UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION & FORMULATION OF BIO-ACTIVE FRACTION OF Moringa oleifera LAM.LEAVES EXTRACT AND ITS PROTECTIVE POTENTIAL AGAINST ACETAMINOPHEN TOXICITY

KARTHIVASHAN GOVINDARAJAN

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KARTHIVASHAN GOVINDARAJAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

September 2016

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DEDICATION

This thesis is dedicated to my beloved mother Mrs. Amutha Govindarajan, my father Mr. Govindarajan Thiruvarasan, my dearest wife Mrs. Asha Karthivashan, my relatives and friends more like family.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

CHARACTERIZATION & FORMULATION OF BIO-ACTIVE FRACTION OF *Moringa oleifera* LAM.LEAVES EXTRACT AND ITS PROTECTIVE POTENTIAL AGAINST ACETAMINOPHEN TOXICITY

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September 2016

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Moringa oleifera (MO) is a well-known and widely distributed tropical species of Moringaceae family. Its leaves possess an excellent nutritional profile and an impressive range of therapeutic properties. Recently, the investigations on pharmaceutical properties of MO leaves get expanded due to its enriched antioxidant potential. Though numerous study reports focused on its therapeutic efficacy, the responsible active compounds and its underlying molecular mechanism of action has not been determined yet which hold a setback for researchers to explore its exact therapeutic potential.

Acetaminophen (APAP) overdose is a worldwide leading cause of acute liver failure and drug-induced hepatotoxicity. During APAP overdose, majority of the drug is converted by the cytochrome P450 (CYP 450 - 2E1/1A2) enzymes to the reactive toxic metabolite, N-acetyl-p-benzoquinoneimine (NAPQI) that depletes GSH level and covalently binds to the other cellular proteins and induce hepatocyte death/acute liver failure. Currently, the most effective therapy for APAP overdose is N-acetylcysteine (NAC), which replenishes glutathione level and enhances hepatic recovery. However, NAC has few significant limitations such as time constraints and reversal of GSH level alone may not be sufficient to arrest progress of APAP hepatotoxicity. This drives scientists/researchers in exploring for an alternative safe and effective therapy.

In this study, the optimal MO gradient leaf extract has been obtained as 90% hydro-ethanolic solution based upon *in vitro* antioxidant assays and the active compounds responsible for its elite activity has been determined as quercetin, kaempferol, apigenin and multiflorin-B through chromatographic analysis. The underlying mechanism of action of 90% hydro-ethanolic MO leaf extract has been evaluated in Balb/c mice inflicted with lethal dose of APAP for hepato- and nephro-toxicity. The MO leaf extract effectively protects the liver through suppression of CYP 450 isoenzymes and in both liver and kidney through regulation of antioxidant enzymes level and modulation of inflammatory cytokines thereby hindering the further exacerbation of necrotic and renal tubular damage respectively. Further, 90% MO leaf crude extract was fractionated through liquid-liquid partition technique. Among the obtained solvent fractions, ethyl acetate (EA) fraction revealed the highest antioxidant activity evidently due to the presence of quercetin, kaempferol and apigenin which has been identified and quantified with commercial standards using HPLC analysis. Wherein, kaempferol was expressed in higher concentration with 263.86 µg, followed by apigenin and quercetin with 82.64 and 66.89 µg respectively, per mg of MO leaves EA fraction. Soy phosphatidylcholine (PC) is a bifunctional complex comprises of lipophilic phosphatidyl moiety and hydrophilic choline moiety. Naturally, flavonoids and phenolic compounds got affinity to bind with PC molecule resulting in a cell like lipid compatible molecular complex. In accordance, the EA fraction and its three major flavonoids quercetin, kaempferol and apigenin has been successfully loaded in PC molecule to retain its synergism and enhance bioavailability. Further its physico-chemical parameters, *invitro* drug release and hepato-protective potential against APAP inflicted hepatotoxicity in HepaRG cell line has been evaluated.

