UNIVERSITI PUTRA MALAYSIA

ROLES OF ANTI-NEUROINFLAMMATION AND ANTI-OXIDATIVE PROPERTIES OF A STANDARDISED Andrographis paniculata BURM. NEES AQUEOUS EXTRACT IN IMPROVING COGNITION IN WISTAR RATS

DAHIRU SANI

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By

DAHIRU SANI

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

November 2016

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DEDICATION

This thesis is dedicated to my late mother, Malama Safiya and sister Salamatu. May Almighty Allah forgive them all their shortcomings and make Jannatul firdaus to be their final abode amin.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

ROLES OF ANTI-NEUROINFLAMMATION AND ANTI-OXIDATIVE PROPERTIES OF A STANDARDISED *Andrographis paniculata* BURM. NEES AQUEOUS EXTRACT IN IMPROVING COGNITION IN WISTAR RATS

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Chairman : Professor Johnson Stanslas, PhD
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The number of people worldwide with cognitive/memory impairment is projected to double every 20 years to 81 million by 2050. The rapid expansion of the worldwide aged population is expected to greatly increase the number of individuals with cognitive impairment and this will ultimately have a significant impact on the healthcare cost. To date there is no medicine to treat or prevent cognitive impairment. This has led people to resort to taking herbal supplements for improving memory. One good example of a herb that is becoming increasingly popular for memory improvement is *Ginkgo biloba*. Substantial studies have shown neuro-inflammatory processes to contribute to the cascade of events eventually leading to neuronal degeneration and subsequently loss of memory. This present study evaluated the cognition improving potential and neuroprotective effects of a standardised *Andrographis paniculata* (locally known as "Hempedu Bumi") aqueous leaf extract (**APAE**).

The cognitive improvement of **APAE** was assessed in an *in vivo* model of lipopolysaccharide (LPS)-induced neuro-inflammation in Wistar rats. Subsequently, the neuroprotective properties of **APAE** and its major phytochemicals (andrographolide (**AGP**), neoandrographolide (**NAG**) and 14-deoxy-11,12-didehydro andrographolide (**DDAG**)) were evaluated using microglial (BV-2) and dopaminergic (N27) cells.

In the *in vivo* pre-treatment study, rats were pre-treated orally with varied doses of **APAE** (50 – 400 mg/kg) and a standardised *Ginkgo biloba* (**GB**, 50 – 200 mg/kg) extract (EGb761, Tanakan™, as a positive control*)* for 7 days before exposure to LPS (1 mg/kg, i.p). Cognitive function was evaluated using a 2-day Morris Water Maze (MWM) protocol with slight modification. Compared with the LPS control group, pre-treatment with **APAE** at the tested doses effectively prevented memory impairment in the rats as demonstrated by significantly decreasing the mean escape

latency of the rats in locating the hidden platform and dose-dependently increasing the number of entries into the target quadrant in the probe. A similar effect was displayed by GB. However, at doses of 50 and 100 mg/kg, APAE was superior to the latter. But at 200 mg/kg both agents produced similar effect. It is interesting to note that GB (200 mg/kg) and APAE (200 and 400 mg/kg) exhibited an improved performance compared to the vehicle-treated control. It was found that **APAE** was better than **GB** in improving cognition. In the post-LPS treatment study, both the normal and treated groups (**APAE** and **GB**) showed a decrease in the escape latency but interestingly, no significant difference was observed in the probe trial except for group treated with the highest dose (**APAE**, 400 mg/kg). It was also demonstrated that LPS administration caused increased production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), oxidative stress markers (reactive oxygen species (ROS), thiobarbituric acid reactive substances (TBARS), cholinesterase (acetylcholinesterase (AChE), butyrylcholinesterase (BChE) activities and decreased antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) activities and depletion of antioxidant glutathione (GSH) level in the hippocampal region of the brain. Pre-treatment with **APAE** or **GB** reversed these effects. Neuropathological evaluation additionally revealed LPS-treated animals to show marked infiltration of inflammatory cells (neutrophils, eosinophils), disorientation of pyramidal cell and loss of small pyramidal cells. However, pre-treatment of rats with **APAE** or **GB** significantly ($P<0.05$) attenuated the LPS effects dose-dependently. Furthermore, TNF-α, IL-1β, IL-6 mRNA levels were increased while SOD, CAT and glutathione peroxidase (GPx) mRNA levels were decreased significantly after LPS administration. Rats pre-treated with **APAE** and **GB** prevented these effects ($P<0.05$), which suggested pre-treatment of **APAE** prevented brain toxicity by inhibiting neuro-inflammation and oxidative stress.

Urine ^{1}H-NMR metabolomics spectra depicted distinct discrimination in urinary metabolite profiles between control and LPS-treated rats with respect to differences in the metabolites (oxoglutarate, creatinine, allantoin, acetate, citrate, taurine, β-xylose and hippurate) associated with oxidative damage induced by LPS. Urinary metabolite profiles of rats pre-treated with **APAE** (400 mg/kg) or **GB** (200 mg/kg) prior to LPS induction showed distribution similar to control animals. This further supported the anti-neuroinflammatory effect of these agents.

In the *in vitro* study, conditioned medium (CM) from LPS-activated glial cells (BV-2) inhibited N-27 viability but the CM from **APAE** or **AGP** pre-treated BV-2 cells did not. This suggested that *in vitro* the agents were exerting a neuroprotective effect. It was also shown that LPS caused increased production of TNF-α, IL-6 and IL-1β as well as ROS, nitric oxide (NO) and TBARS in BV-2 cells. However, pre-treatment of BV-2 cells with **APAE** or **AGP** prior to LPS-stimulation significantly inhibited these effects in a dose-dependent manner, suggesting neuroprotective effect *via* prevention of inflammatory and oxidative stress mediators. The blood-brain barrier study suggested **AGP** in **APAE** has good permeation, further supporting **APAE**'s neuroprotective potential. The current findings indicate that **APAE** prevented LPS-induced neuroinflammation mediated cognitive impairment *via* inhibition of production of pro-inflammatory cytokines, oxidative stress mediators, cholinesterase enzyme activity and improving antioxidant enzyme activity to increase

neuroplasticity; all of these translated into improved cognition in rats. The neuroprotective potential of **APAE** and **AGP** were further supported *in vitro* by inhibition of microglial activation *via* decreased generation of pro-inflammatory and oxidative stress markers. This study provides the mechanistic evidence by which **APAE** exerts its beneficial effects. The outcome serves as a template to establish the anti-inflammatory and anti-oxidative roles of **APAE** for neuroprotection, which could have benefit in retarding cognitive impairment in a normal population and additionally may have a role in inhibiting the progression or development of certain neurological diseases associated with neuroinflammation and oxidative stress. In addition, data presented in this study suggested that APAE is a superior cognition improving supplement than the existing market leader EGb761 (Tanakan™), particularly at lower doses (100 and 50 mg/kg).

ABSTRAK

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

PERANAN ANTI-NEUROKERADANGAN DAN CIRI-CIRI ANTI-OKSIDATIF EKSTRAK AKUEUS *Andrographis paniculata* BURM. NEES DALAM MENINGKATKAN KOGNISI PADA TIKUS WISTAR

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Jumlah populasi seluruh dunia dengan ketidakmampuan kognitif/memori dijangkakan akan berganda setiap 20 tahun kepada 81 juta orang pada tahun 2050. Pertumbuhan pesat penduduk golongan berumur di seluruh dunia dijangka memberi peningkatan yang ketara terhadap bilangan individu yang mempunyai ketidakmampuan kognitif dan akan memberi impak yang besar ke atas kos penjagaan kesihatan.

Setakat ini tiada ubat untuk merawat atau mencegah ketidakmampuan kognitif. Keadaan ini menyebabkan ramai orang memilih haluan sendiri untuk meningkatkan daya ingatan dengan pengambilan suplemen herba. Salah satu contoh herba yang semakin popular untuk peningkatan memori adalah *Ginkgo biloba*. Kajian yang mendalam telah menunjukkan proses neurokeradangan akan menyumbang kepada rentetan peristiwa yang membawa kepada degenerasi neuron dan akhirnya kehilangan memori.

Kajian ini menilai potensi pembaikan kognitif dan kesan neuropelindung ekstrak daun akueus Andrographis paniculata terpiawai (**APAE**) (nama setempat dikenali sebagai "Hempedu Bumi"). Peningkatan kognitif **APAE** dinilai dengan model in vivo neurokeradangan-teraruh lipopolisakarida (LPS) pada tikus Wistar. Seterusnya, sifat-sifat neuropelindung **APAE** dan fitokimia majornya (andrografolid (**AGP**), neoandrografolid (**NAG**) dan 14-deoksi-11,12-didehidro andrografolid (**DDAG**) dinilai menggunakan sel-sel mikroglial (BV-2) dan dopaminergik (N27).

Dalam kajian pra-rawatan in vivo, tikus dipra-rawat secara oral dengan dos **APAE** yang berbeza (50 - 400 mg/kg) dan ekstrak Ginkgo biloba terpiawai (**GB**, 50 – 200 mg/kg) (EGb761,TanakanTM, sebagai kawalan positif) selama 7 hari sebelum pendedahan kepada lipopolisakarida (LPS, 1 mg/kg, i.p.). Fungsi kognitif dinilai

menggunakan protokol 2-hari Morris Water Maze (MWM) dengan sedikit pengubahsuaian. Berbanding dengan kumpulan kawalan LPS, pra-rawatan dengan **APAE** pada dos yang diuji agak berkesan dalam menghalang ketidakmampuan memori tikus. Kesannya dilihat dengan penurunan kependaman lepas diri purata tikus dalam mencari platform tersembunyi dan akan meningkatkan jumlah kemasukan kuadran sasaran dalam kuar mengikut dos kebergantungan. Kesan yang sama telah dipaparkan oleh **GB**. Walau bagaimanapun, pada dos 50 dan 100 mg/kg, **APAE** lebih dominan berbanding dengan **GB** tetapi, pada 200 mg/kg keduadua agen memberi kesan yang sama. Pemerhatian yang menarik adalah **GB** (200 mg/kg) dan **APAE** (200 dan 400 mg/kg) menunjukkan prestasi yang lebih baik berbanding dengan kumpulan kawalan normal. Secara keseluruhanya, **APAE** didapati adalah lebih baik dalam menambah baik kognisi berbanding dengan **GB**.

