	0.72 ± 0.2678 <sup>*c</sup>	1.72 ± 0.3839	2.61 ± 0.5659 <sup>*c</sup>	3.36 ± 0.4923 <sup>*c</sup>

All values are expressed as mean fold changes of mRNA from 7 rats  $\pm$  SEM. Statistical analysis of differences was performed by a two-tailed unpaired student's t test. Means with different superscripts are significantly different. <sup>a</sup> $P < 0.05$  versus Calibrator, <sup>b</sup> $P < 0.05$  versus TAA group, and <sup>c</sup> $P < 0.01$  versus TAA group.

#### 4.6 Immunomodulatory effects on PBMCs

Both ethanol extracts of *O. stamineus* and *M. citrifolia* significantly stimulated the proliferation of PBMCs *in vitro* in a dose-dependent manner, but the *O. stamineus* extract has remarkable activity on PBMCs proliferation. Results showed that the cell viability increased after treatment, it is clear that the extract is not potentially toxic to the immune cells and it is modulating the cellular immune response. In our experiment, after an incubation period of 24 hr, treatment with 200 µg/ml of *O. stamineus* and *M. citrifolia* ethanol extracts significantly increase the number of PBMCs with  $242.53 \pm 4.33$  % and  $123.77 \pm 1.62$  % respectively, compared to the control  $105 \pm 1.30$  % (Figure 4.9). On the other hand, there are no significant differences between 50, 25 and 12.5 µg/ml ethanol extract of both plants and control. As shown in (Figure 4.10) only 200 µg/ml of *O. stamineus* stimulated the PBMCs proliferation to more than double the amount of the initial PBMCs control numbers ( $242.53 \pm 4.33$  %), which may be of value in combination with other therapies in the treatment of immunodeficiency, cancer, infections and even autoimmune disorders.

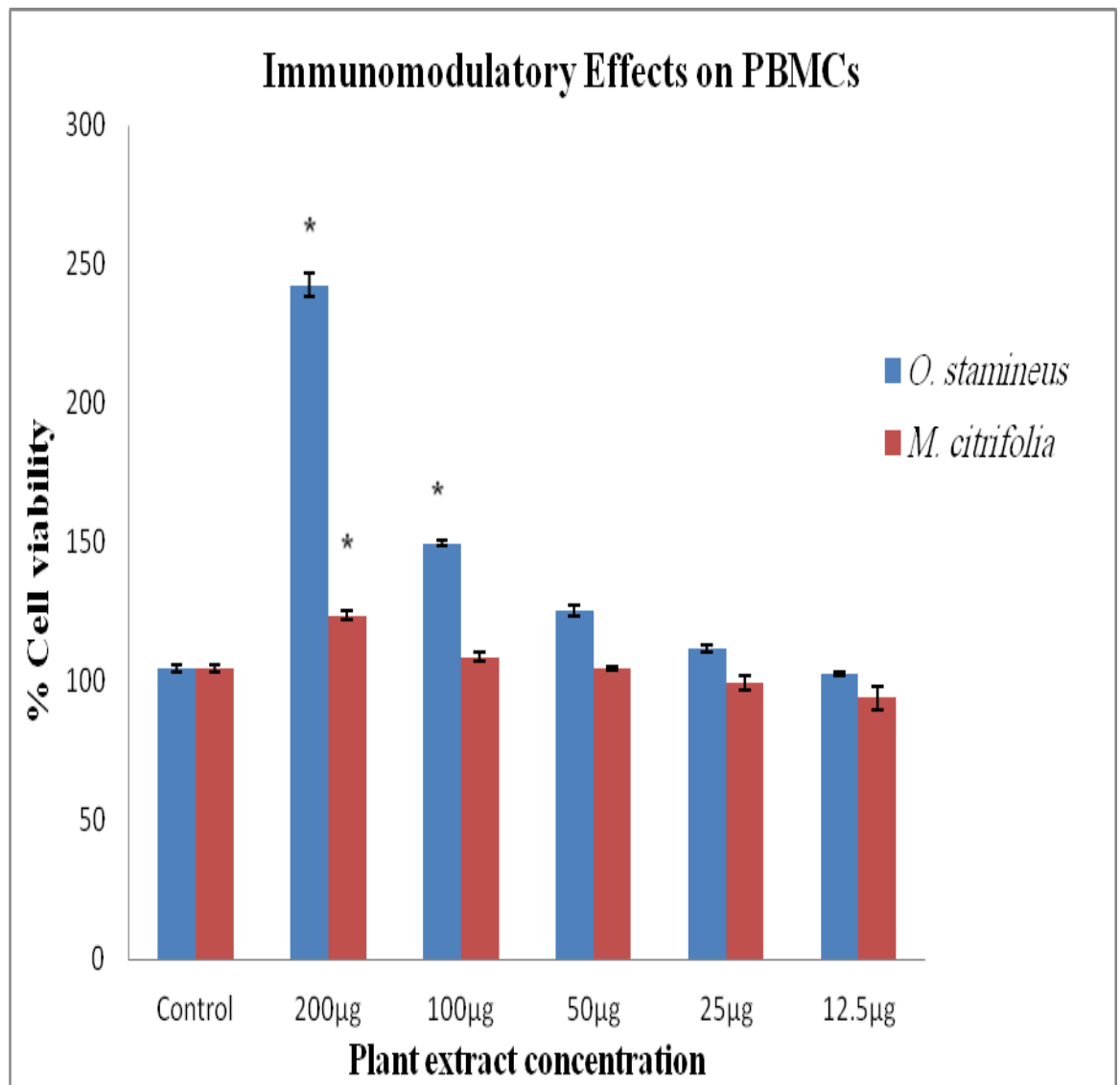
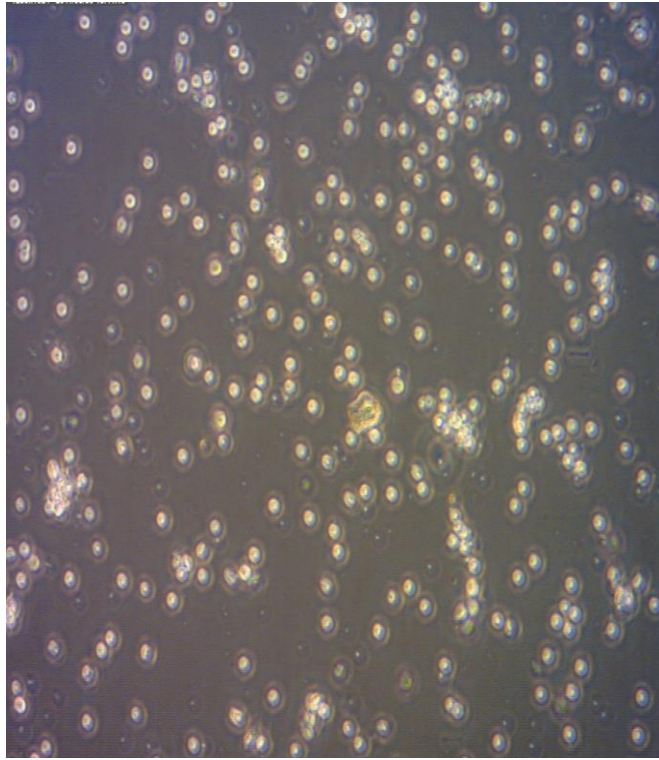
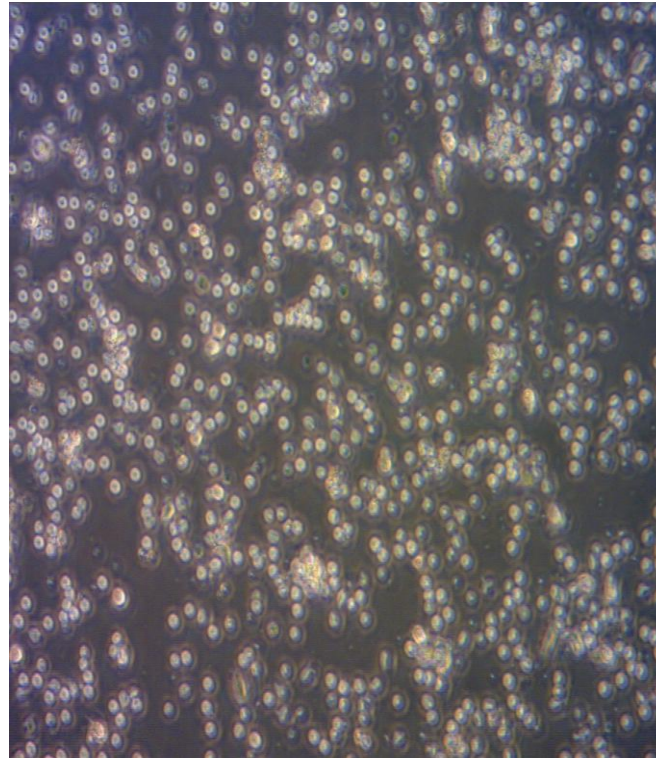


Figure 4.9 Percentage of (PBMCs) cell viability of *O. stamineus* and *M. citrifolia* treated groups compared to control (untreated group). Each value represents the means  $\pm$  S.E.M. of three independent experiments. Means with \* symbol are significantly different *versus* control group, \*  $p < 0.05$ .



Untreated cells (control group)



Treated cells with 200 µg/ml *O. stamineus*



Treated cells with 200 µg/ml *M. citrifolia*

Figure 4.10 Photomicrographs of (PBMCs) cell viability of *O. stamineus* and *M. citrifolia* treated cells compared to control (untreated cells), (magnification 10x).

#### 4.7 *In vitro* hepatoprotective activity of crude extracts and isolated fractions

First, we performed cytotoxicity assay of H<sub>2</sub>O<sub>2</sub> on WRL-68 cells to select the suitable concentration for hepatotoxicity induction experiments. Thus, we estimated the concentration of H<sub>2</sub>O<sub>2</sub> that can inhibit WRL-68 cell viability to 40-50 % comparing with untreated cells. WRL-68 treated cells with H<sub>2</sub>O<sub>2</sub> concentrations from 100 to 1000 µM were tested for cell viability; the viable cells were quantified by MTT assay. After 2 hr of treatment the cell number was reduced in a dose-dependent manner from 100 to 1000 µM (Figure 4.11). Cell line treated with H<sub>2</sub>O<sub>2</sub> concentrations below 600 µM produced a slight decrease in cell viability and has no statistically difference comparing with untreated cells. However, concentrations between 600 – 1000 µM caused a significant decrease in viable cell number (Figure 4.11). Concentration of 1000 µM H<sub>2</sub>O<sub>2</sub> significantly inhibited cell viability to 40.8 % and this concentration were chosen for further investigation. Result of *in vitro* hepatoprotective activity experiment showed that oxidative stress by H<sub>2</sub>O<sub>2</sub> resulted in a decrease of cell viability to 41.9 % after 2 h of treatment as compared with the control group. Nevertheless, cell viability was increased obviously when pre-incubated with plant extracts and its isolated fractions before treatment with H<sub>2</sub>O<sub>2</sub>. *O. stamineus* and *M. citrifolia* crude extracts showed the ability to inhibit cell death induced by H<sub>2</sub>O<sub>2</sub>. The activity of *O. stamineus* and *M. citrifolia* extracts were found to be significantly increased the cell viability to 81.1 % and 76.4 % respectively at the concentration 100 µg/ml. On the other hand, there was almost no variation in cell viability observed between the *O. stamineus* F3 (95.1 %) and *M. citrifolia* F2 (86.1 %) treated cells comparing to the control of untreated cells (Figure 4.12). These results demonstrated that *O. stamineus* F3 and *M. citrifolia* F2 have prevented the WRL-68 death and normalized the oxidative damage generated by H<sub>2</sub>O<sub>2</sub> induction. Figure 4.13 showed morphology and the changes on WRL-68 cell viability

due to the effects of *O. stamineus* F3 and *M. citrifolia* F2 treatment on the H<sub>2</sub>O<sub>2</sub> induced WRL-68 cells in comparison to the untreated cells.

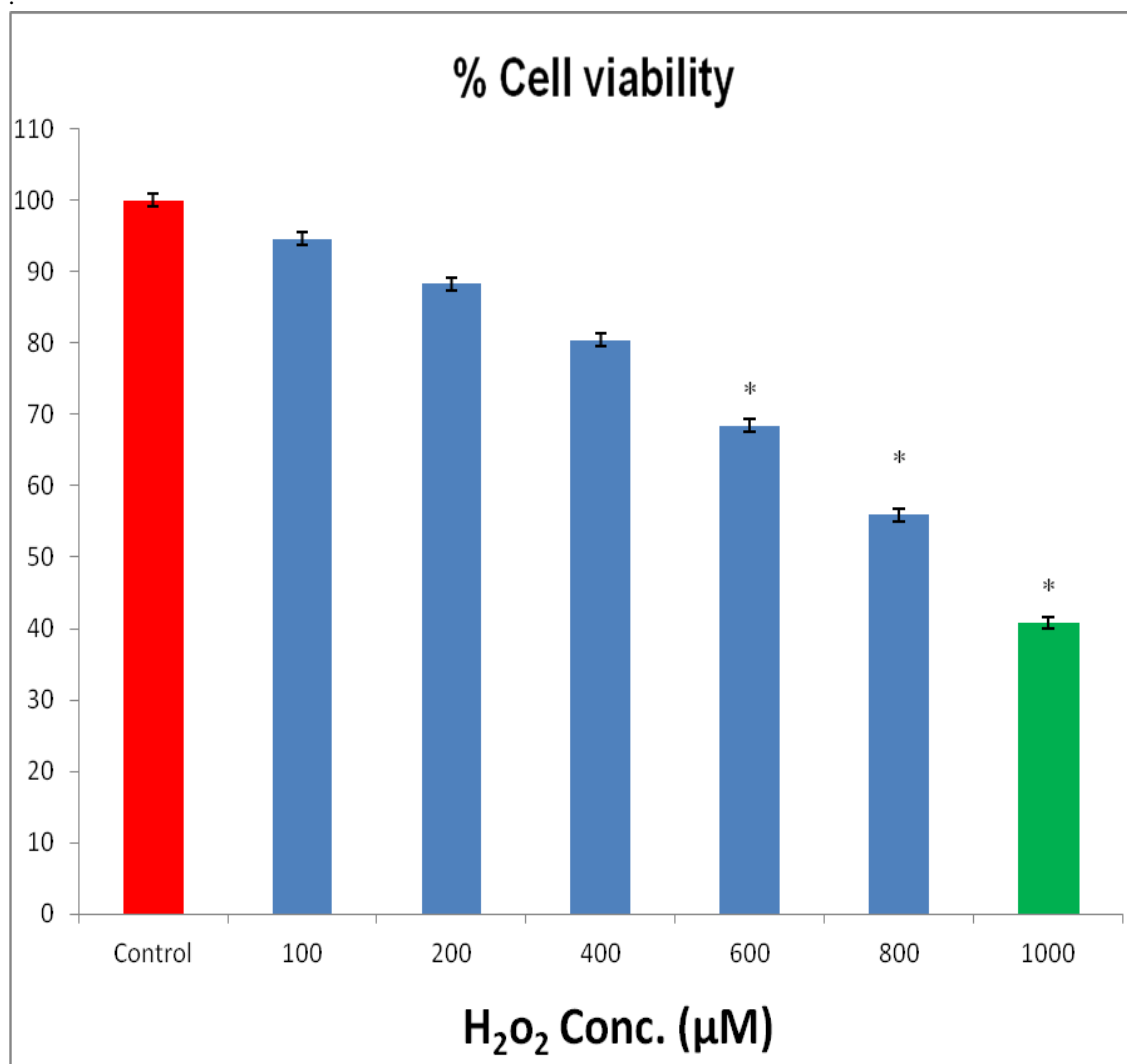


Figure 4.11 Effects of H<sub>2</sub>O<sub>2</sub> on WRL-68 cell viability after exposure to varying concentrations of H<sub>2</sub>O<sub>2</sub>; data are expressed as mean ± SEM; \**P* < 0.05

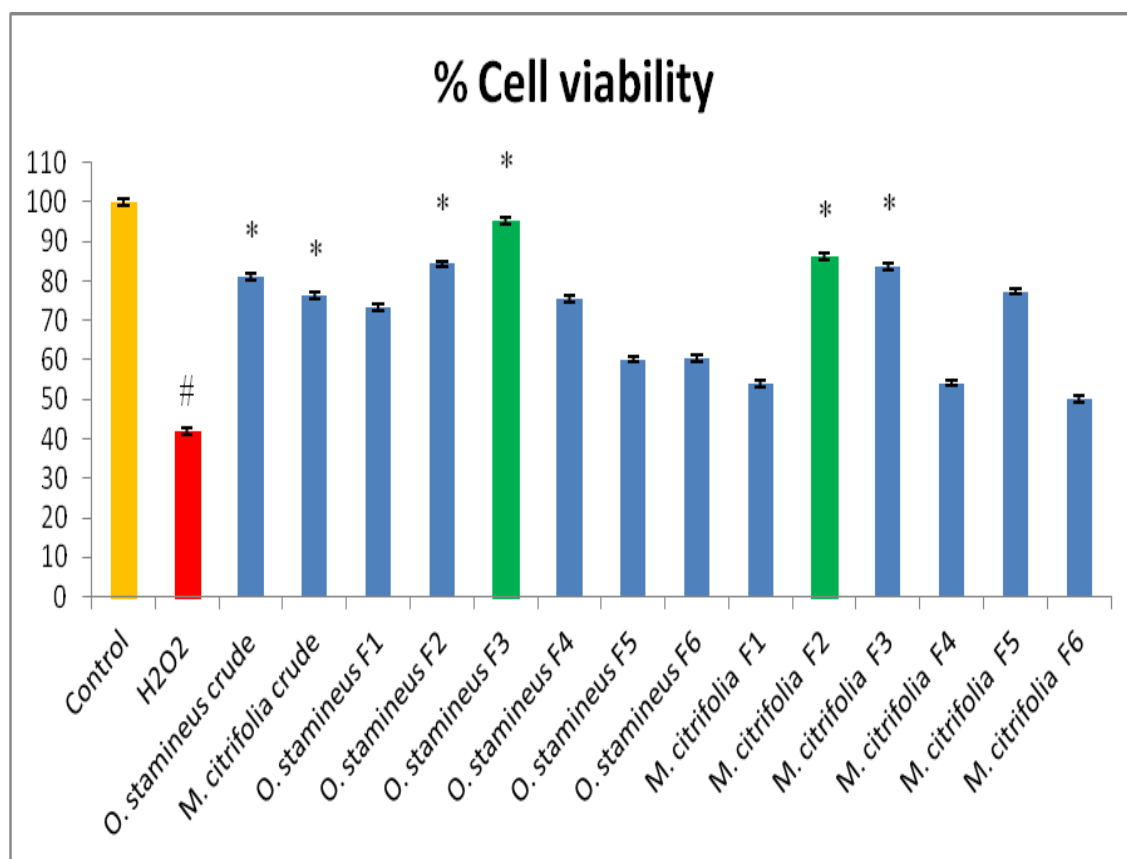
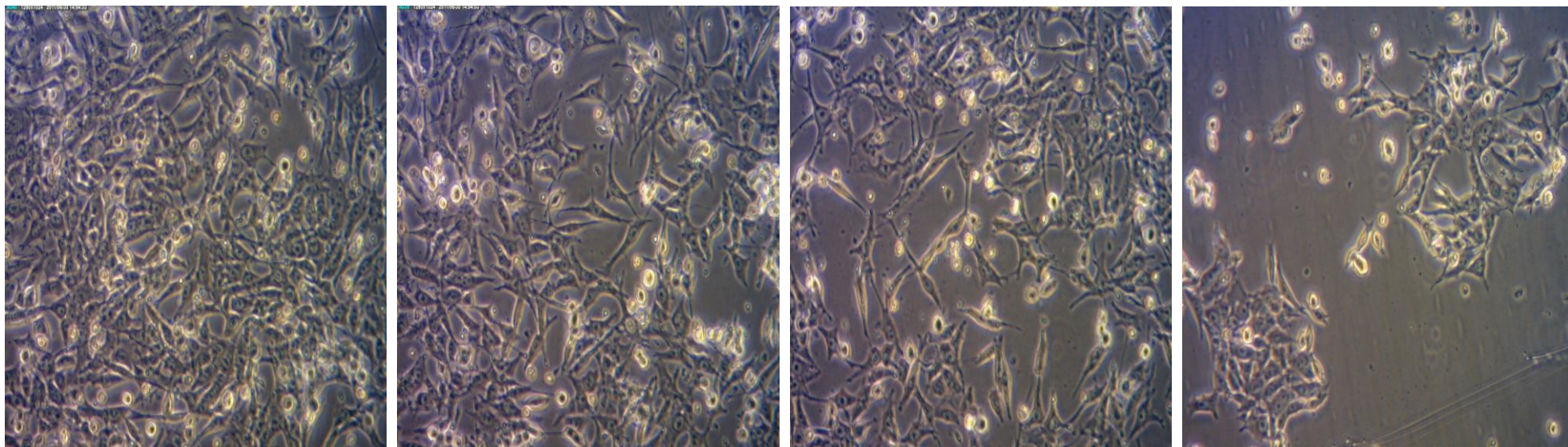


Figure 4.12 Effect of *O. stamineus*, *M. citrifolia* extracts and its fractions pre-incubated treatment on WRL-68 cell viability before H<sub>2</sub>O<sub>2</sub> exposure in comparison to control. All values are expressed as mean  $\pm$  SEM; #  $P < 0.01$  versus control group, \*  $P < 0.01$  versus H<sub>2</sub>O<sub>2</sub> group.





