EFFECTS OF SELECTED MALAYSIAN HERBS ON HEALTHY AGEING

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EFFECTS OF SELECTED MALAYSIAN HERBS ON HEALTHY AGEING

ABSTRACT

Oxidative damage caused by free radicals plays an important role in accelerating ageing and age-related diseases including neurodegenerative diseases, inflammation (rheumatoid arthritis), cardiovascular disorder, immunosenescence and cancer. The life expectancy of the world is rising with an estimated 29% of the world population reaching age 60 by 2025. This would lead to an increase in the number of people acquiring age-related chronic diseases which would reduce the quality of life and further burden society and government. The free radicals can be quenched by endogenous antioxidant enzymes or exogenous antioxidants. Herbal antioxidants have potentially multifunctional targets in quenching free radicals and protecting health. Malaysia is home to many medicinal plants and herbs which have been used traditionally for the treatment of diseases. They however lack scientific evidence proving the acclaimed benefits. In this research, herbs with a long history of traditional use were investigated for their potential as agents for preventive healthcare potentially via their antioxidative properties. *Kesum* (*Polygonum minus*) was established as very high in antioxidant properties with acetylcholinesterase inhibitory activities - the enzyme target for neurological drug therapies. The *Kesum* extract was also confirmed to possess compounds which can permeate cells thus protecting the cell from oxidative damage from within. The extract was able to attenuate scopolamine-induced memory deficit *in vivo* at a dosage of 100 mg/kg body weight. Kesum also has anti-inflammatory properties inhibiting lipoxygenase and cyclooxygenase enzyme. The reduction of paw oedema *in vivo* in just 4 hours, in addition, potentially places the plant as a remedy for arthritis. The *Misai Kuching* (*Orthosiphon stamineus*) which is known to be highly antioxidative was found to possess adenosine 2 receptors antagonist activity which is

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also a target for drug therapy against neurological diseases. This was further qualified *in vivo* whereby a dose of at least 300 mg/kg body weight of extract improved learning and memory. The anti-ageing and antioxidant property of *Tongkat Ali* (*Eurycoma longifolia*) has also been elucidated. Additionally, the immunomodulatory effects were evaluated as immunosenescence is a major contribution to morbidity and mortality among the aged. The *Tongkat Ali* was able to improve the immune state by increasing the number of naive T-cells, lymphocyte and reducing the immunological age which is a patented scoring system which measures T cell proliferation activity and number, which is normally higher in younger persons. *Tongkat Ali* in combination with *Kesum,* improved the mens' quality of life, sexual and physical wellbeing. It was also able to improve testosterone levels which reduce with age and environmental stress. Therefore, the supplementation of antioxidant herbs with its multifunctional pharmacological targets are able to sequester free radicals that cause oxidative stress, thus proving to be agents for preventive healthcare and alternative therapy in healthy ageing as evidenced in this research programme.

Keywords: oxidative stress, antioxidant, herbs, healthy ageing, healthcare

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KESAN HERBA MALAYSIA TERPILIH KE ATAS PENUAAN SIHAT

ABSTRAK

Kerosakan oksidatif yang disebabkan oleh radikal bebas memainkan peranan dalam meningkatkan kadar penuaan dan penyakit yang dihubungkait dengan penuaan seperti neurodegenerasi, inflamasi (artritis reumatoid), gangguan kardiovaskular, penuaanimmun dan kanser. Jangka hayat manusia di dunia semakin meningkat dengan jangkaan 29% populasi dunia akan mencapai umur 60 tahun pada 2025. Ini akan mengakibatkan pertambahan bilangan manusia yang menghidap penyakit kronik yang berkaitan dengan usia, yang mana akan mengurangkan kualiti kehidupan dan meningkatkan bebanan masyarakat dan kerajaan. Radikal bebas dapat dikurangkan dengan enzim antioksidan ataupun antioksidan eksogenus. Antioksidan herba berpotensi mempunyai sasaran bermultifungsi dalam mengurangkan radikal bebas dan melindungi kesihatan. Negara Malaysia mempunyai banyak tumbuhan ubatan dan herba yang bukan sahaja tinggi dengan antioksidan malah digunakan secara tradisional untuk merawat penyakit. Walaubagaimanapun, masih terdapat kekurangan bukti saintifik. Dalam penyelidikan ini, beberapa herba yang telah digunakan secara turun-temurun telah diuji potensinya sebagai agen pencegahan penyakit menerusi keupayaan antioksidannya. Kesum (*Polygonum minus*) telah terbukti mempunyai nilai antioksidan yang tinggi dengan aktiviti penindasan asetilkolinesterase-enzim yang menjadi sasaran terapi dadah neurologi. Ekstrak Kesum juga disahkan mengandungi sebatian yang dapat menembusi sel hidup lalu memberi perlindungan kepada sel daripada kerosakan oksidatif. Ekstrak tersebut dapat mengatasi secara *in vivo* pengurangan daya ingatan yang dihasilkan oleh scopolamin pada dos 100 mg/kg berat badan. Kesum juga mempunyai ciri antiinflamasi menindas enzim *lipoxygenase* dan *cycloxygenase*. Pengurangan edema kaki *in vivo* dalam tempoh 4 jam, tambahan pula, menjadikan Kesum sebagai agen yang berpotensi

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sebagai penawar bagi artritis. Misai Kuching (*Orthosiphon stamineus*) pula yang diketahui beroksidatif tinggi, didapati mempunyai aktiviti antagonis reseptor adenosine 1 dan 2 yang mana merupakan sasaran terapi bagi penyakit neurologi. Ini telah terbukti secara *in vivo* dimana dos ekstrak sekurang-kurangnya 300 mg/kg berat badan dapat meningkatkan daya pembelajaran dan ingatan. Ciri anti-penuaan dan antioksidan Tongkat Ali (*Eurycoma longifolia*) juga telahpun terbukti. Sebagai tambahan, kesan imunomodulator diuji memandangkan degenerasi imun adalah penyumbang utama morbiditi dan mortaliti di kalangan orang tua. Tongkat Ali berupaya meningkatkan tahap imun dengan meningkatkan bilangan sel T naif, limfosit dan mengurangkan usia immunological yang berasaskan kadar penghasilan sel T dan bilangannya pada biasanya lebih tinggi di kalangan orang muda. Tongkat Ali apabila digabungkan dengan Kesum, dapat meningkatkan kualiti kehidupan, kesihatan fizikal dan seksual lelaki. Ia juga didapati boleh memperbaiki tahap testosterone yang berkurangan dengan usia dan tekanan persekitaran. Oleh itu, suplimentasi herba antioksidan multifungsi berupaya mengurangkan radikal bebas penyebab tekanan oksidatif lalu membuktikan ia sebagai agen bagi pencegahan penyakit dan terapi alternatif bagi penuaan sihat seperti yang terbukti dalam program penyelidikan ini.

Kata kunci: tekanan oksidatif, antioksidan, herba, penuaan sihat, penjagaan kesihatan

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LIST OF SYMBOLS AND ABBREVIATIONS

ºC : Degree Celsius

K_D : Dissociation Constant

$<$: Less Than

V_c : Mean OedemaVolume Measured in the Control Group

V_t : Mean Oedema Volume Measured in the Test Group

$>$: More Than

% : Percentage

® : Registered Trademark

B_{max} : Total Density (Concentration) of Receptors

™ : Trademark

A1R : Adenosine A_1 Receptor

A2AR : Adenosine A_{2A} Receptor

AA : Arachidonic Acid

AAPH : 2,2” Azobis 2-Amininopropane Hydrochloride

AChE : Acetylcholinesterase

AD : Alzheimer’s Disease

ADHD : Attention Deficit Hyperactivity Disorder

AE : Adverse Events

ALP : Alkaline Phosphatase

ALT : Alanine Aminotransferase

AMS : Ageing Males Symptom

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ANCOVA	: Analysis of Covariance
ANOVA	: Analysis of Variance
AST	: Aspartate Transaminase
BAI	: Beck Anxiety Disorder
BDI	: Beck Depression Inventory
BHA	: Butylated Hydroxyanisole
BHT	: Butylated Hydroxytoluene
BMI	: Body Mass Index
BUN	: Blood Urea Nitrogen
cAMP	: Cyclic Adenosine Monophosphate
CAP-e	: Cellular Antioxidant Protection of Erythrocytes
CAT	: Catalase
CBC	: Complete Blood Count
cGMP	: Cyclic Guanosine Monophosphate
CHA	: N6-cyclohexyl Adenosine
CMC	: Carboxymethyl Cellulose
CMP	: Complete Metabolic Panel
CNS	: Central Nervous System
COX	: Cyclooxygenase
CPCSEA	: Committee for Purpose of Control and Supervision of Experiments on Animals
DCF-DA	: Dichlorofluorescein Diacetate
DER	: Drug-Extract-Ratio

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DHEA	: Dehydroepiandrosterone
DMSO	: Dimethyl Sulfoxide
DNA	: Deoxyribonucleic Acid
DPBS	: Dulbecco's Phosphate-Buffered Saline
DPPH	: 2,2-Diphenyl-1-Picrylhydrazyl
DTNB	: 5,5' –Dithio-bis-(2-Nitrobenzoic Acid)
EDITS	: Erectile Dysfunction Inventory for Treatment Satisfaction
EDTA	: Ethylenediaminetetraacetic Acid
EGFR	: Estimated Glomerular Filtration Rate
EHS	: Erection Hardness Score
EKG	: Electrocardiogram
F	: *F*-value
FRAP	: Ferric Reducing Ability of Plasma
g/L	: Gram per Litre
GAE	: Gallic Acid Equivalent
GFR	: Glomerular Filtration Rate
GI	: Gastrointestinal
GPCR	: G-Protein-Coupled Receptor
GSH-Px	: Glutathione Peroxidase
H_2O_2	: Hydrogen Peroxide
HAT	: H-Atom Transfer
HDL	: High Density Lipoprotein
HEK-293	: Human Embryonic Kidney Cells 293
HORAC	: Hydroxyl Radical Antioxidant Capacity

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HPLC : High Performance Liquid Chromatography

HTRF : Homogeneous Time Resolved Fluorescence

IAEC : Institutional Animal Ethics Committee

IC_{50} : Inhibitory Concentration at 50% of Maximum Inhibition

IFN : Interferon

IG : Immunological Grade

IIEF : International Index of Erection Function

IL : Interleukin

iNOS : inducible Nitric Oxide Synthase

INR : International Normalized Ratio

IRB : Institutional Review Board

IT-TOFMS : Ion Trap Time of Flight Mass Spectrometer

LC-MS-MS : Liquid Chromatography-Mass Spectrometry-Mass Spectrometry

LDL : Low Density Lipoprotein

LOX : Lipooxygenase

LT : Leukotrienes

$MgCl_2$: Magnesium Chloride

MDA : Malondialdehyde

µg/ml : Microgram per Millilitre

µM : Micromolar

µmole/g : Micromole per Gram

mg/ml: : Milligram per Millilitre

mmHg : Millimetre of Mercury

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mM : Millimolar

MMSQ : Multi Modal Stress Questionnaire

MTT : 3-[4,5-dimethylthiazol-2-yl]-2,5 Diphenyl Tetrazolium Bromide

NACLAR : National Advisory Committee for Laboratory Animal Research

NADPH : Nicotinamide Adenine Dinucleotide Phosphate

ng/dL : Nanogram per Decilitre

NF-ƙB : Nuclear Factor Kappa B

NGDA : Nordihydroguaiaretic Acid

NK cells : Natural Killer Cells

NOAEL : No Observed Adverse Effect Level

NORAC : Peroxynitrite Radical Absorbance Capacity

NOS : Nitric Oxide Synthase

NSAID : Non Steroidal Anti-Inflammatory Drug

$O_2^{\cdot -}$: Superoxide Anion

$\cdot OH$: Hydroxyl Radical

ORAC : Oxygen Radical Absorbance Capacity

$ORAC_{FN}$: Oxygen Radical Absorbance Capacity Food and Nutrition

P : *P*- value

PD : Parkinson Disease

PDA : Photo Diode Array

PDE-5 : Phosphodiesterase-5

PDE-Is : Phosphodiesterase Inhibitors

PG : Prostaglandin

PGT : Propyl Gallate

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PLA2	: Phospholipase A2
pmole/mg	: Picomole/Miligram
POMS	: Profile of Mood State
PUFA	: Polyunsaturated Fatty Acids
PVC	: Polyvinyl Chloride
QoL SF-36	: Quality of Life Short Form-36
QoL	: Quality of Life
RACK-1	: Receptor of Activated Protein C Kinase1
RCT	: Randomized Controlled Trial
RI	: Recognition Index
ROS	: Reactive Oxidative Stress
RT	: Retention Time
SD	: Sprague Dawley
SEAR	: Self-Esteem and Relationship
SEM	: Standard Error Mean
SET	: Single Electron Transfer
SHIM	: Sexual Health Inventory for Men
SIA	: Sexual Intercourse Attempt
SIV	: Scores of Immunological Vigour
SOD	: Superoxide Dismutase
sPLA2	: Phospholipases A_2
SRT	: Social Recognition Test
T:E ratio	: Testosterone to Epitestosterone
TA	: Tongkat Ali

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TC : Total Cholesterol

TCPI : T cell Proliferative Index

TE µmole/g : Trolox Equivalent Micromole/Gram

TEPC : Arachidonic Thioester Phosphatidylcholine

THA : Tetrahydroaminoacridine

TNF-α : Tumor Necrosis Factor-α

Tri-HCL : Tris-Hydrochloride

Tukey 's HSD : Tukey 's Honest Significant Difference

UA : Urinalysis

UFLC : Ultra Fast Liquid Chromatography

UT : Untreated Cells

WA : Wistar Albino

WHO : World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 Oxidative stress and ageing

Ageing in organisms is a progressive decline involving physical function, loss of homeostasis and increased susceptibility to diseases (Ho *et al*., 2010). It is a result of many factors including genetic variations, environmental risk factors, nutritional factors and lifestyle (Harman, 1998). Many theories have been proposed to explain ageing (Armbrecht, 2001; Biesalski, 2002; Finkel & Holbrook, 2000; Sohal *et al*., 2002; Balaban *et al*., 2005). Among these, the free radical theory of ageing by Harman (1956) has gained universal acceptance and is supported by the fact that production of free radicals and free radical damage increases with age leading to a progressive accumulation of cellular damages including DNA, protein, membrane and lipid whereby the damage is termed as "oxidative stress" (Sohal & Weindruch, 1996).