Dalam kajian rawatan pasca-LPS, kedua-dua kumpulan normal dan kumpulan dirawat (**APAE** dan **GB**) menunjukkan penurunan dalam kependaman lepas diri, namun tiada perbezaan ketara diperhatikan dalam percubaan prob kecuali untuk kumpulan dirawat dengan dos yang tertinggi (**APAE**, 400 mg/kg). Ini menunjukkan bahawa pengambilan LPS menyebabkan peningkatan produksi sitokin prokeradangan (TNF-α, IL-1β, IL-6), penanda stres oksidatif (spesies oksigen reaktif (ROS), bahan reaktif asid tiobarbiturik (TBARS), kolinesterase (asetilkolinesterase (AChE), butirilkolinesterase (BChE) dan menunjukkan penurunan aktiviti antioksida enzim dismutase superoksida (SOD), dan kemerosotan paras antioksida glutation (GSH) di rantau hipokampus otak. Pra-rawatan dengan **APAE** atau **GB** dapat memberikankesan berlawanan terhadap aktiviti LPS. Selain itu, penilaian neuropatologi mendedahkan haiwan yang dirawat dengan LPS menunjukkan infiltrasi sel keradangan (neutrofil, eosinofil), disorientasi sel piramid dan kehilangan sel piramid kecil. Tikus pra-rawatan dengan **APAE** atau **GB** ($P < 0.05$) dengan ketara melemahkan kesan LPS kebergantungan terhadap dos bersandar. Tambahan pula, tahap TNF-α, IL-1β, IL-6 mRNA telah meningkat, manakala tahap SOD, CAT dan glutation peroksidase (GPx) mRNA telah menurun dengan ketara selepas pemberian LPS. Tikus yang dipra-rawat dengan **APAE** atau **GB** menghalang kesan-kesan ini ($P < 0.05$). Justeru itu dicadangkan pra-rawatan **APAE** menghalang ketoksikan otak dengan menghalang neurokeradangan dan stress oksidatif.

Spektrum metabolomik urin 1H-NMR menunjukkan diskriminasi yang jelas berbeza bagi profil metabolit urinari di antara tikus kawalan dan tikus terawat LPS, dengan mengambil kira perbezaan dalam metabolit (oksoglutarat, kreatinina, allantoin, asetat, sitrat, taurina, β-xylose dan hippurate) yang dikaitkan dengan kerosakan oksidatif yang dicetuskan oleh LPS. Profil metabolit urinari tikus dipra-rawat dengan **APAE** (400 mg/kg) atau **GB** (200 mg/kg) sebelum induksi LPS menunjukkan pengagihan yang sama terhadap haiwan kawalan. Ia seterusnya menyokong kesan anti-neurokeradangan bagi agen ini.

Dalam kajian in vitro , medium kondisi (CM) daripada sel glia (BV-2) teraktif-LPS menghalang kebolehhidupan sel dopaminergik, sebaliknya diperhatikan untuk sel BV-2 yang diprarawat dengan **APAE** atau **AGP**. Ini mencadangan bahawa dalam in vitro, agen tersebut memberikan kesan neuropelindung. Ia juga menunjukkan yang

LPS menyebabkan peningkatan pengeluaran TNF-α, IL-6 dan IL-1β serta ROS, nitrik oksida (NO) dan TBARS dalam sel BV-2. Walau bagaimanapun, prarawatan sel BV-2 dengan **APAE** atau **AGP** sebelum rangsangan-LPS menghalang dengan ketara kesan-kesan ini secara kebergantungan dos, menunjukkan kesan neuropelindung melalui pencegahan pengantara keradangan dan pengantara tekanan oksidatif. Kajian halangan darah otak mencadangkan **AGP** dalam **APAE** mempunyai penelapan yang baik, seterusnya menyokong potensi neuropelindung **APAE**.

Penemuan terkini menunjukkan **APAE** menghalang ketidakmampuan kognitif berperantara neurokeradangan teraruh LPS melalui pengekangan penghasilan aktiviti sitokin pro-keradangan, perantara stres oksidatif dan enzim kolinesterase serta meningkatkan aktiviti enzim antioksidan untuk meningkatkan neurokeplastikan; semua ini diterjemahkan kepada kognisi yang lebih baik pada tikus. Potensi neuropelindung **APAE** dan **AGP** telah disokong in vitro oleh perencatan pengaktifan microglial melalui penurunan penanda stres prokeradangan dan oksidatif. Kajian ini membuktikan mekanistik di mana **APAE** memberikan kesan yang baik. Hasilnya berfungsi sebagai templat untuk mewujudkan peranan anti-keradangan dan anti-oksidatif **APAE** untuk neuropelindung, dimana berkemungkinan memberi manfaat untuk memperlahankan ketidakmampuan kognitif dalam populasi normal, di samping mungkin mempunyai peranan dalam menghalang perkembangan atau pembangunan penyakit saraf tertentu yang berkaitan dengan neurokeradangan dan tekanan oksidatif. Di samping itu, data yang dikemukakan dalam kajian ini mencadangkan bahawa **APAE** merupakan suplemen membaiki kognisi yang unggul dibandingkan dengan produk ulung sedia ada di pasaran EGb761 (TanakanTM), terutamanya pada dos yang lebih rendah (50 dan 100 mg/kg).

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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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) are adhered to.

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ABM	astrocyte basal medium
ACh	acetylcholine
AChE	acetylcholinesterase
ACN	acetonitrile
AD	Alzheimer's Disease
AGEs	advanced glycation end products
AGM	astrocyte growth medium
AGP	andrographolide
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	analysis of variance
AP	*andrographis paniculata*
APAE	*andrographis paniculata* aqueous extract
ARMD	age related muscular degeneration
ATCC	animal tissue culture collection
BBB	blood brain barrier
BChE	butyrylcholinesterase
BDNF	brain derived neurotrophic factor
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
BV-2	microglia cell
CAT	catalase
cDNA	complementary deoxyribonucleic acid

CM	conditioned medium
CNS	central nervous system
CO_2	carbon dioxide
COX-2	cylo-oxygenase-2
CPT-CAMP	4-chlorophenylthio-adenosine-3',5'-cyclic monophosphate
CREBB	cyclic-AMP response element binding protein
CSF	cerebrospinal fluid
CV	coefficient of variation
DCF	2, 7- dichlorofluorescin
DASM	dehydroandrographolide succinic acid monoester
DCF-DA	2, 7- dichlorofluorescin diacetate
DDAG	14-deoxy-11, 12-didehydroandrographolide
DMEM	dulbecco's modified eagle's medium
DMSO	dimethylsulphoxide
DTNB	5,5'- dithiobis 2-nitrobenzoic acid
D_2O	deuterium oxide
EBM	endothelial basal medium
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
EGM	endothelial growth medium
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FCS	fetal calf serum

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GB/EGb 761	ginkgo biloba
GSH	glutathione
HBSS	hank's balanced salt solution
HD	Houghton's disease
^{1}H NMR	Proton nuclear magnetic resonance
H & E	Haematoxylin and eosin
HCL	Hydrochloric acid
hCMEC/D3	Human cerebral microvascular endothelial cells
HPLC	High performance liquid chromatography
H_2O_2	Hydrogen peroxide
iNOS	Inducible nitric oxide synthase
i.p	Intraperitoneal
IC_{50}	Inhibitory concentration
IL-1β	Interleukin 1-beta
IL-6	Interleukin 6
LOD	Limit of detection
LOQ	Limit of quantitation
LPS	Lipopolysaccharide
MCI	Mild cognition impairment
MDA	Malondialdehyde
min	Minute
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)
MWM	Morris water maze
NaCl	Sodium chloride

NADPH	Nicotinamide adenine dinucleotide phosphate
NAG	Neoandrographolide
NaH_2PO_4	Phosphate buffer
NGF	Nerve growth factor
NHA	Human cerebral astrocyte
NMDA	N-methyl-D-aspartate
NO	Ntric oxide
N27	Dopaminergic cell line
OPLS-DA	Orthogonal partial least square-discriminant analysis
PBS	Phosphate buffer saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PD	Parkinson's disease
PG	Prostaglandin
PGE2	Prrostaglandin E2
PICs	Pro-inflammatory cytokines
ppm	Part per million
RAGE	Receptors for advanced glycation end products
RNA	Rbonucleic acid
ROS	Reactive oxygen specie
RRt	Relative retention time
SD	Standard deviation
SOD	Superoxide dismutase
TAE	Tris-acetate-EDTA
TBA	Thiobarbituric acid

TBARS	Thiobarbituric acid reactive substance
TCA	Trichloroacetic acid
TEA	Triethylamine
TEER	Trans-endothelial electrical resistance
TJ	Tight junction
TMB	Tetramethylbenzidine
TNF- α	Tumour necrosis factor- α
TSP	3-trimethylsilyl- (2,2,3,3-)-1- propionic acid
URTI	Upper respiratory tract infections
UV/VIS	Ultraviolet/visible
WHO	World health organization
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight
µm	micrometre
%	Percentage
^{o}C	Degree celsius

CHAPTER 1

INTRODUCTION

1.1 Background

The worldwide human polulation with cognitive impairment is forecasted at 81 million by 2040 (Mavrodaris & Thorogood 2013; Organisation 2012). There is an increasing prevalence of aging population in both developed and developing nation like Malaysia (Sidik et al. 2004). Malaysia is predicted to experience the full impact of an aging population in 2035 (Yahaya et al. 2010).

