INVESTIGATION OF LIPID-LOWERING MECHANISM OF *TAMARINDUS INDICA* FRUIT PULP IN HEPG2 CELLS USING PROTEOMIC AND TRANSCRIPTOMIC APPROACHES

URSULA CHONG RHO WAN

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ABSTRACT

Tamarindus indica (*T. indica*), or tamarind, is an edible fruit widely used in many applications including culinary, industrial, and medicinal purposes. It has been shown to exhibit hypolipidaemic effects in hamsters and humans. Previous studies have also shown that the methanol extract of *T. indica* fruit pulp regulated genes related to lipid metabolism. However, the lipid-lowering mechanism of the fruit has not been fully understood. The objective of this present study is to determine the lipid-lowering mechanism of *T. indica* fruit pulp using proteomic and transcriptomic approaches. Proteomic analyses were first performed to formulate a hypothesis on the hypolipidaemic action of the fruit. When secreted proteins extracted from control and *T. indica* fruit-treated HepG2 cells were subjected to 2-dimensional gel electrophoresis, the expression of seven proteins was found to be significantly different ($p < 0.03125$). As for the HepG2 cell lysate proteins, 20 spots were found to be significantly altered ($p < 0.05$). Fourteen spots were identified and categorised based on their biological functions, namely the oxidative phosphorylation, metabolism, protein biosynthesis, cell proliferation and differentiation and mRNA splicing. When the altered secreted proteins and cell lysate proteins were co-analysed using Ingenuity Pathway Analysis (IPA) software, lipid metabolism was found to be the top network being regulated, with a score of 31. Further data mining of the proteomic data as well as previously obtained microarray data indicated that the fruit pulp extract modulates its lipid-lowering effect through the activation of PPARα. To further demonstrate the hypolipidaemic effect of the fruit, lipid studies were conducted. DNA microarray analyses were also conducted to elucidate its mechanism of action, and fenofibrate, a hypolipidaemic drug which is a ligand to PPARα, was used as a comparison to *T. indica* fruit which was hypothesised to lower lipids in a similar mode of action. HepG2 cells were first treated with 0.3 mM

palmitic acid to induce hepatic steatosis. The lipid loaded-cells were then treated with different concentrations of *T. indica* fruit pulp extract and the total triglyceride and cholesterol levels were measured. Total cellular RNA was then extracted for DNA microarray analysis and the significantly regulated genes were subjected to IPA software analysis. Results showed that treatment with 0.1 mg/ml *T. indica* fruit pulp extract reduced total triglyceride and total cholesterol by 40 % and 18 % respectively, a level comparable to fenofibrate. DNA microarray analyses showed that treatment of lipid loaded-HepG2 cells with the same concentration of *T. indica* fruit extract regulated 140 genes ($p < 0.05$) when compared to control. Further data mining using IPA analysis showed that 21 genes were involved in lipid metabolism network and PPARα and PPARγ activation could be responsible for the lipid-lowering effects, possibly attributed to proanthocyanidins, the major polyphenol found in *T. indica* fruit extract. As a conclusion, the methanol extract of *T. indica* fruit pulp lowers lipid levels significantly, particularly triglyceride and it does so through the activation of PPARα, a mechanism similar to fenofibrate.

ABSTRAK

Buah *Tamarindus indica* (*T. indica*), atau asam jawa boleh dimakan serta digunakan secara meluas dalam pelbagai aplikasi termasuk masakan, perindustrian, dan untuk tujuan perubatan. Ia telah terbukti mempamerkan kesan hipolipidemik dalam hamster dan manusia. Kajian kami sebelum ini juga telah menunjukkan bahawa ekstrak metanol buah *T. indica* mengawal atur gen yang berkaitan dengan metabolisme lipid. Walau bagaimanapun, mekanisme tindakan hipolipodemik buah *T. indica* masih belum difahami sepenuhnya. Objektif kajian ini adalah untuk menjelaskan mekanisme perendahan lipid oleh buah *T. indica* dengan menggunakan pendekatan proteomik dan transkriptomik. Analisa proteomik dijalankan terlebih dahulu untuk merangka hipotesis mengenai tindakan hipolipidemik buah asam jawa. Apabila dianalisa melalui kaedah elektroforesis 2-dimensi, terdapat perubahan signifikan dikesan bagi ekspresi tujuh tompokan protein ($p < 0.03125$) dalam ekstrak protein dari rembesan sel-sel HepG2 yang dirawat dengan buah *T. indica* berbanding dengan sel-sel kawalan yang tidak menerima rawatan. Bagi protein *lysate* sel HepG2 pula, perubahan ekspresi 20 tompokan protein pada gel didapati signifikan ($p < 0.05$). Empat belas tompokan protein telah dikenal pasti dan dikategori berdasarkan fungsi biologi, iaitu pemfosforilan oksidatif, metabolisme, biosintesis protein, percambahan sel dan pembezaan dan pemotongan mRNA. Apabila rembesan protein dan protein *lysate* sel dianalisasi dengan menggunakan perisian Ingenuity Pathway Analysis (IPA), rangkaian utama yang dikawal atur adalah metabolisme lipid, dengan skor 31. Maklumat lanjut analisis perlombongan data proteomik serta data mikroarray kami yang terdahulu menunjukkan bahawa ekstrak buah asam jawa merendahkan lipid melalui pengaktifan PPARα. Untuk membuktikan kesan hipolipidemik buah tersebut, kajian lipid telah dijalankan untuk mengukur kesan perendahan lipid. Analisis mikroarray DNA juga

telah digunakan untuk menjelaskan mekanisme tindakan, dan fenofibrate, ubat hipolipidemik yang merupakan ligan untuk PPARα telah digunakan sebagai perbandingan dengan buah *T. indica* yang dijangka merendahkan lipid melalui mekanisme tindakan yang sama. Sel-sel HepG2 terlebih dahulu dirawat dengan 0.3 mM asid palmitik untuk mengaruh steatosis pada sel hepar. Sel yang kaya-lipid, kemudian dirawat dengan ekstrak buah *T. indica* pada kepekatan berbeza dan jumlah trigliserida dan paras kolesterol telah diukur. tcRNA sel kemudiannya diekstrak untuk analisis mikroarray DNA dan gen yang dikawal atur dengan ketara telah dianalisa menggunakan perisian IPA. Hasil kajian menunjukkan bahawa rawatan dengan 0.1 mg/ml ekstrak buah *T. indica* mengurangkan paras trigliserida dan kolesterol masing-masing sebanyak 40 % dan 18 %, setanding dengan 0.1 mM fenofibrate. Analisis mikroarray DNA menunjukkan bahawa rawatan sel HepG2 yang kaya-lipid dengan ekstrak buah *T. indica* pada kepekatan 0.1 mg/ml mengubah ekspresi 140 gen ($p < 0.05$) berbanding dengan kawalan. Perlombongan data menggunakan perisian IPA menunjukkan bahawa 21 gen terlibat dalam rangkaian metabolisme lipid, dan pengaktifan PPARα dan PPARγ bertanggungjawab untuk kesan perendahan lipid, mungkin disebabkan oleh proantosianidin, polifenol utama yang terdapat dalam ekstrak buah *T. indica*. Kesimpulannya, ekstrak metanol buah *T. indica* merendahkan lipid dengan ketara, terutamanya trigliserida dan ia merendahkan lipid melalui pengaktifan PPARα, mekanisme yang sama dengan fenofibrate.

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LIST OF SYMBOLS AND ABBREVIATIONS

℃	Degree in Celcius
µA	microampere
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometer
2D	Two-dimensional
2D-GE	Two-dimensional gel electrophoresis
3D	Three dimensional
ABCA1	ATP binding cassette transporter, subfamily A, member 1
ABCG5	ATP-binding cassette, subfamily G (WHITE), member 5
ABCG8	ATP-binding cassette, subfamily G (WHITE), member 8
ACN	Acetonitrile
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
apoA-I	Apolipoprotein A-I
apoA-IV	Apolipoprotein A-IV
apoA-V	Apolipoprotein A-V
BCA	Bicinchoninic acid
bp	Base pair
BSA	Bovine serum albumin
CDC	Centre of Disease Control
cDNA	Complementary DNA
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
cm	centimeter
CS	Complement system
C_T	Threshold cycle
Da	Dalton
dL	decilitre
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ELISA	Enzyme-linked immunosorbent assay
ENO1	Alpha enolase
ER	Endoplasmic reticulum
FA	Fatty acid
FA	Formic acid
FAS	Fatty acid synthase

FBS	Foetal bovine serum
FDA	Food and Drug Administration
FDR	False discovery rate
FF	Fenofibrate
fg	femtogram
g	gram
x *g*	Gravity force
GAE	Gallic acid equivalents
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Guanine and cytosine
GDI-2	Rab GDP dissociation inhibitor beta
h	hour
HCl	Hydrochloric acid
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HNF4	Hepatocyte nuclear factor 4
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
IL-1β	Interleukin-1 beta
IPA	Ingenuity Pathways Analysis
IPG	Immobilised pH gradient
JNK	Janus kinase
kDa	Kilodalton
kg	Kilogram
kV	Kilovolt
L	Litre
LDL	Low density lipoprotein
LXR	Liver X receptor
M	Molar
mA	milliampere
MALDI-TOF MS	Matrix-assisted laser desorption ionisation time of flight mass spectrometry
MAPK	Mitogen-activated protein kinase
mg	milligram
min	minutes
ml	millilitre
mM	millimolar
mmol	millimole
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MTTP	Microsomal triglyceride transfer protein
NaCl	Sodium chloride

NAFLD	Non-alcoholic fatty liver disease
NaOH	Sodium hydroxide
NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 10
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1
ng	nanogram
NH_4HCO_3	Ammonium bicarbonate
NL	Non-linear
nm	Nanometre
OD	Optical density
PA	Palmitic acid
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCA	Principle component analysis
PCR	Polymerase chain reaction
PCYT2	Ethanolamine-phosphate cytidylyltransferase
PE	Phosphatidylethanolamine
PGC-1α	PPARγ coactivator-1α
pI	Isoelectric points
PM	Perfect-match
PPAR	Peroxisome proliferator-activated receptor
PPARA	Locus encoding for PPARα
PPARG	Locus encoding for PPARγ
PPARGC1A/PGC1A	Locus encoding for PPARG coactivator 1 alpha
PPO	2,4-diphenyloxazole
PTM	Post translational modification
PVDF	Polyvinylidene fluoride
PXR	Nuclear receptor subfamily 1, group I, member 2
qRT-PCR	Quantitative real time-polymerase chain reaction
RE	Rutin equivalents
RMA	Robust Multi-array Average
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RXR	Retinoid X receptor
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
TCM	Traditional Chinese Medicine
tcRNA	Total cellular RNA
TEMED	N,N,N’,N’-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TI	*Tamarindus indica*
TNFα	Tumour necrosis factor α
TTR	Transthyretin

TZD	Thiazolidinediones
U.S.	United States of America
UPR	Unfolded Protein Response
UQCRC2	Ubiquinol-cytochrome-c reductase complex core protein 2
V	Volt
v/v	Volume over volume
w/v	Weight over volume
WHO	World Health Organisation

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CHAPTER 1

INTRODUCTION

Lipids are essential building blocks in many biosynthetic pathways. For example cholesterol is an important component of the cell membrane and is a precursor for steroid hormones and bile acids. Triglyceride, an ester derived from glycerol and three fatty acids, functions mainly as energy storage. However, excessive lipid in the blood, a condition known as hyperlipidaemia, could lead to diseases such as coronary artery diseases. Individuals with high total cholesterol have approximately twice the risk of heart disease than those with optimal levels. In fact, ischemic heart disease was the leading cause of death in Malaysia in year 2008 (Malaysia Department of Statistics, 2010) and also the top cause of death in the world in 2012 (WHO, 2012). Other than changing to a healthier lifestyle and diet, the current medication for hyperlipidaemia is mainly through prescription of lipid-lowering drugs like statins and fibrates. However these drugs may cause adverse effects at higher doses and can be costly depending on the types and brands of drugs used. In view of this matter, alternative medicinal research has come into the limelight in the hope of searching for a cheaper and more effective alternative to, or to be used in combination with the existing medications.

Since the ancient time, many natural products were used as medicines and some were well documented for its medicinal properties. Traditional medicines like the Ayurveda from India or the Traditional Chinese Medicine (TCM) are a few traditional medicinal practices that are still being practised until today. While the scientific evidence for the use of many of these medicinal plants still remain to be proven, some have indeed been shown to possess potent medicinal properties. In fact, several prescription drugs are extracted or derived from these plants. For example vinblastine

and vincristine extracted from the flowering plant, *Catharanthus roseus*, are potent anti-cancer drugs used in the treatment of leukaemia and Hodgkin's lymphoma (Moudi, Go, Yien, & Nazre, 2013). Lovastatin, a cholesterol-lowering drug, was initially derived from fungi. It can also be found naturally in red yeast rice (J. Ma et al., 2000) and oyster mushrooms (Bobek, Ozdin, & Galbavy, 1998). This reflects the endless possibilities that can be discovered through the research on natural products.

Tamarindus indica or tamarind is a tropical fruit tree native to the African savannahs but it can now be found in many tropical countries. The sweet and sour taste of its fruit pulp is used to add flavour to local cuisines. Besides culinary, tamarind is also used in traditional medicine as laxative, diuretic, anti-bacterial agents as well as in treatment of fever and malarial infections (Bhadoriya, Ganeshpurkar, Narwaria, Rai, & Jain, 2011; Havinga, et al., 2010). Previous biochemical analyses have demonstrated that extracts of *T. indica* fruit pulp possess high antioxidant activities (Lim, Mat Junit, Abdulla, & Abdul Aziz, 2013; Martinello et al., 2006; Sudjaroen et al., 2005). In addition, *T. indica* extracts have also been shown to reduce the levels of blood cholesterol and triacylglycerol in hypercholesterolaemic hamsters (Lim, et al., 2013; Martinello, et al., 2006), in obese rats (Azman et al., 2012; Jindal, Dhingra, Sharma, Parle, & Harna, 2011), and in humans (Iftekhar, Rayhan, Quadir, Akhteruzzaman, & Hasnat, 2006). However, the mechanisms of action at the molecular levels have yet to be deciphered.

Analysis of the methanol extract of the tamarind fruit pulp by HPLC revealed the presence of (+)-catechin, (–)-epicatechin, procyanidins, naringenin, apigenin, luteolin, taxifolin and eriodictyol (Sudjaroen, et al., 2005). The jasmine green tea epicatechin has been shown to reduce the levels of triacylglycerol and cholesterol in the

sera of hamsters fed with a high-fat diet (Chan et al., 1999). The observed hypolipidaemic effects of epicatechin were postulated to involve inhibition of the absorption of dietary fat and/or cholesterol or through the reabsorption of bile acids since it did not inhibit liver HMG-CoA reductase (Chan, et al., 1999). It was also shown that tea catechins like epigallocatechin gallate (EGCG) and epigallocatechin (EGC) were able to activate PPARα (K. Lee, 2004), a nuclear receptor that promotes fatty acid oxidation and is also a target for the hypolipidaemic fibrates. Naringenin from grapefruit was shown to regulate lipid metabolism through partial activation of PPARα (Goldwasser et al., 2010). Another study showed that the procyanidin B1 flavangenol extracted from pine bark was able to enhance fatty acid oxidation (Shimada et al., 2012). This suggests that *T. indica* fruit extract may have exerted its hypolipidaemic effect through regulating nuclear receptors such as peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR). Earlier studies have also shown that proanthocyanidins, which constitutes more than 73 % of the total phenolic content of *T. indica* extract (Sudjaroen, et al., 2005), were able to modulate the activation of LXR/RXR (Jiao, Zhang, Yu, Huang, & Chen, 2010).

More recently, we have shown that the methanol extract of *T. indica* fruit pulps significantly up-regulated the expression of a total of 590 genes and down-regulated the expression of 656 genes in HepG2 cells (Razali, Aziz, & Junit, 2010). Amongst the genes that were altered in expression were those that encode proteins associated with lipoprotein metabolism, including ApoA-I, ApoA-IV, ApoA-V and ABCG5 but not the HMG-CoA reductase. Both ApoA-I and ABCG5 are involved in the reverse cholesterol transport, where the latter, together with ABCG8, are involved in the hepatobiliary cholesterol secretion.

1.1 Objectives

The main objective of this study is to further investigate the mechanism of action of the lipid-lowering effect of *T. indica* fruit pulp extract. Previous microarray study by Razali et al. (2010) revealed that *T. indica* fruit significantly regulated genes involved in lipid metabolism and antioxidant activities; however the molecular mechanism has yet to be deciphered. Thus this study was designed as a continuation from the previous study.

Therefore, the objectives of this study are to

1) hypothesise a lipid-lowering mechanism of *T. indica* fruit by performing proteomic analyses on cell lysate and secreted proteins of HepG2 cells treated with *T. indica* fruit extract.
2) evaluate the lipid-lowering effect of *T. indica* fruit extract on steatotic HepG2 cells by comparing to a commercial lipid-lowering drug.
3) verify the lipid-lowering hypothesis by examining the global gene expression of *T. indica*-treated steatotic HepG2 cells.

The integration of the proteomic and transcriptomic approaches may help to elucidate and provide a better insight into the relevant molecular pathways associated with the multi-functional effects of *T. indica* fruit pulp.

CHAPTER 2

LITERATURE REVIEW

2.1 Significance of lipid-lowering studies

Hyperlipidaemia is characterised by elevated levels of any or all lipids in the blood. Hyperlipidaemia is generally classified into 2 subtypes, primary or secondary. The primary or familial hyperlipidaemia is usually caused by genetic abnormalities while the secondary or acquired hyperlipidaemia is normally a condition caused by underlying disorders like diabetes that leads to altered plasma lipid or lipoprotein metabolism (Chait & Brunzell, 1990). Hyperlipidaemia is also characterised based on the type of lipid that is elevated in the blood, i.e. hypercholesterolaemia, hypertriglyceridaemia or combined hyperlipidaemia.

Hyperlipidaemia, particularly hypercholesterolaemia is associated with diseases like cardiovascular diseases, which is the leading cause of death in the world (WHO, 2012). In America, 71 million adults (33.5 %) have high LDL cholesterol and only 1 out of every 3 adults with high LDL cholesterol has the condition under control while less than half of adults with high LDL cholesterol got treatment (CDC, 2011). Individuals with high total cholesterol have approximately twice the risk of heart disease than those with optimal levels. The average total cholesterol level for adult Americans is about 200 mg/dL, which is borderline high risk (Roger et al., 2012) while in Malaysia, the number of individuals suffering from high cholesterol rose from 20.7 % in 2006 to 35.1 % in 2011 (NHMS, 2011). This could be attributed to the sedentary lifestyles and consumption of food high in fat content.

The liver is the major site for fatty acid oxidation in mammals. Decreased turnover of hepatic lipid droplets can lead to the development of fatty liver disease in man (Greenberg et al., 2011). Recently, the rapid rise in the prevalence of obesity and diabetes in the general population has contributed to a parallel increase in "non-alcoholic fatty liver disease" (NAFLD) in many parts of the world. It is currently estimated that up to 46 % of the adult U.S. population may have hepatosteatosis (C. D. Williams et al., 2011). Presently, there are no effective drug therapies for NAFLD, now considered a risk factor for Type II diabetes (Anstee, Targher, & Day, 2013).

2.2 Lipid-lowering mechanisms

2.2.1 Commercial lipid-lowering drugs

Other than changing to a healthier lifestyle such as exercising regularly and reducing fat intake in food, drugs are also prescribed to aid in the lipid-lowering process. Generally, the lipid-lowering drugs exert their effects by reducing cholesterol biosynthesis, increasing fatty acid oxidation, increasing lipid clearance or inhibiting lipid uptake from food. The most common lipid-lowering drugs are classified mainly into 2 groups, statins and fibrates.

Statins are a class of lipid-lowering drug that inhibits the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key rate-limiting enzyme involved in the cholesterol biosynthetic pathway. As structural analogues of HMG-CoA, statins inhibit HMG-CoA reductase competitively with an affinity of about 1,000-10,000 times greater than its natural substrate. The first generation of statins, mevastatin and lovastatin, were first discovered in fungi. The second-generation and third-generation statins such as simvastatin, pravastatin, fluvastatin, atorvastatin, rosuvastatin and pitavastatin were either modification from the first-generation statins or chemically

synthesised in the laboratory (Steinmetz, 2002). Besides inhibiting the cholesterol biosynthetic pathway, statins were also reported to lower plasma cholesterol indirectly through the up-regulation of LDL receptor (Vaziri & Liang, 2004).

While statins are mainly used to lower plasma cholesterol level, fibrates are the primary drugs used to treat hypertriglyceridaemia. Fibrates are peroxisome proliferator-activated receptor (PPAR) agonists. PPARs are a group of nuclear receptors that govern many metabolic processes such as lipid metabolism, glucose regulation and energy production. There are three isoforms of PPARs, PPARα, PPARγ and PPARβ/δ. Fibrates exert their lipid-lowering effects by enhancing beta oxidation of fatty acids. The first fibrate, clofibrate was discovered in Japan (Ozawa & Ozawa, 2002), and subsequently other fibrate derivatives such as bezafibrate, ciprofibrate, fenofibrate, and gemfibrozil were developed. Different fibrates act on different PPAR isoforms, for example fenofibrate and clofibrate have a 10-fold selectivity towards PPARα than PPARγ (Willson & Wahli, 1997). On the other hand, bezafibrate is a pan-agonist which activates all three PPARs with similar potency. However, only fenofibrate and gemfibrozil are used in treating humans as their effects were milder as compared to other fibrates, which were reported to cause hepatomegaly and tumour formation in the liver of rodents after prolonged usage (Gray, Beamand, Lake, Foster, & Gangolli, 1982; Lazarow, Shio, & Leroy-Houyet, 1982; Leighton, Coloma, & Koenig, 1975; M. S. Rao, Subbarao, & Reddy, 1986; Reddy & Krishnakantha, 1975).

Besides these two main lipid-lowering drugs, other lipid-lowering drugs are used either stand-alone or with statin/fibrate to treat hyperlipidaemia. Ezetimibe is used to lower cholesterol by inhibiting intestinal cholesterol absorption (Vasudevan & Jones, 2005). Bile acid sequestrants like cholestyramine, colestipol, and colesevelam are also

prescribed to lower plasma cholesterol level. These bile acid sequestrants increase the rate of bile acid excretion and therefore promote cholesterol conversion to bile acid (Steinmetz, 2002). Apart from these, microsomal triglyceride transfer protein (MTTP) inhibitor like lomitapide was also used to treat familial hypercholesterolaemia (Perry, 2013; Raal, 2013). Niacin and its derivatives which are commonly used as supplements are also prescribed to lower lipid by modifying lipoproteins (Vasudevan & Jones, 2005).

2.2.2 Alternative medicine to treat hyperlipidaemia

As mentioned earlier, the mainstay to treat hyperlipidaemia is mainly through weight reduction, dietary changes, exercise and lipid-lowering drugs. However, these oral medications may inevitably lead to adverse effects. High doses of statins have been reported to cause myositis and myalgia, possibly caused by dose-dependent reduction of coenzyme Q10 (Golomb & Evans, 2008). Fibrates are associated with a slightly increased risk (<1.0 %) for myopathy, cholelithiasis, and venous thrombosis (Davidson, Armani, McKenney, & Jacobson, 2007). In view of this matter, medicinal plants have garnered much attention due to their generally milder, if any, adverse effects that may result from the consumption. Epidemiological studies had also shown that high intake of fruits and vegetables coupled with low consumption of trans-fat, cholesterol, saturated fat and salt reduced the risk of CVD (Bazzano et al., 2002; Bendinelli et al., 2011; Joshipura et al., 2001). This finding is also supported by the number of deaths worldwide associated with low fruit and vegetable consumption (WHO, 2009).

2.2.2.1 Flavonoids

Flavonoids are a family of phenolic compounds with strong bioactivities that are present in fruits, vegetables, and herbs. More than 5000 distinct flavonoids have been identified in plants, and several hundreds are known to occur in commonly consumed fruits, vegetables, grains, herbal products, and beverages. Structurally, flavonoids have a common basic chemical structure that consists of 2 aromatic rings linked by a 3-carbon chain that forms an oxygenated heterocyclic ring. Differences in the generic structure of the heterocyclic ring, as well as the oxidation state and functional groups of the ring, classify flavonoids as flavonols, flavan-3-ols (flavans), flavanones, flavones and isoflavones (Erdman et al., 2007).

Flavonols are the most widespread flavonoids in foods, and the most prominent flavonols in food are quercetin and kaempferol (Erdman, et al., 2007). Red wine and tea can also contain a significant amount of flavonols. Flavan-3-ols are present in many fruits such as grape products, teas, cocoa, and chocolate. They are found either as monomers (epicatechin and catechin) or oligomers (e.g., proanthocyanidins). Catechin and epicatechin are the main flavan-3-ols in fruits and cocoa. Flavanones are present in high concentrations in citrus fruits. The main aglycones in citrus are naringenin, hesperetin, and eriodictyol. In contrast, flavones are less common than flavonols in fruits and vegetables. The major flavones in food are luteolin and apigenin. Parsley and celery are the primary food sources. Soy and soybean-derived products are the main sources of isoflavones, which are structurally analogous to oestrogens. The 3 common soybean isoflavones are genistein, daidzein, and glycitein.

Flavonoids are excellent antioxidants (Rice-Evans, 2001), and because of their antioxidant activities as well as their abundance in fruit and vegetables, they may partly

contribute to the health benefits of plant foods (Arts & Hollman, 2005). Many flavonoids are reported to exhibit hypolipidaemic properties. Green tea, which is rich in catechin and epigallocatechin gallate, has been shown to lower triglyceride and cholesterol in humans and rats (Bursill & Roach, 2006; Nagao et al., 2005; Richard et al., 2009; Unno et al., 2005). Naringenin-rich-ethyl acetate fraction of fenugreek seeds was also reported to possess lipid-lowering activities when fed to high-cholesterol-fed rats (Belguith-Hadriche et al., 2010). The famed resveratrol from red wine has also garnered much attention for its cardioprotective and anticarcinogenic potential (Boddicker, Whitley, Davis, Birt, & Spurlock, 2011; Guerrero, Garcia-Parrilla, Puertas, & Cantos-Villar, 2009; Pal et al., 2003; Szmitko & Verma, 2005). Proanthocyanidin-rich grape seed extract has also been reported to lower lipid (Yamakoshi, Kataoka, Koga, & Ariga, 1999).

2.2.2.2 Other plant compounds with hypolipidaemic effect

There are a few well known natural products that possess hypolipidaemic effects and red yeast rice is one of them. It has been traditionally used as food and medicine for centuries in China to lower cholesterol, improve blood circulation and help digestive problems (Monograph. *Monascus purpureus* (red yeast rice), 2004; J. Wang et al., 1997). It is made by fermenting a type of yeast, *Monascus purpureus* over red rice. Several studies had shown that red yeast rice was able to lower high cholesterol (Becker & Gordon, 2011; Cicero et al., 2013; Feuerstein & Bjerke, 2012; Venero, Venero, Wortham, & Thompson, 2010). In a meta-analysis involving 9625 patients in 93 randomised trials, 3 different commercial preparation of red yeast rice produced a mean reduction in total cholesterol of 0.91 mmol/L, LDL-cholesterol of 0.73 mmol/L, triglyceride of 0.41 mmol/L and a mean rise in HDL-cholesterol of 0.15 mmol/L (J. Liu et al., 2006). The active compound that attributes to the lipid-lowering activities is

monacolin K, a compound identical to lovastatin (J. Ma, et al., 2000). As lovastatin is a prescription drug, the Food and Drug Administration (FDA) considered monacolin K as a drug and prohibited selling of red yeast rice that contains monacolin K. However since monacolin K is not listed in the label, the presence of monacolin K in red yeast rice supplements remains ambiguous (Childress, Gay, Zargar, & Ito, 2013).

Berberine is another effective lipid-lowering agent. It is an isoquinoline alkaloid that can be extracted from Goldenseal (*Hydrastis canadensis*), Oregon grape (*Berberis aquifolium*), Barberry (*Berberis vulgaris*), and Chinese Goldthread (*Coptis chinensis*). Two other berberine-containing plants that are familiar to practitioners of Chinese medicine are *Phellodendron chinense* and *Phellodendron amurense*. In 2004, a study on berberine treatment on 32 hypercholesterolaemic patients reduced serum cholesterol by 29 %, triglycerides by 35 %, and LDL-cholesterol by 25 % (Kong et al., 2004). Another study in 2009 reported that berberine was able to prevent the development of fatty liver in rats (W. S. Kim et al., 2009). This was followed by a randomised controlled trial of 60 humans with fatty liver disease. Patients given 0.5 g berberine twice per day showed improvement in their liver ultrasounds and this was accompanied with lowered serum triglyceride and cholesterol (Xie, Meng, Zhou, Shu, & Kong, 2011). The lipid-lowering activities of berberine is attributed to its ability to activate adenosine monophosphate-activated protein kinase (AMPK) (Cheng et al., 2006; Y. S. Lee et al., 2006; Q. Wang et al., 2011). Since AMPK regulates an array of biological activities that normalise lipid, glucose and energy imbalances, its activation is especially useful in treating metabolic syndromes that includes hyperglycaemia, diabetes, lipid abnormalities and energy imbalances (Srivastava et al., 2012).

2.3 *Tamarindus indica* (*T. indica*)

2.3.1 Description of *T. indica*

Tamarindus indica or *T. indica* (Figure 2.1) is a pantropical fruit tree that originates from Africa. It is a large evergreen tree (up to 24 m in height and 7m in girth) that bears fruit with ligneous pod containing sticky flesh with black, hard seeds. Its leaves have alternate, compound, with 10-18 pairs of opposite leaflets and the shape of leaflets are narrowly oblong. It bears small pale yellow or pinkish flowers. The fruit pod can be straight or curved, and is velvety and rusty-brown in colour. The shell of the pod is brittle and the seeds are embedded in a sticky edible pulp. Each pod contains 3-10 seeds, approximately 1.6 cm long, irregularly shaped, and testa hard, shiny, and smooth (Bhadoriya, et al., 2011).

Figure 2.1: (From the left) *Tamarindus indica* fruit pulp seeds, skin, flesh and leaves.

2.3.2 Taxonomical classification

T. indica is categorised as a monospecific genus in the family of Leguminosae (Bhadoriya, et al., 2011). The following is the taxonomical classification of *T. indica*.

Kingdom: Plantae

Phylum: Spermatophyte

Class: Angiosperm

Sub class: Dicotyledone

Family: Leguminosae

Subfamily: Caesalpiniaceae

Genus: *Tamarindus*

Species: *indica*

2.3.3 Chemical composition of *T. indica* fruit pulp

T. indica fruit pulp is characterised by its sweet and sour taste. Its acidity is mostly attributed to tartaric acid (2,3-dihydroxybutanedioic acid, $C_4H_6O_6$, a dihydroxydicarboxylic acid), which remains in the pulp upon ripening. However when added together with the increasing sugar levels during ripening, the fruit tasted simultaneously sweet and acidic (Lewis & Neelakantan, 1964). Other organic acids that were found in the tamarind fruit pulp are malic acid, ascorbic acid, oxalic acid, succinic acid, citric acid and quinic acid (Ishola, Agbaji, & Agbaji, 1990; Lewis & Neelakantan, 1964; Lewis, Neelakantan, & Bhatia, 1961).

The ripe fruit also contains saponins, flavonoids, tannins, invert sugar, pipecolic acid, citric acid, nicotinic acid, 1-malic acid, vitexin, isovitexin, orientin, isoorientin, vitamin B3, volatile oils (geranial, geraniol, limonene), cinnamates, serine, beta-alanine, pectin, praline, phenylalanine, leucine, potassium and lipids (Dalimartha, 2006).

Sudjaroen et al. (2005) reported that the methanol extract of tamarind fruit pulp was dominated by proanthocyanidins (73.4 %) in various forms (+)-catechin (2.0 %), procyanidin B2 (8.2 %), (-)-epicatechin (9.4 %), procyanidin trimer (11.3 %), procyanidin tetramer (22.2 %), procyanidin pentamer (11.6 %), procyanidin hexamer (12.8 %) along with taxifolin (7.4 %), apigenin (2.0 %), eriodictyol (6.9 %), luteolin (5.0 %) and naringenin (1.4 %).

2.3.4 *T. indica* applications

2.3.4.1 Food and product

T. indica fruit pulp is commonly used in culinary to add flavour for its sweet and sour taste. Besides adding the juice of the fruit pulp into cooked dishes, it is also eaten raw or made into food products. In Malaysia, it is most commonly used as a condiment in many local cuisines. Besides this, the fruit is also made into juice, jam, candy and syrup. A sweeter cultivar of the fruit produced mainly in Thailand, is usually eaten fresh.

In other parts of Asia, the immature green pods are often eaten by children and adults dipped in salt as a snack. More commonly, the acidic pulp is used as a favourite ingredient in culinary preparations such as curries, chutneys, sauces, ice cream and sherbet in countries where the tree grows naturally (Little & Wadsworth, 1964). In Sri Lanka, tamarind is widely used in cuisine as an alternative to lime and also in pickles and chutneys. It is also used in India, to make ‘tamarind fish’, a sea-food pickle, which is considered a great delicacy. The juice is also an ingredient of Worcestershire and other barbecue sauces, commonly used in European and North American countries. In the Philippines, Sri Lanka and Thailand, fibres are removed from the fruit pulp, which is mixed with sugar, wrapped in paper and sold as toffees. The pulp is also used to make sweet meats mixed with sugar called ‘tamarind balls’ (Purseglove, 1987); in Senegal,

they are called ‘bengal’. Similarly in India, the pulp is eaten raw and sweetened with sugar.

2.3.4.2 Medicinal uses

The use of plants as herbal medicines had been practiced since the ancient times. In fact, it is still being used as the main medicine in many countries especially in countries where medical treatments are not easily accessible. In other parts of the world, traditional medicine commonly serves as a complementary or alternative treatment to the available medical treatment. Traditional medicine is favoured because it is commonly believed to have minimal side effects as compared to current medical treatments. The use of plants in traditional medicine can be explained by physiologically active phytochemical compounds of a species and also by its ascribed meaning in a culture (Etkin, 1986). Medicinal plants with a long history of safe and effective use are likely to have a pharmaceutical effect (Tabuti, 2008).

T. indica fruit has been used as a traditional medicine to treat several ailments in countries where the tree is indigenous. In Africa, tamarind has been used either on its own or with other plants to treat diseases of the circulatory system, digestive system, genitourinary system, sensory system, infections or infestations, injuries, mental problems and pregnancy-related disorders (Table 2.1). In traditional Thai medicine, the fruit of the tamarind is used as a digestive aid, carminative, laxative, expectorant, and blood tonic (Farnsworth & Bunyapraphatsara, 1992).

Table 2.1: Medicinal uses of tamarind fruit in Africa summarised from a review by Havinga et al. (2010)

Disorder category	Medicinal use	Preparation	Country	Ethnicity
Unspecified	Fortifiant	Add decoction of the leaves and fruits to millet porridge and drink	Benin and Senegal	Dendi, Fulani, Gourmantché, Haussa
	Jaundice	A handful of fruits is macerated with powdered *Cassia siberiana* bark and the extract drunk	Nigeria	Hausa
Circulatory	Heart disease	Chew unripe fruit with onion and swallow to treat palpitations	Benin	Dendi, Fulani, Gourmantché, Haussa
Digestive system	Abdominal pain	Beverage	Madagascar	Veso-Sakalava Malagasy
		Fruit or root and bark soaked in water for stomach disorders	Kenya and Nigeria	Suiei Dorobo Hausa
	Laxative	Decoction of the fruit, administered orally	Togo	-
		Eaten raw or prepared as 'lavement' for constipation	Madagascar	-
		Processed into laxative beverage	Mali	-
		Infusion or decoction of the fruit	Sudan	Several tribes involved
		Drink macerate of fruits in water, for constipation	Nigeria	Fulani
		Sweetmeat called 'bengal' prepared from the fruit pulp by Wolof of Senegal. Used as laxative mixed with honey or lime juice	West Africa	-
		Mix fruit pulp with water and add sugar for taste, then drink as laxative, purgative or for constipation	Benin	Dendi, Fulani, Gourmantché, Haussa

Table 2.1, continued

Disorder category	Medicinal use	Preparation	Country	Ethnicity
		The fruits are welled with leaves of *Combretum micranthum* until the water has taken an acid taste, then drink. Also used to treat nausea.	Mali	Dogon
		Crushed and soaked for half a day in water with a little salt before administration	Cote d'Ivoire and Burkina Faso	-
		Mashed fruit pulp is mixed with water or sangl é, a beverage based on milk, and given to drink with or without salt	Senegal	-
Genitouri-nary system	Aphrodisiac	-	Cote d'Ivoire	-
Infections/ Infestations	Cold	Mix with water and add sugar for taste, then drink	Benin	Dendi, Fulani, Gourmant-ch é, Haussa
	Fever	Fruit pulp used in the treatment of fever for refreshment and to quench thirst. Often followed by rubbing the roughly dehusked pods with some vinegar on to the body of the feverish patient	Senegal	-
		Beverage	Madagascar	Veso-Sakalava Malagasy
		Drink boiled, evaporated pulp that is partly dissolved in water	All over Soudan	-
	Malaria	Infusion or decoction of the fruit	Sudan	Several tribes involved
		Mix fruit with water and add sugar for taste, then drink	Benin	Dendi, Fulani, Gourmant ch é, Haussa
	Helminth infections (parasitic worms)	Vermifuge not specified	Nigeria	-

Table 2.1, continued

Disorder category	Medicinal use	Preparation	Country	Ethnicity
	Microbial infections	Soaked fruit, oral administration to treat infectious diseases including sex transmitted diseases	Guinea	Malinké or Sousou
	Sleeping sickness	Boil leaves and give to animals to drink; grind fruits with raw beans and give animals to feed	Nigeria	Hausa, Fulani
	Leprosy	In leprosy treatment to enhance the emetico-cathartic properties of *Trichilia emetica*; A mixture of Cantharides-powder and tamarind pulp is taken by the patient before the syphilis treatment starts	Senegal	Wolof
Injuries	Wounds	Leaf and fruit decoction used as mouthwash for lesions and sores	Burkina Faso	Mossi
Mental	Sleep	Mix with water and add pepper, then drink	Benin	Dendi, Fulani, Gourmantché, Haussa
Nutritional	Scurvy	Not specified	Kenya	-
Pregnancy, birth, puerperium	Lactation	To increase lactation, eat Kunu (a kind of porridge) prepared with fruit of tamarind and *Ximenia americana* or drink a macerate of tamarind fruits in water	Nigeria	Fulani
	Pregnancy	Drink macerate of fruits in water to relieve pain upon labour	Nigeria	Fulani
Sensory system	Vertigo	Mix with water and add sugar for taste, then drink	Benin	Dendi, Fulani, Gourmantché, Haussa

2.3.5 Current studies of *T. indica*

T. indica fruit pulp has been shown to have good antioxidant activities. Sudjaroen et al. (2005) reported that the methanolic extract of *T. indica* fruit pulp has better antioxidant capacity than the seeds even though the total phenolic content of the seed is much higher than the *T. indica* fruit pulp. Lim et al. (2013) showed that the fruit had significant amount of phenolic (244.9 ± 10.1 mg GAE/extract) and flavonoid (93.9 ± 2.6 mg RE/g extract) content and possessed considerable antioxidant activities. Similarly, Martinello et al. (2006) observed that the fruit pulp extract of *T. indica* showed radical scavenging ability *in vitro*, and improved the efficiency of the antioxidant defense system *in vivo* when the tamarind extract was administered at a concentration of 5 %. Other studies were also in agreement with the considerable anti-oxidant activities of tamarind fruit extract (Khairunnuur et al., 2009; Lamien-Meda et al., 2008; Ramos et al., 2003).

Besides this, the fruit has been shown to have anti-bacterial properties. Nwodo et al. (2011) reported that the aqueous and ethanolic extracts of *T. indica* fruit pulp exhibited wide spectrum of antibiotic properties. The methanol and hexane extracts of the fruit had antibacterial effect too (Adeola, Adeola, & Dosumu, 2010). Others studies (Dabur et al., 2007; Melendez & Capriles, 2006) also supported the antibacterial activities of tamarind fruit extract. In a recent study, the fruit has also been demonstrated to reduce the secretion of aflatoxin from *Aspergillus flavus* and *A. parasiticus*, although the aqueous extract did not inhibit their growth (El-Nagerabi, Elshafie, & Elamin, 2013). Koudouvo et al. (2011) reported that the aqueous extract of tamarind fruit exhibit antiplasmodial activity.

T. indica fruit has also been shown to exhibit hypolipidaemic effects. The crude extracts from the fruits had been shown to have a lipid-lowering effect in hypercholesterolaemic hamsters (Martinello, et al., 2006), and in cafeteria diet- and sulpiride-induced obese rats (Jindal, et al., 2011). Azman et al. (2012) had reported that the aqueous extract of *T. indica* fruit pulp improved obesity-related parameters in blood, liver, and adipose tissue in a rat model and suppressed obesity induced by a high-fat diet, possibly by regulating lipid metabolism and lowering plasma leptin and fatty acid synthase (FAS) levels. A similar effect was reported by Lim et al. (2013) in which the methanol extracts of *T. indica* fruit had high content of phenolic and flavonoid compounds and possessed antioxidant activities and lowered triglyceride, cholesterol and LDL but not HDL in hyperlipidaemic hamsters. The hypolipidaemic results were in agreement with the human study where the dried and pulverised *T. indica* fruits, significantly reduced both total cholesterol and LDL-cholesterol levels in humans (Iftekhar, et al., 2006). *In vitro* study on HepG2 cells by Razali et al. (2010) showed that the methanol extract of *T. indica* fruit pulp significantly regulated thousands of genes and many of which were involved in cholesterol synthesis and lipoprotein metabolism.

Tamarind fruit was shown to ameliorate fluoride toxicity in rats (Dey, Swarup, Saxena, & Dan, 2011) and in rabbits (Ranjan, Swarup, Patra, & Chandra, 2009). It has also been shown to delay progression of fluorosis by enhancing urinary excretion of fluoride (Khandare, Rao, & Lakshmaiah, 2002). Besides this, it also exhibits anti-spasmolytic effect in rabbits (Ali & Shah, 2010) and anti-nociceptive activities in rodents (Khalid et al., 2010). Rimbau et al. (1999) had also reported anti-inflammatory effect of the fruit. Landi Librandi et al. (2007) determined the effect of *T. indica* fruit extract on the complement system (CS) *in vitro* and *in vivo*; the hydroalcoholic extract increased complement components and complement lytic activity *in vitro*, but had no

effect on the CS *in vivo*. Additional analysis and efforts to isolate the compounds of the extract that act on the CS could lead to its therapeutic use as an inflammation modulator.