Reactive oxygen species (ROS) is defined as oxygen-containing molecules that are more reactive than triplet state oxygen in the air (Noguchi & Niki, 1999). Generally ROS are the form of reactive free radicals containing one or more unpaired electron (Halliwell *et al*., 1995). Reactive oxygen species (ROS) are produced by the human body to carry out physiological functions (Halliwell *et al.,* 1995). However, oxidative stress caused by the imbalance between excessive formation of ROS and limited antioxidant defences is said to be involved in the pathology of degenerative diseases and conditions such as atherosclerosis, cancer, rheumatoid arthritis, neurodegeneration, ageing, immunosenescence and inflammation (Pappas, 1998; Rahman, 2003). The human body deals with the pathological effects of ROS by utilising endogenous antioxidant system (eg. enzymes such as superoxide dismutase and glutathione peroxidase) and by the ingestion of exogenous antioxidants in the diet (eg. flavonoids, vitamins and minerals) (Rahman, 2007). Plant antioxidants such as carotenoids and

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plant polyphenols (flavonoids, phenolic acids, cathechins, coumarins, tannins and anthocyanins) are commonly consumed as part of food composition and can act as a protective defence mechanism against oxidative damage from ROS (Pandey & Rizvi, 2009). As an example, the antioxidant tocopherols found in oil palm, cereal grains and kale have the ability to scavenge free radicals (Watkins *et al.*, 1999; Niki, 1996) and have been associated with the reduction of heart disease, delay of Alzheimer's disease and prevention of cancer (Meydani, 2000). Similarly, carotenoids such as lycopene found in tomatoes and β-carotene in carrots inhibit the formation of oxidized products of LDL cholesterol, which are associated with coronary heart disease (Weisburger, 1999).

The World Health Organization reported that the percentage of the world's population of people over 60 years of age will double from 11 to 22% between 2000 and 2050 (WHO, 2014) which is 605 million to 2 billion people. According to WHO, the low- and middle-income countries such as Malaysia will experience the most rapid and dramatic demographic change and more people will live to see their 80s or 90s than ever before. The increase in ageing population is associated with acquiring age-related chronic disease of the cardiovascular, brain and immune systems which can cause a loss of autonomy, increased dependence, high social costs and a greater financial burden on individuals and the health services worldwide. Furthermore, use of drugs to treat diseases associated with ageing is costly; it often comes with side effects. Drugs can have adverse effects on any part of the gastrointestinal (GI) tract from mouth to colon (Makins & Ballinger, 2003). Eventually, this leads to malnutrition as a result of poor absorption of nutrients. Hence, steps towards healthy ageing with increased longevity and a decrease in number of elderly depending on high-tech and expensive medicine, are fast gaining momentum. With healthy ageing, one can have a better quality of life

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(QOL) and financial health while ensuring that the typical symptoms of ageing are either delayed or kept to a manageable minimum.

Medicinal plants have been consumed for centuries as an alternative therapy and in preventive healthcare and have played an important role in the development and progress of many modern drugs, directly and indirectly (Parvathy *et al.*, 2014). A systematic review of the efficacy and safety of anti-ageing herbs in animals and humans, categorised the mechanism of the tested herbs into four categories: antioxidant and immunomodulatory; memory, cognition and mood; sex hormones balance and anti-inflammation (Hasani-Ranjbar *et al.*, 2012). Herbal therapies most commonly used are the *Gingko biloba* for neurodegeneration, St. Johns's Wort for depression, Saw Palmetto for benign prostrate hyperplasia, Kava for short term anxiety and Echinaceae for upper respiratory tract infection (Ernst, 2002). Wolfberry (*Lycium barbarum*), which is a food ingredient used in Asian cooking recipe and is high in antioxidants, is reported to be multi-functional and can protect the body through different mechanisms (Ho *et al.,* 2010).

In Malaysia, several popular herbs have been traditionally used to treat and prevent diseases or eaten as food such as *Kesum* (*Polygonum minus*)*, Tongkat Ali* (*Eurycoma longifolia*) and *Misai Kuching* (*Orthosiphon stamineus*) also known as java tea. These plants have been reported to be high in antioxidants (Huda-Faujan *et al.*, 2009; Maizura *et al.*, 2010; Sumazian *et al.*, 2010; Qader *et al.*, 2010; Urones *et al.*, 1990; Christapher *et al.*, 2013; Akowuah *et al.*, 2004; Yam *et al.*, 2007). The *Polygonum minus* Huds. is from the family Polygonaceae and is commonly referred to as *Kesum* or *laksa* leaf in Malaysia. It is used as a flavouring ingredient in culinary dishes and also consumed as an *ulam* (salad) for preventive healthcare (Jaganath & Teik, 2000; Vimala *et al.*, 2011). The leaves of the herb are traditionally consumed as a decoction to treat indigestion, a tonic for after childbirth and for good eyesight (Vimala

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et al., 2011) and a treatment for dandruff (Wan Hassan & Mustaffa, 2007). The herb is superior in antioxidants compared to other popular herbs such as the *Ulam Raja* (*Cosmos caudatus), Selom* (*Oenanthe javanicus*)*, Pegaga* (*Centella asiatica*)*,* curry leaves (*Murraya koenigii*) (Huda-Faujan *et al.,* 2009), ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*) (Maizura *et al.,* 2010) and possess 98.3% of lipid peroxidation inhibitory activities (Vimala *et al.,* 2011). Phytochemical screening of *P. minus* has shown the presence of flavonoids, flavones, cathecin, epicatechin gallate and terpenoids (Urones *et al.,* 1990). Flavonoid and polyphenols have long been studied for their strong antioxidant capacities, and their ability to scavenge reactive oxygen species thus preventing ageing and oxidative stress-related diseases (Gutteridge, 1993; Hartman *et al.*, 2006). Studies also show that flavonoids have an effect on memory, cognition and against neurodenegeration and the ability to improve cerebrovascular blood flow (Spenser, 2009). Potential anti-inflammatory effects of *P. minus* have been reported (Wasman *et al*., 2010; Christapher *et al*., 2015) and is further elaborated in literature review.

Another popular local plant, *Misai Kuching* (*Orthosiphon stamineus)* is traditionally used in South East Asia for a variety of ailments such as bladder and kidney disease, relieving joint stiffness and inflammation including arthritis and rheumatism, diabetes mellitus and has been reported to possess antioxidant activity (Jaganath & Teik, 2000; Yuliana *et al.*, 2009; Wan Hassan & Mustaffa, 2006). More than a hundred compounds classified as flavonoids, monterpenes, diterpenes, triterpenes, saponins, sterols organic acids, caffeic acids derivatives, chromenes, and oleanic and ursolic acid have been identified in *O. stamineus* (Hossain & Ismail, 2013; Malterud *et al*., 1989; Masuda *et al*., 1992; Olah *et al*., 2003; Sumaryono *et al*., 1991). Terpenoids from natural products such as *G. biloba* and Asian ginseng (*Panax ginseng*) have been investigated as potential therapeutics in Alzheimer's disease (Yoo & Park,

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2012). A study on the adenosine receptor 1 (A1R) antagonist effects, a target for new drug development for the treatment of cognition related impairments, has been reported, presenting *O. stamineus's* possible protective effect on cognition (Yuliana *et al*., 2009).

The *Tongkat Ali* (*Eurycoma longifolia*) root have been used in indigenous traditional medicine for its unique anti-malarial, anti-pyretic, anti-ulcer, cytotoxic, energy tonic and aphrodisiac properties (Gimlette & Thomson, 1977; Jaganath & Ng, 2000; Ismail *et al.*, 1999; Perry & Metzger, 1980) and is termed the cure for a thousand diseases in Vietnam (Goreja, 2004). It has been reported to possess antioxidant properties (Christapher *et al*., 2016). It has been clinically reported to increase serum testosterone levels in aged or hypogonadic subjects thus modulating healthy hormonal levels (Tambi *et al.*, 2012; Henkel *et al*., 2014) which resulted in improvements in strength (Hamzah & Yusof, 2003), mood (Talbott *et al.,* 2013), symptoms of ageing, quality of life (Tambi *et al.*, 2012) and erectile function (Ismail *et al*., 2012). Modulation of natural killer cells, a function of immunity, in an endurance running trial among recreational athletes was also reported (Muhamad *et al*., 2015).

A study by Hong *et al*. (2017) demonstrated a traditional Japanese herbal medicine (Kampo) containing a combination of ten herbs to have a synergistic effect in improving memory accompanied by an improvement in antioxidant enzymes such as superoxidase dismutase in prefrontal cortex implying protection from free radicals as a consequence of ageing. Plant antioxidants could therefore act as agents in scavenging ROS which eventually leads to healthy ageing.

1.2 Problem statement

Healthy-ageing is not only prolonging lifespan but also increasing health span, which emphasizes more on the quality of life. This is the concept of healthy ageing and prevention of pathological ageing, associated with diseases which are sometimes

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contributed by oxidative stress. Medicinal herbs have been traditionally used for intervention or prevention of age-associated diseases. However, there is lack of scientific evidence on Malaysian herbs in that regard.

1.3 Objectives

This research is intended to investigate local Malaysian herbs with antioxidant activity for ability to alleviate age-related diseases involving cognition, inflammation, immunity, overall quality of life and liver health and the possible pharmacological mode of action. *Kesum* (*Polygonum minus*), *Misai Kuching* (*Orthosiphon stamineus*) and *Tongkat Ali* (*Eurycoma longifolia*) are antioxidant herbs with a long history of use as food and medicinal tonic/tea and have been selected in that order to be scientifically substantiated for prevention or slowing down age-related disease. The objectives of the study are therefore:

1. to investigate the cell-based antioxidative effect of *Polygonum minus* and memory enhancing effects of *Orthosiphon stamineus* and *Polygonum minus*.
2. to determine the antioxidative protection of *Polygonum minus* against inflammation.
3. to evaluate the antioxidative protection of *Eurycoma longifolia* in combination of *Polygonum minus* for hormonal balance and improving the quality of life.
4. to determine the antioxidative effect of *Eurycoma longifolia* in modulating and improving immunity.

1.4 Hypothesis

It is suggested that some herbs which are traditionally used as a tonic and "ulam" in Malaysia are rich in antioxidants, may be pharmacologically active and have

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preventive healthcare properties which can be potential candidates for healthy ageing and prevention of age-associated diseases and limitations. Herbs may have phytochemicals other than antioxidants to prevent degeneration. The hypothesises tested are listed below:

1. The antioxidative properties of *P. minus* and *O. stamineus* are able to exert a protective and restorative effect on cognition, one of the parameters associated with oxidative stress.
2. The antioxidative properties of *P. minus* are able to exert a protective and preventive effect on inflammation one of the parameters associated with oxidative stress.
3. The antioxidative effect of *E. longifolia* in combination with *P. minus* is able to modulate healthy hormonal levels and improve quality of life.
4. The antioxidative effect of *E. longifolia* is able to affect and improve the immunological state associated with oxidative stress related decline called immunosenescence.

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CHAPTER 2: LITERATURE REVIEW

2.1 The interaction between free radicals and antioxidants in maintaining cellular intergrity.

The free radicals that contribute to oxidative stress are molecules such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), singlet oxygen O^- and the hydroxyl radical ($^{\cdot}OH$) (Winterbourn, 2008). Reactive nitrogen, iron, copper and sulphur species also contribute to reactive oxygen stress (Riley, 1994; Halliwell *et al*., 1992).

Reactive oxygen species (ROS) function in normal physiological cell processes at low to moderate concentrations but at high concentrations, they produce adverse modifications to cell components (Ames, 2004). Sources of free radicals (a one electron reduction product) include Fenton reaction, Haber-Weiss reaction, NADPH oxidases, xanthine oxidase, cytochrome P450 and mitochondria (Radi *et al.,* 2001; Lipinski, 2011). Reactive oxygen species are also produced by living organisms as a result of normal cellular metabolism such as cyclooxygenation, lypooxygenation and lipid peroxidation (Shahidi & Zhong, 2010).

Free radicals attack biomolecules mainly the polyunsaturated fatty acids (PUFA) of the cell membrane in a process known as lipid peroxidation and are associated with tissue injuries and diseases (Esterbauer *et al.*, 1991). The hydroxyl radicals ($^{\cdot}OH$), generated from oxygen metabolism react with biomolecules such as DNAs, proteins and lipids. Oxidative damage to DNA is highly correlated to mutagenesis, carcinogenesis and ageing (Breen & Murphy, 1995). Malondialdehyde (MDA) is a major end product of lipid peroxidation (Devasagayam *et al.*, 2003; Negre-Salvayre *et al*., 2008). It cross-links with DNA causing significant cellular damage as a result of oxidative stress (Sivalokanathan *et al.*, 2006). Most oxidized proteins that are functionally inactive,

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formed as part of the normal regulatory process as a defence mechanism against oxidative stress are rapidly removed. But some gradually accumulates with time contributing to damage associated with ageing and disease. Lipofuscin for example is an aggregate of peroxidised lipids and proteins which is known to accumulate in the lysosomes of aged cells (Stadman, 1992) and brain cells of patients with Alzheimer's disease (Perry *et al.*, 2002).

The enzymatic antioxidants SOD, catalytically converts $O_2^{\cdot -}$ into oxygen and H_2O_2 whereas CAT converts H_2O_2 to water and oxygen and gluthathione peroxidise (GSH-Px) converts H_2O_2 to water and peroxyredoxin catalyse the reduction of H_2O_2 thus preventing oxidative damage (Gough & Cotter, 2011; Cabiscol *et al.*, 2000). The non-enzymatic antioxidants are either natural such as polyphenols, carotenoids, vitamins A, C and E or synthetic such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PGT) (Wojcik *et al.*, 2010). Alleviation of symptoms associated with oxidative stress and ageing through regular consumption of antioxidants have been reported (Eastwood, 1999; Muellenbach *et al.*, 2008; Curtis *et al.*, 2010) either passively through dietary intake or stimulation of the intrinsic antioxidant machinery of the body and in some cases having a pharmacological activity (Anekonda & Reddy, 2005).

As reactive oxidative stress is required for normal physiological processes, a delicate balance between antioxidants and reactive oxygen speciesis necessary in preventing cell damage. The scientific validation and use of natural antioxidants with not only free radical scavenging ability but with additional pharmacological properties are limited among local herbs and plants though the traditional use of herbs in preventive heathcare has been documented (Gimlette & Thomson, 1977).

2.2 The case for antioxidative herbs as candidates for the improvement of cognition

The brains especially consume high levels of oxygen and at the same time produce high levels of free radicals (Prasad *et al.*, 1999). The brain is therefore particularly susceptible to oxidative stress and easily succumbs to premature ageing. Recent evidence has indicated that oxidative damage is a potential cause for Alzheimer's disease (Nunomura *et al.*, 2006). Subjects with dementia showed altered balance between oxidant and antioxidant levels (Sinclair *et al.*, 1998). Alzheimer's disease involves a chronic inflammatory response associated with brain injury and beta-amyloid formation in the brain. Beta amyloids are plaques formed in the brain as a result of lipid peroxidation and protein oxidation from oxidative stress (Butterfield *et al.*, 2013).

Apart from assessing reactive oxygen scavenging properties, several pharmacological targets can be used to additionally qualify the antioxidant herb for pro-cognitive effects. Inhibition of acetylcholinesterase is presently the most accepted and recognized therapeutic marker for the development of cognitive enhancers (Kim *et al.*, 2009). Acetylcholine is a neurotransmitter related to learning and memory (Blokland, 1995). It is metabolized by the enzyme acetylcholinesterase (AChE). The reduction in the activity of AChE correlates with increased lipid proxidation (mark of oxidative stress) and a decrease in Ferric Reducing Ability of Plasma (FRAP) values (the test for antioxidant levels) (Jha & Rivzi, 2009). AChE is found mainly at neuromuscular junctions and in chemical synapses of the cholinergic type (synapse between nerve cells and muscle cells) where its activity serves to terminate synaptic transmission by hydrolyzing acetylcholine. For a cholinergic neuron to receive impulse, ACh must be released from the ACh receptor. Screening for herbal plants with acetylcholinesterase inhibitory activity would open new possibilities for cognition-improving herbals.