The findings of this study has evidently suggested that MO leaves extract and its EA fraction loaded phospholipid complex can be implied as an effective antidote against APAP intoxication as it hinders/suppresses/modulates various key biomarkers involved in APAP hepatotoxicity pathway.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENCIRIAN & FORMULASI BAGI PECAHAN BIO-AKTIF EKSTRAK DAUN *Moringa oleifera* LAM.DAN POTENSI PERLINDUNGAN TERHADAP KETOKSIKAN ACETAMINOPHEN

Oleh

KARTHIVASHAN GOVINDARAJAN

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Moringa oleifera (MO) adalah spesies tropika yang terkenal dan tergolong di dalam keluarga Moringaceae yang didapati secara meluas. Daunnya mempunyai profil nutrisi yang sangat baik dan memiliki pelbagai kesan terapeutik yang mengagumkan. Kini, siasatan ke atas keupayaan farmaseutikal daun MO telah berkembang akibat daripada berkembang kerana potensi antioksidan yang diperkaya. Walaupun banyak laporan kajian memberi tumpuan kepada keberkesanan terapeutik, sebatian aktif yang bertanggungjawab serta mekanisme molekul yang disebalik keberkesanannya masih belum dikenalpasti yang mmenyebabkan mengehadkanpenerokaan potensi terapeutik yang tepat tidak dapat dijalankan.

Acetaminophen (APAP) berlebihan adalah punca utama kegagalan hati akut dan hepatoksisiti disebabkan oleh dadah di seluruh dunia. Apabila APAP diambil secara berlebihan, majoriti dadah ini ditukarkan oleh P450 cytochrome (CYP 450 – 2E1/1A2) enzim menjadi metabolit toksik yang reaktif, N-acetyl-p-benzoquinoneimine (NAPQI) yang merendahkan tahap GSH dan mengikat kepada protein selular secara kovalen dan mendorong kematian sel-sel hati / kegagalan hati akut. Pada masa ini, terapi yang paling berkesan untuk APAP berlebihan adalah N-acetylcysteine (NAC), yang menggantikan semula semula tahap glutathione dan meningkatkan pemulihan hepatik. Walau bagaimanapun, NAC mempunyai beberapa batasan yang ketara seperti kekangan masa dan kesan pembalikan tahap

kepekatan GSH yang mana tidak mencukupi untuk mengekang perkembangan toksisiti hati. Hal ini telah mendorong saintis / penyelidik untuk meneroka kaedah alternatif yang selamat dan berkesan.

Di dalam kajian ini, pengekstrakan daun MO secara optimal telah diperoleh pada 90 % larutan hidro-etanolik berdasarkan in vitro antioksidan asai dan sebatian aktif yang bertanggungjawab untuk aktiviti elit telah ditentukan sebagai quercetin, kaempferol, apigenin dan multiflorin-B melalui analisis kromatografi. Mekanisme asas bagi 90% pecahan hidro-etanolik bagi ekstrak daun MO telah dinilai menggunakan tikus Balb/ c yang diberikan dos maut APAP untuk ketoksikan hati dan ginjal. Ekstrak daun MO berkesan melindungi hati melalui penindasan CYP 450 isoenzim manakala dalam kedua-dua hati dan ginjal melalui regulasi tahap enzim antioksidan dan modulasi sitokin yang menghalang menghalang nekrosis serta kerosakan tiub buah pinggang.Di samping itu, 90% daun ekstrak MO mentah telah difraksinasi melalui teknik pemecahan berperingkat cecair-cecair. Antara pecahan pelarut yang diperolehi, etil asetat (EA) pecahan telah menunjukkan aktiviti antioksida yang paling tinggi kerana kehadiran quercetin, kaempferol dan apigenin yang telah dikenal pasti dan dinilai dengan piawaian komersil menggunakan analisis HPLC. Kaempferol telah dinyatakan dalam kepekatan yang paling tinggi dengan 263,86 µg, diikuti oleh apigenin dan quercetin dengan 82.64 dan 66.89 µg masing-masing, bagi setiap mg pecahan EA daun MO. Posfatidilkolin daripada soya merupakan kompleks dwifungsi daripada moiety fosfatidil lipofilik dan moiety kolin hidrofilik. Secara semulajadinya, flavonoid dan sebatian fenolik mempunyai afiniti yang tinggi untuk berikatan dengan molekul PC menyebabkan sel seperti kompleks lipid molekul yang serasi. Selaras dengan itu, pecahan EA dan tiga flavonoid utama quercetin, kaempferol dan apigenin telah berjaya dimuatkan dalam molekul PC bagi mengekalkan sinergi dan meningkatkan tahap bioavailabiliti. Selanjutnya, parameter fiziko-kimia, pelepasan dadah secara in vitro dan potensi perlindungan-hepa terhadap ketoksikan hati disebabkan APAP di dalam sel HepaRG telah dinilai.