The rapid expansion of the worldwide aged population could greatly increase the number of individuals with cognition impairment and the number of dependent older adults and this will overall have a significant impact on the healthcare cost. The total population of Malaysia based on the 2010 census is 28.28 million with 4.7 % of the population aged 65 years old and above and this figure is projected to rise to 13 % by 2025 (Rosdinom et al. 2011).

Mild cognitive impairment (MCI), a distinct state between normal ageing and early dementia (Cooper et al. 2013) has been suggested to denote an initial, but distinct state of cognitive impairment as well as generated bundle of research interest from both clinical and research perspectives (Bondi & Smith 2014; Petersen 2004). About 19% of people aged 65 and over has been shown to be affected with MCI (Lopez et al. 2007) with approximately 46% of people with MCI developing dementia within 3 years compared with 3% of the population of the same age (Tschanz et al. 2006). The rate of diagnosed individuals with MCI is rapidly increasing in Western countries as people are inspired to present early problem with memory to prevent crisis (Cooper et al. 2013). Thus, it is projected that, by 2040, if growth in the older population continues, and there are no changes in mortality or burden reduction by preventive measures, 71% of 81.1 million dementia cases will be in the developing world (Ferri et al. 2005).

Although age is an important factor associated with development of memory loss, other non-age related factors mainly dysregulation in the inflammatory network and oxidative imbalance are key components in the pathogenesis of the condition. It is hypothesised that down-regulation of these oxidative stress markers and inflammatory mediators offers protection against development and progression of the condition.

Cognition is the process of thinking, learning and memory (Sharifah et al. 2011). It is a natural function that decline with ageing due to impairment of memory, judgment, language, and attention (Woodford & George 2007). Cognitive disability is a common feature associated primarily with normal process of aging but could also be linked with physical or mental disorders (Sidik et al. 2004). Other risk factors includes brain injury, introduction to pesticides or toxins, metabolic syndromes, tobacco, depression as well as varieties of cardiovascular diseases including stroke, diabetes, hypertension and dysphoria condition (Plassman et el. 2010; Cicconetti et al. 2004).

To date there is no definitive drug use in treating cognition impairment leading to increased rate of depression and hospitalization among patients. This might be due to a gap in understanding of the precise pathomechanisms associated with this condition. However, cumulative reports have suggested that neuroinflammation and oxidative stress are pivotal in the cascade of events ultimately leading to neurodegeneration and subsequently affecting cognition *via* production of toxic pro inflammatory cytokines and oxidative stress mediators (Lee et al. 2008). Recently, it was reported that dementia could hypothetically be abolished with neuroprotection, treating vascular risk factors or increasing cognitive reserves, and could be targeted at specific people with MCI who are at high risk of developing it (Cooper et al. 2013).

Neuroplasticity illustrate an intrinsic property of the nervous system maintained throughout life that enables change in synaptic transmission efficiency (Pascual-Leone et al. 2011). There is an increasing interest in recent times on exploration of natural products with antioxidant and anti-inflammatory activities for prevention and treatment of cognitive impairment (Odubanjo 2013). Inhibition of oxidative damage and inflammatory conditions has been reported to prevent neurodegenerative conditions (Khanna et al. 2007; Fusco et al. 2007). Activation of the brain immune cells produces enormous amounts of reactive oxygen species (ROS), nitric oxide (NO) and inflammatory cytokines such as tumour necrotic factor (TNF)-α, interleukin-1 (IL-1) which eventually results in neuronal damage and death (Liu et al. 2003; Liu & Hong 2003; Liu et al. 2002; Jeohn et al. 1998; Chao et al. 1992).

Oxidative stress has been linked with brain damage and cognitive abilities associated with inflammatory conditions (Markesbery & Lovell 2008). It was also shown that environmental stimuli, emotions, and thought may cause alteration in neuroplasticity (Pascual-Leone et al. 2011), thereby having significant implications for healthy growth, learning, memory, and recovery from brain injury. This therefore necessitates the crucial need to explore a more effective alternative therapy capable of targeting and negating the underlying abnormal mechanisms in neuroinflammatory process to promote neuroprotection to reduce the burden posed by neuroinflammatory complication.

One of such strategy is the use of plants as source for a new therapeutic approach capable of targeting and preventing the toxic inflammatory cytokines and oxidative stress mediators linked with neuroinflammation to enhance protection thereby preventing memory loss.

Andrographis paniculata (AP) is a herb from the family Acanthaceae, found throughout Southeast Asia, and known locally as "Hempedu Bumi" or akarcerita". This is an annual herbaceous plant (Jarukamjorn & Nemoto 2008), well-known as 'king of bitter' (Subramanian & Asmawi 2006) and is reputed in Malaysia as remedy for diabetes and hypertension. It was also reported to have analgesic, antimalarial, anti-inflammatory, anti-oxidant, antineoplastic, antiulcerogenic, antibacterial, febrifuge, antiplatelet, anti-diarrhoeal properties (Jarukamjorn & Nemoto 2008). The plant has also been shown to possess protective activity against various liver disorders (Jarukamjorn & Nemoto 2008).

However, there is very limited information available about its central nervous system (CNS) action, mainly on cognition as well as on neuroprotection. Some important phytochemicals have been isolated from this plant. The aerial part contains mainly 3 major diterpenoids such as andrographolide (AGP), neoandrographolide (NAG) and 14-deoxy-11, 12-didehydroandrographolide (DDAG) (Fig. 1.1).

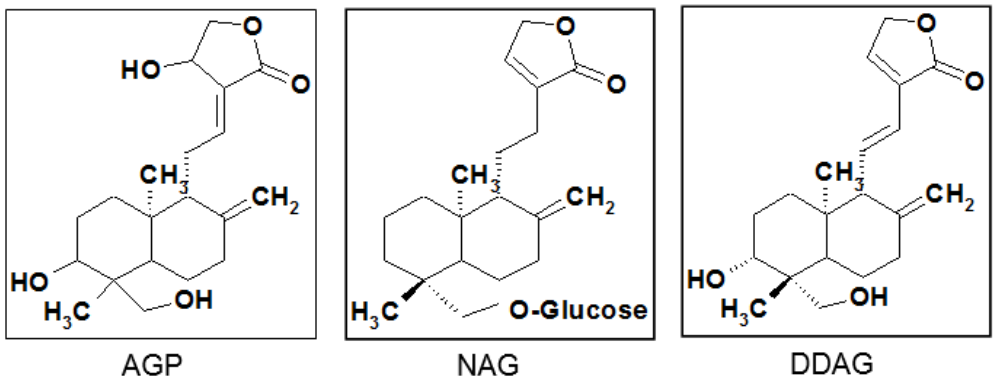

Figure 1.1: Major diterpenoids found in *Andrographis paniculata.*

AGP is the most abundant diterpene lactone and is mainly responsible for its bitter taste (Mishra et al. 2007). AP and AGP have many pharmacological properties, out of which anti-inflammatory and anti-oxidant properties play important roles in in many disorders (Lee et al. 2011; Sheeja et al. 2006).

Experimental studies have revealed that plant could enhanced cognition traditionally. In this regard, AP, which is bestowed with many biological properties, including anti-inflammation and anti-oxidative properties is expected to exert a similar effect on the brain since AGP was shown to distribute into rat brain (Zheng 1982). See Fig. 1.2 for concept map.

Hence, this study aimed to evaluate an aqueous extract of AP in *in vivo* and *in vitro* models to establish its neuroprotective role by maintenance and improve neuroplasticity via its anti-inflammatory and anti-oxidative mechanisms, expected to enhance cognition.

In addition, natural compounds from AP were also evaluated in the same *in vitro* models. To date, one natural product that has been extensively studied for its neuro-protective effects against free radical as well as improving learning and memory is "*Ginkgo biloba*" (EGb761®). However, EGB761 (GB), marketed, as "Tanakan" is a highly purified, standardised pharmaceutical grade *Ginkgo biloba* extract obtained through a patented manufacturing process in France that generates the quality and concentration of its content. Thus making it not only expensive but also difficult to prepare in high quantities.

The concept map (Fig. 1.2), illustrate that factors such as neuronal death, resulting from stimuli (neuro-inflammation, neurotoxicity, oxidative stress) and altered neuro transmitter function could affect synaptic plasticity which consecutively will lead to cognitive decline and memory loss. Conversely, it is expected that treatment with APAE could prevent this neuronal death by inhibition of neuroinflammatory and oxidation processes to improve integrity of neuroplasticity as well as enhancing learning and memory.