Another receptor targeted in the screening for pro-cognitive effects are the adenosine receptors which have been associated with sleep and arousal, cognition, memory, protection from neuronal damage and neurodegeneration (Chen *et al.*, 2007). Endogenous adenosine is generally known to modulate cognition through the activation of adenosine A1 receptors (A1R). Evidence is now emerging on a possible role of adenosine A2 receptors (A2AR) in learning and memory (Gutteridge, 1993). Antagonist actions on adenosine receptors A1 and A2A receptors produce CNS-enhancing effects facilitating learning and memory *in vivo* (Takahashi *et al.*, 2008). They might also protect against memory dysfunction shown in experimental models of ageing such as Alzheimer's disease.

Oxidative stress in the brain has been reported to increase with daily Wi-Fi induced electromagnetic exposure (Celik *et al.*, 2016). Lipid peroxidation levels in the brain and liver were increased whereas the glutathione peroxidase activity, and vitamin A, vitamin E and β-carotene concentrations were decreased in the brain and liver. The study demonstrated that Wi-Fi-induced oxidative stress in the brain and liver of developing rats was the result of reduced GSH-Px and antioxidant vitamin concentrations. Vitamin E, an antioxidant has the ability to detoxify free radicals, such as hydroxyl, superoxide, and peroxyl radicals, into repairable and harmless radical forms. In a study where rats were subjected to chronic oxidative stress, the induced reduced memory in young rats which were equivalent to normal aged were reversed by vitamin E supplementation in the young and old rats in a water and radial maze model (Fukui *et al.*, 2002). The impaired memory was related to an onset of apoptosis in the hippocampus following stress. Vitamin E effectively blocks H_2O_2 formation and prevents its cytotoxic effects (Behl *et al.*, 1994).

The *P. minus* has been reported to be rich in vitamins such as carotenes, retinol equivalents and vitamin C, α-tocopherol (vitamin E) and minerals (Jaganath & Teik,

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2000; Ching & Mohamed, 2001) and is a potential source of passive antioxidants. The aqueous extract of whole plant of *P. minus* showed significant protection from ulcers in rats pretreated with *P. minus* and was shown to be equally effective as omeprazole (20 mg/kg) (Wasman *et al.*, 2010) linking it to not only gastroprotective but possibly anti-inflammatory benefits. Christapher *et al*. (2015) reported analgesic and anti-inflammatory activity in a carrageenan induced paw oedema *in vivo* whereby a reduction in pain and ulcer was observed. Web searches using Google Scholar and Pubmed applying *P. minus* and anti-inflammatory and *P. minus* with cognition, memory, neuro as keywords, the pharmacological effects of *P. minus in vivo* and clinical are illustrated in Table 2.1.

There is one clinical trial reported on *P. minus*. The study evaluated the effect of a multi-herb comprising of *Sireh* (*Piper betle*), *Turmeric* (*Curcuma longa*), *Pegaga* (*Centella asiatica*), curry leaf (*Murraya koenigii*), *Selasih* (*Ocimum basilicum*), *Kesum* (*P. minus*) and, *Ulam Raja* (*Cosmos caudatus*) in improving memory in a crossover-group trial, randomized, double-blind, placebo-controlled, on 20 healthy individuals aged 35-65 years (Udani, 2013). Supplementation with the multi-herb containing *P. minus* significantly improved cognitive function and mood. The antioxidant properties of *P. minus* have been demonstrated and the neuroprotective benefits alluded in the clinical trial of the multi-herb supplementation. However, reports on its anti-inflammatory effects (which are influenced by antioxidative states) are limited hence the mechanism of action is not known. The nootropic effects and mechanism of action of the singular herb are also not known.

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Table 2.1 Summary of pharmacological effects of *P. minus* relating to anti-inflammatory and cognition, demonstrated in *in vivo* and clinical studies.

Author (year)	Purpose/ Indication	Study Design	Dose	Main Results
Pre-clinical				
Wasman *et al*. (2010)	Gastroprotective (anti-ulcer activity) of *P. minus*.	Ethanol-induced gastric ulcer in rats.	Standard 20 mg/kg omeprazole. Rats were orally pre-treated with 250 and 500 mg/kg of *P. minus* extract.	Decreases in ulcer areas, reduction of oedema and leucocytes infiltration of the submucosal layer with *P. minus* aqueous extract.
George *et al*. (2014)	Immunomodulatory effects of *P. minus*	*In vivo* carbon clearance assay.	200 mg/kg and 400 mg/kg b.w; standard drug Levamisole at 2.5 mg/kg body weight.	Dose dependent increase in Phagocytic Index with *P. minus*.
Christapher *et al.* (2015)	Anti-inflammatory and analgesic activity of *P. minus*.	Analgesic activity in formalin test, acetic acid-induced writhing, tail immersion test; anti-inflammatory activity in carrageenan induced paw oedema.	100 mg/kg and 200 mg/kg body weight.	Significant analgesic effect against acetic acid writing, tail immersion and formalin induced pain methods, but the effect was not equivalent to that of standard. Aqueous extract showed significant anti-inflammatory action.
Clinical				
Udani (2013)	Improvement of concentration and mood with *P. minus*.	Randomized; double-blind; placebo-controlled; crossover study.	One tablet/d containing extracts of: *sireh* (*Piper betle*) 150 mg, turmeric (*Curcuma longa*) 50 mg, *pegaga* (*Centella asiatica*) 100 mg, curry leaf (*Murraya koenigii*) 50 mg, *selasih* (*Ocimum basilicum*) leaf 50 mg, *Kesum* (*Polygonum minus*) 150 mg and *Ulam Raja* (*Cosmos caudatus*) 25 mg for duration of one day.	Significant improvement in executive functioning, cognitive flexibility, reaction time, and working memory ($p<0.05$). Significant decreases in tension, depression, and anger ($p<0.05$) in POMS.

POMS=Profile of Mood Scores

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There are several antioxidant assays. The Oxygen Radical Absorbance Capacity (ORAC) was identified as one of the best standardized antioxidant assay for the natural products industry in measuring antioxidant levels in nutritional and natural products (Ou *et al.*, 2006; Ou *et al.*, 2001; Prior *et al.*, 2003). The ORAC unlike the DPPH which uses artificial free radical and methanol as solvent is performed using a biologically relevant free radical, the peroxyl radical, at a physiological pH which is closest to biological conditions. Further explanation of ORAC is provided in Publication No. 1 on page 28. The Amazonian fruit acai (*Euterpe oleracea* Mart.) has been found to contain compounds with potent anti-inflammatory and with antioxidant properties (Kang *et al.*, 2012). The cell-based antioxidant protection in erythrocytes (CAP-e) bioassay was employed on the isolated antioxidant compounds, including luteolin, quercetin, and dihydrokaempferol demonstrating that they had the capacity to enter live cells and protect them from oxidative damage. The CAP-e assay, is a cell-based anti-oxidant protection assay using erythrocytes to address the question of whether antioxidants in complex natural products enter the cytosol and contribute to the reduction of oxidative damage within the cell (Honzel *et al.*, 2008). In a randomized, double-blind, placebo-controlled, crossover study, significant reductions in lipid peroxidation and increased free radical scavenging *in vivo* were observed when healthy participants age 19–52 years were fed an acai pulp–rich juice (Jensen *et al.*, 2008). Consumption of a single acute dose of 4 ounces of an acai-rich juice resulted in a rapid increase in antioxidant activity in the serum, as measured by the CAP-e assay, pointing towards an appropriate screening of biologically effective antioxidant by this assay method.

Apart from assessing reactive oxygen scavenging properties, several targets can be used additionally to qualify the antioxidant herb for pro-cognitive effects since antioxidant herbs may also possess pharmacological activities. Plants such as seeds of *Amorpha fruticosa* L. from Mississippi river basin and *Phytolacca americana* berries

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from Iran have been screened for antioxidant and AChE inhibition properties in order to discover new natural sources of natural compounds for treatment of neurodegenerative disorders (Zheleva-Dimitrova, 2013). The highly antioxidant plant *Amorpha fruticosa* L., exhibited strong AChE inhibition activity. Similarly, the flavonoids in a traditional medicinal herb, *Marchantia polymorpha* L, exhibited strong antioxidant potential, superoxide anion scavenging properties accompanied with acetylcholinesterase inhibitory activity.

Tetrahydroaminoacridine (THA) and donepezil which are popular AChE inhibitors are FDA-approved to improve cognitive function in Alzheimer's disease. The effect of an extract in improving cognition may be tested in a rodent model of cognition using scopolamine-induced cognition deficits in the Barnes maze (Barnes, 1979) which is a sensitive tool for testing hippocampus-dependent spatial memory. Scopolamine, a muscarinic receptor antagonist, produces deficits in spatial navigation tasks in rodents (Bontempi *et al.*, 2003). It significantly increases AChE and MDA levels in the cortex and hippocampus (Kim *et al.,* 2009) and causes increased oxidative stress seen by an increase in lipid peroxidation, nitric oxide and a decrease in gluthathione antioxidant enzyme (El-Khadragy *et al.*, 2014). An extract that can negate the effects of scopolamine would see a benefit and restoration in memory in an animal model such as the Barnes maze.

Another receptor targeted in the screening for pro-cognitive effects are the adenosine receptors which are a class of purinergic G protein-coupled receptors with adenosine as endogenous ligand (Fredholm *et al.*, 2001). The adenosine receptors 1 and A2A functions by regulating neurotransmitters in the brain (Kalda *et al.*, 2006). This protein is a member of the G protein-coupled receptor (GPCR) family. Inactivation of adenosine A2A receptors reversed working memory deficits at early stages of Huntington's disease in animal models providing a proof-of-principle for A2AR as

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novel targets to reverse cognitive deficits in Huntington's disease (Li *et al.*, 2015). While *in vitro* studies provide the initial target of potential herb, its effect in a life system needs to be confirmed. To demonstrate the effect *in vivo*, social recognition test (SRT) has been used in studies with caffeine, an adenosine A2A receptor antagonist, in reversing cognitive decline in age-related deficits in olfactory discrimination, Parkinson's disease, and attention deficit hyperactivity disorder (ADHD) (Hartman, *et al.,* 2006). This memory test probes short-term recognition/working memory relevant to cognitive impairment including neuropsychiatric disorders such as dementia, Alzheimer's disease (AD), schizophrenia, and Parkinson's disease (PD).

Another recent target for drugs with memory enhancement potential as possible targets for treatment of age-related cognitive decline as well as Alzheimer's disease is the phosphodiesterase inhibitors (PDE-Is) (Heckman *et al.*, 2015). It enhances cAMP and/or cGMP signaling via reducing the degradation of these cyclic nucleotides. Both cAMP and cGMP signaling are essential in a variety of cellular functions, including neuroplasticity and neuroprotection. However, the demonstration of clinical proof of concept for cognition enhancing effects of PDE-Is need to be established before developing safe and efficacious novel PDE-Is for the treatment of age-associated cognitive decline or AD.

While the adenosine receptors is the target for diuretic activity, recently its' effect in modulating cognitive functions of the central nervous system have also been implicated (Fredholm *et al.*, 1999). The primary cause of Alzheimer's disease is the formation of beta amyloids in the brain as a result of inflammation which can be caused by oxidative stress (Cai *et al.*, 2011). Pre-clinical studies of *O. stamineus* have confirmed the anti-inflammatory (Awale *et al*., 2003), antioxidant (Abdelwahab *et al.*, 2011; Akowuah *et al*., 2004), antibacterial (Ho *et al*., 2010), hepatoprotective (Han *et al.*, 2008), diuretic (Arafat *et al.*, 2008) antihypertensive (Ohashi *et al.*, 2000),

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hypoglycemic effects (Sriplang *et al.*, 2007) and vasodilative (Beaux *et al.*, 1999) properties of the herb. The free radical scavenging activity using models like 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, superoxides and xanthine oxidase were demonstrated with antioxidant potency comparable with quercetin and butylated hydroxylanisole (BHA) (Akowuah *et al.*, 2005a; Akowuah *et al.*, 2005b; Akowuah *et al.*, 2006). Two clinical studies of the herb demonstrated the anti-hypertensive property of the herb (Cicero *et al*., 2012; Adnyana *et al.*, 2013). The *O. stamineus*, a well known diuretic, has been reported to possess A1R antagonist activity (Yuliana *et al.,* 2009). Web searches using Google Scholar and Pubmed applying *O. stamineus* with memory, cognition and neuro as keywords, the effect of *O. stamineus* on cognition is illustrated in Table 2.2. Although the *O. stamineus* is rich in terpenoids, has A1R antagonist activity and reported to enhance antioxidant enzymes, its' effect on cognition upto 2015 was yet to be studied. Taken together, this herb could potentially be a candidate for procognitive benefits.

2.3 The case for antioxidative herbs as candidates for the modulation of inflammation

Inflammation occurs to eradicate an irritant or stimuli and repair the surrounding tissue. However, prolonged and excessive inflammatory process can induce tissue damage and chronic diseases. Inflammatory cells like neutrophils, monocytes, macrophages, eosinophils and lymphocytes infiltrate the site of stimulus. At the site of inflammation, the activated inflammatory cells release many enzymes such as proteases, collagenase and elastase and lipases, reactive species and chemical mediators such as

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Table 2.2 Summary of pharmacological effects of *O. stamineus* on cognition demonstrated in *in vivo* studies.

Author (year)	Purpose/ Indication	Study Design	Dose	Main Results
Pre-clinical				
Shaik *et al*. (2016)	Learning and memory improving effects of *O. stamineus*.	Induced dementia model by permanent, bilateral occlusion of the common carotid arteries (PBOCCA) in an animal model to investigate cognitive decline by chronic cerebral hypoperfusion evaluated in a passive avoidance test and Morris water maze test.	Acute treatment with extract at 100, 200 and 400 mg/kg body weight.	Significant decrease in escape latency with 100 & 200 mg/kg extract was observed in both testsindicating a significant improvement in learning and memory functions.
Retinasamy *et al*. (2016)	Learning and memory improving effects of *O. stamineus*.	Induced dementia model in a Morris water maze test.	Rats were pre-treated with 100, 200 and 400 mg/kg body weight extract. Standards were 1 mg/kg donepezil and dementia induced with 1 mg/kg scopolamine.	Significant decrease in escape latency in the Morris water maze test and an increase in the amount of time spend in the target quadrant for doses 100 and 200mg/kg when compared with the negative control group denoting an improvement in learning and memory.
Choo *et al*. (2018)	Improvement of neurological disorder with *O. stamineus* extract.	Scoring of seizure in chemically induced seizure in zebrafish model.	Zebrafish was treated with 50 mg/L, 100 mg/L and 200 mg/L of *O. stamineus* extract. Controls were water, diazepam and Pentylenetetrazol which induced seizure.	The extract demonstrated anti-convulsive effect in zebrafish at higher doses and is comparable to diazepam.