Untreated cells (normal control)

*O. stamineus* F3 treated cells after  
H<sub>2</sub>O<sub>2</sub> exposure

*M. citrifolia* F2 treated cells after  
H<sub>2</sub>O<sub>2</sub> exposure

H<sub>2</sub>O<sub>2</sub> exposure cells (Oxidative  
damage group)

Figure 4.13 Photomicrographs showing the effects of H<sub>2</sub>O<sub>2</sub> and plant fractions treatment on (WRL-68) cell viability in comparison to the untreated cells, (magnification 10x).

#### 4.8 *In vitro* antioxidant activity for cell line experiment

*O. stamineus* and *M. citrifolia* and their fractions that prevent the oxidative damage in the *in vitro* hepatoprotective activity experiment namely; *O. stamineus* F3 and *M. citrifolia* F2 were used for antioxidant assay experiment. The oxidative stress was induced by exposing cells to 1000  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 2 hr, while the protective effect of the plants and fractions reduced the oxidative stress. Cells were first pre-incubated with *O. stamineus*, *M. citrifolia*, *O. stamineus* F3, *M. citrifolia* F2 and Gallic acid as reference standard for 2 hr and then treated with 1000  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . The antioxidant activities of *O. stamineus*, and *M. citrifolia* and their fractions were positively correlated with the improvement of the cell viability. It is obvious that  $\text{H}_2\text{O}_2$  lead to the production of ROS, which in consequence reduced the antioxidant enzymes such as catalase and GPx. However, pre-treatment with plant extracts and the fractions decreased the free radical formation; therefore the antioxidant enzymes level became higher. Our results revealed that  $\text{H}_2\text{O}_2$  exposed cells caused a statistically significant decrease in catalase and GPx activity to 1.81 and 10.88 U/mg protein respectively, whereas showed significantly increase in *O. stamineus* extract and *O. stamineus* F3. On the other hand, lipid peroxidation value, measured as MDA production, significantly increased in  $\text{H}_2\text{O}_2$  induce oxidative stress group 48.84 nmol/mg protein comparing to untreated cells 18.00 nmol/mg protein. Whereas in cells pre-incubated with *O. stamineus* and *O. stamineus* F3 were significant prevent lipid peroxidation by reduction of MDA to 24.17 nmol/mg protein and 24.67 nmol/mg protein respectively, which are near to the MDA result of untreated cells 18.00 nmol/mg protein and even better then the gallic acid result (Table 4.7).

Table 4.7 Effects of H<sub>2</sub>O<sub>2</sub>, *O. stamineus*, *M. citrifolia* extracts and its fractions on the antioxidant enzymes and MDA in H<sub>2</sub>O<sub>2</sub> – induced WRL-68 cell line

Group	CAT (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)	MDA (nmol/mg protein)
Normal Control	4.88 ± 0.025	9.91 ± 0.010	26.15 ± 0.200	18.00 ± 1.015
H <sub>2</sub> O <sub>2</sub>	1.81 ± 0.030 <sup>*a</sup>	9.65 ± 0.065	10.88 ± 0.185 <sup>*a</sup>	48.84 ± 1.835 <sup>*a</sup>
<i>O. stamineus</i>	3.13 ± 0.115 <sup>*c</sup>	9.00 ± 0.150	22.05 ± 0.195 <sup>*c</sup>	24.17 ± 2.165 <sup>*b</sup>
<i>M. citrifolia</i>	2.53 ± 0.110 <sup>*c</sup>	8.34 ± 0.025	19.01 ± 1.110 <sup>*b</sup>	28.5 ± 6.830
<i>O. stamineus</i> F3	3.07 ± 0.120 <sup>*c</sup>	9.85 ± 0.020	21.27 ± 0.625 <sup>*c</sup>	24.67 ± 5.000 <sup>*b</sup>
<i>M. citrifolia</i> F2	2.99 ± 0.040 <sup>*c</sup>	8.91 ± 0.015	14.86 ± 2.295	27.00 ± 2.330
Gallic acid	3.93 ± 0.040 <sup>*c</sup>	9.27 ± 0.010	22.51 ± 0.540 <sup>*c</sup>	26.33 ± 1.665

Values are presented as means ± SEM, <sup>a</sup> *P* < 0.05 versus normal control group, <sup>b</sup> *P* < 0.05 versus H<sub>2</sub>O<sub>2</sub> group, and <sup>c</sup> *P* < 0.01 versus H<sub>2</sub>O<sub>2</sub> group.

#### 4.9 Identification of active constituents of the active fractions

We applied UPLC-DAD and LC-MS using positive and negative ionization mode in order to know the chemical structural information and to identify the phenolic constituents and other active compounds of *O. stamineus* F3 and *M. citrifolia* F2 fractions. Approximately seven compounds (Table 4.8) were detected in *O. stamineus* F3 and four of them were characterized on the basis of the UV spectra and MS fragmentation patterns in comparison with literature or by searching the dictionary of natural products on DVD, Version 20:2 (2011) (CRC Press, Taylor & Francis Group, London, UK). In addition, three active compounds were detected in *M. citrifolia* F2 and two of them were identified. Typical HPLC-TOF/MS peaks and UV diode array chromatograms of the *O. stamineus* F3 and *M. citrifolia* F2 fractions are shown in (Figure 4.14 and Figure 4.19) respectively. Tables 4.8 and 4.9 showed all peaks detected with their retention time, UV max, observed *m/z* and the *m/z* of fragment ions.

The *O. stamineus* F3 compounds include ponkanetin at  $m/z$  373.1287 (Figure 4.15), eupatorin at  $m/z$  345.0950 (Figure 4.16), TMF (3-hydroxy-5, 7, 3, 4-tetramethoxy flavone) at  $m/z$  359.1123 (Figure 4.17) and salvigenin at  $m/z$  329.1025 (Figure 4.18), while the compounds of *M. citrifolia* F2 comprise scopoletin at  $m/z$  193.0498 (Figure 4.20) and *P*- coumaric acid at  $m/z$  163.0390 (Figure 4.21).

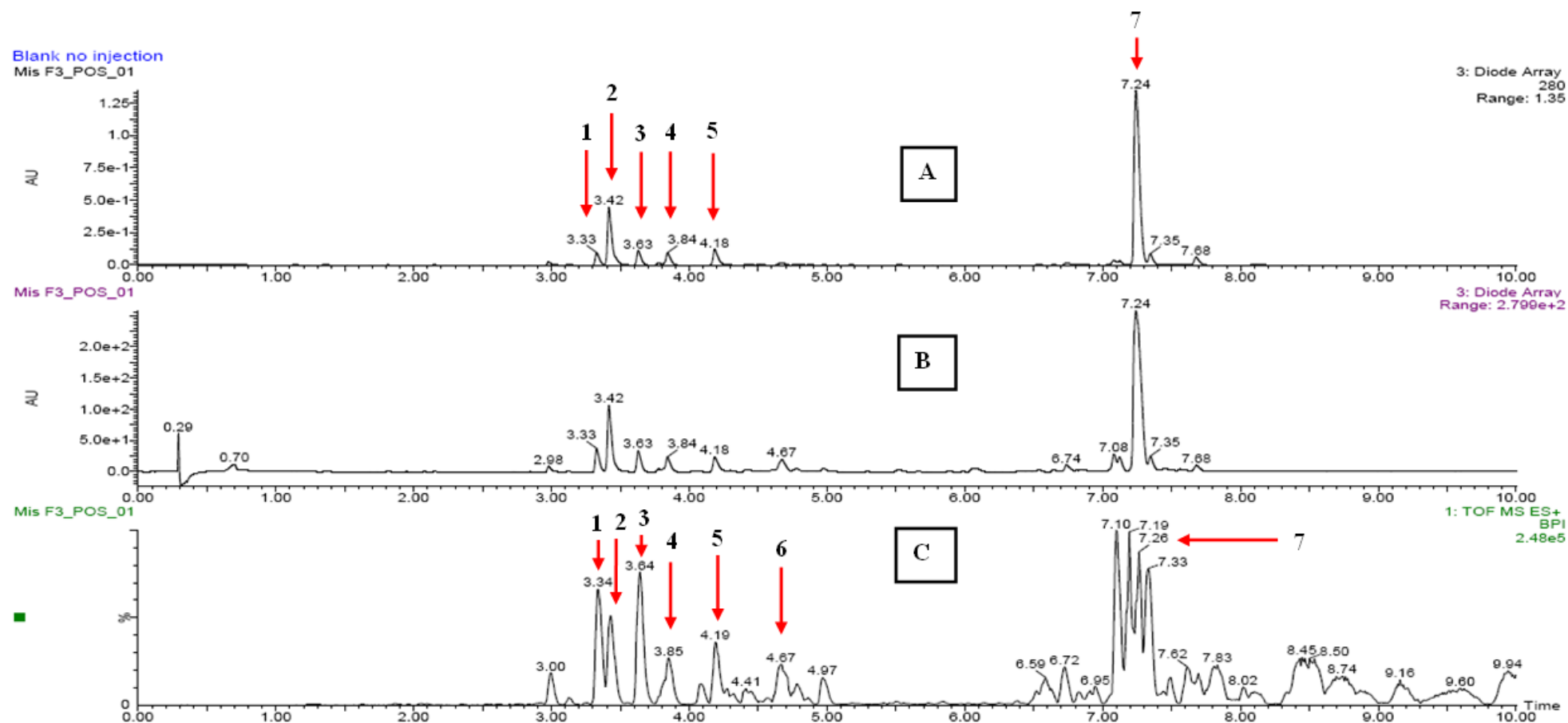


Figure 4.14 HPLC-TOF/MS and UV diode array chromatograms of *O. stamineus* fraction 3 (F3); (A) Diode array detection UV spectra at 280 nm, (B) Diode array detection UV spectra at range between 190 and 800 nm, (C) TOF MS peaks in positive mode ionization

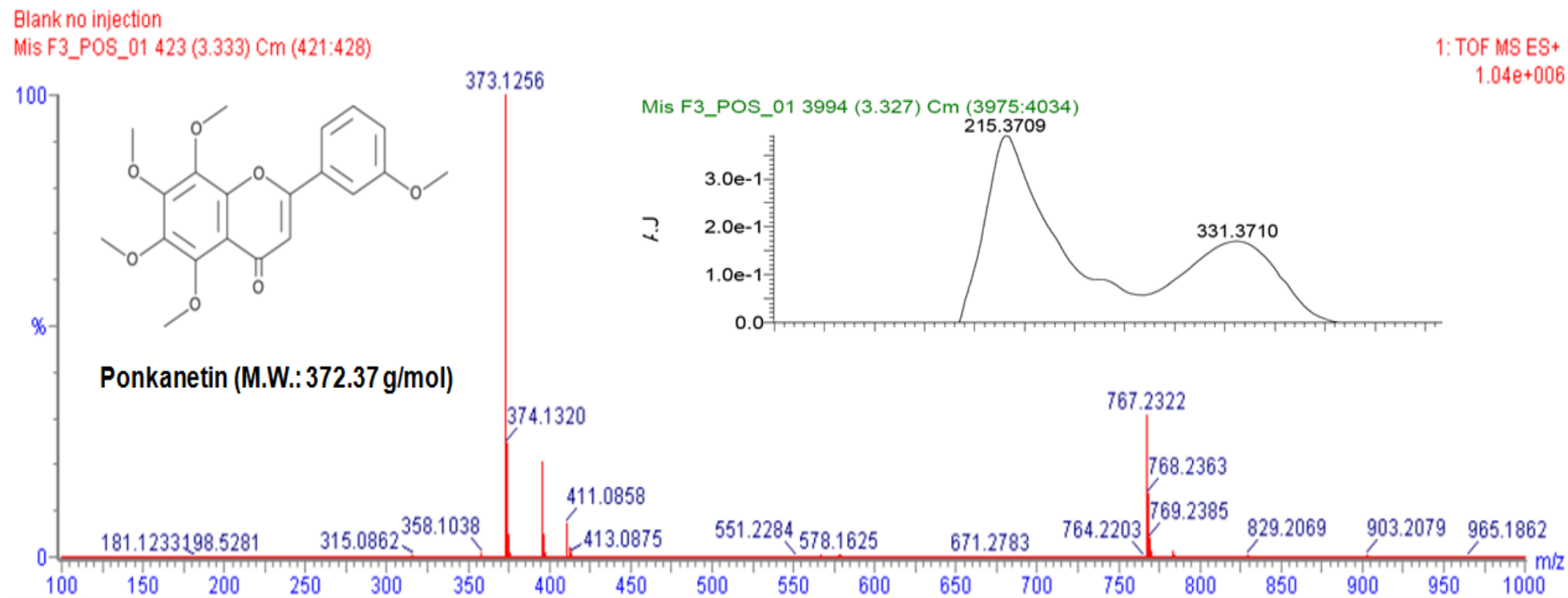


Figure 4.15 Mass spectrum (TOF MS ES+), chemical structure and UV max spectra of ponkanetin (peak No. 1) identified in *O. stamineus* F3

Mis F3\_POS\_01 436 (3.429) Cm (433:439)

1: TOF MS ES+  
6.83e+005

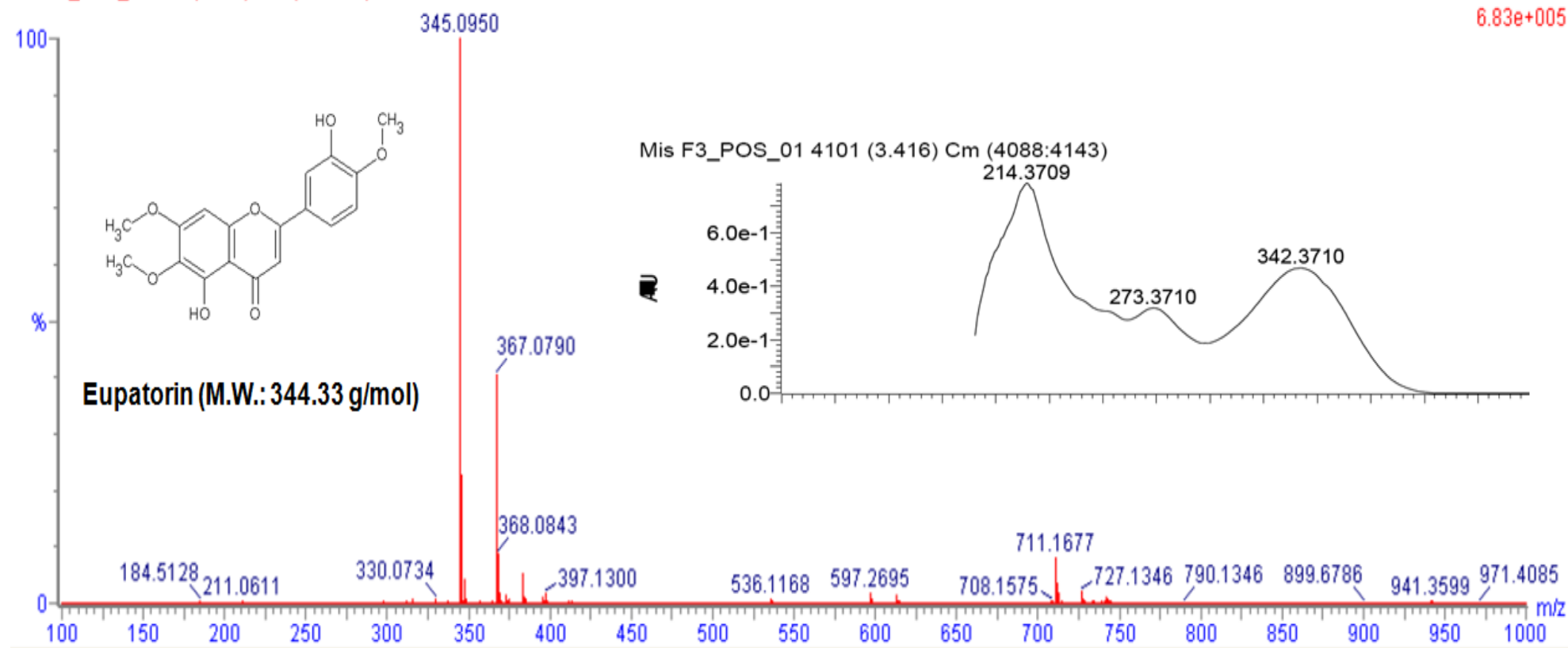


Figure 4.16 Mass spectrum (TOF MS ES+), chemical structure and UV max spectra of eupatorin (peak No. 2) identified in *O. stamineus* F3

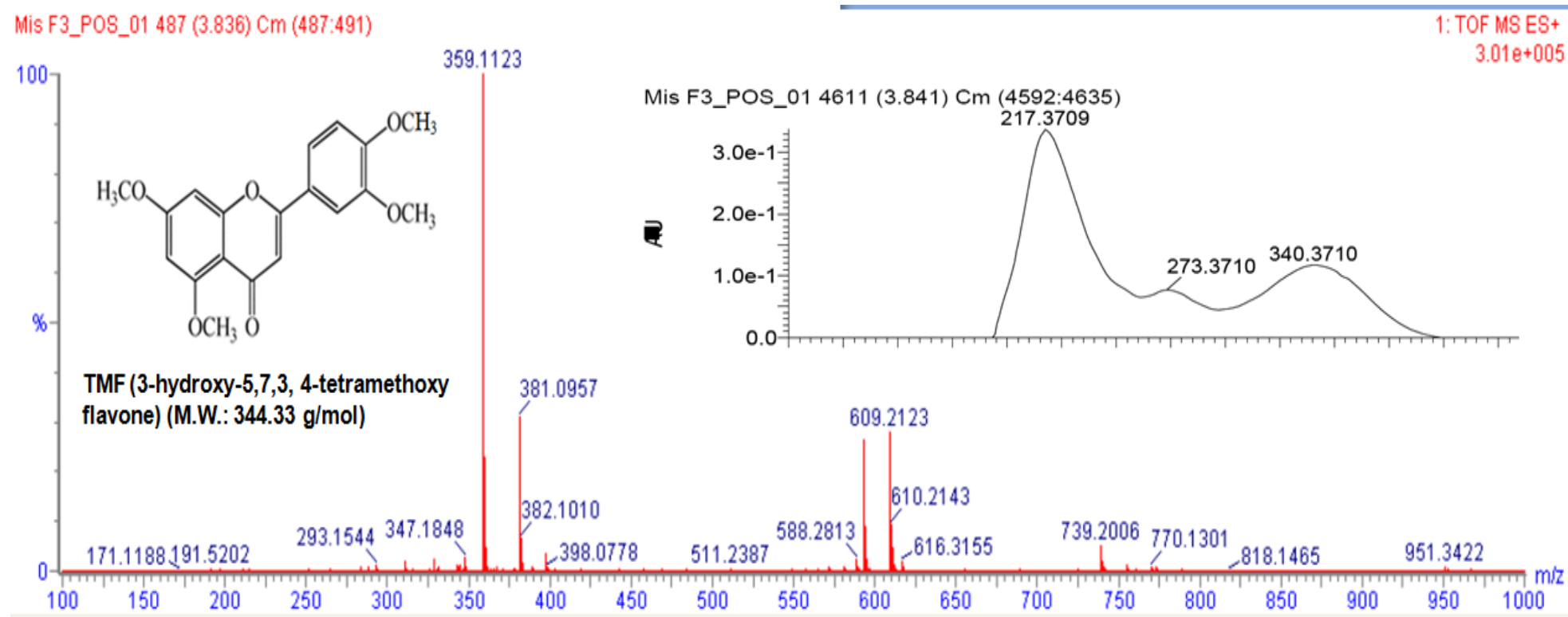


Figure 4.17 Mass spectrum (TOF MS ES+), chemical structure and UV max spectra of TMF (3-hydroxy-5, 7, 3, 4-tetramethoxy flavone) (peak No. 4) identified in *O. stamineus* F3