Hasil kajian ini telah jelas menunjukkan bahawa ekstrak daun MO dan pecahan EA yang dimuatkan di dalam kompleks phospholipid yang dapat digunkakan sebagai penawar yang berkesan terhadap kesan buruk APAP kerana ia menghalang / menyekat / memodulatkan pelbagai bio-penanda utama yang terlibat dalam ketoksikan hati yang disebabkan oleh APAP.

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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^{1}H NMR	Nuclear Magnetic Resonance
3-OH-APAP	3′Hydroxyacetaminophen
AAEAC	Ascorbic Acid Equivalent Antioxidant Capacity
AAS	Atomic absorption spectrometer
AIP-1	Actin-interaction protein 1
AKT	Protein kinase B
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AP-1	Activator protein 1
APAP	Acetaminophen/N-acetyl-p-aminophenol
APC	Antigen presenting cells
ARE	Antioxidant response element
ASK-1	Apoptosis signal–regulating kinase 1
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CIDM	Centre for International Drug Monitoring
DAMP	Damage-associated molecular pattern

DC	Dendritic cells
DCM	Dichloromethane
DLS	Dynamic light scattering
DMSO	Dimethylsulfoxide
DPPH	1-diphenyl-2-picrylhydrazyl
DSC	Differential scanning calorimeter
ECL	Enhanced chemi-luminescence
ERK	Extracellular signal-regulated kinases
FDA	The Food and Drug Administration
FRAP	Ferric reducing antioxidant power
FTIR	Fourier-Transformed Infrared
GCL-c	Glutamate cysteine ligase catalytic subunit
GCLM	Glutamate-Cysteine Ligase Modifier Subunit
GI	Gastrointestinal
GSH	Glutathione
GSK-3β	Glycogen synthase kinase-3beta
GSTA2	Glutathione S-transferase alpha 2
GSTA2	Glutathione S-transferase A2
H_2O_2	Hydrogen Peroxide
HMGB1	High-mobility group box 1
HO-1	Heme oxygenase
HPLC	High Performance Liquid Chromatography
HRP	Horseradish peroxidase

IFN	Interferon
IL-1β	Interleukin 1 beta
JNK	c-Jun N-terminal kinases
KC	Kupffer cells
LC-MS/MS	Liquid chromatography–mass spectrometry
MAPK	Mitogen-activated protein kinases
Mcl-1	myeloid cell leukemia-1
MHC	Major histocompatibility complex
MIP-2	Macrophage inflammatory protein 2
MKK-4/7	Mitogen activated protein kinase kinase -4/7
MLK3	Mixed-lineage kinase-3
MO	*Moringa oleifera*
MPT	Mitochondrial permeability transition
NAC	N-acetyl-cysteine
NAFLD	Non-alcoholic fatty liver disease
NAPQI	N-acetyl-p-benzo-quinone imine
NEDD	n-(1-naphthyl) ethylenediamine dihydrochloride
NF-κβ	Nuclear factor kappa of activated B cells
NK/NKT	Natural Killer/Natural Killer -T cells
NO	Nitric oxide
NQO1	NAD(P)H dehydrogenase [quinone] 1
Nrf-2	Nuclear factor erythroid 2-related factor 2
OATP	Organic anion-transporting polypeptide