2.4 Foodomics

Foodomics is a relatively recent scientific discipline that studies food and nutrition with the use of advance "omics" technologies (Herrero, Simo, Garcia-Canas, Ibanez, & Cifuentes, 2012). Traditionally, the main aim of food analysis is to ensure food safety. Analytical chemistry was, and is still the main tool being used to achieve this goal. However there is also a general trend in food science to link food and health. Therefore, food is considered not only a source of energy but also an alternative to prevent diseases. This has led to the introduction of food analysis using advanced "omics" approaches known as foodomics. Foodomics enables us to study the effect of food ingredient(s) at the genomic/transcriptomic/proteomic and/or metabolomic level, making possible new investigations at the molecular level on food bioactivity and its effect on human health. Generally, foodomics is presented as a global discipline in which food (including nutrition), advanced analytical techniques (mainly omics tools), and bioinformatics are combined (Figure 2.2). This opens up a whole new window to study food once thought to require multidisciplinary expertise to achieve, as traditional food analysis mostly involve analytical chemistry. This had also permitted us to address issues like understanding the beneficial or adverse effects of a certain bioactive food compound on cellular or molecular level using nutrigenomic approaches (Wittwer et al., 2011); determining the effect of bioactive food constituents on crucial molecular pathways (Corella et al., 2011); understanding the gene-based differences among individuals in response to a specific dietary pattern following nutrigenetic approaches

(C. M. Williams et al., 2008); establishing the global role and functions of gut microbiome (Kau, Ahern, Griffin, Goodman, & Gordon, 2011) and etc.

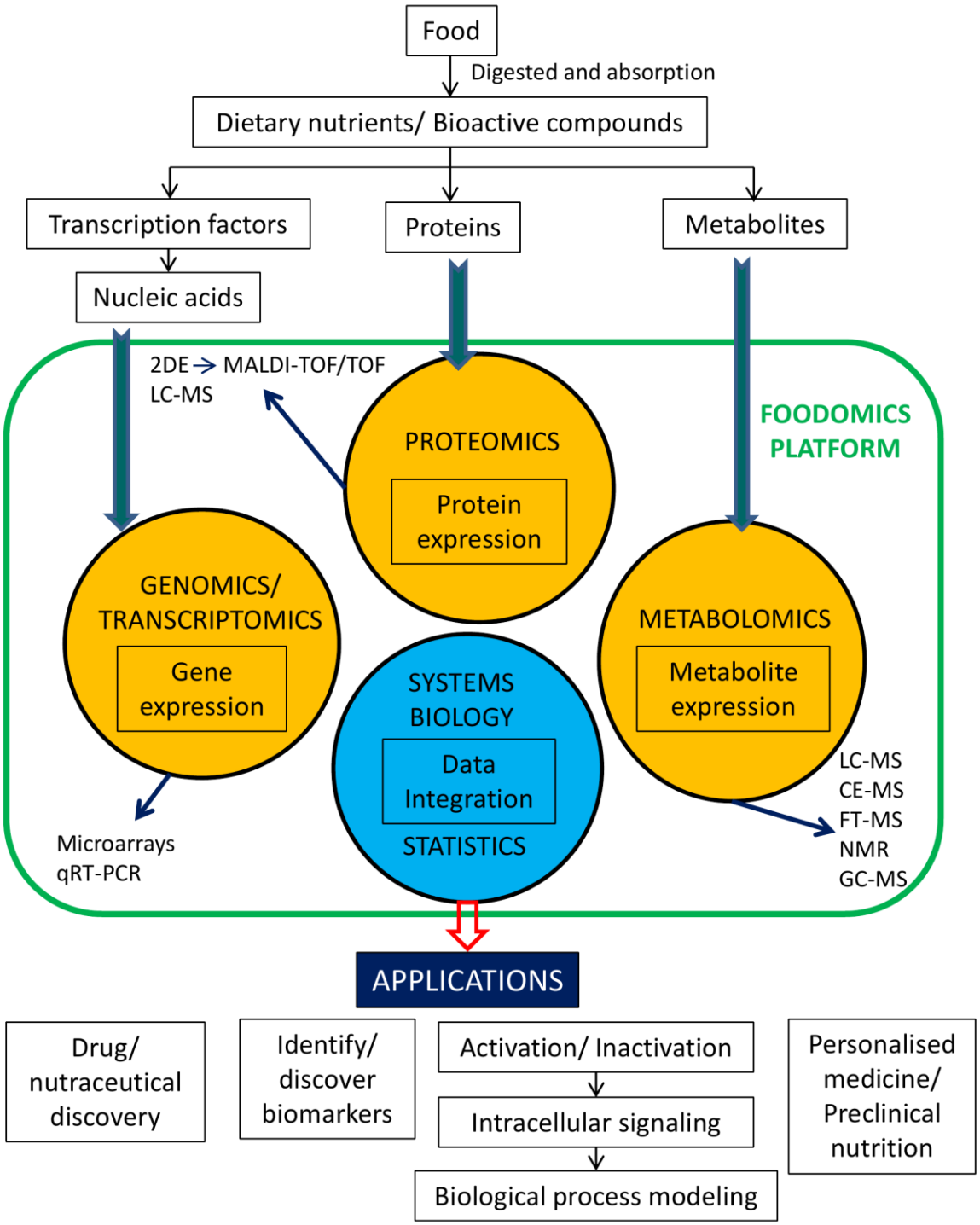

Figure 2.2: Effects of dietary nutrients on nucleic acids, proteins and metabolites, a typical representation of tools used in foodomics analyses, and the major applications of foodomics. (Adapted from Ganesh & Hettiarachchy, 2012 and Garcia-Canas, et al., 2012)

2.4.1 Proteomics

Nutriproteomics is one of the "omics" technologies used in foodomics studies. Proteomics is the study of nature of proteins and the correlation with their underlying biological processes, therefore allowing the identification of the proteins, their expressional changes, levels of production, post translational modifications (PTM), amino acid substitution and polymorphisms to be determined (Dutt & Lee, 2000; Pandey & Mann, 2000). Physiological, pathological and nutritional alterations can play a pivotal role in altering the proteome of an individual (Fuchs et al., 2005; H. Kim, Page, & Barnes, 2004; J. Wang, Li, Dangott, & Wu, 2006). A key step in proteomics analysis is to separate the protein mixtures in order to quantify or characterise it, depending on the objective of the study. This can be accomplished either as gel-based or gel-free approaches. In the gel-based approach, two-dimensional gel electrophoresis and mass spectrophotometry (MS) are used. Basically, the proteins are separated using two dimensions, i.e. to separate them based on their isoelectric points and molecular mass, in which the latter involves running an SDS-PAGE. Gel-free approach involves the direct digestion of proteins in solution and the resulting peptides are resolved using liquid chromatography coupled with mass spectrophotometer. More than one type of column is often used in the separation of peptides to give better separation, for example a strong cation exchange column and reverse-phase HPLC coupled with MS is a common setup for gel-free approach. This is also known as the multi-dimensional protein identification technology (MudPIT). Both approaches have their pros and cons. Gel-based approach allows high throughput analysis and the ability to detect isoforms and PTMs, both of which are not achievable through gel-free approach. Gel-free approach is more sensitive and needs only a small amount of sample. It also separates protein with extreme characteristics (extremes in isoelectric points, molecular weights, quantities and hydrophobicity) better (Ganesh & Hettiarachchy, 2012).

2.4.1.1 Two-dimensional gel electrophoresis (2D-GE)

2D-GE was first introduced by O'Farrell in 1975 (O'Farrell, 1975). It is a robust and common technique used to separate protein mixture from cells, tissues, or other biological samples. This technique separates proteins in two steps, the first-dimension separation, isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pI); and the second-dimension separation, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular mass. The advent of 2D-GE has revolutionised proteomic analyses, however its application had only started to gain significance with a number of developments. In the original technique introduced by O'Farrell, the first-dimension separation was carried in carrier-ampholyte-containing polyacrylamide gels cast in narrow tubes. This method was improved with the introduction of immobilised pH gradient (IPG) strips which significantly increased the resolution and reproducibility of first-dimension separation. The improvement in protein identification from the protein spot has also contributed to the robustness in 2D-GE application. This includes more sensitive mass spectrometry techniques for rapid identification of small amount of peptides or proteins; more powerful, less expensive computers and software rendering thorough computerised evaluations of highly complex 2D patterns to become economically feasible; and protein sequences are being added on a daily basis to databases available on the public domain, thus increasing the possibility to identify a protein.

2.4.1.2 Protein sample preparation

A good protein sample is crucial for any downstream proteomic applications. Therefore, protein sample preparation is the key to successful proteomic analysis. Generally, a good protein sample should have minimal degradation and this can be

achieved by extracting protein at low temperature, the addition of protease inhibitor in lysis buffer and avoiding freeze-thawing protein samples multiple times. The protein sample should also be free of contaminating substances that will interfere with the protein separation such as salts, detergents, nucleic acids and lipids. Besides this, in order to achieve a well-focused first-dimensional separation, the protein sample should also be completely solubilised, disaggregated, denatured and reduced. This ensures that each protein is present in only one conformation and that aggregation and intermolecular interaction is avoided. Therefore, all sample preparation solution for first-dimension separation will include denaturant, detergent, reducing agents and solubilising agent. Urea is a common denaturant used to solubilise and unfold most proteins to their fully random conformation, with all ionisable groups exposed to solution. Studies have shown that the use of thiourea in addition to urea can improve solubilisation, especially membrane proteins (Molloy et al., 1998; Musante, Candiano, & Ghiggeri, 1998; Rabilloud, 1998; Rabilloud, Adessi, Giraudel, & Lunardi, 1997). A non-ionic or zwitterionic detergent like 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) is commonly used to ensure complete sample solubilisation and to prevent aggregation through hydrophobic interactions. CHAPS is more effective for solubilising a wide range of samples than NP-40 or Triton X-100 (Perdew, Schaup, & Selivonchick, 1983), both of which were used originally as non-ionic detergent before CHAPS (Bjellqvist et al., 1982; O'Farrell, 1975). SDS, a powerful anionic detergent, was not recommended as it is charged and forms complexes with proteins. Dithiothreitol (DTT) is used as reducing agents to break any disulfide bonds present and to maintain all proteins in their fully reduced state. Lastly, carrier ampholytes or IPG Buffer is added to enhance solubility by minimising protein aggregation due to charge-charge interactions.

2.4.1.3 First dimension separation (IEF) and second-dimension separation (SDS-PAGE)

In first-dimension separation, also known as isoelectric focusing (IEF), proteins are separated according to their isoelectric points (pI) using immobilised pH gradient (IPG) strips. Proteins are amphoteric in nature, i.e. they are positively charged, negatively charged or neutral depending on the pH of their surrounding environment. Isoeletric point (pI) is the specific pH at which the net charge of the protein is zero. By applying high voltage, typically 8000V, the proteins will move to pH points where their net charges are zero, thus separating the protein mixture.

After IEF, the IPG strip is then equilibrated by saturating the IPG strip with SDS buffer system required for the second-dimension separation. The equilibration solution typically contains buffer, urea, glycerol, reductant, SDS, and dye. An additional equilibration step replaces the reductant with iodoacetamide to stabilise the reduced proteins. The function of urea and glycerol is to reduce the effects of electroendosmosis which will interfere with protein transfer from the strip to the second-dimension gel by increasing the viscosity of the buffer (Gorg, Postel, & Gunther, 1988). SDS functions to denature proteins and forms negatively charged protein-SDS complexes. This is important as the proteins in the IPG strips are neutral in charge and SDS confers the proteins negative charge for separation in SDS-PAGE.

After equilibration, the strip is ready for second-dimension separation, or SDS-PAGE, which separates proteins according to their molecular mass. The technique is performed in polyacrylamide gels containing SDS. The SDS in the sample and gel denatures the proteins and confers them in negative charge which will then be separated through polyacrylamide gel electrophoresis. Besides SDS, a reducing agent such as

DTT is also added to break any disulfide bonds present in the proteins. When proteins are treated with both SDS and a reducing agent, the degree of electrophoretic separation within a polyacrylamide gel depends largely on the molecular mass of the protein. In fact, there is an approximately linear relationship between the logarithm of the molecular mass and the relative distance of migration of the SDS-polypeptide complex. It should be noted that this linear relationship is only valid for a certain molecular mass range, which is determined by the polyacrylamide percentage. The most commonly used buffer system for second-dimension SDS-PAGE is the Tris-glycine system described by Laemmli (1970). This buffer system separates proteins at high pH, which confers the advantage of minimal protein aggregation and clean separation even at relatively heavy protein loads. The Laemmli buffer system has the disadvantage of a limited gel shelf life. Other buffer systems can also be used, particularly the Tris-tricine system of Schagger and von Jagow (1987) for improving resolution of polypeptides with molecular mass values below 10 kDa.

2.4.1.4 Visualisation of 2-dimensional (2D) gels

There are a few methods that can be employed to visualise a 2D gel. Generally, the desirable features of the gel staining method include high sensitivity, wide linear range for quantification, compatibility with mass spectrometry, low toxicity and environmentally friendly.

The two most common methods used are silver staining and Coomassie staining. Silver staining is a sensitive non-radioactive method that is able to detect protein amount of below 1 ng. However, it is a complex, multi-step process utilising numerous reagents for which quality is critical. By omitting glutaraldehyde from the sensitiser and formaldehyde from the silver nitrate solution, the method becomes compatible with

mass spectrometry analysis (Shevchenko, Wilm, Vorm, & Mann, 1996), although at the expense of sensitivity. Coomassie staining, although 50- to 100-fold less sensitive than silver staining, is a relatively simple method compared to silver staining. Coomassie blue is preferable when relative amounts of protein are to be determined by densitometry. An improved method of Coomassie blue, colloidal staining methods has a higher sensitivity which can detect down to 100 ng/protein spot (Neuhoff, Arold, Taube, & Ehrhardt, 1988; Neuhoff, Stamm, & Eibl, 1985).

Other staining methods like negative zinc-imidazole staining has a detection limit of approximately 15 ng protein/spot (Fernandez-Patron et al., 1998) and is compatible with mass spectrometry, but is a poor quantitation technique. Fluorescent labelling (Unlu, Morgan, & Minden, 1997) and fluorescent staining (Mackintosh et al., 2003) provide significant advantages over Coomassie blue or silver staining. Fluorescent detection offers increased sensitivity, simple, robust staining protocols, and quantitative reproducibility over a broad dynamic range. The method is also compatible with mass spectrometry. Autoradiography and fluorography are the most sensitive detection methods (down to 200 fg of protein). To employ these techniques, the sample must contain protein radiolabeled *in vivo* using either ^{35}S, ^{14}C, ^{3}H or, in the case of phosphoproteins, ^{32}P or ^{33}P. For autoradiographic detection, the gel is simply dried and exposed to X-ray film or- for quicker results and superior dynamic range of quantitation- to a storage phosphor screen. Fluorography is a technique that provides extra sensitivity by impregnating the gel in a scintillant such as 2,4-diphenyloxazole (PPO) prior to drying.

2.4.1.5 Further analysis of protein spots

After staining, the gels are scanned using a gel scanner. Dedicated software for 2D-GE application is used to compare and contrast 2D gels. The protein spot of interest can be excised and digested using enzyme, typically trypsin, into peptides. The peptides are then mixed with matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) matrix material, and spotted onto MALDI-TOF MS plates. Time-of-flight mass spectrometry is a technique for analysing molecular weights based on the motion of ionised samples in an electrical field. In MALDI-TOF MS, a matrix-bound sample is bombarded with a pulsed laser beam to generate ions for subsequent detection. The MS spectrum generated from these peptides can then be identified using public databases such as SWISS-PROT and NCBI.

2.4.2 Transcriptomics

Nutritranscriptomics is another "omics" technology used in the foodomics studies. Transcriptomics examines the mRNA expression levels in a cell population. The most common method used in transcriptomic studies is DNA microarray technology. Recently, a technique named RNA-seq which employs next-generation sequencing technology emerged as a method of choice to measure the transcriptomes in an organism (Z. Wang, Gerstein, & Snyder, 2009), although DNA microarray is still being used. In the nutrition field, the global analysis of transcripts could elucidate the effect of a nutrient or diet on metabolic pathways; identify potential biomarkers in chronic diseases; and determine the impact of a diet and/or a single nutrient on a human pathology (Garcia-Canas, et al., 2012; Wittwer, et al., 2011).

2.4.2.1 DNA microarray

DNA microarray is a useful tool to simultaneously and rapidly examine the expression of thousands of genes. Before the advent of DNA microarray, Northern blot was used to visualise gene expression of targeted genes. Ever since the establishment of microarray two decades ago (DeRisi et al., 1996; Lockhart et al., 1996; Schena, Shalon, Davis, & Brown, 1995), it has become one of the most widely used technique to study transcriptomes of an organism. A microarray is a glass slide or silicon chip onto which DNA molecules (probes) are bound at fixed locations called spots. This allows the labelled cDNA transcribed from mRNA of sample of interest to hybridise to the probes, which can then be quantified by measuring the fluorescence intensity emitted by the labelled cDNA. Generally there are two types of microarray: spotted microarrays like cDNA microarray and oligonucleotide microarray, and the Affymetrix GeneChip system, which involves direct synthesis of oligonucleotides onto a chip.

2.4.2.2 Types of DNA microarray

Spotted cDNA microarray was the first widely used microarray technique. In a typical spotted microarray, cDNA fragments, representing different genes, are amplified using PCR and printed at high density onto microscope glass slides with special surface chemistry that allows binding of the spotted DNA. Two different cDNA populations derived from independent RNA samples are labelled with red (Cy5) and green (Cy3) fluorescent dyes, respectively, and hybridised to the slide. The array is subsequently washed and scanned by lasers that excite the different dyes. A fluorescent signal is computed for each spot on the array and the ratio of Cy3:Cy5-induced fluorescence for each spot corresponds to the relative amount of transcript in the samples. While this method is especially useful for examining transcriptomes in organisms that have little or no genome sequences, its uniformity across each microarray slides remains challenging

due to probes of varying length. This method also measures only one gene per probe as compared to Affymetrix GeneChip which has multiple probes for a gene, this impacts on the sensitivity of spotted microarray in detecting the genes.

The next microarray technology to emerge involved *in situ*-synthesised oligonucleotide arrays using photolithographic technology (Affymetrix). Affymetrix GeneChip technology employs a series of 25-mer oligonucleotides, which are designed using a computer algorithm to represent known or predicted open reading frames (Lipshutz, Fodor, Gingeras, & Lockhart, 1999). However, it is only limited to organisms with a significant amount of genome information. There are between 10 and 20 different oligonucleotides representing each gene to control for variation in hybridisation efficiency due to factors such as GC content. A control for cross-hybridisation with similar short sequences in transcripts other than the one being probed for is a mismatch oligonucleotide next to each oligonucleotide with a single base pair change at its centre. Under stringent hybridisation conditions, this control should not hybridise to the exact match cDNA. The level of expression of each gene is calculated using a procedure provided by the Affymetrix software, which computes the weighted average of the difference between the perfect match and mismatch. The high density arrays are constructed on silicon wafers using a technique called photolithography and combinatorial chemistry. The process used to prepare the arrays is expensive and processing requires a proprietary hybridisation station, scanner and software, thus is more expensive than the spotted microarrays. The target cDNA is labelled using amplified RNA and only a single sample is hybridised to each chip.

Another technique that uses *in situ* synthesis oligonucleotide arrays is the ink-jet technology by Agilent. Instead of using 25-mer probes like in Affymetrix GeneChips,

this technique uses longer oligonucleotide probes (60-mer). Unlike the Affymetrix counterpart, this technique is analogous to the spotted cDNA microarray in the sense that it only detects one gene per probe as oppose to multiple probes detecting a gene in Affymetrix GeneChip technique and it also uses a 2-dye system. Agilent microarrays have improved selectivity over conventional spotted cDNA microarray due to increased probe length and sequence optimisation. However, this advantage may be compromised by relatively high background fluorescence.

2.4.2.3 Normalisation and data analysis

DNA microarray data were obtained as scanned image files. Before subjecting those files for expression analysis, they have to be normalised to standardise microarray data and to differentiate between real (biological) variations in gene expression levels and variations due to the measurement process. Generally, normalisation of data from two-colour microarray systems like spotted cDNA microarray is achieved by linear regressions. This process requires raw data manipulation, owing to differences in the chemistry of the dyes, before differences in transcript levels can be identified. It is necessary to normalise the fluorescence ratios in order to compensate for systematic variations (Bilban, Buehler, Head, Desoye, & Quaranta, 2002). Normalisation of Affymetrix GeneChip array expression data is often done by utilising the Robust Multi-array Average (RMA) algorithm. RMA is a method for normalising and summarising probe-level intensity measurements from Affymetrix GeneChips. Starting with the probe-level data from a set of GeneChips, the perfect-match (PM) values are background-corrected, normalised and finally summarised resulting in a set of expression measures. The background correction used in RMA is a non-linear correction, done on a per-chip basis. It is based on the distribution of PM values amongst probes on an Affymetrix array. The normalisation used in RMA is quantile

normalisation, which is non-linear method. This usually gives very sharp normalisations. Once the probe-level PM values have been background-corrected and normalised, they are summarised into a single expression measure per probe-set, per chip. The summarisation used is motivated by the assumption that observed log-transformed PM values follow a linear additive model containing a probe affinity effect, a gene specific effect and an error term. For RMA, the probe affinity effects are assumed to sum to zero, and the gene expression level is estimated using median polishing. Median polishing is a robust model fitting technique that protects against outlier probes.

After normalising the microarray expression data, the data are ready to be analysed. The most basic method to analyse the huge amount of microarray expression data is to filter them by fold-change when comparing samples with two or more different parameters. Often changes of ≥ 1.5-fold may be sufficient to identify a list of candidate genes. To further strengthen the fact that the changes in genes identified are likely to be due to the defined parameter rather than random fluctuations, one- or two-way analysis of variance (ANOVA) is performed. ANOVA is performed separately for each gene, and only those genes that pass the significance level are retained for further analysis. Another method is based on grouping genes that have similar patterns of gene expression throughout the whole experiment. There are two ways of grouping, class discovery analysis or class prediction analysis. Class discovery analysis is also known as unsupervised classification or knowledge discovery. The idea of clustering genes together in groups based on iterative pattern recognition or statistical learning methods to find an optimal number of clusters in data allows the discovery of new groups that otherwise were not reported (Peterson, 2013). Examples of class discover analysis include hierarchical cluster analysis, k-means cluster analyses, self-organising maps and

model-based cluster analysis (Rimbach, Fuchs, & Packer, 2005). Another clustering method is the class prediction analysis or supervised classification. It uses sample associated parameters or classes to identify gene lists that can associate unknown samples with these parameter or classes (Soinov, 2003). Principle component analysis (PCA) is a common method used to reduce the number of dimensions prior to data analysis (Peterson, 2013). It identifies the correlations between gene expression profiles and attempts to explain a majority of the variance in the entire data set. It is a decomposition technique that produces a set of expression patterns that are known as principle components. Diagonal or 3D combinations of these patterns can be assembled to represent the behaviour of all of the genes in a given data set (Remy & Michnick, 2003).

2.4.2.4 Validation of microarray analysis

As mentioned earlier, DNA microarray is an extremely powerful tool for the analysis of gene expression. However it should be noted that there are technical limitations that may distort the data analysis. The type (cDNA or oligonucleotide), the size (in bp), and the location in the target gene of microarray probes may affect efficiency of the hybridisation reaction and thus the ability of the technique to accurately detect differences in gene expression levels. Therefore, an additional test or assay is normally performed to confirm and increase confidence of the microarray expression results.

Quantitative real-time polymerase chain reaction or qRT-PCR is the most common method used to validate microarray results. qRT-PCR allows the continuous measurement of products produced during the course of PCR reaction. This can be done by using either TAQman based fluorescent probe method or by using Sybr Green to detect double stranded DNA products. Both chemistries rely on the detection of product

molecule present throughout the numerous cycles occurring during a complete PCR reaction. The exponential growth in PCR products is related to the cycle threshold point and from this crossing point (C_T value), the number of targets present in the input sample can be determined.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Merck, Germany:

i. Sodium carbonate

ii. Silver nitrate

iii. Ammonium peroxodisulphate

iv. EDTA disodium

v. Potassium hexacyanoferrate (III)

vi. Sodium hydrogen carbonate

vii. N,N,N',N'-Tetramethylethylenediamine (TEMED)

viii. Iodoacetamide

ix. Dithiothreitol (DTT)

x. Urea

xi. Ammonium hydrogen carbonate

xii. Acetonitrile

xiii. Glycine

xiv. Thiourea

xv. Isopropanol

xvi. Sodium acetate trihydrate

xvii. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)

xviii. Sodium thiosulphate pentahydrate

xix. Sodium hydroxide

xx. Formaldehyde solution 37%

xxi. Acrylamide

xxii. Trifluoroacetic acid (TFA)

Sigma-Aldrich Chemical Company, USA:

i. Fenofibrate

ii. Palmitic acid

iii. Oil Red O

iv. Gluteraldehyde

v. Albumin from bovine serum, fatty acid free

vi. Tris-base

vii. Trypsin-EDTA

viii. Trypan blue

ix. Orange G

x. Glycerol

xi. Sterile dimethylsulphoxide (DMSO)

xii. N,N'-methylenebisacrylamide

GE Healthcare, USA:

i. Sodium dodecyl sulphate (SDS)

ii. Glycine

iii. Drystrip cover fluid

iv. Immobiline pH gradient (IPG) strips, pH 3-10 NL, 13 cm

v. IPG buffer, pH 3-10 NL

Hyclone, Australia:

i. Dulbecco's modified Eagle medium (DMEM)

ii. Foetal bovine serum (FBS)

iii. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)

Thermo Scientific, USA:

i. Halt protease inhibitor cocktail 100X

ii. Trypsin

Fisher Scientific, UK:

i. Methanol analytical grade

ii. Ethanol

Invitrogen, UK:

i. Ethidium bromide

Vivantis, USA:

i. Agarose

Flowlab, Australia:

i. Penicillin and streptomycin

Bio-rad, USA:

i. Bradford reagent

ii. Beta-mercaptoethanol

SRL, India:

i. Deoxycholic acid sodium salt

Oxoid, UK:

i. Phosphate buffered saline (Dulbecco A)

Calbiochem, USA:

i. 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)

Acros Organics, USA:

i. Bromophenol blue

Applichem, Germany:

i. Triton X-100

Applied Biosystems, USA:

i. Fast SYBR® Green Master Mix

3.1.2 Apparatus

i. Rotary evaporator (Buchi, Switzerland)

ii. Weighing machine (Denver Instrument, USA)

iii. Vortex mixer (Labnet, USA)

iv. Orbital shaker (Biometra, Germany)

v. Sorvall Legend Micro 17 centrifuge (Thermo Scientific, USA)

vi. JOUAN CR3i Multifunction centrifuge (Thermo Scientific, USA)

vii. Sorvall Biofuge Primo R centrifuge (Thermo Scientific, USA)

viii. Magnetic stirrer (Thermo Scientific, USA)

ix. PCR Thermocycler (Biometra, Germany)

x. Dry Block Heating Thermostat (Biosan, Latvia)

xi. ELISA Plate Reader (Bio-Rad, USA)

xii. Hybridization Oven 640 (Affymetrix, USA)

xiii. Fluidics Station 450 (Affymetrix, USA)

xiv. GeneQuantpro Spectrophotometre (GE Healthcare, USA)

xv. Gel Doc 1000/2000 (Bio-Rad, USA)

xvi. StepOne Real-Time PCR System (Applied Biosystems, USA)

xvii. Ettan IPGphor III Isoelectric Focusing System (GE Healthcare, Sweden)

xviii. SE 600 Ruby electrophoresis system (GE Healthcare, Sweden)

xix. ImageScanner III (GE Healthcare, Sweden)

xx. Applied Biosystems 4800 Plus MALDI TOF/TOF (Applied Biosystems, USA)

xxi. Vivaspin® 20 ultrafiltration device with 5 kDa molecular weight cut off PES membrane (Sartorius Stedim, Germany)

3.1.3 Kits

i. RNeasy Mini Kit (Qiagen, Germany)

ii. High Capacity RNA-to-cDNA kit (Applied Biosystems, USA)

iii. RNase-free DNase set (Qiagen, Germany)

iv. BCA assay (Pierce, Thermo Scientific, USA)

v. 2D-cleanup kit (GE Healthcare, USA)

vi. Western Dot 625 Goat Anti-Rabbit Western Blot kit (Invitrogen, USA)

vii. Triglyceride quantification kit (Abcam, UK)

viii. Cholesterol/cholesteryl ester quantification kit (Abcam, UK)

ix. Applause WT-AMP ST System (NuGEN Technologies, USA)

x. Encore Biotin Module (NuGEN Technologies, USA)

xi. MinElute Reaction Cleanup Kit (Qiagen, Germany)

3.1.4 Software

i. Image Master 2D Platinum V 7.0 software (GE Healthcare, Sweden)

ii. GPS Explorer software (Applied Biosystems, USA)

iii. MASCOT program (Matrix Science, UK)

iv. ImageJ software (http://rsb.info.nih.gov/ij/)

v. Ingenuity Pathways Analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com)

vi. Partek Genomics Suite TM (Partek, USA)

vii. Microarray Suite 5 software (Affymetrix, USA)

viii. GeneChip Operating Software (GCOS) (Affymetrix, USA)

ix. Sequence Detection System (SDS) software (Applied Biosystems, USA)

x. Quantity One Software (Bio-Rad, USA)

xi. StepOne Software (Applied Biosystems, USA)

xii. Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)

3.1.5 Cell culture

i. Human hepatoblastoma cell line, HepG2 (American Type Culture Collection, ATCC)

3.2 Methods

3.2.1 Nomenclature

Human gene symbols are italicised with all letters in uppercase while mouse or rat gene symbols are italicised with the first letter in uppercase followed by all lowercase letters. Protein symbols are at uppercase and not italicised. With regards to proteomics, in the context of 2D-GE, the terms "protein abundance" and "differentially modulated" are used to reflect the variations in protein levels, including transcription (up-regulation and down-regulation), post-translational modifications, translocation, degradation, accumulation and trafficking (Cohen et al., 2008, Godovac-Zimmermann et al., 2005, Nesvizhskii & Aebersold, 2005) that occur when proteomes are perturbed in various conditions especially. In contrast, the term "differentially expressed" is more tractable and suitable for describing detected protein from MS-based proteomics.

3.2.2 Sampling and sample preparation

Whole, ripe *T. indica* fruits were collected from Kedah in the northern region of Malaysia. The voucher specimen of the sample with an identification number, KLU 45976, was deposited in the Rimba Ilmu Herbarium, the University of Malaya. A measure of 10 g of *T. indica* fruit pulp powder was suspended in 200 ml of methanol at room temperature. The mixture was then stirred with a magnetic stirrer for 1 h and incubated in the dark for 24 h. The supernatant was filtered with a filter paper. The crude extracts of *T. indica* fruit pulp were obtained after evaporating methanol to dryness in a rotary evaporator.

3.2.3 Cell culture and treatment

HepG2 cells were cultured in Dulbecco's modified Eagle medium (DMEM), with 5 mM glucose, supplemented with 10 % foetal bovine serum (HyClone, Australia),

0.37 % (w/v) sodium bicarbonate and 0.48% (w/v) HEPES, pH 7.4, in a CO_2 humid incubation chamber at 37 ℃. To study the effects of methanol extract of *T. indica* fruit pulp on HepG2 cells, the cells were seeded at a density of 9.0 $\times 10^6$ in 75 cm^2 flask for 18–24 h, then the cells were extensively washed with PBS to remove any remaining serum. The cells were then incubated in serum-free DMEM with a final concentration of 0.02 % DMSO (vehicle) as control and 60 µg/ml methanol extract of *T. indica* fruit pulp as treatment. After 24 h, the secreted proteins and the cell lysate were harvested.

3.2.4 Recovery of secreted proteins from cell culture media

The medium containing the secreted proteins was collected and centrifuged at 1000 x *g* for 5 min to remove cellular debris. The medium was further filtered through 0.22 µm syringe filter. The supernatant was then concentrated by ultrafiltration through a spin column with 5 kDa molecular weight cut off membrane (Sartorius Stedim, Germany) at 7000 x *g* for 2 h. The protein concentration in the concentrated medium was then determined using Bradford assay kit (Bio-Rad).

3.2.5 Cell lysate extraction

The cells were detached using trypsin-EDTA. They were then spun at 261 x *g* to pellet the cells. The pellet was washed with ice-cold PBS twice and thiourea rehydration solution (7 M urea, 2 M thiourea, 2 % w/v CHAPS, 0.5% v/v IPG buffer, orange G, protease inhibitor) was added to lyse the cells. Vigorous pipetting and sonication were performed to ensure the cells were thoroughly ruptured. The mixture was then incubated on ice and vortexed every 10 min for a time span of 30 min. After incubation, the mixture was centrifuged at 17,000 x *g* for 20 min at 4 ℃ to remove the cell debris. The supernatant containing the proteins were then aliquoted and kept at -80

℃ for downstream application. Protein concentration was determined using Bradford assay.

3.2.6 Cell viability

To ensure that the cell was still above 90% viable after the treatment, the percentage of viable cells during secretion period and after treatment was estimated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, cells were plated at a density of 1.5×10^4 cells per well in a 6-well plate and cultured exactly as mentioned. Medium was then removed and 100 µl of 5 mg/ml MTT (Merck, Germany) was added to each well. Cells were incubated for 4 h and the MTT solution was discarded and the precipitate in each well was resuspended in 2 ml of isopropanol. The optical density (OD) of the samples was read at 570 nm.

3.2.7 Two-dimensional gel electrophoresis (2D-GE)

2D-GE is a method employed to separate proteins based on two dimensions; the first dimension separates proteins based on their isoelectric focusing points (pI) using immobilised pH gradient (IPG) gel strip, while in the second dimension the proteins were separated based on their molecular mass through SDS-polyacrylamide gel electrophoresis. The gel can then be stained using Coomassie blue or silver staining to visualise the protein spots. The gel is then scanned and image can be analysed for downstream application.

3.2.7.1 Sample preparation and rehydration of IPG gel strips

The optimum amount of protein to load per gel and sample preparation was optimised for better separation of proteins. Forty micrograms of secreted protein or cell lysate was first cleaned-up using 2D clean-up kit (GE Healthcare, Piscataway, USA).

The resulting protein pellet after cleaning up was reconstituted in 20 µl of thiourea rehydration solution (7 M urea, 2 M thiourea, 2% w/v CHAPS, 0.5% v/v pH 3-10 NL IPG buffer, Orange G), 80 µl of sample buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% v/v pH 3-10 NL IPG buffer, 40 mM DTT) and topped up to 250 µl with thiourea rehydration solution. For cell lysate sample, the sample buffer was slightly modified with DTT reduced by half and the addition of DeStreak reagent (GE Healthcare, USA). Treated and untreated samples, containing the same protein amount, were rehydrated passively for 18 h at room temperature on immobilised pH gradient strips (13 cm, non-linear, pH 3-10, GE Healthcare, Uppsala, Sweden).

3.2.7.2 First dimension separation of protein through IEF

First dimension separation was carried out with Ettan IPGphor III Isoelectric Focusing System and a standard strip holder (GE Healthcare, Uppsala, Sweden). Isoelectric focusing (IEF) was performed under the following conditions: (i) 500 V, 1 h 10 min, step and hold; (ii) 1000 V, 1 h, gradient; (iii) 8000 V, 2 h 30 min, gradient and (iv) 8000 V, 55 min, step and hold. The temperature was maintained at 20℃ and the current was kept at 50 µA per strip. The strips were either used immediately for second dimension separation or kept in -80 ℃ for further use.

3.2.7.3 Second dimension separation of protein through SDS-PAGE

Upon completion of IEF, strips were equilibrated in equilibration buffer (6 M urea, 75 mM Tris-HCl, pH 8.8, 29.3 % v/v glycerol, 2 % w/v SDS, 0.002 % w/v bromophenol blue, 1 % w/v DTT) for 15 min. The solution was then discarded and replaced with equilibration buffer containing 4.5 % w/v iodoacetamide instead of DTT for another 15 min. The second dimension separation was carried out at 15 ℃ on 12.5 % SDS slab gels using a SE 600 Ruby electrophoresis system (GE Healthcare, Uppsala,

Sweden), with the IPG strips sealed on the top of the gels with 0.5 % (w/v) agarose in SDS-electrophoresis buffer (25 mM Tris base, 192 mM glycine, 0.1 % w/v SDS and a trace amount of bromophenol blue). SDS-PAGE was run at constant power of 50 V and 40 µA/gel for 20 min, and then switched to 500 V and 40 µA/gel until the bromophenol blue marker was 1 mm away from the bottom of the gel.

3.2.8 Silver staining of gel

After SDS-PAGE, the gel was fixed in fixing solution (40 % (v/v) ethanol, 10 % acetic acid) for 40 min. The solution was discarded and the gel was immersed in incubation solution (30 % (v/v) ethanol, 0.5 M sodium acetate, 8 mM sodium thiosulphate, 0.13 % (v/v) glutaraldehyde) for 40 min. Gluteraldehyde was added freshly prior to gel immersion. The gel was then washed three times in distilled water for 5 min. Silver staining solution (5.9 mM silver nitrite, 0.02 % (v/v) formaldehyde) was then used to stain the gel. Formaldehyde was only added prior to use. To develop the stained gel, developing solution (0.24 M sodium carbonate, 0.2 % (v/v) formaldehyde) was added. Formaldehyde was added immediately before use. After developing the gel, the reaction was halted by adding the stop solution containing 40 mM EDTA-$Na_2.2H_20$. All the silver staining steps were performed on orbital shaker at 60 rpm. After staining, the gels were scanned with ImageScanner III (GE Healthcare, Uppsala, Sweden).

For gels to be used for mass spectrophotometry analyses, gluteraldehyde and formaldehyde were omitted from the silver staining protocol, but at the expense of reduced sensitivity. To compensate with that, the protein amount loaded was doubled for gels that were used in mass spectrophotometry analyses.

3.2.9 Gel image and data analysis

The 2D gel images were analysed using the Image Master 2D Platinum V 7.0 software (GE Healthcare, Uppsala, Sweden). In brief, the 2D gel images were subjected to spot detection and quantification in the differential in-gel analyses module. To minimise the variations in between gels within the same groups, the protein spots were normalised using percentage of volume. Statistically significance ($p < 0.05$, Student's *t*-test) and presence in all 4 gels were two criteria for the acceptance of the differentially-expressed protein spots. The selected spots were filtered based on an average expression level change of at least 1.5-fold.

3.2.10 In-gel tryptic digestion

Differentially expressed protein spots were excised manually from the 2D gels, and washed with 100 mM NH_4HCO_3 for 15 min. The gel plugs were then destained twice with 15 mM potassium ferricyanide/50 mM sodium thiosulphate with shaking until gel plugs became clear. After destaining, the gel plugs were reduced with 10 mM DTT at 60 ℃ for 30 min and alkylated with 55 mM iodoacetamide in the dark at room temperature for 20 min. The gel plugs were later washed thrice with 500 µl of 50 % ACN/ 50 mM NH_4HCO_3 for 20 min, dehydrated with 100 % ACN for 15 min and SpeedVac the gel plugs till dry. Finally the gel plugs were digested in 6 ng/µl trypsin (Pierce, Rockford, IL USA), in 50 mM NH_4HCO_3 at 37 ℃ for at least 16 h. Peptide mixtures were then extracted twice with 50 % ACN and 100 % ACN respectively and finally concentrated using Speedvac until completely dry. The dried peptide was then kept in -20 ℃ or reconstituted with 10 µL of 0.1 % FA prior to desalting using Zip Tip C18 micropipette tips (Millipore, Billerica, MA, USA).

3.2.11 Mass spectrometry and database searching

Peptide mixtures were analysed by using a mass spectrometer (Applied Biosystems 4800 Plus MALDI TOF/TOF, Foster City, CA, USA). The trypsin digest were crystallised with alpha-cyano-4-hydroxycinnamic acid matrix solution (10 mg/ml, 70 % ACN in 0.1 % (v/v) TFA aqueous solution) and spotted onto a MALDI target (192-well) plate. The MS results were automatically acquired with a trypsin autodigest exclusion list and the 20 most intense ions selected for MS/MS analysis, with a minimum S/N (signal/noise) of at least 25. The collision gas was nitrogen gas and the energy was 4.3 kV. Interpretation was carried out using the GPS Explorer software (Applied Biosystems, Foster City, CA, USA) and database searching using the in-house MASCOT program (Matrix Science, London, UK). Both combined MS and MS/MS searches were conducted with the following settings: Swiss-Prot database, *Homo sapiens*, peptide tolerance at 200 ppm, MS/MS tolerance at 0.4 Da, carbamidomethylation of cysteine (variable modification) and methionine oxidation (variable modifications).

3.2.12 Western blot

Three of the altered proteins, ethanolamine-phosphate cytidylyltransferase (PCYT2), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 10 (NDUFA10) and ubiquinol-cytochrome-c reductase complex core protein 2 (UQCRC2) were selected to be validated using Western blotting. HepG2 cells (3×10^6) were treated with 0.06 mg/ml *T. indica* fruit extract for 24 h. The cells were then trypsinised and lysed with RIPA buffer (150 mM NaCl; 50 mM Tris-HCl, pH 7.4; 1 mM EDTA; 1 % Triton X-100; 1 % sodium deoxycholate; 0.1 % SDS). The total cell lysate proteins were quantified using BCA assay kit (Pierce, Rockford, IL, USA). Forty micrograms of cell lysate protein was separated on a 12.5 % SDS-PAGE, and the separated proteins

were transferred onto a PVDF membrane with 0.45 µm pore size (Thermo Scientific, IL, USA) at 100 V, 110 mA for 90 min. The blot was then blocked overnight and developed against anti-PCYT2 (ab126142, rabbit polyclonal, Abcam, UK), anti-NDUFA10 (ab103026, rabbit polyclonal, Abcam, UK), anti-UQCRC2 (ab103616, rabbit polyclonal, Abcam, UK) and anti-beta actin (ab8227, rabbit polyclonal, Abcam, UK), the loading control using the WesternDot 625 Goat Anti-Rabbit Western Blot kit (Invitrogen, Oregon, USA). Quantification of the band intensity was calculated using ImageJ software.

3.2.13 Data mining using Ingenuity Pathways Analysis (IPA) software

The proteomics data were further analysed using the Ingenuity Pathways Analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com) to predict networks that are affected by the differentially expressed proteins. Details of proteins identified to be differentially released, their quantitative expression values (fold change difference of at least 1.5) and *p*-values ($p < 0.05$) were imported into the IPA software. Each protein identifier was mapped to its corresponding protein object and was overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. Network of proteins were then algorithmically generated based on their connectivity. Right-tailed Fischer's exact test was used to calculate a *p*-value indicating the probability that each biological function assigned to the network is due to chance alone.

3.2.14 Lipid Studies

3.2.14.1 Cell culture and treatment to study lipid-lowering effects of *T. indica* fruit extract in HepG2 cells

HepG2 cells were cultured in Dulbecco's modified Eagle medium (DMEM), with 5 mM glucose, supplemented with 10 % foetal bovine serum (HyClone, Australia), 0.37 % (w/v) sodium bicarbonate and 0.48 % (w/v) HEPES, pH 7.4, in a CO_2 humid incubation chamber at 37 ℃. To study the lipid-lowering effects of methanol extract of *T. indica* fruit pulp on HepG2 cells, steatosis was first induced in the cells by culturing them in palmitic acid-containing media. The cells were seeded and incubated for 18–24 h, after which they were treated with 0.3 mM palmitic acid complexed to fatty acid-free bovine serum albumin and *T. indica* fruit extract or 0.1 mM fenofibrate (a positive control) or 0.02 % DMSO (vehicle, a negative control). The cells were then incubated for 24 or 48 h before various assays were performed.