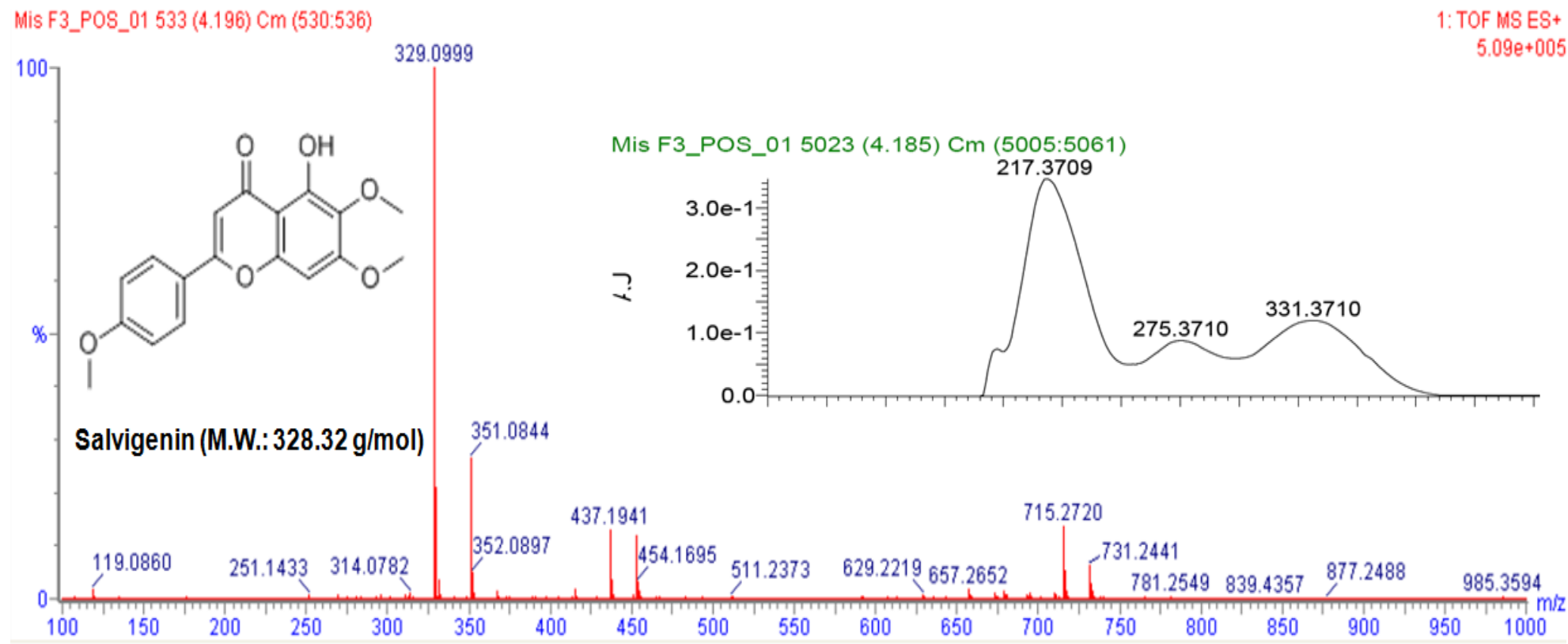


Figure 4.18 Mass spectrum (TOF MS ES+), chemical structure and UV max spectra of salvigenin (peak No. 5) identified in *O. stamineus* F3

Table 4.8 Identification of phenolic compounds in *O. stamineus* F3 by UPLC-DAD and LC-MS data using positive ionization mode

Peak No.	RT (min)	UV $\lambda$ max (nm)	Suggested formula	Observed $m/z$	$m/z$ of fragment ions observed	Tentative identification	M.W. (g/mol)
1	3.34	215, 331	C <sub>20</sub> H <sub>21</sub> O <sub>7</sub>	373	411, 767, 181, 198, 315, 358	Ponkanetin	372.37
2	3.43	214, 342	C <sub>18</sub> H <sub>17</sub> O <sub>7</sub>	345	367, 711, 184, 211, 330	Eupatorin	344.33
3	3.64	217, 321	C <sub>19</sub> H <sub>19</sub> O <sub>6</sub>	343	365, 381, 707, 176, 282, 328	Unknown	-----
4	3.85	217, 340	C <sub>19</sub> H <sub>19</sub> O <sub>7</sub>	359	381, 593, 609, 171, 191, 347	TMF (3-hydroxy-5,7,3, 4-tetramethoxy flavone)	358.11
5	4.19	217, 331	C <sub>18</sub> H <sub>17</sub> O <sub>6</sub>	329	351, 437, 453, 715, 119	Salvigenin	328.32
6	4.67	192, 226	C <sub>35</sub> H <sub>43</sub> N <sub>2</sub> O <sub>13</sub>	699	694, 677, 715, 295, 313, 555, 267	Unknown	-----
7	7.26	202, 234	C <sub>36</sub> H <sub>45</sub> O <sub>9</sub>	621	413, 149, 279	Unknown	-----

RT: Retention Time, M.W.: Molecular weight

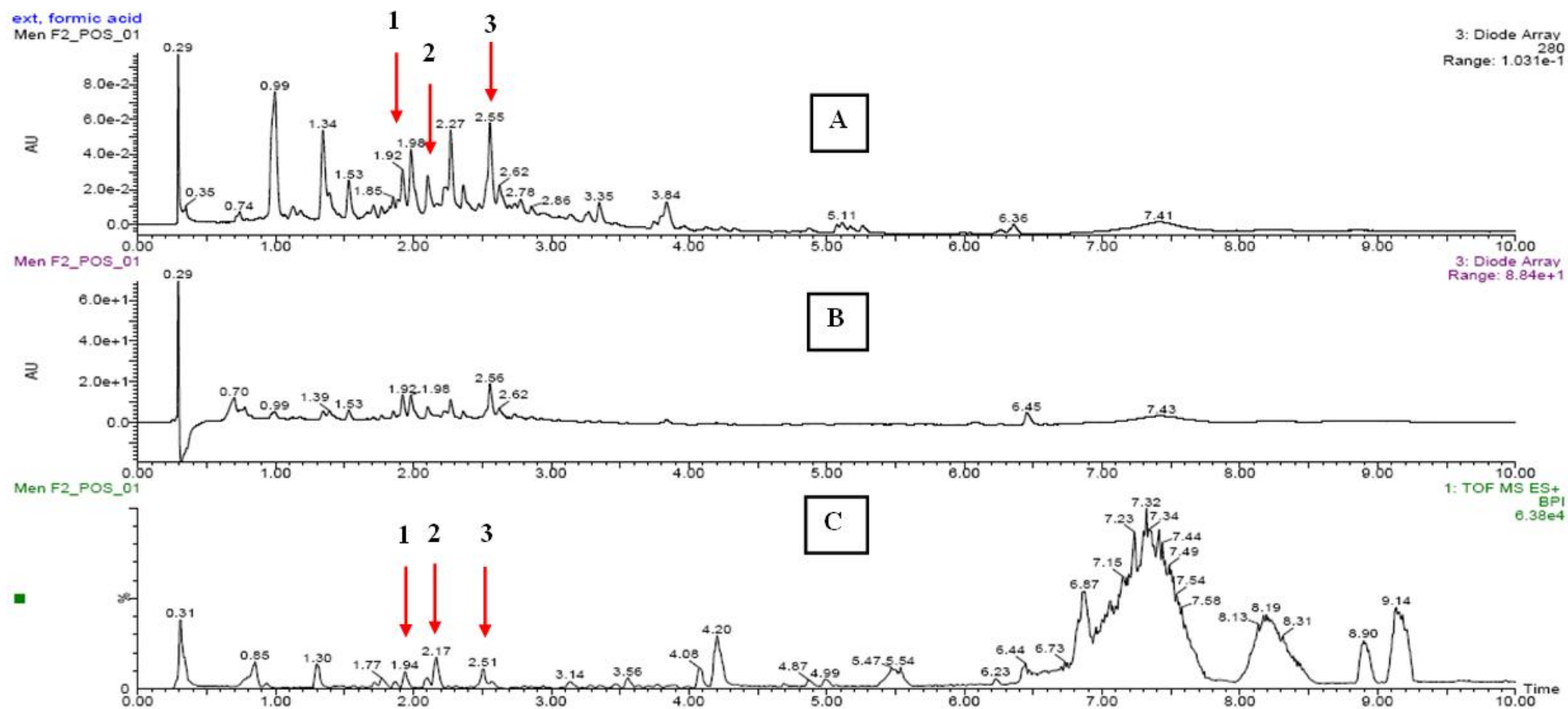


Figure 4.19 HPLC-TOF/MS and UV diode array chromatograms of *M. citrifolia* fraction 2 (F2); (A) Diode array detection UV spectra at 280 nm, (B) Diode array detection UV spectra at range between 190 and 800 nm, (C) TOF MS peaks in positive mode ionization



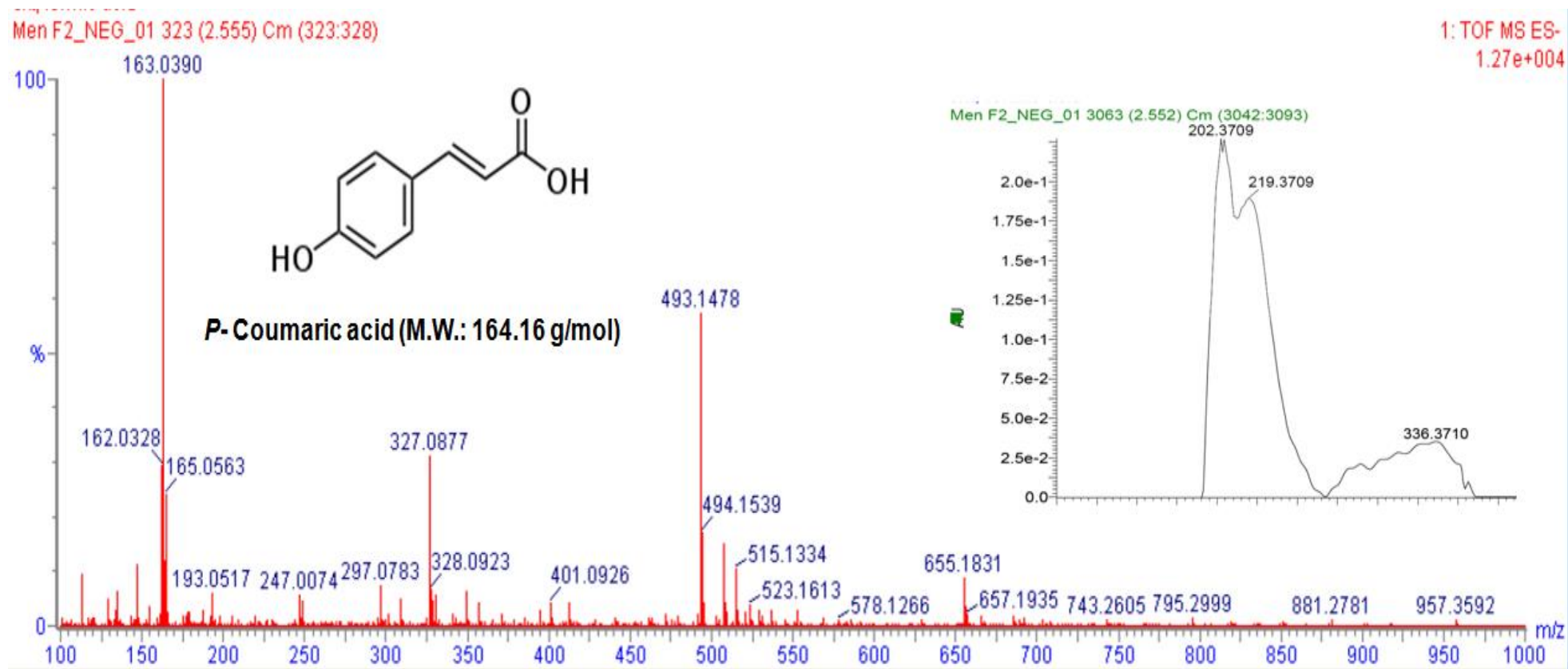


Figure 4.21 Mass spectrum (TOF MS ES-), chemical structure and UV max spectra of *P*-coumaric acid (peak No. 2) identified in *M. citrifolia* F2

Table 4.9 Identification of phenolic compounds in *M. citrifolia* F2 by UPLC-DAD and LC-MS data using positive and negative ionization mode

Peak No.	Mode	RT (min)	UV $\lambda$ max (nm)	formula	Observed $m/z$	$m/z$ of fragment ions observed	Tentative identification	M.W. (g/mol)
1	+ve	1.94	204	C <sub>10</sub> H <sub>9</sub> O <sub>4</sub>	193	133, 273, 371, 399, 517	Scopoletin	192.16
2	+ ve	2.17	202	C <sub>14</sub> H <sub>23</sub> N <sub>6</sub> O <sub>5</sub>	355	371, 161, 285, 301, 463, 527, 603	Unknown	-----
3	- ve	2.55	202, 219	C <sub>9</sub> H <sub>7</sub> O <sub>3</sub>	163	327, 493, 515, 655	<i>P</i> - Coumaric acid	164.16

RT: Retention Time, M.W.: Molecular weight, (+ve) positive ionization mode, (–ve) negative ionization mode,

#### 4.10 Antibacterial activity

The antibacterial activity results of the investigated extracts are shown in (Table 4.10). Among the investigated extracts, the aqueous extract of *O. stamineus* showed significant activity against *Staphylococcus aureus* and *Streptococcus agalactiae*, with inhibition zones 10.5 and 8.1 mm, respectively, while there were no activities against gram negative bacteria (*Escherichia coli* and *Klebsilla pneumonia*). Moreover, it was found that aqueous extract has minimum inhibitory concentration (MIC) value of 1.56 mg/ml, while the minimum bactericidal concentration (MBC) was 3.13 mg/ml against *Staphylococcus aureus*. On the other hand, the same aqueous extract has only moderate activity against *Streptococcus agalactiae*, with a MIC value of 3.13 mg/ml and MBC value of 6.25 mg/ml (Table 4.11).

In addition, the ethanol crude extract of *O. stamineus* exhibited a weak antibacterial activity against Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus agalactiae*) with inhibition zones of 6.8 mm and 6.5 mm, respectively. The aqueous and ethanol crude extracts of *M. citrifolia* has no antibacterial activity against both gram positive and negative bacteria (Table 4.10).

Table 4.10 Antibacterial activity of *O. stamineus* and *M. citrifolia* extracts in disk diffusion method

Plants and Control	Extracts	Average Inhibition Zone Diameter (mm)			
		<i>S. aureus</i>	<i>S. agalactiae</i>	<i>E. coli</i>	<i>K. pneumonia</i>
<i>O. stamineus</i>	Ethanollic	6.8±0.09	6.5±0.09	-----	-----
	Aqueous	10.5±0.20	8.1±0.07	-----	-----
<i>M. citrifolia</i>	Ethanollic	-----	-----	-----	-----
	Aqueous	-----	-----	-----	-----
Amoxicillin 2 µg/disc		NT	13.1±0.15	NT	NT
Gentamicin 30 µg/disc		NT	NT	22.5 ± 0.29	21.5 ± 0.26
Vancomycin 5 µg/disc		13.0 ± 0.12	NT	NT	NT

NT, not tested; —, no inhibition; Inhibition zones including the diameter of the paper disc (6 mm); Values are represented the mean inhibition zone (mm) ± SEM of triplicates. *S. aureus*, *Staphylococcus aureus*; *S. agalactiae*, *Streptococcus agalactiae*; *E. coli*, *Escherichia coli*; *K. pneumonia*, *Klebsilla pneumonia*.

Table 4.11 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the investigated extracts

Plants	Extracts	<i>S. aureus</i>		<i>S. agalactiae</i>	
		MIC	MBC	MIC	MBC
		(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)
<i>O. stamineus</i>	Ethanollic	NT	NT	NT	NT
	Aqueous	1.56	3.13	3.13	6.25
<i>M. citrifolia</i>	Ethanollic	NT	NT	NT	NT
	Aqueous	NT	NT	NT	NT

MIC, Minimum Inhibitory Concentrations; MBC, Minimum Bactericidal Concentrations; NT, not tested (because the inhibition zone less than 8 mm or there are no antibacterial activity)



## CHAPTER V

### DISCUSSION AND CONCLUSION

#### 5.1 Liver cirrhosis mechanism and hepatoprotective activity of medicinal plants

Hepatic cirrhosis is a wound healing process characterized by excessive accumulation of ECM especially collagen I in response to severe liver damage that occurs in many patients with chronic liver injury of any etiology and associated with inflammation and cell death with the tendency to progress into sclerosis. Toxic injury occurs in the liver more often than that in any other organ. When a drug is used widely, drug-induced liver injury has become a serious health problem. Thus research on the mechanism of drug-induced liver injury is very useful in therapy and prevention of drug-induced liver injury (Xin & Cai-qin, 2008). Liver cirrhosis represents the end-stage process of liver fibrotic degeneration of the most chronic liver diseases. Complex cellular and molecular mechanisms resulting from chronic activation of tissue repair mechanism following tissue injury have been characterized. The fibrosis process initiated upon liver tissue injury occurred, followed by the inflammatory reaction with activation of Kupffer cells and stellate cells, which in turn leading to increased expression of pro-inflammatory and pro-fibrotic cytokines and the recruitment and activation of fibroblast responsible for increased production of ECM proteins (Flier *et al.*, 1993). Activation of HSC is regulated by several soluble factors, including cytokines such as TGF- $\beta$ 1 and IL- 6 and products of oxidative stress as well as by extensive changes in composition and organization of ECM components (Gressner, 1991; Stalnikowitz & Weissbrod, 2003). The production of ECM protein certainly represents the most typical function of activated HSC. In addition, along the progression of the fibrotic process, qualitative and quantitative changes in ECM are also favoured by the fact that HSC express MMP

which lead to disruption of normal matrix. As scarring progresses from bridging fibrosis to the formation of complete nodules it results in architectural distortion and ultimately liver cirrhosis.

On the other hand, many liver diseases are accompanied by unbalanced increase of ROS and related products of lipid peroxidation resulting from oxidative stress, which represent one aspect of a very complex series of events able to affect the liver cell structure and function. Moreover, in alcohol intoxication, viral hepatitis infection and metabolic disorders, ROS are now increasingly recognized to have significant role in both initiation and sustaining of liver fibrosis. During the development of fibrosis, kupffer cells and HSC are the actual key-players in such chronic disease process; exert a series of effects through the enhancement of the intracellular and extracellular levels of oxidants (Poli, 2000). Oxidative stress is essentially a consequence of a necrotic event, but not withstanding it, ROS produced by activated macrophages as well as reactive aldehydes stemming from membrane lipid peroxidation can stimulate the progression of collagen deposition in the inflamed tissue or organ. Production of ROS may be cause and consequence of cellular damage. For instance, many hepato-toxins lead to increased concentrations of ROS that cannot be handled in a normal way by the protective machinery of the cells (Marí *et al.*, 2001). Excessive production of ROS results in lipid peroxidation leading to an increase in highly reactive aldehydic end products, altered signal transduction, modulation of gene expression, alteration of the redox state including decrease of glutathione levels, and induction of apoptosis and necrosis (Dalton *et al.*, 1999).

Based on understanding of the cellular and molecular basis of hepatofibrogenesis, hepatoprotective agents can be categorized as follows; agents for scavenging of free radicals and reducing oxidative stress, agents for cytoprotection and reduction of

inflammation and cell death, agents for inhibition of HSC activation and agents for fibrolysis and promotion of matrix degradation. These strategies include the curing of primary disease to prevent injury; reducing inflammation; down-regulating stellate cell activation directly or by neutralizing proliferative, fibrogenic, contractile, and/or pro-inflammatory responses of stellate cells; stimulating apoptosis of stellate cells; or increasing the degradation of scar matrix. Reducing oxidative stress is another type of intervention and silymarin (our reference drug) are very good candidates in this area (Li & Friedman, 1999). Even traditional drugs such as pentoxifylline (Windmeier & Gressner, 1997), as well-known phosphodiesterase inhibitor, were unexpectedly found to block HSC activation by interfering with the oxidative stress cascade suggesting new mechanisms for their anti-fibrotic activity.

Popularity of herbal remedies is increasing worldwide and at least one quarter of patients with liver diseases use medicinal plants for the prevention and treatment of liver diseases. More efforts need to be directed towards methodological scientific evaluation for their safety and efficacy by subjecting to vigorous pre-clinical studies followed by clinical trials to scientifically prove their traditional uses on evidence-based findings (Stickel & Schuppan, 2007). Recently, some researches has confirmed the efficacy of several plants as hepatoprotective agents and evaluated their mechanisms of action. *Silybum marianum* (milk thistle) has been shown to have clinical applications in the treatment of liver cirrhosis via its antioxidative, antifibrotic, anti-inflammatory, immune-modulating, and liver regenerating effects. *Picrorhiza kurroa*, appears to have similar applications and mechanisms of action (Luper, 1998). In addition *Curcuma longa*, *Camellia sinensis* (green tea), and *Glycyrrhiza glabra* have been approved to exhibit hepatoprotective effects (Luper, 1999). Using different experimental models some other plant extracts were evaluated for their hepatoprotective activity such as *Andrographis lineate*, *Azadirachta indica*, *Cassia fistula*, *Cleome viscosa*, *Polygala*

*arvensis*, *Pterocarpus santalinus*, *Solanum nigrum*, and *Wedelia calendulacea* (Bhawna & Kumar, 2009).