ORAC	Oxygen Radical Absorbent Capacity
OTC	Over-the-counter
PBS	Phosphate buffer saline
PC	Phosphotidylcholine
PGES	Prostaglandin endoperoxidase synthase
PI3K	Phosphoinositide 3-kinase
PVDF	Polyvinylidene fluoride
ROS/RNS	Reactive oxygen species/ Reactive nitrogen species
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Scanning electron microscope
SOD	superoxide dismutase
STAT1	Signal transducer and activator of transcription-1
TEM	Transmission electron microscope
TGA	Thermogravimetric analysis
TGF- β	Transforming growth factor beta
THF	Tetrahydrofuran
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor – alpha
TPTZ	Tris(2-pyridyl)-s-triazine
TRAF-2	TNF receptor associate factor -2
TRX	Thioredoxin
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Introduction

Acetaminophen (N-acetyl-p-aminophenol; APAP) is commonly used over-the-counter (OTC) analgesic and antipyretic drug, due to its rapid absorption in the gastro-intestinal tract and minimal risk of gastric ulceration. However, its overdose leads to both liver and kidney damage (Bagheri et al., 2013; Ghosh et al., 2010). Centre for International Drug Monitoring - World Health Organization (CIDM-WHO) has categorized APAP as the ultimate amongst the top 10 drugs associated with fatal liver injury (Björnsson et al., 2006). According to the US Food and Drug Administration, each week approximately 50 million adults in the United States consume APAP /related OTC products and were reported as the leading cause for acute liver failure. APAP has also been reported as the most common drug induced toxicity either accidentally or intentionally in the United Kingdom (UK), with an estimated 70 000 cases annually; Canada had a yearly occurrence of 46 per 100 000 population between 1997–2002 and a low profile of around 2% among the European countries. Amidst the reported APAP toxicity cases, few are intentional (suicide attempts) whereas around 50% of deaths and emergency cases are due to unintended overdoses of APAP (Marzilawati et al., 2012; Clark et al., 2012).

At normal therapeutic dose APAP acts as an effective analgesic, whereby the reactive toxic metabolite (NAPQI, N-acetyl-p-benzo-quinone imine) which is produced during the metabolic process was detoxified by liver enzymes and flushed out through bile. During APAP over dosage the rate of NAPQI formation overwhelms the rate of detoxification activity by the liver enzymes and leads to necrotic cell death. The resultant necrosis ultimately leads to organ dysfunction (Hinson et al., 2004). Despite liver, APAP also induce kidney toxicity, however its mechanism of action is not extensively explored as APAP hepatotoxicity. Retrospective case series documented approximately 1-2% of patients with APAP overdose revealed renal impairment. When significant APAP hepatotoxicity occurs, renal injury is commonly seen with notable elevation of creatinine levels in 43-57% of a prospective study of 275 patients with encephalopathy and coagulopathy secondary to APAP-induced fulminant hepatic failure (Eguia et al., 1997; Cekmen et al., 2009).

The occurrence of APAP overdose in population with other medical conditions such as hypertension, dyslipidemia, obesity and diabetes mellitus has been reported to further aggravate the primary medical complication (Wang et al., 2013; Shertzer et al., 2010). APAP overdose induce elevated oxidative stress environment and associated exacerbation of tissue damage due to activation of inflammatory cascade in obese, type 2 diabetic and NAFLD induced animals (Kon et al., 2010; Kučera et al., 2011). Therefore, previous reports have substantiated the influence of APAP overdose in both general and vulnerable populations with other medical complications and hence drives the FDA to propose ways to limit the hepatotoxicity of APAP via reducing its therapeutic index/ minimizing combinational therapy.