3.2.14.2 Preparation of palmitic acid/fatty acid-free bovine serum albumin complex

Ten percent fatty acid-free bovine serum albumin (FA free-BSA) was prepared by dissolving the FA free-BSA powder in phosphate buffered saline (PBS) and the pH was adjusted to 7.0 using 7.5 % (w/v) sodium bicarbonate. The solution was then sterile-filtered using a 0.2 μm filter syringe.

A stock solution of 100 mM palmitic acid was prepared by dissolving the palmitic acid in 0.1 M NaOH at 70 ℃. Before complexing the palmitic acid to the FA free-BSA, the 10 % FA free-BSA solution was first incubated at 55 ℃, and palmitic acid solution was added slowly to the FA free-BSA solution while swirling to achieve

the desired concentration. The solution was then sterile-filtered using a 0.45 µm filter syringe and kept in -20 ℃.

3.2.14.3 Cell viability

To assess the cell viability of HepG2 during the treatment, MTT assay was performed. Briefly, cells were plated at a density of 216,000 cells per well in a 24-well plate. After 24 h, the media was removed and the wells were washed gently with PBS twice. The cells were then incubated for 24 or 48 h in DMEM supplemented with different concentrations of palmitic acid/10 % FA-free BSA and treatments with 0.1 mM fenofibrate or different concentrations of *T. indica* fruit extract or 0.02 % DMSO (vehicle). After incubation, 50 µL of 5 mg/ml MTT (Merck, Germany) was added to each well and incubated for another 4 h. The media was then removed and 1 mL of isopropanol was added to each well to dissolve the formazan crystal. The optical density of the sample was read at 570 nm.

3.2.14.4 Oil Red O staining

Oil Red O staining was performed to stain the lipid droplets in the cells. Cells were seeded in a 24-well plate and cultured as described in Section 3.2.14.1. After removing the media, the plate was rinsed gently with PBS twice and fixed in 10 % formalin for 1 h. The formalin was then removed and each well was rinsed gently with double distilled water. Sixty percent (v/v) isopropanol was added to each well to fix the cells for 5 min. After that, the isopropanol was removed and Oil Red O working solution was added to each well to stain the lipid droplets in the cells for 5 min. Before viewing, Oil Red O was removed and the plate was rinsed with water until the water rinsed off clear.

3.2.14.5 Triglyceride quantification

After treatment, three million of cells were homogenised in 300 µl of 5 % Triton-X100 in water and were slowly heated to 90 ℃ for 5 min. The samples were cooled down to room temperature before heating them again to solubilise all triglycerides. The samples were then centrifuged for 5 min to remove any insoluble materials. Samples were diluted 10-fold with double distilled water before the quantification assay using the triglyceride quantification kit (Abcam, UK) was performed. Assay was done in triplicate and the data were normalised to the amount of protein for each sample to eliminate statistical bias caused by cell death.

3.2.14.6 Cholesterol quantification

After treatment, three million of cells were dissolved in 500 µl of chloroform:isopropanol:Triton X-100 (7:11:0.1) solution. The samples were then centrifuged for 5 min at 17,000 x *g*. The supernatant was then transferred to a new tube and air-dried at 50 ℃ to remove chloroform. After removing the chloroform, the samples were put under vacuum to remove traces of organic solvents or until dry. Prior to the quantification assay, the dried lipid was reconstituted with 200 µl of cholesterol assay buffer and quantitated using the cholesterol/cholesteryl ester quantification kit (Abcam, UK). The quantification assay was performed in triplicate and the data were normalised to the amount of protein for each sample to eliminate statistical bias caused by cell death.

3.2.15 DNA microarray analyses

3.2.15.1 Total cellular RNA (tcRNA) extraction

HepG2 cells were cultured as described in Section 3.2.14.1. The cells were then harvested and total cellular RNA (tcRNA) was extracted using the RNeasy mini kit

(Qiagen, Germany), and DNase I (Qiagen, Germany) was used to remove contaminating DNA. The extracted tcRNA was then quantitated using GeneQuantpro Spectrophotometre (GE Healthcare, USA) and the integrity of the tcRNA extracted was assessed using Agilent Bioanalyzer 2100 and agarose gel electrophoresis. The extracted tcRNA was then kept at -20 ℃ until use.

3.2.15.2 tcRNA to cDNA conversion

The tcRNA was converted to cDNA for the microarray analyses using the Applause WT-AMP ST System (NuGEN Technologies, USA) in accordance to the manufacturer's instructions. Briefly, 200 ng of tcRNA was used to prepare the first strand cDNA using a unique first strand DNA/RNA chimeric primer mix and reverse transcriptase. The resulting cDNA/mRNA hybrid molecule was then used to generate a DNA/RNA heteroduplex double-stranded cDNA. This was then amplified and cDNA with sequence complementary to the original mRNA was generated. After purifying the resulting cDNA with MinElute Reaction Cleanup Kit (Qiagen, Germany), 2.5 µg of cDNA was then fragmented and labelled with biotin using Encore Biotin Module (NuGEN Technologies, USA). The biotinylated fragments were then hybridised to Affymetrix Human Gene 1.0 ST array at 45 ℃ for 16 h in Hybridization Oven 640 (Affymetrix, USA). After this, the gene chips were stained and washed in the Affymetrix GeneChip Fluidics Station 450 using Affymetrix protocol FS450_0007. The stained arrays were scanned at 532 nm using Affymetrix GeneChip Scanner 3000 7G and CEL files were generated using Affymetrix GeneChip Operating Software (GCOS). The quality of the data was assessed using Affymetrix GeneChip Command Console Version 3.

3.2.15.3 Data analyses using Partek Genomic Suite (GS) software

The CEL files generated from each array were imported into Partek Genomic Suite software for statistical analyses and visualisation of the microarray data. The CEL files were normalised using the Robust Multi-array Average (RMA) algorithm and GC content was adjusted for pre-background adjustment. The gene list produced was then filtered for statistically significant genes based on the one-way analysis of variance (ANOVA) of *P* value less than 0.05 and fold-change of at least 1.5-fold. The significantly regulated genes were then subjected to Gene Ontology (GO) Enrichment tool in the Partek Genomic Suite Software to categorise the genes based on their involvement in biological processes, molecular function and cellular component.

3.2.15.4 Functional analyses using IPA software

Significantly regulated genes identified using Partek GS software were subjected to functional analyses using Ingenuity Pathways Analysis (IPA) software. The application was used to query databases for interactions between sets of differentially expressed genes and all other genes stored in the Ingenuity Knowledge Base to generate a set of interactive networks taking into consideration canonical pathways, relevant biological interactions as well as cellular and disease processes. The IPA system computes a score for each network according to the fit of the set of the supplied ‘‘focus genes’’. The *p*-value scores for such a set indicate the likelihood of focused genes to belong to a network versus those obtained by chance.

3.2.15.5 DNA microarray data validation using quantitative real-time polymerase chain reaction (qRT-PCR)

Eight significantly regulated genes from DNA microarray analysis were chosen to be validated using qRT-PCR. Primer sets for qPCR were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) with the following settings: PCR product size, 70-180 bp; optimum melting temperature, 60 ℃; exon junction spanning; organism: *Homo sapiens*. The primer sets were further analysed using online nucleotide tool, BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure the specificity of the amplification product. The list of primers is listed in Table 3.1.

Two micrograms of tcRNA extracted from the control and treated cells were converted to cDNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, USA). qPCR was carried out using the StepOne Real-Time PCR System (Applied Biosystems, USA) with 40 amplification cycles. Melting curve analyses were performed for all primers to ensure single amplification product. Each reaction consisted 20 ng of cDNA, 0.2 mM forward and reverse primers, 2X Fast SYBR® Green Master Mix (Applied Biosystems, USA) in a final volume of 20 µl. All qRT-PCR experiments were repeated with 3 biological replicates from different experiments and reactions were performed in triplicates. Gene expressions were normalised against beta actin, a housekeeping gene and fold change was expressed as mean ± SEM.

Table 3.1: Primer sequences of genes selected for verification of DNA microarray analysis in qRT-PCR.

Gene name	GenBank ID	Primer sequences	Product size (bp)	Melting temperature (℃)	%GC content
Cytochrome P450, family 7,subfamily A, polypeptide 1 (*CYP7A1*)	NM_000780	Forward: 5'-TTGCTACTTCTGCGAAGGCA-3' Reverse: 5'-TCCGTGAGGGAATTCAAGGC-3'	124	56.9 57.4	50.0 55.0
Starch binding domain 1 (*STBD1*)	NM_003943	Forward: 5'- GAGAAAGACGCCCCTCTTGG-3' Reverse: 5'- GAAGATGCTCTGGTTTGGTGAC-3'	115	57.9 56.0	60.0 50.0
Solute carrier family 2, member 1 (*SLC2A1*)	NM_006516	Forward: 5'- CTGGCATCAACGCTGTCTTC-3' Reverse: 5'- GTTGACGATACCGGAGCCAA-3'	98	56.5 57.2	55.0 55.0
Carnitine palmitoyltransferase 1A (*CPT1A*)	NM_001876	Forward: 5'- TGCTGATGACGGCTATGGTG-3' Reverse: 5'- TGAGAATCCGTCTCAGGGCA-3'	99	57.4 58.1	55.0 55.0
Amphiregulin (*AREG*)	NM_001657	Forward: 5'- GTGGTGCTGTCGCTCTTGATAC-3' Reverse: 5'-AGAGTAGGTGTCATTGAGGTCCAAT-3'	72	58.0 57.4	54.5 44.0
Prominin 1 (*PROM1*)	NM_001145847	Forward: 5'-TCAGCGTCTTCCTATTCAGG-3' Reverse: 5'-AAAAATCACGATGAGGGTCA-3'	163	53.9 51.9	50.0 40.0
Phospholipase A2, group IIA (*PLA2G2*)	NM_000300	Forward: 5'-GAAAGGAAGCCGCACTCAGTT-3' Reverse: 5'-CAGACGTTTGTAGCAACAGTCA-3'	122	57.9 55.3	52.3 45.4
Keratin 23 (*KRT23*)	NM_015515	Forward: 5'-CGGCAGAACAATGAATACCA-3' Reverse: 5'-GCCTTGATCTTTGGAGTTGC-3'	155	53.0 54.3	45.0 50.0
Beta actin (*ACTB*)	NM_001101	Forward: 5'- ACAGAGCCTCGCCTTTGCCG-3' Reverse: 5'- ACATGCCGGAGCCGTTGTCG-3'	104	62.9 63.1	65.0 65.0

CHAPTER 4

RESULTS

4.1 Cell viability in serum-free medium

MTT analysis was carried out to determine the influence of serum-free medium and methanol extract of *T. indica* fruit pulp on viability of HepG2 cells. Our results showed no significant difference in viability of cells that were grown in complete or serum-free media (Figure 4.1). Viability of HepG2 cells that were grown in the presence of the methanol extract of the *T. indica* fruit pulp at a final concentration of 60 µg/ml was also not significantly different from those grown in the control serum-free medium in the presence of 0.02 % (v/v) DMSO (Figure 4.1).

4.2 Proteomic analyses of secreted proteins and cell lysate of HepG2 cells

4.2.1 Optimisation of 2D-GE for secreted proteins and cell lysate

In order to obtain a good representation of the HepG2 proteome, the optimum protein quantity to be loaded per gel was optimised. Ideally the proteins should be well separated and of high resolution with minimal streaking. Based on these, the optimum protein amount to be loaded per gel was determined to be 40 µg for both secreted proteins (Figure 4.2) and cell lysate (Figure 4.3).

The sample was first cleaned-up using the 2D-cleanup kit (GE Healthcare, USA) to minimise the streaking at the acidic pH region of the gel caused by excessive salt in the cell lysate protein sample (Figure 4.4A). DeStreak reagent (GE Healthcare, USA) was also added to the rehydration solution to reduce the gap in the basic pH region of the gel which was caused by non-specific oxidation of the proteins (Figure 4.4B).

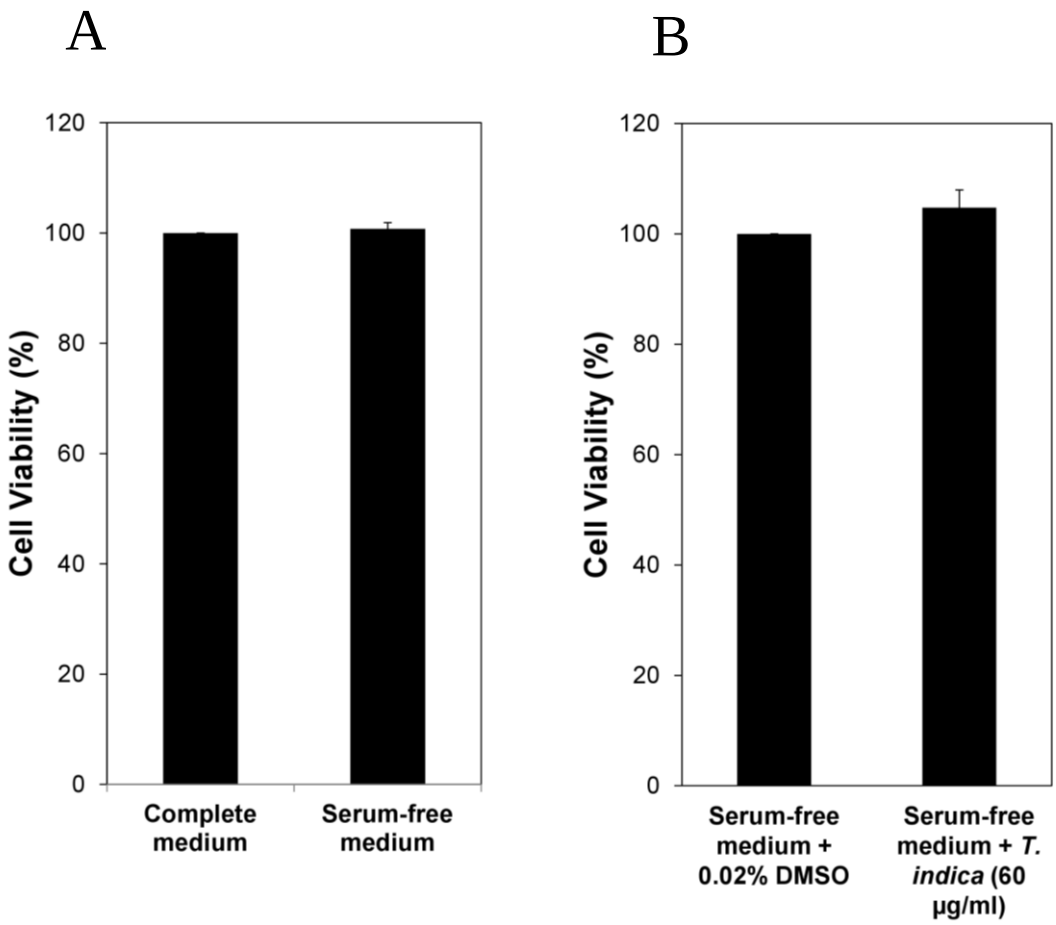

Figure 4.1: MTT analysis to assess the cell viability of HepG2 cells in A) serum and serum-free media B) control (serum-free medium + 0.02% DMSO) and 0.06 mg/ml methanol extract of *T. indica* fruit pulp in serum-free condition. All data are expressed as mean ± S.E.M. (standard error of means).

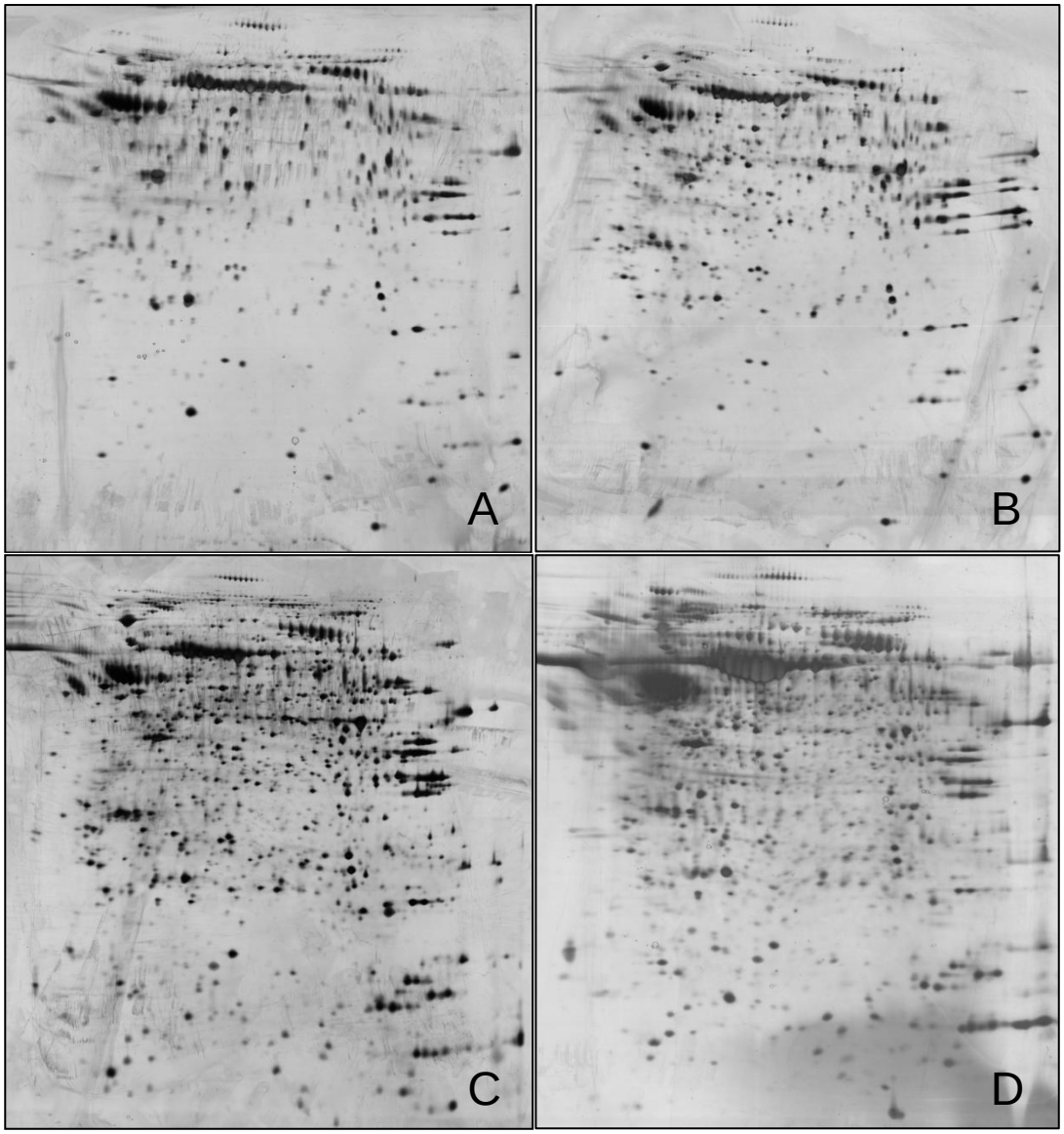

Figure 4.2: Optimisation of protein amount to be loaded per gel for secreted protein

A) 20 µg; B) 30 µg; C) 40 µg (optimum); D) 50 µg.

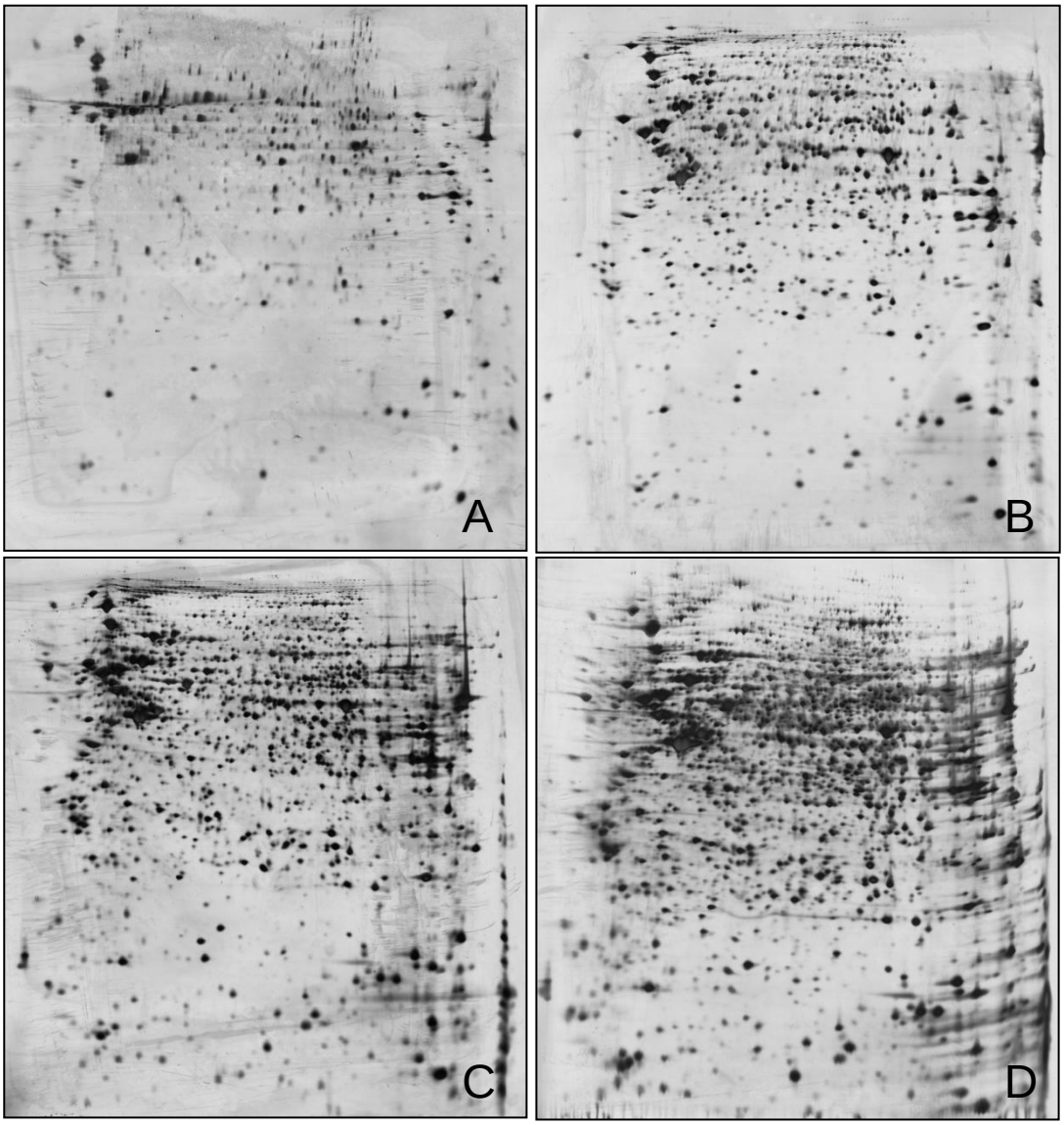

Figure 4.3: Optimisation of protein amount to load per gel for cell lysate protein

A) 20 µg; B) 30 µg; C) 40 µg (optimum); D) 60 µg.

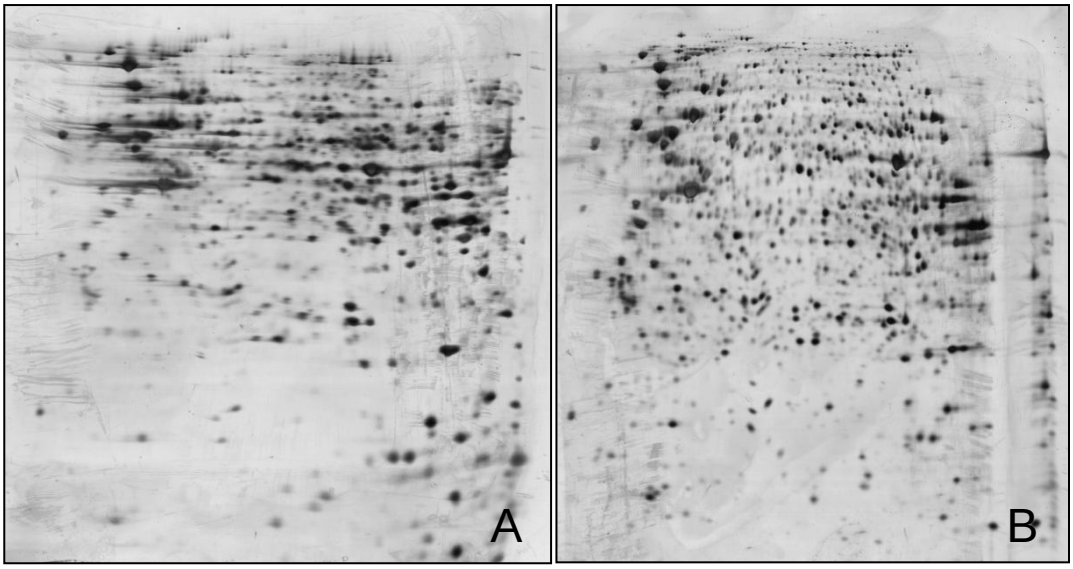

Figure 4.4: Minimising streaking in the 2D-GE of HepG2 cell lysate proteins

A) Streaking at the acidic pH region of the gel caused by excessive salt in protein sample; B) Gap at the basic pH region of the gel caused by non-specific oxidation of proteins.

4.2.2 2D-GE of secreted proteins and cell lysate proteins

4.2.2.1 2D-GE of secreted proteins

Forty micrograms of secreted proteins extracted from control and treated HepG2 cells were separated on a 13 cm pH 3-10 non-linear IPG strips for the first dimension and subsequently on a 12.5 % SDS-PAGE for the second dimension (n = 4 gels derived from 4 individual culture flasks for each group; total n = 8 gels). A total of approximately 1500 spots were detected for each gel (Figure 4.5).

4.2.2.2 2D-GE of cell lysate proteins

Similarly, 40 µg of cell lysate extracted from control and treated HepG2 cells were separated using the same running profiles and conditions as the secreted proteins (n = 6 gels derived from 3 individual culture flasks for each group, 2 technical replicates of each individual culture flasks; total n = 12 gels). A total of approximately 2500 spots were detected for each gel (Figures 4.6 and 4.7).

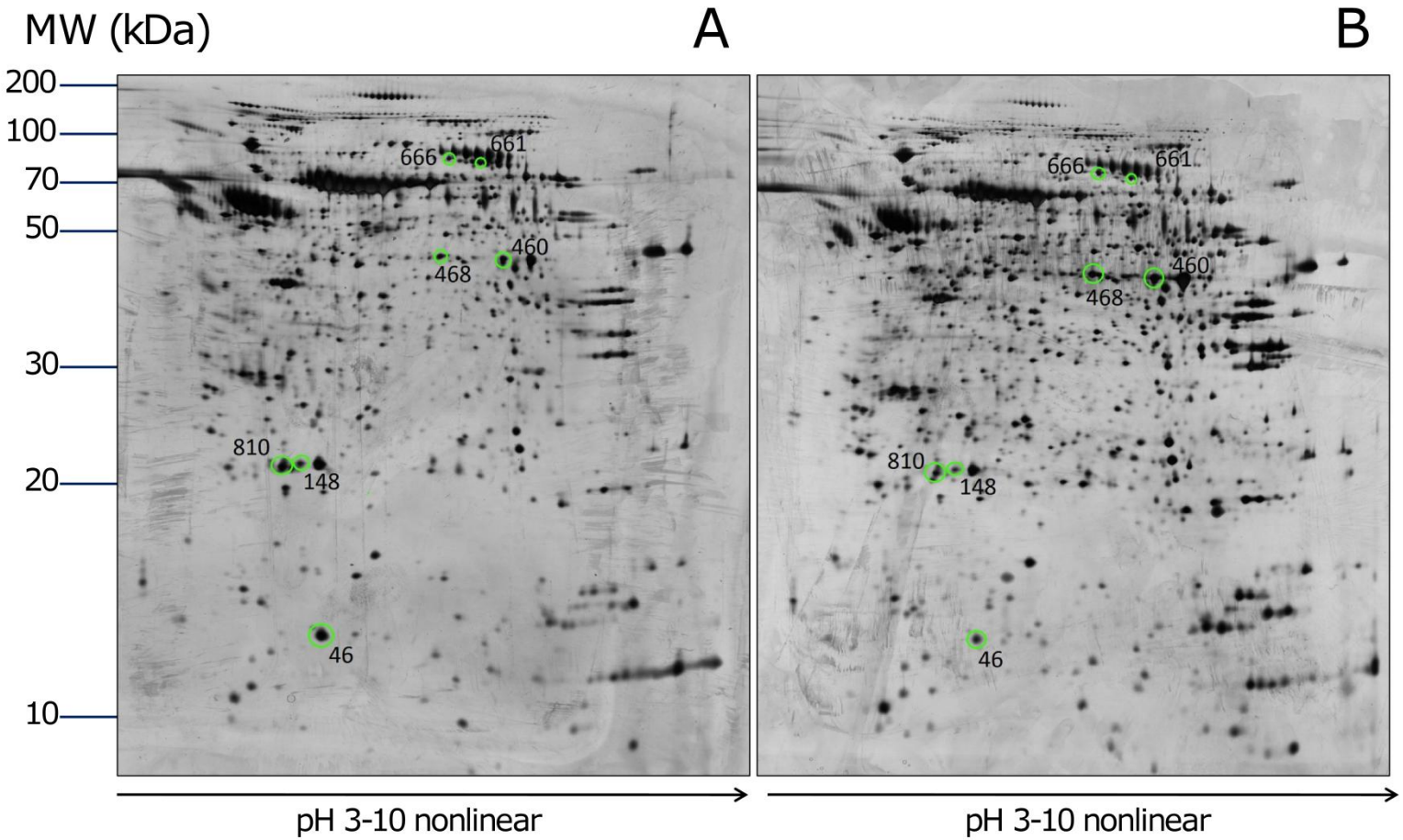

Figure 4.5: 2D-GE of secretomes of HepG2 cells of A) control; B) treatment with 0.06 mg/ml methanol extract of *T. indica* fruit pulp. Approximately 1500 spots per gel within the pH 3-10 range for secretome of HepG2 cells were detected. Seven spots (circled and labelled) were differentially expressed (adjusted $p < 0.03125$) in which 4 were significantly up-regulated and 3 were significantly down-regulated.

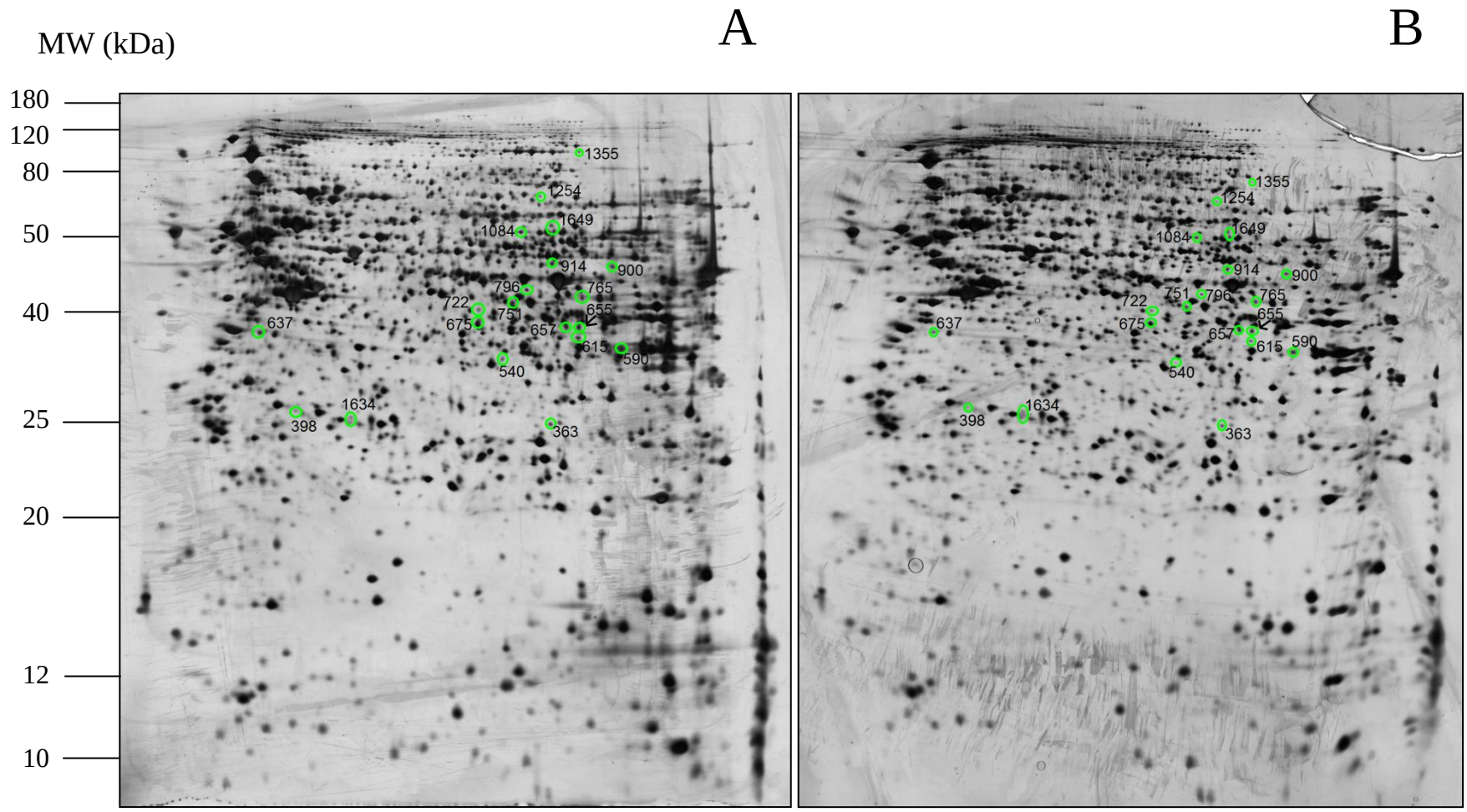

Figure 4.6: 2D-GE of cell lysate of HepG2 cells of A) control; B) treatment with 0.06 mg/ml methanol extract of *T. indica* fruit pulp. Approximately 2500 spots per gel within the pH 3-10 range for cell lysate of HepG2 cells were detected. Twenty spots (circled and labelled) were all significantly down-regulated ($p < 0.05$).

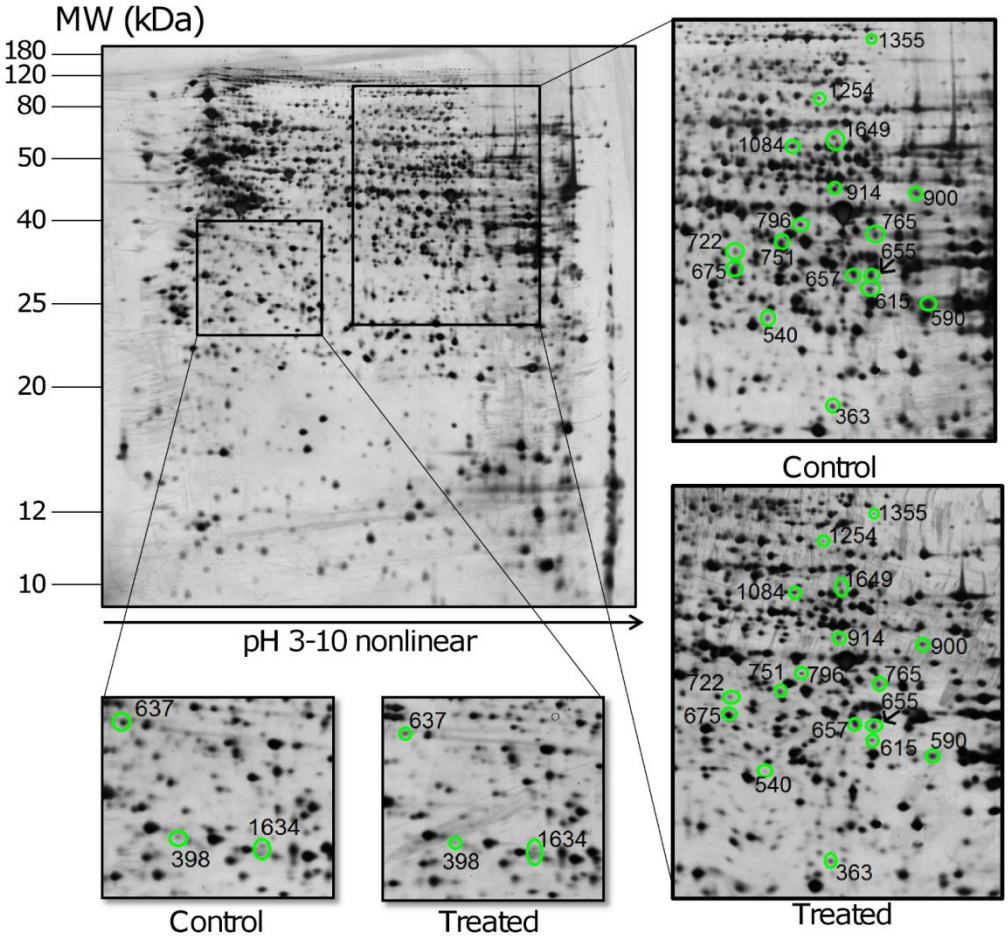

Figure 4.7: An enlarged proteome map of HepG2 cell lysate.

4.2.3 Gel image analyses

4.2.3.1 Secretome analysis

Using ImageMaster 2D Platinum V 7.0 software, the percentage of volume for each spot was obtained and the fold change was acquired. Spots that were significantly regulated at more than 1.5 fold ($p < 0.05$) and must be present in all 4 gels were filtered out. Based on these criteria, 9 protein spots were differentially expressed in which 4 were significantly up-regulated and 5 were significantly down-regulated. The spots were further subjected to false discovery rate (FDR) analysis and two spots were found to be false-positive based on the Benjamini-Hochberg's method (Benjamini, 1995), leaving only 7 spots to be truly differentially expressed (Table 4.1).

4.2.3.2 Cell lysate analysis

Using the same software, approximately 2500 spots were detected in each gel. After acquiring the percentage of volume for each spot, the fold change for each spot was obtained. Spots that are significantly regulated at more than 1.5 fold ($p < 0.05$) and must be present in at least 5 gels were filtered out. Based on these criteria, 20 spots were significantly altered and they were all down-regulated (Table 4.2).

Table 4.1: Average percentage of volume of spots, adjusted *p*-values and the fold change of secreted proteins in *T. indica*-treated cells versus control.

Spot ID	Average Percentage of Volume ± SEM		Adjusted *p*-value ($p < 0.03125$)	Fold Change
	Control	Treated		
46	0.731 ± 0.205	0.289 ± 0.136	0.0113	-2.5
148	0.214 ± 0.014	0.096 ± 0.014	< 0.001	-2.2
460	0.292 ± 0.103	0.613 ± 0.194	0.0265	+2.1
468	0.175 ± 0.023	0.349 ± 0.098	0.0136	+2.0
661	0.034 ± 0.014	0.070 ± 0.012	0.0084	+2.1
666	0.045 ± 0.021	0.113 ± 0.014	0.0018	+2.5
810	0.475 ± 0.095	0.278 ± 0.088	0.0227	-1.7

Table 4.2: Average percentage of volume of spots, *p*-values and the fold change of cell lysate proteins in *T. indica*-treated cells versus control.

Spot ID	Average Percentage of Volume ± SEM		*p*-value ($p < 0.05$)	Fold Change
	Control	Treated		
363	0.0273 ± 0.0016	0.0187 ± 0.0019	0.0052	-1.5
398	0.0214 ± 0.0021	0.0134 ± 0.0020	0.0173	-1.6
540	0.0128 ± 0.0019	0.0073 ± 0.0011	0.0291	-1.8
590	0.0892 ± 0.0073	0.0609 ± 0.0039	0.0066	-1.5
615	0.0240 ± 0.0023	0.0157 ± 0.0016	0.0133	-1.5
637	0.0254 ± 0.0033	0.0156 ± 0.0011	0.0158	-2.0
655	0.0499 ± 0.0033	0.0328 ± 0.0056	0.0250	-1.5
657	0.0655 ± 0.0088	0.0360 ± 0.0035	0.0109	-1.8
675	0.0660 ± 0.0067	0.0440 ± 0.0017	0.0094	-1.5
722	0.0223 ± 0.0022	0.0144 ± 0.0014	0.0127	-1.5
751	0.0420 ± 0.0038	0.0235 ± 0.0027	0.0028	-1.8
765	0.0470 ± 0.0031	0.0315 ± 0.0040	0.0123	-1.5
796	0.0162 ± 0.0011	0.0093 ± 0.0009	0.0006	-1.7
900	0.0394 ± 0.0048	0.0206 ± 0.0044	0.0163	-1.9
914	0.0279 ± 0.0015	0.0177 ± 0.0012	0.0003	-1.6
1084	0.0305 ± 0.0015	0.0209 ± 0.0017	0.0017	-1.5
1254	0.0154 ± 0.0018	0.0078 ± 0.0007	0.0023	-2.0
1355	0.0107 ± 0.0018	0.0054 ± 0.0010	0.0253	-2.0
1634	0.0746 ± 0.0125	0.0435 ± 0.0031	0.0359	-1.7
1649	0.0639 ± 0.0069	0.0416 ± 0.0032	0.0149	-1.5

4.2.4 Identification of differentially expressed proteins

4.2.4.1 Secretome

Among the seven protein spots that were altered in expression, five spots (protein spot ID: 46, 148, 460, 468 and 810) were identified by mass spectrometry and database search. Spots 661 and 666 were not successfully identified by MS/MS analysis as their scores were lower than the cut off value for positive inclusion criteria. This could probably be due to their close proximity to the high abundant proteins which hinder the detection of their peptides. The five differentially expressed proteins that were identified include transthyretin (TTR – spot 46), two isoforms of apolipoprotein A-I (ApoA-I – spots 148 and 810), alpha enolase (ENO1 – spot 460) and rab GDP dissociation inhibitor beta (GDI-2 – spot 468) (Table 4.3). ENO1 and GDI-2 were apparently up-regulated by approximately 2-fold, while TTR was down-regulated by 2.5-fold. The two spots identified as ApoA-1 was down-regulated by 2.2- and 1.7-folds.

4.2.4.2 Cell lysate

Among the 20 protein spots that were significantly reduced in abundance, 14 were successfully identified by mass spectrometry and database search (Table 4.4). Six spots (protein spot ID 398, 615, 637, 1254, 1355 and 1649) were considered not successfully identified as their scores were lower than the cut-off value for positive inclusion criteria. The 14 identified proteins may be grouped according to their biological processes using the UniProt Protein Knowledgebase (UniProtKB). NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 10, ubiquinol-cytochrome-c reductase complex core protein 2 and NADH dehydrogenase (ubiquinone) flavoprotein 1 were grouped under “mitochondrial respiratory chain” category. Three proteins, i.e. eukaryotic translation initiation factor 3 subunit 3, elongation factor Tu and tyrosyl-tRNA synthetase are involved in “protein biosynthesis”. Another six proteins were

categorised under “metabolism”, with glyceraldehyde-3-phosphate dehydrogenase and GDP-L-fucose synthetase being involved in carbohydrate metabolism, GMP reductase 2 and UMP synthase in nucleotide and nucleoside metabolism, S-methyl-5-thioadenosine phosphorylase in polyamine metabolic process, and ethanolamine-phosphate cytidylyltransferase in biosynthesis of phospholipids. Prohibitin, on the other hand, was categorised under “cell proliferation and differentiation”, while heterogeneous nuclear ribonucleoprotein H3 did not belong to any of the above groups and therefore categorised as “others”.