## 5.2 Antioxidant properties of crude extracts

*O. stamineus* ethanol extract exhibited high antioxidant activity, as proven by FRAP result and its scavenging activity towards ABTS and DPPH radicals, while ethanol extract of *M. citrifolia* were found to have weaker antioxidant properties. The antioxidant activity was expressed as IC<sub>50</sub>; it is the measure of concentration in µg/ml of extract that inhibits 50 % of DPPH radicals. The DPPH IC<sub>50</sub> of *O. stamineus* was found to be 21.4±0.104 µg/ml, which is almost the same as that of the standard BHT 21.1±0.031 µg/ml. In the same study as shown in (Table 4.1) the FRAP result of *O. stamineus* (1692.8± 85.09 µmol Fe (II)/g) was significantly much higher than that of BHT (988.8± 24.83 µmol Fe (II)/g) but a little lower than ascorbic acid (1987.4± 34.98 µmol Fe (II)/g) and there is no significant difference between them. In addition, correlation analysis between the antioxidant properties and total phenolic contents of extracts indicated that there was a linear relationship between antioxidant potency, free radical-scavenging ability and the content of phenolic compounds of plant extracts (Figure 4.4). The calculated coefficients of correlations between antioxidant activity, scavenging effects on radicals and contents of phenolic compounds are shown in (Figure 4.4). Therefore, the high antioxidant activity of *O. stamineus* which led to more potent radical scavenging effects is certainly associated with the high content of phenolic and flavonoids components. The total phenolic contents of *O. stamineus* and *M. citrifolia* are in accordance with the DPPH, ABTS and FRAP results.

The results of this study are consistent with the results published by Yam *et al.* (2007), who reported that water extract of *O. stamineus* has antioxidant and free radical scavenging properties. *O. stamineus* has been reported to possess antioxidant activity.

Furthermore, the plant exhibited significant radical-scavenging activity probably due to the higher concentration of caffeic acid derivatives, especially rosmarinic acid (Akowuah *et al.*, 2005a; Ho *et al.*, 2010). Furthermore, Akowuah found that *O. stamineus* exhibited antioxidative potency greater than a synthetic antioxidant BHA and almost equal to that of pure quercetin (Akowuah *et al.*, 2005b). Rosmarinic acid and caffeic acid derivatives have been reported as predominant in *O. stamineus* extracts, which represent 67% of total identified phenolics (Sumaryono *et al.*, 1991). A quantitative HPLC analysis for *O. stamineus* ethanol extracts showed that the rosmarinic acid concentration ranged between 0.117 and 0.091 mg/ml, depending on ethanol concentration (Olah *et al.*, 2003). In another study the antioxidant activity of rosmarinic acid was greater than that of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Lu & Yeap Foo, 2002). Moreover, rosmarinic acid was reported to have a number of biological activities *in vitro*, such as anti-carcinogenic, antioxidant, antibacterial, and anti-inflammatory properties (Huang & Zheng, 2006). This plant could represent a potent source for antioxidant agent, which provides prophylaxis against many diseases like arteriosclerosis, heart diseases, and cancers. According to Sumaryono *et al.* (1991).

The antioxidant capabilities of phenolic compounds are important for the human body to destroy the free radicals that exist in our body. Many of the polyphenols such as flavonoids have been identified as powerful antioxidants; moreover, play a significant role in the treatment of many diseases, including liver cirrhosis (Hollman & Ilja, 2000). In addition to that, a study by Madani *et al.* (2008) suggested that the hepatoprotective effect of *Silybum marianum* and *Cichorium intybus* extracts were attributed to the presence of flavonoids and their antioxidant effects.

### 5.3 *In vivo* hepatoprotective and antioxidant activities of plant extracts

Thioacetamide is a known hepatotoxic, which produces hepatic necrosis in high doses by producing free radicals during TAA metabolism resulting in oxidative stress mediated acute hepatitis and induces apoptosis of hepatocytes in the liver (Sun *et al.*, 2000). In the *in vivo* hepatoprotective experiment, TAA model was used as hepatotoxicants to induce liver cirrhosis. It has been reported that long term taken of TAA induced cirrhosis in rats; on account of this, it is proven that TAA through cytochrome p-450 pathway is converted into a highly toxic metabolite N-acetyl-p-benzoquinone imine (NAPBI) (Fontana *et al.*, 1996). Meanwhile NAPBI is accompanied with glutathione and excreted in the urine as a conjugates. The acute hepatic necrosis induced by TAA, which activates cytochrome p450 and produces a highly reactive NAPBI that, by the way, combines with sulphahydryl groups of proteins and causes a rapid reduction of intracellular glutathione (Fontana *et al.*, 1996). In addition, TAA promotes oxidative stress, both by increased formation of ROS and by depletion of oxidative defences in the cell. Furthermore, liver cells from TAA-treated animals are more susceptible to the cytotoxic effects of TNF- $\alpha$  and other cytokines than cells from normal animals. Mitochondria play a critical role in the apoptotic response, and alterations in mitochondrial function after chronic TAA treatment may contribute to enhanced cell death by apoptosis or necrosis (Hoek & Pastorino, 2002). Therefore, increases the oxygen free radical causing an oxidative stress and initiates apoptosis; consequently, the elevated liver enzymes (ALT, AST) are an indicator of cellular liver necrosis (Fontana *et al.*, 1996). In addition, TAA interferes with the movement of RNA from the nucleus to the cytoplasm which may cause membrane injury resulting in a rise in serum liver markers (Saraswat *et al.*, 1996). TAA is responsible for many changes occur for hepatocytes such as an increase in nuclear volume and enlargement of nucleoli, cell permeability changes, rise in intracellular concentration of Ca<sup>++</sup>, and

effects on mitochondrial activity, which leads to cell death (Ahmad *et al.*, 2002). On the other hand, the preclinical studies using different hepatotoxic substances showed that silymarin has multiple actions as a hepatoprotective agent. The antioxidant property, inhibition of the transformation of stellate hepatocytes into myofibroblasts, stimulation of protein synthesis, the anti-inflammatory and antifibrotic actions considered as most important leading to cell-regenerating (Ghosh *et al.*, 2010; Kosina *et al.*, 2002).

The plasma concentration of ALT, AST, ALP, bilirubin, albumin and total protein determines the functionality and cellular integrity of the liver (Gowda *et al.*, 2009). ALT and AST are biomarkers of the hepatocytes. Under pathological conditions of the liver including, cirrhosis, adverse effects of some drugs (e.g., TAA, paracetamol), there is a leak of these enzymes in to the plasma, thus raising their activity (Nyblom *et al.*, 2004). ALT is specific for the liver but AST is also found in other tissues including the red blood cells, the cardiac and the skeletal muscle. ALP is located in the biliary duct of the liver (Nyblom *et al.*, 2006). Obstruction of this duct increases the level of the enzyme in the plasma. Albumin and globulin constitutes the total plasma proteins and are in ratio 2:1. Thus, albumin constitutes the major component of the Total Plasma protein. It is synthesized in the liver and therefore a diagnostic tool for the determination of liver functionality. It has a half life of 120 days and its level is lowered in chronic liver disease such as cirrhosis and in poor diet or states of impaired protein catabolism. Bilirubin is a catabolic intermediate of haem. High concentration of this molecule results in jaundice. However, the liver plays an important role in mopping bilirubin from the plasma. In this study TAA administration for eight weeks led to induced liver fibrosis, which has been proven by the significantly difference of biochemical markers between the TAA control and normal control groups. Thus, serum levels of liver function parameters like ALT, AST, bilirubin, alkaline phosphatase are elevated. The mechanism of liver fibrosis is not fully understood, but no doubt that

oxidative stress and ROS play an important role in pathological changes in the liver. At the same time, the hepatoprotective effect exhibited by *O. stamineus* and *M. citrifolia* at high dose 200 mg/kg was comparable to silymarin at dose 50 mg/kg in TAA-induced liver injury rats. Treatment with the ethanolic extracts of *O. stamineus* and *M. citrifolia* (200 mg/kg) has accelerated the return of the altered levels of liver function enzyme to the near normal profile. The abnormal reconstruction of the lobular architecture, the appearance of widespread fibrosis; in addition, nodular lesions of the hepatic parenchyma are the main characteristics of liver cirrhosis (Li & Crawford, 2004). Our histological findings prove that the ethanol extracts of *O. stamineus* and *M. citrifolia* affected the recovery of liver structure in TAA-induced liver cirrhosis rats. Indeed there was Remarkable reduction in fibrosis extent and a decrease of stellate infiltration in rats treated with plant extract compared to control TAA group. Histological studies confirmed the hepatoprotective effect of *O. stamineus* and *M. citrifolia* ethanolic extract. TAA treated rat liver sections showed fatty degeneration of hepatocytes and necrosis of cells. The *O. stamineus* extract treatment (200 mg/kg) almost normalized these effects in the histo-architecture of liver with much better than that of *M. citrifolia* (200 mg/kg). Furthermore, the severe fatty changes in the livers of rats caused by TAA were treated in the HD treatment of both plant groups. Therefore, the ethanol extracts of both *O. stamineus* and *M. citrifolia* could be promising hepatoprotective agents against thioacetamide induced liver damage in rats. The high dose (200 mg/kg) of *O. stamineus* and *M. citrifolia* improve the cellular integrity and functionality of the liver as demonstrated by the significantly decrease in the level of ALT, AST, ALP and bilirubin concentrations, moreover increase in albumin and total protein.

Furthermore, the *in vivo* antioxidant activity investigated the effect of the plant extracts on SOD, CAT, GPx and MDA concentrations in rats liver homogenate and it was demonstrated that, liver damage is associated with significant increases of tissue lipid



peroxidation expressed as MDA level, which caused by oxidative stress and depletion in the tissue GSH levels. In addition, the extent of oxidative stress was demonstrated to be directly correlated with the amount of ALT detectable in the blood (De Maria *et al.*, 1996). SOD, CAT and GPx are components of the intrinsic antioxidant defence system, which is responsible for dissemination of superoxide radicals and other free radicals, during oxidative stress the body uses its defence mechanism to minimize the process of lipid peroxidation by using the antioxidant enzymes. Thus, the activity of these enzymes become higher in early stages of insult, but if the insult continue, the enzymes become depleted as shown in (Table 4.4) and unable to scavenge the free radicals, which means that in advance stages of peroxidation the activity of enzymes declined (Chin *et al.*, 2008a). However, in the *O. stamineus* and *M. citrifolia* treatment groups showed significant increase in these endogenous enzymes activities. In the current study reduced lipid peroxidation was revealed by a significant decrease in MDA level in groups treated with ethanol extracts of both plants. The results of the hepatoprotective effects of both extracts can be attributed to the presence of high content of phenolic and flavonoid compounds and their antioxidant effects besides the free radical scavenging property. Likewise, the hepatoprotective activity of the extracts could be due to neutralization of the toxic compounds produced by converting TAA to a highly toxic metabolite during cytochrome P-450 pathway as mentioned above. On account of this, *O. stamineus* extract has been reported recently to affect cytochrome P-450 enzyme system through its inhibition. Consequently, the toxic metabolite of TAA is affected by the *O. stamineus* extract that might lead to reduce the progress of liver necrosis (Hanapi *et al.*, 2010). The high antioxidant activity of *O. stamineus* is certainly associated with the high content of phenolic components. Furthermore, antioxidants from the herbal medicine were recently found to act as hepatoprotective in different animal models (Shimizu *et al.*, 1999). It has been reported that the antioxidant defence system in the

liver of mice is activated after exposure to oxidative stress, but after prolonged time of exposure it causes failure of the antioxidant defence system resulting in initiation of liver injury (Santra *et al.*, 2000).

Taken together these findings, suggest that the ethanol extracts of *O. stamineus* and *M. citrifolia* exhibit hepatoprotective effect which was demonstrated by a significant decrease in the liver biomarker level in TAA-induced rat liver cirrhosis. Moreover, the plant extracts enhanced the activities of antioxidant enzymes (SOD, CAT and GPx) against the TAA-induced hepatotoxicity in these animals, suggesting that the reduction of oxidative stress in this scenario likely plays a role in the mechanism of its hepatoprotective effects. In agreement with our result, the bilirubin lowering potential of *O. stamineus* was evaluated by Faizul *et al* (2009) in jaundiced rats. Treatment of these rats with aqueous extract of *O. stamineus* for three days reduced the bilirubin level significantly to the normal value. Whereas lower dose (50 mg/kg body weight) resulted in the reduction in bilirubin level nearly half when compared to the control. Therefore, *O. stamineus* aqueous extract can be used to reduce bilirubin concentration to a normal level. Furthermore, recent study evaluated the protective effects of *M. citrifolia* fruit juice on acute liver injury induced by carbon tetrachloride (CCl<sub>4</sub>) in female SD rats. They found that *M. citrifolia* juice is effective in protecting the liver from extrinsic toxin exposure (Wang *et al.*, 2008). However, caution should be taken when animal studies are extrapolated to human, because there are significant qualitative and quantitative differences in the major drug metabolism enzyme cytochrome P-450 mixed function mono-oxygenases. Some subfamilies of P-450 in human versus animal catalyze xenobiotics biotransformation differently leading to unlike metabolites and it need further clinical evaluation (Guengerich, 1997).

## 5.4 Genes expression

Gene expression represents a key role in the liver tissue response to pro-inflammatory and pro-fibrotic stimuli, which expressed by an intense transcriptional activation of a wide variety of genes, in different way involved in tissue repair. Oxidative stress and lipid peroxidation derived aldehydes, appears able to activate AP-1, a transcription factor which has been demonstrated to be essential for optimal transcription of many genes, whose some of primary interest in liver fibrosis, like TGF- $\beta$ 1, collagen type I and MMP2 (Armendariz-Borunda *et al.*, 1994; Feinberg *et al.*, 2000). In the current study all four target genes i.e. TGF $\beta$ 1, MMP2, TIMP-1 and Coll  $\alpha$  were significantly up-regulated in TAA-induced liver cirrhosis group compared to the normal group, whereas three of them namely; TGF $\beta$ 1, MMP2, and Coll  $\alpha$ ; were highly significant ( $P < 0.01$ ) down-regulated in *O. stamineus* treatment group. At the same time the fourth target gene TIMP-1 showed no significant difference in mRNA expression between plants extract treatment groups and TAA group (Figure 4.8). In normal rat liver, TGF- $\beta$ 1 is mainly produced by Kupffer cells with some contribution by endothelial cells. However, in the drug-induced fibrotic rat liver, evidence was obtained in support of the involvement of all sinusoidal cells in TGF- $\beta$ 1 expression, in particular of stellate and endothelial cells (De Bleser *et al.*, 1997). TGF- $\beta$ 1 plays a central role in the process of liver fibrosis, contributing to influx and activation of Kupffer cells as well as activation of HSC. TGF- $\beta$ 1 is produced by Kupffer cells and HSC, it up-regulates the transcription of collagen I and II genes and induces the expression of TIMP-1, a tissue inhibitor of MMP2 involved in collagen degradation (Bedossa & Paradis, 1995; Epstein *et al.*, 1994). Recently reported that the presence of the large TGF- $\beta$  complex was conclusively demonstrated in rat liver stellate cells but also in parenchymal cells (Roth-Eichhorn *et al.*, 1998). Our result is in accordance with many reports mentioned that the fibrotic liver is characterised by an increased of TGF- $\beta$ 1. Supporting evidence were

obtained in rodent models of liver fibrosis (De Bleser *et al.*, 1997; Parola *et al.*, 1993; Pietrangelo *et al.*, 1995; Poli & Parola, 1997). Furthermore, as reported by Leonarduzzi *et al.* (1997), such up-regulated TGF- $\beta$ 1 mRNA level led to a consistent and statistically significant rise of active TGF- $\beta$ 1 cytokine. Relevant to our finding, studies demonstrated that in rat fibrotic liver as induced by chronic treatment with CCl<sub>4</sub> or by iron overload, suitable antioxidant treatment (vitamin E or silybin) was significantly able to afford almost complete prevention of the marked up-regulation of hepatic TGF $\beta$ 1 and collagen type I genes (Parola *et al.*, 1992b; Pietrangelo *et al.*, 1995). Moreover, extracellular degradation of matrix proteins is regulated by matrix metalloproteinase (MMPs) produced by HSC (Benyon & Arthur, 2001). In fact, level and activity of MMP are the result of a complex regulation of the expression of the coding genes, extracellular cleavage of pro-enzyme forms and specific inhibition by tissue inhibitors of metalloproteinase (TIMPs) (Arthur, 1998; Cawston, 1998). In case of liver injury, namely in its early stages, an increased expression of MMP has been reported, mainly dependent upon activation of stellate cells (Milani *et al.*, 1994). On the other hand, a great bulk of evidence exists in support of an over-expression of TIMP-1 and TIMP-2 in hepatic fibrosis (Arthur, 1998). Both TIMP-1 and TIMP-2 are released by fully activated stellate cells mainly through up-regulated transcription of the respective gene (Bahr *et al.*, 1997; Roeb *et al.*, 2005). Such increased expression of these two forms of MMP inhibitors is prominent in advanced liver fibrosis both in the experimental animal and in humans (Herbst *et al.*, 1997). This hypothesis is supported by studies of experimental hepatic fibrosis and in human liver disease, in which TIMP-1 expression is significantly up-regulated in cirrhotic liver compared with normal liver (Knittel *et al.*, 2000). In consistent to our result, study by Clement *et al.* (1988) evidence has been obtained of mRNA expression for collagen types I in progressive liver fibrosis. Collagen type III is first increased after liver damage, later on largely substituted by

collagen type I. The amount of the latter type keeps increasing with the progression of the fibrotic process to sclerosis and cirrhosis, up to represent even 60-70 % of total collagen (Milani *et al.*, 1990; Ramadori *et al.*, 1998).

### **5.5 Immunomodulatory Effects on PBMCs**

Many medicinal plants used in folk medicines have been shown to stimulate or suppress immune responses. Results from other studies have indicated non-specific immune-stimulating or immune-modulating activities of a number of medicinal plants (Sriwanthana *et al.*, 2007). Generally, any immune-stimulant agent has the ability to enhance the body's defence against infections and cancer. Consequently these agents may be used as adjuncts to chemotherapy in immune-compromised patients such as cancer chemotherapy patients to alleviate infections, as well as to remove the residual cancer cells. It is well known that macrophages and other PMMCs play an important role in the defence mechanism against host infection and the killing tumour cells. The immunomodulatory activity of herbs and their active compounds is an area of research in cancer treatment (Kang *et al.*, 2002). MTT assay was used for assaying PBMCs cell survival and proliferation. MTT is utilized by all living, metabolically active cells and the amount of MTT formazan generated is directly proportional to the cell number (Mosmann, 1983).

Results of the present study have shown that both *O. stamineus* and *M. citrifolia* extracts at the serial concentration of 12.5 µg/ml to 200 µg/ml show promising PBMCs stimulating activities in a dose dependent manner. Furthermore 200 µg/ml treatments of both plant extracts significantly enhanced and increased the PMMCs cell viability, while 12.5, 25 and 50 µg/ml doses of both plants did not show significant changes as shown in (Figure 4.9). Our results suggested that the extracts of those plants have

stimulating activity on human PMMCs and could be useful to prevent the onset of cancer. *O. stamineus* contains high content of phenolic compounds and these compounds could affect the immune system due to the hydroxyl groups in their structure. The hydroxyl groups can stimulate the enzyme or electron-transferring system, thus resulting in immune-modulating properties, particularly in proliferation of macrophages and lymphocytes (Manosroi *et al.*, 2003). Moreover, our results are in accordance with those reported by other authors in which the immunological activity of *M. citrifolia* fruit at various concentrations inhibited the production of tumour necrosis factor-alpha (TNF- $\alpha$ ), which is an endogenous tumour promoter (Hirazumi *et al.*, 1996). On the other hand, scopoletin is a phenolic compound isolated from *M. citrifolia*; was found induced cell proliferation of normal T-lymphocytes; this immunomodulatory effect was reported due to the interaction with kinase C (PKC) protein. These results indicate that scopoletin and some other phenolic and flavonoid compounds have immune-modulating activities (Manuele *et al.*, 2006).

### **5.6 *In vitro* hepatoprotective and antioxidant activities of plant crude extracts and isolated fractions**

H<sub>2</sub>O<sub>2</sub> has been identified to have potential to generate free radicals in cell line and biological system. However, the cellular antioxidant enzymes SOD, CAT and GPx limit the effects of oxidant molecules on tissues and are active in the defence against oxidative cell injury by means of their being free radical scavengers (Kyle *et al.*, 1987). These enzymes work together to eliminate active oxygen species and small deviations in physiological concentrations may have a dramatic effect on the resistance of cellular lipids, proteins and DNA to oxidative damage. The increased activities of antioxidant enzymes may be a compensatory regulatory response to increased oxidative stress. The

WRL-68 normal liver cell line was used, due to the fact that, although this cell line expresses some hepatic characteristics, it does not express alcohol dehydrogenase or cytochrome P450 activity, and the results could be considered as the toxic effect of thioacetamide and other chemicals induced liver cirrhosis in animals (Gutiérrez-Ruiz *et al.*, 1994). In addition, studies showed that chemicals can produce oxidative stress and hepatocyte toxicity on liver cells line. Moreover, all of these damage could be blocked by the administration of antioxidants (Bailey & Cunningham, 2002). *In vitro* experiments with human liver cells used for hepatoprotective evaluation have many potential advantages compared with conventional animal testing. One such advantage is that the cell line does not required large quantity of the examined compound as in animal experiment and this is useful for the evaluation of isolated compounds, which are usually in very small amount. The other advantage is the possibility of using human cellular models, thereby avoiding the difficulty of extrapolating animal data to humans (Olivares *et al.*, 1997).