The current and foremost effective treatment for APAP toxicity is supplemental therapy using the clinically accepted antidote, N-acetyl-cysteine (NAC), a precursor of GSH. NAC replenishes glutathione level and enhances hepatic recovery, however its role in APAP induced renal toxicity remains unclear and thereby limits its function towards APAP nephrotoxicity (Eguia et al., 1997; Mazer et al., 2008). Clinically, if the patient presents less than eight hours after APAP overdose, the NAC treatment (either IV or oral dosage) significantly reduces the risk of serious hepatotoxicity and guarantees survival. If the NAC is administered after the stipulated time limits, a sharp decline in its effectiveness was observed, with increased risk of acute toxicity mediated fatality (Sfetcu, 2014).

Moringa oleifera (MO) Lam is a well-known widely distributed species of Moringaceae family, and holds high nutritional value and a remarkable range of therapeutic properties. MO leaves have been reported to be a rich source of micro- and macronutrients, thus nurturing both animal and human as an excellent nutritive supplement (Siddhuraju et al., 2003). MO leaves have been enduringly used as a traditional medicinal source and employed for treatment of many diseases, thus coined as "the miracle tree." A recent report revealed that MO leaves is the source of the highest antioxidant content among natural food resources, whose leaves powder measured over 157000 µmol trolox equivalent/100 g, using an oxygen radical absorbent capacity system of measurement developed by the National Institute of Health (Jodi patkin steel peach communication, 2012).

The leaves of MO have been reported for its various therapeutic properties such as antimicrobial, antiinflammatory, anti-cancer, anti-diabetic effects (Anwar et al., 2007; Coppin et al., 2013) and has recently been evaluated and demonstrated to show hepatoprotective effects (Das et al., 2012; Sharifudin et al., 2013). Hydro-alcoholic pod extract and aqueous leaves extract of MO at a dosage of 150 to 300 mg/kg significantly exerted a protective effect

against streptozotocin induced diabetic rats (Jaiswal et al., 2009; Gupta et al., 2012). MO leaves extract also exhibited strong anticancer potential (Gupta et al., 2012), and its hydro-alcoholic leaves extract at a dose of 1000 mg/kg improved the activities of antioxidant enzymes and reduced peroxidation of lipids in CCl4-induced hepatotoxicity mice model (Rakesh et al., 2010). Despite their extensive pharmacological properties the active plant extracts / their phyto-constituents reveal poor water solubility, inadequate bio-permeability, limited bioavailability due to rapid first past metabolism before entering the systemic circulation and thereby limiting their extensive potential in clinical applications (Mohan et al., 2014; Lee et al., 2004).

Nanoscience is an interdisciplinary field that has its early beginning in 1980s. In medicine, nanoparticles as drug carriers are showing to have vast potentials (De Jong at al., 2008). Phytosome technology is attained by preparing complexes of active plant extracts / their phyto-constituents with soy derived phosphotidylcholine (PC) has been established as an appropriate drug delivery system to protect the active principle, improve its membrane permeability, sustain release and enhance its bioavailability (Kumar et al., 2010). Phytosomes revealed better absorption profile and enhances delivery of phenolic phyto-constituents to the tissues. The chemo-bonding interaction of active phyto-constituents and PC makes the active ingredients more stable in the complex form (Bhattacharya et al., 2009). Thus, several bioactive candidates has been successfully formulated and delivered with remarkable therapeutic efficacy compared to its natural dosage form (Singh et al., 2011) using this technology. Recently, numerous phytosomal products have been commercially introduced and gain remarkable turnover to the pharma / nutraceutical and cosmetic market (Di Pierro et al., 2009; Maiti et al., 2006; Morazzoni et al., 2001; Naik et al., 2006; Semalty et al., 2010).

In this study, we optimized the gradient hydro-ethanolic solvent for effectual extraction from MO leaves and also identified the possible compounds responsible for its enhanced antioxidant activity. The best gradient MO leaves crude extract was further administered for the treatment of APAP induced toxicity in mice model, subsequently, establishing potential underlying molecular mechanism of MO leaves extract against APAP induced liver and kidney toxicity. The MO leaves crude extract was further fractionated using various organic solvents with increasing polarity. The bio-active fraction and possible active compounds responsible for its enhanced bioactivity were determined.