Table 4.3: List of differentially expressed secreted proteins in *T. indica* fruit extract-treated cells identified by MALDI-MS/MS.

Spot no.	Protein description	SWISS-PROT Accession No.	MASCOT score	pI/MW (kDa)	Av. % of Vol. ratio[a]	% Cov[b]	Matched Peptide Sequences
46	Transthyretin precursor (TTR)	P02766	252	5.52/15.88	-2.5	18	42-54; 55-68; 56-68
148	Apolipoprotein A-I precursor (ApoA-I)	P02647	216	5.56/30.76	-2.2	35	121-130; 132-140; 185-195
460	Alpha enolase (ENO1)	P06733	257	7.01/47.14	+2.1	16	16-28; 33-50; 184-193; 240-253; 270-281; 407-412
468	Rab GDP dissociation inhibitor beta (GDI-2)	P50395	618	6.11/50.63	+2.0	47	36-54; 56-68; 69-79; 90-98; 143-156; 194-208; 211-218; 279-288; 300-309; 310-328; 391-402; 403-418; 424-436
810	Apolipoprotein A-I precursor (ApoA-I)	P02647	437	5.56/30.76	-1.7	33	52-64; 121-130; 132-140; 165-173; 185-195; 231-239; 251-262

[a] Positive value signifies up-regulation against control samples and negative value signifies down-regulation in terms of fold-differences. All ratio is statistically significant with $p < 0.05$ (Student's *t*-test).
[b] % Coverage of the identified sequence.

Table 4.4: List of cell lysate proteins of altered abundance in *T. indica* fruit extract-treated cells identified by MALDI-TOF/TOF MS/MS.

Spot no.	Protein description	Acc. No.	Score	*p*I/MW (kDa)	Av. % FC[a]	% Cov[b]	Matched peptide sequences	Functional category
657	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 10 (CI-42 kD)	O95299	194	8.67/ 40.73	–1.8	44	117-122; 13-139; 140-161; 253-261; 290-295	Mitochondrial respiratory chain
765	Ubiquinol-cytochrome-c reductase complex core protein 2, mitochondrial precursor (Coreprotein II)	P22695	537	8.74/ 48.41	–1.5	28	71-84; 163-183; 184-196; 200-217; 232-241	
900	NADH dehydrogenase (ubiquinone) flavoprotein 1, mitochondrial precursor (CI-51 kD)	P49821	275	8.51/ 50.79	–1.9	33	72-81; 153-159; 160-174; 376-386; 441-449	
796	Ethanolamine-phosphate cytidylyltransferase	Q99447	339	6.44/ 43.81	–1.7	38	185-199; 262-271; 333-348	Phospholipid biosynthesis

Table 4.4, continued

Spot no.	Protein description	Acc. No.	Score	*p*I/MW (kDa)	Av. % FC[a]	% Cov[b]	Matched peptide sequences	Functional category
590	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	P04406	192	8.57/ 36.03	–1.5	18	235-248; 310-323	Carbohydrate metabolism
722	GDP-L-fucose synthetase	Q13630	280	6.13/ 35.87	–1.5	49	26-44; 82-88; 90-107; 200-214; 291-297; 307-320	
655	GMP reductase 2	Q9P2T1	386	6.79/ 37.85	–1.5	52	70-78; 178-189; 192-213; 277-286; 278-286; 292-298; 292-306	Nucleotide and nucleoside metabolism
914	Uridine 5'-monophosphate synthase (UMP synthase)	P11172	379	6.81/ 52.20	–1.6	37	6-22; 30-41; 146-155; 353-363; 389-405; 460-467; 469-477	
363	S-methyl-5-thioadenosine phosphorylase (MTAP)	Q13126	253	6.75/ 31.23	–1.5	43	12-29; 72-82; 83-99; 100-116; 134-147; 181-187; 272-282	Polyamine metabolism
1634	Prohibitin	P35232	66	2.27/ 29.79	–1.7	21	134-143	Cell proliferation and differentiation

Table 4.4, continued

Spot no.	Protein description	Acc. No.	Score	*p*I/MW (kDa)	Av. % FC[a]	% Cov[b]	Matched peptide sequences	Functional category
675	Eukaryotic translation initiation factor 3 subunit 3 (elF3h)	O15372	279	6.09/ 39.91	–1.5	43	52-75; 242-249; 260-265; 304-313; 314-331	Protein biosynthesis
751	Elongation factor Tu, mitochondrial precursor (EF-Tu)	P49411	114	7.26/ 49.51	–1.8	29	105-120; 239-252	
1084	Tyrosyl-tRNA synthetase, cytoplasmic (TyrRS)	P54577	96	6.61/ 59.11	–1.5	21	85-93; 179-189; 433-450	
540	Heterogenous nuclear ribonucleoprotein H3 (hnRNP H3)	P31942	409	6.37/ 36.90	–1.7	39	56-67; 85-90; 98-104; 206-222; 223-232; 262-287; 288-301	Others

[a]Negative value signifies down-regulation in terms of fold-differences. All ratios are statistically significant with $p < 0.05$ (Student's *t*-test).

[b] % Coverage of the identified sequence.

4.2.5 Western blot analyses

To validate the effects of the *T. indica* fruit pulp extract on HepG2 proteins, Western blotting was performed using antisera raised against the cellular proteins. In view of the scarce amount of HepG2 cell lysate protein extract that was generated in this study, three proteins, i.e. ethanolamine-phosphate cytidylyltransferase (PCYT2), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 10 (NDUFA10) and ubiquinol-cytochrome-c reductase complex core protein 2 (UQCRC2) that represent those involved in the mitochondrial and metabolic activities were selected. Densitometry scanning of bands detected by the antisera showed that the abundance of PCYT2, NDUFA10 and UQCRC2 was indeed lower in HepG2 cells-treated *T. indica* fruit pulp extract compared to the controls, with fold differences of –1.7, –1.5 and –1.5, respectively (Figure 4.8).

4.2.6 Pathway interactions and biological process analysis

When the differentially expressed secreted proteins were analysed using IPA, the software identified "Lipid Metabolism, Molecular Transport and Small Molecule Biochemistry" as the sole putative network linking three of the differentially expressed proteins with other interactomes, with a score of 9. A score of 2 or higher indicates at least a 99% confidence of not being generated by random chance and higher scores indicate a greater confidence. When both the differentially expressed secreted proteins and cell lysate proteins were included in the analysis, the same network showed an even higher score, with a score of 31. The combination of both sets of data signifies that lipid metabolism may be the main system being regulated after treatment with *T. indica* fruit extract. Figures 4.9 and 4.10 show a graphical representation of the predicted molecular relationships between the regulated proteins. A canonical pathway analysis ranked the LXR/RXR activation with the highest significance ($p < 1.29 \times 10^{-04}$; Figure 4.11).

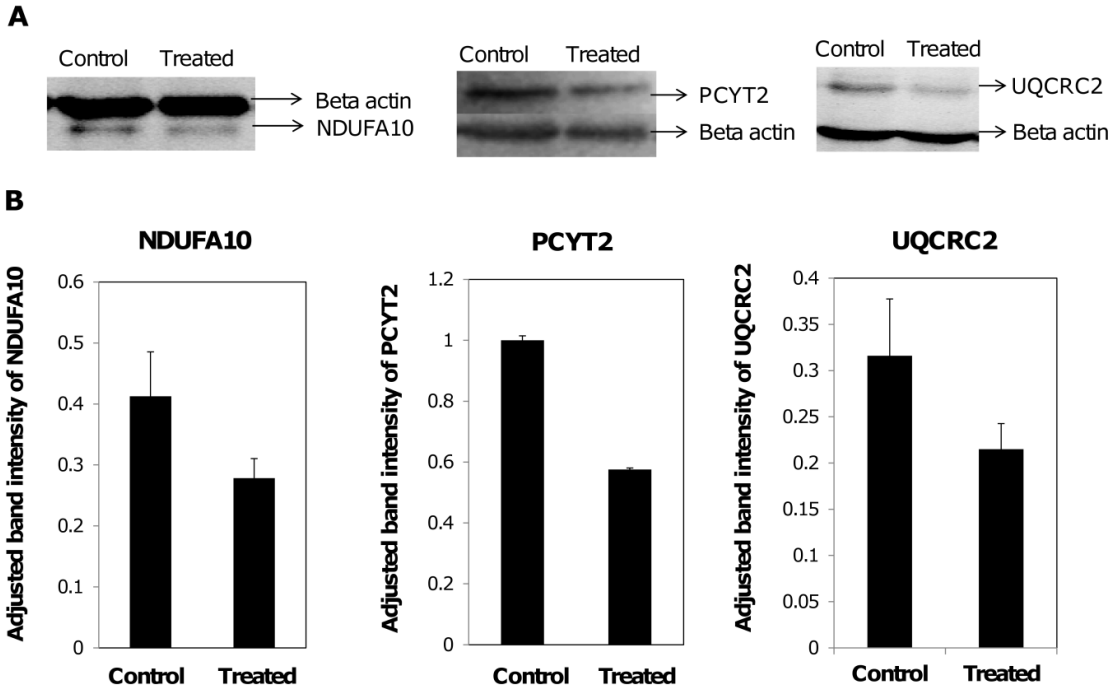

Figure 4.8: Western blot analyses of NDUFA10, PCYT2 and UQCRC2

A) Western blot cropped images of NDUFA10, PCYT2, UQCRC2 and beta actin bands detected by antisera against the respective proteins; B) Densitometry analyses of Western blot using ImageJ software. Assay was done in triplicate and data were represented as mean ± standard deviation.

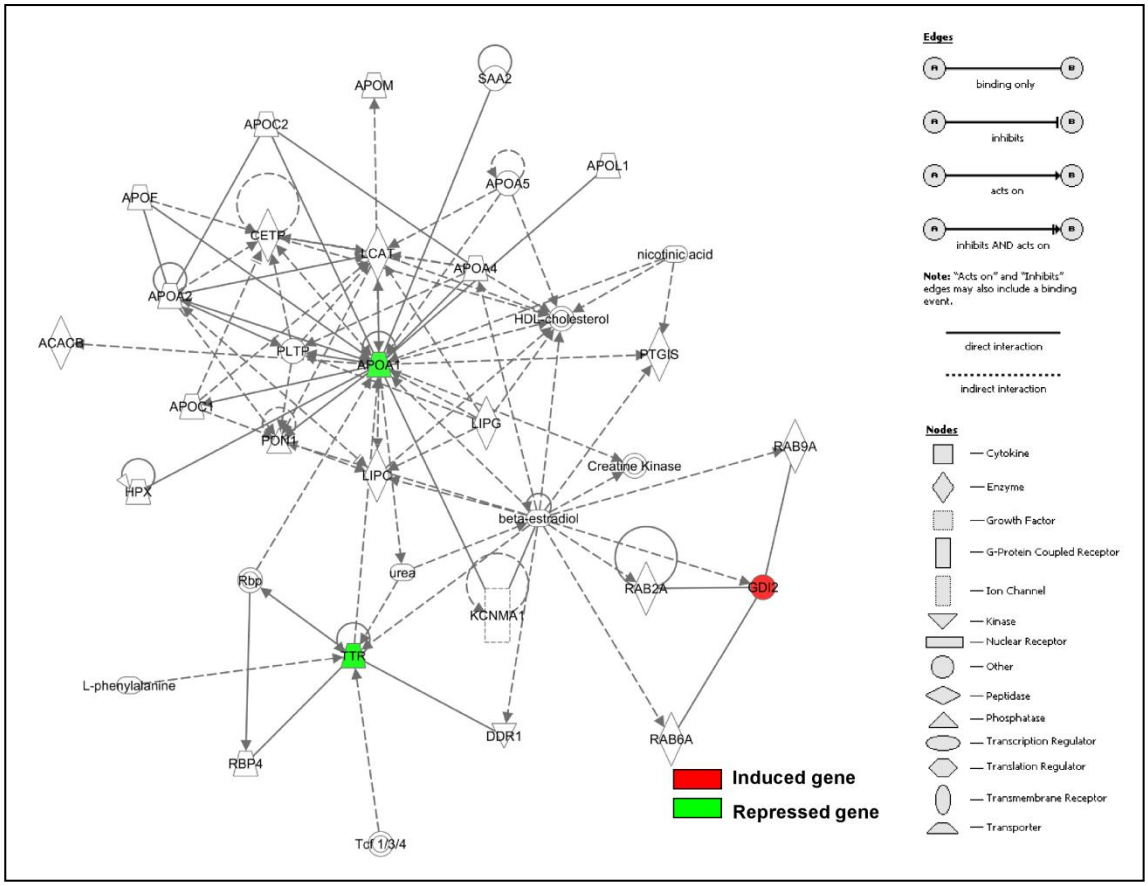

Figure 4.9: IPA graphical representation of the molecular relationships between differentially expressed secreted proteins in HepG2 cells treated with *T. indica* fruit extract

The network is displayed graphically as nodes (proteins) and edges (the biological relationships between the nodes). Nodes in red indicate up-regulated proteins while those in green represent down-regulated proteins. Nodes without colours indicate unaltered expression. Various shapes of the nodes represent the functional class of the proteins. The different arrow shapes represent different types of interactions. Edges are displayed with various labels that describe the nature of the relationship between the nodes. Names of proteins corresponding to the abbreviations are as follows: APOA1, Apolipoprotein A-1; APOA2, Apolipoprotein A-2; APOA4, Apolipoprotein A-4; APOA5, Apolipoprotein A-5; APOC1, Apolipoprotein C-1; APOC2, Apolipoprotein C-2; APOE, Apolipoprotein E; APOM, Apolipoprotein M; APOL1, Apolipoprotein L-1; ACACB, acetyl-CoA-carboxylase 2; SAA2, serum amyloid A2; LCAT, lecithin

cholesterol acyltransferase; PLTP, phospholipid transfer protein; CETP, cholesterylester transfer protein; PTGIS, prostaglandin I synthase; RAB9A, Ras-related protein Rab 9A; RAB6A, Ras-related protein Rab 6A; GDI2, Rab GDP dissociation inhibitor beta; RAB2A, Ras-related protein Rab 2A; KCNMA1, Potassium large conductance calcium-activated channel, subfamily M, alpha member 1; DDR1, discoidin domain receptor tyrosine kinase 1; TTR, transthyretin; RBP4, retinol binding protein 4; LIPC, hepatic triglyceride lipase; LIPG, endothelial lipase; PON1, paraoxonase 1; HPX, haemopexin; Tcf 1/2/3, T-cell factor -1, -2, -3; Rbp, retinol binding proteins.

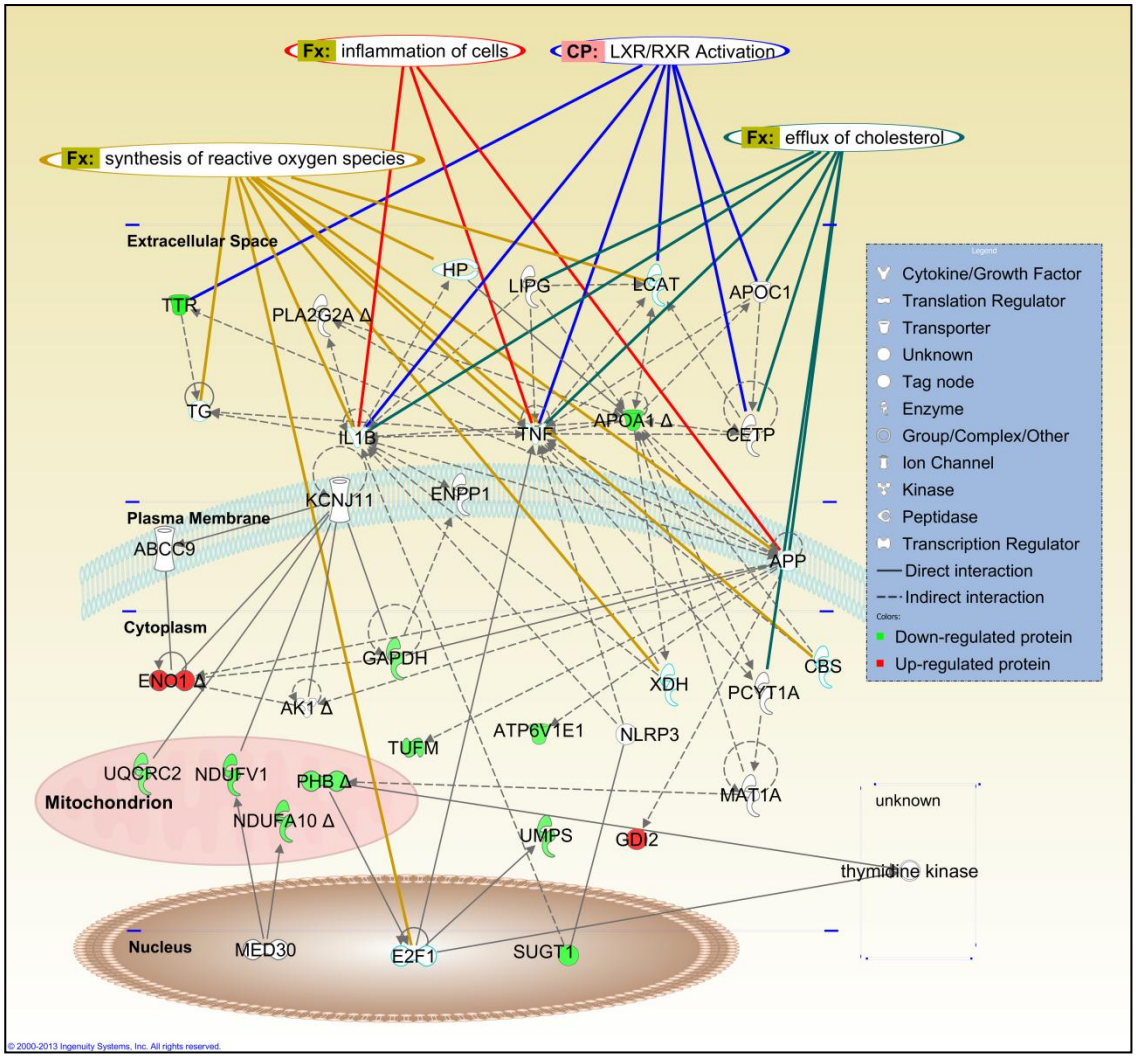

Figure 4.10: IPA graphical representation of the molecular relationships between HepG2 secreted and cytosolic proteins after treatment

The network is displayed graphically as nodes (proteins) and edges (the biological relationships between the nodes). Nodes in red indicate up-regulated proteins while those in green represent down-regulated proteins. Nodes without colours indicate unaltered expression. Various shapes of the nodes represent functional class of the proteins. Edges are displayed with various labels that describe the nature of the relationship between the nodes. Transthyretin, TTR; thyroglobulin, TG; interleukin-1 beta, IL1B; tumour necrosis factor, TNF; apolipoprotein A-1, APOA1; apolipoprotein C-1, APOC1; lecithin cholesterol acyltransferase, LCAT; endothelial lipase, LIPG; haptoglobin, HP; phospholipase A2, PLA2G2A; cholesterylester transfer protein, CETP; ATP-binding cassette transporter sub-family C member 9, ABCC9; ATP-

sensitive inward rectifier potassium channel 11, KCNJ11; ectonucleotide pyrophosphatase/phosphodiesterase family member 1, ENPP1; amyloid precursor protein, APP; glyceraldehyde-3-phosphate dehydrogenase, GAPDH; alpha enolase, ENO1; adenylate kinase, AK1; ubiquinol-cytochrome-c reductase complex core protein 2, UQCRC2; xanthine dehydrogenase, XDH; cystathionine beta-synthase, CBS; methionine adenosyltransferase I, alpha, MAT1A; rab GDP dissociation inhibitor beta, GDI2; NLR (nucleotide-binding domain and leucine rich repeat containing family) family, pyrin domain containing 3, NLRP3; Vacuolar ATP synthase subunit E, ATP6V1E1; elongation factor Tu, TUFM; prohibitin, PHB; choline-phosphate cytidylyltransferase A, PCYT1A; uridine 5'-monophosphate synthase, UMPS; NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 10, NDUFA10; NADH dehydrogenase (ubiquinone) flavoprotein 1, NDUFV1; mediator of RNA polymerase III transcription subunit 30, MED30; transcription factor E2F1, E2F1; suppressor of G2 allele of SKP1 homolog, SUGT1.

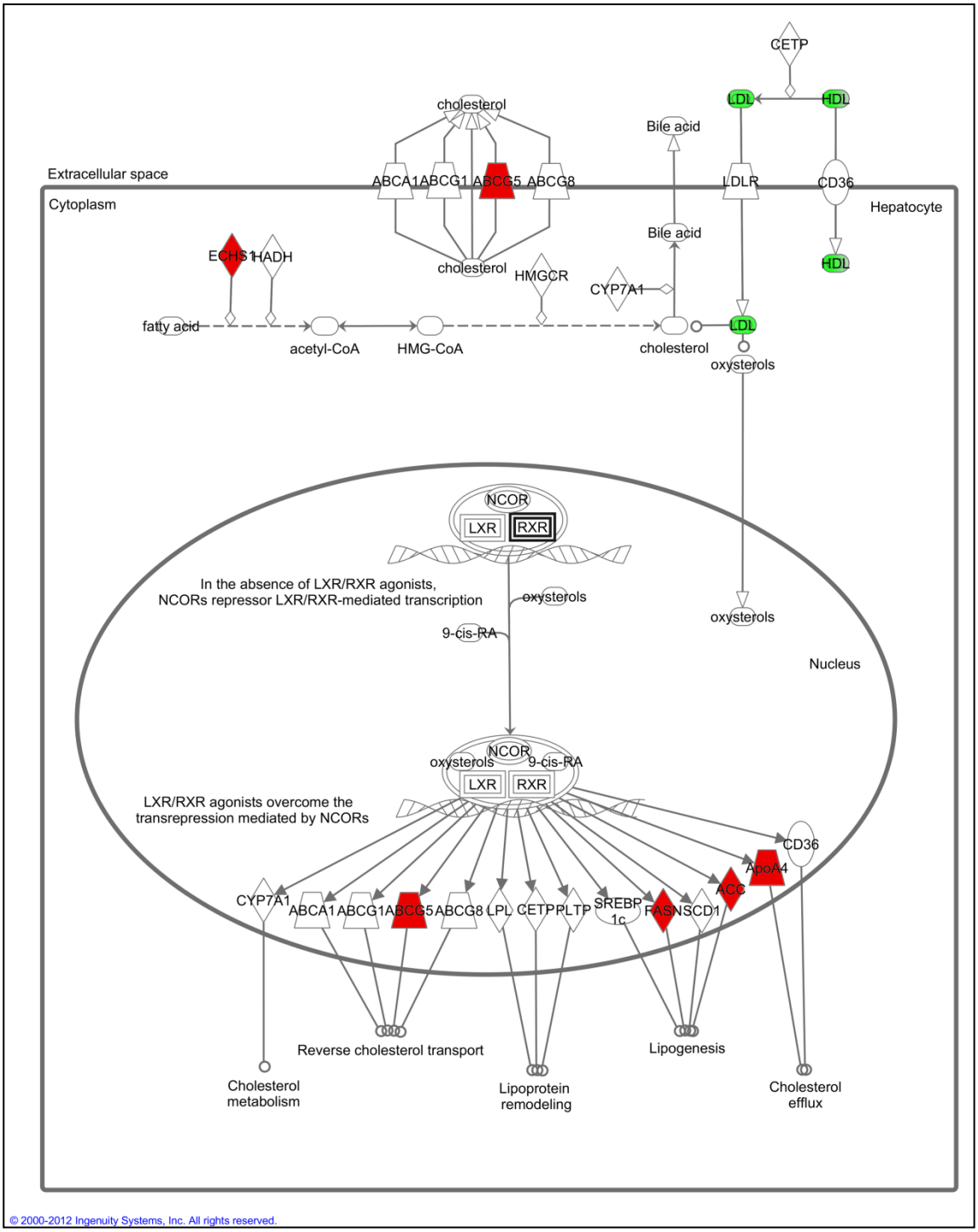

Figure 4.11: Predicted canonical pathway affected by *T. indica* fruit extract

IPA identified 'LXR/RXR activation' as the canonical pathway with the highest predicted potential/significance of being affected by the altered levels of TTR, ApoA-I and GDI-2 in HepG2 cells that were treated with *T. indica* fruit extract. Lines between the proteins represent known interactions. Red nodes indicate over expression of genes induced by the extract, which was based on our previous report (Razali, et al., 2010).

Abbreviation: ABCA1, ATP-binding cassette sub-family A member 1; ABCG1, ATP-binding cassette sub-family G member 1; ABCG5, ATP-binding cassette sub-family G member 5; ABCG8, ATP-binding cassette sub-family G member 8; ECHS, enoyl CoA hydratase; HADH, hydroxyacyl-CoA dehydrogenase; HMGCR, HMG-CoA reductase; CYP7A1, cytochrome P450, family 7, subfamily A, polypeptide 1; LDLR, low density lipoprotein receptor; CETP, cholesterylester transfer protein; CD36, cluster of differentiation 36; NCOR, nuclear receptor corepressor; LXR, liver X receptor; RXR, retinoid X receptor; 9-cis-RA, 9-cis-retinoic acid; LPL, lipoprotein lipase; PLTP, phospholipid transfer protein; SREBP-1c, sterol regulatory element-binding protein 1c; FASN, fatty acid synthase; SCD1, stearoyl-conzyme A desaturase 1; ACC, acetyl-CoA carboxylase; APOA4, apolipoprotein A-4; LDL, low density lipoprotein; HDL, high density lipoprotein.

4.3 *In vitro* evaluation of hypolipidaemic properties of *T. indica* fruit pulp extract

To further investigate the lipid-lowering effects of *T. indica* fruit pulp, the following lipid studies were conducted where the lipid amount was quantified enzymatically as well as examined optically with Oil Red O staining after treatments. The mechanism of action was investigated by analysing the gene expression incurred by the treatment using DNA microarray and software analyses.

In this study, HepG2 cells were induced to develop steatosis by treatment with palmitic acid. There were 4 treatment groups in this study, HepG2 cells treated with palmitic acid only (PA), cells treated with a lipid-lowering drug, fenofibrate and palmitic acid (FF+PA), cells treated with methanol extract of *T. indica* fruit pulp and palmitic acid (TI+PA) and cells treated with 0.02 % DMSO (vehicle) only (control). The triglyceride and cholesterol levels were quantified using enzyme assays and the lipid droplets in the cells were visualised using Oil Red O staining. The total cellular RNA was then extracted for transcriptomic studies using DNA microarray. The gene expressions were then analysed using data mining software to elucidate the mechanism of action of *T. indica* lipid-lowering effect.

4.3.1 Cell viability and Oil Red O staining of HepG2 cells in different concentrations of palmitic acid and *T. indica* fruit extract

In order to determine the concentration of palmitic acid to best induce steatosis in HepG2 cells without causing extensive cell death, MTT assay was performed and lipid droplets were stained using Oil Red O staining. In this study, different concentrations of palmitic acid ranging from 0.2 to 1 mM were tested for 24 and 48 h. The MTT assay showed a concentration-dependent reduction in cell viability upon treatment with palmitic acid, with 48 h treatment showing a more prominent cell death than 24 h (Figure 4.12). Oil Red O staining of the lipid droplets in HepG2 cells showed a dose-dependent increase of lipid droplet size and number (Figure 4.13).

Similarly, the viability of HepG2 cells treated with 0.3 mM palmitic acid and different concentrations of *T. indica* fruit extract was also determined using MTT assay (Figure 4.14). The assay showed that generally the cells were more than 90 % viable when they were treated with concentrations of less than 0.2 mg/ml *T. indica* fruit extract. The Oil Red O staining of lipid droplets of HepG2 cells showed that the lipid droplets were fewer in number and smaller in size in fenofibrate-treated HepG2 cells compared to cells treated with palmitic acid only. As for the *T. indica* fruit-treated cells, Oil Red O staining showed a dose-dependent reduction in lipid droplets. However, when the cells were treated with 0.3 mg/ml *T. indica* fruit extract, the lipid droplets appeared to be more abundant as compared to the 0.2 mg/ml *T. indica* fruit-treated cells (Figure 4.15).

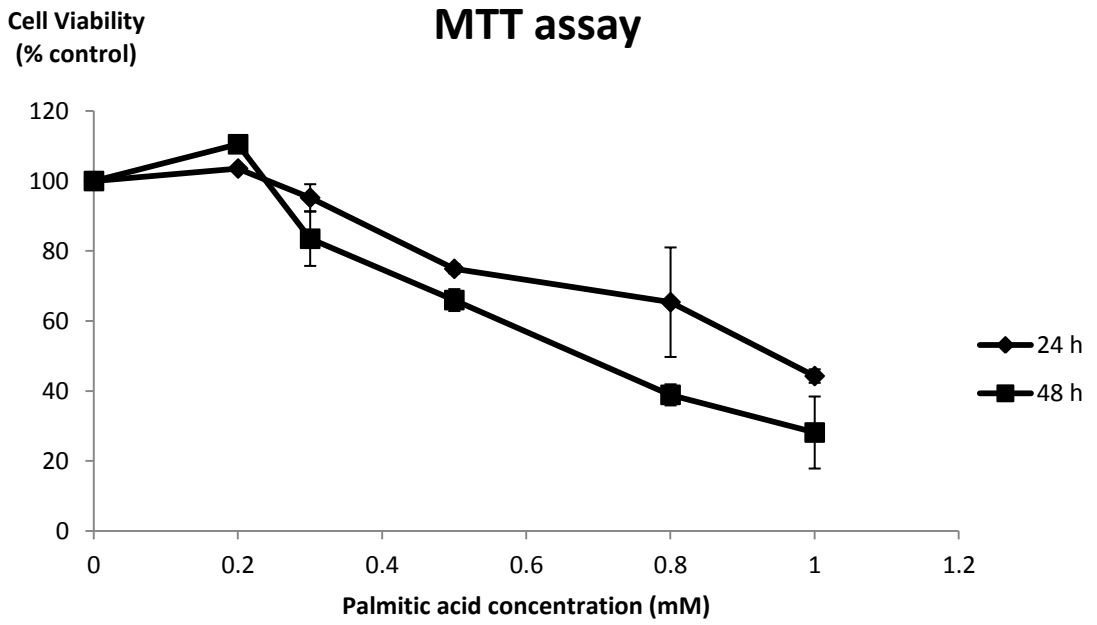

Figure 4.12: MTT analysis to assess the viability of HepG2 cells treated with different concentrations of palmitic acid after 24 and 48 h.

Assay was done in triplicate and all data were expressed as mean ± SD (standard deviation).

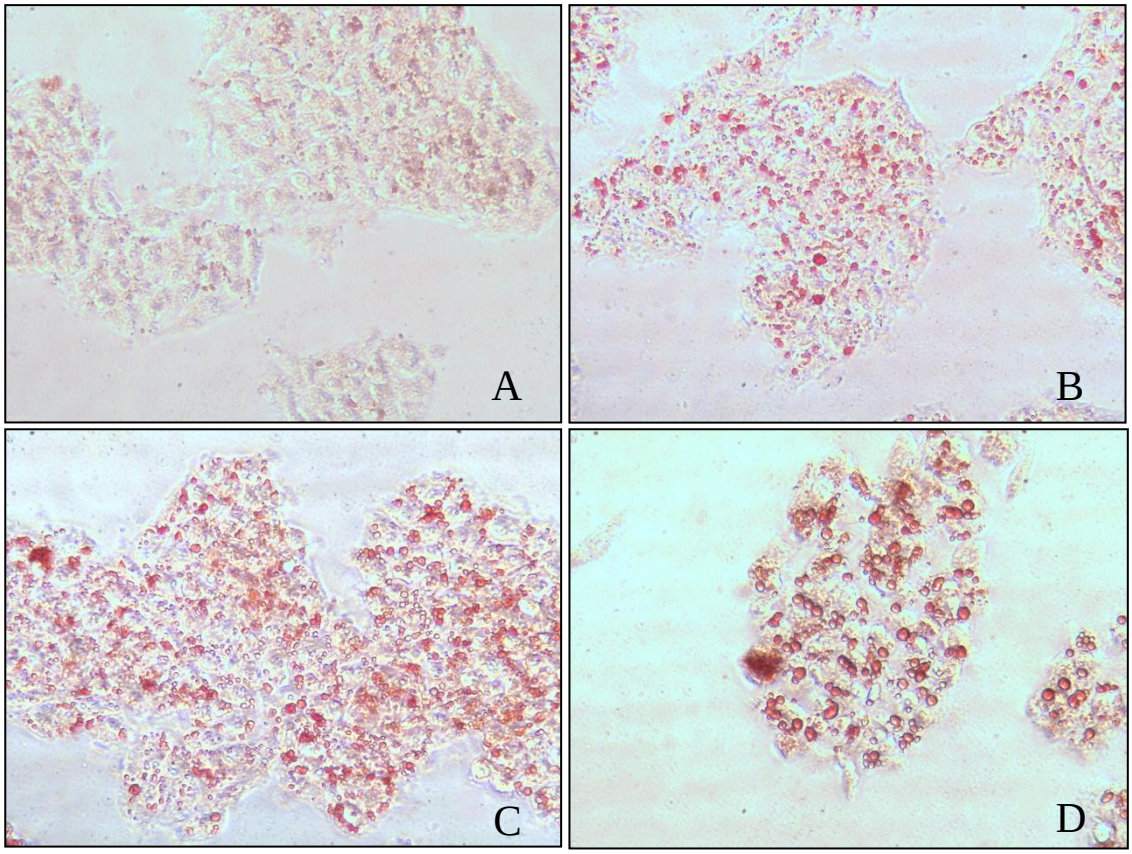

Figure 4.13: Oil Red O staining of lipid droplets in HepG2 cells treated with different concentrations of palmitic acid

HepG2 cells were treated with A) fatty acid-free bovine serum albumin only; B) 0.2 mM palmitic acid; C) 0.3 mM palmitic acid and D) 0.8 mM palmitic acid for 24 h. There was a dose-dependent increment in number of lipid droplets as well as the sizes.

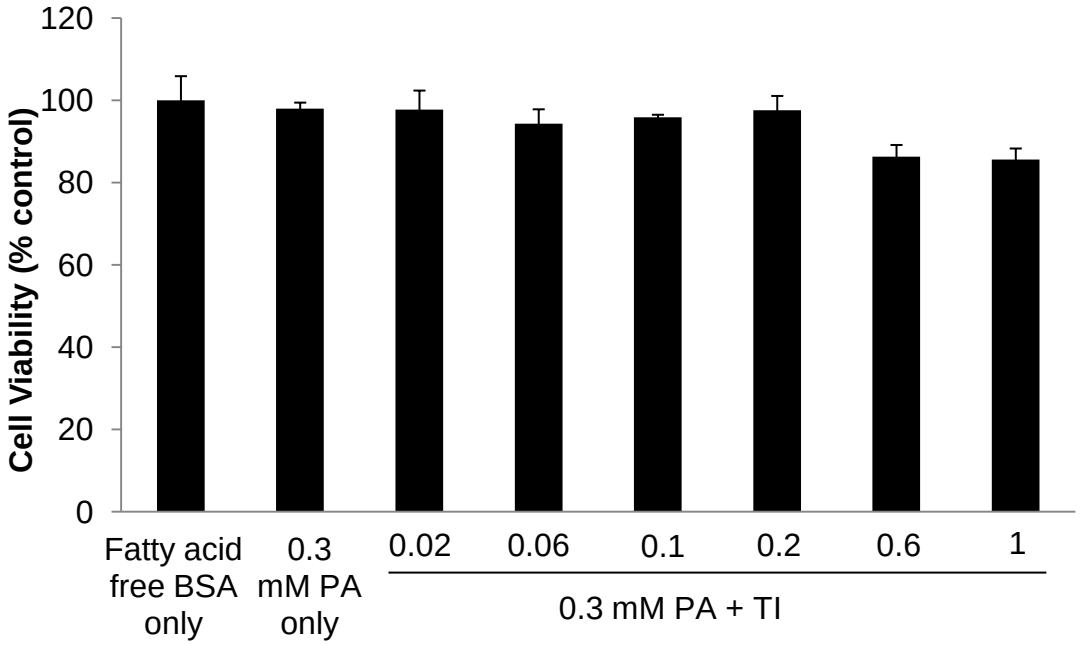

Figure 4.14: MTT assay of HepG2 cells treated with different concentrations of *T. indica* fruit extract (TI) and 0.3 mM palmitic acid (PA) for 24 h.

Assays were done in triplicates and data were represented as mean ± standard deviation.

Abbreviation: PA- Palmitic acid, TI- *T. indica* fruit extract

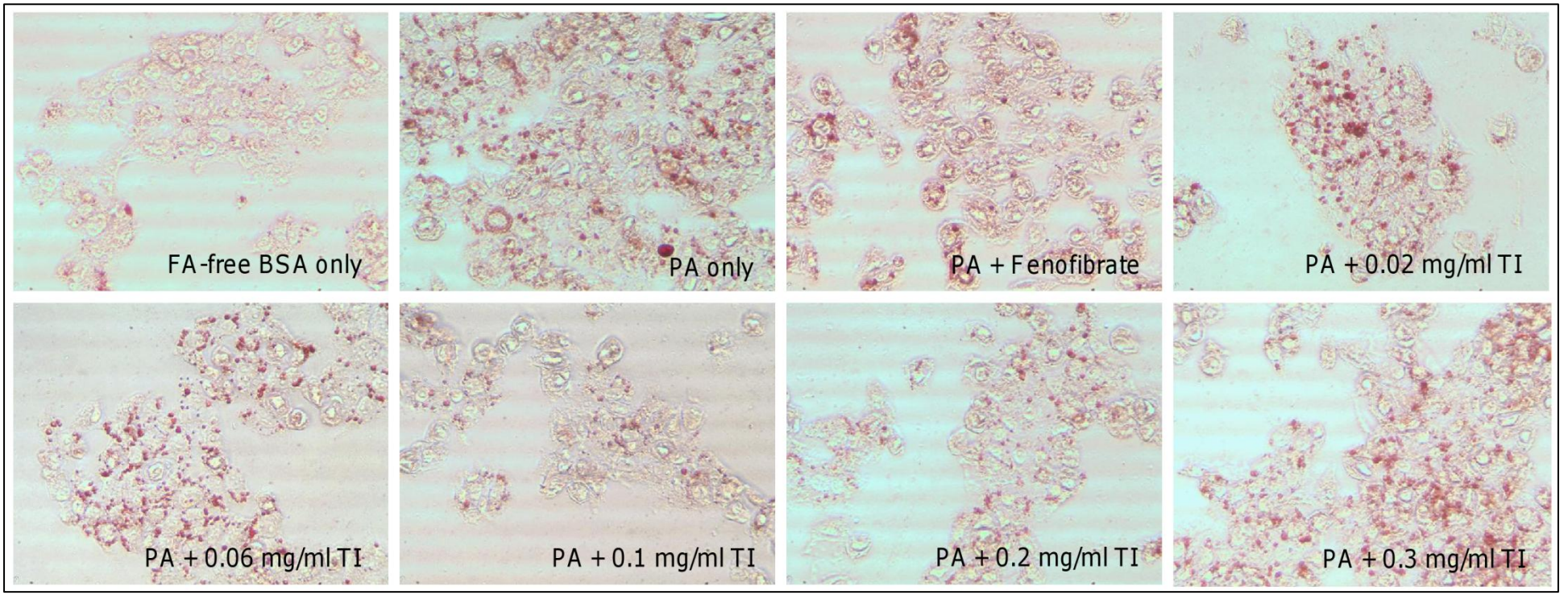

Figure 4.15: Oil Red O staining of lipid droplets in HepG2 cells treated with fenofibrate and different concentrations of *T. indica* fruit extract

Lipid droplets were visible after treatment with palmitic acid (PA only), and the amount of droplets reduced after treatment with fenofibrate (PA+fenofibrate). The reduction in droplet amount was dose dependent in treatment with 0.02 mg/ml to 0.2 mg/ml *T. indica* (PA+0.02 mg/ml TI – PA+0.2 mg/ml TI). At 0.3 mg/ml *T. indica* treatment (PA+0.3 mg/ml TI), the lipid droplets increased in size and quantity.

Abbreviation: PA- palmitic acid; TI- *T. indica* fruit extract

4.3.2 Total triglyceride and cholesterol quantification

Total triglyceride and total cholesterol were also quantified in HepG2 cells treated with *T. indica* fruit extract and palmitic acid, as shown in Figures 4.16 and 4.17, respectively. Total triglyceride in palmitic acid-treated HepG2 cells was reduced significantly ($p < 0.05$) by around 35 % in the presence of fenofibrate, a commonly used hypolipidaemic drug. The *T. indica* fruit pulp extract at the concentrations of 0.1 mg/ml and 0.2 mg/ml, were able to significantly ($p < 0.01$) reduce the total triglyceride level to a level comparable to that of fenofibrate. However, at the concentration of 0.3 mg/ml of *T. indica* fruit extract, the total triglyceride level was increased by 36 % when compared to 0.2 mg/ml *T. indica* fruit-treated cells (Figure 4.15).

Total cholesterol in HepG2 cells treated with 0.3 mM palmitic acid and different concentrations of *T. indica* fruit extract also showed a similar trend whereby the total cholesterol increased at higher concentrations of *T. indica* fruit extract treatment (Figure 4.17). Total cholesterol was reduced by around 25 % after treatment with fenofibrate. The cholesterol lowering effect of the fruit was not significant as compared to the triglyceride lowering effect. At 0.05 mg/ml *T. indica* fruit treatment, the total cholesterol was reduced by around 9 %, while at 0.1 mg/ml *T. indica* fruit treatment, the total cholesterol was reduced by around 18 %, and decreased to 8 % at 0.2 mg/ml *T. indica* fruit extract treatment. At 0.3 mg/ml *T. indica* fruit concentration, the total cholesterol increased by 12 %.

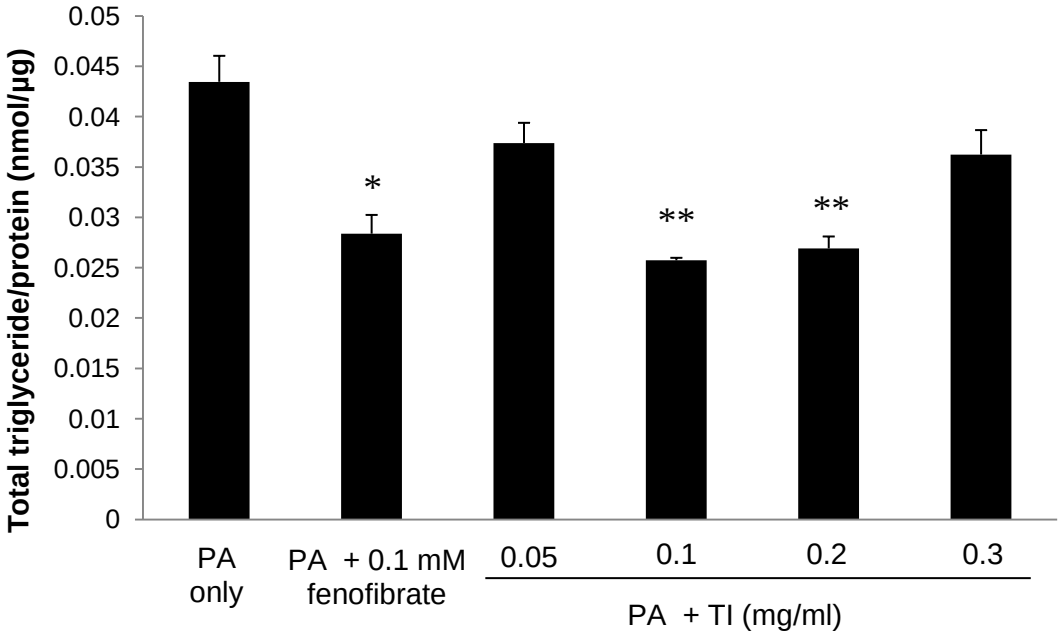

Figure 4.16: Measurement of total triglyceride in HepG2 cells after treatment

Assays were done in triplicates and data were represented as mean ± standard deviation.