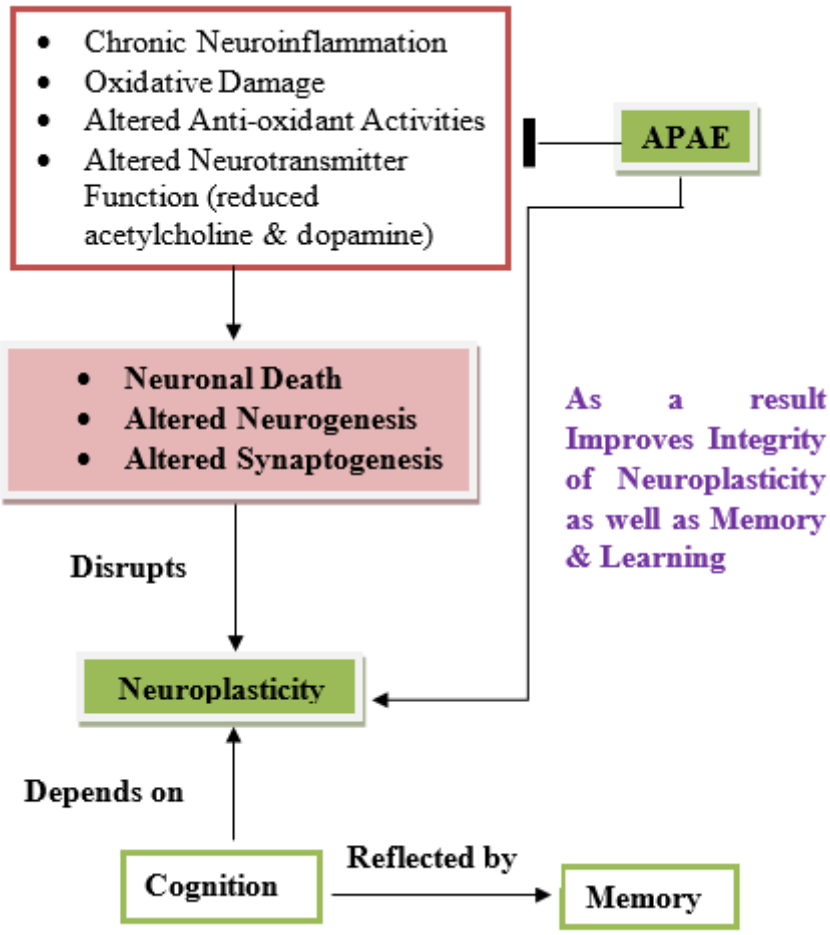

Figure 1.2: Concept map of research.

1.2 Statement of the problem

- The incidence of aging and other non-age related memory decline are rapidly increasing in both developed and developing countries thus forming an increasing clinical problem and public burden.
- It is projected that the number of people worldwide with cognitive impairment to reach 81 million by 2040.
- There is rapid growth of aged population in Malaysia which is anticipated to greatly influence occurance of memory impairment in the country
- Most memory impairments are associated with increased neuroinflammation and oxidative stress
- At present, the only available agent that has shown some improvement in cognition decline is GB. However, it is very expensive and not affordable by most. As such, a cheaper alternative with better efficacy is urgently needed.

1.3 Justification for the Study

- Neuroinflammation remain an unresolved issue despite extensive studies on inflammatory process. Thus, owing to the anti-inflammatory property of AP, it can be an agent for improvement of memory loss due to neuroinflammation and aging related memory decline.
- Despite the increasing studies on AP or AGP, their potential application as neuroprotective agents and cognitive enhancer has not been previously reported as can be ascertained from the survey of scientific literature.
- In order to prevent neurodegeneration and curtail its burden on individuals health/healthcare cost, it is imperative to clinically develop novel cost effective therapies for prevention and treatment of neuro-inflammatory conditions
- There is still paucity in the concept of neuroprotection for memory loss therapy. Thus, to date, there is no definitive medicine used clinically to prevent or cure cognitive impairment, leading to normal people to resort to taking herbal supplements for improving memory.

1.4 Hypothesis

- H_{01}- hypothesis —APAE do not improve memory in LPS-induced animal model of neuroinflammation and cognitive impairment.
- H_{A1}- hypothesis — APAE improve memory in LPS-induced animal model of neuroinflammation and cognitive impairment.
- H_{02}- hypothesis — APAE and its major phytochemicals (AGP, NAG and DDAG) do not ensure neuroprotection in an *in vitro* model of lipopolysaccharide (LPS) – induced neurotoxicity.
- H_{A2}- hypothesis — APAE and its major phytochemicals (AGP, NAG and DDAG) ensure neuroprotection in *in vitro* models of LPS – induced neurotoxicity.

1.5 Objectives

1.5.1 General Objective

To determine the potential of a standardised *Andrographis paniculata* aqueous extract (APAE) in preventing and treating memory loss *via* its neuro-protective properties.

1.5.2 Specific objectives

- To prepare a standardised APAE.
- To determine the memory enhancing potential of standardised APAE in LPS-induced animal model of neuroinflammation.
- To assess APAE effect based on histopathological changes and on the expression level of inflammatory and oxidative stress markers in the brain tissue sections of LPS-induced neuroinflammation model.
- To investigate the NMR metabolic profile associated with anti-neuroinflammation property of APAE *via* analysis of urine samples of rat model of LPS-induced neuroinflammation to determine potential biomarkers of anti-neuroinflammation of APAE
- To determine the *in vitro* neuroprotective effect of APAE and its major phytochemicals *via* analysis of inflammatory and oxidative stress mediators as well as to evaluate the blood-brain barrier penetrability of the major phytochemicals found in APAE.

1.6 Research outlook

This research thesis is summarised into six main interconnected parts (Fig. 1.3). First, an extraction method for preparation of a standardised APAE involving drying and heating technique. The second part, an *in vivo* study involving animal model of cognitive impairment induced by a bacterial toxin, lipopolysaccharide (LPS) to mimic neuro-inflammation and subsequently neuronal death. Pre and post-treatment with a standardised APAE was to prevent the neuro-inflammatory processes and Morris Water Maze test was to assess the changes in cognitive function in the rats following the LPS-induction. The third part examines the effect of a standardised APAE on the expression of antioxidant enzymes and pro-inflammatory genes associated with neuro-inflammation induced rats.

The fourth aspect involves a metabolomics study, to evaluate urine metabolomics in LPS-induced rat model of neuro-inflammation using ^{1}H NMR-based metabolomics approach to identify urinary metabolites that discriminate between control, LPS and APAE + LPS rats providing further insights into the effect of AP on inflammation and oxidative stress processes within the brain. In the fifth part, the neuro-protective potential of the standardized APAE and AGP was evaluated in an LPS-induced model of microglial (BV-2) and dopaminergic (N27) cell lines. The final part involves *in vitro* blood-brain barrier penetrability determination of major phytochemicals in APAE.

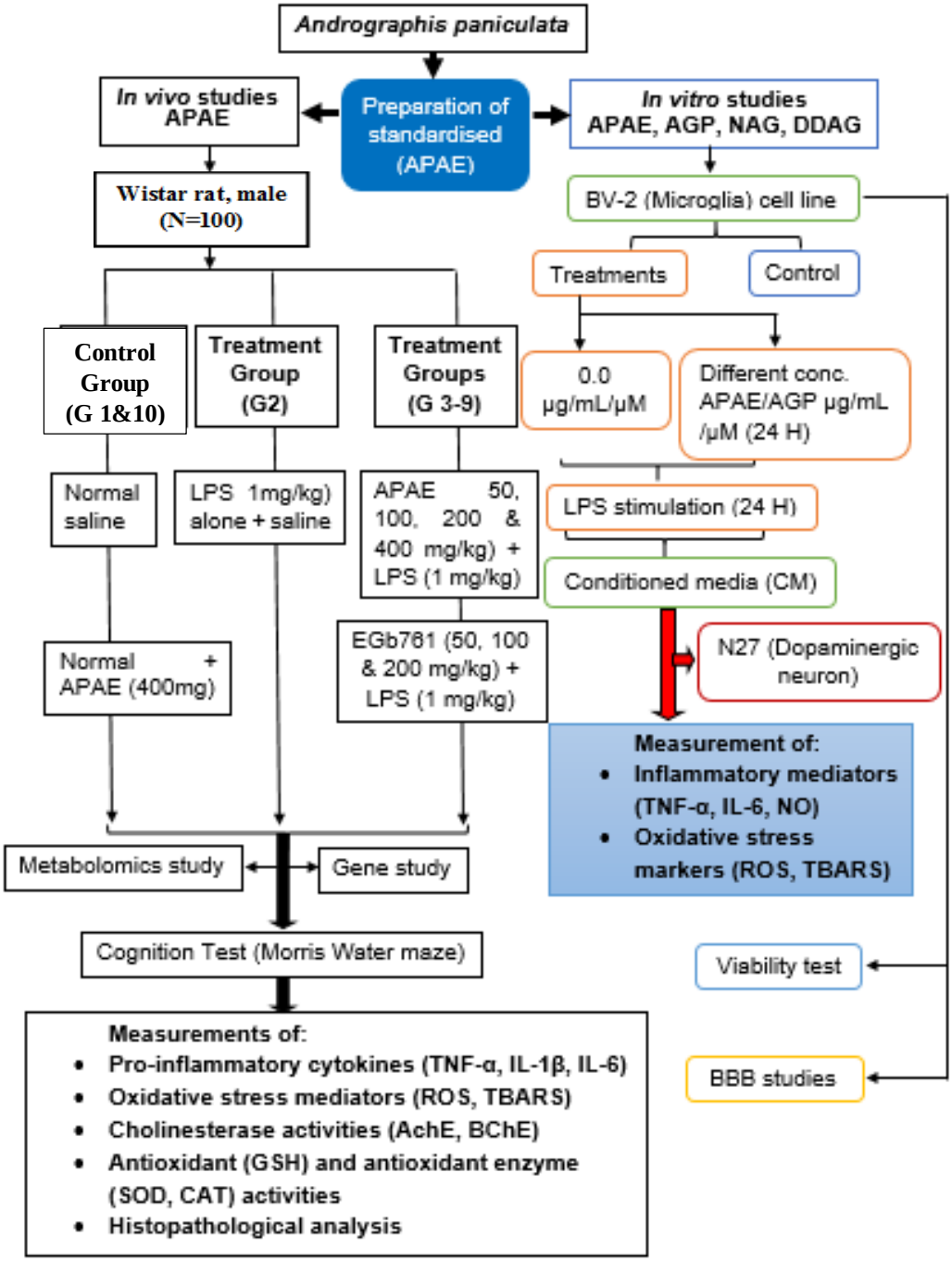

Figure 1.3: Research outlook.