The crude extracts and all isolated fractions were screened for their *in vitro* hepatoprotective activity against H<sub>2</sub>O<sub>2</sub>-induced liver cell line. Results indicate that both plant extracts and the two fractions; *O. stamineus* F3 and *M. citrifolia* F2 have the best ability to fight against H<sub>2</sub>O<sub>2</sub> cell oxidative damage and enhanced the cell viability. The activity of *O. stamineus* and *M. citrifolia* crude extracts was found to be are in accordance with that of the *in vivo* evaluation, whereas better result obtained by *O. stamineus* F3 and *M. citrifolia* F2, in term of prevent the WRL-68 death and normalized the oxidative damage generated by H<sub>2</sub>O<sub>2</sub> induction. Other fractions did not show highly significant difference when compared to H<sub>2</sub>O<sub>2</sub> group. For the *in vitro* antioxidant studies, we noticed that *O. stamineus* F3 showed significant increase in cellular antioxidant enzymes; CAT and GPX compared to H<sub>2</sub>O<sub>2</sub> group; at the same time

significantly reduced the MDA, which represent the lipid peroxidation. The crude extracts and *M. citrifolia* F2 have the same ability to induce the antioxidant enzymes but with less effect (Table 4.7). *O. stamineus* and *M. citrifolia* are rich in phenolic compounds and exhibits antioxidant capacity against oxidative stress, which in turn lead to increased cellular antioxidant enzymes. Nevertheless, SOD showed no significant difference between all groups and this may be explained by that the SOD has no action on H<sub>2</sub>O<sub>2</sub> radicals, while CAT and GPx are the most abundant enzymes in the liver cell responsible for the catalytic decomposition of H<sub>2</sub>O<sub>2</sub> to oxygen and water. SOD is an extremely effective antioxidant enzyme, but it is responsible for catalytic dismutation of superoxide radicals to H<sub>2</sub>O<sub>2</sub> (Reiter *et al.*, 2000).

### 5.7 Phytochemicals investigation of the active fractions

The LC-MS investigation of *O. stamineus* F3 showed the presence of ponkanetin, eupatorin, TMF (3-hydroxy-5, 7, 3, 4'-tetramethoxy flavone) and salvigenin, while *M. citrifolia* F2 showed the presence of scopoletin and *p*-coumaric acid compounds. The literature survey showed that compounds such as eupatorin and 3'-hydroxyl-5, 7, 3, 4'-tetramethoxy flavone were previously isolated from *O. stamineus* (Akowuah *et al.*, 2005a; Olah *et al.*, 2003; Tezuka *et al.*, 2000). On the other hand, a number of studies have identified many phenolic compounds in *M. citrifolia* including scopoletin and *P*-coumaric acid (Jayaraman *et al.*, 2008; Levand & Larson, 1979; Wang *et al.*, 2002).

The therapeutic effects of *O. stamineus* have been referred mainly to its polyphenols, which are the most dominant constituents in the leaf that has been reported by Hollman & Katan (1999) to be effective in reducing oxidative stress by inhibiting the formation of lipid peroxidation products in biological systems which could lead to some of the chronic diseases such as coronary heart disease and cancer. The leaves were reported to



have the highest antioxidant properties whereby the phenolic fraction is the most active principle among the phytochemicals studied (Pietta *et al.*, 1998), so the leaves of this herb are often used in traditional medicine compared to other parts of the plant. According to Sumaryono *et al.* (1991), the derivatives of caffeic acid, were reported to constitute 67 % of total identified phenolics in methanol extract and about 94.6 % in hot water extract.

Recent findings showed that the flavonoid compounds; ponkanetin, eupatorin, tetramethoxy flavone and salvigenin, as well as scopoletin and *P*-coumaric acid were also effective as antioxidant, free radical scavenging, anticancer, anti-proliferative, anti-fibrotic and hepatoprotective agents (Akowuah *et al.*, 2004; Androutsopoulos *et al.*, 2008; Nagao *et al.*, 2002; Okafor & Dioka, 2011; Ponce *et al.*, 2004; Rafatian *et al.*, 2012; Tezuka *et al.*, 2000; Yam *et al.*, 2009; Zang *et al.*, 2000). Thus, the plant-derived antioxidants represent valuable anti-fibrotic drugs.

### **5.8 Antibacterial activity**

The antibacterial assay showed that both aqueous and ethanol extracts of *O. stamineus* exhibited activity against Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus agalactiae*) but not against Gram-negative bacteria (*Escherichia coli* and *Klebsilla pneumonia*). Among the investigated extracts, the aqueous extract of *O. stamineus* showed the best antibacterial efficacy against *Staphylococcus aureus* and *Streptococcus agalactiae*, while *M. citrifolia* did not show any antibacterial activity (Table 4.10).

In general, plants are rich in a wide variety of chemical compounds, such as alkaloids, tannins, flavonoids and terpenoids, with antimicrobial activities (Cowan, 1999). The reported antibacterial activities of *O. stamineus* extracts were related to their high

content of rosmarinic acid (Ho *et al.*, 2010; Huang & Zheng, 2006). The results of this study are in agreement with the results published by Ho *et al.* (2010) who reported the antimicrobial activities of methanol extracts of the *O. stamineus* against selected food-borne bacteria. Moreover, *O. stamineus* showed markedly inhibition of the growth of *Vibrio parahaemolyticus* and *Streptococcus mutans*. It was claimed that saponins and caffeic acid derivatives could be responsible for the antimicrobial activity of this herb (Ho *et al.*, 2010).

## 5.9 Conclusion

In conclusion, this study showed that the ethanol extracts of *O. stamineus* and *M. citrifolia* have hepatoprotective effects and antioxidant properties that were proven *in vivo* against thioacetamide-induced liver cirrhosis in rats and *in vitro* against WRL-68 normal liver cell line. In addition, the active constituents identified in both plants have confirmed and correlate to the antioxidant and hepatoprotective activity. Furthermore, this study revealed that the high dose 200 mg/kg of *O. stamineus* exerted a significant hepatoprotective and antioxidant effect as proven by biochemical, antioxidant and histopathological analysis. Accordingly, *O. stamineus* could be an effective herbal and efficient remedy for chemical-induced hepatic cirrhosis. Consequently, this activity could be claimed to the following identified flavonoids compounds; ponkanetin, eupatorin, TMF and salvigenin as confirmed by the *in vitro* assays. On the other hand, the high dose 200 mg/kg of *M. citrifolia* exhibited antioxidant and hepatoprotective activity but less than that of *O. stamineus* and this activity may be attributed to the presence of scopoletin and *P*-coumaric acid. *O. stamineus* treatment resulted in significant increase in the levels of total antioxidants, reduced oxidative stress and enhanced the activities of antioxidant enzymes (SOD, CAT and GPx) suggesting that

the reduction of oxidative stress likely plays a role in the mechanism of its hepatoprotective effects. The other possible mechanism of hepatoprotective and liver regeneration action of both plant extracts could be due to the free radical scavenging, antioxidant properties, high phenolic and flavonoid contents and anti-lipid peroxidation activities, furthermore may be as consequence of a significant down-regulation of the liver fibrosis involved genes expression; namely; TGF $\beta$ 1, MMP2 and Coll  $\alpha$  genes, in contrast to the over-expression of those three genes in liver cirrhosis.

The obtained results indicate that *O. stamineus* exhibits potent antioxidant activity and stimulating activity on human PBMCs which might be useful for therapeutic purposes to prevent ROS disorders and enhance the immune system, and could be used as a potential immunomodulatory agent for tumor immunotherapy. Moreover *O. stamineus* has some interesting antibacterial efficacy, especially against Gram positive bacteria. These findings indicate that *O. stamineus* showed high antioxidant, antibacterial and immune-modulating activity and may be considered as an immunomodulatory and antibacterial agent.

In summary, the results of our study indicate that *O. stamineus* and *M. citrifolia* are safe and effective alternative chemo-preventive. Thus, they could be promising candidate drugs that merits further preclinical development and pharmacological studies with a view to determining its eventual suppressive and therapeutic potential. Furthermore, our results offer a scientific basis for the traditional use of these medicinal plants.

### **5.10 Future work**

Further studies need to confirm the potential of active constituents isolated from these plant extracts such as the bioavailability, pharmacodynamics, pharmacokinetics and other pharmacological evaluations. In addition, the amount of the antioxidant should be compared to what actually biologically available in the blood after the plant extract administration. Moreover, study the mechanism of action for the prevention of the oxidative stress applied on cell line such as apoptosis and necrosis using flow cytometry method.

Finally, future studies to be undertaken in relation with these results to screen the medicinal plants that are used in traditional medicine to prevent the liver diseases, should be done for screening of potential active constituents.

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## APPENDIXES

### APPENDIX I Forms and photos

#### A. Acute toxicity ethic approval

*The Leader In Research and Innovation*

PEJABAT KETUA

01 November 2011

Professor Dr. Mahmood Ameen Abdulla  
Jabatan Molekul Perubatan  
Fakulti Perubatan  
Universiti Malaya

Tuan/Puan,

**PERLANJUTAN NOMBOR ETIKA: SCREENING OF PURE COMPOUNDS AND MEDICINAL  
PLANT EXTRACT FOR ACUTE AND SUB-CHRONIC TOXICITY TEST**


Dengan sukacitanya Jawatankuasa Institusi Penjagaan dan Penggunaan Haiwan, Universiti Malaya telah meluluskan permohonan untuk perlanjutan nombor etika bagi penyelidikan tersebut di atas.

No rujukan etika: **PM/07/05/2008/1111/MAA (a)(R )**

Sila ambil perhatian bahawa nombor rujukan etika yang diberi adalah sah untuk tempoh **dua (2) tahun iaitu sehingga 1 November 2013.**

Sekian, terima kasih.

Yang benar,

  
**Dr. Haji Azizuddin Bin Haji Kamaruddin**  
Ketua  
Pusat Haiwan Makmal  
Fakulti Perubatan  
Merangkap Setiausaha Jawatankuasa Institusi Penjagaan dan Penggunaan Haiwan, Universiti Malaya



MS ISO 9001:2000 REG. NO. AR 2760



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Laboratory Animal Centre  
Faculty of Medicine, University of Malaya, 50603, Kuala Lumpur, Malaysia  
Tel: (603) 7967 4792 Faks: (603) 7955 9886  
E-mail: [azizud@um.edu.my](mailto:azizud@um.edu.my) Website: <http://www.um.edu.my>



## B. LC/MC analysis request form (CARIF)



Cancer Research Initiatives Foundation (CARIF)

Tel: 03-5639 1874; F: 03-5639 1875

UPLC/MS analysis request form

Ref. No:

Date received:

Received by:

Billing detail:

### (A) Details of applicant

Name : Prof. Mahmood Ameen Abdulla / Mohammed Alshawsh  
(Prof./ Assoc. Prof./ Dr./ Mr./ Ms.)  
e-mail : alshaweshmam@yahoo.com  
Address : Molecular Medicine Department, Faculty of Medicine, University of Malaya.  
Phone number : 0172593425

### (B) Details of sample. (please tick where ever required)

- 1) Sample label: 2 samples (F3 and F2)      2) Molecular weight; formula (if pure): \_\_\_\_\_  
3) Sample purity: ☒ Crude    ☐ Pure      4) Storing condition: \_\_\_ 4-8 C \_\_\_\_\_  
5) Sample toxicity (If known. Please specify at what concentration & which cell-line): \_\_\_\_\_

### (C) LC details. (please attach the chromatogram if available)

- 1) Type & length of column: Thermo Scientific      2) PDA detector (nm): \_\_\_ 280nm \_\_\_\_\_  
Hypersil Gold, C18, Dim. (mm) (250x4.6)  
3) Injection volume: (a) HPLC: \_\_\_ 20µl \_\_\_\_\_      4) Temperature: (a) Column: \_\_\_ 27C \_\_\_\_\_  
(b) UPLC: \_\_\_\_\_      (b) Sample: \_\_\_\_\_  
5) HPLC profile: (flow rate: 1ml/min )      UPLC profile: (flow rate: )

Time	% A - <del>0.1% TFA</del> <b>0.1% TFA (PH 2.6)</b>	% B - <del>Acetonitrile</del> <b>Acetonitrile + 0.1% TFA (PH 2.6)</b>	Time	% A -	% B -
0min	90	10			
5min	55	45			
20min	50	50			
21min	40	60			
22min	90	10			
23min	stop				

### (D) Analysis required. (please tick where ever required)

- 1) Type of analysis: ☐ Nominal mass only      ☒ **Accurate mass only**  
☐ Nominal mass + MS/MS      ☐ Accurate mass + MS/MS  
2) Ionization mode: ☒ **+** ~~ve~~    ☐ ~~+~~ **-**    ☐ **Both**  
3) Method: ☐ LCMS      4) MS/ MS parent: \_\_\_\_\_

### (E) Result & Comment.



**C. Some photos of different steps in the *in vivo* hepatoprotective an animal model experiment**







## **APENDIX II Reagents preparation and laboratory protocols**

### **A. Phosphate buffer saline (PBS)**

A total of 10 Tablet of phosphate buffer saline (Sigma-Aldrich, UK) were added to 1000 ml of distilled water (as described by manufacture) and mixed well. The pH was adjusted between 7-7.4 if necessary and the solution was autoclaved at 120°C for 15 min.

### **B. 10 % buffered formalin solution**

To make a solution of 10% Formalin, nine parts of water were added to one part of 40% (aqueous) Formaldehyde and the pH was adjusted between 7-7.4 by adding PBS Tablet and mixed gently. Therefore, to prepare 1000 ml:

Formaldehyde (37-40%) -----100 ml

Distilled water ----- 900 ml

PBS Tablet (Sigma-Aldrich, UK) -----10 Tablets

Mix to dissolve.

### **C. Tissue processing (automated machine procedures)**

Process	Fixation	Dehydration	Clearing	Infiltration
Reagents	10% Neutral Buffered Formalin	70% Ethanol 95% Ethanol 100% Absolute	Xylene	Paraffin
Action	Stabilize Proteins	Removal of water	Removal of alcohol	Interpenetration

A. Dehydration:

- a. Station 1: 70 % Dehydrating agent, about 1 hour.
- b. Station 2: 95 % Dehydrating agent, about 1 hour.
- c. Station 3: 100 % Dehydrating agent, 45 minutes to about 1 hour.
- d. Station 4: 100 % Dehydrating agent, 45 minutes to about 1 hour.
- e. Station 5: 100 % Dehydrating agent, 45 minutes to about 1 hour.

B. Clearing:

- a. Station 6: Clearing agent, 30 min to about 1 hour.
- b. Station 7: Clearing agent, 30 min to about 1 hour.
- c. Station 8: Clearing agent, 30 min to about 1 hour.

C. Infiltration:

- a. Station 9: Embedding media, about 1 hour.
- b. Station 10: Embedding media, at least one hour till embedded.

**D. Hematoxylin-eosin (H&E) staining protocol**

Basic steps of staining:

- De-waxing with xylene
- Re-hydration
- Staining
- Dehydration with xylene
- Mounting

To bring section to water (de-wax and re-hydrate) before staining:

- Place slide in xylene 3 minutes x2
- Drain off excess xylene
- Transfer slide to absolute alcohol 2 minutes x1
- Transfer slide to 95 % alcohol 2 minutes x2
- Transfer slide to 70 % alcohol 2 minutes x1
- Leave in slow running tap water 3 minutes
- Section now is ready for staining

## HAEMATOXYLIN AND EOSIN (H&E) STAIN

### Reagents required:

- 1) Harris' haematoxylin working solution
- 2) Eosin working solution
- 3) 0.5% acid alcohol
- 4) 2% sodium 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 colour goes off.
4. Differentiation:  
Dip 2 to 3 X in 0.5% acid alcohol  
Wash well in running tap water. 2 min  
*Check microscopically – Nuclear structure should be purplish blue and cytoplasm colourless.*  
Repeat differentiation if necessary.
5. Wash well in running tap water 2-3 min.
6. "Blue" section with 2% sodium acetate 2 sec.
7. Wash again in running tap 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 xylene 2 min x 3
12. Mount with DPX
13. Wipe slide to remove excess xylene.
14. Label slide appropriately.

### RESULT:

Nuclei

blue

Cytoplasm, connective tissue, RBC

various shades of pink and red

### **E. 2 % denaturing agarose gel electrophoresis and ethidium bromide staining**

To prepare agarose gel (2 % agarose), mix:

2 gm of agarose

100 ml 1x TEA (Tris-acetate-EDTA) buffer (2 ml 10x TEA buffer + 98 ml nuclease-free water)

If smaller or larger gels are needed, adjust the quantities of components proportionately.

Heats the mixture in microwave to melt agarose until become dissolve completely. Cool to 65°C in a water bath. Add 1 µl of a 10 mg/ml ethidium bromide stock solution.

Mix thoroughly and pour onto gel support. Before running the gel, equilibrate in 1x TEA gel running buffer for at least 30 minutes.

## APENDIX III Manufacturer kits instruction and procedures

### A. Antioxidant Cayman assay

#### 1. Antioxidant assay kit (Item No. 709001 Cayman)

##### 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).

	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 is 210  $\mu$ l 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

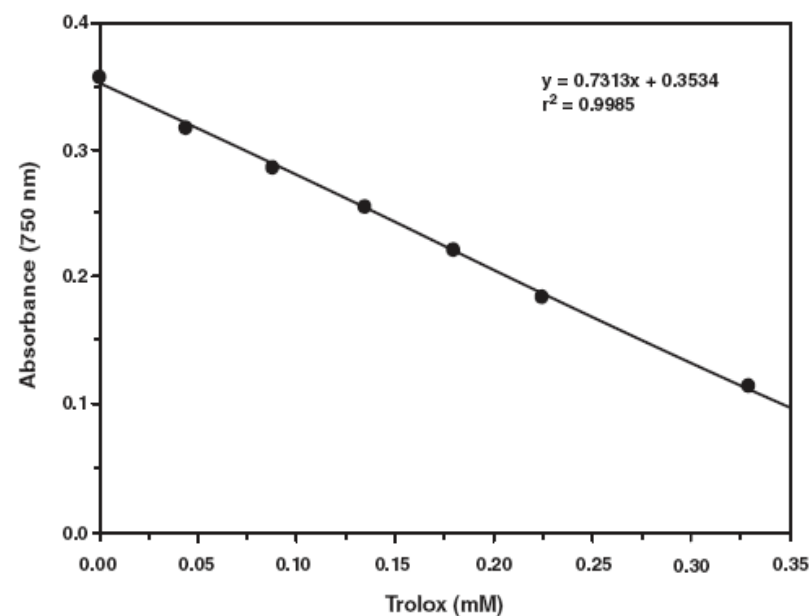
**Table 1. Trolox standard preparation**

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**

## 2. Catalase assay kit (Item No. 707002 Cayman)

### 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  $\mu$ l 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  $\mu$ l 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 ( $\mu$ l)	Sample Buffer ( $\mu$ l)	Final Concentration ( $\mu$ M 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  $\mu$ l reaction.

## Performing the Assay

1. **Formaldehyde Standard Wells** - Add 100  $\mu$ l of Assay Buffer (dilute), 30  $\mu$ l of methanol, and 20  $\mu$ l 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  $\mu$ l of Assay Buffer (dilute), 30  $\mu$ l of methanol, and 20  $\mu$ l of diluted CAT (control) to two wells.
3. **Sample Wells** - Add 100  $\mu$ l of Assay Buffer (dilute), 30  $\mu$ l of methanol, and 20  $\mu$ l 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  $\mu$ l 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  $\mu$ l of potassium hydroxide to each well to terminate the reaction and then add 30  $\mu$ l 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  $\mu$ l 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.

### 3. Superoxide dismutase assay kit (Item No. 706002 Cayman)

#### 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  $\mu$ l 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  $\mu$ l 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

- 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).
- Sample Wells** - add 200 μl of the diluted radical detector and 10 μl of sample to the wells. *NOTE: If using an inhibitor, add 190 μl of the diluted radical detector, 10 μl of inhibitor, and 10 μl of sample to the wells. The amount of sample added to the well should always be 10 μl. Samples should be diluted with Sample Buffer (dilute) or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 10,000 to bring the enzymatic activity to fall within the standard curve range.*
- 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.*
- Carefully shake the 96-well plate for a few seconds to mix. Cover with the plate cover.
- 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

- Calculate the average absorbance of each standard and sample. If assayed, subtract sample background absorbance from the sample.
- 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).
- 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.
- 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

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

### 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.

#### 4. Glutathione peroxidase assay kit (Item No. 703102 Cayman)

##### 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 Ceramic

- 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.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	B	7	7	7	15	15	15	23	23	23
B	C	C	C	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

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

Figure 2. Sample plate format

### Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Use different tips to pipette the assay buffer (dilute), co-substrate mixture, enzymes, and cumene hydroperoxide.
- 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 190  $\mu$ l 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.
- Use the Assay Buffer (dilute) in the assay.
- Monitor the decrease in absorbance at 340 nm using a plate reader.