*$p < 0.05$ and ** $p < 0.01$ when compared to PA only treatment

Abbreviation: PA- palmitic acid; TI- *T. indica* fruit extract

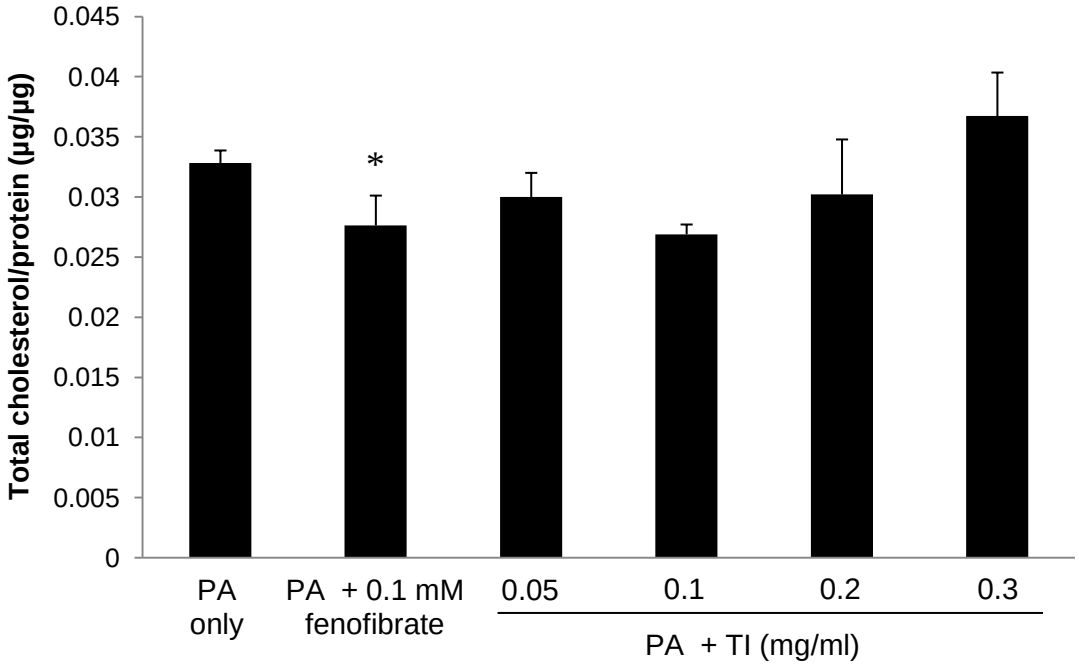

Figure 4.17: Measurement of total cholesterol in HepG2 cells after treatment

Assays were done in triplicates and data were represented as mean ± standard deviation.

*$p < 0.05$ when compared to PA only treatment

Abbreviation: PA- palmitic acid; TI- *T. indica* fruit extract

4.4 Transcriptomic studies

4.4.1 Assessment of the integrity of tcRNA extracted from HepG2 cells

Total cellular RNA (tcRNA) extracted from the HepG2 cells were assessed to ensure that the tcRNA quality and integrity was high. This is important as the quality and integrity of the tcRNA extracted is essential to obtain reproducible results in the downstream applications, especially in microarray analysis. Figure 4.18A shows the denaturing agarose gel electrophoresis of the tcRNA extracted from HepG2 cells. The presence of two distinct bands with 28S band approximately twice the intensity of 18S band with no smearing indicates that the tcRNA is intact (Figure 4.18A). Besides the conventional use of denaturing agarose gel electrophoresis, Agilent Bioanalyzer 2100 was also used to determine the integrity of the tcRNA. RNA integrity number (RIN) of more than 8 shows that the tcRNA is intact. The two peaks generated in the chromatogram represents 18S and 28S respectively (Figure 4.18B).

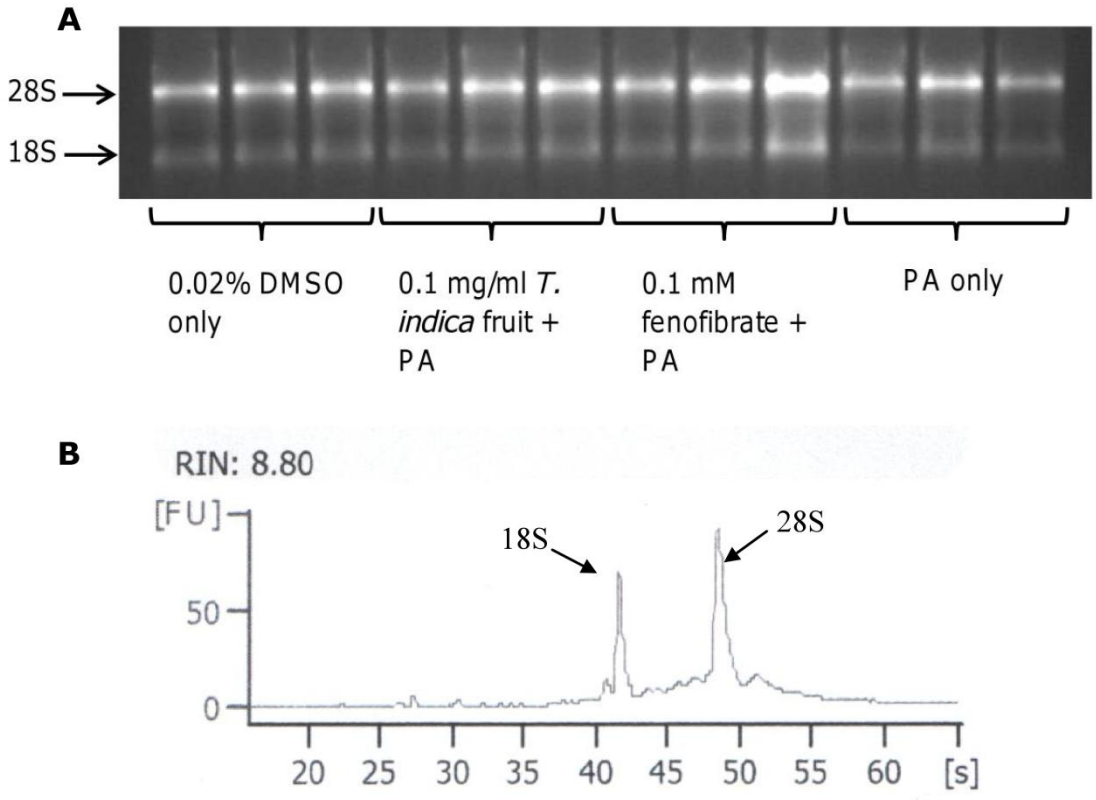

Figure 4.18: Assessment of tcRNA integrity using denaturing agarose gel electrophoresis and Agilent Bioanalyzer 2100

A) Denaturing agarose gel electrophoresis of tcRNA extracted from HepG2 cells. B) An example of a tcRNA sample analysed using Agilent Bioanalyzer 2100.

4.4.2 Principal component analysis (PCA) mapping of different treatment groups

PCA is a statistical technique for determining the key variables in a multidimensional data set that explain the differences in the observations. Figure 4.19 demonstrates the PCA mapping of different treatment groups in the DNA microarray analysis. It can be seen that the control group is furthest from all other treatments, indicating that the gene expression differed the most. Other treatments were closer to each other, forming 3 clusters but remained separated, signifying different sets of gene were being regulated.

4.4.3 Identification of significantly regulated genes using Partek Software

A total of 160, 77 and 169 genes were significantly regulated ($p < 0.05$) by at least 1.5-fold in HepG2 cells treated with 0.1 mg/ml *T. indica* fruit extract (TI+PA vs control), fenofibrate (FF+PA vs control) and palmitic acid (PA vs control), respectively when compared to control (Figure 4.20). Thirty seven genes were significantly altered in all 3 treatments (Figure 4.20) and the names of the genes are listed in Table 4.5. Twenty four genes were commonly regulated in *T. indica* and fenofibrate treatments (Table 4.6) while 34 genes were commonly regulated in *T. indica* treatment and palmitic acid treatment alone (Table 4.7). Only 3 genes were commonly altered in fenofibrate treatment and palmitic acid treatment (Table 4.8). Sixty five, 13 and 95 genes were uniquely regulated in *T. indica* treatment, fenofibrate treatment and palmitic acid treatment respectively (Tables 4.9, 4.10 and 4.11).

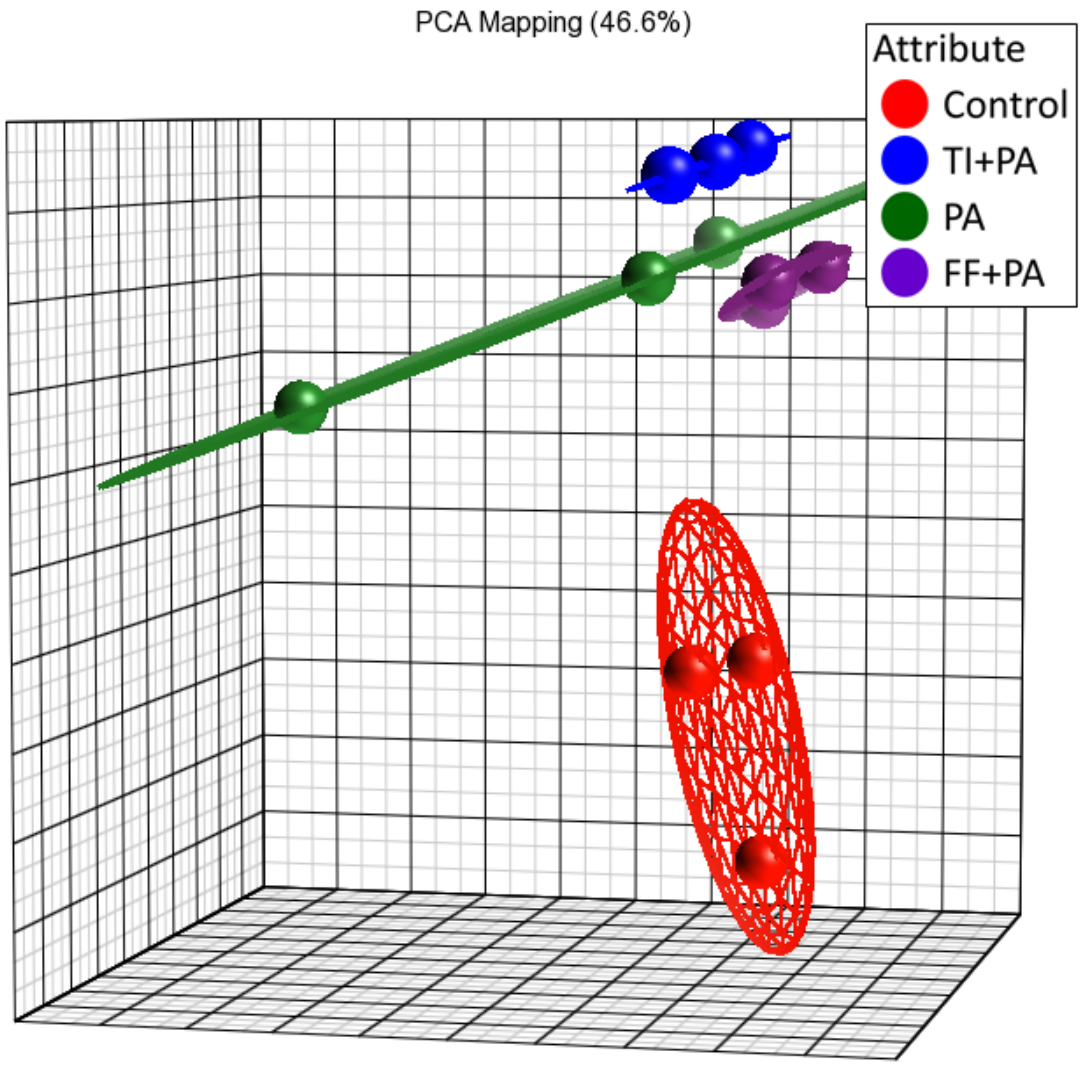

Figure 4.19: PCA mapping of 4 different treatment groups in DNA microarray analysis

Abbreviation: TI- *T. indica* fruit extract; PA- palmitic acid; FF- fenofibrate

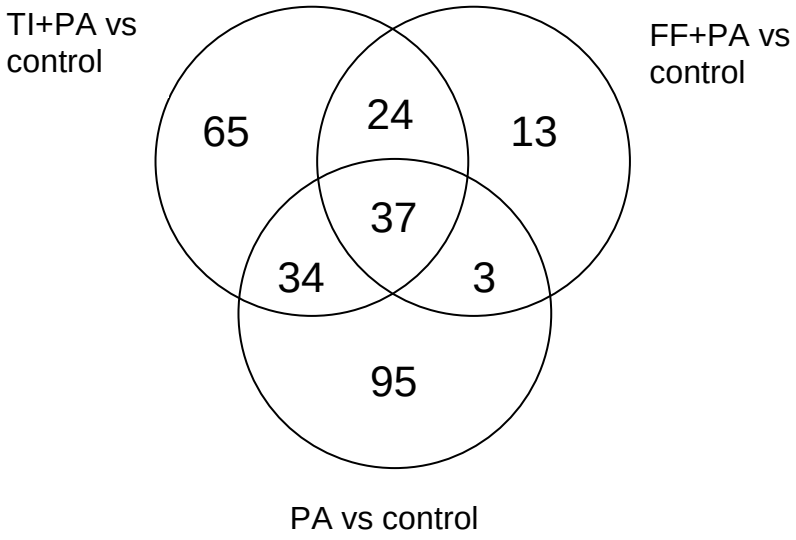

Figure 4.20: Venn diagram of number of genes that were significantly regulated (*p* < 0.05) by at least 1.5-fold in DNA microarray analyses

Abbreviation: TI+PA- *T. indica* fruit extract and palmitic acid; FF+PA- fenofibrate and palmitic acid; PA- palmitic acid

Table 4.5: Genes commonly regulated in all treatment groups (TI+PA vs control, PA vs control and FF+PA vs control)

Gene ID	Symbol	Gene Name	Fold Change		
			TI+PA vs control	FF+PA vs control	PA vs control
8095744	*AREG/AREGB*	amphiregulin	11.6	6.4	12.0
8095736	*AREG/AREGB*	amphiregulin	6.6	3.8	6.8
8123651	*TUBB2B*	tubulin, beta 2B class IIb	3.1	1.7	1.9
8015133	*KRT23*	keratin 23 (histone deacetylase inducible)	2.5	1.8	2.6
7964460	*DDIT3*	DNA-damage-inducible transcript 3	2.5	1.8	1.7
8138381	*AGR2*	anterior gradient 2 homolog (Xenopus laevis)	2.4	2.2	2.6
8106098	*MAP1B*	microtubule-associated protein 1B	2.4	1.8	2.6
8007429	*G6PC*	glucose-6-phosphatase, catalytic subunit	2.3	1.6	1.5
8117630	*ZNF165*	zinc finger protein 165	2.3	1.8	1.7
7965979	*ALDH1L2*	aldehyde dehydrogenase 1 family, member L2	2.3	1.8	1.7
7958262	*TCP11L2*	t-complex 11, testis-specific-like 2	2.3	1.6	2.2
7995895	*HERPUD1*	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	2.2	1.7	1.5
8042503	*MXD1*	MAX dimerization protein 1	2.2	1.8	1.7
8135480	*DNAJB9*	DnaJ (Hsp40) homolog, subfamily B, member 9	2.2	1.6	1.8
8021653	*SERPINB8*	serpin peptidase inhibitor, clade B (ovalbumin), member 8	2.1	1.7	1.5
8158167	*LCN2*	lipocalin 2	2.1	1.7	1.5
8131996	*CREB5*	cAMP responsive element binding protein 5	1.9	1.7	1.6
7917530	*GBP2*	guanylate binding protein 2, interferon-inducible	1.8	1.8	2.5
8147145	*ATP6V0D2*	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2	1.8	1.8	2.1
7962559	*SLC38A4*	solute carrier family 38, member 4	1.8	2.1	2.7
8142307	*PNPLA8*	patatin-like phospholipase domain containing 8	1.8	1.5	1.7
8113064	*LYSMD3*	LysM, putative peptidoglycan-binding, domain containing 3	1.8	1.6	1.7
8103951	*ACSL1*	acyl-CoA synthetase long-chain family member 1	1.6	1.7	1.6

Table 4.5, continued

Gene ID	Symbol	Gene Name	Fold Change		
			TI+PA vs control	FF+PA vs control	PA vs control
8150002	*FBXO16*	F-box protein 16	1.6	1.7	1.6
8175288	*MOSPD1*	motile sperm domain containing 1	1.6	1.6	1.5
7997740	*MAP1LC3B*	microtubule-associated protein 1 light chain 3 beta	1.5	1.6	1.6
8092169	*TNFSF10*	tumor necrosis factor (ligand) superfamily, member 10	1.5	1.6	1.6
8008885	*mir-21*	microRNA 21	-2.4	-2.9	-4.5
8122144	*SNORA33*	small nucleolar RNA, H/ACA box 33	-2.0	-1.7	-2.0
7985317	*KIAA1199*	KIAA1199	-1.8	-1.8	-1.9
8114797	*SPRY4*	sprouty homolog 4 (Drosophila)	-1.7	-1.6	-1.7
8160213	*TTC39B*	tetratricopeptide repeat domain 39B	-1.7	-1.7	-1.5
8048864	*CCL20*	chemokine (C-C motif) ligand 20	-1.7	-1.8	-1.6
7982597	*THBS1*	thrombospondin 1	-1.6	-1.7	-1.8
7934979	*ANKRD1*	ankyrin repeat domain 1 (cardiac muscle)	-1.6	-2.1	-1.7
7995783	*MT2A*	metallothionein 2A	-1.5	-1.6	-1.6
8122265	*TNFAIP3*	tumor necrosis factor, alpha-induced protein 3	-1.5	-1.6	-1.6

Table 4.6: Significantly regulated genes in *T. indica* treatment group and fenofibrate treatment group (TI+PA vs control and FF+PA vs control)

Gene ID	Symbol	Gene Name	Fold change	
			TI+PA vs control	FF+PA vs control
8030128	*PPP1R15A*	protein phosphatase 1, regulatory subunit 15A	2.5	1.8
8115831	*DUSP1*	dual specificity phosphatase 1	2.4	1.6
8126629	*GTPBP2*	GTP binding protein 2	2.0	1.8
8095826	*STBD1*	starch binding domain 1	1.9	1.7
7952145	*HYOU1*	hypoxia up-regulated 1	1.9	1.6
7912750	*FBXO42*	F-box protein 42	1.9	1.6
8018264	*HID1*	HID1 domain containing	1.8	1.5
7970301	*TMCO3*	transmembrane and coiled-coil domains 3	1.8	1.6
8154381	*LURAP1L*	leucine rich adaptor protein 1-like	1.8	1.6
8124365	*SLC17A2*	solute carrier family 17 (sodium phosphate), member 2	1.8	1.5
7924450	*DUSP10*	dual specificity phosphatase 10	1.7	1.6
8040211	*KLF11*	Kruppel-like factor 11	1.7	1.6
7949971	*CPT1A*	carnitine palmitoyltransferase 1A (liver)	1.7	1.6
7909610	*ATF3*	activating transcription factor 3	1.6	1.5
8174239	*BEX2*	brain expressed X-linked 2	1.6	1.6
8103535	*GK3P*	glycerol kinase 3 pseudogene	1.6	1.6
7922773	*NCF2*	neutrophil cytosolic factor 2	1.5	1.6
7952339	*HSPA8*	heat shock 70kDa protein 8	-3.0	-2.3
8146115	*C8orf4*	chromosome 8 open reading frame 4	-2.0	-2.5
8118322	*SNORD52*	small nucleolar RNA, C/D box 52	-1.8	-1.6
7922416	*SNORD75*	small nucleolar RNA, C/D box 75	-1.7	-1.5
7915472	*SLC2A1*	solute carrier family 2 (facilitated glucose transporter), member 1	-1.6	-1.6
8034390	*ZNF799*	zinc finger protein 799	-1.6	-1.6
8169504	*SLC6A14*	solute carrier family 6 (amino acid transporter), member 14	-1.5	-1.7

Table 4.7: Significantly regulated genes in *T. indica* treatment group and palmitic acid treatment group (TI+PA vs control and PA vs control)

Gene ID	Symbol	Gene Name	Fold change	
			TI+PA vs control	PA vs control
8041644	*PLEKHH2*	pleckstrin homology domain containing, family H (with MyTH4 domain) member 2	2.1	1.7
7965423	*BTG1*	B-cell translocation gene 1, anti-proliferative	1.8	1.8
8052798	*AAK1*	AP2 associated kinase 1	1.8	1.6
8162276	*NFIL3*	nuclear factor, interleukin 3 regulated	1.7	1.5
8020847	*DTNA*	dystrobrevin, alpha	1.7	1.6
8069532	*HSPA13*	heat shock protein 70kDa family, member 13	1.7	1.5
7988767	*CYP19A1*	cytochrome P450, family 19, subfamily A, polypeptide 1	1.6	1.6
8105908	*OCLN*	occludin	1.6	1.6
7971388	*SLC25A30*	solute carrier family 25, member 30	1.6	1.6
8044450	*ZC3H6*	zinc finger CCCH-type containing 6	1.6	1.5
7946089	*TRIM5*	tripartite motif containing 5	1.6	1.5
8115397	*FAXDC2*	fatty acid hydroxylase domain containing 2	1.6	1.6
8094719	*N4BP2*	NEDD4 binding protein 2	1.6	1.5
8084219	*KLHL24*	kelch-like 24 (Drosophila)	1.6	1.7
7925342	*ERO1LB*	ERO1-like beta (*S. cerevisiae*)	1.5	1.6
7916112	*RAB3B*	RAB3B, member RAS oncogene family	1.5	1.5
8102848	*SETD7*	SET domain containing (lysine methyltransferase) 7	1.5	1.5
8106516	*JMY*	junction mediating and regulatory protein, p53 cofactor	1.5	1.9
8005202	*SNORD49A*	small nucleolar RNA, C/D box 49A	-1.9	-1.9
7948902	*SNHG1*	small nucleolar RNA host gene 1 (non-protein coding)	-1.8	-2.1
7948904	*SNORD28*	small nucleolar RNA, C/D box 28	-1.8	-1.8
7948900	*SNORD30*	small nucleolar RNA, C/D box 30	-1.8	-2.2

Table 4.7, continued

Gene ID	Symbol	Gene Name	Fold change	
			TI+PA vs control	PA vs control
7948896	*SNHG1*	small nucleolar RNA host gene 1 (non-protein coding)	-1.7	-2.1
7948898	*SNHG1*	small nucleolar RNA host gene 1 (non-protein coding)	-1.7	-2.4
8005951	*SNORD42B*	small nucleolar RNA, C/D box 42B	-1.7	-2.9
7914212	*SNHG12*	small nucleolar RNA host gene 12 (non-protein coding)	-1.7	-1.6
7948908	*SNHG1*	small nucleolar RNA host gene 1 (non-protein coding)	-1.6	-2.4
8059538	*SLC19A3*	solute carrier family 19, member 3	-1.6	-1.9
7948906	*SNHG1*	small nucleolar RNA host gene 1 (non-protein coding)	-1.5	-2.0
8084704	*EIF4A2*	eukaryotic translation initiation factor 4A2	-1.5	-1.6
8061564	*ID1*	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	-1.5	-1.5
7995797	*MT1E*	metallothionein 1E	-1.5	-1.5
7910134	*MIXL1*	Mix paired-like homeobox	-1.5	-1.5
7952335	*SNORD14E*	small nucleolar RNA, C/D box 14E	-1.5	-1.6

Table 4.8: Significantly regulated genes in fenofibrate treatment group and palmitic acid treatment group (FF+PA vs control and PA vs control)

Gene ID	Symbol	Gene Name	Fold change	
			FF+PA vs control	PA vs control
8014342	*CCL16*	chemokine (C-C motif) ligand 16	1.6	1.8
8108370	*EGR1*	early growth response 1	-1.8	-2.4
8178435	*IER3*	immediate early response 3	-1.7	-1.6

Table 4.9: Genes exclusively regulated in fenofibrate treatment group (FF+PA vs control)

Gene ID	Symbol	Gene Name	Fold change
			FF+PA vs control
8024754	*CREB3L3*	cAMP responsive element binding protein 3-like 3	1.6
7953943	*GABARAPL1*	GABA(A) receptor-associated protein like 1	1.6
8087224	*SLC25A20*	solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	1.5
8049534	*LRRFIP1*	leucine rich repeat (in FLII) interacting protein 1	-1.9
8124848	*IER3*	immediate early response 3	-1.6
8179704	*IER3*	immediate early response 3	-1.6
8142270	*NRCAM*	neuronal cell adhesion molecule	-1.5
8095362	*MT2A*	metallothionein 2A	-1.5
8053278	*EVA1A*	eva-1 homolog A (*C. elegans*)	-1.5
8084880	*HES1*	hairy and enhancer of split 1, (Drosophila)	-1.5
7943413	*BIRC3*	baculoviral IAP repeat containing 3	-1.5

Table 4.10: Genes exclusively regulated in *T. indica* treatment group (TI+PA vs control)

Gene ID	Symbol	Gene Name	Fold change
			TI+PA vs control
8132694	*IGFBP1*	insulin-like growth factor binding protein 1	2.2
8077441	*BHLHE40*	basic helix-loop-helix family, member e40	2.2
7956426	*INHBE*	inhibin, beta E	2.2
7931810	*KLF6*	Kruppel-like factor 6	2.0
7916609	*JUN*	jun proto-oncogene	1.9
8000574	*NUPR1*	nuclear protein, transcriptional regulator, 1	1.8
7991587	*VIMP*	VCP-interacting membrane protein	1.7
7953291	*CD9*	CD9 molecule	1.7
8157933	*ZBTB43*	zinc finger and BTB domain containing 43	1.7
8155630	*LOC286297*	uncharacterized LOC286297	1.7
8045289	*RHOQ*	ras homolog family member Q	1.6
7953135	*TULP3*	tubby like protein 3	1.6
8060344	*TRIB3*	tribbles homolog 3 (Drosophila)	1.6
8153002	*NDRG1*	N-myc downstream regulated 1	1.6
8110055	*CPEB4*	cytoplasmic polyadenylation element binding protein 4	1.6
7947496	*SLC1A2*	solute carrier family 1 (glial high affinity glutamate transporter), member 2	1.6
8160297	*PLIN2*	perilipin 2	1.6
8018902	*DNAH17*	dynein, axonemal, heavy chain 17	1.6
8174103	*GK*	glycerol kinase	1.6
8037071	*RABAC1*	Rab acceptor 1 (prenylated)	1.6
7905938	*SLC50A1*	solute carrier family 50 (sugar transporter), member 1	1.6
7969677	*MBNL2*	muscleblind-like splicing regulator 2	1.6
8072876	*LGALS1*	lectin, galactoside-binding, soluble, 1	1.6
8053648	*KRCC1*	lysine-rich coiled-coil 1	1.6

Table 4.10, continued

Gene ID	Symbol	Gene Name	Fold change TI+PA vs control
8005171	*TRPV2*	transient receptor potential cation channel, subfamily V, member 2	1.6
7927082	*HSD17B7P2*	hydroxysteroid (17-beta) dehydrogenase 7 pseudogene 2	1.5
8163002	*KLF4*	Kruppel-like factor 4 (gut)	1.5
8117045	*RBM24*	RNA binding motif protein 24	1.5
8040340	*LPIN1*	lipin 1	1.5
8137709	*ZFAND2A*	zinc finger, AN1-type domain 2A	1.5
7990080	*LARP6*	La ribonucleoprotein domain family, member 6	1.5
7927146	*CSGALNACT2*	chondroitin sulfate N-acetylgalactosaminyltransferase 2	1.5
8147206	*RIPK2*	receptor-interacting serine-threonine kinase 2	1.5
7981290	*WARS*	tryptophanyl-tRNA synthetase	1.5
8113073	*ARRDC3*	arrestin domain containing 3	1.5
8088167	*SELK*	selenoprotein K	1.5
8135576	*TES*	testis derived transcript (3 LIM domains)	1.5
8124040	*ATXN1*	ataxin 1	1.5
8110032	*CREBRF*	CREB3 regulatory factor	1.5
8003939	*TM4SF5*	transmembrane 4 L six family member 5	1.5
8130211	*SYNE1*	spectrin repeat containing, nuclear envelope 1	1.5
8127072	*GSTA1*	glutathione S-transferase alpha 1	-2.0
8127065	*GSTA2*	glutathione S-transferase alpha 2	-2.0
7922406	*SNORD79*	small nucleolar RNA, C/D box 79	-1.9
7967107	*HNF1A-AS1*	HNF1A antisense RNA 1	-1.7
8014755	*SNORA21*	small nucleolar RNA, H/ACA box 21	-1.7
7922807	*COLGALT2*	collagen beta(1-O)galactosyltransferase 2	-1.7
7922807	*GLT25D2*	glycosyltransferase 25 domain containing 2	-1.7
8117594	*HIST1H2BM*	histone cluster 1, H2bm	-1.6
7901048	*SNORD46*	small nucleolar RNA, C/D box 46	-1.6

Table 4.10, continued

Gene ID	Symbol	Gene Name	Fold change TI+PA vs control
7927631	*DKK1*	dickkopf 1 homolog (*Xenopus laevis*)	-1.6
7981943	*PAR5*	Prader-Willi/Angelman syndrome-5	-1.6
8117288	*SCGN*	secretagogin, EF-hand calcium binding protein	-1.6
8034393	*ZNF443*	zinc finger protein 443	-1.6
7951030	*TAF1D*	TATA box binding protein (TBP)-associated factor, RNA polymerase I, D, 41kDa	-1.6
8139482	*SNORA5A*	small nucleolar RNA, H/ACA box 5A	-1.6
7961026	*LOC728715*	ovostatin homolog 2-like	-1.6
7903022	*RPL5*	ribosomal protein L5	-1.5
7922402	*GAS5*	growth arrest-specific 5 (non-protein coding)	-1.5
8044212	*SULT1C2*	sulfotransferase family, cytosolic, 1C, member 2	-1.5
7922408	*SNORD78*	small nucleolar RNA, C/D box 78	-1.5
7953873	*OVOS/OVOS2*	ovostatin 2	-1.5
8095390	*UGT2B10*	UDP glucuronosyltransferase 2 family, polypeptide B10	-1.5
8066254	*LOC388796*	uncharacterized LOC388796	-1.5
7942527	*POLD3*	polymerase (DNA-directed), delta 3, accessory subunit	-1.5

Table 4.11: Genes exclusively regulated in palmitic acid treatment group (PA vs control)

Gene ID	Symbol	Gene Name	Fold change
			PA vs control
7920873	*SNORA42*	small nucleolar RNA, H/ACA box 42	3.0
7951077	*SESN3*	sestrin 3	2.1
8114964	*SPINK1*	serine peptidase inhibitor, Kazal type 1	2.1
7961524	*ERP27*	endoplasmic reticulum protein 27	2.0
8150920	*CYP7A1*	cytochrome P450, family 7, subfamily A, polypeptide 1	2.0
8156160	*KIF27*	kinesin family member 27	1.9
8151890	*TP53INP1*	tumor protein p53 inducible nuclear protein 1	1.8
8156060	*TLE4*	transducin-like enhancer of split 4 (E(sp1) homolog, Drosophila)	1.8
8156905	*TMEFF1*	transmembrane protein with EGF-like and two follistatin-like domains 1	1.7
8028194	*ZNF382*	zinc finger protein 382	1.7
8102877	*CLGN*	Calmegin	1.7
8151559	*SLC10A5*	solute carrier family 10 (sodium/bile acid cotransporter family), member 5	1.7
7947156	*MUC15*	mucin 15, cell surface associated	1.7
8113602	*CCDC112*	coiled-coil domain containing 112	1.7
8121277	*AIM1*	absent in melanoma 1	1.7
8002283	*TMED6*	transmembrane emp24 protein transport domain containing 6	1.7
8058477	*KLF7*	Kruppel-like factor 7 (ubiquitous)	1.7
7989037	*CCPG1*	cell cycle progression 1	1.7
7954729	*FGD4*	FYVE, RhoGEF and PH domain containing 4	1.7
8047401	*CFLAR*	CASP8 and FADD-like apoptosis regulator	1.6
8142098	*ATXN7L1*	ataxin 7-like 1	1.6
7989069	*PYGO1*	pygopus homolog 1 (Drosophila)	1.6
8083779	*SERPINI1*	serpin peptidase inhibitor, clade I (neuroserpin), member 1	1.6
7936419	*C10orf118*	chromosome 10 open reading frame 118	1.6

Table 4.11, continued

Gene ID	Symbol	Gene Name	Fold change PA vs control
8127234	*DST*	Dystonin	1.6
7990879	*EFTUD1*	elongation factor Tu GTP binding domain containing 1	1.6
8155794	*C9orf85*	chromosome 9 open reading frame 85	1.6
8078033	*CCDC174*	coiled-coil domain containing 174	1.6
8099476	*PROM1*	prominin 1	1.6
8163839	*C5*	complement component 5	1.6
7919780	*GOLPH3L*	golgi phosphoprotein 3-like	1.6
8004184	*XAF1*	XIAP associated factor 1	1.6
8047078	*MFSD6*	major facilitator superfamily domain containing 6	1.6
8162006	*GKAP1*	G kinase anchoring protein 1	1.6
8098084	*ETFDH*	electron-transferring-flavoprotein dehydrogenase	1.6
8128553	*BVES*	blood vessel epicardial substance	1.6
8030908	*ZNF480*	zinc finger protein 480	1.5
8058509	*PLEKHM3*	pleckstrin homology domain containing, family M, member 3	1.5
7974697	*DAAM1*	dishevelled associated activator of morphogenesis 1	1.5
7909332	*CD55*	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	1.5
8154416	*CCDC171*	coiled-coil domain containing 171	1.5
7965060	*BBS10*	Bardet-Biedl syndrome 10	1.5
8056060	*BAZ2B*	bromodomain adjacent to zinc finger domain, 2B	1.5
8069565	*BTG3*	BTG family, member 3	1.5
8022666	*CHST9*	carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 9	1.5
8124492	*HIST1H2BJ/ HIST1H2BK*	histone cluster 1, H2bk	1.5
8043995	*IL1R1*	interleukin 1 receptor, type I	1.5
8113403	*GIN1*	gypsy retrotransposon integrase 1	1.5

Table 4.11, continued

Gene ID	Symbol	Gene Name	Fold change PA vs control
8154670	*IFT74*	intraflagellar transport 74 homolog (Chlamydomonas)	1.5
8096733	*SGMS2*	sphingomyelin synthase 2	1.5
8146427	*PCMTD1*	protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 1	1.5
8061780	*BPIFB2*	BPI fold containing family B, member 2	-2.1
8127989	*SNORD50B*	small nucleolar RNA, C/D box 50B	-2.0
8127987	*SNORD50A*	small nucleolar RNA, C/D box 50A	-1.9
8001547	*PLLP*	Plasmolipin	-1.9
7966749	*TESC*	Tescalcin	-1.8
8063345	*SNORD12C*	small nucleolar RNA, C/D box 12C	-1.8
8019273	*ALYREF*	Aly/REF export factor	-1.8
8178059	*LY6G5B*	lymphocyte antigen 6 complex, locus G5B	-1.8
8084708	*EIF4A2*	eukaryotic translation initiation factor 4A2	-1.8
8019772	*ALYREF*	Aly/REF export factor	-1.8
7902400	*RABGGTB*	Rab geranylgeranyltransferase, beta subunit	-1.7
8006655	*DHRS11*	dehydrogenase/reductase (SDR family) member 11	-1.7
8123562	*GMDS*	GDP-mannose 4,6-dehydratase	-1.7
7969574	*KRT18*	keratin 18	-1.7
7969574	*mir-622*	microRNA 622	-1.7
8170863	*RPL10*	ribosomal protein L10	-1.7
8023259	*SNORD58A*	small nucleolar RNA, C/D box 58A	-1.7
8024900	*UHRF1*	ubiquitin-like with PHD and ring finger domains 1	-1.6
8043187	*MAT2A*	methionine adenosyltransferase II, alpha	-1.6
8025498	*RPL10*	ribosomal protein L10	-1.6
7995362	*GPT2*	glutamic pyruvate transaminase (alanine aminotransferase) 2	-1.6
8122142	*SNORD101*	small nucleolar RNA, C/D box 101	-1.6

Table 4.11, continued

Gene ID	Symbol	Gene Name	Fold change PA vs control
7976783	*DLK1*	delta-like 1 homolog (Drosophila)	-1.6
8106278	*FAM169A*	family with sequence similarity 169, member A	-1.6
8003298	*SLC7A5*	solute carrier family 7 (amino acid transporter light chain, L system), member 5	-1.6
7945573	*POLR2L*	polymerase (RNA) II (DNA directed) polypeptide L, 7.6kDa	-1.6
7904433	*PHGDH*	phosphoglycerate dehydrogenase	-1.6
8001457	*CES1*	carboxylesterase 1	-1.6
7938291	*RPL27A*	ribosomal protein L27a	-1.6
7942594	*SNORD15B*	small nucleolar RNA, C/D box 15B	-1.6
8120315	*TINAG*	tubulointerstitial nephritis antigen	-1.6
7995825	*MT1F*	metallothionein 1F	-1.6
8064978	JAG1	jagged 1	-1.6
8079294	*EXOSC7*	exosome component 7	-1.5
7973896	*GSTM2*	glutathione S-transferase mu 2 (muscle)	-1.5
7994928	*PHKG2*	phosphorylase kinase, gamma 2 (testis)	-1.5
8136336	*AKR1B10*	aldo-keto reductase family 1, member B10 (aldose reductase)	-1.5
8065868	*EIF6*	eukaryotic translation initiation factor 6	-1.5
8046488	*CDCA7*	cell division cycle associated 7	-1.5
8163328	*PTGR1*	prostaglandin reductase 1	-1.5
8001531	*MT1G*	metallothionein 1G	-1.5
8154725	*KRT18*	keratin 18	-1.5
8162744	*CORO2A*	coronin, actin binding protein, 2A	-1.5
7928705	*TSPAN14*	tetraspanin 14	-1.5

4.4.4 Functional analyses of significantly regulated genes using IPA software

The significantly regulated genes were subjected to functional analyses using IPA Core Analysis software. The genes were mapped to the Ingenuity Knowledge Base creating molecular networks which were generated *de novo*. The software also determines over represented signalling and metabolic canonical pathways and identifies the cascade of upstream transcriptional regulators that can explain the observed gene expression changes in the study, from which illuminates the biological activities occurring in the cells being studied.

4.4.4.1 Network and functional analysis

Network analysis of significantly regulated genes in all treatments revealed that different network functions were involved (Table 4.12). In all three treatments, cancer, cell growth and proliferation, as well as cell death-related networks appeared as the dominant associated network functions. Lipid metabolism-related network was shown to be regulated in TI+PA vs control treatment group only, with a score of 40. In the FF+PA vs control group, carbohydrate metabolism was generated as one of the associated network functions, with a score of 42 and 28. On the other hand, in the PA vs control group, cellular growth and proliferation-related networks were dominant.

Figure 4.21 illustrates the molecular relationships in “Lipid Metabolism, Small Molecule Biochemistry, Metabolic Disease” network generated in TI+PA vs control group. It can be seen from the network that the nodes converged to a few central nodes, namely the Rxr, ERK1/2, MAP2K1/2, CYP19A1, FSH and LH, indicating that these genes may be of significant importance in regulating this particular network.

Functional analysis showed that oxidation of fatty acid was activated in the TI+PA vs control group, with an activation Z-score of 2.175. Activation Z-score of more than 2 indicates activation while less than -2 indicates inhibition. Table 4.13 shows the genes related to oxidation of fatty acid that were significantly regulated in the microarray analyses of the different treatments on HepG2 cells. Four out of 6 genes were involved in the "Lipid Metabolism, Small Molecule Biochemistry, Metabolic Disease" network, which were overlaid in the network figure in Figure 4.21.

Table 4.12: Top three networks generated by Ingenuity Pathways Analysis (IPA) software when significantly regulated genes from different treatments were analysed

Treatment	Associated network functions	Score[a]
TI+PA vs control	1) Cancer, Cell Morphology, Cellular Function and Maintenance	51
	2) Lipid Metabolism, Small Molecule Biochemistry, Metabolic Disease	40
	3) Tissue Development, Cellular Development, Cell Death and Survival	31
FF+PA vs control	1) Cell Death and Survival, Renal Necrosis/Cell Death, Carbohydrate Metabolism	42
	2) Endocrine System Disorders, Gastrointestinal Disease, Hereditary Disorder	36
	3) Cell Death and Survival, Carbohydrate Metabolism, Drug Metabolism	28
PA vs control	1) Cellular Development, Cellular Growth and Proliferation, Tumor Morphology	44
	2) Cell Morphology, Organ Morphology, Tissue Morphology	44
	3) Cell-To-Cell Signalling and Interaction, Cellular Growth and Proliferation, Connective Tissue Development and Function	32

[a]A score of 2 or higher indicates at least a 99 % confidence of not being generated by random chance and higher scores indicate a greater confidence.

Table 4.13: Genes related to oxidation of fatty acid that were significantly regulated in the microarray analyses of the different treatments on HepG2 cells

Gene Symbol	Literature findings on gene regulation when oxidation of fatty acid is activated	References	Gene Name	Fold change		
				TI+PA vs control[a]	PA vs control	FF+PA vs control
ACSL1	↑	(Caviglia et al., 2004; J. H. Kim, Kim, Kim, & Hwang, 2011)	Acyl-CoA synthetase long-chain family member 1	1.6	1.6	1.7
CPT1A	↑	(Akkaoui et al., 2009; S. Fu et al., 2012; Perdomo et al., 2004; Rubi et al., 2002; Sebastian et al., 2009)	Carnitine palmitoyltransferase 1A (liver)	1.7		1.6
CYP19A1	↑	(Egawa et al., 2003; Nemoto et al., 2000)	Cytochrome P450, family 19, subfamily A, polypeptide 1	1.6	1.6	
LPIN1	↑	(Finck et al., 2006)	Lipin 1	1.5		
PNPLA8	Affected	(Mancuso et al., 2007; Mancuso et al., 2010)	Patatin-like phospholipase domain containing 8	1.8	1.7	1.5
SLC2A1/ GLUT1	↓	(J. Yan et al., 2009)	Solute carrier family 2 (facilitated glucose transporter), member 1	-1.6		-1.6

[a] Oxidation of fatty acid was predicted to be activated in TI+PA vs control group based on the IPA software. (Activation z-score in TI+PA vs control = 2.175)

↑ indicates up-regulation of the gene while ↓ indicates down-regulation of the gene in literature findings; affected indicates that the gene is shown to be up-regulated and down-regulated in literature findings.