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cytokines, chemokines and nitric oxide which induce tissue damage and oxidative stress (Collins, 1999). Inflammatory substances are divided to two main categories: pro- and anti-inflammatory mediators (Vignali & Kuchroo, 2012). Some of the inflammatory mediators and cellular pathways that have been extensively studied are cytokines (e.g., interferons, interleukins and tumor necrosis factor), chemokines (e.g., monocyte), eicosanoids (e.g., prostaglandins and leukotrienes) and the potent transcription factor nuclear factor ƙB (NF-ƙB) (Azab *et al.*, 2016). The transcription factor nuclear factor ƙB regulates the gene expression of proinflammatory cytokines, chemokines and adhesion molecules (Tak & Firestein, 2001). Oxidative stress through the production of H_2O_2 have been found to activate NF-ƙB and antioxidants are reported to block its' activation (Anderson *et al.*, 1994).

Tumour necrosis factor (TNF-α) which is secreted from various cells is an important pro-inflammatory cytokine (Montgomery & Bowers, 2012). TNF-α has been associated with multiple illness states in humans, including immune and inflammatory diseases, cancer, psychiatric disorders, to name a few (van Horssen *et al.*, 2006; Berthold-Losleben & Himmerich, 2008). Other cytokines which mostly exerts a pro-inflammatory activity is IL-1α and IL-6 (Fenton, 1992). Both IL-1α and IL-6 are also associated with anti-inflammatory activity as well. Many of the interleukins possess both pro and anti-inflammatory functions. The IL-10, a potent anti-inflammatory cytokine, impedes the action of many pro-inflammatory mediators and helps to maintain tissue homeostasis and attenuates the damage that may result from an exaggerated inflammatory response by weakening and controlling the inflammatory response (Sabat, 2010; Ng *et al.*, 2013; Kwilasz *et al.*, 2015). The transcription factor nuclear factor ƙB (NF-ƙB) is a prominent regulator of immune and inflammatory responses and is highly involved in the pathophysiology of cancer (Rayet & Gélinas, 1999).

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Prostaglandin (PG) E2, another inflammatory mediator functions in the regulation of normal body temperature, gastric mucosal integrity, renal blood flow and the function of female reproductive system (Chizzolini & Brembilla, 2009). Alterations in PGE2 activity are associated with pathological conditions such as inflammatory diseases, abnormal changes in body temperature, colorectal cancer, to name a few. The pathway of PGs synthesis starts with generation of arachidonic acid (AA) from cell membrane phospholipids by phospholipase A2 (PLA2) followed by arachidonic acid being converted to PGs by the enzyme cycloogygenase (COX) (Goetzl *et al.*, 2005). Among the three known COX isoforms (COX-1, COX-2 and COX-3), the inducible enzyme COX-2 is recognized as the most active during inflammatory processes (Lee *et al.*, 2005).

Leukotrienes (LTs) such as LTB4 is also linked to human illness states including inflammation, asthma and depression (Hallstrand & Henderson, 2010; Peters-Golden & Henderson, 2007). LTs are produced by the enzyme 5-lipooxygenase (5-LOX). Another enzyme that is highly associated with inflammatory conditions is nitric oxide synthase (NOS) where similar to COX-2, inducible NOS (iNOS) is the most pro-inflammatory NOS isoform (Freeman *et al.*, 1998).

In cases of severe inflammation such as joint damage, the AA is produced in excess (Ricciotti & FitzGerald, 2011). Excess AA is converted into powerful inflammatory substances such as PG and LT, respectively (Namgaldaze & Bruene, 2006; Oestvang & Johansen, 2006) by the cyclooxygenase (COX) and lipooxygenase (LOX) pathways. In recent years, research has focused on the LOX pathway as controlling this pathway can play an important role in the relief of joint pain. The LOX pathway is a parallel inflammatory pathway to the COX pathway, where the AA produced is converted to LT, one of the strongest chemotactic agent produced in the body (Xie *et al.*, 1992; Jia *et al.*, 2003). If left unregulated, these inflammatory

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pathways can cause joint damage. The COX-1 is constitutively expressed in many tissues and PGs produced by the action of COX-1 mediate housekeeping functions such as cytoprotection of gastric mucosa, regulation of renal blood flow and platelet aggregation, mostly through formation of eicosanoids from AA (Zarghi & Arfaei, 2011). The 5-LOX enzyme plays a key role in the metabolism of AA to produce leukotrienes. Several studies suggest that there is a link between 5-LOX and carcinogenesis due to inflammation in human and animals (Aggarwal *et al.*, 2006). Leukotrienes also play crucial roles as mediators in allergy and inflammation (Miyahara *et al.*, 2006). Additionally, there has been some concern over the use of current COX-2 inhibitors ie. rofecoxib and valdecoxib for therapeutic interventions, causing some of these therapeutic products to be either withdrawn or made to carry a warning by the Food and Drug Authority (FDA) of the USA (Naesdal & Brown, 2006; Salmon, 2006).

In the recent years, the use of herbal remedies for the treatment of inflammatory disease has been gaining momentum as an alternative source of therapy (Chrubasik *et al.*, 2007). Inhibitors of the 5-LOX enzyme that are of herbal origin are reported to offer significant relief and do not appear to have any adverse effects, thus becoming the preferred choice of treatment for some of the diseases caused by chronic inflammation (Oliver, 2007; Sengupta *et al.*, 2008). Inflammation could be due to reactive oxygen species, trauma, genetic predisposition, stress and age causing damage to cells of the body, thus releasing various membrane components that can activate the inflammatory process. Acute and/or chronic inflammation can lead to acute or chronic inflammatory diseases such as septic shock, rheumatoid arthritis, gastritis, and atherosclerosis (Michaelsson *et al.*, 1995; Stuhlmuller *et al.*, 2000). The LOX pathway is said to play an important role in inflammation associated with joint pain (Lascelles *et al.*, 2009).

Plant compounds such as Curcumin, a yellow pigment from *Curcuma longa* has antioxidant and anti-inflammatory properties through its ability of inhibiting COX-2,

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LOX, and inducible nitric oxide synthase (iNOS). The COX-2, LOX, and iNOS are important enzymes that mediate inflammatory processes and preventing cancerous tumor proliferation (Menon & Sudheer, 2007). While *P. minus* have reported to possess antioxidant, anti-pyretic, analgesic and gastroprotective activity, the anti-inflammatory effects and mechanism have not been investigated. Furthermore, flavonoids have been found to have anti-inflammatory activity. Targeting reduction of chronic inflammation is a good strategy to combat several human diseases including rheumatoid arthritis (Pan *et al.*, 2010). The *P. minus* contains high amounts of flavonoids (Urones *et al*., 1990) and is thus a potential candidate for anti-inflammatory investigation.

2.4 The case for antioxidative herbs as candidates for addressing quality of life and immunosenescence

Hormonal levels in men and women decline with age and is exacerbated by environmental stress and oxidative stress (Alonso-Alvarez *et al.*, 2007). From about 40 years of age, serum testosterone concentrations in men decline with annual rates of between 0.4 and 2.6% for total testosterone and 0.8 and 1.7% for free testosterone (Harman *et al.*, 2001; Feldman *et al.*, 2002; Kaufman & Vermeulen, 2005). Eventually, this decline results in serum testosterone levels being 40–50% lower at the age of 60 than at younger age. A decline in testosterone levels affects muscle, immunity, fertility, sexual health, mental and physical energy.

Treatment with testosterone using human blood neutrophils produced a significant decrease of superoxide (free radical) and lipid peroxidation (Marin *et al.*, 2010). In another animal study, an increase was observed in the activities of SOD and GSH-Px enzyme as well as a decrease in MDA levels and the proportion of mtDNA mutations in the mice that had received testosterone therapy in a castrated mice model (Zhang *et al.*, 2011) indicating antioxidant-promoting effects. The testosterone

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deficiency that had induced oxidative stress was alleviated with testosterone therapy by suppressing oxidative stress mediated via the AR-independent pathway.

Oxidative stress can affect Leydig cells that produce testosterone. The Leydig cells which produce testosterone are exposed to oxidative stress which can reduce activities of antioxidant enzymes (e.g., catalase, superoxide dismutase (SOD), and glutathione peroxidase and increase secretion of the inflammatory MDA - a product of lipid peroxidation (Cao *et al.*, 2004; Gautam *et al.*, 2006). The damage in Leydig cells leads to a reduction of testosterone by the inactivation of enzymes in the testosterone biosynthetic pathway. Decline in serum testosterone contributes to alterations in body composition, diminished energy, muscle strength, sexual function and depressed mood (Matsumoto, 2002) and is caused by ageing and oxidative stress. This phenomenon produces a lowered quality of life. The *E. longifolia* root extract is able to modulate healthy serum testosterone levels (Sambandan *et al.*, 2006; Tambi *et al.*, 2011; Henkel *et al.*, 2014), improve quality of life (Ismail *et al.,* 2012), reduce the symptoms of ageing (Tambi *et al.*, 2012) and improve reproductive health through improved semen and sperm profiles (Ismail *et al.*, 2012; Ma *et al.*, 2017). Preclinical and clinical studies have demonstrated the ability of *E. longifolia* to improve mood (Ang & Cheang, 1999), physical & sexual well-being, reduce stress (Talbott *et al.*, 2013) and increase testosterone. Web searches using Google Scholar and Pubmed applying *E. longifolia* and randomized, double-blind, placebo-controlled as keywords, the pharmacological effects related to ageing is presented in Table 2.3. As the optimal functions of the Leydig cells which produces testosterone is dependent on the management of free radicals and oxidative stress, the *P. minus* which has been reported to be highly antioxidative, could provide the much needed protective benefit for the optimal functioning of Leydig cells. Hence, *E. longifolia* in combination with *P. minus* may be

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able to improve the sexual performance, physical and mental wellbeing of men with poor reproductive health as a result of improved hormonal functions.

Deficiencies in nutrients contribute to stress and depression, confusion and fatigue (Kaplan *et al.*, 2007; Kaplan *et al.*, 2015; Long & Benton, 2013). Recent evidence suggests that chronic fatigue syndrome is attributed to excessive free radicals (Kennedy *et al.,* 2005). Stress induces the catabolic/anabolic hormone imbalance characterised by high cortisol, glucose and insulin and low androgens like testosterone and growth hormones (Epel *et al*., 2009). Studies in animal (Liu & Mori, 1999) and humans (Irie *et al*., 2003) have linked phychological stress with oxidative stress. In fact telomere shortening has been associated with life stress (Epel *et al*., 2004). Immunological functions decrease with factors such as ageing, stress, and infection rendering the host vulnerable to infectious diseases. In fact, immune system efficiency is said to decline with age through a process known as immunosenescence (Gruver *et al*., 2007). A contributing factor to this process is a reduction in the number of naïve T cells (Hawkley & Cacioppo, 2004). The reduction of naïve T cells reduces adaptive immunity which is acquired immunity upon occurrence of an infection. The age-related decline in T cells renders the body less able to defend itself against current and new infections. In addition to ageing, oxidative stress plays an important role in the progression of immunological decline. In the immune system, high levels of ROS can be beneficial; neutrophils generate ROS and release them intracellularly and extracellularly in the form of an “oxidative burst” to defend itself against pathogens, thus providing anti-microbial protection (Dahlgren & Karlsson, 1999). However, excessive ROS are generated in the presence of immune complexes with auto-antigens where further macromolecular damage is induced. Prolonged exposure to high ROS concentrations can inhibit T-cell proliferation and lead to apoptosis (Thoren *et al.*, 2007).

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Antioxidant compounds can enhance antioxidant-producing enzymes to slow immunological decline (Hirokawa *et al.*, 2009). The *E. longifolia* has anti-cancer properties (Thu *et al*., 2018) and pre-liminary immunomodulatory effect seen by the increase of natural killer (NK) cells in a clinical study (Muhamad *et al*., 2015) of *E. longifolia* supplementation of 200 mg/day for seven days. While the plant has been reported to possess many anti-ageing properties, its' effect in modulating immunity and stress is only very preliminary hence rendering conclusion difficult.

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Table 2.3: Summary of selected randomized, double-blind and placebo-controlled RCTs with *E. longifolia* (*Tongkat Ali*) for various indications related to ageing: muscle, psychological, physical & sexual well-being, stress, sexual/erectile dysfunction and immunity

Author (year)	Purpose Indication	Subjects/Study Design	Herbal Extract Intervention, Duration	Main Results
Hamzah & Yusof (2003)	Ergogenic and performance enhancing effects of *E. longifolia* on body composition and muscle strength in males compared to placebo.	14 healthy males. Parallel study.	*E. longifolia* extract 100 mg/d. Trial duration: 5 weeks.	Increase in lean body mass with *E. longifolia* (P=0.012), reduced body fat, and increased muscle strength and size. None with placebo.
Ismail *et al*. (2012)	Effects of *E. longifolia* on sexual well-being and quality of life in healthy males compared to placebo.	109 healthy males. Parallel study.	300 mg/d *E. longifolia* extract. Trial duration: 12 weeks.	Benefits in IIEF (p<0.001), sexual libido (p=0.009), sperm motility (P=0.01) and semen volume (p=0.096) with *E. longifolia* from baseline to end of study. No significant changes for placebo. No group difference in overall QoL, but "physical functioning" domain improved compared to placebo (P=0.028). No changes in hormone profile.
Talbott *et al*. (2013)	*E. longifolia* on stress hormone, testosterone and psychological mood states in moderately stressed adults compared to placebo.	64 males and female subjects. Parallel study.	*E. longifolia* extract 200 mg/d. Trial duration: 4 weeks.	Cortisol decreased, Testosterone increased with *E. longifolia* compared to placebo. Significant reduction in anger, confusion and tension domains (p<0.05) evaluated by POMS in *E. longifolia* as compared to placebo from baseline to end of study.
George *et al*. (2013)	Investigation of anabolic effects using the ratio of Testosterone:Epitestosterone (T:E) with *E. longifolia*.extract in healthy non-athletic males compared to placebo.	40 males. Parallel study.	300 mg/d *E. longifolia* extract. Trial duration: 12 weeks.	T:E ratios for *E. longifolia* and PL in "normal" range ie.< 1. No differences between groups. Hormones - no changes within and between groups. Weight lifting force (strength) increased with *E. longifolia* (p=0.0166) from baseline to end of study as compared to placebo.
Muhamad *et al*. (2015)	Immune response and endurance running with *E. longifolia* in males compared to placebo.	9 healthy males. Cross-over design.	200 mg/d *E. longifolia* extract. Trial duration: 7 days.	No significant group difference in running capacity (P=0.139) as well in all immune parameters, except for natural killer cells (P=0.02) which were significantly higher in only the *E. longifolia* group.

Note: **IIEF** International Index of Erection Function, **POMS** Profile of Mood State, **QoL SF-36** Quality of Life Short Form-36, **T:E** ratio Testosterone to Epitestosterone.

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CHAPTER 3: PUBLISHED PAPERS

3.1 Publication 1

George, A., Ng, C. P., O'Callaghan, M., Jensen, G. S., & Wong, H. J. (2014). *In vitro* and *ex vivo* cellular antioxidant protection and cognitive enhancing effects of an extract of *Polygonum minus* Huds. (Lineminus™) demonstrated in a Barnes Maze animal model for memory and learning. *BMC Complementary and Alternative Medicine*, *14*, 161.

3.1.1 Contribution of co-authors

Name	Contribution
Annie George	Involved in the identification of appropriate *in vitro* and *ex vivo* enzyme and cell-based assay; animal study design, interpretation of data and manuscript writing.
Chee Perng Ng	Involved in the animal study design and conduct; statistics, interpretation of data and manuscript writing.
Matthew O'Callaghan	Involved in the animal study design and conduct; statistics, interpretation of data and manuscript writing.
Gitte S Jensen	Involved in the *ex vivo* cell based anti-oxidant study (e-CAP) and revision of manuscript.
Wong Hoi Jin	Involved in the characterisation and standardisation of the extract used in the study.