### Performing the Assay

1. **Background or Non-enzymatic Wells** - add 120  $\mu$ l of Assay Buffer and 50  $\mu$ l of co-substrate mixture to three wells.
2. **Positive Control Wells (bovine erythrocyte GPx)** - add 100  $\mu$ l of Assay Buffer, 50  $\mu$ l of co-substrate mixture, and 20  $\mu$ l of diluted GPx (control) to three wells.
3. **Sample Wells** - add 100  $\mu$ l of Assay Buffer, 50  $\mu$ l of co-substrate mixture, and 20  $\mu$ l of sample to three wells. To obtain reproducible results, the amount of GPx added to the well should cause an absorbance decrease between 0.02 and 0.135/min. When necessary, samples should be diluted with Sample Buffer or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 10,000 to bring the enzymatic activity to this level. *NOTE: The amount of sample added to the well should always be 20  $\mu$ l. To determine if an additional sample control should be performed see the Interferences section (page 14).*
4. Initiate the reactions by adding 20  $\mu$ l of cumene hydroperoxide to all the wells being used. Make sure to note the precise time the reaction is initiated and add the cumene hydroperoxide as quickly as possible.
5. Carefully shake the plate for a few seconds to mix.
6. Read the absorbance once every minute at 340 nm using a plate reader to obtain at least 5 time points. *NOTE: The initial absorbance of the sample wells should not be above 1.2 or below 0.5.*

## 5. TBARS (MDA) assay kit (Item No. 10009055 Cayman)

### 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  $2 \times 10^7$  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



### 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<sup>7</sup> 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

### Fluorometric Standard Preparation

Dilute 25  $\mu$ l of the MDA standard (Catalog No. 10009202) with 975  $\mu$ l of water to obtain a stock solution of 12.5  $\mu$ M. Take eight clean glass test tubes and label them A-H. Add the amount of 12.5  $\mu$ M MDA stock solution and water to each tube as described in Table 2.

Tube	MDA ( $\mu$ l)	Water ( $\mu$ l)	MDA Concentration ( $\mu$ M)
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  $\mu$ l of sample or standard to appropriately labeled 5 ml vial.
3. Add 100  $\mu$ l 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  $\mu$ l (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.



## B. Gene expression kits

### 1. QIAamp® RNA Blood Mini Handbook, for total RNA purification

#### 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 *RNA/later 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.
- $\beta$ -Mercaptoethanol ( $\beta$ -ME) must be added to Buffer RLT before use. Add 10  $\mu$ l  $\beta$ -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 described in Appendix D (page 41).

## 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  $\beta$ -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  $\mu$ l 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 $\mu$ l
20 to 30 mg, if tissue is difficult to lyse	600 $\mu$ l

As a guide, 3 mm<sup>3</sup> 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 RNA/later 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  $\mu$ l 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  $\mu$ l or 600  $\mu$ l) 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  $\mu$ l 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  $\geq 8000 \times g$  ( $\geq 10,000$  rpm). If sample volume exceeds 700  $\mu$ l, 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  $\mu$ l Buffer RW1 onto the QIAamp spin column and centrifuge for 15 s at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash.**

Discard flow-through\* and collection tube.

**5. Transfer QIAamp spin column to a new 2 ml collection tube (provided). Pipet 500  $\mu$ l of Buffer RPE onto the QIAamp spin column and centrifuge for 15 s at  $\geq 8000 \times g$  ( $\geq 10,000$  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  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to elute. Repeat if the expected RNA yield is  $>30 \mu\text{g}$ .**

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.
- $\beta$ -Mercaptoethanol ( $\beta$ -ME) must be added to Buffer RLT before use. Add 10 µl of  $\beta$ -ME per 1 ml of Buffer RLT. Buffer RLT is stable for 1 month after addition of  $\beta$ -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 described 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:**  $\beta$ -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  $\geq 8,000 \times g$  ( $\geq 10,000$  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  $\geq 8,000 \times g$  ( $\geq 10,000$  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 ( $20,000 \times g$ , 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.

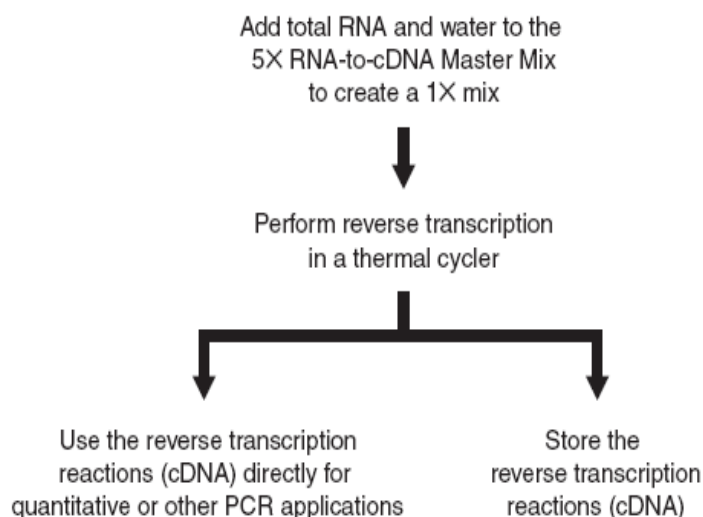
7. Transfer the QIAamp spin column to a new 1.5 ml collection tube (provided) and pipet 30–50  $\mu$ l of RNase-free water (provided) directly onto the QIAamp membrane. Centrifuge for 1 min at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to elute. Repeat if the expected RNA yield is  $>30 \mu$ g.

A second elution step into the same collection tube with a further 30–50  $\mu$ l RNase-free water is recommended when the expected RNA yield is  $>30 \mu$ g.

## 2. High Capacity RNA-to-cDNA Master Mix Kit (Reverse Transcription)

### Using the High Capacity RNA-to-cDNA Master Mix

**Overview** 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  $\mu$ L per reaction):

1.	Place the Master Mix (or No-RT Control) components on ice, then mix and briefly centrifuge them.															
2.	<p>Calculate the total volume of components needed to prepare the required number of reactions. Use the table below.</p> <table><tr><th>Component</th><th>Volume per Reaction</th><th>Final Concentration</th></tr><tr><td>Master Mix (or No-RT Control)</td><td>4.0 <math>\mu\text{L}</math></td><td>1X</td></tr><tr><td>RNA template</td><td>up to 16 <math>\mu\text{L}</math></td><td>1 pg - 1 <math>\mu\text{g}</math></td></tr><tr><td>Nuclease-free <math>\text{H}_2\text{O}</math></td><td>sufficient to 20 <math>\mu\text{L}</math></td><td>—</td></tr><tr><td>Total</td><td>20.0 <math>\mu\text{L}</math></td><td>—</td></tr></table> <p><b>IMPORTANT!</b> Include additional reactions in the calculations to provide excess volume for the loss that occurs during reagent transfers.</p>	Component	Volume per Reaction	Final Concentration	Master Mix (or No-RT Control)	4.0 $\mu\text{L}$	1X	RNA template	up to 16 $\mu\text{L}$	1 pg - 1 $\mu\text{g}$	Nuclease-free $\text{H}_2\text{O}$	sufficient to 20 $\mu\text{L}$	—	Total	20.0 $\mu\text{L}$	—
Component	Volume per Reaction	Final Concentration														
Master Mix (or No-RT Control)	4.0 $\mu\text{L}$	1X														
RNA template	up to 16 $\mu\text{L}$	1 pg - 1 $\mu\text{g}$														
Nuclease-free $\text{H}_2\text{O}$	sufficient to 20 $\mu\text{L}$	—														
Total	20.0 $\mu\text{L}$	—														
3.	<p>Prepare the reaction mix according to your calculations in step 2.</p> <div> <b>WARNING</b> <b>CHEMICAL HAZARD.</b> 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.</div> <p><b>IMPORTANT!</b> Prepare the reaction mix on ice.</p>															
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  $\mu$ L per reaction):  
(continued)

6.	Place the plate or tubes on ice until you are ready to load the thermal cycler.
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**Performing  
Reverse  
Transcription with  
High Capacity  
RNA-to-cDNA  
Master Mix**

To perform reverse transcription:

1.	<p>Program the thermal cycler conditions as shown below, using one of the thermal cyclers listed in <a href="#">Table 4 on page 16</a>.</p> <p><b>IMPORTANT!</b> These conditions are optimized for the High Capacity RNA-to-cDNA Master Mix.</p> <table><tr><td></td><td><b>Step 1</b></td><td><b>Step 2</b></td><td><b>Step 3</b></td><td><b>Step 4</b></td></tr><tr><td>Temperature (°C)</td><td>25</td><td>42</td><td>85</td><td>4</td></tr><tr><td>Time</td><td>5 min</td><td>30 min</td><td>5 min</td><td>∞</td></tr></table>		<b>Step 1</b>	<b>Step 2</b>	<b>Step 3</b>	<b>Step 4</b>	Temperature (°C)	25	42	85	4	Time	5 min	30 min	5 min	∞
	<b>Step 1</b>	<b>Step 2</b>	<b>Step 3</b>	<b>Step 4</b>												
Temperature (°C)	25	42	85	4												
Time	5 min	30 min	5 min	∞												
2.	Set the reaction volume to 20 μL.															
3.	Load the reaction plates or tubes into the thermal cycler.															
4.	<p>Start the reverse transcription run.</p> <p>Perform PCR amplification with one-tenth of the first-strand reaction.</p>															

### 3. TaqMan® Fast Advanced Master Mix Protocol



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	4444558	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 µg
Nuclease-free water	3.5	7.0	
<b>Total volume per reaction</b>	<b>10.0</b>	<b>20.0</b>	—

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

Product Insert  
06/2010

- 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).

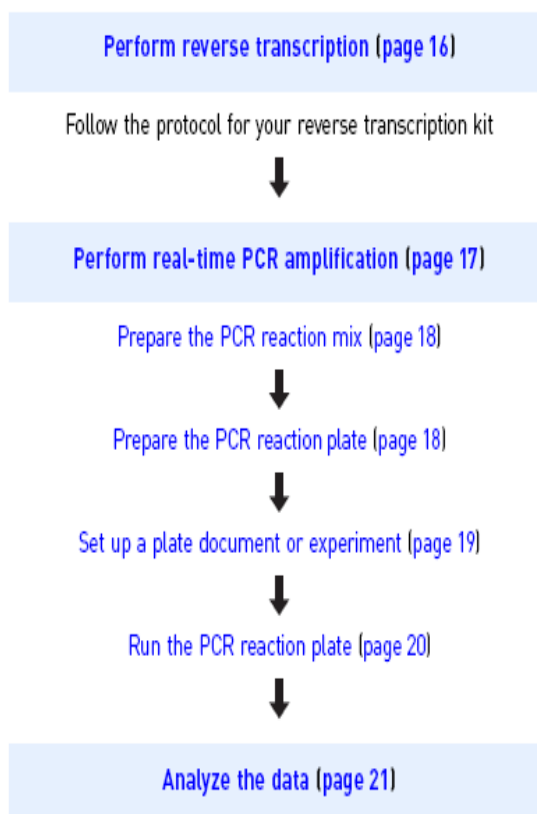
## Obtaining SDSs

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](http://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**.

## 4. TaqMan® Gene Expression Assay protocol

### Workflow



### Perform reverse transcription

Synthesis of single-stranded cDNA from total RNA samples is the first step in the two-step RT-PCR.


### Recommended kits and reagents

To obtain cDNA from RNA samples, Applied Biosystems recommends the reverse transcription kits and reagents listed in [“Reagents for reverse transcription” on page 14](#).

---

## Prepare the PCR reaction mix

1. Determine the total number of PCR reactions to perform. On each reaction plate, include:
  - A gene expression assay for each cDNA sample
  - No template controls (NTCs) for each assay on the plate

 **IMPORTANT!** You can run multiple assays on one reaction plate. Include controls for each assay that you run on a plate.

Applied Biosystems recommends that you perform 3 replicates of each reaction.

2. Per the table below, calculate the total volume required for each reaction component.



**Note:** Include extra volume to compensate for the volume loss that occurs during pipetting.

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 (2×)	5.0	10.0	1×
TaqMan® Gene Expression Assay (20×) or Custom TaqMan® Gene Expression Assay (20×)†	0.5	1.0	1×
Nuclease-free Water	3.5	7.0	—
<b>Total volume per reaction</b>	<b>9.0</b>	<b>18.0</b>	<b>—</b>

† See [aligenes.com](https://www.aligenes.com) for assay information.

3. Label a 1.5-mL microcentrifuge tube, add all components to the labeled tube, then cap the tube.
4. Vortex the tube briefly to mix the components.
5. Centrifuge the tube briefly to spin down the contents and eliminate air bubbles.

## Prepare the PCR reaction plate

1. Transfer the appropriate volume of PCR reaction mix to each well of an optical reaction plate:

Reaction plate format	Reaction volume (μL)
384-well plate	9.0
96-well and 48-well plates (both Standard and Fast)	18.0

2. Cover the reaction plate with an optical adhesive film.
3. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles.

- Remove the optical adhesive film.
- Per the table below, add cDNA template or water to each well.

Component	Volume (μL) for 1 reaction	
	384-well plate	96-well and 48-well plates (both Standard and Fast)
cDNA template + Nuclease-free Water†	1.0	2.0
Nuclease-free Water (for the NTC reactions)	1.0	2.0
<b>Total volume per reaction</b>	<b>10.0</b>	<b>20.0</b>

† Use 100 ng to 1 pg of cDNA diluted in Nuclease-free Water.

- Cover the reaction plate with a new optical adhesive film.
- Centrifuge the plate briefly to spin down the contents and eliminate air bubbles.
- If required by your real-time PCR system, apply a compression pad to the plate.

## Set up a plate document or experiment

When you set up a plate document or experiment, use the following thermal-cycling conditions:

- Thermal-cycling profile:

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.

- Run mode:

Applied Biosystems Real-Time PCR System	Default run mode
7900HT system	Standard
7900HT Fast system (384-Well and Standard 96-Well Block Modules)	
7500 system	
7300 system	
ViiA™ 7 system	Fast
StepOne™ system	
StepOnePlus™ system	
7900HT Fast system (Fast 96-Well Block Module)	
7500 Fast system	

- Sample volume:

Reaction plate format	Reaction volume (µL)
384-well plate	10.0
96-well and 48-well plates (both Standard and Fast)	20.0

## Run the PCR reaction plate

1. In the system software, open the plate document or experiment that corresponds to the reaction plate.
2. Load the reaction plate into the real-time PCR system.
3. Start the run.



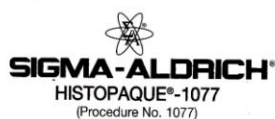
## Analyze the data

Data analysis varies depending on the real-time PCR system that you use. The general process for analyzing gene expression quantitation data involves:

1. Viewing the amplification plots for the entire reaction plate.
2. Setting the baseline and threshold values to determine the threshold cycles ( $C_T$ ) for the amplification curves.
3. Using the relative standard curve method or the comparative  $C_T$  method to analyze the data.

For detailed analysis information, refer to the appropriate documentation for your instrument. See [“Product documentation” on page 75](#).

## C. Histopaque-1077 kit



### INTENDED USE

Sigma-Aldrich HISTOPAQUE®-1077 is intended for the isolation of mononuclear cells. HISTOPAQUE®-1077 is for "In Vitro Diagnostic Use".

In 1968, Boyum<sup>1</sup> described methods for isolation of mononuclear cells from circulating blood and bone marrow.

In general, these procedures employed mixtures of polysaccharide and a radiopaque contrast medium. HISTOPAQUE®-1077 is a solution of polysucrose and sodium diatrizoate, adjusted to a density of  $1.077 \pm 0.001$  g/ml. This medium facilitates rapid recovery of viable mononuclear cells from small volumes of blood. The HISTOPAQUE®-1077 procedure is suitable for studying cell-mediated lympholysis<sup>2</sup> and for human lymphocyte antigen (HLA) typing.<sup>3</sup> It may also be employed as the initial isolation step prior to enumeration of T-, B- and "null" lymphocytes.<sup>4</sup>

Anticoagulated venous blood is layered onto HISTOPAQUE®-1077. During centrifugation, erythrocytes and granulocytes are aggregated by polysucrose and rapidly sediment; whereas, lymphocytes and other mononuclear cells remain at the plasma-HISTOPAQUE®-1077 interface. Erythrocyte contamination is negligible. Most extraneous platelets are removed by low speed centrifugation during the washing steps.

### REAGENT

HISTOPAQUE®-1077, Catalog No. 1077-1

Polysucrose, 5.7 g/dl, and sodium diatrizoate, 9.0 g/dl.

#### STORAGE AND STABILITY:

Store HISTOPAQUE®-1077 in refrigerator (2-8°C). Protect from light. Reagent label bears expiration date.

#### DETERIORATION:

A cloudy appearance indicates deterioration of the product.

#### PREPARATION:

HISTOPAQUE®-1077 is ready for use. Warm to 18-26°C before use.

If the intended use of the separated cells involves subsequent culturing, the HISTOPAQUE®-1077 must be sterile filtered prior to cell separation. For application where sterility is required, sterile filter the HISTOPAQUE®-1077 prior to use.

#### PRECAUTIONS:

Normal precautions exercised in handling laboratory reagents should be followed. Upon contact with human source substances, treat all reagents and equipment as potentially biohazardous. Dispose of waste observing all local, state, provincial or national regulations. Refer to Material Safety Data Sheet for any updated risk, hazard or safety information.

#### US Risks and Safety Statements

HISTOPAQUE®-1077-1 solutions are HARMFUL. May cause sensitization by inhalation and skin contact. Wear suitable protective clothing. Target organ: Blood.

#### EU Risks and Safety Statements

HISTOPAQUE®-1077-1 solutions are HARMFUL. May cause sensitization by inhalation and skin contact. Do not breathe vapor. Wear suitable protective clothing and gloves. In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).

### PROCEDURE

#### SPECIMEN COLLECTION:

It is recommended that specimen collection be carried out in accordance with NCCLS document M29-A2. No known test method can offer complete assurance that blood samples or tissue will not transmit infection. Therefore, all blood derivatives or tissue specimens should be considered potentially infectious.

Collect venous blood in preservative-free heparin or EDTA. For best results, blood should be processed within 2 hours.

#### SPECIAL MATERIALS REQUIRED BUT NOT PROVIDED:

Centrifuge (swinging bucket rotor) capable of generating 400 x g

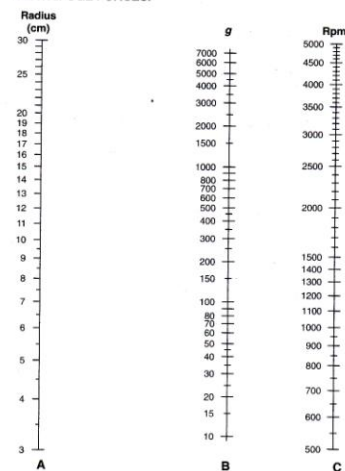
Isotonic phosphate buffered saline solution or appropriate tissue culture medium

Centrifuge tubes, 15-ml plastic, conical

#### NOTES:

1. If the intended use of the separated cells involves subsequent culturing, the HISTOPAQUE®-1077 must be sterile filtered prior to cell separation. For application where sterility is required, sterile filter the HISTOPAQUE®-1077 prior to use. This product is only for "In Vitro Use". Cells isolated using HISTOPAQUE® should not be used for "In Vivo" procedures.
2. 50 ml centrifuge tubes may be used. Alter stated procedure by using 15 ml of HISTOPAQUE®-1077-1, and 15 ml of whole or diluted blood.
3. On occasion it may be necessary to dilute blood 1:2 or 1:4, depending upon absolute cell numbers. The possibility of overloading the gradient exists.
4. Avoid use of powdered gloves. Glove powder will activate monocytes and cause lower yields.
5. Avoid use of high binding plastics such as polystyrene. Polystyrene may bind cells to the centrifuge tube walls.
6. Other anti-coagulants may be used; however the choice of anti-coagulant may affect cell recovery. As blood ages the cell recoveries will drop.
7. The procedure describes separation of mononuclear cells using isotonic phosphate buffered saline as a diluent and washing fluid. In many circumstances, balanced salt solutions such as Hank's or cell culture medium such as RPMI 1640 supplemented with fetal bovine serum are preferred.
8. The purity of cell population obtained by this procedure may be determined by performing a Romanowsky stain (e.g., Wright Stain) on a film or cytopsin slide of material from Step 10. Viability may be determined by treatment with trypan blue.
9. If cell viability is <80%, replacement of Phosphate Buffered Saline Solution with a tissue culture medium, such as RPMI 1640 fortified with fetal bovine serum, 5% of the total volume of RPMI 1640, may be helpful.
10. If T- and B-cell enumeration is to be performed, blood should not be stored more than several hours.<sup>5</sup> Addition of blood to certain tissue culture media may allow storage up to 72 hours without alteration in T- or B-cell values.<sup>6</sup>
11. Prediluted blood may be used with this procedure. Blood may be diluted with phosphate buffered saline or other appropriate medium, recommended ratio, 1 part blood to 1 part medium. Equal volumes of HISTOPAQUE®-1077 and blood should be used to optimize mononuclear cell recovery. Dilution is appropriate for specimens with hematocrits above normal.
12. Removing excess amounts of HISTOPAQUE®-1077 with the mononuclear band increases granulocyte contamination from residual granulocytes which may remain below the mononuclear interface.
13. Removing excess amounts of supernatant with the mononuclear band may promote contamination by plasma proteins and platelets.
14. Use of volumes of prediluted or whole blood other than those recommended may result in decreased recovery.
15. To remove all contaminating platelets, a second centrifugation in a 4 to 20% sucrose gradient layered over HISTOPAQUE®-1077 can be performed. The sucrose gradient will effectively isolate the platelets while the mononuclear cells will penetrate to the HISTOPAQUE®-1077 layer.
16. Failure to bring ACCUSPIN System-HISTOPAQUE®-1077 to room temperature may present limited recovery of mononuclear cells.
17. The use of a "normal" patient is recommended as a control for each run.