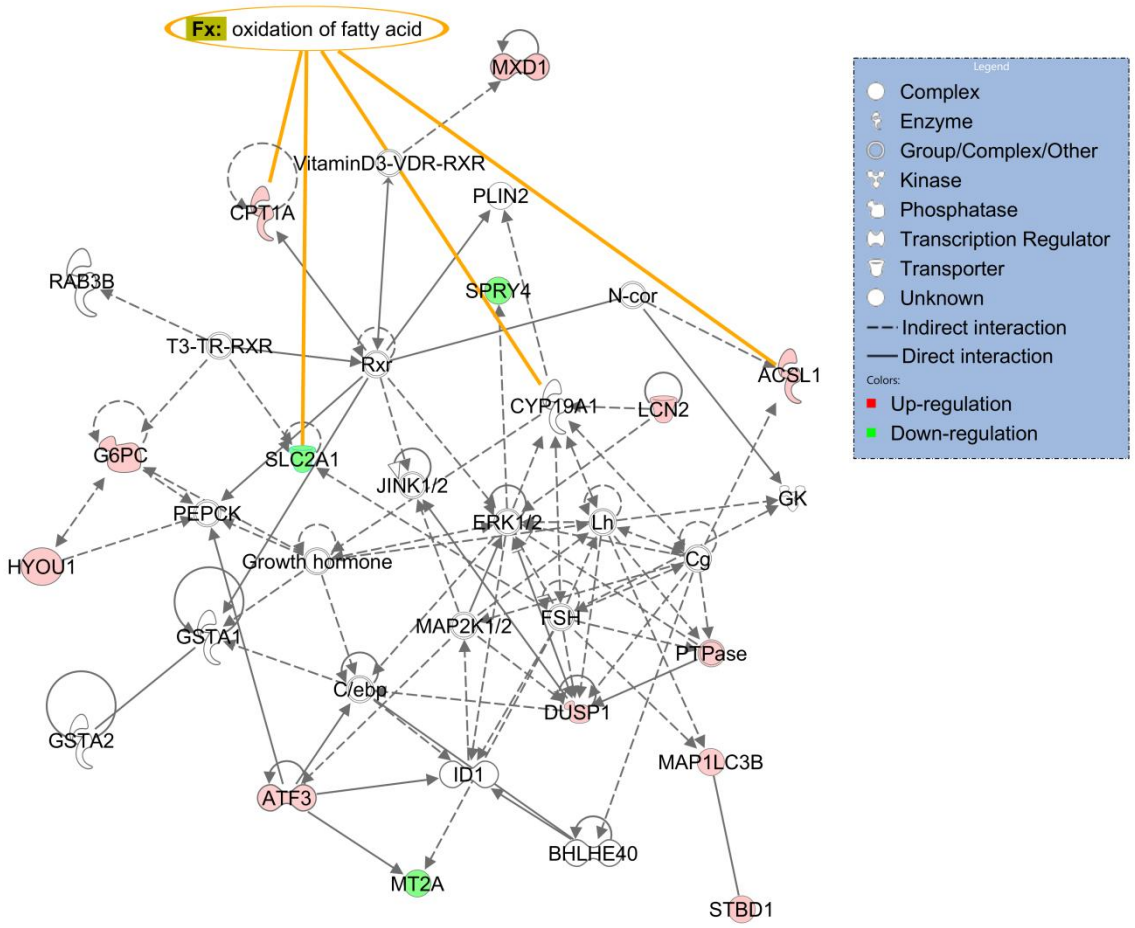

Figure 4.21: IPA graphical representation of the molecular relationships in "Lipid Metabolism, Small Molecule Biochemistry, Metabolic Disease" network in TI+PA vs control treatment overlaid with oxidation of fatty acid function

The network is displayed graphically as nodes (genes) and edges (the biological relationships between the nodes). Nodes in red indicate up-regulated genes while those in green represent down-regulated genes. Nodes without colours indicate unaltered expression. Various shapes of the nodes represent the functional class of the proteins. The different arrow shapes represent different types of interactions. Edges are displayed with various labels that describe the nature of the relationship between the nodes. Names of genes corresponding to the abbreviations are as follows: MAX dimerization protein 1, MXD1; carnitine palmitoyltransferase 1A, CPT1A; perilipin 2, PLIN2;

sprouty homolog 4, SPRY4; retinoid receptor, Rxr; glucose-6-phosphatase, G6PC; hypoxia up-regulated 1, HYOU1; phosphoenolpyruvate carboxykinase, PEPCK; glutathione S-transferase alpha 1, GSTA1; glutathione S-transferase alpha 2, GSTA2; JNK p54, JINK1/2; lutenizing hormone, Lh; chorionic gonadotrophin, Cg; glycerol kinase, GK; acyl-coA synthetase long-chain family member 1, ACSL1; follicle-stimulating hormone, FSH; lipocalin 2, LCN2; activating transcription factor 3, ATF3; metallothionein 2A, MT2A; inhibitor of DNA binding 1, dominant negative helix-loop-helix protein, ID1; dual specificity phosphatase 1, DUSP1; phosphotyrosine phosphatase, PTPase; microtubule-associated protein 1 light chain 3 beta, MAP1LC3B; starch binding domain 1, STBD1; basic helix-loop-helix family e40, BHLHE40; cytochrome p450, family 19, subfamily A, polypeptide 1, CYP19A1; solute carrier family 2 (facilitated glucose transporter), member 1, SLC2A1.

4.4.4.2 Canonical pathway analysis

The top three canonical pathways generated by IPA software are shown in Table 4.14. PXR/RXR activation was shown as the top canonical pathway in TI+PA vs control and PA vs control treatment groups with p-values of 1.03E-04 and 1.47E-02 respectively. Figure 4.22 illustrates the relationship between the regulated genes with other interactomes in the PXR/RXR pathway and their implications on gluconeogenesis and lipid metabolism.

Mitochondrial L-carnitine shuttle pathway was one of the top three canonical pathways in TI+PA vs control and FF+PA vs control treatment groups with p-values of 6.97E-03 and 1.86E-03, respectively. Figure 4.23 depicts the molecular relationships of the regulated genes and other interactomes in the pathway.

Other pathways like LPS/IL-1 mediated inhibition of RXR function was the second canonical pathway generated in the TI+PA vs control treatment. P38 MAPK signalling and TNFR2 signalling were involved in FF+PA vs control treatment while alanine degradation III and alanine biosynthesis II were predicted to be regulated in PA vs control treatment.

Table 4.14: Top three canonical pathways generated by Ingenuity Pathway Analysis (IPA) software when significantly regulated genes from different treatments were analysed.

Treatment	Top Canonical Pathways	p-value
TI+PA vs control	1) PXR/RXR Activation	1.03E-04
	2) LPS/IL-1 Mediated Inhibition of RXR Function	1.2E-03
	3) Mitochondrial L-carnitine Shuttle Pathway	6.97E-03
FF+PA vs control	1) p38 MAPK Signalling	1.08E-03
	2) Mitochondrial L-carnitine Shuttle Pathway	1.86E-03
	3) TNFR2 Signalling	5.27E-03
PA vs control	1) PXR/RXR Activation	1.47E-02
	2) Alanine Degradation III	1.73E-02
	3) Alanine Biosynthesis II	1.73E-02

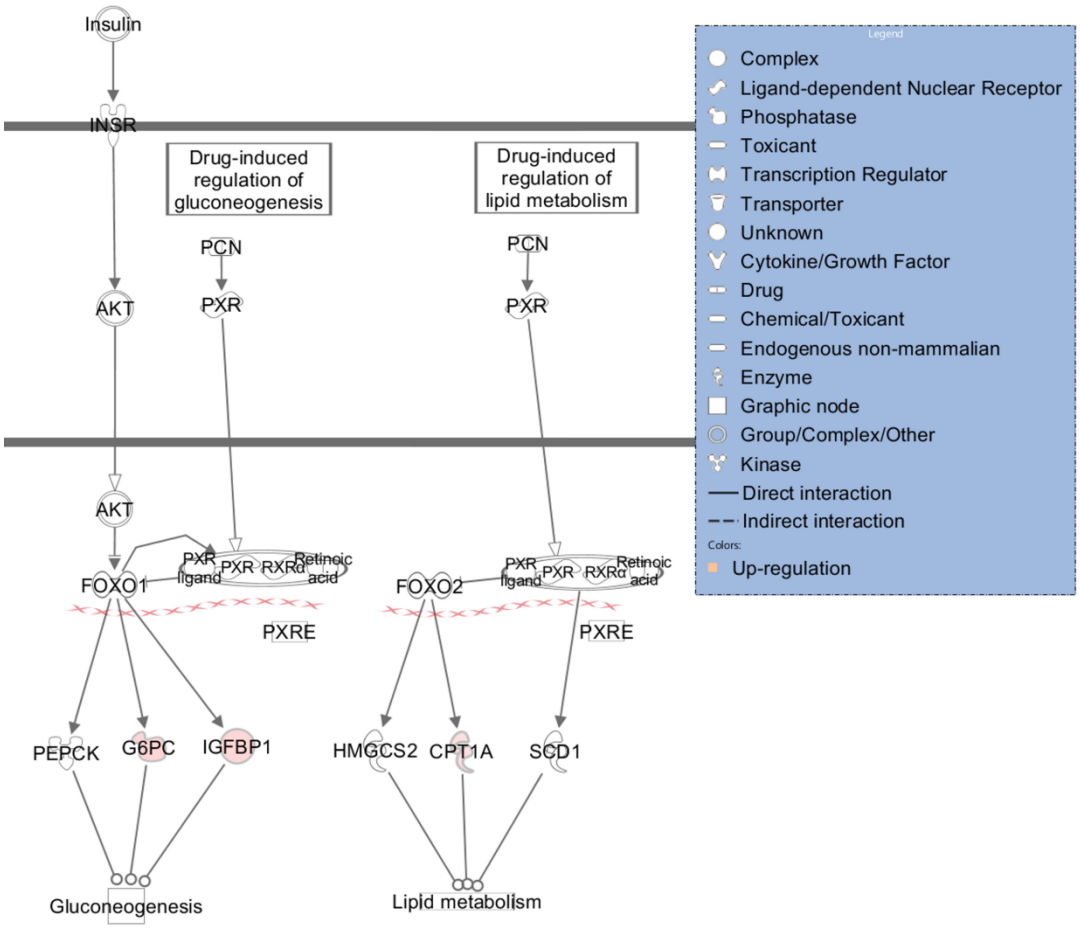

Figure 4.22: PXR/RXR activation pathway generated by IPA software in the canonical pathway analysis

This figure demonstrates that G6PC, IGFBP1 and CPT1A are involved in the PXR/RXR activation pathway generated by IPA software. This pathway was shown to be one of the top three canonical pathways in the TI+PA vs control and PA vs control treatment groups, with p-values of 1.03E-04 and 1.47E-02, respectively.

Names of genes corresponding to the abbreviations are as follows: insulin receptor. INSR; forkhead box O1, FOXO1; nuclear receptor subfamily 1, group I, member 2, PXR; retinoid X receptor, alpha, RXRα; phosphoenolpyruvate carboxykinase 2 (mitochondrial), PEPCK; glucose-6-phosphatase, G6PC; insulin-like growth factor binding protein 1, IGFBP1; forkhead box O3, FOXO2; HMG-CoA synthetase, HMGCS2; carnitine palmitoyltransferase 1A, CPT1A; stearoyl CoA desaturase, SCD1.

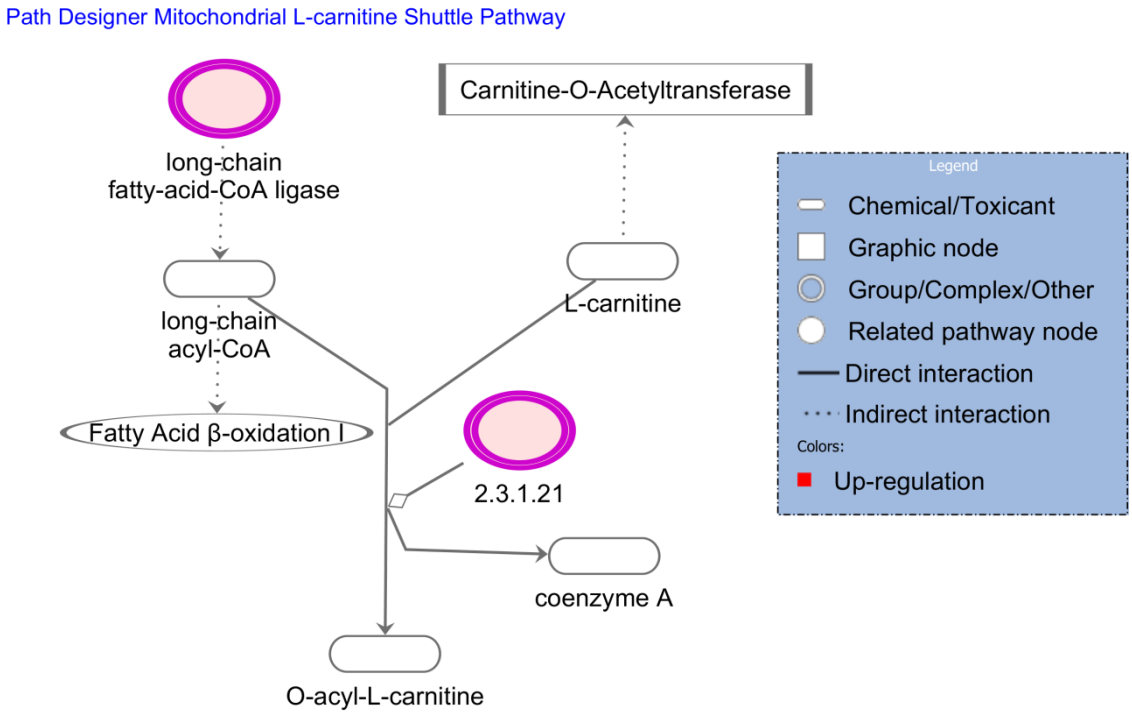

Figure 4.23: Mitochondrial L-carnitine shuttle pathway generated by IPA software in the canonical pathway analysis

Mitochondrial L-carnitine shuttle pathway were predicted to be involved in both TI+PA vs control and FF+PA vs control treatment groups, with p-values of 6.97E-03 and 1.86E-03, respectively. Two genes were shown to be up-regulated in this pathway, namely the long-chain fatty-acid-CoA ligase (*ACSL*) and 2.3.1.21 or carnitine palmitoyltransferase 1A (*CPT1A*), both of which were highlighted in red in the figure.

4.4.4.3 Upstream regulators analysis

The upstream regulator analysis is based on prior knowledge of expected effects between transcriptional regulators and their target genes stored in the Ingenuity Knowledge Base. The analysis examines how many known targets of each transcription regulator are present in the dataset, and also compares their direction of change to what is expected from the literature in order to predict likely relevant transcriptional regulators.

Table 4.15 shows the upstream regulators predicted to be regulated in HepG2 cells exposed to the different treatments. Activation Z-score of more than 2 indicates activation while less than -2 indicates inhibition. Twelve transcription factors were predicted to be regulated in the TI+PA vs control treatment, in which 11 were up-regulated and 1 was down-regulated. In the FF+PA vs control treatment, 5 were predicted to be altered while 3 were predicted to be activated in the PA vs control treatment group. XBP1 was shown to be commonly activated in all treatments. PPARA and FOXO3 were predicted to be activated in TI+PA vs control group and FF+PA vs control group.

Tables 4.16 and 4.17 show the significantly regulated genes that were involved in the downstream mechanism of the predicted activation of PPARA and PPARG. Figure 4.24 displays the molecular interactions between the 4 upstream regulators and the downstream molecules.

Table 4.15: Upstream regulators predicted to be regulated in different treatments using IPA software

Upstream regulators	Gene symbol	Activation z-score[a]		
		TI+PA vs control	FF+PA vs control	PA vs control
Peroxisome proliferator-activated receptor alpha	PPARA	2.745	2.379	
Peroxisome proliferator-activated receptor gamma	PPARG	2.309		
Forkhead box O3	FOXO3	2.038	2.151	
X-box binding protein 1	XBP1	2.961	2.200	2.607
Activating transcription factor 4	ATF4	2.602		
Nuclear protein, transcriptional regulator, 1	NUPR1	2.065		
Progesterone receptor	PGR	2.345		
cAMP responsive element binding protein 1	CREB1	2.219		
Activating transcription factor 2	ATF2	2.199		
Tumor protein p53	TP53	2.185		
Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	PPARGC1A	2.394		
DNA-damage-inducible transcript 3	DDIT3	2.408		
v-myc myelocytomatosis viral oncogene homology	MYC	-2.460		
CCAAT/enhancer binding protein, beta	CEBPB		2.228	
Nuclear factor of kappa light polypeptide gene enhancer in B cells 1	NFKB1		-2.224	
Interferon regulatory factor 7	IRF7			2.000
Signal transducer and activator of transcription 1	STAT1			2.219

[a] Activation z-score of more than 2 is considered **activated** (orange) while less than -2 is considered **inhibited** (blue). This score was generated by the IPA software.

Table 4.16: Genes related to PPARA activation that were significantly regulated in the microarray analyses of the different treatments on HepG2 cells

Gene Symbol	Literature findings on gene regulation when PPARA is activated	References	Gene Name	Fold change		
				TI+PA vs control[a]	FF+PA vs control[a]	PA vs control
STBD1	↑	(Sanderson, Boekschoten, Desvergne, Muller, & Kersten, 2010)	Starch binding domain 1	1.9	1.7	
PLIN2	↑	(Bindesboll, Berg, Arntsen, Nebb, & Dalen, 2013; Nielsen, Grontved, Stunnenberg, & Mandrup, 2006; Tachibana et al., 2006)	Perilipin 2	1.6		
OCLN	↑	(Huang, Eum, Andras, Hennig, & Toborek, 2009)	Occludin	1.6		1.6
NCF2	↑	(Teissier et al., 2004)	Neutrophil cytosolic factor 2	1.5	1.6	
KRT23	↑	(Sanderson, et al., 2010)	Keratin 23 (histone deacetylase inducible)	2.5	1.8	2.6
CPT1A	↑	(Ammerschlaeger, Beigel, Klein, & Mueller, 2004; Begriche, Igoudjil, Pessayre, & Fromenty, 2006; Clemenz et al., 2008; M. H. Hsu, Savas, Griffin, & Johnson, 2001; Lawrence et al., 2001; Vega, Huss, & Kelly, 2000)	Carnitine palmitoyltransferase 1A (liver)	1.7	1.6	

Table 4.16, continued

Gene Symbol	Literature findings on gene regulation when PPARA is activated	References	Gene Name	Fold change		
				TI+PA vs control[a]	FF+PA vs control[a]	PA vs control
ACSL1	↑	(Finck et al., 2002; Schoonjans et al., 1995; Tachibana, et al., 2006)	Acyl-CoA synthetase long-chain family member 1	1.6	1.7	1.6
G6PC	Affected	(Bandsma et al., 2004; Y. Fan et al., 2011)	Glucose-6-phosphatase, catalytic subunit	2.3	1.6	1.5
IGFBP1	Affected	(Degenhardt, Matilainen, Herzig, Dunlop, & Carlberg, 2006)	Insulin-like growth factor binding protein 1	2.2		
GK	↑	(Patsouris et al., 2004)	Glycerol kinase	1.6		

[a] PPARα was predicted to be activated in *T. indica* treated group and fenofibrate treated group based on the IPA software. (Activation z-score in TI+PA vs control= 2.745; Activation z-score in FF+PA vs control = 2.379)

↑ indicates up-regulation of the gene while ↓ indicates down-regulation of the gene in literature findings; affected indicates that the gene is shown to be up-regulated and down-regulated in literature findings.

Table 4.17: Genes related to PPARG activation that are significantly regulated in the microarray analyses of the different treatments on HepG2 cells

Gene Symbol	Literature findings on gene regulation when PPARG is activated	References	Gene Name	Fold change		
				TI+PA vs control[a]	PA vs control	FF+PA vs control
TRIB3	↑	(Hu et al., 2012; Koo et al., 2004)	Tribbles homolog 3 (Drosophila)	1.6		
TNFSF10	↑	(Ho et al., 2011)	Tumor necrosis factor (ligand) superfamily, member 10	1.5	1.6	1.6
PLIN2	↑	(Bhalla et al., 2011; Nielsen, et al., 2006; Schadinger, Bucher, Schreiber, & Farmer, 2005)	Perilipin 2	1.6		
OCLN	↑	(Huang, et al., 2009)	Occludin	1.6	1.6	
NDRG1	↑	(Pino, Wang, McDonald, Qiang, & Farmer, 2012)	N-myc downstream regulated 1	1.6		
KLF4	↑	(Drori et al., 2005; S. Li, Zhou, He, Zhao, & Liu, 2013; Rageul et al., 2009)	Kruppel-like factor 4 (gut)	1.5		
CYP19A1	↑	(W. Fan et al., 2005)	Cytochrome P450, family 19, subfamily A, polypeptide 1	1.6	1.6	
ACSL1	↑	(Francis, Fayard, Picard, & Auwerx, 2003; Schoonjans, et al., 1995; Way et al., 2001)	Acyl-CoA synthetase long-chain family member 1	1.6	1.6	1.7
KLF6	↓	(Schupp et al., 2009)	Kruppel-like factor 6	2.0		

Table 4.17, continued

Gene Symbol	Literature findings on gene regulation when PPARG is activated	References	Gene Name	Fold change		
				TI+PA vs control[a]	PA vs control	FF+PA vs control
CPT1A	Affected	(Begriche, et al., 2006)	Carnitine palmitoyltransferase 1A (liver)	1.7		1.6
G6PC	Affected	(Way, et al., 2001; S. Yu et al., 2003)	Glucose-6-phosphatase, catalytic subunit	2.3	1.5	1.6
GSTA1	Affected	(E. Y. Park, Cho, & Kim, 2004)	Glutathione S-transferase alpha 1	-2.0		
HYOU1	Affected	(M. Jiang et al., 2010)	Hypoxia up-regulated 1	1.9		1.6
IGFBP1	Affected	(Degenhardt, et al., 2006)	Insulin-like growth factor binding protein 1	2.2		
JUN	Affected	(Anghel et al., 2007)	Jun proto-oncogene	1.9		
SLC2A1/ GLUT1	Affected	(Anghel, et al., 2007)	Solute carrier family 2 (facilitated glucose transporter), member 1	-1.6		-1.6

[a] PPARγ was predicted to be activated in *T. indica* treated group based on the IPA software. (Activation Z-score for TI+PA vs control= 2.309).
↑ indicates up-regulation of the gene while ↓ indicates down-regulation of the gene in literature findings; affected indicates that the gene is shown to be up-regulated and down-regulated in literature findings.

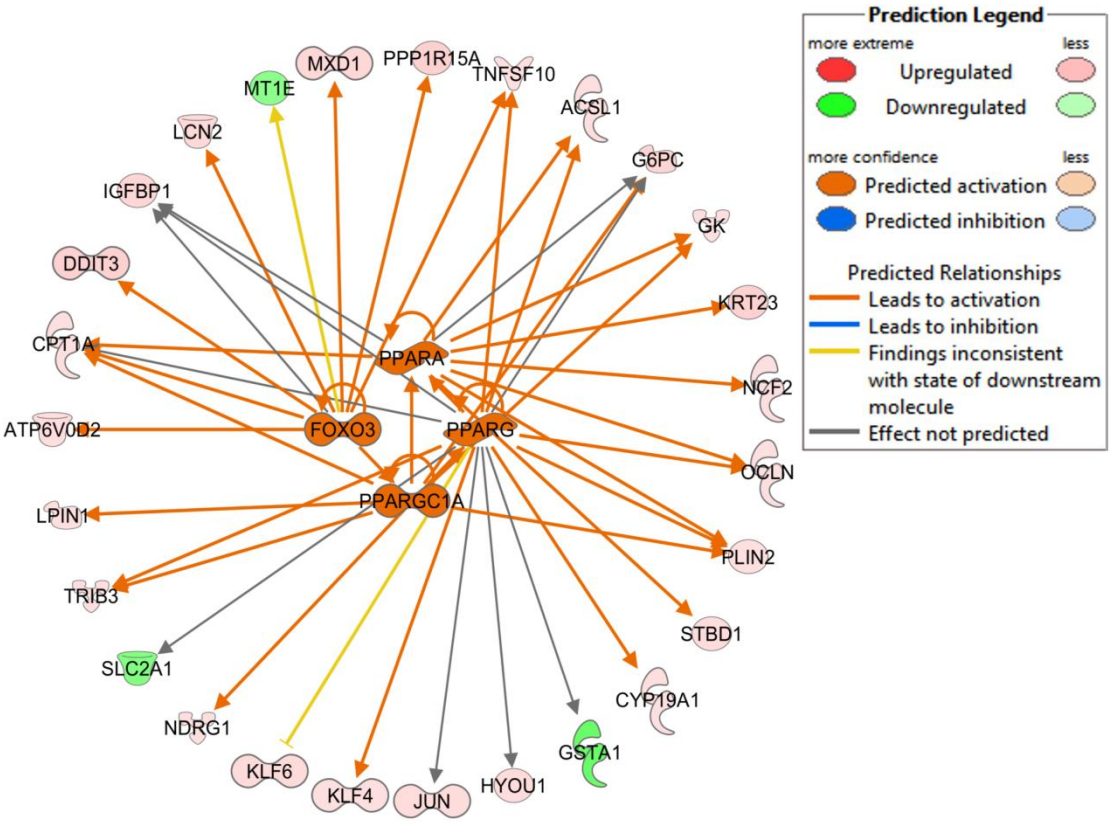

Figure 4.24: IPA illustration of upstream analysis of the genes dataset linked to PPARA, PPARG, PPARGC1A and FOXO3 in TI+PA vs control treatment

This figure shows the molecular relationship between the significantly regulated genes to the predicted upstream regulators, i.e. PPARG, PPARGC1A, PPARA and FOXO3. PPARG, PPARGC1A, PPARA and FOXO3 were predicted to be activated based on the direction of downstream gene expressions which was expressed as Z-score.

4.4.5 Identification of significantly regulated genes that were reverted to expression level similar to control

A set of significantly regulated genes were found to revert to control level after treatment with either TI+PA or FF+PA or both when compared to PA only treatment. Nine genes in which 7 were down-regulated and 2 were up-regulated reverted to the expression level similar to control after treatment with TI+PA and FF+PA (Table 4.18). In the TI+PA treatment, six genes were reverted to level similar to control where 3 were up-regulated and 3 were down-regulated (Table 4.19). All 9 genes but 1 were up-regulated to control level in the FF+PA treatment (Table 4.20).

Table 4.18: Significantly regulated genes that were reverted to a level similar to that of a control after treatment with TI+PA and FF+PA

Symbol	Gene Name	Fold Change		
		TI+PA vs PA	FF+PA vs PA	PA vs control
AIM1	Absent in melanoma 1	-1.8	-1.8	1.7
CES1	Carboxylesterase 1	1.6	1.7	-1.6
CFLAR	CASP8 and FADD-like apoptosis regulator	-1.6	-1.5	1.6
CYP7A1	Cytochrome P450, family 7, subfamily A, polypeptide 1	-1.8	-1.6	2.0
ERP27	Endoplasmic reticulum protein 27	-1.8	-1.5	2.0
MUC15	Mucin 15, cell surface associated	-2.2	-1.7	1.7
PHGDH	Phosphoglycerate dehydrogenase	1.7	1.7	-1.6
PROM1	Prominin 1	-1.6	-1.9	1.6
SERPINI1	Serpin peptidase inhibitor, clade 1 (neuroserpin), member 1	-1.7	-1.5	1.6

Table 4.19: Significantly regulated genes that were reverted to a level similar to control after treatment with TI+PA

Symbol	Gene Name	Fold Change	
		TI+PA vs PA	PA vs control
CHST9	Carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 9	-1.7	1.5
EGR1	Early growth response 1	1.5	-2.4
HIST1H2BJ	Histone cluster 1, H2bk	-1.7	1.5
JAG1	Jagged 1	1.5	-1.6
SLC7A5	Solute carrier family 7 (amino acid transporter light chain, L system), member 5	1.7	-1.6
SPINK1	Serine peptidase inhibitor, Kazal type 1	-1.7	2.1

Table 4.20: Significantly regulated genes that were reverted to a level similar to control after treatment with FF+PA

Symbol	Gene Name	Fold Change	
		FF+PA vs PA	PA vs control
ALYREF	Aly/REF export factor	1.7	-1.8
DHRS11	Dehydrogenase/reductase (SDR family) member 11	1.6	-1.7
EIF6	Eukaryotic translation initiation factor 6	1.5	-1.5
GSTM2	Glutathione S-transferase mu 2 (muscle)	1.6	-1.5
LY6G5B	Lymphocyte antigen 6 complex, locus G5B	1.6	-1.8
PHKG2	Phosphorylase kinase, gamma 2 (testis)	1.6	-1.5
PTGR1	Prostaglandin reductase 1	1.6	-1.5
RPL10	Ribosomal protein L10	1.5	-1.7
SESN3	Sestrin 3	-1.6	2.1

4.4.6 Validation of microarray data using qRT-PCR

Transcript levels of selected genes were measured by quantitative real-time polymerase chain reaction (qRT-PCR) to validate the gene expressions in the DNA microarray analysis. Three biological replicates of each treatment group were used in the analysis and was normalised against beta actin, an endogenous control. Eight genes were chosen based on the function in energy metabolism and their magnitude of fold change. *PROM1* and *CYP7A1* were chosen to validate the reversion of gene expression to control in different treatments as compared to PA only treatment. *CPT1A*, *STBD1*, *SLC2A1* and *KRT23* were chosen because of their involvements in PPARA and PPARG pathways. *PLA2G2A* was selected for its function in inflammation process and lastly, *AREG* was selected due to its large magnitude of fold change in the microarray data. Generally, the results were in good agreement with the microarray data in terms of direction and magnitude of change, thus validating the microarray gene expression data (Figure 4.25).

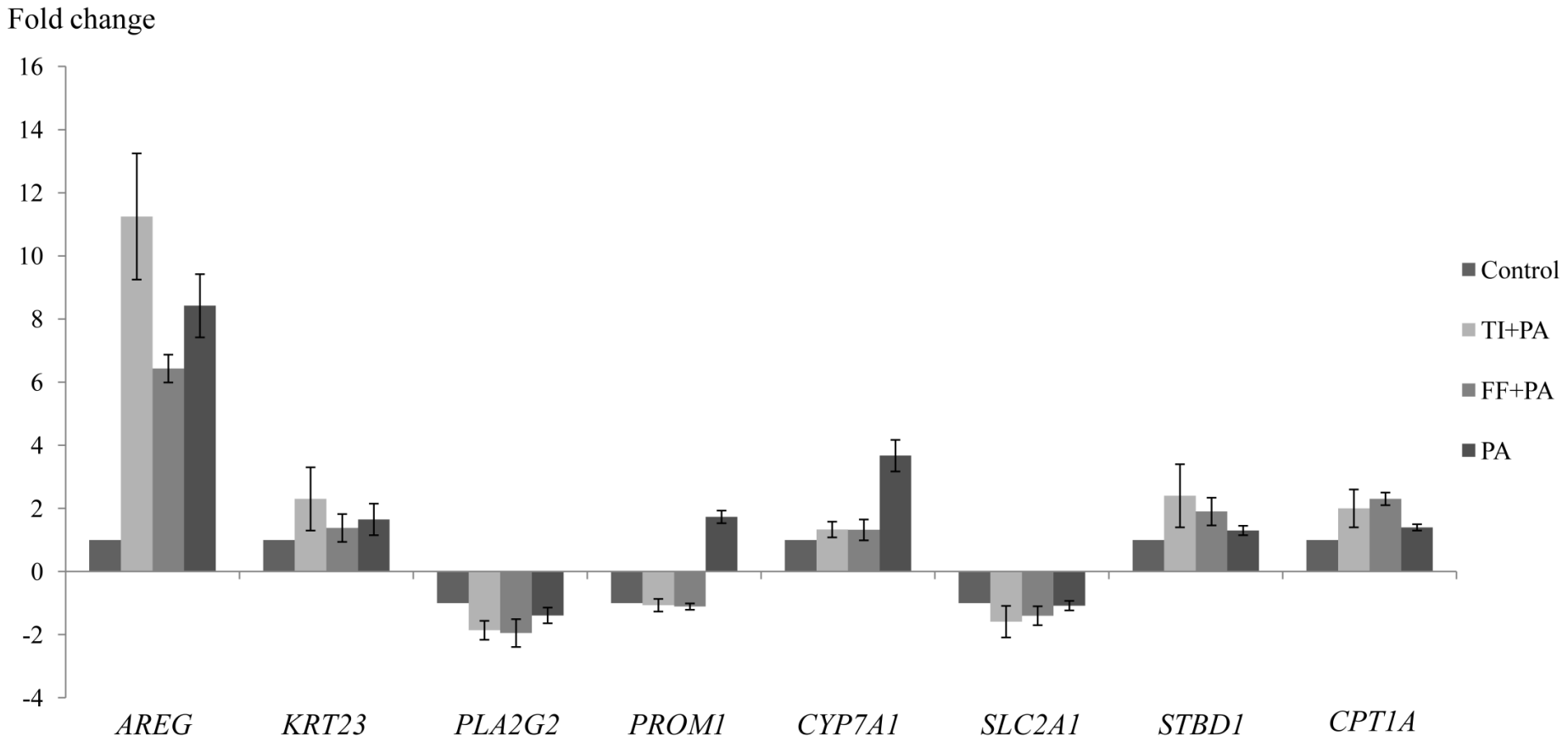

Figure 4.25: Validation of microarray data using quantitative real-time polymerase chain reaction (qRT-PCR)

Assays were done using three biological replicates and three technical replicates and data were represented as mean ± standard error.

Legend abbreviations: TI- *T. indica* fruit extract; PA- palmitic acid; FF- fenofibrate

Gene name abbreviations: AREG, amphiregulin; KRT23, keratin 23; PLA2G2, phospholipase A2, group IIA; PROM1, prominin 1; CYP7A1, cytochrome P450, family 7, subfamily A, polypeptide 1; SLC2A1, solute carrier family 2, member 1; STBD1, starch binding domain 1; CPT1A, carnitine palmitoyltransferase 1A.

CHAPTER 5

DISCUSSIONS

Previous microarray study by Razali et al. (2010) revealed that *T. indica* fruit extract significantly regulated genes that are involved in lipid metabolism and antioxidant activities; however the molecular mechanism has yet to be deciphered. Therefore, this is a continuation study from Razali et al. (2010), with the main objective is to further investigate the mechanism of action of the lipid-lowering effect of the *T. indica* fruit pulp extract. In view of the extensive possibilities that could attribute to the hypolipidaemic effect, proteomic analyses were first performed on the cell lysate and secretome of HepG2 cells treated with *T. indica* fruit extract to narrow down the possible mechanisms. Based on the proteins that were significantly altered in proteomic study and genes that were differentially expressed in the previous microarray data, a proposed mode of action was formulated, which was then tested using DNA microarray. The lipid-lowering activities of *T. indica* fruit were also measured in HepG2 cells.

5.1 Proteomic studies

In this study, proteomic techniques were used to analyse the expression of cytosolic proteins as well as proteins that were released by HepG2 cells in response to treatment with the methanol extract of *T. indica* fruit pulp. Serum-free DMEM culture media from HepG2 cells grown for 24 h in the absence and presence of *T. indica* fruit pulp extract were initially subjected to 2D-GE. The use of serum-free medium was necessary for the proteomic analysis to avoid masking of the proteins released by the cells as opposed to the highly abundant proteins present in FBS. The results of our MTT assays showed that viability of the HepG2 cells was not affected by use of serum-free

medium and neither was it significantly different when the cells were exposed to the *T. indica* fruit pulp extract.

5.1.1 Methanol extract of *T. indica* fruit pulp altered the secretion of proteins from HepG2 cells

Among the thousands of protein spots that were detected in the 2D-GE profiles of culture media isolated from HepG2 cells grown in the absence or presence of the methanol extract of *T. indica* fruit pulp, only seven were found to be altered in expression. Five of the protein spots were identified by using mass spectrometry analysis and found to be those of TTR, ENO1, GDI-2 and ApoA-I (2 isoforms), whilst two spots were not successfully identified. Exposure of the HepG2 cells to the *T. indica* fruit pulp extract appeared to have caused the increased release of ENO1 and GDI-2 but decreased secretion of TTR and ApoA-I. While the two latter proteins are known to be secretory proteins, ENO1 and GDI-2 are apparently cytosolic proteins (Pfeffer, Dirac-Svejstrup, & Soldati, 1995). However, several earlier studies had also detected the presence of ENO1 and GDI-2 in the culture media of HepG2 cells (Bottoni, Giardina, Vitali, Boninsegna, & Scatena, 2009; Higa et al., 2008).

When the differentially expressed proteins were subjected to analysis using IPA, all but ENO1 were found to be interconnected with interactomes in lipid metabolism. ENO1, although more popularly known as a glycolytic enzyme, is apparently a multifunctional protein that also acts as a receptor, activator and regulator molecule (Pancholi & Fischetti, 1998; Subramanian & Miller, 2000). Hence, the ENO1 that was detected in the culture media in this study may not be involved in glycolysis. Due to the multiple roles played by ENO1, it is difficult to speculate the reason why the release of

the protein was increased when HepG2 cells were exposed to the *T. indica* fruit pulp extract.

Interestingly, the three differentially expressed proteins that are involved in lipid metabolism appeared to be commonly associated with the same hormonal regulation, i.e., estradiol, and the homeostasis of cholesterol. GDI-2 functions to translocate prenylated Rab proteins from the cytosol to the membrane to form nascent transport vesicles. The protein also assists the subsequent retrieval of Rab proteins (Pfeffer, et al., 1995; Stenmark & Olkkonen, 2001), which are key regulators for the transport of lipids and proteins between cell organelles from target membranes (Pfeffer, 2001; Stenmark & Olkkonen, 2001; Zerial & McBride, 2001). To date, approximately 70 Rab proteins had been identified but their specific functions are still largely unknown (Agola, Jim, Ward, Basuray, & Wandinger-Ness, 2011). These include Rab11, whose over expression has been shown to block the recycling of cholesterol from the endosome recycling compartment to the plasma membrane (Holtta-Vuori, Tanhuanpaa, Mobius, Somerharju, & Ikonen, 2002; Soccio & Breslow, 2004). On the other hand, Rab8 has been shown to assist the redistribution of cholesterol from late endosomes to the cell periphery and stimulate cholesterol efflux through the ABCA1/ApoA-I pathway (Linder et al., 2007). The increased release of GDI-2 by HepG2 cells when they were exposed to the methanol extract of *T. indica* fruit pulp was probably to enable the recycling of Rab proteins that are involved in the cholesterol homeostasis in the cell.

TTR is a protein that is mainly synthesised in the liver and the choroid plexus of the brain (Aleshire, Bradley, Richardson, & Parl, 1983). Its two main functions are to transport thyroxine and retinol through binding to the hormone and retinol-binding protein, respectively (Hagen & Solberg, 1974; Raz & Goodman, 1969). However, a

small fraction of plasma TTR (1-3 %) is apparently associated with ApoA-I, the major apoprotein found in the anti-atherogenic lipoprotein HDL. ApoA-I is synthesised in the liver (as well as the intestine) and its secretion is believed to be either in a lipid-free/poor or pre-lipidated forms (intracellularly assembled nascent HDL) (Chisholm, Burleson, Shelness, & Parks, 2002). Ohnsorg (Ohnsorg et al., 2011), Liz (Liz, Gomes, Saraiva, & Sousa, 2007) and their co-workers showed that TTR cleaves the C-terminus of ApoA-I, which is necessary for the transport of lipid-free ApoA-I through the aortic endothelial cells. When HepG2 cells were exposed to the tamarind extract in this study, secretion of both TTR and ApoA-I was reduced by more than 2-fold. The two proteins were also shown to be interconnected via our IPA analysis. This alteration reflects the indirect effects of *T. indica* fruit pulp extract in regulating the function of HDL in the reverse transport of cholesterol and also in line with the earlier findings on the cholesterol and triacylglycerol lowering effects of the fruit extract in hypercholesterolaemic hamsters (Martinello, et al., 2006) and in humans (Iftekhar, et al., 2006).

Earlier report by Razali et al. (2010), described the up-regulated expression of the *APOA1* gene by 1.2-fold when HepG2 cells were exposed to the same concentration of *T. indica* fruit pulp extract (Razali, et al., 2010). The high ApoA-I mRNA levels that were detected in HepG2 cells upon exposure to the extract as opposed to the low levels of ApoA-I that appeared in the media could be either due to the decrease in the rate of export of the apoprotein or that the mRNA may not be translated into ApoA-I. Previous study by the same group also showed that the *T. indica* fruit pulp extract induced the expression of the *ABCG5* gene. ABCG5 apparently forms a dimer with ABCG8 to assist the secretion of cholesterol into the bile and its subsequent excretion via the faeces. Therefore, the lower ApoA-I secretion that was detected in the present study

suggests that the *T. indica* fruit pulp extract may be promoting the excretion of cholesterol via the ABCG5 instead of the ApoA-I transport system. Interestingly, the ABCG5/8-mediated cholesterol excretion and absorption and ABCA-1-mediated cholesterol efflux are apparently controlled by the liver X receptors, LXRs, which was also in agreement to the canonical pathway generated in IPA analysis (Figure 4.11).

5.1.2 Methanol extract of *T. indica* fruit pulp altered the abundance of cytosolic proteins in HepG2 cells

In this study, the abundance of 20 cell lysate proteins was found to be significantly reduced when HepG2 cells were exposed to the *T. indica* fruit pulp extract. Fourteen of the proteins were identified by mass spectrometry and database search, and the reduced abundance of three representative identified HepG2 mitochondrial and metabolic proteins were subsequently validated by Western blotting.

Among the identified HepG2 proteins, three components of the mitochondrial respiratory chain, i.e. ubiquinol-cytochrome-c reductase complex core protein 2 (UQCRC2), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 10 (NDUFA10) and NADH dehydrogenase (ubiquinone) flavoprotein 1 (NDUFV1), were found to be reduced in abundance when HepG2 cells were exposed to *T. indica* fruit pulp extract. UQCRC2 belongs to complex III of the mitochondrial respiratory chain while NDUFA10 and NDUFV1 are components of complex I. In mitochondria, only complex I (Barja & Herrero, 1998; Genova et al., 2001) and complex III (Boveris, Cadenas, & Stoppani, 1976) of the respiratory chain are known to produce reactive oxygen species (ROS). Hence, the decreased amount of the three mitochondrial components may indicate reduced production of free radicals, although the functionality of the mitochondria is yet to be confirmed. Nevertheless, earlier studies in obese rat

muscles have shown that reduced levels of respiratory chain complex I and diminished ROS production that were induced by chronic supplementation with grape seed proanthocyanidins did not affect the function of the mitochondria (Pajuelo et al., 2011).