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APPENDICES

Appendix 1: Ethical approval letter for animal studies

UPM
UNIVERSITI PUTRA MALAYSIA
BERILMU BERBAKTI

PEJABAT TIMBALAN NAIB CANSELOR (PENYELIDIKAN DAN INOVASI)
OFFICE OF THE DEPUTY VICE CHANCELLOR (RESEARCH AND INNOVATION)

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

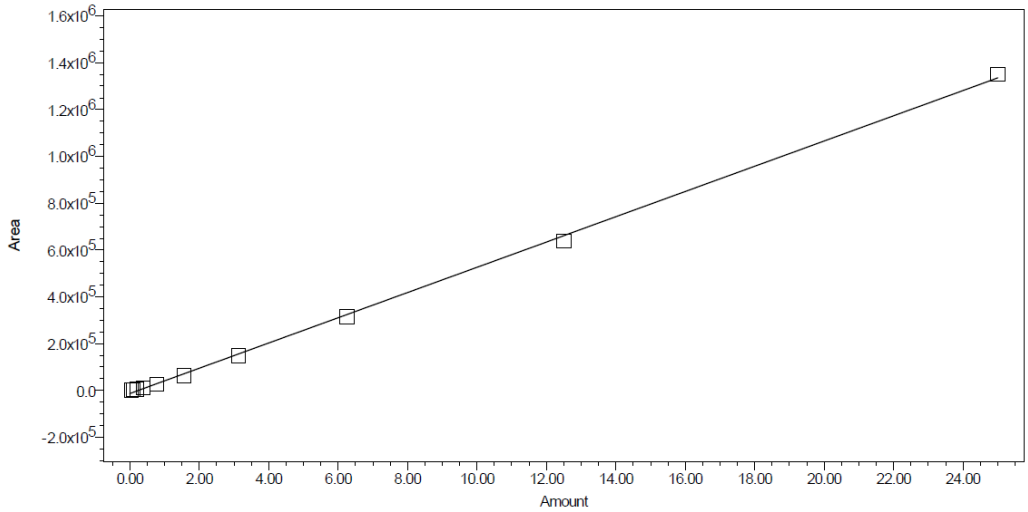
Date:	14 March 2014
Ref.:	UPM/IACUC/AUP- R046/2013
Project Title:	Potential of a standardized aqueous extract of *Andrographis paniculata*, its natural and semisynthetic compounds, as neuroprotective agents in male Wistar rats.
Principal Investigator:	Prof. Dr Johnson Stanslas
Associates:	Dr Md. Shariful Hasan Sumon, Dr. Sreenivasa Rao Sagineedu, Mohd. Al-Saufreen Bin Akhiruddin
Student:	Dr Dahiru Sani
Committee Decision:	The committee has reviewed and approved the proposed animal utilization protocol
AUP No.:	R046/2013
Project Classification:	Chronic
Category of Invasiveness:	B
Source of Animals:	Takrif Bistari Enterprise, No 5 Jalan 1/4, Taman Kembangsari, Seri Kembangan, 43300 Seri Kembangan, Selangor.
Number of Animals Approved:	140 rats
Accommodation:	Animal House, Faculty of Medicine and Health Sciences, UPM
Duration:	1 April, 2014 – 31 March, 2015

(Prof. Dr. Mohd Hair Bejo)
Chairman,
Institutional Animal Care and Use Committee
Universiti Putra Malaysia

Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
Pejabat Timbalan Naib Canselor (P&I) 603-8947 1293 603-8945 1646, Pejabat Pentadbiran TNCPI 603-8947 1608 603-8945 1673,
Pejabat Pengarah, Pusat Pengurusan Penyelidikan (RMC) 603-8947 1601 603-8945 1596, Pejabat Pengarah, Putra Science Park (PSP)
603-8947 1291 603-8946 4121 http://www.tncpi.upm.edu.my

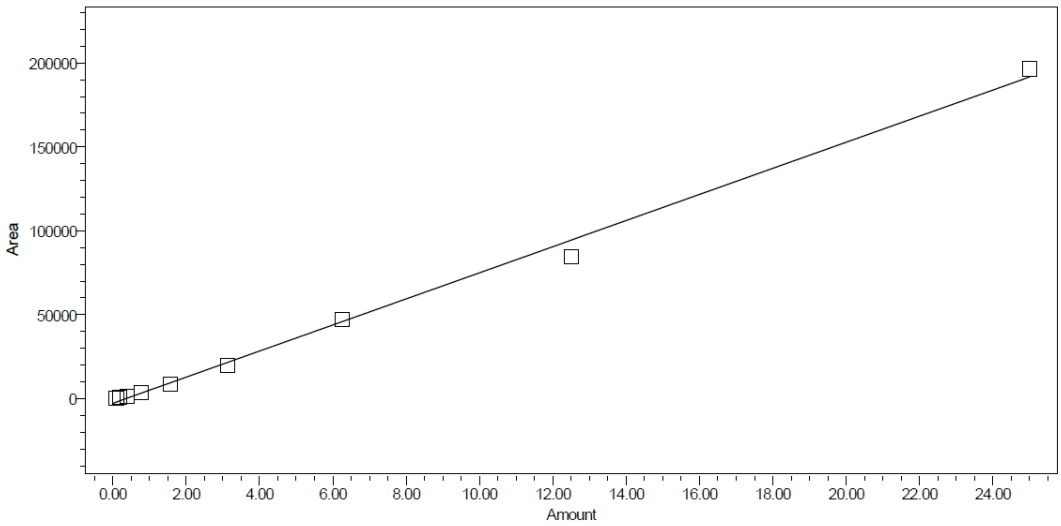
Appendix 2. Phytochemical Analysis by HPLC

Calibration curve of AGP



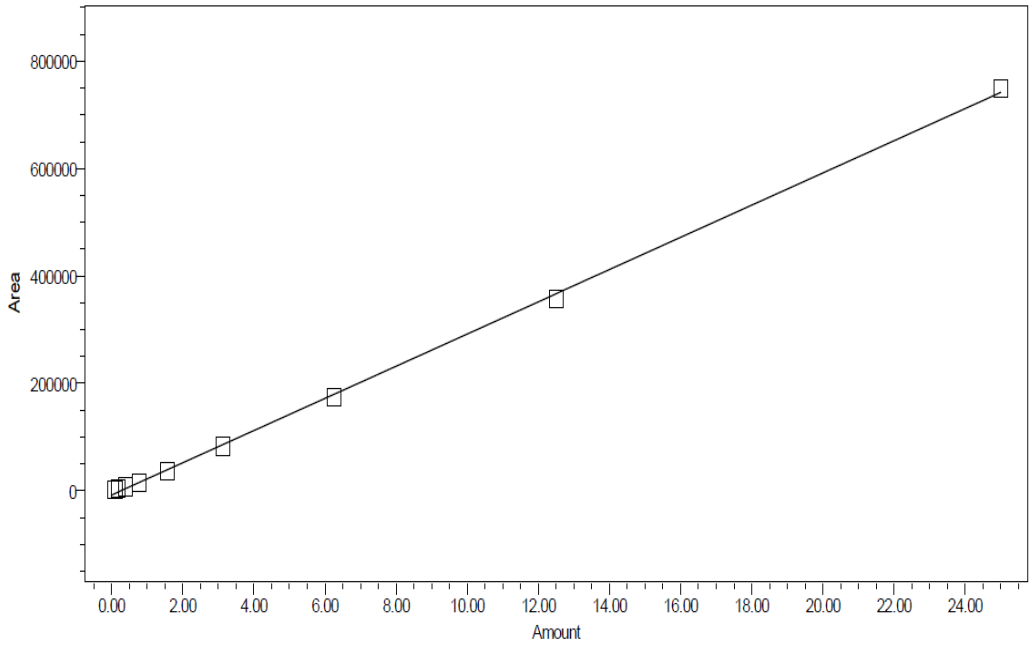
Peak Name: AGP; RT: 3.290; Fit Type: Linear (1st Order); Cal Curve Id: 3992; R: 0.999641; R^2: 0.999283; Weighting: None; Equation: Y = 5.39e+004 X - 1.33e+004; Normalized Intercept/Slope: -0.019680; RSD(E): 4.818792

Calibration curve of NAG



Peak Name: NAG; RT: 5.729; Fit Type: Linear (1st Order); Cal Curve Id: 3993; R: 0.997943; R^2: 0.995890; Weighting: None; Equation: Y = 7.78e+003 X - 2.79e+003; Normalized Intercept/Slope: -0.028636; RSD(E): 11.047645