#### NOMOGRAM FOR DETERMINING RELATIVE CENTRIFUGE FORCES:



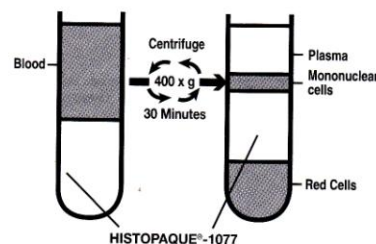
A nomogram can be used to derive the rpm setting for your centrifuge.

How to establish the rpm required to obtain 400 x g for Procedure No. 1077.

1. Measure the radius (cm) from the center of the centrifuge spindle to the end of the test tube carrier. Mark this value on scale A.
2. Mark the relative centrifugal force (e.g., 400) on scale B.
3. With a ruler, draw a straight line between points on columns A and B, extending it to intersect column C. The reading on column C is the rpm setting for the centrifuge.

#### PROCEDURE:

1. To a 15-ml conical centrifuge tube, add 3.0 ml HISTOPAQUE®-1077 and bring to room temperature.
2. Carefully layer 3.0 ml whole blood onto the HISTOPAQUE®-1077. Centrifuge at 400 x g for exactly 30 minutes at room temperature. Centrifugation at lower temperatures, such as 4°C, may result in cell clumping and poor recovery.
3. After centrifugation, carefully aspirate, with a Pasteur pipet, the upper layer to within 0.5 cm of the opaque interface containing mononuclear cells. Discard upper layer.
4. Carefully transfer the opaque interface, with a Pasteur pipet, into a clean conical centrifuge tube.



5. Add to this tube (Step 4), 10 ml Isotonic Phosphate Buffered Saline Solution and mix by gentle aspiration.
6. Centrifuge at 250 x g for 10 minutes.
7. Aspirate the supernatant and discard.

8. Resuspend cell pellet with 5.0 ml Isotonic Phosphate Buffered Saline Solution and mix by gentle aspiration with a Pasteur pipet.
9. Centrifuge at 250 x g for 10 minutes.
10. Repeat Steps 7, 8 and 9, discard supernatant and resuspend cell pellet in 0.5 ml Isotonic Phosphate Buffered Saline Solution.

#### PERFORMANCE CHARACTERISTICS

Erythrocytes and granulocytes should pellet to the bottom of the centrifuge tube. Mononuclear cells should band at the interface between the HISTOPAQUE®-1077 and the plasma.

In a comparison study, 42 paired heparinized blood samples were isolated simultaneously on Sigma-Aldrich HISTOPAQUE®-1077 and a competitive product according to respective procedures. Recovery of lymphocytes was calculated from the initial number layered on the gradients compared to the number appearing at the plasma-gradient interface. Trypan blue exclusion was used to assess cell viability. The two products produced similar results as shown by the following data:

	Lymphocytes at Interface	Recovery of Lymphocytes	Viability (Trypan Blue)
HISTOPAQUE®-1077	84 ± 14%	68 ± 13%	98 ± 4%
Competitive Product	83 ± 14%	60 ± 13%	98 ± 4%

If observed results vary from expected results, please contact Sigma-Aldrich Technical Service for assistance.

#### REFERENCES

1. Boyum A: Separation of leukocytes from blood and bone marrow. Scand J Clin Lab Invest 21 (Suppl 97):77, 1968
2. Lightbody J: Use of the Cell-mediated Lympholysis Test in Transplantation Immunity. IN Manual of Clinical Immunology, NR Rose, H Friedman, Editors. American Society for Microbiology, Washington (DC) 1976, pp 851-857
3. Amos DB, Pool P: HLA Typing. *ibid.* pp 797-804
4. Winchester RJ, Ross G: Methods for Enumerating Lymphocyte Populations. *ibid.* pp 64-76
5. Hofman FM, Kanesberg B, Smith D, et al: Stability of T- and B-cell numbers in human peripheral blood. Am J Clin Pathol 77:710, 1982

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Procedure No. 1077  
Previous Revision: 2003-09  
Revised: 2009-11



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## D. WRL-68 (ATCC Catalog)

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				<a href="#">See New Benefits of ATCC Culture</a>
Designations:	WRL 68			<b>Related Links</b> ▶
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<a href="#">Biosafety Level:</a>	2 [Cells contain Human Papilloma Viral (HPV) DNA sequences ]			<a href="#">Make a Deposit</a>
Shipped:	frozen			<a href="#">Frequently Asked Questions</a>
Medium & Serum:	<a href="#">See Propagation</a>			<a href="#">Material Transfer Agreement</a> New!
Growth Properties:	adherent			<a href="#">Technical Support</a>
Organism:	Homo sapiens			<a href="#">Related Cell Culture Products</a>
Morphology:				
Source:	<b>Organ:</b> HeLa contaminant			<a href="#">BioProducts</a>
	In addition to the <a href="#">MTA</a> mentioned above, other <a href="#">ATCC and/or regulatory permits</a> may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please <a href="#">click here</a> for information regarding the specific requirements for shipment to your location.			<a href="#">Cell microbial and molecular genomics products for the life sciences</a>
Permits/Forms:				<b>BioServices</b>
	Amelogenin: X CSF1PO: 9,10 D13S317: 12,13.3 D16S539: 9,10			<a href="#">Bio-materials management: basic repository to complex partnership-level services</a>
DNA Profile (STR):	D5S818: 11,12 D7S820: 8,12 TH01: 7 TPOX: 8,12 vWA: 16,18			<b>BioStandards</b>
Isoenzymes:	G6PD, A			<a href="#">Biological Reference Material and Consensus Standards for the life science community</a>
Comments:	A culture submitted to the ATCC in July of 1969 was found to be contaminated with mycoplasma, and was cured by treatment with Ciprofloxacin			
Propagation:	<b>ATCC complete growth medium:</b> The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. <b>Temperature:</b> 37.0°C			
Subculturing:	<b>Subcultivation Ratio:</b> A subcultivation ratio of 1:2 to 1:6 is recommended <b>Medium Renewal:</b> Every 2 to 3 days Remove medium, and rinse with 0.25% trypsin, 0.03% EDTA solution. Remove the solution and add an additional 1 to 2 ml of trypsin-EDTA solution. Allow the flask to sit at room temperature			



## APENDIX IV Raw data and result

### A. Blood analysis result form (Central Diagnostic Laboratory, University of Malaya Medical Centre)



#### Division of Laboratory Medicine

Doctor:	Dr Mahmood Ameen	Name:	T3
Location:	Shawsh Study	RN:	
Address:		IC No:	
		Age:	Sex: unknown
		Date collected:	DOB: unknown
		Date received:	11/02/2010 01:02 PM

#### CLINICAL CHEMISTRY

Test	Result	Unit	Ref. Range
<b>CLINICAL CHEMISTRY (GENERAL)</b>			
Glucose	5.6	mmol/L	3.9 - 6.1
Uric Acid	72	umol/L	
<b>ELECTROLYTES/ RENAL FUNCTION TESTS</b>			
Sodium	H 151	mmol/L	136 - 145
Potassium	H 5.9	mmol/L	3.6 - 5.2
	Sample not lysed		
Chloride	H 114	mmol/L	100 - 108
Carbon Dioxide	24.9	mmol/L	21 - 30
Anion Gap	18	mmol/L	10 - 20
Urea	H 6.8	mmol/L	2.5 - 6.4
Creatinine	56	umol/L	
<b>BONE PROFILE TESTS</b>			
Calcium	H 2.61	mmol/L	2.2 - 2.6
Phosphate	H 2.57	mmol/L	0.85 - 1.45
<b>LIVER FUNCTION TESTS</b>			
Total Protein	73	g/L	64 - 82
Albumin	L 12	g/L	35 - 50
Globulin	H 61	g/L	23 - 35
Total Bilirubin	8	umol/L	3 - 17
Conjugated Bilirubin	3	umol/L	0 - 3
Alanine Aminotransferase (ALT)	H 323	IU/L	30 - 65
Aspartate Aminotransferase	H 584	IU/L	15 - 37
Alkaline Phosphatase	H 536	IU/L	50 - 136
G-Glutamyl Transferase	24	IU/L	15 - 85
<b>CARDIAC ENZYMES</b>			
Lactate Dehydrogenase (LDH)	H 892	IU/L	100 - 190

Validated By: PUTRI JUNAIDAH BINTI MEGAT YUNUS

Lab No: 2508329  
Report Date: 26/07/2010  
Report Time: 04:24 PM

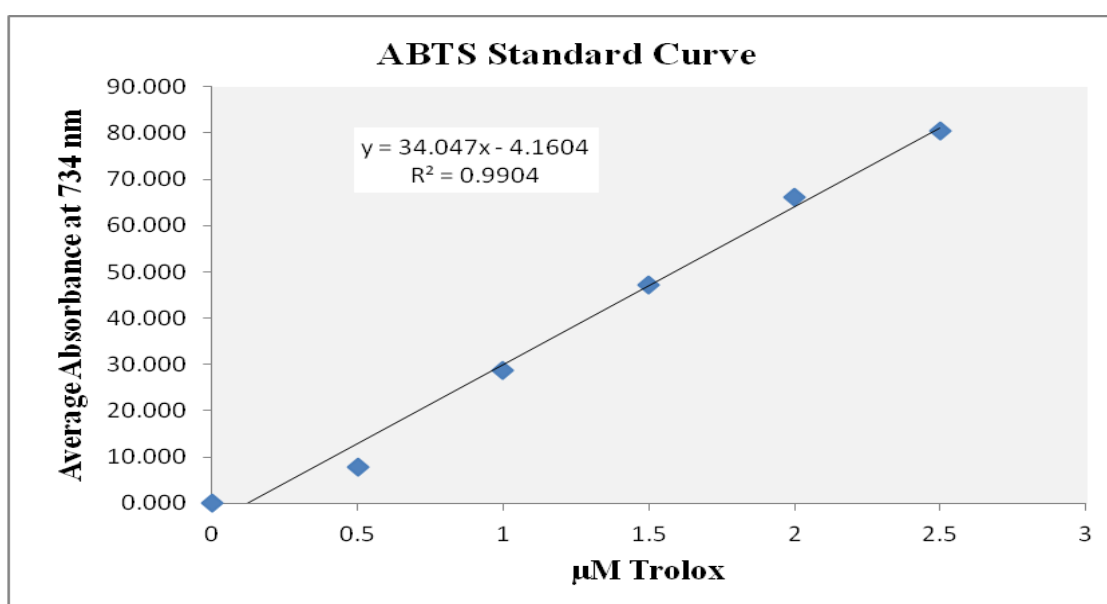
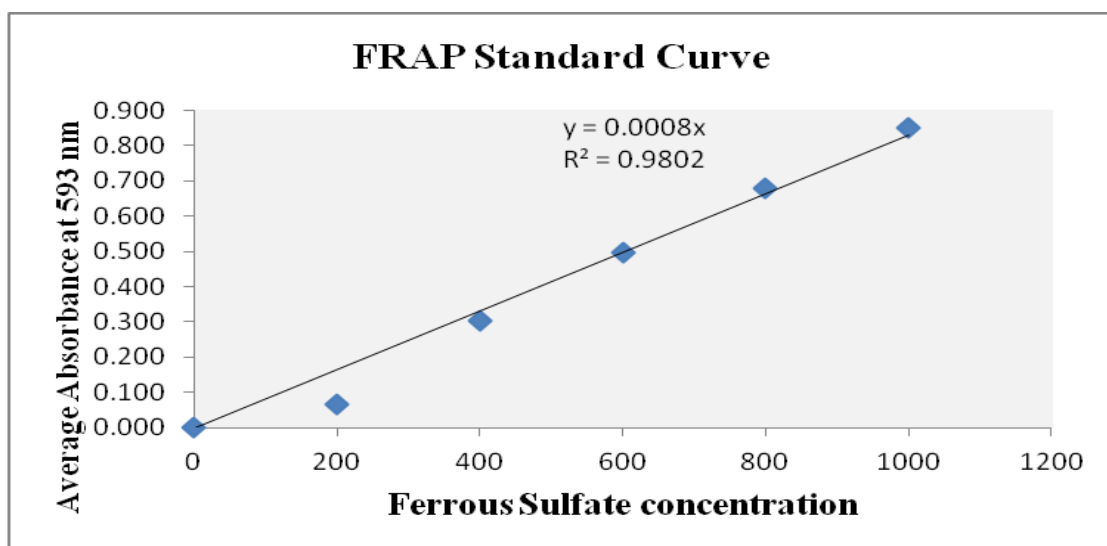
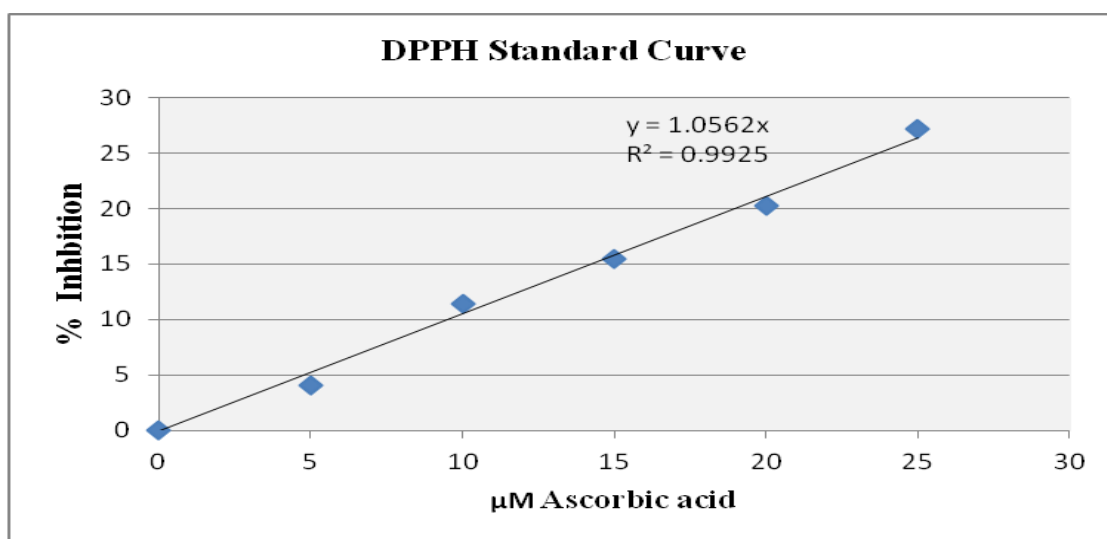
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LEMBAH PANTAI, 59100 KUALA LUMPUR, MALAYSIA  
Tel: 603-7949 2925  
Fax: 603-7949 2818  
Web Site: www.ummc.edu.my

Page: 1



## B. DPPH, FRAP and ABTS standard curves



### C. RNA concentration and purity measured by NanoDrop 2000 spectrophotometer

Sample	Conc. ng/μl	260/280	260/230	A 230	A 260	A 280	A 320
Calibrator 1	647	1.974	2.052	0.788	1.618	0.820	0.005
Calibrator 2	329	2.013	2.075	0.396	0.882	0.409	0.003
Calibrator 3	503	2.000	2.054	0.612	1.257	0.628	0.006
Calibrator 4	464	1.997	2.125	0.545	1.159	0.580	0.004
Calibrator 5	641	1.974	1.781	0.900	1.602	0.812	0.005
Calibrator 6	516	2.003	2.056	0.637	1.290	0.644	0.004
Calibrator 7	879	1.776	1.867	1.177	2.197	1.237	0.007
TAA 1	225	2.017	1.777	0.409	0.563	0.279	0.002
TAA 2	577	1.991	1.949	0.740	1.443	0.725	0.005
TAA 3	281	2.020	1.685	0.494	0.703	0.348	0.003
TAA 4	522	2.009	1.683	0.776	1.305	0.650	0.005
TAA 5	884	1.836	1.880	1.236	2.211	1.300	0.007
TAA 6	724	1.950	1.952	0.927	1.809	0.928	0.005
TAA 7	284	2.017	2.039	0.348	0.710	0.352	0.002
<i>O. stamineus 1</i>	76.9	2.031	1.864	0.222	0.192	0.095	0.000
<i>O. stamineus 2</i>	130	2.012	1.302	0.249	0.324	0.161	0.001
<i>O. stamineus 3</i>	276	2.032	1.750	0.600	0.690	0.340	0.002
<i>O. stamineus 4</i>	279	2.034	1.783	0.590	0.699	0.343	0.002
<i>O. stamineus 5</i>	482	2.016	1.946	0.619	1.204	0.597	0.005
<i>O. stamineus 6</i>	364	2.022	1.698	0.537	0.911	0.451	0.003
<i>O. stamineus 7</i>	248	2.016	1.948	0.318	0.620	0.307	0.002
<i>M. citrifolia 1</i>	554	1.990	2.105	0.658	1.384	0.696	0.006
<i>M. citrifolia 2</i>	551	1.993	2.122	0.649	1.377	0.691	0.005
<i>M. citrifolia 3</i>	556	1.999	1.834	0.758	1.390	0.696	0.004
<i>M. citrifolia 4</i>	575	1.984	2.103	0.684	1.438	0.725	0.004
<i>M. citrifolia 5</i>	316	2.012	1.715	0.489	0.789	0.392	0.003
<i>M. citrifolia 6</i>	629	1.988	1.754	0.897	1.573	0.791	0.005
<i>M. citrifolia 7</i>	541	1.999	1.984	0.682	1.353	0.677	0.004

#### D. Expression stability of four endogenous reference genes using NormFinder and geNorm algorithm

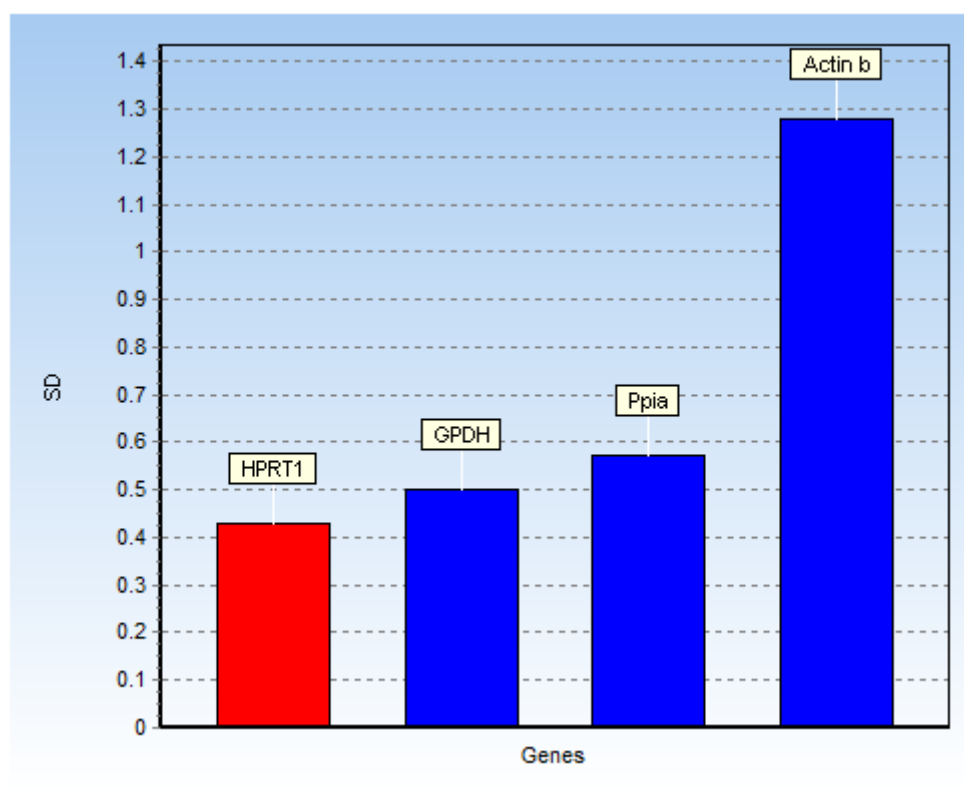
##### 1. NormFinder analysis:

Gene Name	SD	SD	0.4304
HPRT1	0.4304		
GPDH	0.5031	<b>Best Gene</b>	HPRT1
Ppia	0.5706		
Actin $\beta$	1.28		

Output summary

Best Genes: HPRT 1

SD : 0.4304 (lowest variability)





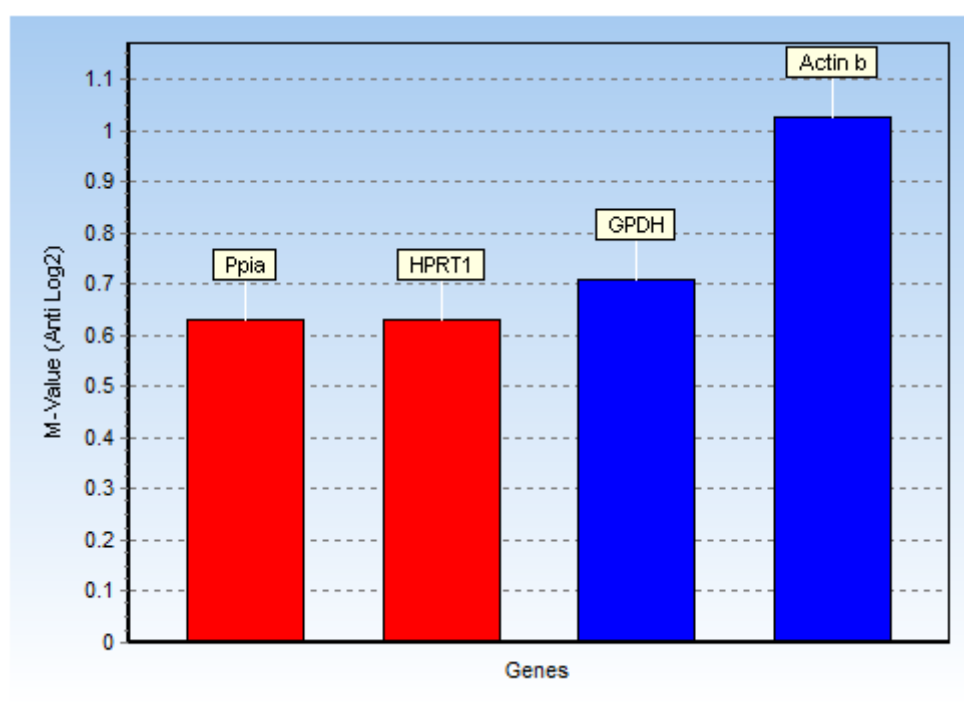
## 2. geNorm analysis:

Gene Name	M-Value
Actin $\beta$	1.02737671033742
GPDH	0.7103238761604
HPRT1	0.631837272682082
Ppia	0.631837272682082

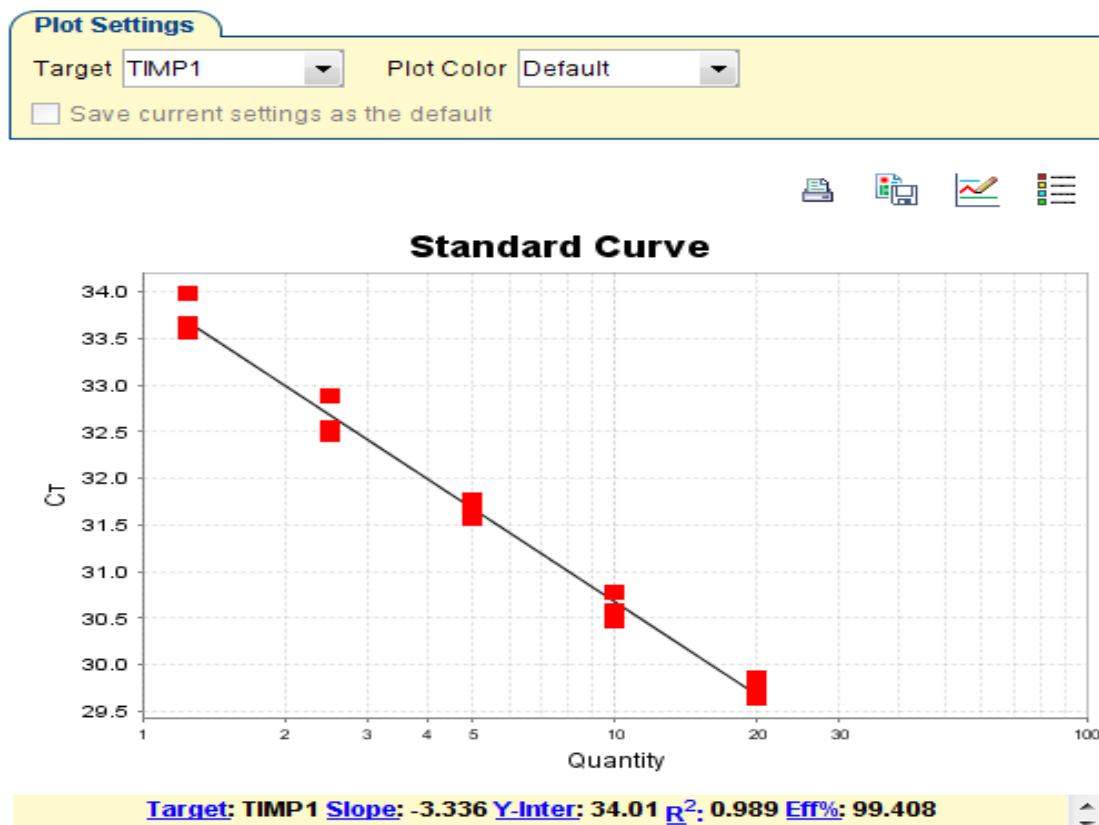
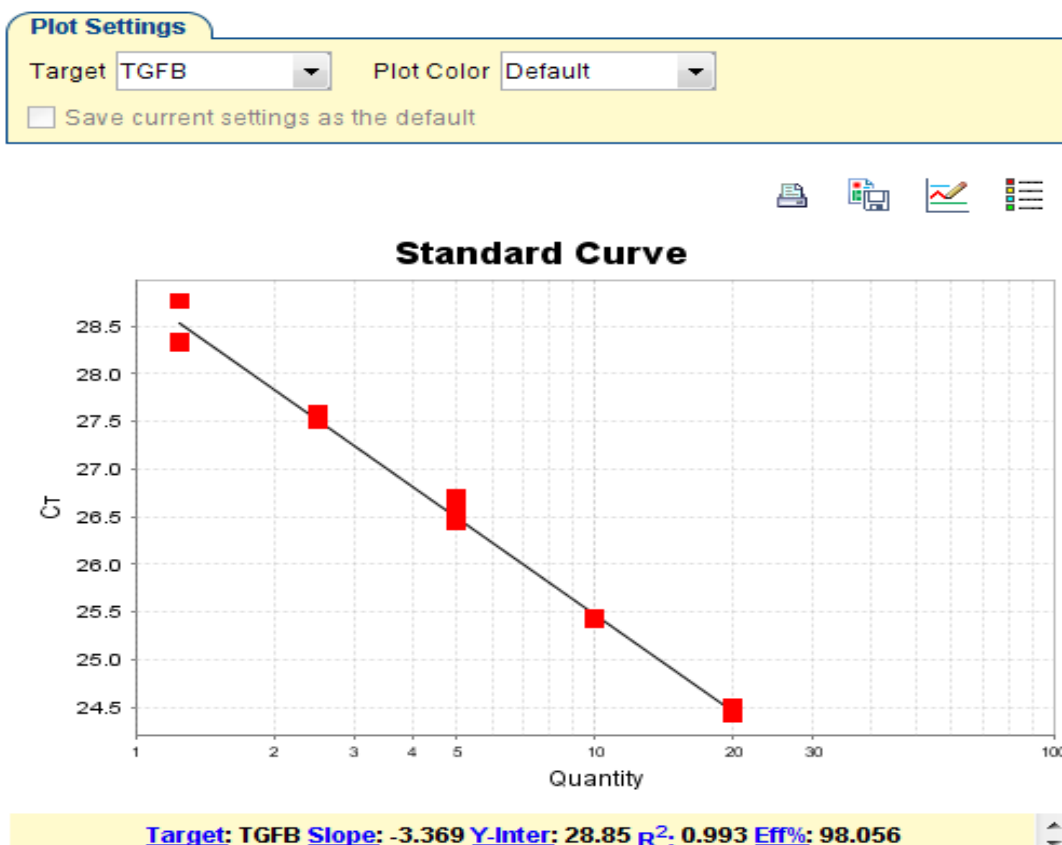
Output summary

Best Genes combination: Ppia and HPRT-1

M value: 0.6318 (stable expression)



## E. Standard Curve and efficiency for target and endogenous reference genes



### Plot Settings

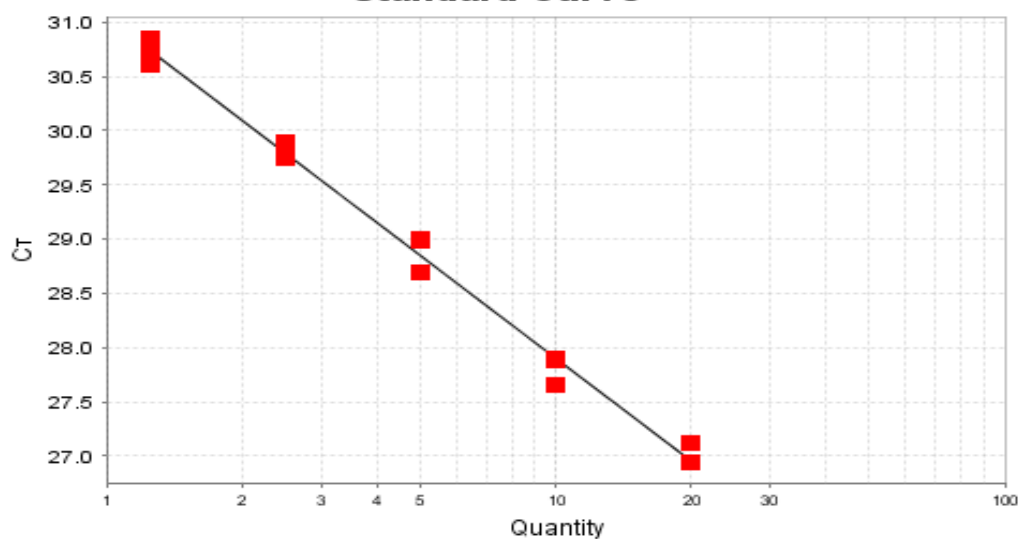
Target **MMP2**

Plot Color **Default**

☐ Save current settings as the default



### Standard Curve



**Target:** MMP2 **Slope:** -3.136 **Y-Inter:** 31.042 **R<sup>2</sup>:** 0.991 **Eff%:** 108.376

### Plot Settings

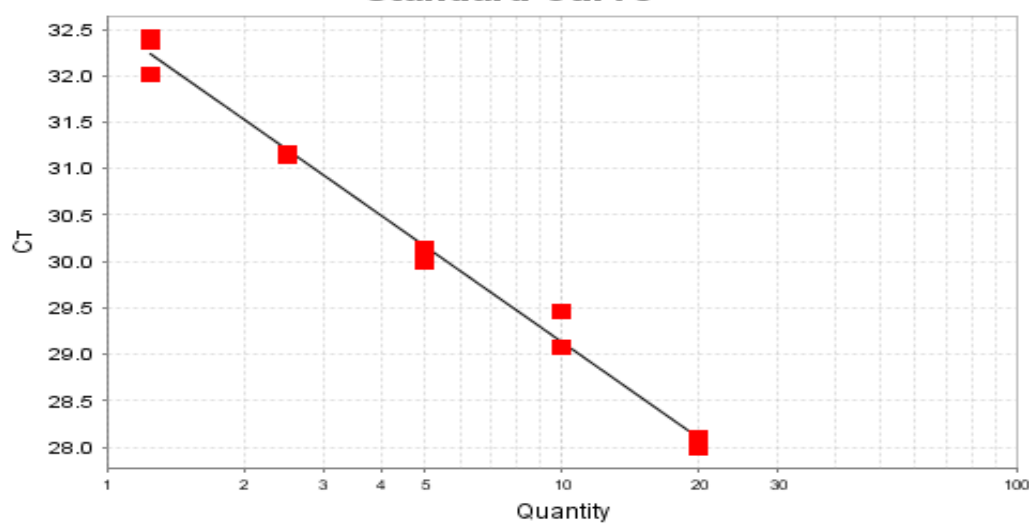
Target **COLLa**

Plot Color **Default**

☐ Save current settings as the default



### Standard Curve



**Target:** COLLa **Slope:** -3.439 **Y-Inter:** 32.567 **R<sup>2</sup>:** 0.99 **Eff%:** 95.351

### Plot Settings

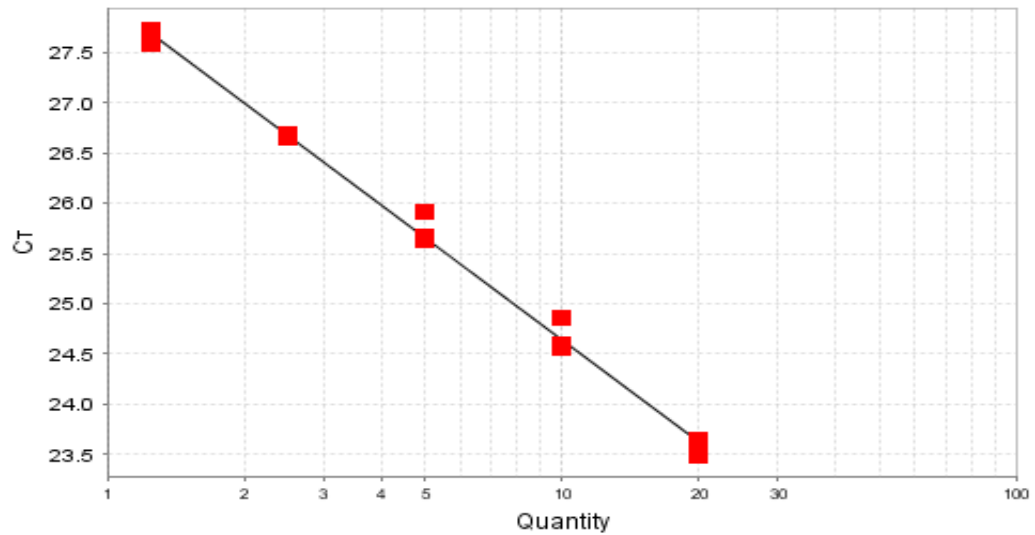
Target **HPRT1**

Plot Color **Default**

☐ Save current settings as the default



### Standard Curve



**Target:** HPRT1 **Slope:** -3.376 **Y-Inter:** 28.026 **R<sup>2</sup>:** 0.995 **Eff%:** 97.78

### Plot Settings

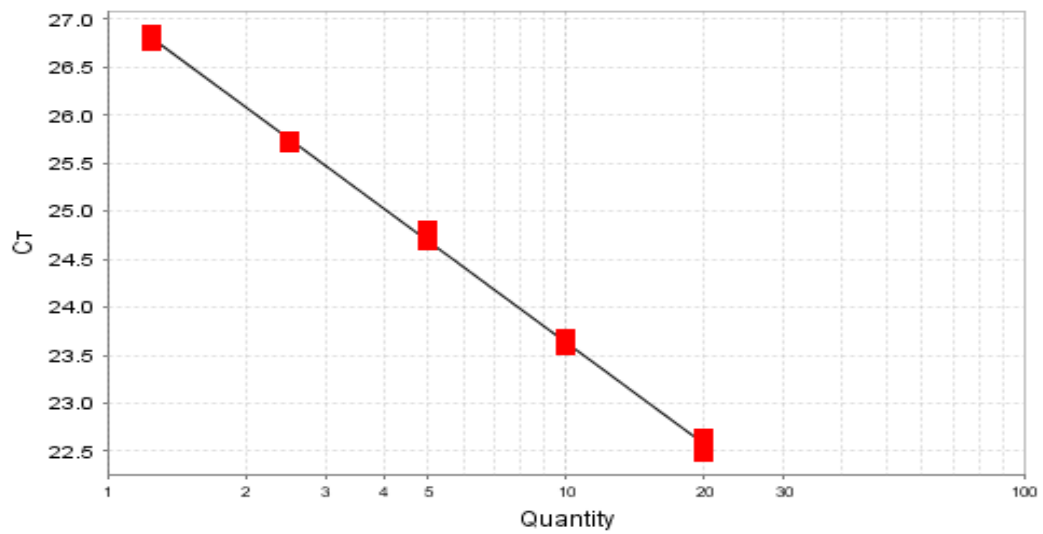
Target **PPIA**

Plot Color **Default**

☐ Save current settings as the default



### Standard Curve



**Target:** PPIA **Slope:** -3.511 **Y-Inter:** 27.154 **R<sup>2</sup>:** 0.998 **Eff%:** 92.679

## **APENDIX V List of grants, conferences and publication**

- This work was supported by two research Grants from University of Malaya, Malaysia (PS182/2009C) and (PV047-2011B).
- Mohammed A. Alshawsh, Mahmood Ameen Abdulla, Salmah Ismail , and Zahra A. Amin, “Hepatoprotective Effects of *Orthosiphon stamineus* Extract on Thioacetamide-Induced Liver Cirrhosis in Rats,” *Evidence-Based Complementary and Alternative Medicine*, vol. 2011, Article ID 103039, 6 pages, 2011. doi:10.1155/2011/103039. (ISI Cited Journal, IF = 4.774, Q1 Journal).
- Alshawsh M.A., Abdulla M.A., Ismail S., Amin Z.A., Qader S.W., Hadi H.A., Harmal N.S. Free Radical Scavenging, Antimicrobial and Immunomodulatory Activities of *Orthosiphon stamineus*. *Molecules*. 2012; 17(5):5385-5395. (ISI Cited Journal, IF = 2.386, Q 2 Journal)

## **Manuscripts under review**

- Hepatoprotective Effects of *Morinda citrifolia* Extract on Thioacetamide - Induced Liver Cirrhosis in Rats
- *In vitro* and *in vivo* antioxidant properties of *Orthosiphon stamineus* and *Morinda citrifolia* Extracts.

## **List of conferences and medals**

- Poster presentation in the Malaysia Technology Expo2010 (Malaysian Association of Research Scientists) held from 4<sup>th</sup> - 6<sup>th</sup> Feb 2010, PWTC, Kuala Lumpur, Malaysia.

- Poster presentation in the 21<sup>st</sup> International Invention, Innovation & Technology Exhibition ITEX 2010 held from 14<sup>th</sup> - 16<sup>th</sup> May 2010 Kuala Lumpur, Malaysia.
- Poster presentation in the BioMalaysia 2010 Conference & Exhibition held from 1<sup>st</sup> – 3<sup>rd</sup> Nov 2010, at Convention Centre, Kuala Lumpur, Malaysia.
- Poster presentation in the 15<sup>th</sup> Biological Sciences Graduate Congress 2010 held from 15<sup>th</sup> – 17<sup>th</sup> Dec 2010 at Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.
- Oral presentation in the 3<sup>rd</sup> International Conference on Natural Products for Health and Beauty (NATPRO-3) held from 16<sup>th</sup> – 18<sup>th</sup> Mar 2011, Bangkok, Thailand.
- Oral presentation in the International Conference on Natural Products 2011 for Metabolomics: A new frontier in Natural products Science held from 14<sup>th</sup> – 16<sup>th</sup> Nov 2011, Palm Garden Hotel, IOI Resort, Putrajaya, Malaysia.
- Oral presentation in the 16<sup>th</sup> Biological Sciences Graduate Congress 2011 held from 12<sup>th</sup> – 14<sup>th</sup> Dec 2011 at Department of Biological Sciences, Faculty of Science, National University of Singapore, Singapore.
- Oral presentation in the 4<sup>th</sup> International Conference on Natural Products for Health and Beauty (NATPRO 4) held from 28<sup>th</sup> – 30<sup>th</sup> Nov 2012, Chiang Mai, Thailand.

- **Silver Medal**, INVENTION & INNOVATION Award, 21<sup>st</sup> International Invention, Innovation & Technology Exhibition ITEX 2010 held from 14<sup>th</sup> - 16<sup>th</sup> May 2010 Kuala Lumpur, Malaysia.
- **Bronze Medal**, BioInno Awards BioMalaysia 2010 Conference & Exhibition held from 1<sup>st</sup> – 3<sup>rd</sup> Nov 2010 at Convention Centre, Kuala Lumpur, Malaysia.