In addition to the mitochondrial respiratory chain proteins, the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as well as GDP-L-fucose synthetase (TSTA3), also appeared to be down-regulated after treatment with *T. indica* fruit pulp extract. Oxidative stress is known to induce up-regulation of GAPDH levels (De Marco et al., 2012; Ito, Pagano, Tornheim, Brecher, & Cohen, 1996). Hence, the reduced amount of GAPDH in this case may possibly indicate a state of repressed oxidative stress. This, together with the earlier data on the reduced mitochondrial respiratory chain proteins, may shed some light on molecular mechanisms involved in the well acclaimed antioxidant properties of *T. indica* (Martinello, et al., 2006; Sudjaroen, et al., 2005).

The fruit pulp extract of *T. indica* also appeared to cause decreased abundance of proteins involved in the metabolism of nucleic acids and polyamines in HepG2 cells. To the best of our knowledge, these have not been previously reported and their rationale is not quite understood. On the other hand, decreased abundance of ethanolamine-phosphate cytidylyltransferase (PCYT2), the rate-limiting enzyme which catalyses conversion of phosphoethanolamine to cytidylylphosphoethanolamine in the biosynthesis of phosphatidylethanolamine, in HepG2 cells exposed to the fruit extract of *T. indica* may compromise availability of the phospholipid, which stores arachidonic acid for the production of prostaglandins. This could possibly explain the anti-inflammatory action of *T. indica* that was earlier reported (Rimbau, et al., 1999).

Prohibitin is another mitochondrial protein that was shown to be of reduced abundance in HepG2 cells exposed to *T. indica* fruit pulp extract. The depletion of prohibitin has been reported to promote the lifespan of an organism, although it has not been shown on higher organisms and it is dependent on the metabolic state of the organism (Artal-Sanz & Tavernarakis, 2010). Prohibitin is an evolutionary highly conserved protein that is located in the mitochondria. Many roles have been suggested for this protein such as acting like a chaperone (Nijtmans et al., 2000), protease (Steglich, Neupert, & Langer, 1999) and scaffolding proteins (Osman et al., 2009). Certain review papers also mentioned that it plays a role in cell cycle regulation, regulation of transcription and cell surface signalling (Mishra, Murphy, & Murphy, 2006; Rajalingam & Rudel, 2005). However, the true biochemical function of prohibitin remains elusive. While some studies showed that the depletion of prohibitin shortened the lifespan of wild-type *Caenorhabditis elegans* (Artal-Sanz & Tavernarakis, 2009) and yeast (Berger & Yaffe, 1998), a study by Artal-Sanz and Tavernarakis (2009) had shown that under certain metabolic conditions, the reduced prohibitin can prolong the lifespan of the animal. It is interesting that the result from the study coincides with this study in certain manner, for example prohibitin depletion results in reduced fat content, in all genetic backgrounds where prohibitin deficiency extends lifespan (Artal-Sanz & Tavernarakis, 2009). *T. indica* has been used traditionally to treat hyperlipidaemia, and studies have shown that it possesses hypolipidaemic effect *in vivo* (Lim, et al., 2013; Martinello, et al., 2006). In this study, prohibitin was down-regulated by 1.7-fold. While there may be no direct relationship in between prohibitin depletion and lipid-lowering-effect, the depletion of prohibitin in HepG2 cells upon exposure to the fruit pulp extract of *T. indica* appears to suggest a similar mechanism in attempt to extend lifespan of the cells.

Adding to that, the reduced amount of proteins involved in protein biosynthesis, i.e. eukaryotic translation initiation factor 3 subunit 3 (eIF3H), tyrosyl-tRNA synthetase (YARS) and elongation factor Tu (EFTU), which may indicate lowered protein synthesis rate, had been shown to increase lifespan of *C. elegans* (Hansen et al., 2007; Pan et al., 2007; Syntichaki, Troulinaki, & Tavernarakis, 2007). Hipkiss, AR (2007) pointed out that this could be due to lesser error proteins such as misfolded proteins being produced, therefore increasing the availability of proteases and chaperones for the removal of erroneous proteins that may lead to amyloidal accumulation. These reduced proteins involved in protein biosynthesis may be key regulatory points of action of the fruit pulp extract of *T. indica.* Suppression of these proteins by the extract may reflect the underlying epigenetic mechanism that ultimately caused the reduced expression of all the mitochondrial and metabolic proteins and enzymes.

Subjecting the altered abundance proteins to IPA analysis generated a single network on "Hereditary disorder, metabolic disease, molecular transport", which ranked mitochondrial dysfunction with the highest significance ($p < 3.65 \times 10^{-4}$). However, "Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry" became the top network involved when IPA was reanalysed to include proteins that were previously shown to be differentially secreted by HepG2 cells treated with the same fruit extract. This network was not generated in the earlier analysis as PCYT2 was the sole cellular protein involved in lipid metabolism that was affected when HepG2 cells were exposed to *T. indica* fruit pulp extract. In the earlier IPA analysis of secreted proteins of altered abundance from HepG2 exposed to the *T. indica* fruit pulp extract, a score of 9 was obtained and this improved to 31 when the data were reanalysed to include cell lysate proteins of reduced abundance, signifying markedly higher probability. In addition, the IPA software also identified tumour necrosis factor (TNF) and interleukin-1 beta (IL-

1β), both of which are potent inflammatory mediators, as interactomes in the network affected by *T. indica*. This further supports our earlier speculation of the molecular mechanism involved in anti-inflammatory effects of *T. indica*.

5.1.3 PPARα activation: possible mode of action of lipid-lowering effect of *T. indica* fruit pulp extract

Peroxisome proliferator-activated receptors or PPARs are nuclear receptors. There are 3 isoforms identified in humans: PPARα, PPARβ/δ and PPARγ. PPARα is highly expressed in liver, brown adipose tissue, heart, skeletal muscle, kidney, and at lower levels in other organs. PPARγ is highly expressed in adipose tissues and is present in the colon and lymphoid organs while PPARβ/δ is expressed ubiquitously, but its levels may vary considerably.

In this study, we proposed that the *T. indica* fruit extract exerts its lipid-lowering effects through the activation of PPARα. In the liver, PPARα promotes fatty acid oxidation and it is the target for the hypolipidaemic fibrates, such as fenofibrate, clofibrate and gemfibrozil, which are used in the treatment of hypertriglyceridaemia. This was shown by the up-regulation of genes involved in the mitochondrial β-oxidation in the microarray analysis after *T. indica* fruit treatment (Figure 5.1) (Razali, et al., 2010). Besides promoting fatty acid oxidation, it also mediates its action through the activation of liver X receptor alpha (LXRα) (Johnston & Waxman, 2008), which was also derived as the one of the canonical pathways generated in Ingenuity Pathway Analysis (IPA) software (Figure 4.11). LXRs are oxysterol-activated nuclear receptors, which control cholesterol homeostasis by modifying expression of genes involved in cholesterol absorption and efflux from peripheral tissues. This process is mediated through ABCA1-mediated cholesterol efflux and ABCG5/8-mediated cholesterol

excretion and absorption. In the microarray study, ABCG5 was up-regulated by 1.8-fold (Razali, et al., 2010).

LXRs also regulate genes essential in lipogenesis, glucose metabolism, and inflammation. A possible explanation for the anti-inflammatory action of *T. indica* fruit pulp is that it reduces the arachidonic acid reservoir pool, the principal substrate of the prostanoid inflammatory mediators by inhibiting phospholipid synthesis. Ethanolamine-phosphate cytidylyltransferase (PCYT2) is the rate-limiting enzyme that catalyses the conversion of phosphoethanolamine to cytidylylphosphoethanolamine in the Kennedy's pathway or the biosynthesis of phosphatidylethanolamine. After the treatment with *T. indica* fruit extract, cytosolic PCYT2 expression was down-regulated by 1.7-fold which is hypothesised to lead to lower production of phosphatidylethanolamine (PE). As one of the component of the HDL phospholipids, its reduction could enhance the ABCA1-mediated efflux and reduce the SR-BI- mediated efflux (Yancey et al., 2004). ABCA1-mediated efflux has a higher efflux potential compared to SR-BI-mediated efflux. Microarray study by Razali et al. (2010) also showed that choline kinase alpha (CHKA) was down-regulated by 1.8-fold, which may lead to a decreased level of phosphatidylcholine (PC). The lowered amount of phospholipids (PE and PC) could reduce the availability of arachidonic acid, which will in turn reduce the prostaglandin produced. This could possibly explain the anti-inflammatory action of this plant as reported by many. Besides this, the network generated by the IPA software also identified tumour necrosis factor (TNF) and interleukin-1 beta (IL1B) as two of the interactomes in the network, both of which are potent inflammatory mediators (Figure 4.10). This suggests that the fruit may also play a role in mediating the inflammation pathway, besides regulating the lipid metabolism.

Although studies have shown that PPARα raises the HDL in human plasma, some researchers showed that it was otherwise (Berthou et al., 1996; Duez et al., 2002). Huuskonen et al. (2006) reported that the LXR agonist, T0901317 inhibited the synthesis of apoA-1. The lowered amount of transthyretin and apoA-1 is possibly due to the inhibition of orphan nuclear receptor hepatocyte nuclear factor 4 (HNF4) by PPARα activation. It has been shown that the PPARα agonist, Wy 14643, reduced the expression of HNF4 in HepG2 cells (Marrapodi & Chiang, 2000). HNF4 is a liver-enriched transcription factor that controls embryonic liver development and regulates tissue-specific gene expression in adult liver cells. HNF4 activates several hepatocyte-specific genes, including the gene encoding apoA-1 (Malik & Karathanasis, 1996) and transthyretin (J. W. Park, Lee, Choi, Park, & Jung, 2010; Z. Wang & Burke, 2007). Therefore, the inhibition of HNF4 by PPARα may account for the lowered amount of apoA-1 and transthyretin after the treatment with *T. indica* fruit extract.

The lipid-lowering effects induced by plant polyphenols have been reported by many. In fact, there is an upcoming trend of researches revealing the potentials of plant polyphenols in regulating metabolic processes *in vitro* and *in vivo*. Methanolic extract of *T. indica* fruit pulp contains catechin, epicatechin, procyanidins, naringenin, apigenin, luteolin, taxifolin and eriodictyol (Sudjaroen, et al., 2005). It was shown that tea catechins like epigallocatechin gallate (EGCG) and epigallocatechin (EGC) were able to activate PPARα (K. Lee, 2004). Naringenin from grapefruit was shown to regulate lipid metabolism through partial activation of PPARα (Goldwasser, et al., 2010). Another study showed that flavangenol extracted from pine bark was able to enhance fatty acid oxidation, mainly attributed to procyanidin B1 (Shimada, et al., 2012). This shows that a number of polyphenols were able to regulate nuclear receptors such as PPAR and LXR. Earlier studies have also shown that proanthocyanidins, which

constitutes more than 73 % of the total phenolic content of *T. indica* extract (Sudjaroen, et al., 2005), were able to modulate the activation of LXR/RXR (Jiao, et al., 2010). Hence, the data of this study, when taken together with that of our earlier report by Razali et al. (2010), suggest that the *T. indica* fruit pulp extract exerts its lipid-lowering effects and anti-inflammatory actions through the modulation of the LXRs, a conception that was similarly derived from the canonical pathway analysis; or at a higher order, that is the activation of PPARα, both of which were highly interrelated in terms of gene regulated by their activations.

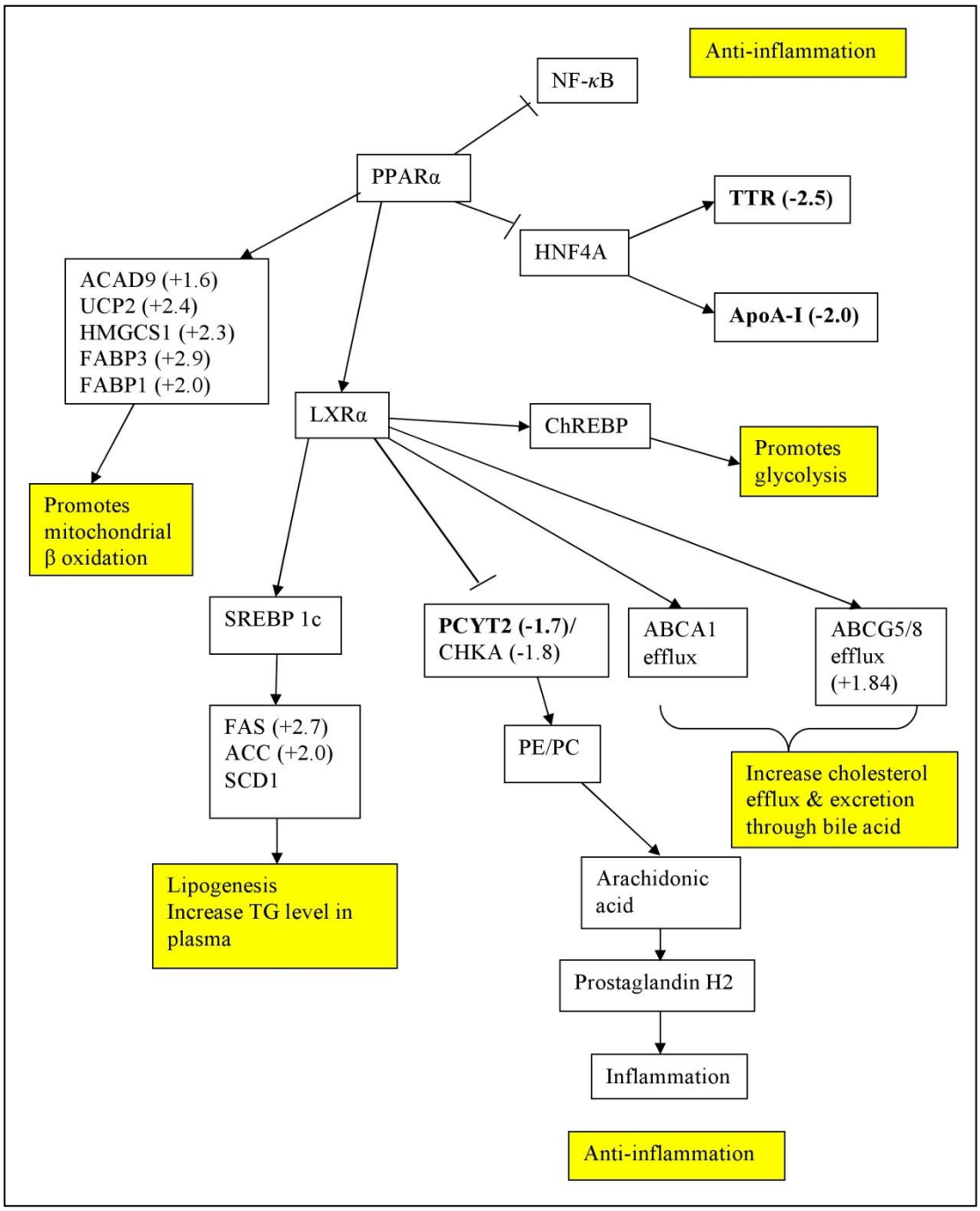

Figure 5.1: Proposed mechanism of action induced by *T. indica* fruit pulp through activation of peroxisome proliferator-activated receptor alpha (PPARα)

The gene names in **bold** are proteins regulated in this proteomic study; while the gene names in normal font are genes significantly regulated in the previous microarray study (Razali, et al., 2010).

5.2 Transcriptomic studies

To further investigate the earlier hypothesis that *T. indica* fruit pulp extract exerts its lipid-lowering effects through the activation of PPARα, transcriptome profiles in steatotic HepG2 cells treated with either *T. indica* fruit extract or fenofibrate, a PPARα agonist and a hypolipidaemic drug, were compared. HepG2 cells were treated with palmitic acid to simulate steatotic condition, and the total triglyceride and cholesterol were quantitated after treatment with the fruit. Cell viability was assessed using MTT assay in order to determine the concentration of palmitic acid to best induce steatotic effect in HepG2 cells without causing extensive cell death. A cell viability of more than 90 % after treatment was considered to be appropriate for this study. Based on this, treatment with 0.3 mM palmitic acid for 24 h was considered to induce hyperlipidaemic effect in HepG2 cells. Oil Red O staining of the lipid droplets also showed that at 0.3 mM of palmitic acid treatment, the lipid droplets were clearly visible. Although at a higher concentration of palmitic acid treatment (0.8 mM palmitic acid) the lipid droplets were much bigger and abundant, the cell death was quite extensive; only 65 % of cells were still viable after 24 h.

The total triglyceride and cholesterol in HepG2 cells after treatment with different concentrations of *T. indica* fruit extract was also quantified. At 0.1 mg/ml *T. indica* fruit concentration, both total triglyceride and cholesterol content were reduced. The lipid-lowering effect of the fruit extract was more prominent in reducing total triglyceride than total cholesterol; even to the level comparable to that of fenofibrate at the concentration of 0.1 mg/ml *T. indica* fruit extract. This is also in agreement to the fact that fenofibrate was more efficient in lowering triglyceride than cholesterol, probably signifying that the fruit may exert a similar mechanism to that of fenofibrate, that is through the activation of PPARα. However, further analysis is still needed to

confirm the hypothesis. At higher concentrations of *T. indica* fruit treatment, the lipid levels were increased. This was also supported by the Oil Red O staining of HepG2 cells which showed a more abundant lipid droplet in cells treated with high concentration of *T. indica* fruit extract. The adverse effects of *T. indica* fruit extract at high concentration could be due to the excessive oxidative stress exerted by compounds like proanthocyanidin, the major polyphenol in *T. indica* fruit extract. It has been shown that grape seed proanthocyanidins can induce pro-oxidant toxicity in cardiomyocytes at high dose (Shao et al., 2003). However at low dose, proanthocyanidins are anti-oxidants and were shown to be cardioprotective (Corder et al., 2006).

5.2.1 *T. indica* fruit extract regulated genes that are involved in fatty acid oxidation

Hepatic steatosis or fatty liver disease is characterised by accumulation of triglycerides in the vacuoles of liver cells. Fatty liver disease can progress from simple steatosis with no symptoms, through non-alcoholic steatohepatits (NASH), fibrosis and cirrhosis, which can result in liver cancer, liver failure and death. The current treatment for fatty liver disease is using drugs that are able to lower lipid levels particularly triglyceride to reduce the accumulation of triglycerides in the liver for example the fibrate drugs. The use of fibrate drug, which is a PPARα ligand induces fatty acid oxidation, which in turn increases lipid catabolism and thus reducing lipid levels in the liver. In this study, it was shown that the fruit exerts its lipid-lowering effect through the activation of fatty acid oxidation, which was supported by the up-regulation of genes involved in the process (*ACSL1*, *CPT1A*, *CYP19A1*, *LPIN1*, *PNPLA8* and *SLC2A1*). This was also supported by the IPA analysis in which the process was shown to be activated with activation Z-score of 2.175.

Fatty acid oxidation is a process that breaks down fatty acids by beta-oxidation to produce acetyl-CoA and it takes place in the mitochondrion. Before catabolising long-chain fatty acids which are unable to pass through the inner mitochondrial membrane, the fatty acids are first converted to acyl-CoA to be transported into the mitochondria for the oxidation of fatty acid to occur. This process is mediated by the mitochondrial L-carnitine shuttle pathway, which was one of the top canonical pathways generated in the *T. indica* fruit and fenofibrate treatment groups. Two key genes involved in this pathway, *CPT1A* and *ACSL1* were up-regulated in both treatment groups. *ACSL1* gene codes for a rate limiting enzyme, acyl-CoA synthetase long-chain family member 1. It converts long chain fatty acid to acyl-coA, which is then transported across the outer mitochondrial membrane into the inner mitochondrial membrane. *CPT1A* gene codes for carnitine palmitoyltransferase 1A, which is an enzyme that catalyses the conversion of acyl-CoA into acylcarnitine. Acylcarnitine is then translocated into the mitochondrial matrix for beta-oxidation to occur. Therefore, the up-regulation of these 2 genes is indicative of increased fatty acid being shuttled into the mitochondria for beta-oxidation of fatty acid.

CYP19A1 encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyse many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein localises to the endoplasmic reticulum and catalyses the last steps of estrogen biosynthesis, three successive hydroxylations of the A ring of androgens. It has been shown that homozygous mutant mouse aromatase (*Cyp19a1*) gene knockout in mouse decreases beta-oxidation of palmitic acid in a cell-free system (Nemoto, et al., 2000). Egawa et al. (2003) had also reported that pitavastatin increases beta-oxidation of lauric acid in homogenate from mouse liver that is decreased by homozygous mutant mouse

Cyp19a1 gene knockout. This shows that the up-regulation of *CYP19A1* is associated to increased beta-oxidation of fatty acid.

LPIN1 was up-regulated by 1.5-fold in *T. indica* treatment group. This gene encodes lipin 1, a magnesium-ion-dependent phosphatidic acid phosphohydrolase enzyme that catalyses triglyceride synthesis including the dephosphorylation of phosphatidic acid to yield diacylglycerol. Expression of this gene is required for adipocyte differentiation, activation of hepatic fatty acid oxidation genes during fasting conditions and it also functions as a nuclear transcriptional coactivator with peroxisome proliferator-activated receptor a (PPARα) and PPARγ coactivator 1a (PPARGC1A) in a complex that modulates fatty acid oxidation gene expression (Finck, et al., 2006). Mutations in this gene are associated with metabolic syndrome, type 2 diabetes, and autosomal recessive acute recurrent myoglobinuria (ARARM) (Reue & Dwyer, 2009).

SLC2A1 codes for GLUT 1, a major glucose transporter in the mammalian blood-brain barrier and it was down-regulated in both *T. indica* and fenofibrate treatment groups. Yan et al. (2009) reported that transgenic human SLC2A1 protein decreased oxidation of fatty acid in mouse heart that was increased by high fat diet. The down-regulation of this gene could imply an increase in fatty acid oxidation. However in a recent study the down-regulation of *SLC2A1* was linked to increased lipid accumulation and oxidative stress in NAFLD induced liver. Despite the results, the group was unsure that the down-regulation of *SLC2A1* induces triglyceride accumulation in liver or *vice versa* (Vazquez-Chantada et al., 2013). Nevertheless, given that this gene was also down-regulated in fenofibrate treated group in this study, which is a hypolipidaemic drug, the lowered expression of *SLC2A1* is more likely to be involved in the oxidation of fatty acid.

PNPLA8 encodes a member of the patatin-like phospholipase domain containing protein family. Members of this family are phospholipases which catalyse the cleavage of fatty acids from membrane phospholipids. The up-regulation of this gene is correlated to increased fatty acid oxidation and this is in agreement with Mancuso et al. (2007) whom reported that transgenic human PNPLA8 protein in cardiac myocytes increased oxidation of palmitic acid in working isolated perfused heart from 4-7 month-old adult mouse. However in 2010, the same author reported that homozygous mutant mouse PNPLA8 gene knockout increased the rate of oxidation of palmitic acid in epididymal adipose tissue explants from male mouse (Mancuso, et al., 2010).

5.2.2 *T. indica* fruit extract regulated genes that are involved in gluconeogenesis

T. indica fruit treatment had also led to the regulation of genes involved in gluconeogenesis (*G6PC*, *GK* and *SLC2A1*). IPA analyses had also shown that *T. indica* fruit decreased polysaccharide synthesis particularly glycogen based on the regulation of genes involved in polysaccharide synthesis (*G6PC*, *DKK1*, *VIMP*, *IGFBP1* and *CSGALNACT2*), with an activation z-score of -2.000.

Gluconeogenesis is a metabolic process which produces glucose from non-carbohydrate sources including glycerol derived from the breakdown of triglyceride. *G6PC* gene encodes glucose-6-phosphatase, which catalyses the hydrolysis of D-glucose 6-phosphate to D-glucose and orthophosphate. It is a key enzyme in glucose homeostasis, functioning in gluconeogenesis and glycogenolysis. The up-regulation of this gene is implicated with the increased activity of glycogenolysis and this is supported by Aiston et al. (1999) whom reported that G6PC protein decreased synthesis of glycogen in hepatocytes. The up-regulation of *G6PC* gene in this study could

indicate reduced glycogen synthesis. *GK* gene codes for glycerol kinase, an enzyme that catalyses the conversion of glycerol to dihydroxyacetone phosphate, which will be utilised in gluconeogenesis or glycolysis pathway. *SLC2A1* codes for glucose transporter 1 which facilitates transport of glucose across plasma membrane of mammalian cells. The reduced expression of this gene is linked to increased glucose levels (Hahn, Barth, Weiss, Mosgoeller, & Desoye, 1998). This may be a result of increased glucose production by the cell. Increased expressions of *IGFBP1* and *VIMP* genes were also associated with decreased synthesis of glycogen (Gao et al., 2003; Menuelle, Binoux, & Plas, 1995). Taken together, the regulation of these genes indicates an increased production of glucose through gluconeogenesis or glycogenolysis.

5.2.3 *T. indica* fruit extract lowers lipid through the activation of PPARα

Peroxisome proliferator activator receptor alpha or PPARα is a nuclear receptor that regulates lipid metabolism and glucose homeostasis. In the body, it is activated in fasting state and its activation will lead to regulations in an array of downstream genes that eventually increase lipid metabolism, gluconeogenesis, glycogenolysis and ketone bodies production.

In this study, PPARα was predicted to be activated in both *T. indica* fruit and fenofibrate treatment groups in the IPA upstream regulator analysis, with activation z-score of 2.745 and 2.379 respectively. While PPARα was expected to be activated in the fenofibrate group since it is a ligand to the PPARα receptor, the activation of PPARα by *T. indica* fruit treatment proved the hypothesis that was made prior to the microarray analyses, that is PPARα activation could be responsible for the lipid-lowering effect of

the fruit. This was shown by the simultaneous regulation of downstream genes involved in PPARα activation in both *T. indica* fruit and fenofibrate treatments.

Fatty acid oxidation is the hallmark of PPARα activation and it was indeed shown to be activated in both *T. indica* and fenofibrate treatment groups. Two genes, *CPT1A* and *ACSL1* which are involved in the mitochondrial L-carnitine shuttle pathway were shown to be up-regulated in both *T. indica* fruit and fenofibrate treatment. Besides this, they were also shown to be increased in PPARα activation (Ammerschlaeger, et al., 2004; Begriche, et al., 2006; Clemenz, et al., 2008; Finck, et al., 2002; M. H. Hsu, et al., 2001; Lawrence, et al., 2001; Schoonjans, et al., 1995; Tachibana, et al., 2006; Vega, et al., 2000). The up-regulation of both *CPT1A* and *ACSL1* genes indicates that PPARα was being activated in *T. indica* fruit treatment group. Besides this, fatty acid oxidation genes were also shown to be up-regulated in the presence of lipin-1 gene, which was up-regulated in *T. indica* fruit treatment. In the cytoplasm, lipin-1 is an enzyme responsible in triglyceride accumulation and phospholipid synthesis; however when translocated to the nucleus, it functions as a transcriptional co-activator together with PPARGC1A to PPARα that leads to the induction of fatty acid oxidation genes (Finck, et al., 2006).

PPARα activation also modulates glucose homeostasis. While contradictory findings regarding the regulation of glucose by PPAR activation had been reported (Peeters & Baes, 2010), it was certain that PPARα activation leads to the regulation of glucose levels in fasting conditions. This was supported by studies showing that fasting PPARα knockout mice displayed marked hypoglycaemia (Bandsma, et al., 2004; Chakravarthy et al., 2005; Kersten et al., 1999; Leone, Weinheimer, & Kelly, 1999; Patsouris, et al., 2004). As mentioned earlier, treatment with *T. indica* fruit regulated

genes involved in gluconeogenesis and it was supported by the IPA analysis which showed that the synthesis of polysaccharide particularly glycogen was reduced. Since glucose level or regulation is not studied in this experiment, a clear conclusion cannot be drawn at this juncture. However a few genes regulated in this study pointed to the activation of PPARα. For example, a key enzyme involved in gluconeogenesis and glycogenolysis, *G6PC* was demonstrated to be modulated in the event of PPARα activation. While it was shown that homozygous mutant mouse *Ppara* gene knockout decreased expression of mouse *G6pc* mRNA that involves fasting by mouse (Bandsma, et al., 2004), Fan et al. (2011) reported that inhibition of active human PPARA protein increases expression of human *G6PC* mRNA in serum-deprived HepG2 cells and a similar observation was seen in *Ppara* gene knockout mutant mouse. Another gene involved in PPARα activation is the *GK* gene, which codes for glycerol kinase. Patsouris et al. (2004) reported that PPARα induced gluconeogenic genes including glycerol kinase during fasting in wild-type mice but not in PPARα null mice, indicating that this gene is a target of PPARα activation.

PPARα activation is also implicated in the bile acid metabolism, although the mechanism of action by which PPARα controls bile acid homeostasis remains unclear. In this study, *CYP7A1*, a key gene responsible in the bile acid biosynthesis pathway, was reduced in expression to the level similar to control. It has been shown that *Cyp7a1* gene is down-regulated in PPARα null mice in fasting condition (Rakhshandehroo et al., 2007). Ironically, synthetic PPARα agonists reduce Cyp7a1 expression in both mice and human (Bertolotti et al., 1995; Post et al., 2001; Stahlberg, Angelin, & Einarsson, 1989; Stahlberg et al., 1995). In agreement with the latter observation, fibrate treatment leads to decreased bile acid synthesis. The mechanism that led to change in Cyp7a1 level is unclear as PPARα also modulates the expression of other nuclear hormone

receptors such as FXR and LXR. It has also been suggested that PPARα can antagonise LXR signaling and LXR-dependent activation of *Cyp7a1* gene promoter (Gbaguidi & Agellon, 2002; Miyata, McCaw, Patel, Rachubinski, & Capone, 1996; Yoshikawa et al., 2003).

Other genes involved in PPARα activation that were also regulated in both *T. indica* fruit and fenofibrate treatment groups are *STBD1*, *KRT23* and *NCF2*. *STBD1* encodes starch binding domain 1 while *KRT23* codes for keratin 23. It was shown that in male mouse, homozygous mutant mouse *Ppara* gene knockout decreases expression of mouse *Stbd1* and *Krt23* mRNA in liver from male mouse that involves fasting (Sanderson, et al., 2010). Similarly, the up-regulation of *NCF2* was reported to be associated with activation of PPARα in macrophages nuclei (Teissier, et al., 2004). Perilipin 2 (*PLIN2*), another target gene of PPARα in liver was also up-regulated in the treatment with *T. indica* fruit. A study showed that PPARα-induced-Plin2 prevents the formation of VLDL by diverting fatty acids from the VLDL assembly pathway into cytosolic triglycerides (Magnusson et al., 2006). Figure 5.2 depicts the significantly regulated genes involved in PPARα activation in *T. indica* fruit treatment.

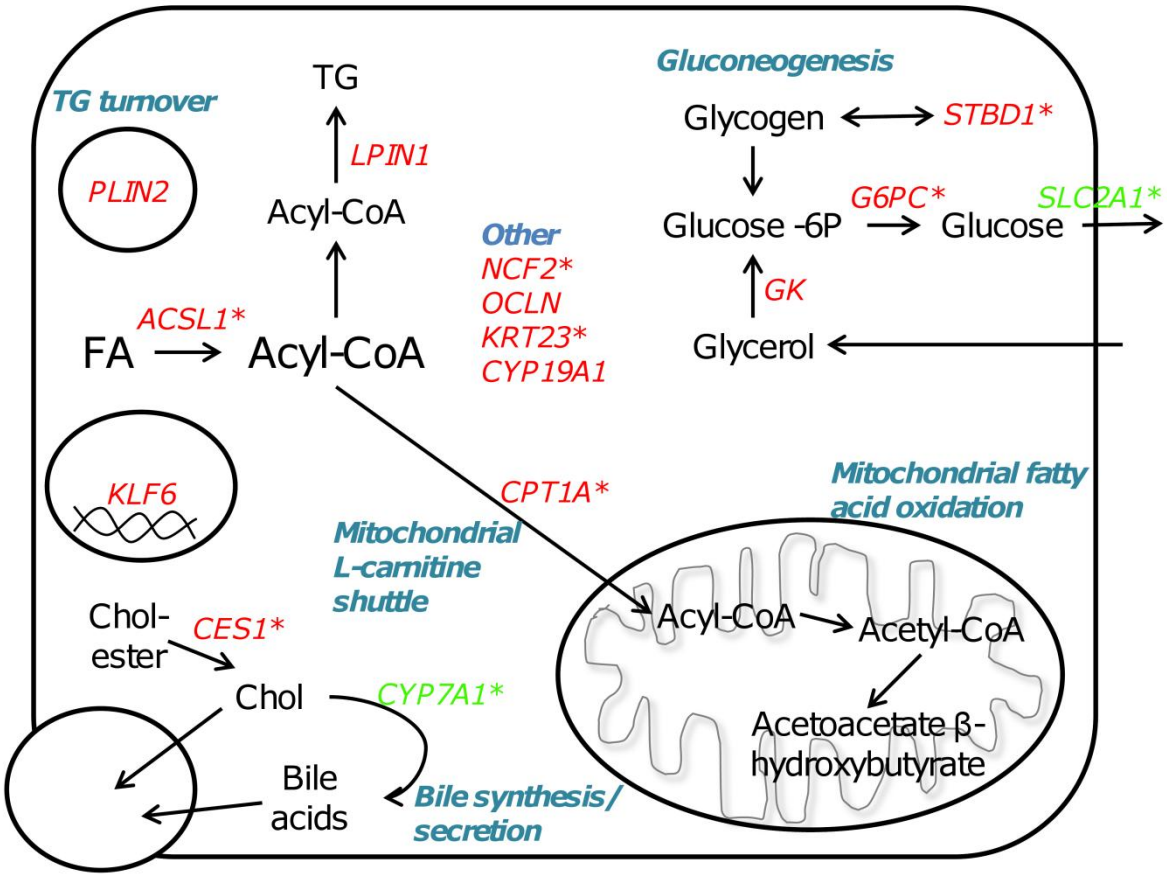

Figure 5.2: Significantly regulated genes involved in PPARα activation in hepatocyte

Red colour genes indicate up-regulated genes while green colour genes indicate down-regulated genes in *T. indica*-treated HepG2 cells. Asterisk indicates that the gene was regulated in both *T. indica* fruit and fenofibrate treatment groups.

5.2.4 PPARGC1A or PGC1A: the key regulator of multiple nuclear receptors

Based on the genes that were significantly regulated after treatment with *T. indica* fruit in the microarray study, it was shown that many transcription factors were predicted to be activated and many of which are involved in lipid and glucose homeostasis (PPARA, PPARG, CREB1 and PPARGC1A). This implies that the fruit may have exerted its lipid-lowering effects through the activation of one or more of these transcription factors. It should be noted that cross-talk can occur between transcription factors, as seen in the overlapping of genes regulated by different transcription factors (Figure 4.24). This further complicates the identification of a precise mode of action of the fruit in lowering lipid. However the activation of PPARA by the fruit was eminent based on the fact that it regulated genes involved in fatty acid oxidation and was comparable to fenofibrate, both of which were predicted to activate PPARA. However, the prediction about the activation of other transcription factors could indicate that the fruit probably mediates its hypolipidaemic effects through regulation at a higher order, or at an entirely different mechanism of action altogether.

The activation of multiple transcription factors could be explained by the involvement of PPARGC1A. PPARGC1A or PGC1A is a transcriptional coactivator responsible for the regulation of many metabolic processes like gluconeogenesis, adaptive thermogenesis, positive regulator of mitochondrial biogenesis and respiration. (Handschin & Spiegelman, 2006). PGC-1 coactivators functionally interact with transcription factors like PPARG, PPARA, ERR, LXR and HNF-4a (Nagai et al., 2009; Puigserver & Spiegelman, 2003; Yang, Williams, & Kelly, 2009) and non-nuclear receptor transcription factors and regulatory elements including cAMP response element-binding protein (CREB), sterol regulatory element-binding protein-1c (SREBP-1c) and forkhead box O1 (FOXO1) (Gupta et al., 2005; Nakae et al., 2002; Puigserver

et al., 2003; Yamagata et al., 1996; J. C. Yoon et al., 2001). In a review by Sugden et al. (2010), PGC1A works in orchestration with PPARA, FOXO1, HNF4A and CREB in inducing hepatic gluconeogenesis. It was also shown that lipin-1 (LPIN1), which was up-regulated in the microarray study, induces fatty acid oxidation by forming a complex with PGC1A and PPARA (Figure 5.3). The authors also proposed that SIRT1 may be the key molecule regulating the PPARs, PGCs and lipin-1 in modulating the metabolic responses in tissues including liver and adipose tissues in varying nutrient and physiological signals.

The transactivation of PPARα can be mediated by PPARα ligands or by the presence of high levels of *PPARGC1A* (Sanderson et al., 2009). PPARGC1A was predicted to be activated in the *T. indica* treatment group in the IPA upstream regulator analysis, with an activation z-score of 2.394. The result was supported by the fact that genes that were involved in the *PPARGC1A* activation were up-regulated, i.e. *TRIB3*, *PLIN2*, *LPIN1*, *G6PC* and *CPT1A*. PPARGC1A is a transcription coactivator that modulates lipid metabolism, energy production and glucose metabolism. It was shown that lipin-1 (*LPIN1*) which was up-regulated in the *T. indica* fruit treatment enhanced fatty acid oxidation by forming a complex with PPARGC1A and PPARA (Finck, et al., 2006) (Figure 5.3).

The activation of PPARGC1A is also implicated in increased glucose production in the liver (Herzig et al., 2001). In this study CREB1, FOXO1 and PPARGC1A were predicted to be activated in the IPA analyses (Table 4.15). CREB was activated by TORC2 in the presence of glucagon during fasting. The activated CREB subsequently induces PPARGC1A and the formation of a complexion consisting of GR, FOXO1, HNF4A and PPARα induces genes involved in gluconeogenesis (Dentin et al., 2007; X.

Li, Monks, Ge, & Birnbaum, 2007). PPARGC1A was also reported to be involved in inducing gluconeogenesis through a non-classical pathway that does not involve glucocorticoids and glucagon by coactivating FOXO1 and HNF4A to induce gluconeogenesis-related genes (Rodgers et al., 2005).

PPARα activation has been indicated in lipid-lowering activities mainly through fatty acid oxidation, which was indeed shown to be activated in both *T. indica* fruit and fenofibrate treatment groups. However its involvement in glucose homeostasis remains ambiguous. While two studies reported that the fruit exhibited glucose lowering properties in hyperglycaemic mice (Koyagura et al., 2013; Roy et al., 2010), our previous treatment using *T. indica* ethanolic extract on obese hamsters showed no significant changes in glucose level (Lim, et al., 2013).

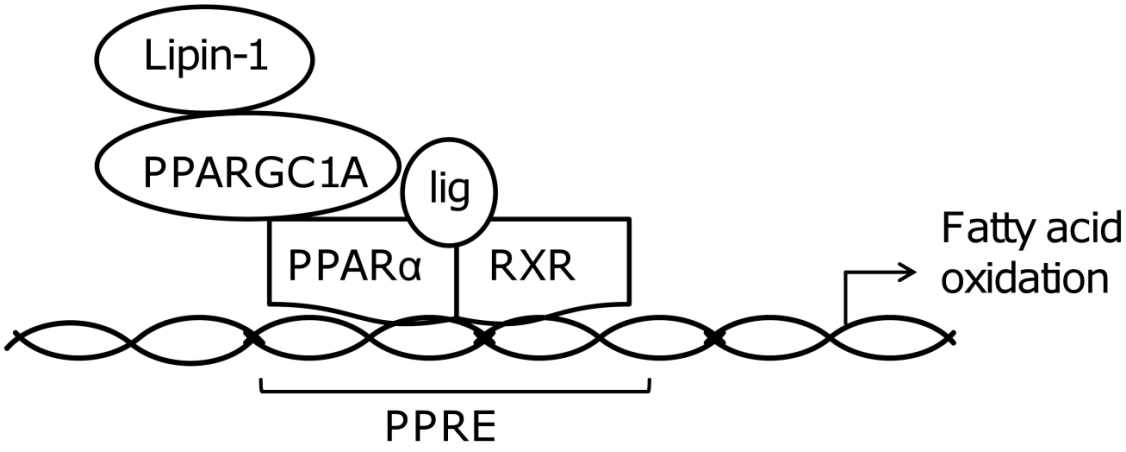

Figure 5.3: Lipin-1 (*LPIN1*) enhances fatty acid oxidation by forming a complex with PPARGC1A and PPARA

5.2.5 *T. indica* fruit activates PPARγ

PPARγ, like PPARα is a member of the nuclear receptor superfamily of ligand-activated transcription factors (Cornelius, MacDougald, & Lane, 1994) highly expressed in adipocytes (Chawla, Schwarz, Dimaculangan, & Lazar, 1994; Tontonoz, Hu, Graves, Budavari, & Spiegelman, 1994) and plays a role in improving glucose homeostasis and adipocyte differentiation (Kallwitz, McLachlan, & Cotler, 2008). Mutations of PPARγ results in the development of severe insulin resistant, type-2 diabetes, hypertension in the absence of obesity, elevated triglycerides and low HDL levels and a number of components of the metabolic syndrome. Like other PPARs, free fatty acid and their derivatives can bind and activate PPARγ. However, specific mechanism of action related to fatty acids and their metabolites is still unclear as identification of specific endogenous PPARγ ligands remains ambiguous (Forman, Chen, & Evans, 1996; Forman et al., 1995). In contrast, synthetic ligands, such as thiazolidinediones or TZDs, are potent activators of PPARγ with robust insulin-sensitising activities (Kung & Henry, 2012). While TZDs are highly effective hypoglycaemic drugs, side effects like weight gain, fluid retention and osteoporosis were reported (Kung & Henry, 2012).

The protein encoded by *TRIB3* gene is a putative protein kinase that is induced by the transcription factor NF-kappaB. The encoded protein is a negative regulator of NF-kappaB and can also sensitise cells to TNF- and TRAIL-induced apoptosis. In addition, this protein can negatively regulate the cell survival serine-threonine kinase AKT1. The up-regulation of this gene could probably explain the anti-inflammatory action of this fruit (Rimbau, et al., 1999). Other than its involvement in apoptosis and inflammation processes, it has been shown that human *TRIB3* decreased biosynthesis of fatty acid (Qi et al., 2006). Mouse *Trib3* is involved in transport of glucose by

inhibiting Akt/PKB activation by insulin in liver (Du, Herzig, Kulkarni, & Montminy, 2003).

PLIN2, a gene up-regulated in the presence of PPARα and PPARγ transactivation (Nielsen, et al., 2006), was shown to reduce fatty acid uptake, oxidation and lipolysis when PLIN2 protein was down-regulated (Faleck et al., 2010). However, a study using homozygous mutant mouse *Plin2* gene knockout in obese mouse decreased quantity of triglyceride and glucose in mouse liver (B. H. Chang, Li, Saha, & Chan, 2010). *ACSL*, which was shown to be up-regulated in fatty acid oxidation and PPARα activation, was also up-regulated in PPARγ activation (Finck, et al., 2002; Schoonjans, et al., 1995; Tachibana, et al., 2006).