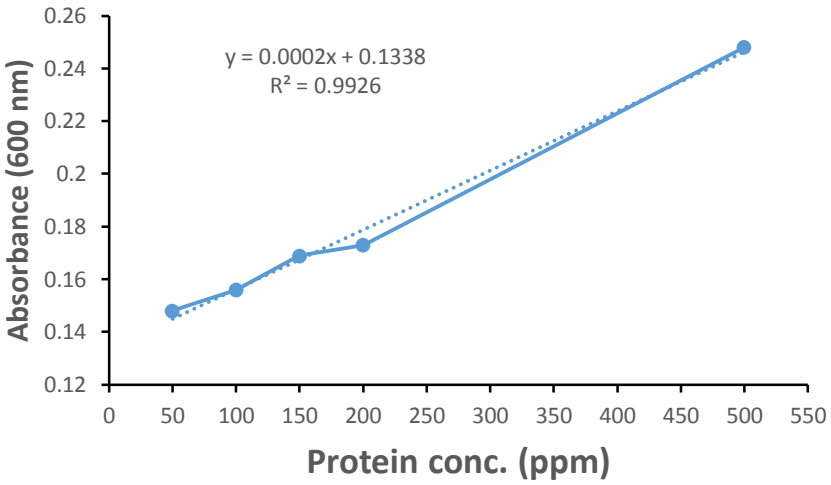
Calibration curve of DDAG



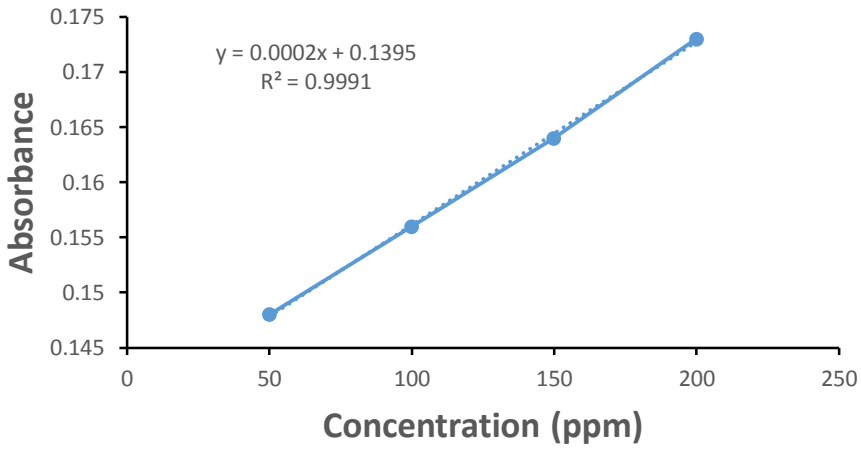
Peak Name: DDAG; RT: 10.659; Fit Type: Linear (1st Order); Cal Curve Id: 3994; R: 0.999728; R^2: 0.999457; Weighting: None; Equation: Y = 3.00e+004 X - 8.12e+003; Normalized Intercept/Slope: -0.021571; RSD(E): 3.942851

Appendix 3. Primary metabolites content of APAE

Calibration curve of protein content in the APAE

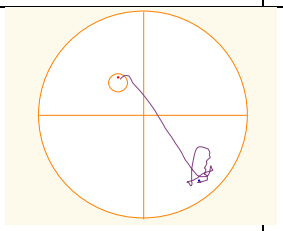
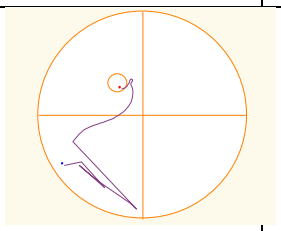
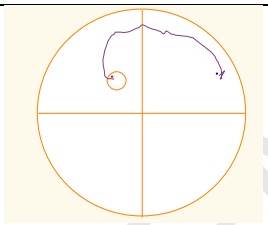
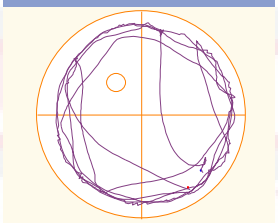
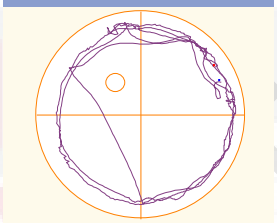
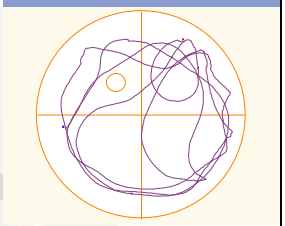
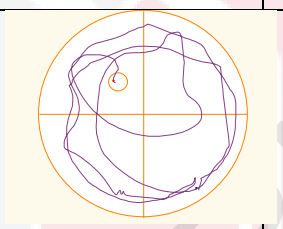
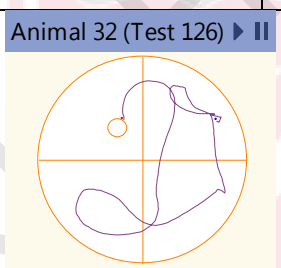
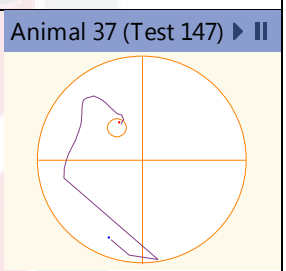
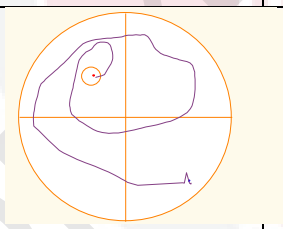
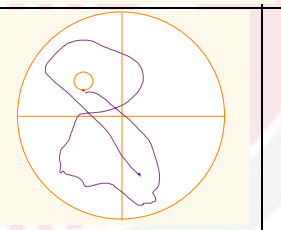
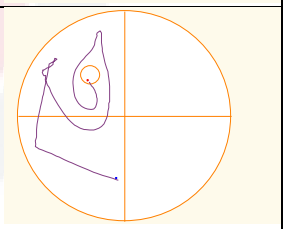
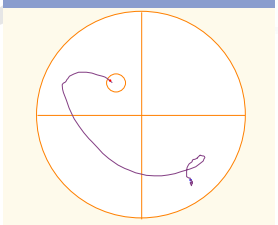
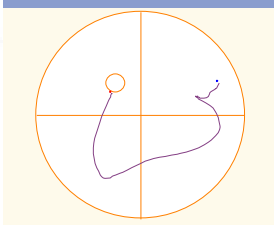
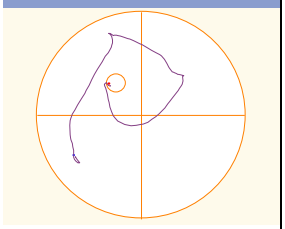
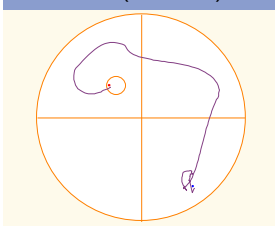
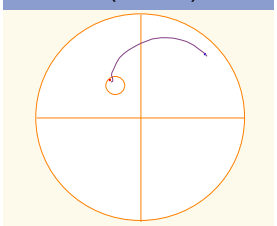
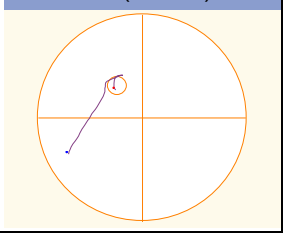


Calibration curve of glucose content in the APAE



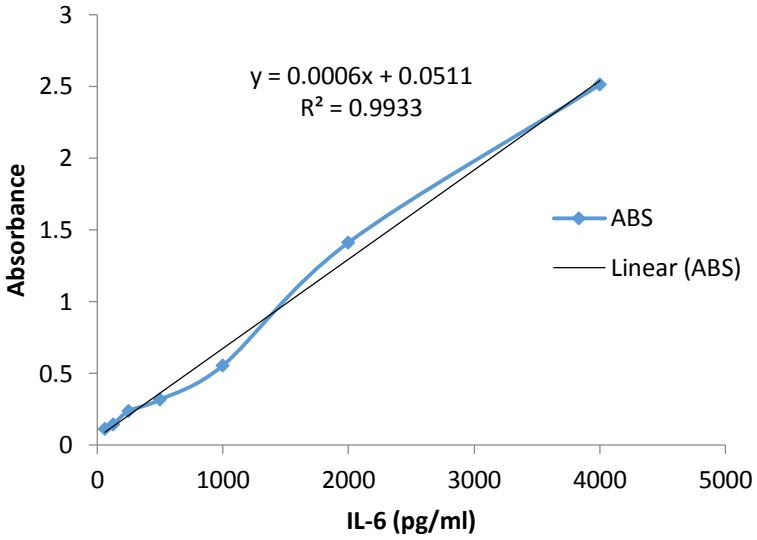
Appendix 4. Morris Water Maze Test

Track Plot Hidden Platform (D2H1)

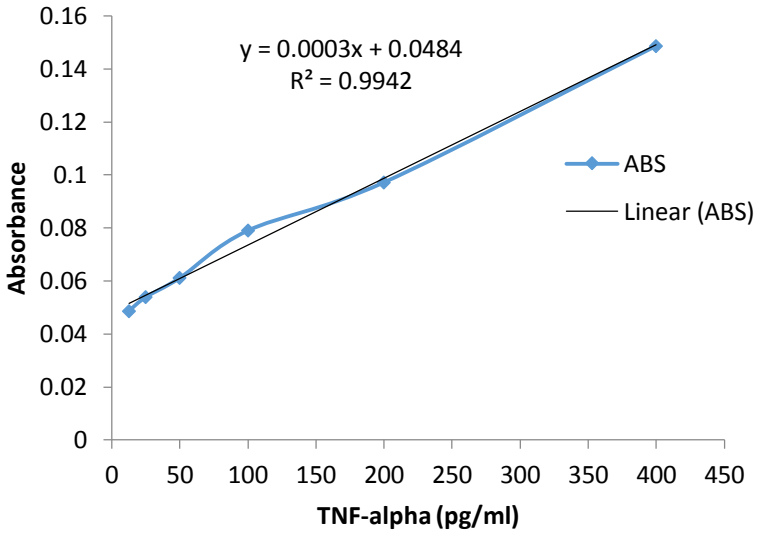
Groups			
Normal Control			
LPS 1mg/kg	Animal 17 (Test 65) 	Animal 17 (Test 66) 	Animal 17 (Test 67) 
APAE 200 mg/kg + LPS 1mg/kg		Animal 32 (Test 126) 	Animal 37 (Test 147) 
Ginkgo 200 mg/kg + LPS 1mg/kg			
APAE 400 mg/kg + LPS 1mg/kg	Animal 2 (Test 5) 	Animal 3 (Test 10) 	Animal 3 (Test 11) 
Control + APAE 400 mg/kg	Animal 28 (Test 109) 	Animal 6 (Test 22) 	Animal 16 (Test 63) 