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RESEARCH ARTICLE Open Access

In vitro and *ex-vivo* cellular antioxidant protection and cognitive enhancing effects of an extract of *Polygonum minus* Huds (Lineminus™) demonstrated in a Barnes Maze animal model for memory and learning

Annie George[1*], Chee Perng Ng[2], Matthew O'Callaghan[2], Gitte S Jensen[3] and Hoi Jin Wong[1]

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Abstract

Background: *Polygonum minus* Huds.is a culinary flavouring that is common in South East Asian cuisine and as a remedy for diverse maladies ranging from indigestion to poor eyesight. The leaves of this herb have been reported to be high in antioxidants. Flavonoids which have been associated with memory, cognition and protection against neurodegeneration were found in *P. minus*.

Method: This study examined a *P. minus* aqueous extract (Lineminus™) for its antioxidant activity using the Oxygen Radical Absorbance Capacity (ORAC) assay, the *ex vivo* Cellular Antioxidant Protection of erythrocytes (CAP-e) assays and for potential anticholinesterase activity *in vitro*. Cognitive function and learning of Lineminus™ was evaluated using scopolamine induced cognition deficits in a Barnes maze, rodent model of cognition.

Results: The extract displayed *in vitro* antioxidant activity with a total ORAC value of 16,964 μmole TE/gram. Cellular antioxidant protection from free radical damage using the CAP-e assay, with an IC50 of 0.58 g/L for inhibition of cellular oxidative damage, was observed. The extract inhibited cholinesterase activity with an IC50 of 0.04 mg/ml with a maximum inhibition of 68%. In a rodent model of cognition using scopolamine induced cognition deficits in the Barnes maze, the extract attenuated scopolamine induced disruptions in learning at the higher dose of 100 mg/kg.

Conclusion: These data shows that *P. minus* possesses antioxidant and anticholinesterase activity and demonstrated enhanced cognition *in vivo*. The data suggest neuroprotective properties of the extract.

Keywords: Scopolamine, Antioxidant, Barnes maze, *Polygonum minus*, Cognition

Background

Polygonum minus Huds. synonymous to *Persicaria minor* is from the family Polygonaceae and is commonly referred to as Kesum or laksa leaf in Malaysia. It is used as a flavouring ingredient in culinary dishes and also consumed as an ulam (salad) for preventive healthcare [1,2]. Traditionally, the decoction of leaves is taken for indigestion, after childbirth, to warm the body up and to promote good eyesight [3]. It is possible that *P. minus* has the ability to increase blood circulation [4].

Several studies have shown that leaves of *P. minus* are high in antioxidants [5-9]. It has been reported that water extracts of *P. minus* have shown superior antioxidant activity when compared to other popular herbs such as ulam raja, selom, pegaga and curry leaves, where antioxidant activity was similar to the synthetic antioxidant butylhydroxytoluene (BHT) [6]. Water extracts of *P. minus* had higher antioxidant activity, measured by the total phenolic content (TPC), when comparing to ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*) [9],

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2,2-diphenyl-11-picryhydrazyl free radical (DPPH) and ferric reducing antioxidant power (FRAP) [7]. *Polygonum minus* was shown to possess 98.3% of lipid peroxidation inhibitory activities and is proposed as a candidate for nutraceutical and cosmeceutical product [2]. It has also been reported to be rich in vitamins such as carotenes, retinol equivalents and vitamin C, α-tocopherol (vitamin E) and minerals such as calcium, phosphorus, iron, sodium, potassium, magnesium, copper and zinc [10].

Free radicals, including reactive oxygen species, have been shown to cause aging and several degenerative diseases such as atherogenesis, cardiovascular and neurodegenerative diseases [11,12]. Although the production of free radicals is a normal by-product of metabolism and environmental stress, the over production leads to cell damage. For this reason, antioxidants such as vitamins A, C, E, carotenoids and plant polyphenols (flavonoids, phenolic acids, cathechins, coumarins, tannins and anthocyanins) are commonly consumed as part of the food composition as a protective defence mechanism against such damage. Plant antioxidants can therefore act as agents in scavenging reactive oxygen species.

Phytochemical screening of *P. minus* have shown the presence of flavanoids, flavones, cathecin, epicatechingallate and terpenoids [5]. Flavonoid and polyphenols have been long studied for their strong antioxidant capacities, and their ability to scavenge reactive oxygen species thus preventing aging and oxidative stress related diseases [13,14]. Studies also show that flavonoids have an effect on memory, cognition and against neurodenegeration and the ability to improve cerebrovascular blood flow [15].

The nutraceutical industry uses a standardized chemical antioxidant method, ie. the Oxygen Radical Absorbance Capacity (ORAC) test to evaluate antioxidant strength. The ORAC is a HAT-based assays measuring the capability of an antioxidant to quench free radicals (generally, peroxyl radicals) by H-atom donation [16]. In addition, the ORAC assay measures the degree and length of time the extracts take to inhibit the action of an oxidizing agent unlike the DPPH assay. The ORAC was identified as one of the best standardized antioxidant assay for the natural products industry in measuring antioxidant levels in nutritional and natural products [17-19]. The U.S. Department of Agriculture developed a database of biological materials and foods to provide the basis for comparing antioxidant strength based on ORAC values [20]. This enables one to compare the antioxidant levels against other popularly known antioxidant foods hence making it easier to compare the antioxidant effect of *P. minus* as a food with other more established high antioxidant foods. The ORAC assay has since been commercialized by Brunswick Labs, Wareham, MA, USA. The DPPH and FRAP method on the other hand is a SET method, which measures the ability of the antioxidant to transfer one electron to reduce a specific oxidant. In the case of DPPH assay, it measures direct reactions with the DPPH radical, which is dependent on the structure of an antioxidant compound therefore only giving a general indication of the radical scavenging abilities of antioxidants.While DPPH method is a rapid and convenient method to measure antioxidant activity it diverges from biological conditions the most, by the use of an artificial DPPH radical and methanol as the solvent [21]. This is in contrast to ORAC which is performed at a physiological pH producing a biologically relevant radical, the peroxyl radical [22].

However, there is a need to demostrate the antioxidant capacity in serum to show bioavailibity. Since a clinical trial is costly and time consuming, a more intermediary cell-based study was developed to test to what extent a substance protects against oxidative stress in a biologically relevant sytem. The Cellular Antioxidant Protection of erythrocytes (CAP-e) assay is a cell-based antioxidant protection assay using erythrocytes to address the question of whether antioxidants in complex natural products enter the cytosol and contribute to the reduction of oxidative damage within the cell [23] and was successfully applied in other antioxidant rich herb and fruit such as the Acai berry [24]. The current study is an attempt to draw a parallel between the antioxidant property of the herb, its' relevance in a biological system, using the CAP-e assay (ex vivo) and the improvement of learning and memory *in vivo* as one of the manifestation of the antioxidant property.

As improvements in cogniton may be multi-pronged, the herb is also tested for antiacetylcholinesterase activity. Acetylcholine is a neurotransmitter related to learning and memory [25]. It is metabolised by the enzyme acetylcholinesterase. Inhibition of acetylcholinesterase is presently the most accepted and recognized therapeutic marker for the development of cognitive enhancers [26]. Anti-cholinesterase activity has never been tested for *P. minus.* Screening for herbal plants with acetylcholinesterase inhibitory activity would open new possibilities for cognition improving herbal products.

Several *in vivo* models have been used to investigate learning and memory in animal models of which Scopolamine, a muscarinic receptor antagonist, produce deficits in spatial navigation tasks in rodents [27]. Scopolamine significantly increases acetylcholinesterase (AChE) and malondialdehyde (MDA) levels in the cortex and hippocampus [26].

The Barnes maze was developed as a sensitive tool for testing hippocampus-dependent spatial memory in rats [28] and is the model adopted for this study. In addition, for mice the Barnes maze is better, as they swim less well than rats. The Barnes maze is similar to the Morris water maze task, but does not utilize a strong aversive stimulus (stress induced by swimming as reinforcement).

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Behavioral tasks involving high levels of stress can influence the animal's performance [29].

This study was performed to investigate *P. minus* in protecting against oxidative stress in a cell-based study and in memory improvement *in vivo.* Currently the plant extract most popularly researched for the ability to enhance cognition is *Gingko biloba*. Extracts of *G. biloba* were shown to improve memory and normalizing cognitive deficits in animal models [30], and in treatment of cognitive improvement in Alzheimer's patients [31]. In this study, the antioxidant activity of *P. minus* was tested *in vitro* and the protection against oxidative damage demonstrated in red blood cells. The paper attempts to draw a parallel between the protective antioxidant affect of the herbal extract to the cognition enhancing effect, in an animal model induced with cognitive deficits by scopolamine, whose activity can also be attenuated by an anti acetylcholinesterase. The activity of the herb was compared to the more traditionally and scientifically documented, *G. biloba* which has been reported to possess anti-acetylcholinesterase activity and improve cognition *in vivo*.

Methods

Plant material

Polygonum minus was procured from Biotropics Malaysia Berhad, Malaysia. The plant material was identified based by a Taxonomist from Institute Bio Science, University Putra Malaysia (UPM) based on their exomorphic characters and literature review of the plant. The voucher specimen of the plant (SK 2077/12) was deposited in the Herbarium Institute Bioscience, UPM Malaysia. The aerial parts of plants including stem and leaves were extracted to produce an aqueous extract for *in vitro* and *in vivo* assays.

Plant extract

Fresh plant material was oven-dried to below 10% moisture content. The dried leaves were chopped into fragments and the extraction was performed by immersing these leaves in water at a ratio of 1:20 and percolated for 2 cycles for 4 hours at 80°C. The liquid was then filtered and evaporated. The liquid concentrate was subsequently freeze dried until it reached a moisture content of below 8% w/w. The extract was then vacuum packed in aluminum foil to preserve it in a cool low humidity with no direct exposure to sunlight. The water extract of *P. minus*, standardised to Quercetin-3-glucuronide 0.59% and 0.27% Quercitrin was prepared by Biotropics Malaysia Berhad according to process outlined in Malaysian Patent Pending No. PI2012003882 [32]. The HPLC fingerprint (Figure 1A) of *P. minus* water extract was obtained according to the HPLC method using Kinetex 1.7 μm C18 (2.1 × 150 mm) column. The mobile phase consisted of solvent A-0.10% formic acid in water and B-0.10% formic acid in acetonitrile mixed according to a linear gradient program of between 5-89% of solvent A and 95-11% of solvent B. Two major peaks in the fingerprint profile were isolated and identified to be quercetin-3-glucuronide and quercitrin based on their mass(es) and MS fragmentations. LC-MS-MS was performed using a Shidmadzu UFLC system equipped with a PDA and IT-TOFMS. Peaks at retention times 7.15 and 13.96 min identified as Quercetin glucuronide and Quercitrin respectively were further confirmed by comparing their retention time values and the obtained UV max with those of the standards. (Figure 1A-C).

The comparative plant extract of *Gingko biloba* was based on commercially available standardised extract of dried leaf from Shanghai Novanat Co. Ltd. The extract was standardised to 27.25% Gingkoflavoglycosides, 6% Terpene lactones and ≤ 5 ppm Ginkgolic acid determined through HPLC methods and passed microbial and heavy metal test (based on the Certificate of Analysis).

Determination of antioxidant capacity using ORAC assay

Extract of *P. minus* was shipped to Brunswick Laboratories, Norton, MA, an independent contract laboratory specialising in standardised natural product assays, to test for ORAC values. Data were obtained for ORAC hydrophilic testing using fluorescein as the fluorescent probe and 2,2′-azobis (2-amidinopropane) dihydrochloride as a peroxyl radical generator, ORAC lipophilic testing for lipid antioxidants capable of quenching peroxyl free radicals, HORAC testing for antioxidants capable of quenching hydroxyl free radicals, NORAC testing for antioxidants capable of quenching peroxynitrite, and SORAC testing for superoxide dismutase-like activity (based on Certificate of Analysis released by the lab) [33].

Determination of CAP-e antioxidant capacity

The CAP-e antioxidant capacity was estimated according to the modified method of Honzel [23], modified for a more sensitive and accelerated protocol [33]. An amount of 0.5 g of plant extract was mixed with 5 mL 0.9% saline at physiological pH, mixed by inversion, vortexed and allowed to incubate on a rocker for 20 minutes. The solids were removed by centrifugation at 2400 rpm for 10 minutes. The supernatant was removed and then filtered through a 0.22 micron cellulose acetate syringe filter before use in the CAP-e assay. Serial dilutions were prepared from the filtered supernatant in 0.9% saline at physiological pH. Red blood cells were treated in duplicate with serial dilutions of the test product. Samples of untreated red blood cells (negative controls) and samples of red blood cells treated with oxidizing agent but not with an antioxidant-containing test product (positive controls) were prepared in hexaplicate. The antioxidants

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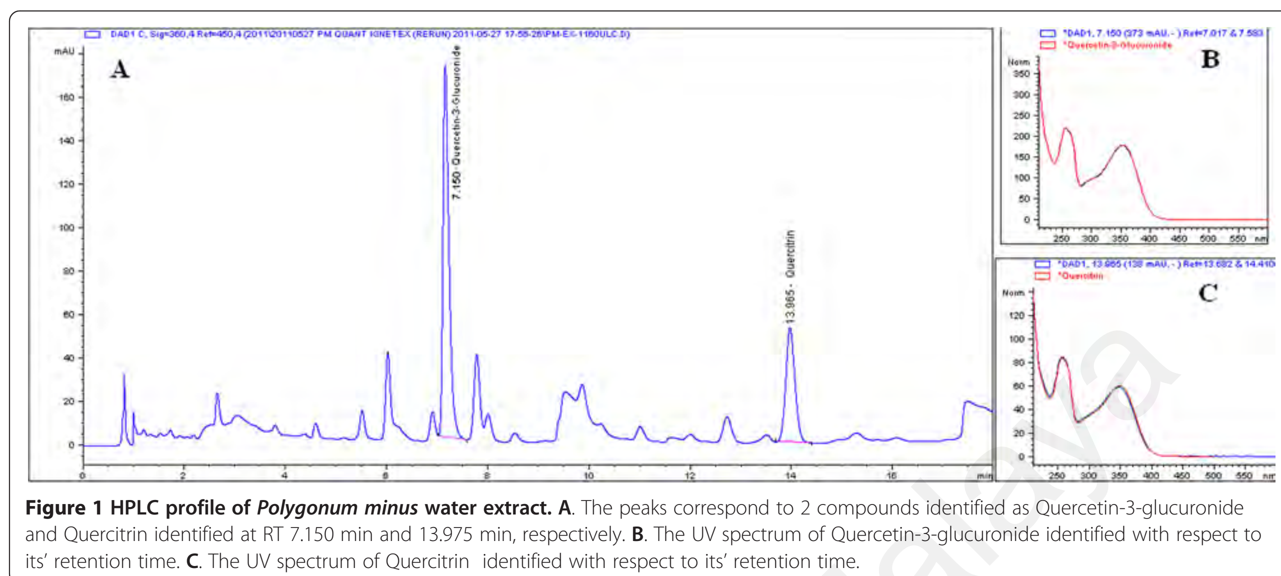

Figure 1 HPLC profile of *Polygonum minus* water extract. A. The peaks correspond to 2 compounds identified as Quercetin-3-glucuronide and Quercitrin identified at RT 7.150 min and 13.975 min, respectively. **B**. The UV spectrum of Quercetin-3-glucuronide identified with respect to its' retention time. **C**. The UV spectrum of Quercitrin identified with respect to its' retention time.

not able to enter the cells were removed by centrifugation and aspiration of supernatant above the cell pellet.