In second part of the study, the bio-active compounds of the elite solvent fraction have been loaded in PC complex using various formulation

methods. The optimal formulation method was chosen based on its physico-chemical characteristics and antioxidant kinetics. Further its toxicity potential towards human hepatoma (HepaRG) cell line has also been determined. Lastly, the synthesized soy-lecithinated bio-active compounds and bioactive fraction of MO leaves has been comparatively evaluated based on their physico-chemical properties, *in vitro* antioxidant potential and hepatoprotective potential against APAP induced liver injury in HepaRG cell line.

1.2 Problem Statement

Since the commencement on the era of systematic scrutinization on MO leaves extract, researchers randomly pick various gradient solvents for maceration/extraction. Choosing the best solvent gradient for maceration is one of the major factors for effectual extraction process in plants. Knowledge of a drug's mechanism of action enables better dosing and target precise pathway in the clinical treatment. However, most of the reported studies have focused on the evaluation of pharmaceutical potential of MO against APAP toxicity but not at its molecular level mechanism of action. Though numerous bio-active phytoconstituents are naturally available in MO leaves, its maximum efficacy could not be fully attained, since they get degrade before they reaches its target organ. It has also been often observed that the isolation and purification of the constituents from any extract loses the synergistic effect of the active principle(s).

1.3 Justification

The best gradient solvent for extraction / maceration can be achieved by screening the obtained gradient MO leaves extracts through various *in vitro* antioxidant assays and identification of the compound responsible for its elite activity through HPLC and LC-MS/MS analysis. Further deployment of the obtained optimal MO leaves extract against APAP induced toxicity in animal model and analysis of its impact on various key biomarkers, enables us to establish its underlying molecular mechanism of action. Loading of bioactive fraction of MO leaves extract or its bioactive constituents in soy-derived phospholipid carrier enhances its bio efficacy and sustained release. Despite their comparative evaluation on physico-chemical and therapeutic properties reveal the impact of synergism in drug delivery system.

1.4 General objective

To develop a potential soy-phospholipid delivery system, loaded with bioactive fraction/ associated active flavonoids of MO leaves extract to combat against acetaminophen toxicity.

1.5 Specific objectives

1. To obtain the optimal hydro-ethanolic gradient solvent for effectual extraction from MO leaves and identify the potential bioactive compounds responsible for its elite antioxidant activity.
2. To investigate the underlying molecular mechanism of MO leaves extract against APAP induced hepato- and renal- toxicity.
3. To obtain the bioactive fraction of MO leaves crude extract through bioassay guided fractionation and identify the responsible active flavonoids through chromatographic analysis.
4. To determine an optimal formulation method for loading multiple flavonoids in a single soy-phospholipid molecule (flavonosome) based on its physico-chemical characteristics and *in vitro* DPPH kinetics.
5. To formulate bioactive flavonoids (flavonosome) and bioactive MO fraction (fractionosome) loaded soy-phospholipid complex and comparatively evaluate their physico-chemical characteristics, antioxidant potential and hepato-protective potential against APAP induced liver injury in HepaRG cell line.

1.6 Hypotheses

- Based on the availability of enriched bioactive phyto-constituents profile of MO leaves extract, it shall target APAP toxicity pathway via multiple mechanism of action.
- Loading more than one flavonoid within one PC molecule might improve its effectiveness by retaining synergism and enhance its bio efficacy due to their sustain release.
- Multiple - active flavonoids loaded flavonosome might show effective pharmacological activity compare to the MO active fraction loaded fractionosome, as the phospholipid carrier loaded with only active candidates might reveal best activity.

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Conferences

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Participated - Seminar on "Toxicologic Pathology: A Basis for Drug Development and Chemical Safety Assessment" organized by Faculty of Engineering, UPM. 05th – 07th Nov 2012, Malaysia.

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