Appendix 5. Inflammatory markers

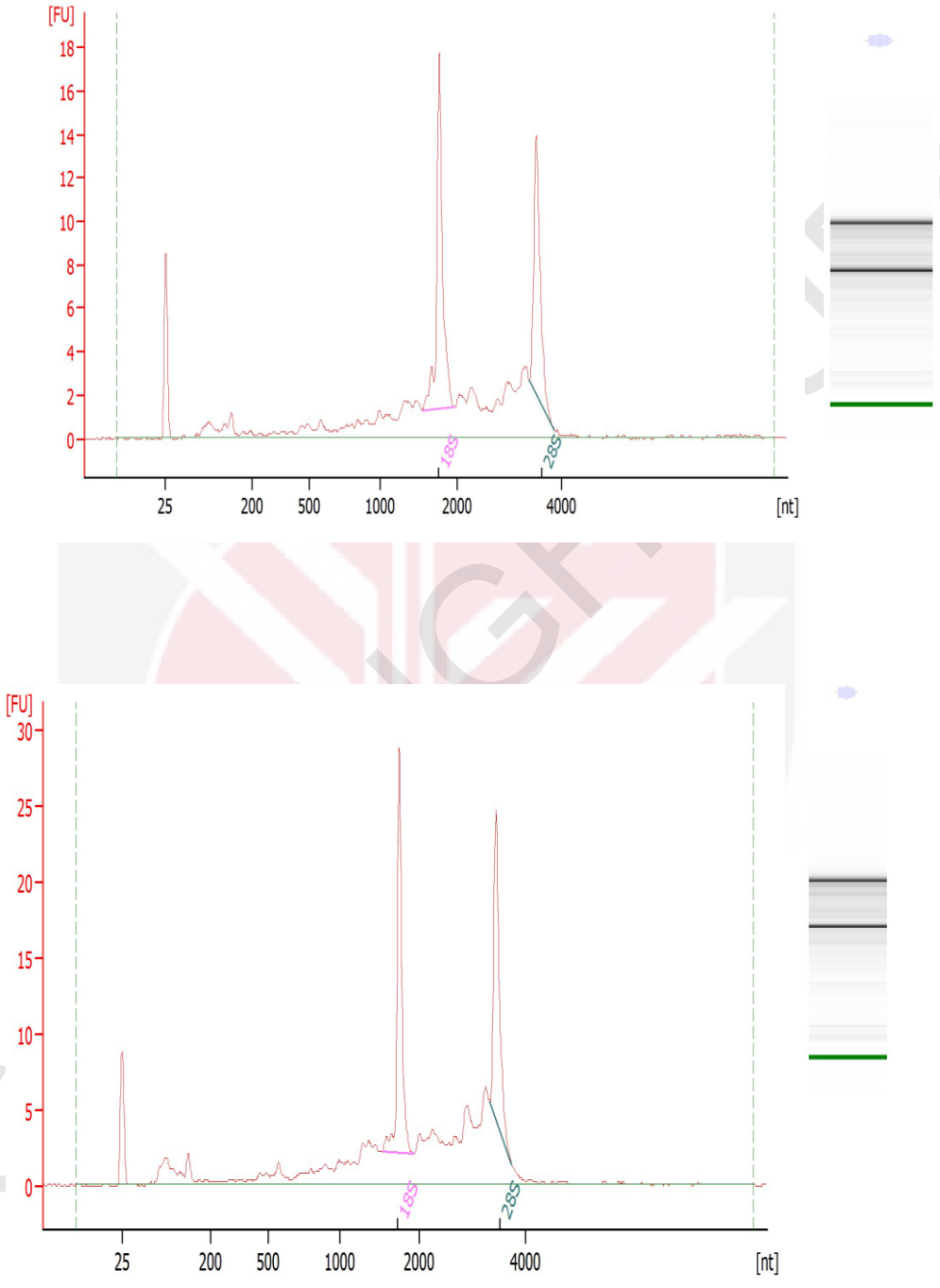
Calibration curve of IL-6 concentration



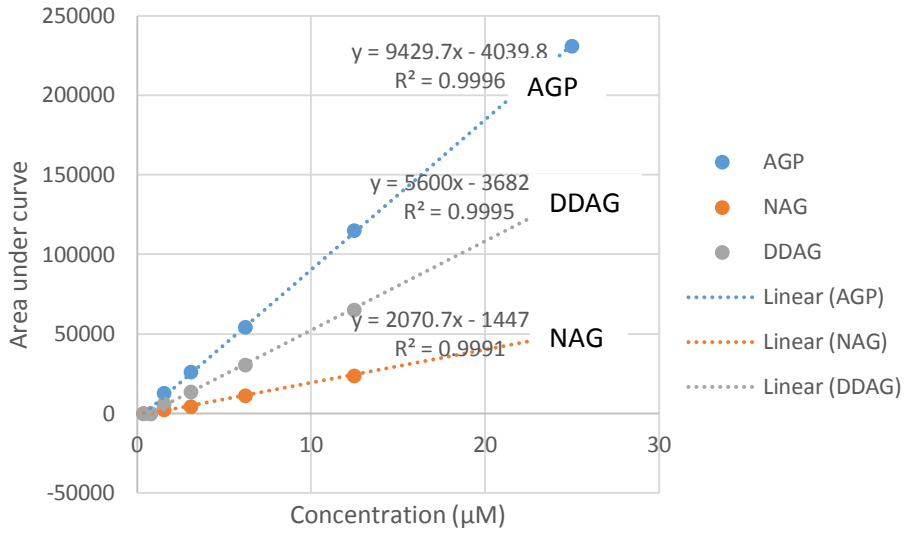
Calibration curve of TNF-α concentration



Appendix 6. Electropherogram and gel electrophoresis showing RNA integrity

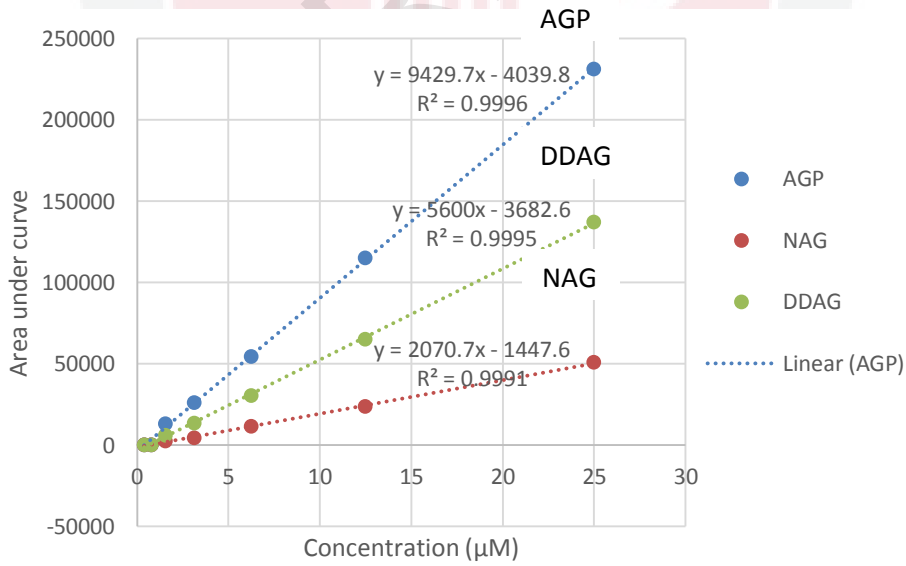


Appendix 7. Calibration curve of AGP, NAG and DDAG spiked in media at different concentration for compound penetration through Blood Brain Barrier.



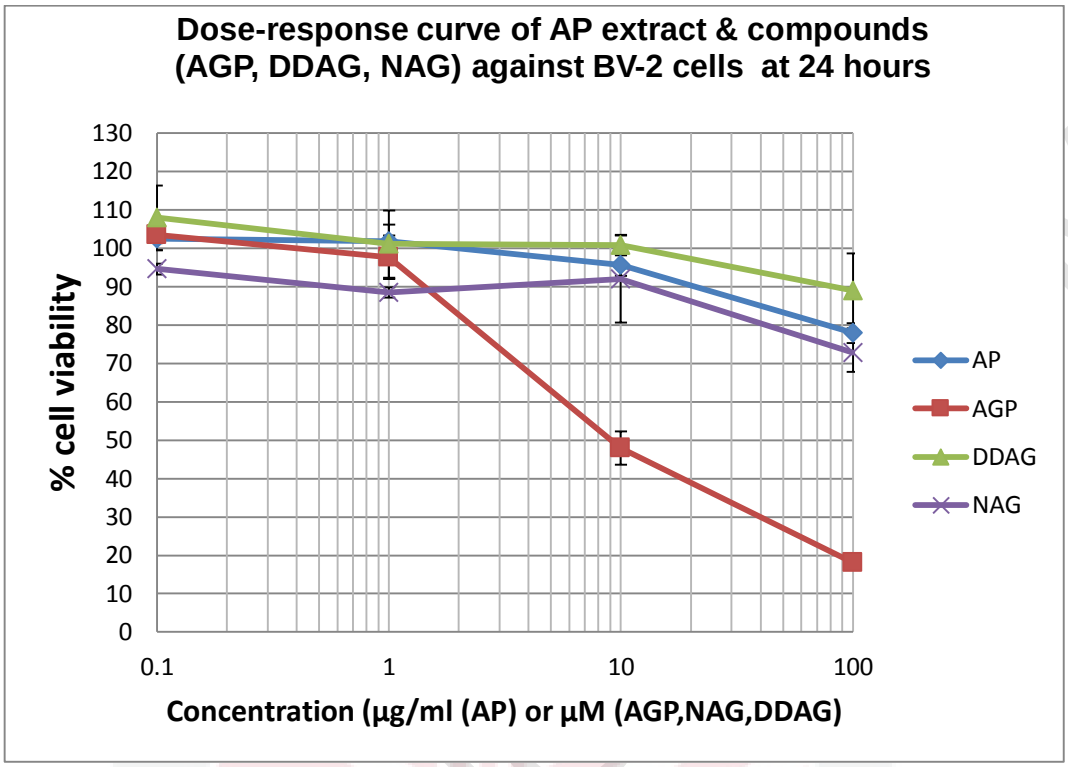
For 10 µg/ml AP extract

For 100 µg/ml AP extract.