The other two genes that were involved in lipid and glucose metabolism, *CPT1A* and *G6PC*, were found to be regulated in the event of PPARγ transactivation. Begriche et al. (2006) reported that CREB protein increased expression of CPT1A protein that involves PPARγ protein. In a study using diabetic rats, it was found that the activation of rat Pparg protein decreased expression of rat *G6pc* mRNA in fatty rat liver (Way, et al., 2001). However in another study using mutant mouse with a homozygous knockout of mouse *Ppara* gene, mouse PPARγ 1 protein increased the expression of mouse *G6pc* mRNA (S. Yu, et al., 2003).

The effects of TZDs against NAFLD are controversial, according to a review by Ables (2012). Studies using different experimental models showed either beneficial or undesirable effects when TZDs were used. It was shown that TZDs improved insulin sensitivity but with the concomitant development of hepatosteatosis, while some showed otherwise (Ables, 2012). In this study PPARγ was predicted to be activated in *T. indica* treatment, and the same effect was not shown in other groups like fenofibrate

and palmitic acid treatment groups based on the genes involved in PPARγ activation. This indicates that the transactivation of PPARγ was attributed to *T. indica* fruit pulp extract. While studies pointed that PPARγ can improve insulin sensitivity but induce fatty liver, the lipid study in this experiment showed otherwise. Treatment with *T. indica* fruit extract lowered triglyceride and cholesterol and the effect is comparable to fenofibrate at 0.1 mg/ml *T. indica* fruit extract and was supported by the Oil Red O staining showing lowered amount of lipid droplets in the cells after treatment. Taken together, PPARγ activation was shown to regulate genes related to lipid and glucose metabolism but without the adverse effect of lipid accumulation in liver cells.

It has been shown that PPARγ ligands have an anti-tumour effect in humans as these compounds decrease cell growth and induce apoptosis in several malignant human cell types, including hepatocellular carcinoma, breast adenocarcinoma and colon adenocarcinoma (Boitier, Gautier, & Roberts, 2003). This effect was also observed in the microarray study in which genes involved in apoptosis and cell death were significantly regulated. For example, up-regulation of *TNFSF10* gene is linked to apoptosis (Baader et al., 2005; Ichikawa et al., 2001) and cell death (Di Pietro & Zauli, 2004). The protein encoded by this gene is a cytokine that belongs to the tumour necrosis factor (TNF) ligand family. This protein preferentially induces apoptosis in transformed and tumour cells, but does not appear to kill normal cells although it is expressed at a significant level in most normal tissues. It has been shown that inhibition of active mouse Pparg protein by GW9662 prevented transactivation of human *TNFSF10* gene in HuH7 cells that is dependent on human PPARG protein (Ho, et al., 2011).

Other genes like *OCLN*, *NDRG1*, *GSTA1*, *HYOU1* and *JUN* involved in apoptosis were significantly regulated in the microarray study too. Occludin, the protein encoded by *OCLN* gene was shown to be increased by human PPARG protein in co-cultured U937 cells (Huang, et al., 2009). *Ndrg1* gene encode N-myc downstream regulated 1. This gene is a member of the N-myc downregulated gene family which belongs to the alpha/beta hydrolase superfamily. The protein encoded by this gene is a cytoplasmic protein involved in stress responses, hormone responses, cell growth, and differentiation. The encoded protein is necessary for p53-mediated caspase activation and apoptosis. Interference of mouse *Pparg* mRNA by siRNA was shown to decrease expression of mouse *Ndrg1* mRNA in mature 3T3-L1 adipocytes that is increased by hypoxia (Pino, et al., 2012). *GSTA1* and *HYOU1* genes were found to be involved in activation of PPARγ. 9-cis-retinoic acid and prostaglandin J2 increased binding of PPRE from rat *Gsta2* gene and a heterodimeric protein-protein complex consisting of rat Pparg and of rat Rxr (E. Y. Park, et al., 2004). As for *HYOU1*, the findings by Jiang et al. (2010) were contradictory. They reported that homozygous mutant mouse *Pparg* gene knockout increased expression of mouse *Hyou1* mRNA in epithelium from mouse prostate gland. However, interference of mouse *Pparg2* mRNA by siRNA decreased expression of mouse *Hyou1* mRNA in epithelial cells from mouse prostate gland.

KLF4 and *KLF6* were both up-regulated by 1.5- and 2.0-fold respectively. These genes encode members of the Kruppel-like family of transcription factors. The zinc finger protein is a transcriptional activator, and functions as a tumour suppressor. It was demonstrated that activation of PPARγ led to the increased expression of KLF4 protein and mRNA (Drori, et al., 2005; S. Li, et al., 2013; Rageul, et al., 2009). While for KLF6, it was shown that interference of mouse *Pparg* mRNA by siRNA increased expression of mouse *Klf6* mRNA in mature terminally differentiated 3T3-L1 adipocytes

(Schupp, et al., 2009). Taken together, *T. indica* fruit was shown to activate PPARγ based on the genes regulated in microarray study.

5.2.6 *T. indica* fruit modulates apoptosis and cell death

T. indica fruit pulp is known to exhibit lipid-lowering effects and it has been shown in this study that it could be mediated by fatty acid oxidation through the activation of PPARα. While the regulations of lipid metabolism and to a certain extent, carbohydrate metabolism were being modulated, the number of genes involved in apoptosis and cell death were interestingly high. *T. indica* fruit extract has never been reported to exhibit anti-proliferative effect to the best of our knowledge, and the MTT assay in this study was in agreement to that fact. The extensive regulation of genes involved in apoptosis and cell death in all treatments could probably explain that cancer, cell growth and proliferation, as well as cell death-related networks as being the dominant associated network functions in the IPA network analysis.

5.2.6.1 Induction of endoplasmic reticulum (ER) stress

Among the many mechanisms that could lead to apoptosis, one is associated with the induction of ER stress. ER is a site for Ca^{2+} storage and also responsible for synthesis, folding and maturation of secreted and transmembrane proteins. Pathological or physiological conditions that interrupt protein folding in the ER can cause stress in the endoplasmic reticulum and lead to the activation of signalling pathway known as the Unfolded Protein Response (UPR) pathway.

In the IPA upstream regulator analysis, three transcription factors associated to ER stress, DDIT3, ATF4 and XBP1 were predicted to be activated. While DDIT3 and XBP1 were predicted to be activated in all treatment groups based on the genes

regulated, ATF4 was exclusively predicted to be activated in *T. indica* treatment group only.

DDIT3 or CHOP is one of the key markers in ER stress induced pathway, UPR pathway. It codes for C/EBP homologous protein, a member of the CCAAT/enhancer-binding protein family. CHOP is implicated in adipogenesis and erythropoiesis, and is activated by ER stress, and promotes apoptosis. In this study, it was up-regulated in all treatments and its activation is supported by the IPA upstream regulator analysis based on the genes regulated the *T. indica* fruit treatment. There are 6 DDIT3 target genes that were significantly altered in this study, *WARS*, *TRIB3*, *PPP1R15A*, *LCN2*, *ATF3*, and *ANKRD1*. Out of these 6 genes, 4 genes (*WARS*, *TRIB3*, *PPP1R15A* and *ATF3*) were also shown to be regulated in the transactivation of ATF4, another transcription factor that was shown to be activated based on the genes regulated (J. Han et al., 2013).

Activating transcription factor 4 or ATF4 is also induced by ER stress through the UPR pathway. According to the IPA upstream regulator analysis, 9 genes have expression direction consistent with the activation of ATF4, namely *WARS*, *NDRG1*, *MAP1LC3B*, *KLF4*, *HERPUD1*, *DDIT3*, *ATF3*, *TRIB3* and *PPP1R5A*. In a microarray analysis done by Jousse et al. (2007), it was shown that amino acid starvation of Mef cells increased expression of mouse genes like *Wars*, *Herpud1*, *Ddit3*, *Areg* and *Trib3* that involves mouse Atf4 protein. The activation of ATF4 was also supported by the regulations of genes like *WARS*, *NDRG1*, *KLF4*, *HERPUD1* and *ATF3* which were found to be decreased in expression when *Atf4* gene was knockout in mouse (Harding et al., 2003). However in a recent study, tribbles 3 or *TRIB3* which was up-regulated in this study was shown to be up-regulated in high-fat feeding mice and humans with

obesity and type-2 diabetes. When the gene was knockout in high-fat feeding mice, the mice showed improved insulin resistance in skeletal muscle (Koh et al., 2013).

Although these results were linked to the induction of ER stress, it was shown that ER stress suppressed genes involved in maintaining energy and lipid homeostasis such as PPARα, PGC1a and FOXO1. (Rutkowski et al., 2008). This finding is in contrast with our results which were predicted to be activated based on the genes regulated in the microarray study. It should be noted that classical pro-apoptotic molecules like Bax, Bak, and caspases were not significantly regulated in this study, indicating that apoptosis may not occur in the cells. Besides this, *T. indica* fruit was able to lower lipid levels in HepG2 cells, which also defies the fact that ER stress leads to hepatosteatosis. Furthermore, the MTT assay did not exhibit anti-proliferative activity nor extensive cell death, thus eliminating the possibilities of ER stress-induced apoptosis.

5.2.6.2 Tumour suppressing genes involved in TP53, FOXO3 and c-MYC downstream pathways

Besides this, the microarray results also showed regulation of genes involved in tumour suppressing activities. *TP53* codes for tumour suppressor 53, which was predicted to be activated in the IPA upstream regulator analysis based on the genes regulated. As the name implies, it is a potent tumour suppressor in humans, based on the fact that most of the human tumours have mutations or deletions in *p53* gene itself or in the p53 pathway (Vogelstein, Lane, & Levine, 2000). It responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. Mutations in *p53* have been linked to poor prognosis in a variety of human cancers, including lung (Quinlan,

Davidson, Summers, Warden, & Doshi, 1992), breast (Deng et al., 1994) and gastric cancers (Scott et al., 1991), as well as lymphomas (Gaidano et al., 1991; Lo Coco et al., 1993). Other than its involvement in tumour suppressing activities, it was also reported to regulate fatty acid oxidation through the induction of lipin-1 (*LPIN1*), which was up-regulated in the microarray study (Assaily et al., 2011).

Another tumour suppressor, FOXO3 is responsible for the expression of a program of genes involved in cell cycle arrest, DNA repair, hypoxia response and apoptosis (Bakker, Harris, & Mak, 2007; Brunet et al., 1999; Medema, Kops, Bos, & Burgering, 2000; Tran et al., 2002). It is negatively regulated in response to insulin and growth factors through phosphorylation-dependent nuclear export (Brunet, et al., 1999; Nakae, Park, & Accili, 1999), while positive regulation occurs in the presence of oxidative stress through JNK activity (Brunet et al., 2004; Essers et al., 2004). In recent studies, it was shown that transactivation of FOXO3 is hepatoprotective against acute and chronic alcohol-induced liver injury by inducing autophagy, a protective mechanism against alcohol-induced liver injury by removing damaged mitochondria (Ni, Du, You, & Ding, 2013; Tumurbaatar et al., 2013).

MYC is a transcription factor regulating genes involved in cell growth, cell proliferation, cell cycle and apoptosis (Dang, 1999). Its activation is associated with many types of cancers and its inhibition is a therapeutic target for anti-cancer research. In this study it was predicted to be inhibited based on the genes regulated in *T. indica* fruit treatment group (*THBS1, TES, TAF1D, SNHG12, SLC2A1, RPL5, PPP1R15A, NDRG1, LGALS1, ID1, DUSP1, DDIT3, CPT1A, CD9, KLF6 and MT1E)*. Down-regulation of c-Myc has been reported to mediate anti-cancer effect. In fact, a number of

plant extracts were shown to inhibit cancer cell proliferation by inhibiting c-Myc (Giessrigl et al., 2012; Othman et al., 2012; Unger et al., 2012).

The overall activation of tumour suppressors and inhibition of oncogene in this microarray study may suggest anti-cancer properties of the fruit. However it should be noted that the fruit did not affect cell viability significantly, as shown in the MTT assay results. Moreover, there has been no studies reporting any anti-cancer activities in relation to this fruit, although individual polyphenolic compounds found in the methanol extract of *T. indica* has been shown to exhibit anti-proliferative effects.

5.3 Polyphenols in *T. indica* fruit that may attribute to the activities

The lipid-lowering effects induced by plant polyphenols have been reported by many. In fact, there is an upcoming trend of researches revealing the potentials of plant polyphenols in regulating metabolic processes *in vitro* and *in vivo*. Methanolic extract of *T. indica* fruit pulp contains catechin, epicatechin, procyanidins, naringenin, apigenin, luteolin, taxifolin and eriodictyol (Sudjaroen, et al., 2005). It was shown that tea catechins like epigallocatechin gallate (EGCG) and epigallocatechin (EGC) were able to activate PPARα (K. Lee, 2004). Naringenin from grapefruit was shown to regulate lipid metabolism through partial activation of PPARα (Goldwasser, et al., 2010). This was also supported by Mulvihill et al.(2009) who had shown that naringenin was able to correct VLDL overproduction, ameliorate hepatic steatosis, and attenuate dyslipidemia without affecting caloric intake or fat absorption in LDL receptor null mice through the activation of PGC1A/PPARα activation. Interestingly, naringenin from black elder flowers was shown to increase activation of PPARγ too (Christensen, Petersen, Kristiansen, & Christensen, 2010). Another study showed that flavangenol extracted from pine bark was able to enhance fatty acid oxidation, mainly

attributed to procyanidin B1 (Shimada, et al., 2012). Proanthocyanidins from hawthorn had also been reported to lower lipid and glucose level by activating AMPK and PPARα (Shih, Lin, Lin, & Wu, 2013). Tart cherries rich in anthocyanins had also been shown to ameliorate hepatic steatosis and hyperlipidaemia through the activation of PPARα (Seymour et al., 2008). This shows that a number of polyphenols in the methanolic extract of *T. indica* fruit were able to regulate nuclear receptors such as PPARα.

Although *T. indica* fruit did not exhibit anti-proliferative effect, genes related to apoptosis were seen significantly regulated. The polyphenols in the fruit could be accountable for the phenomenon. For instance procyanidins from grape seed and apple had been shown to induce apoptosis through the induction of caspases (C. P. Hsu et al., 2009; Miura et al., 2008). Similarly, catechins and green tea catechins which consist of (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG) and (−)-epicatechin (EC) were also reported to induce apoptosis in cancer cells (Al-Hazzani & Alshatwi, 2011; Alshatwi, 2010; Chung et al., 2001; Nakazato et al., 2005; Philips, Coyle, Morrisroe, Chancellor, & Yoshimura, 2009). However in a recent study, catechin was shown to increase viability and decrease apoptosis and proliferation of epithelial cells and vascular smooth muscle cells (Negrao et al., 2013). Apigenin is another polyphenol that exhibit anti-proliferative properties (Budhraja et al., 2012; C. C. Lin et al., 2012; Zbidah et al., 2012). Yan et al. (2012) reported that luteolin exhibited anti-cancer effect in non-small cell lung cancer xenograft mouse model through the increased activity of TRAIL (TNFSF10), which was up-regulated in the microarray study.

CHAPTER 6

CONCLUSION

In the present study, the lipid-lowering effect of *T. indica* fruit pulp was investigated systematically. The study was performed in three stages, i) proteomic analysis to corroborate with the previous microarray analysis and to formulate a hypothesis; ii) measurement of lipid levels in hepatosteatotic HepG2 cells and iii) transcriptomic analysis on *T. indica* fruit treated-hepatosteatotic HepG2 cells and comparing the transcriptomic profile with fenofibrate treated cells. The first stage of this study suggested that *T. indica* fruit could have exerted its hypolipidaemic effect through the activation of PPARα. Lipid studies showed that 0.1 mg/ml methanolic extract of *T. indica* fruit pulp was able to lower lipid (triglyceride and cholesterol) to a level comparable to fenofibrate treatment. Based on the transcriptomic studies, *T. indica* fruit mediates its hypolipidaemic effects by increasing fatty acid oxidation through the transactivation of PPARα, a method similar to fenofibrate. In summary, the results from this study suggest that such an integrated approach has led to a better understanding of the lipid-lowering effects of *T. indica* fruit pulp.

6.1 Future study

The findings from this study demonstrated that *T. indica* fruit pulp is a promising lipid-lowering agent and more in-depth studies should be warranted in the future. To further validate the lipid-lowering action of *T. indica* fruit pulp through the activation of PPARα, PPARα reporter assay can be performed. This assay allows the monitoring of PPARα transcriptional activities in cells. Besides PPARα, other transcription factors that were predicted to be activated or inhibited can also be validated by using reporter assays for the respective transcription factors. The

hypothesis can also be tested in animal models by observing the effect of *T. indica* fruit pulp in PPARα-knockout mice.

The individual compounds in *T. indica* fruit pulp extract can also be isolated and assessed for bioactive properties. While pure compound is often more potent than crude extract, it should be noted that complex mixture of compounds found in natural products often interacts with each other either synergistically or antagonistically, thus affecting the potency of the bioactive properties.

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LIST OF ISI-PUBLICATIONS AND CONFERENCE PAPERS PRESENTATION

List of ISI-publications

1) **Ursula Rho Wan Chong**, Puteri Shafinaz Abdul-Rahman, Azlina Abdul-Aziz, Onn Haji Hashim, Sarni Mat-Junit. *Tamarindus indica* Extract Alters Release of Alpha Enolase, Apolipoprotein A-I, Transthyretin and Rab GDP Dissociation Inhibitor Beta from HepG2 Cells. PLoS ONE **2012**, 7(6), e39476. doi:10.1371/journal.pone.0039476. (*ISI-Cited Publication*) (Impact factor: 3.73, Q1)
2) **Ursula R.W. Chong**, Puteri S. Abdul-Rahman, Azlina Abdul-Aziz, Onn H. Hashim, Sarni Mat-Junit. Effects of *Tamarindus indica* fruit pulp extract on abundance of HepG2 cell lysate proteins and their possible consequential impact on metabolism and inflammation. BioMed Research International, vol. **2013**, Article ID 459017, 9 pages, 2013. doi:10.1155/2013/459017 (*ISI-Cited Publication*) (Impact factor: 2.88, Q2)
3) **Ursula Rho Wan Chong**, Puteri Shafinaz Abdul-Rahman, Azlina Abdul-Aziz, Sarni Mat-Junit. Transcriptomic profiling of HepG2 cells treated with *Tamarindus indica* fruit extract. (Manuscript in preparation)

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PLoS one

Tamarindus indica Extract Alters Release of Alpha Enolase, Apolipoprotein A-I, Transthyretin and Rab GDP Dissociation Inhibitor Beta from HepG2 Cells

Ursula Rho Wan Chong[1], Puteri Shafinaz Abdul-Rahman[1,2], Azlina Abdul-Aziz[1,2], Onn Haji Hashim[1,2], Sarni Mat Junit[1,2]*

1 Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia, **2** University of Malaya Centre for Proteomics Research, Medical Biotechnology Laboratory, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Abstract

Background: The plasma cholesterol and triacylglycerol lowering effects of *Tamarindus indica* extract have been previously described. We have also shown that the methanol extract of *T. indica* fruit pulp altered the expression of lipid-associated genes including *ABCG5 and APOAI* in HepG2 cells. In the present study, effects of the same extract on the release of proteins from the cells were investigated using the proteomics approach.

Methodology/Principal Findings: When culture media of HepG2 cells grown in the absence and presence of the methanol extract of *T. indica* fruit pulp were subjected to 2-dimensional gel electrophoresis, the expression of seven proteins was found to be significantly different ($p<0.03125$). Five of the spots were subsequently identified as alpha enolase (ENO1), transthyretin (TTR), apolipoprotein A-I (ApoA-I; two isoforms), and rab GDP dissociation inhibitor beta (GDI-2). A functional network of lipid metabolism, molecular transport and small molecule biochemistry that interconnects the three latter proteins with the interactomes was identified using the Ingenuity Pathways Analysis software.

Conclusion/Significance: The methanol extract of *T. indica* fruit pulp altered the release of ENO1, ApoA-I, TTR and GDI-2 from HepG2 cells. Our results provide support on the effect of *T. indica* extract on cellular lipid metabolism, particularly that of cholesterol.

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* E-mail: sarni@um.edu.my

Introduction

Tamarindus indica, also known as tamarind, is a tropical fruit tree that grows naturally in many tropical and subtropical regions. Due to the sour taste, its fruit pulp is widely used to add flavour in cooking. Many claims have been made on the medicinal use of tamarind fruit pulps including as gentle laxative, expectorant, antipyretic and antimicrobial agents [1,2,3]. Biochemical experiments have also shown that tamarind extracts possess high antioxidant activities [4,5]. In addition, the fruit pulp extract of *T. indica* has also been shown to cause a decrease in the levels of serum total cholesterol and triacylglycerol but an increase in the HDL cholesterol levels in hypercholesterolaemic hamsters [4] and in humans [5]. However, the precise mechanisms of action at the molecular levels have yet to be deciphered.

Analysis of the methanol extract of the tamarind fruit pulp by HPLC revealed the predominant presence of proanthocyanidins, including (+)-catechin and (−)-epicatechin [6]. The jasmine green tea epicatechin has been shown to reduce the levels of triacylglycerol and cholesterol in the sera of hamsters fed with a high-fat diet [7]. The observed hypolipidaemic effects of epicatechin were postulated to involve inhibition of the absorption of dietary fat and/or cholesterol or through the reabsorption of bile acids since it did not inhibit liver HMGCoA reductase [7]. More recently, we have shown that the methanol extract of *T. indica* fruit pulps significantly up-regulated the expression of a total of 590 genes and down-regulated 656 genes expression in HepG2 cells [8]. Amongst the genes that were altered in expression were those that encode proteins associated with lipoprotein metabolism, including ApoA-I, ApoA-IV, ApoA-V and ABCG5 but not the HMGCoA reductase. Both ApoA-I and ABCG5 are involved in the reverse cholesterol transport, where the latter, together with ABCG8, are involved in the hepatobiliary cholesterol secretion.

In the present study, we have investigated the effects of *T. indica* fruit pulp extract on the release of proteins from HepG2 cells as a mean to validate previously reported gene expression data at the protein level. Identification of proteins that were differently altered in the cell culture media may help to improve our understanding of the metabolic pathways that are affected and the molecular mechanisms involved. Secreted proteins were specifically targeted in this study as they may be involved in regulating the many

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Research Article

Effects of *Tamarindus indica* Fruit Pulp Extract on Abundance of HepG2 Cell Lysate Proteins and Their Possible Consequential Impact on Metabolism and Inflammation

Ursula R. W. Chong,[1] Puteri S. Abdul-Rahman,[1,2] Azlina Abdul-Aziz,[1,2] Onn H. Hashim,[1,2] and Sarni Mat-Junit[1,2]

[1] *Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[2] *University of Malaya Centre for Proteomics Research, Medical Biotechnology Laboratory, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia*

Correspondence should be addressed to Sarni Mat-Junit; sarni@um.edu.my

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The fruit pulp extract of *Tamarindus indica* has been reported for its antioxidant and hypolipidemic properties. In this study, the methanol extract of *T. indica* fruit pulp was investigated for its effects on the abundance of HepG2 cell lysate proteins. Cell lysate was extracted from HepG2 cells grown in the absence and presence of the methanol extract of *T. indica* fruit pulp. Approximately 2500 spots were resolved using two-dimensional gel electrophoresis and the abundance of 20 cellular proteins was found to be significantly reduced. Among the proteins of reduced abundance, fourteen, including six proteins involved in metabolism (including ethanolamine phosphate cytidylyltransferase), four mitochondrial proteins (including prohibitin and respiratory chain proteins), and four proteins involved in translation and splicing, were positively identified by mass spectrometry and database search. The identified HepG2 altered abundance proteins, when taken together and analyzed by Ingenuity Pathways Analysis (IPA) software, are suggestive of the effects of *T. indica* fruit pulp extract on metabolism and inflammation, which are modulated by LXR/RXR. In conclusion, the methanol fruit pulp extract of *T. indica* was shown to cause reduced abundance of HepG2 mitochondrial, metabolic, and regulatory proteins involved in oxidative phosphorylation, protein synthesis, and cellular metabolism.

1. Introduction

Tamarindus indica or tamarind is a tropical fruit tree native to the African savannahs but it can now be found in many tropical countries. It is categorized as a monospecific genus in the family of Leguminosae. The sweet and sour taste of its fruit pulp is used to add flavor to local cuisines. Besides culinary, tamarind is also used in traditional medicine as laxative, diuretic, antibacterial agents as well as in treatment of fever and malarial infections [1, 2]. Previous biochemical analyses have demonstrated that extracts of *T. indica* possess high antioxidant activities [3, 4]. In addition, *T. indica* extracts have also been shown to reduce the levels of blood cholesterol and triacylglycerol in hypercholesterolemic hamsters [3] and in humans [5]. However, the molecular mechanisms of the anti-inflammatory and hypolipidemic effects of the fruit remain elusive.

In our previous study, the methanol extract of *T. indica* fruit pulp was shown to alter the expression of more than a thousand genes in HepG2 cells, many of which are associated with lipid metabolism [6]. Our recent study also showed that the fruit pulp extract was able to alter the secretion of alpha enolase, apolipoprotein A-1, transthyretin, and rab GDP dissociation inhibitor beta from HepG2 cells, which may account for the lipid-lowering effects of the fruit. These effects were hypothesized to occur via activation of LXR/RXR [7].

The lipid-lowering properties of *T. indica* fruit pulp are likely attributed to the presence of polyphenols in its

List of conference paper presentations

International Scientific Conference

1) Ursula Chong Rho Wan, Puteri Shafinaz Akmar Abdul-Rahman, Azlina Abdul Aziz, Onn Haji Hashim and Sarni Mat Junit. *Tamarindus indica fruit extract modulates metabolism and inflammation in HepG2 cells, possibly through LXRα activation* (2013). International Conference on Natural Products and Health 2013, School of Biological Sciences, Nanyang Technological University, Singapore.

National Scientific Conference

1) Ursula Chong Rho Wan, Puteri Shafinaz Akmar Abdul-Rahman, Azlina Abdul Aziz, Onn Haji Hashim and Sarni Mat Junit. *Preliminary analysis of the secretome of HepG2 cells treated with methanol extracts of Tamarindus indica fruit pulps* (2011). Proc of the 36th Annual Conference of the Malaysian Society for Biochemistry and Molecular Biology, Selangor, Malaysia.
2) Ursula Chong Rho Wan, Puteri Shafinaz Akmar Abdul-Rahman, Azlina Abdul Aziz, Onn Haji Hashim and Sarni Mat Junit. *Tamarindus indica fruit pulp extract alters the secretion of lipid-associated proteins from HepG2 cells* (2012). Proc of the 37th Annual Conference of the Malaysian Society for Biochemistry and Molecular Biology, Selangor, Malaysia.
3) Ursula Chong Rho Wan, Puteri Shafinaz Akmar Abdul-Rahman, Azlina Abdul Aziz and Sarni Mat Junit. *Tamarindus indica fruit pulp extract reduces palmitic acid-induced lipid accumulation in HepG2 cells by modulating genes related to lipid metabolism, possibly through PPARα and PPARγ activation* (2013). Proc of the 38th Annual Conference of the Malaysian Society for Biochemistry and Molecular Biology, Putrajaya, Malaysia.

APPENDIX

Supp. Table 1: Genes related to PPARGC1A activation that are significantly regulated in the microarray analyses of the different treatments on HepG2 cells.

Gene Symbol	Literature findings on gene regulation when PPARGC1A is activated	References	Gene Name	Fold change		
				TI+PA vs control[a]	FF+PA vs control[a]	PA vs control
TRIB3	↑	(Koo, et al., 2004)	Tribbles homolog 3 (Drosophila)	1.6		
PLIN2	↑	(Koves et al., 2013)	Perilipin 2	1.6		
LPIN1	↑	(Finck, et al., 2006; D. K. Kim et al., 2011)	Lipin 1	1.5		
GK	↑	(Feige et al., 2007; Tiraby et al., 2003)	Glycerol kinase	1.6		
G6PC	↑	(J. Lin et al., 2004; Puigserver, et al., 2003; Rhee et al., 2003; J. C. Yoon, et al., 2001)	Glucose-6-phosphatase, catalytic subunit	2.3	1.6	1.5
CPT1A	↑	(K. Ma, Zhang, Elam, Cook, & Park, 2005; Rhee, et al., 2003; Vega, et al., 2000; Y. Zhang et al., 2004)	Carnitine palmitoyltransferase 1A (liver)	1.7	1.6	

Supp. Table 2: Genes related to CREB1 activation that are significantly regulated in the microarray analyses of the different treatments on HepG2 cells.

Gene Symbol	Literature findings on gene regulation when CREB1 is activated	References	Gene Name	Fold change		
				TI+PA vs control[a]	FF+PA vs control[a]	PA vs control
PPP1R15A	↑	(Lemberger, Parkitna, Chai, Schutz, & Engblom, 2008)	Protein phosphatase 1, regulatory subunit 15A	2.5	1.8	
NFIL3	↑	(Lemberger, et al., 2008)	Nuclear factor, interleukin 3 regulated	1.7		1.5
CPT1A	↑	(Begriche, et al., 2006)	Carnitine palmitoyltransferase 1A (liver)	1.7	1.6	
BHLHE40	↑	(Lemberger, et al., 2008)	Basic helix-loop-helix family, member e40	2.2		
ATF3	↑	(Lemberger, et al., 2008)	Activating transcription factor 3	1.6	1.5	
CYP19A1	Affected	(Morales et al., 2003)	Cytochrome P450, family 19, subfamily A, polypeptide 1	1.6	1.6	
DUSP1	Affected	(Abramovitch et al., 2004; Du, Asahara, Jhala, Wagner, & Montminy, 2000)	Dual specificity phosphatase 1	2.4	1.6	
G6PC	Affected	(Carriere et al., 2005; B. Lin, Morris, & Chou, 1997)	Glucose-6-phosphatase, catalytic subunit	2.3	1.6	1.5

Supp. Table 2, continued

HERPUD1	Affected	(Abramovitch, et al., 2004)	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	2.2	1.7	1.5
ID1	Affected	(San-Marina, Han, Suarez Saiz, Trus, & Minden, 2008)	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	-1.5		-1.5
JUN	Affected	(Lamph, Dwarki, Ofir, Montminy, & Verma, 1990; A. Rao, Luo, & Hogan, 1997)	Jun proto-oncogene	1.9		

Supp. Table 3: Genes related to ATF4 activation that are significantly regulated in the microarray analyses of the different treatments on HepG2 cells.

Gene Symbol	Literature findings on gene regulation when ATF4 is activated	References	Gene Name	Fold change		
				TI+PA vs control[a]	FF+PA vs control[a]	PA vs control
WARS	↑	(J. Han, et al., 2013; Harding, et al., 2003; Jousse, et al., 2007)	Tryptophanyl-tRNA synthetase	1.5		
NDRG1	↑	(Harding, et al., 2003)	N-myc downstream regulated 1	1.6		
MAP1LC3B	↑	(Rouschop et al., 2010; Rzymski et al., 2010; Verfaillie, Salazar, Velasco, & Agostinis, 2010)	Microtubule-associated protein 1 light chain 3 beta	1.5	1.6	1.6
KLF4	↑	(Harding, et al., 2003)	Kruppel-like factor 4 (gut)	1.5		
HERPUD1	↑	(Carter, 2007; Harding, et al., 2003; Jousse, et al., 2007; Y. Ma & Hendershot, 2004)	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	2.2	1.7	1.5
DDIT3	↑	(Carter, 2007; Jousse, et al., 2007; Y. Ma & Hendershot, 2004; Rouschop, et al., 2010; Verfaillie, et al., 2010)	DNA-damage-inducible transcript 3	2.5	1.8	1.7

Supp. Table 3, continued

ATF3	↑	(Carter, 2007; Harding, et al., 2003; H. Y. Jiang et al., 2004; G. Liu et al., 2012; Shan, Ord, Ord, & Kilberg, 2009)	Activating transcription factor 3	1.6	1.5	
AREG/AREGB	Affected	(Jousse, et al., 2007)	Amphiregulin	11.6	6.4	12.0
JUN	Affected	(L. Fu, Balasubramanian, Shan, Dudenhausen, & Kilberg, 2011)	Jun proto-oncogene	1.9		
MT2A	Affected	(Hai & Curran, 1991)	Metallothionein 2A	-1.5	-1.6	-1.6
PPP1R15A	Affected	(Carter, 2007; J. Han, et al., 2013)	Protein phosphatase 1, regulatory subunit 15A	2.5	1.8	
TRIB3	Affected	(J. Han, et al., 2013; Jousse, et al., 2007)	Tribbles homolog 3 (Drosophila)	1.6		

Supp. Table 4: Genes related to DDIT3 activation that are significantly regulated in the microarray analyses of the different treatments on HepG2 cells.

Gene Symbol	Literature findings on gene regulation when DDIT3 is activated	References	Gene Name	Fold change		
				TI+PA vs control[a]	FF+PA vs control[a]	PA vs control
PPP1R15A	↑	(J. Han, et al., 2013; Verfaillie, et al., 2010)	Protein phosphatase 1, regulatory subunit 15A	2.5	1.8	
LCN2	↑	(Hsin et al., 2012)	Lipocalin 2	2.1	1.7	1.5
TRIB3	↑	(J. Han, et al., 2013; Verfaillie, et al., 2010; X. Yu, Lv, Zhu, Duan, & Ma, 2013)	Tribbles homolog 3 (Drosophila)	1.6		
ANKRD1	↓	(X. J. Han et al., 2005)	Ankyrin repeat domain 1 (cardiac muscle)	-1.6	-2.1	-1.7

Supp. Table 5: Genes related to XBP1 activation that are significantly regulated in the microarray analyses of the different treatments on HepG2 cells.

Gene Symbol	Literature findings on gene regulation when XBP1 is activated	References	Gene Name	Fold change		
				TI+PA vs control[a]	FF+PA vs control[a]	PA vs control
RABAC1	↑	(Sriburi et al., 2007)	Rab acceptor 1 (prenylated)	1.6		
MAP1LC3B	↑	(Margariti et al., 2013)	Microtubule-associated protein 1 light chain 3 beta	1.5	1.6	1.6
KLF4	↑	(Sugiura et al., 2009)	Kruppel-like factor 4 (gut)	1.5		
HYOU1	↑	(Sriburi, et al., 2007)	Hypoxia up-regulated 1	1.9		1.6
HSPA13	↑	(Sriburi, et al., 2007)	Heat shock protein 70kDa family, member 13	1.7		1.5
HERPUD1	↑	(Sriburi, et al., 2007)	Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	2.2	1.7	1.5
ERO1LB	↑	(Sriburi, et al., 2007)	ERO1-like beta (*S. cerevisiae*)	1.5		1.6
DNAJB9	↑	(Carter, 2007; A. H. Lee, Iwakoshi, & Glimcher, 2003; Sriburi, et al., 2007; H. M. Zhang et al., 2010)	DnaJ (Hsp40) homolog, subfamily B, member 9	2.2	1.6	1.8
DDIT3	↑	(Verfaillie, et al., 2010)	DNA-damage-inducible transcript 3	2.5	1.8	1.7

Supp. Table 6: Genes related to TP53 activation that are significantly regulated in the microarray analyses of the different treatments on HepG2 cells.

Gene Symbol	Literature findings on gene regulation when TP53 is activated	References	Gene Name	Fold change		
				TI+PA vs control[a]	FF+PA vs control[a]	PA vs control
TNFSF10	↑	(Hussain et al., 2004; Lima et al., 2011)	Tumor necrosis factor (ligand) superfamily, member 10	1.5	1.6	1.6
SLC2A1	↓	(Daoud et al., 2003; Schwartzenberg-Bar-Yoseph, Armoni, & Karnieli, 2004; Zawacka-Pankau et al., 2011)	Solute carrier family 2 (facilitated glucose transporter), member 1	-1.6		-1.6
PPP1R15A	↑	(Su et al., 2003)	Protein phosphatase 1, regulatory subunit 15A	2.5	1.8	
NDRG1	↑	(Burrows, Smogorzewska, & Elledge, 2010; Stein et al., 2004)	N-myc downstream regulated 1	1.6		
LPIN1	↑	(Assaily, et al., 2011; Lotem, Benjamin, Netanely, Domany, & Sachs, 2004; Ongusaha et al., 2003)	Lipin 1	1.5		
KLF4	↑	(W. Zhang et al., 2000)	Kruppel-like factor 4 (gut)	1.5		
HSPA8	↓	(Daoud, et al., 2003; Ginsberg, Mechta, Yaniv, & Oren, 1991)	Heat shock 70kDa protein 8	-3.0	-2.3	

Supp. Table 6, continued

DUSP1	↑	(Begum, Hockman, & Manganiello, 2011; M. Li, Zhou, Ge, Matherly, & Wu, 2003)	Dual specificity phosphatase 1	2.4	1.6	
DDIT3	↑	(T. Liu et al., 2007; Su, et al., 2003)	DNA-damage-inducible transcript 3	2.5	1.8	1.7
BTG1	↑	(Amundson et al., 2005; Campaner et al., 2011)	B-cell translocation gene 1, anti-proliferative	1.8		1.8
BHLHE40	↑	(Qian, Zhang, Yan, & Chen, 2008)	Basic helix-loop-helix family, member e40	2.2		
ATXN1	↑	(Boiko et al., 2006)	Ataxin 1	1.5		
ATF3	↑	(Amundson et al., 1999; Campaner, et al., 2011; Jeong, Hu, Belyi, Rabadan, & Levine, 2010; H. Yoon et al., 2002)	Activating transcription factor 3	1.6	1.5	
AREG/AREGB	↑	(B. D. Chang et al., 2002; Hammond et al., 2006)	Amphiregulin	11.6	6.4	12.0
THBS1	↑	(Dameron, Volpert, Tainsky, & Bouck, 1994; Holmgren, Jackson, & Arbiser, 1998; Y. Liu et al., 1999; Vikhanskaya et al., 2001)	Thrombospondin 1	-1.6	-1.7	-1.8
NUPR1	↓	(Vasseur et al., 2002)	Nuclear protein, transcriptional regulator, 1	1.8		
ID1	↑	(Komarova et al., 1998; Qian & Chen, 2008; Wilson et al., 2001)	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	-1.5		-1.5
DKK1	↑	(Caricasole et al., 2004; Harms & Chen, 2007; J. Wang, Shou, & Chen, 2000)	Dickkopf 1 homolog (*Xenopus laevis*)	-1.6		
JUN	Affected	(Ginsberg, et al., 1991; Komarova, et al., 1998)	Jun proto-oncogene	1.9		
MBNL2	Affected	(T. Liu, et al., 2007)	Muscleblind-like splicing regulator 2	1.6		

Supp. Table 7: Genes related to FOXO3 activation that are significantly regulated in the microarray analyses of the different treatments on HepG2 cells.

Gene Symbol	Literature findings on gene regulation when FOXO3 is activated	References	Gene Name	Fold change		
				TI+PA vs control[a]	FF+PA vs control[a]	PA vs control
TNFSF10	↑	(Ghaffari, Jagani, Kitidis, Lodish, & Khosravi-Far, 2003; Modur, Nagarajan, Evers, & Milbrandt, 2002; Sakoe, Sakoe, Kirito, Ozawa, & Komatsu, 2010; van Grevenynghe et al., 2011)	Tumor necrosis factor (ligand) superfamily, member 10	1.5	1.6	1.6
PPP1R15A	↑	(Porcu & Chiarugi, 2005)	Protein phosphatase 1, regulatory subunit 15A	2.5	1.8	
MXD1	↑	(Delpuech et al., 2007)	MAX dimerization protein 1	2.2	1.8	1.7
LCN2	↑	(S. Park, Guo, Kim, & Cheng, 2009)	Lipocalin 2	2.1	1.7	1.5
DDIT3	↑	(Greer et al., 2007)	DNA-damage-inducible transcript 3	2.5	1.8	1.7
CPT1A	↑	(Nakamura, Moore, Negishi, & Sueyoshi, 2007)	Carnitine palmitoyltransferase 1A (liver)	1.7	1.6	
ATP6V0D2	↑	(Greer, et al., 2007)	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d2	1.8	1.8	2.1
MT1E	↑	(Greer, et al., 2007)	Metallothionein 1E	-1.5		-1.5
IGFBP1	Affected	(Brunet, et al., 1999; Datta, Brunet, & Greenberg, 1999)	Insulin-like growth factor binding protein 1	2.2		

Supp. Table 8: Genes related to MYC inhibition that are significantly regulated in the microarray analyses of the different treatments on HepG2 cells.

Gene Symbol	Literature findings on gene regulation when MYC is inhibited	References	Gene Name	Fold change		
				TI+PA vs control[a]	FF+PA vs control[a]	PA vs control
THBS1	↓	(Baudino et al., 2002; Cairo et al., 2005; Oster, Ho, Soucie, & Penn, 2002; Thomas-Tikhonenko et al., 2004)	Thrombospondin 1	-1.6	-1.7	-1.8
TES	↑	(Rounbehler et al., 2012)	Testis derived transcript (3 LIM domains)	1.5		
TAF1D	↓	(D. Wang, Wengrod, & Gardner, 2011)	TATA box binding protein (TBP)-associated factor, RNA polymerase I, D, 41kDa	-1.6		
SNHG12	↓	(D. Wang, et al., 2011)	Small nucleolar RNA host gene 12 (non-protein coding)	-1.7		-1.6
SLC2A1	↓	(Osthus et al., 2000; R. Wang et al., 2011)	Solute carrier family 2 (facilitated glucose transporter), member 1	-1.6		-1.6
RPL5	↓	(Guo et al., 2000; McConnell et al., 2003)	Ribosomal protein L5	-1.5		
PPP1R15A	↑	(Amundson, Zhan, Penn, & Fornace, 1998)	Protein phosphatase 1, regulatory subunit 15A	2.5	1.8	
NDRG1	↑	(Oster, et al., 2002)	N-myc downstream regulated 1	1.6		

Supp. Table 8, continued

LGALS1	↑	(Frye, Gardner, Li, Arnold, & Watt, 2003; S. Yan et al., 2009)	Lectin, galactoside-binding, soluble, 1	1.6		
ID1	↓	(Murphy, Swigart, Israel, & Evan, 2004; Swarbrick et al., 2005)	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	-1.5		-1.5
DUSP1	↑	(Louro et al., 2002; O'Connell et al., 2003; Thomas-Tikhonenko, et al., 2004)	Dual specificity phosphatase 1	2.4	1.6	
DDIT3	↑	(Amundson, et al., 1998; Babcock et al., 2013; Barsyte-Lovejoy, Mao, & Penn, 2004; Oster, et al., 2002)	DNA-damage-inducible transcript 3	2.5	1.8	1.7
CPT1A	↑	(Riu, Bosch, & Valera, 1996)	Carnitine palmitoyltransferase 1A (liver)	1.7	1.6	
KLF4	↓	(O'Connell, et al., 2003; Yilmazer, de Lazaro, Bussy, & Kostarelos, 2013)	Kruppel-like factor 4 (gut)	1.5		
G6PC	↓	(Collier et al., 2003)	Glucose-6-phosphatase, catalytic subunit	2.3	1.6	1.5
DKK1	↑	(Cowling, D'Cruz, Chodosh, & Cole, 2007)	Dickkopf 1 homolog (*Xenopus laevis*)	-1.6		
CD9	Affected	(Mu, Yin, & Prochownik, 2002)	CD9 molecule	1.7		
KLF6	Affected	(Terragni et al., 2011)	Kruppel-like factor 6	2.0		
MT1E	Affected	(Frye, et al., 2003; Oster, et al., 2002)	Metallothionein 1E	-1.5		-1.5