The cells were exposed to oxidative damage by addition of the peroxyl free-radical generator 2,2' azobis (2-amidinopropane hydrochloride) (AAPH). Using the indicator dye Dichlorofluorescein diacetate (DCF-DA), which becomes fluorescent as a result of oxidative damage, the degree of antioxidant damage was recorded by measuring the fluorescence intensity of each sample in a TECAN Spectrafluor plate reader. The inhibition of oxidative damage was calculated as the reduced fluorescence intensity of product-treated cells, compared to cells treated only with the oxidizing agent, in reference to the baseline levels of oxidation in untreated cells. The CAP-e value which is in Gallic Acid Equivalent (GAE) units, reflects the IC50 dose of the test products, i.e. the dose that provided 50% inhibition of oxidative damage. This is then compared to the IC50 dose of the known antioxidant Gallic acid.

Determination of anticholinesterase activity

The extracts was screened for anticholinesterase activity using ProfilingScreen procured from Ricerca Pharmacology Services, Taiwan. The extract was tested in duplicates at a concentration of 10, 30 and 100 μg/ml. Concurrent vehicle 1% DMSO and reference standard Physostigmine were conducted with each assay using Human Recombinant HEK-293 cells.

Animals

Two to six month old adult male C57BL/6 mice (20–25 g), (n = 12–14) were supplied by BioLASCO (Taiwan). The mice were group-housed under a 12/12-h light/dark (200–300 Lux) cycle (lights on 07:00 h) with free access to food (Labdiet, formulated laboratory chow) and water and humidity kept between 50%–70%. The experiment was approved by the Institutional Animal Care and Use Committee of Cerca Insights Sdn Bhd and was conducted in accordance with the Singapore NACLAR Guide (2004) for the care and use of laboratory animals.

Treament

Herbal extract treatments of 50 mg/kg *P. minus*, 100 mg/kg *P.minus*, 50 mg/kg *G.biloba* or vehicle (water) were given daily via oral gavage for fourteen days prior to Barnes maze testing. This treatment continued during the five days of Barnes maze testing. During the Barnes maze testing these mice received i.p. injections of either scopolamine (0.5 mg/kg) or saline vehicle. A further group received i.p. injections of donepezil (1 mg/kg) and scopolamine (0.5 mg/kg). The dose and time of scopolamine administration has been previously shown to produce deficits in spatial navigation tasks in rodents [27].

Barnes assay

The Barnes maze (BM) was created to evaluate spatial learning [18,26]. The Barnes maze consisted of a PVC circular platform with 21 holes placed 6 cm from the edge and equally distributed around the surface. The platform was 122 cm in diameter and 92 cm from the ground. The maze uses rodents natural aversion to open illuminated places and so the subjects were motivated by bright light (Lux level 300 – 500) to locate an escape hole which leads to a dark box (5.4 × 23 × 4.5 cm). Room design and equipment around the maze were used as fixed spatial cues (extra-maze cues) for navigational purposes.

Barnes maze testing consisted of three phases, an adaptation period, an acquisition period and a probe trial. A pre-trial (adaptation period) was given prior the

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start of trial on days one and two. Each subject underwent four trials per day for four days (acquisition period) and then a probe trial was performed twenty-four hours after the final acquisition trial. Thirty minutes prior to the first trial the test subjects received an injection of either scopolamine, saline vehicle, or scopolamine and donepezil.

Adaptation period

The subject was placed in the center zone of the maze, shrouded in a chamber for 10 seconds. The chamber was removed and the subject was allowed to explore the maze for 30 seconds, then gently guided to the escape hole. If the subject did not then enter the escape hole, it was placed inside. The hole was then covered and the subject remained there for 3 minutes. The subject was then returned to its home cage and the platform cleaned with 70% ethanol.

Acquisition period

The chamber was removed and the subject was allowed to explore the maze for 5 minutes and then gently guided to the escape hole. If the subject did not then enter the escape hole, it was placed inside. The hole was then covered and the subject remained there for 1 minute. The subject was then returned to its home cage and the platform cleaned with 70% ethanol. The next trial was run after following an inter-trial interval of two minutes.

Probe trial

For the probe trial, an identical white disc was placed on the platform that covered all the holes. The subject was placed in the center zone of the maze, shrouded in a chamber for 10 sec. The chamber was removed and the subject was allowed to explore the area for 90 seconds. The subject was then returned to its home cage and the platform cleaned with 70% ethanol.

The behavior of the experimental subjects was captured by video camera and recorded on the hard drive of a desktop PC. An analysis of these recordings was performed using EthoVision® XT tracking system for the automatic tracking and analysis of animal movement. For each parameter, the performance in the trials on each day were averaged. The following parameters were measured during the test and processed - (i) Total path length, the distance moved by the subject during the entire session. Total path length is the total distance moved over the whole time of the experimental trial including the distance moved after the first encounter with the escape-hole (when the mouse has encountered the escape hole but failed to enter it). An increase in path length demonstrates a decrease in performance; (ii) Total errors, the count of the number of errors made by the subject throughout the trial. Total errors are the number of approaches to the non-escape hole, when the mouse has interacted with the escape hole but not escaped and further explored the maze. Both total path length and total errors measure learning; (iii) Total latency, the latency for subject to complete the task. Total latency describes the time taken by the mouse to enter the escape hole; (iv) For the probe trial the arena was divided into 8 right equal segments and duration in each segment by the subject was measured. The mice are allowed to explore the maze and the time the mice stay in various areas on the maze is recorded. This 'probe' trial is used to assess memory.

Data was analyzed using two-way repeated measures ANOVA, with 'day' as repeated-measure factor within subjects and 'treatment group' as between-subject's factor. Post-hoc pairwise comparisons between groups using Tukey HSD test were carried out if significant effect was found. Data analysis was performed using Sigma Plot statistical software.

Results

Antioxidant capacity as measured by the ORAC assay and anti-cholinesterase activity

There are five predominant reactive species found in the body: peroxyl radicals, hydroxyl radicals, peroxynitrite, super oxide anion and singlet oxygen. Total $ORAC_{FN}$ provides a measure of the total antioxidant power of a food/nutrition product against the five predominant reactive species. The $ORAC_{FN}$ values for the extract are shown in Table 1.

The extract inhibited cholinesterase activity with an IC50 of 0.0405 mg/ml with maximum inhibition of 68%.

CAP-e assay

The CAP-e assay was used to test whether the extract contained antioxidants capable of protecting cells from oxidative damage. The inhibition of oxidative damage was calculated as the reduced fluorescence intensity of product-treated cells, compared to cells treated only with the oxidizing agent in the absence of antioxidant protection. The CAP-e value reflects the IC50 dose of the extract, i.e. the dose that provided 50% inhibition of oxidative damage. The CAP-e value for the *P. minus*

Table 1 Total $ORAC_{FN}$ showing a measure of the total antioxidant power of *P.minus* aqueous extract against the five predominant reactive species

Antioxidant power against peroxyl radicals	2,591 μmole TE/gram
Antioxidant power against hydroxyl radicals	8,973 μmole TE/gram
Antioxidant power against peroxynitrite	222 μmole TE/gram
Antioxidant power against super oxide anion	4,039 μmole TE/gram
Antioxidant power against singlet oxygen	1,139 μmole TE/gram
Total $ORAC_{FN}$ (sum of above)*	16,964 μmole TE/gram

*Brunswick Laboratories, USA [34].

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extract was shown to be 55 gallic acid equivalents per gram extract, based on an IC50 value of 0.58 g/L (Figure 2A-B).

Learning phase (acquisition training)

A two way repeated measures ANOVA of total path length indicated a significant effect of both treatment ($F_{5,72} = 4.4$, $p < 0.01$) and day ($F_{3,70} = 65.99$,$p < 0.001$) as shown in Table 2. The days one, two, three or four on which the animal was tested is a significant factor in performance. Post-hoc analysis showed a significant increase in path length in the scopolamine treated control mice on days one, two and three ($p < 0.05$) compared to vehicle treated mice indicating a scopolamine induced deficit in learning. Hence, an increase in path length demonstrates a decrease in performance. This increase in path length was not seen in the mice treated with *G. biloba*, donepezil or 100 mg/kg *P. minus*. The scopolamine induced deficit was not reversed by treatment with 50 mg/kg *P. minus*. These mice showed a significant decrease in performance when compared to vehicle treated mice on day two and three. However, the donepezil, *G. biloba* and the 100 mg/kg *P. minus* treated mice did not show a scopolamine induced deficit. There was a significant effect of day ($F_{3,70} = 62.56$, $p < 0.0001$) and treatment ($F_{5,72} = 6.13$, $p < 0.001$) on total errors. A significant increase in total errors was seen in the scopolamine treated mice on days one and two ($p < 0.05$). A significant increase in total errors was also seen in *P. minus* (50 mg/kg) treated mice on days two and three ($p < 0.05$). The deficits for total errors were not observed after treatment with *P. minus* at 100 mg/kg, *G. biloba* or donepezil for days one, two and three (Table 2). There was a significant effect of day ($F_{3,70} = 111.1$,$p < 0.0001$) and treatment ($F_{5,72} = 4.94$, $p < 0.001$) on total latency. A significant increase ($p < 0.05$) in total latency was see in the scopolamine and *P. minus* (50 mg/kg) treated mice on days one, two and three (Figure 3).

Memory phase (probe trial)

One-way ANOVA demonstrated significant duration i.e. time spent in the target segment in the vehicle treated control ($F_{7,103} = 6.86$, $p < 0.0001$); the scopolamine with donepezil treated ($F_{7,111} = 6.94$, $p < 0.0001$); the *G. biloba* treated ($F_{7,100} = 4.2333$, $p < 0.001$) and the *P. minus* –100 mg/kg ($F_{7,95} = 6.6852$, $p = 0.01$) mice, but not the scopolamine alone nor the scopolamine plus *P. minus*- 50 mg/kg treated mice (Table 3). This suggests that scopolamine has induced a deficit in memory which was not reversed by the low dose of *P. minus*. Both *G. biloba*, donepezil and higher dose of 100 mg/kg *P.minus* reversed scopolamine induced memory deficits.

Discussion

The extract was shown to possess strong antioxidant capacity, as measured by the oxygen radical absorbance (ORAC assay). The ORAC value of *P. minus* water extract can now be compared to the more popularly known high antioxidant foods such as Granny Smith apples, cranberry and blueberry at ORAC values of 5381, 8983 and 9019 μmol TE/100 g respectively [20]. In addition, the antioxidants of the standardised extract are capable of entering into and protecting cells from oxidative damage, as shown by the cellular antioxidant protection (CAP-e) assay. These properties may contribute to the effects seen in the animal model of cognitive function, since the brain has a high level of metabolism and oxygen use and so is susceptible to oxidative attack by free radicals. Additionally, it has a relatively low concentration of anti-oxidative enzymes and free radical scavengers [35]. Previous reports have suggested that a water extract of *P. minus* promotes high antioxidant

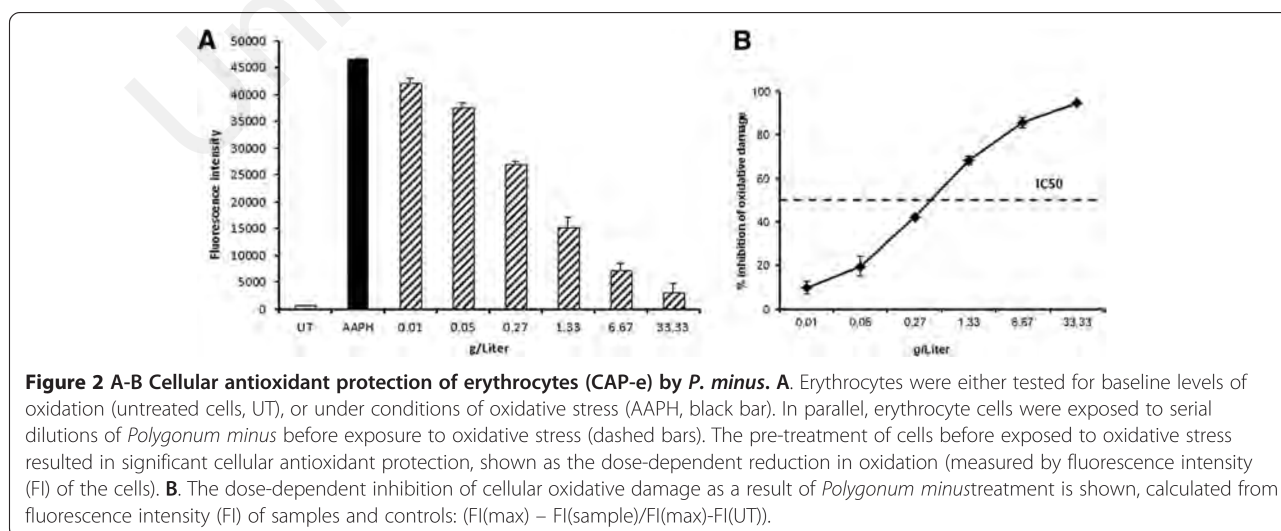

Figure 2 A-B Cellular antioxidant protection of erythrocytes (CAP-e) by *P. minus*. A. Erythrocytes were either tested for baseline levels of oxidation (untreated cells, UT), or under conditions of oxidative stress (AAPH, black bar). In parallel, erythrocyte cells were exposed to serial dilutions of *Polygonum minus* before exposure to oxidative stress (dashed bars). The pre-treatment of cells before exposed to oxidative stress resulted in significant cellular antioxidant protection, shown as the dose-dependent reduction in oxidation (measured by fluorescence intensity (FI) of the cells). **B**. The dose-dependent inhibition of cellular oxidative damage as a result of *Polygonum minus*treatment is shown, calculated from fluorescence intensity (FI) of samples and controls: (FI(max) – FI(sample)/FI(max)-FI(UT)).