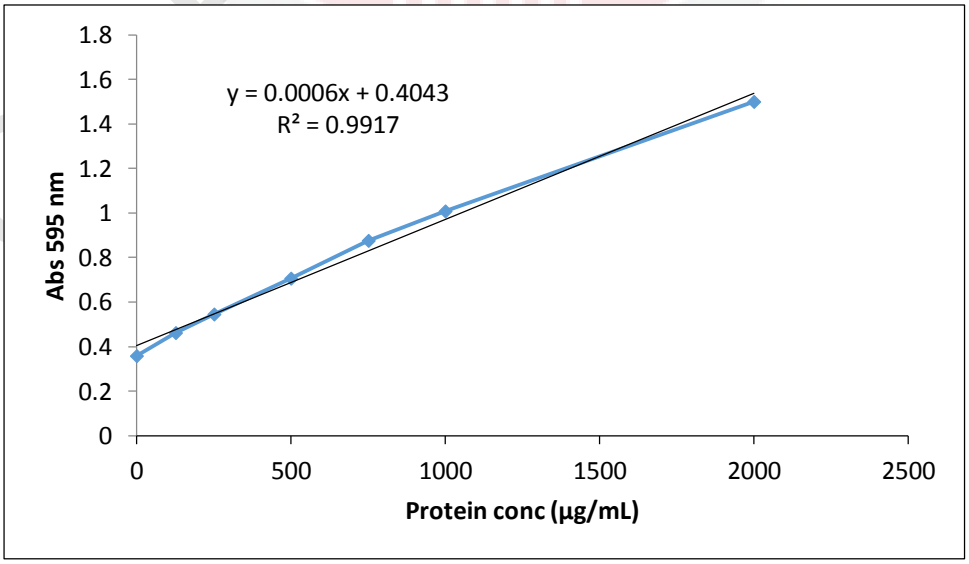


Calibration curve of AGP, NAG and DDAG spiked in media at different concentrations showing the R^2 value and equation

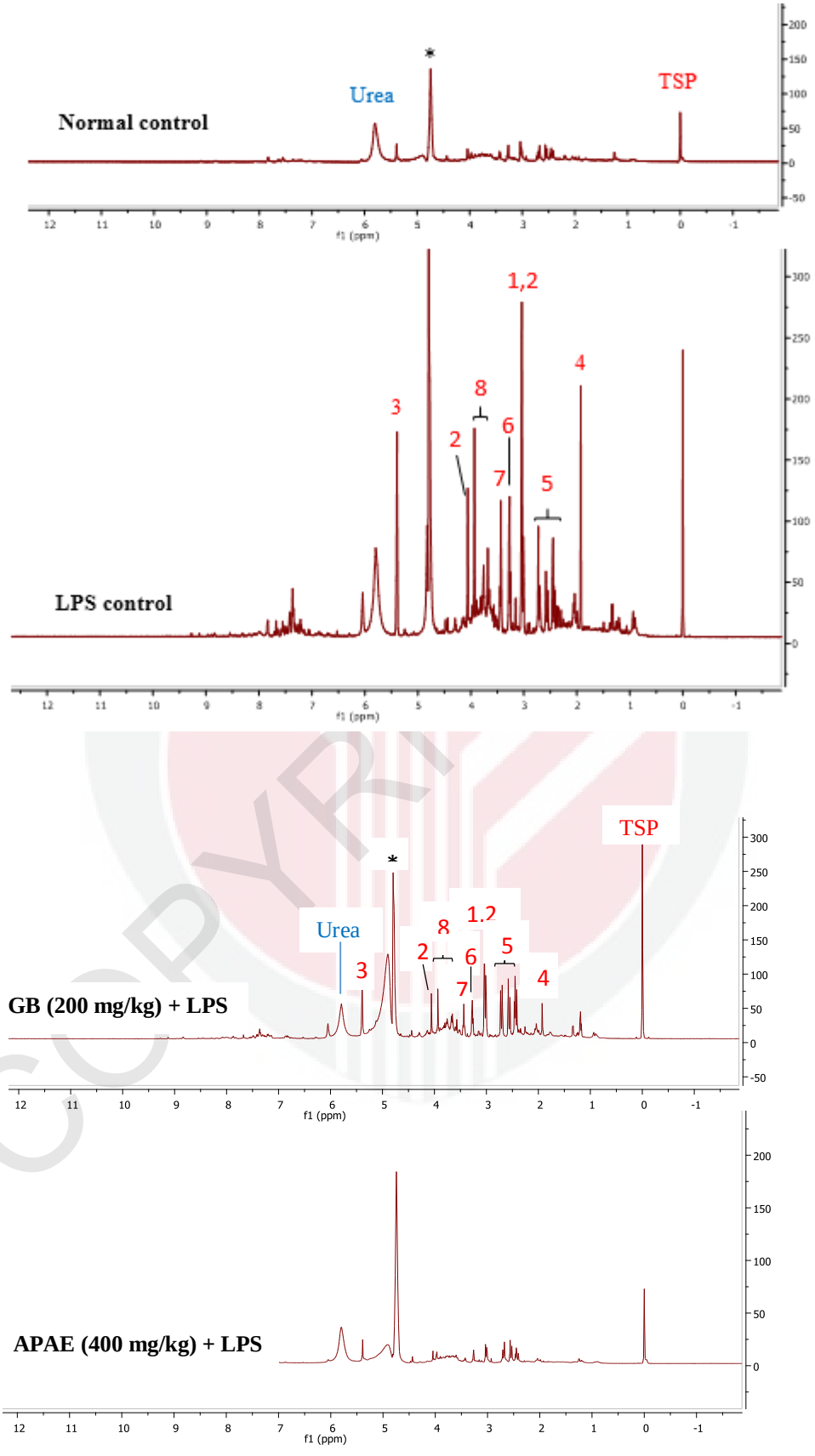
Appendix 8. Dose-response curve of AP extract & compounds (AGP, DDAG, NAG) against BV-2 cells at 24 hours

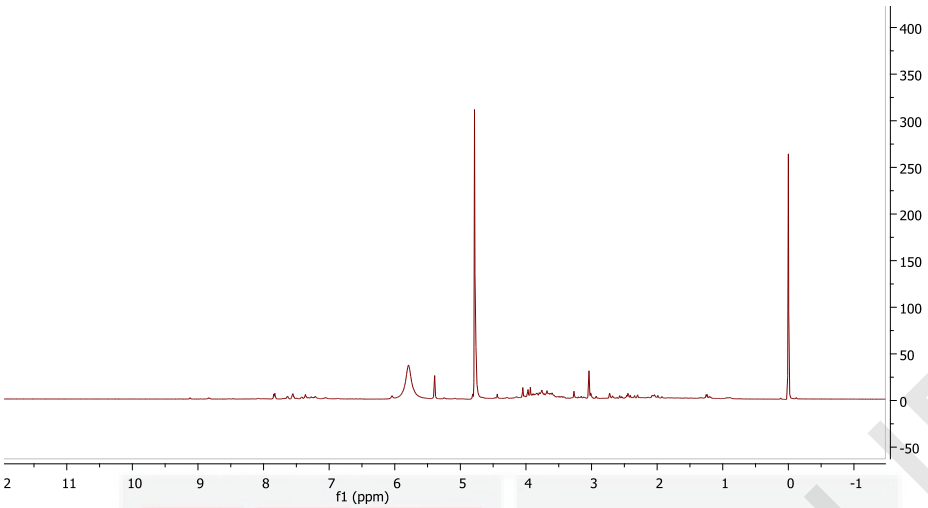


Appendix 9: Calibration curve of protein concentration



Appendix 10: NMR spectra showing both X and Y axes





Appendix 11: Photographs of AP plot

AP plot at Field 2 of Universiti Putra Malaysia, Serdang, Selangor.

Height of plant of 36 cm normally seen at week 12-14.

Well growing plants on field plot (6-7 weeks).

LIST OF PUBLICATIONS

Sim Ling Tan, Johnson Stanslas, Mahiran Basri, Abedi Karjiban R.A., Brian P. Kirby, **Dahiru Sani** and Hamidon Bin Basri (2015). Nanoemulsion-based Parenteral Drug Delivery System of Carbamazepine: Preparation, Characterization, Stability Evaluation and Blood-Brain Pharmacokinetics. *Current Drug Delivery*, 12(6), pp.795-804

Conferences

Experimental Biology, 2014. San Diego conference centre, CA, USA.

ASPET, 2014. Graduate student best abstract award competition. San Diego marriott marquis and marina hotel, CA, USA.

Malaysian society of neuroscience, 20-22nd June, 2014. Berjaya Time square, Kuala Lumpur. MY

Patent

Extract of *andrographis paniculata* for cognitive enhancement. No. PI 2016701854. File reference: UPM/100-45/2 (A) JS1

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