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Table 2 Total path length travelled in the Barnes maze and total errors

Treatment	Total path length (Mean ± SE)				Total errors (Mean ± SE)			
	Day-1	Day-2	Day-3	Day-4	Day-1	Day-2	Day-3	Day-4
***G. biloba* 50 mg/kg**	1538.99	497.76	324.95	212.4	32.35	11.9	9.46	8.00
	±185.09	±73.89	±82.76	±25.16	±3.54	±1.69	±2.77	±1.34
***P. minus* 50 mg/kg**	1420.44	732.61	556.98±	340.38	33.77	22.03	18.77	12.18
	±171.2	±137.06[a]	117.97[a]	±65.71	±3.67	±4.25[a]	±3.83[a]	±2.41
***P. minus* 100 mg/kg**	1436.37±	602.87	356.95±	244.47	31.92	15.71	10.85	7.88
	160.44[b]	±96.82	51.28	±26.89	±3.06	±2.2	±1.78	±1.18
Donepezil 1 mg/kg	1031.39	559.37±	396.59±	305.24	20.79	12.95	11.36	9.00
	±173.06[b]	137.29	71.57	±36.59	±3.65	±2.5	±1.6	±1.09
Veh/Sco	1841.41±	817.31 ± 1	537.84±	365.66	44.06	21.65	16.58	13.00
	238.12[a]	90.27[a]	111.44[a]	±86.44	±5.22[a]	±3.91[a]	±3.27	±2.74
Veh/Veh	926.06 ± 1	234.06 ± 2	202.36±	211.82	19.09	6.75	5.98	6.40
	96.13[b]	7.8[b]	24.26	±30.61	±3.19	±1.24	±0.87	±1.27

[a]Significance when compared to control vehicle + vehicle, p < 0.05.
[b]Significance when compared to control vehicle + scopolamine, p < 0.05.
Veh = vehicle; Sco-Scopolamine; Don = Donepezil.

levels determined by free radical scavenging activity of DPPH radical and the ability of antioxidant in this plants extract to reduce ferric (III) iron to ferrous (II) iron in FRAP reagent, probably as a result of high flavonoid and total phenolic content [6,9]. Using different methods to measure antioxidant property, the antioxidant property for water extracts of *P. minus* was demonstrated and by evaluating ORAC values, the standardised *P. minus* extract-Lineminus™ could be compared with other antioxidant foods.

In this study, we managed to evaluate antioxidant effect *ex-vivo* by the use of erythrocyte cells (in the CAP-e bioassay study), where oxidative stress was reduced in a dose dependent manner by the presence of the extract. Natural products including flavonoids that exhibit anti-oxidative effects have been found to attenuate memory impairments [24]. Some isolated antioxidant compounds, including luteolin, quercetin, and dihydrokaempferol, from Acai fruit pulp had the capacity to enter live cells and protect them from oxidative damage, demonstrated by using the same Cellular Antioxidant Protection in erythrocytes (CAP-e) bioassay [24]. Some flavonoids in fact have been reported to cross blood brain barrier *in vitro* [36]. The *in vivo* studies showed that flavonoids are able to be absorbed after oral administration, pass the blood–brain barrier and do have various effects on the CNS [37]. Derivatives of quercetin and flavonoids were identified in extracts of *P. minus* [8], possibly leading to the CAP-e effect observed in this study. However, the concentrations of flavonoids and their metabolites which reach the brain in the current study have to be assessed. If this antioxidant activity is confirmed *in vivo*, this could help reduce increased oxidative stress such as that reported to occur in the aging brain and so may be therapeutically useful. Hence the possibility of flavonoids of *P. minus* to cross blood brain barrier thus qualifying the procognitive effect in the animal study conducted here, should be further investigated.

In the present study, scopolamine treatment induced deficits during the early part of the acquisition (learning) phase of the Barnes maze task. The scopolamine induced deficits in learning were evidenced by an increase in the number of errors, latency, and path length. Considering the learning curve, the impairment of scopolamine treated animals seemed most pronounced early in training, after which some constancy was reached between the treatment groups. This is best illustrated by the increased path

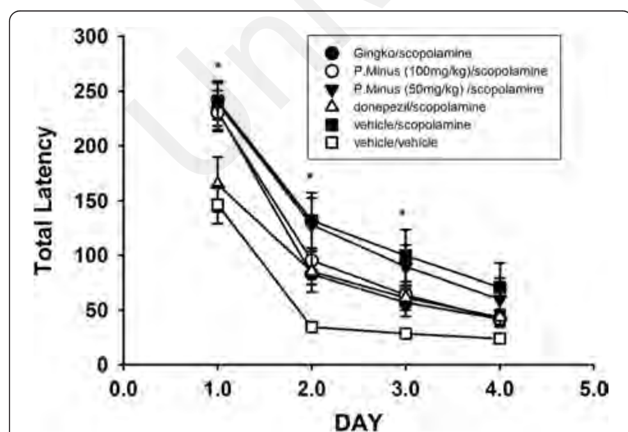

Figure 3 Total latency in the time taken to escape. Scopolamine treated mice showed a significant increase in latency when compared to vehicle treated mice (*P > 0.05). Total latency decreases for all treatments over time.

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Table 3 Duration in target segment during probe trial

		Duration in target segment during probe trial (sec)			
Day	Treatment	Mean	SE	F value	P value
5	***G.biloba* 50 mg/kg**	20.5938462	3.44090282	(7,100) = 4.2333	p = 0.0004***
5	***P.minus* 50 mg/kg**	18.3257143	3.70012143	(7,105) = 1.7621	p = 0.1036 n.s
5	***P.minus* 100 mg/kg**	19.7230769	2.53861445	(7,95) = 6.6852	p = 0.0015**
5	**Donepezil 1 mg/kg**	16.6314286	1.65977983	(7,111) = 6.9417	p < 0.0001***
5	**Veh/Sco**	16.5533333	2.48504781	(7,88) = 1.7926	p = 0.0999 n.s
5	**Veh/Veh**	19.7384615	1.69910151	(7,103) = 6.8633	p < 0.0001***

***, One-way ANOVA demonstrated significant duration in the target segment p < 0.0001.
**, One-way ANOVA demonstrated significant duration in the target segment p < 0.001.
n.s, non significant.

length in the scopolamine treated mice on days one, two and three, but not day four. These deficits were attenuated by the positive controls – donepezil and the *G. biloba* extract.They have both been reported to reverse scopolamine induced deficits in learning [29]. The *P. minus* extract, when administered at 100 mg/kg, attenuated scopolamine induced deficits in the acquisition phase of the Barnes maze task.

The dose of 100 mg/kg *P. minus* extract also reversed scopolamine induced deficits in the retention (probe trial) aspect of the task. In the probe trial, all the treatments except the lower dose of 50 mg/kg of *P. minus* attenuated scopolamine induced deficits. These deficits were described by no significant preference for the target segment by the mice. The lack of effect of the lower dose and the significant effect of the higher dose would suggest that there was a dose dependent action of the extract. The results suggest that an extract of *P. minus* can attenuate scopolamine induced learning and memory deficits in mice.

Decreases in cholinergic tone are associated with cognitive dysfunction and are reported in neurodegenerative diseases such as Alzheimer's [38,39]. Increasing cholinergic tone ie the levels of acetylcholine with the use of cholinesterase inhibitors such as donepezil has been used to address cognitive decline in mild to moderate Alzheimer's disease. The Barnes maze has been used to assess learning and memory in rodents [35]. It has several advantages over the more commonly used water maze in that it is less stressful for mice. It is an extensively used tool in behavioural neuroscience to investigate spatial learning and memory. Scopolamine which causes impairments in Barnes maze testing can be reversed by increasing cholinergic tone by the administration of a cholinesterase inhibitor such as donepezil [35]. Memory can be divided to short-term or long term memory where short term memory refers to holding information in conscious awareness for a duration of seconds whereas long term memory holds a larger amount of information for a longer period of time [40]. Working memory is a subset of short-term memory, required to perform certain mental operations during retention [41]. The Barnes radial maze has been used to assess learning and memory including working memory [42] seen in the animals of this study when locating the correct escape hole. Working memory errors are scored in this task as revisits to "incorrect" holes which subjects have already investigated within a probe trial. Cholinergic (acetylcholine) systems influence long term and working memory [43] as seen in the higher dose of *P. minus* and *G. biloba* group where the animals spent a longer duration in the target segment during probe trial as an indicator that the target segment location is remembered. The mice in lower doses of *P. minus*, the donepezil and scopolamine group demonstrated a shorter period within the target segment suggesting poorer memory for the target segment location that it was exposed to initially during the probe trial. The *in vitro* data from the present study, demonstrates that the extract has measurable anticholinesterase activity (68%), hence it may be that the extract induced increases in cholinergic tone, additionally providing an explanation for the attenuation of scopolamine induced deficits.

Scopolamine memory impairments have also been associated with brain oxidative stress [44] and scopolamine has been shown to trigger the induction of reactive oxygen species and to cause free radical injuries [45]. Herbal extracts with high antioxidant activity have been reported to scavenge free radicals and prevent scopolamine induced lipid peroxidation [26]. *P. minus* has been reported to possess up to 98.3% of lipid peroxidation inhibitory activities [2]. The antioxidant property of *P. minus* [8] may have contributed to the reactive oxygen species scavenging activity thus improving cognition and protecting against cognition decline [15]. It has been suggested that some of these antiamnesic effects are a direct result from antioxidant activity.

In considering human dosage, safety has been demonstrated in the acute toxicity test where oral administration of 2000 mg/kg of the standardised *P. minus* extract used in this study produced neither mortality nor changes in

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behavior or any other physiological activities [46]. The same paper reported in subacute 28-days for the dose of 1000 mg/kg, the no-observed-adverse-effect-level (NOAEL) of the extract was found to be more than 1000 mg/kg body weight in Wistar rats. Blood chemistry analysis including total protein, albumin, globulin, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), glucose, creatinine, urea nitrogen, total billirrubin, calcium, phosphorous, cholesterol, triglycerides, sodium and potassium and haemotological analysis in animals of both sexes, showed no significant changes at 1000 mg/kg.

In a recent randomized, double-blind, placebo-controlled crossover study of a propriety herbal blend (Super-Ulam) containing 150 mg of *P. minus* water extract as one of its ingredient, natural ingredients in brain health of individuals aged 35–65 years of age was evaluated [47]. There was an improvement in cognitive function based on computer assisted testing, demonstrated by a significant improvement from baseline in executive functioning, cognitive flexibility, reaction time, and working memory in subjects on the propriety herbal blend. There was a significant decrease in tension, depression, and anger measured by the Profile of Mood Scores (POMS) in subjects that consumed the blend when compared to placebo. It is possible that the improvement in cognition was a result of *P. minus* which was one of its major ingredient.

Conclusion

The present study confirms that water extract of *P. minus* has antioxidant activity with a high ORAC value of 16,964 μmole TE/gram and was able to reduce oxidative stress in a dose dependent manner. Higher dose of *P minus* (100 mg/kg) was able to attenuate scopolamine induced deficit in cognition *in vivo* by a reduction of total path length travelled and total errors prior to finding escape hole and increased duration in target segment during probe trial, indicating improved memory. These properties suggest that further investigations into the therapeutic potential of this extract for cognition could be a fruitful endeavour.

Competing interests

The authors declare that Annie George and Wong Hoi Jean are employees of Biotropics Malaysia Bhd. Biotropics Malaysia Bhd funded this study and the article processing fee. Chee Perng Ng, Matthew O'Callaghan and Gitte S. Jensen declare they have no conflict of interest. The findings of the study have been applied for patent by Biotropics Malaysia Bhd.

Authors' contributions

AG was responsible for the conception of the study and participated in the *in vivo* design and worked on the draft of manuscript. CPN and MOC designed, conducted and interpreted the research outcome of the *in vivo* study, performed the statistical analysis and worked on the draft of manuscript. GSJ performed the cell based antioxidant assay and made contribution to the revision of the draft manuscript. HJW worked on the standardisation of the herbal extract used in this study. All authors read and approved the final manuscript.

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3.2 Publication 2

George, A., Chinnappan, S., Choudhary, Y., Choudhary, V. K. Bommu, P., & Wong, H. J. (2015). Effects of a proprietary standardized *Orthosiphon stamineus* ethanolic leaf extract on enhancing memory in Sprague Dawley rats possibly via blockade of adenosine A2A receptors. *Evidence-Based Complementary and Alternative Medicine*, *2015,* ID375837.

3.2.1 Contribution of co-authors

Name	**Contribution**
Annie George	Involved in identification of *in vitro* target enzyme assay, design of animal study, interpretation of data and manuscript writing.
Sasikala Chinnappan	Assisted in animal study identification and manuscript.
Yogendra Choudhary	Assisted in interpretation of animal study and manuscript.
Vandana Kotak	Assisted in monitoring and coordinating the animal study.
Praveen Bommu	Assisted in animal study and interpretation of animal study.
Wong Hoi Jin	Involved in the characterisation and standardisation of the extract used in the study.

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Research Article

Effects of a Proprietary Standardized *Orthosiphon stamineus* Ethanolic Leaf Extract on Enhancing Memory in Sprague Dawley Rats Possibly via Blockade of Adenosine A_{2A} Receptors

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Th aim of the study was to explore a propriety standardized ethanolic extract from leaves of *Orthosiphon stamineus* Benth in improving impairments in short-term social memory *in vivo*, possibly via blockade of adenosine A_{2A} receptors (A2AR). Th ethanolic extract of *O. stamineus* leaves showed signific nt *in vitro* binding activity of A2AR with 74% inhibition at 150 μg/ml and signific nt A2AR antagonist activity with 98% inhibition at 300 μg/mL. A signific nt adenosine A_1 receptor (A1R) antagonist activity with 100% inhibition was observed at 300 μg/mL. Its effect on learning and memory was assessed via social recognition task using Sprague Dawley rats whereby the ethanolic extract of *O. stamineus* showed signific nt ($p < 0.001$) change in recognition index (RI) at 300 mg/kg and 600 mg/kg p.o and 120 mg/kg i.p., respectively, compared to the vehicle control. In comparison, the ethanolic extract of *Polygonum minus* aerial parts showed small change in inflexi n; however, it remained insignific nt in RI at 200 mg/kg p.o. Our findings suggest that the ethanolic extract of *O. stamineus* leaves improves memory by reversing age-related defic ts in short-term social memory and the possible involvement of adenosine A_1 and adenosine A_{2A} as a target bioactivity site in the restoration of memory.

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1. Background

Orthosiphon stamineus Benth (Lamiaceae) is a herbaceous perennial plant, widely distributed throughout the tropical regions, especially in Southeast Asia. It is commonly known as cat's whiskers. It is also known as misai kuching in Malaysia and kumis kuching in Indonesia [1]. It is referred to as java tea and consumed as an herbal tea in Europe for urinary flushing (*European Herbal Pharmacopoeia*). Th leaves of *O. stamineus* are traditionally used in South East Asia for a variety of ailments such as bladder and kidney disease (due to its strong diuretic effect), detoxific tion, relieving joint stiffness and inflammation including arthritis and rheumatism, gout, treating catarrh of the bladder, eliminating stones from the bladder, and treating diabetes mellitus [2, 3]. Scientifi studies have further reported the herb to possess anti-inflammatory [4], antioxidant [5, 6], antibacterial [7], hepatoprotective [8], diuretic [9], antihypertensive [10], and hypoglycemic effects [11].

Several classes of bioactive compounds such as fl vonoids, diterpenes, triterpenes, saponins, sterols organic acids, caffeic acids derivatives, chromenes, and oleanic and ursolic acid are known for *O. stamineus* [12–16]. Recent studies have emerged on the flavonoids of *O. stamineus* possessing antagonist activity on adenosine A_1 receptors (A1R) [17]. While the study focused more on the role of the receptors in diuretic activity, adenosine receptors in the central nervous system have also been implicated in the modulation of cognitive functions [18]. While the A1R antagonist activity has been reported in *O. stamineus*, A2AR antagonist activity was not.

Th adenosine receptors have been associated with sleep and arousal, cognition, and memory and with protecting from neuronal damage and degeneration as well as influencing neuronal maturation [19]. Endogenous adenosine is generally known to modulate cognition through the activation of adenosine A_1 receptors. Evidence is now emerging on a possible role of A_{2A} receptors in learning and memory [20]. Th adenosine receptors A_1 and A_{2A} belong to the G-protein-coupled receptor family [18] and antagonist actions on these receptors produced CNS-enhancing effects. Selective blockade of A_1 and A_{2A} receptors were shown to facilitate learning and memory *in vivo* [21, 22]. Th y might also protect against memory dysfunction shown in experimental models of aging such as Alzheimer's disease.

Th social recognition test (SRT) has been used in studies with caffeine, an adenosine A_{2A} receptor antagonist, in reversing cognitive decline in age-related defic ts in olfactory discrimination, Parkinson's disease, and attention defic t hyperactivity disorder (ADHD) [21, 23]. Using the social recognition test, an adenosine A_{2A} receptor antagonist demonstrated the ability to reverse short-term memory loss in Spontaneously Hypertensive Rats (SHR) which have impairments across several cognitive domains such as attention, short-term memory, and spatial reference memory [20].

Th social recognition test was fi st introduced by Th r and Holloway [24] and is based on the premise that rodents spend more time with unfamiliar juveniles than familiar ones. Memory-enhancing drugs are used in this model to investigate whether the duration of investigation is reduced when the juvenile rat is presented twice. The social recognition test in rats has become increasingly popular for the pharmaceutical industry as a tool to evaluate compounds for procognitive activity. This memory test probes short-term recognition/working memory to investigate novel target mechanisms relevant to cognitive impairment including neuropsychiatric disorders such as dementia, Alzheimer's disease (AD), schizophrenia, and Parkinson's disease (PD). Importantly the test uses spontaneous naturalistic behavior of an adult rat when exposed to a juvenile conspecifi on two occasions to access cognition, where the output measured (recognition index (RI)/ratio of investigation duration between the two sessions) involves an assessment of social exploration, strongly influenced by an olfactory component. As a result, SRT animal model was selected in this study.

Antagonists to A2AR are not the only target when seeking cognition enhancing treatment. Th inhibitory effects on other target sites such as acetylcholinesterase and serotonin have shown improvement in memory and cognition. One such plant preparation shown to possess antiacetylcholinesterase activity, a neurotransmitter related to learning and memory, is the standardized extract of *Ginkgo biloba*. Standardised extracts of *G. biloba* were shown to improve memory and normalized cognitive defic ts in animal models [25, 26]. Meanwhile, leaves of another Malaysian herb, *Polygonum hydropiper*, have been reported to also possess anti-acetylcholinesterase activity and recently its related species *P. minus* demonstrated enhanced memory in rats study using the Barnes maze test and demonstrated anticholinesterase activity [27]. The purpose of this study is to evaluate *O. stamineus* leaf ethanolic extract for cognition-improving benefits and adenosine A_{2A} receptor as a possible target. Th effect is compared with *G. biloba* and *P. minus* extract, for memory improvement in an SRT animal model.

2. Materials and Methods

2.1. Extract and Drug

2.1.1. O. stamineus Leaves. *O. stamineus* leaves, of white flower variety, procured from Biotropics Malaysia Berhad, Malaysia, were harvested at maturity approximately 3 months after planting. The plant material was identifi d on the basis of exomorphic characters and literature review by a taxonomist from the Institute of Bioscience, Universiti Putra Malaysia (UPM). Th voucher specimen of *O. stamineus* (SK 2083/12) was deposited in the Herbarium, Institute of Bioscience, UPM of Malaysia.

2.1.2. Ethanolic Extract of O. stamineus Leaves. 1000 g of *O. stamineus* leaves was dried by oven at a temperature of 40°C for 48 hours and ground into a fine powder using a lab mill (Retsch ZM200, Haan, Germany) and was extracted twice with 2 L and 1.5L of 70% ethanol in water (v/v) using ultrasonic treatment for a period of 30 min at room temperature. Th solution was separated from the remaining material. The organic solvent was removed under reduced pressure at 40°C and dried.

2.1.3. HPLC Analysis of O. stamineus Ethanolic Extract. Th extract was characterized using HPLC techniques based on seven known compounds of *O. stamineus* used as reference standards [28]. Th compounds were 3′-hydroxy-4′,5,6,7-tetramethoxyflavone, sinensetin, orthosiphol B, orthosiphol A, staminol A, orthosiphonone A, and ombuin (3,3′,5-trihydroxy-4′,7-dimethoxyflavone). HPLC analysis of the extract was performed using Agilent 1200 Liquid Chromatography (LC) with a photodiode array detector on Zorbax Eclipse XDB-C18, 4.6 × 150 mm, 5 μm column. Th mobile phase consisted of solvent A: water and solvent B: acetonitrile. Th following gradient was used: 0–8 min, 70% A; 8–15min, 70–53% A; 15–30 min, 53–49% A, hold for 10 min; 40–42 min, 49–0% A, hold for 4 min; 46–48 min, 0% A for fin l washing and equilibrium of the column for the next run. Operating conditions were set at fl w rates of 1 mL/min, column temperature at 25°C, UV detection at 230 nm, and injection volume of 5 uL. The extract at the concentration of 50 mg/mL was first injected followed by the mixture of the standards. Identific tion of the marker compounds was achieved by comparing with retention times of reference standards and their UV spectra.

2.1.4. Aqueous Extract of P. minus. 1000 g of aerial parts including stem and leaves of the plant was harvested at maturity approximately 2 months aft r planting and was dried by oven drying at the temperature of 40°C for 48 hours and shredded to 2 to 5 cm in size. Th dried leaves were extracted according to the method described in George et al. [27]. Th

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Table 1: Adenosine receptor A_{2A} binding assay parameters.

Adenosine A_{2A}	
Source	Human recombinant HEK-293 cells
Ligand	0.05 μM [3] CGS-21680
Vehicle	1% DMSO
Incubation time/temp.	90 minutes at 25°C
Incubation buffer	50 mM Tris-HCl, pH 7.4, 10mM $MgCl_2$, 1mM EDTA, 2 U/mL adenosine Deaminase
Nonspecifi ligand	50 μM NECA (5-N-ethylcarboxamide adenosine)
KD	0.064 μM
B_{max}	7 pmole/mg protein
Specifi binding	85%
Quantitation method	Radioligand binding
Signific nce criteria	≥50% of max stimulation
Reference	CGS-21680

Table 2: Adenosine receptor A_{2A} functional assay parameters.

Adenosine A_{2A} adenylyl cyclase	
Source	Human recombinant HEK-293 cells
Control	0.1 μM NECA
Vehicle	0.40% DMSO
Incubation time/temp.	10 minutes at 37°C
Incubation buffer	Modifie Hank's balanced salt solution (HBSS) pH 7.4
Quantitation method	HTRF quantitation of cAMP accumulation
Signific nt criteria for agonist	≥50% increase in cAMP relative to NECA response
Signific nt criteria for antagonist	≥50% inhibition of NECA-induced cAMP increase

Table 3: Adenosine receptor A_1 functional assay parameter.

Adenosine A_1	
Source	Wistar rat vas deferens
Control	0.3 μM CHA (N6-cyclohexyladenosine)
Vehicle	0.10% DMSO
Incubation time/temp.	5 minutes at 32°C
Incubation buffer	KREBS pH 7.4
Quantitation method	Isometric (gram changes)
Signific nt criteria for agonist	≥50% reduction of neurogenic twitch relative to 0.3 μM CHA response
Signific nt criteria for antagonist	≥50% inhibition of 0.3 μM CHA-induced relaxation

dried leaves were then subjected to percolation using purifi d water and extracted at a temperature of about 80°C with an extraction ratio of approximately 1:10. Th extract was further filtered, concentrated using rotary evaporator with the water bath temperature of 65°C, and freeze-dried. Th voucher specimen of the plant (SK 2077/12) was deposited in the Herbarium, Institute of Bioscience, UPM, Malaysia.

2.2. In Vitro Adenosine Receptors A_{2A} and A_1 Assays. Th adenosine A_{2A} receptor (A2AR) and A_1 receptor (A1R) assays were performed to determine test item's A2AR and A1R blockade activity. *O. stamineus* extract was tested at 15 and 150 μg/mL for A_{2A} binding assay, and the method employed was adapted from the one described by Varani et al. [29]. Adenosine A_{2A} and adenosine A_1 functional assays were performed at 3, 30 and 300 μg/mL and the method was adapted from Paucher et al. [30] and Taylor et al. [31], respectively. Adenosine A_{2A} binding assay, selective adenosine A_{2A}, and adenosine A_1 antagonist assays were conducted by Eurofi Panlabs (previously known as Ricerca) with test catalog numbers of 200610, 300500, and 401000, respectively. Reference standards were run as an integral part of all three assays to ensure the validity of the results. Th assays were performed under conditions described in Tables 1–3.

2.3. Animals. Ninety adult male SD rats (3-month-old, 200–250 g) and juvenile male rats of the same strain (35–40-day-old, 75–100 g) from the National Institute of Nutrition, Tarnaka, Hyderabad, were used as described in Table 4. The diet comprised standard pellet diet by Provimi (Nutrilab Rodent). Juvenile rats were kept in groups of ten per cage and served as social stimuli for the adult rats. The animals were maintained in a room under controlled temperature (22 ± 2°C), with relative humidity of between 50 and 70% and were subjected to a 12 h light cycle (lights on 8:00 a.m.) with free access to food and water. All the experimental procedures (IAEC/CPCSEA approval number 1412/a/11 in February 2012) were performed according to the guidelines on animal care of the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted on November 26th, 1997, by decision of the OECD Council [C(97)186/Final].

Table 4: Animal grouping according to test materials, dose, and route of administration.

Group number	Dose level	Route	Number of animals
1	Vehicle control	p.o.	10
2	BT 00119 (200 mg/kg)	p.o.	10
3	BT 00119 (300 mg/kg)	p.o.	10
4	BT 00119 (600 mg/kg)	p.o.	10
5	PME 00012 (200 mg/kg)	p.o.	10
6	GBE 000120 (120mg/kg)	p.o.	10
7	Donepezil (3 mg/kg)	i.p.	10
8	BT 00119 (60 mg/kg)	i.p.	10
9	BT 00119 (120 mg/kg)	i.p.	10

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2.4. Treatment. Th plant extract of *O. stamineus* (doses 60, 120, 200, 300, and 600 mg/kg b.w.), a commercial extract of *G. biloba* (120 mg/kg, standardised to 27.25% Ginkgo fl vonglycosides, 6% Terpene lactones, and ≤ 5 ppm ginkgolic acid determined through HPLC methods), water extract of *P. minus* (200 mg/kg), and the drug donepezil (ARICEPT tablet, Zydus Cadila Ltd., 3 mg/kg) were dissolved in distilled water. Th control solution consisted of distilled water (vehicle). Th extract of *O. stamineus* was tested i.p. and orally. Extracts of *O. stamineus* at doses of 60 and 120 mg/kg b.w. and donepezil at 3 mg/kg b.w. were administered i.p. for a direct comparison to donepezil activity, 120 min before the second encounter C2. In addition, extracts of *O. stamineus* at doses of 200, 300, and 600 mg/kg b.w., *G. biloba* extract at a dose of 120 mg/kg, a concentration derived from past animal studies of *G. biloba* in cognition-related investigations [32], and 200 mg/kg water extract of *P. minus* (as a direct comparison with the lower dose of the test extract) and vehicle were administered orally, 120min before the second encounter C2.

2.5. Social Recognition Test. Short-term social memory was assessed with the SRT described by Mondadori et al. [33]. Nine groups of rats, each consisting of 10 males, were used for the study. Adult Sprague Dawley (SD) rats were housed individually in polycarbonate cages and they were used only aft r at least 7 days of habituation to their new environment. Th test was scored in a consistent manner in an observation room, where the rats had been habituated for at least 1 h before the beginning of the test. All juveniles were isolated in individual cages for 30 min prior to the beginning of the experiment. Th SRT consisted of two successive presentations (5–10 min each) separated by a short period of time where a juvenile rat was placed in the home cage of the adult rat and the time (s) spent by the adult in investigating the juvenile (nosing, sniffing, grooming, or pawing) was recorded (C1). At the end of the fi st presentation, the juvenile was removed and kept in an individual cage during the delay period and reexposed to the adult rat after 120 min and time (s) spent by the adult in investigating the juvenile was recorded (C2). In this paradigm, a reduction in the investigation time during the second encounter refle ts the recognition ability of the adult rat. A pretest was performed for verific tion that the test compounds themselves do not have effects on social investigation per se. In this experiment, a different juvenile to the one used in the fi st presentation was exposed to the adult rat during the second encounter, with a similar duration of social investigation time being expected. RI was calculated using the formula (RI = C2/C1) for social recognition assay.

All values are expressed as means ± SEM (n equals the number of rats included in each analysis). Th RI (RI = C2/C1) was calculated for social recognition assay. The data was analyzed by comparing control versus treatment and standard and changes in activity before and aft r treatment (C1 versus C2) and RI versus control, standard, and treatment using Student's t-test by Graph Pad Prism 4.0 software.

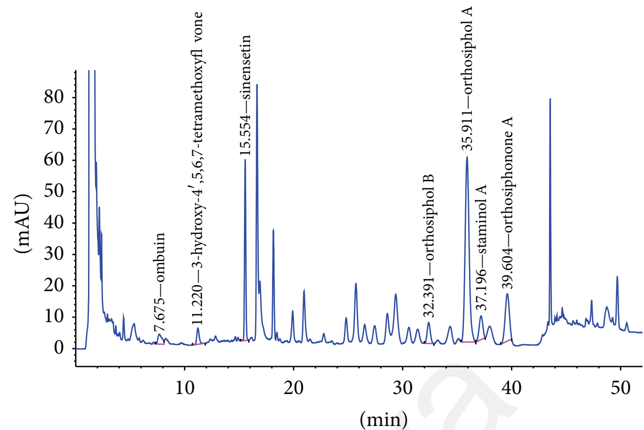

Figure 1: HPLC chromatograms of *O. stamineus* leaf ethanolic extract.

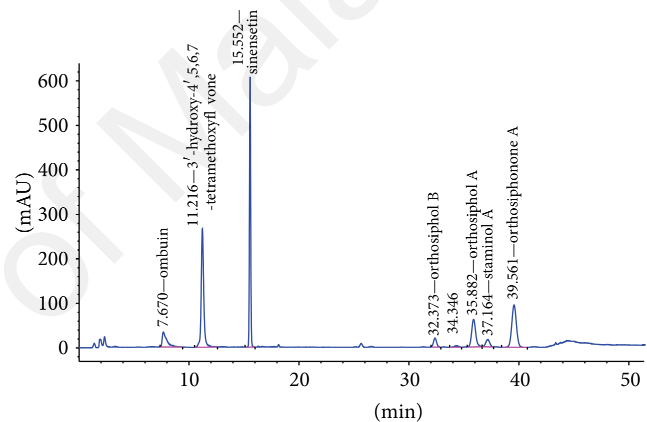

Figure 2: HPLC chromatograms of reference standard compounds. Th identifie peaks are ombuin (3,3′,5-trihydroxy-4′,7-dimethoxy-fl vone), 3′-hydroxy-4′,5,6,7-tetramethoxyfl vone, sinensetin, orthosiphol B, orthosiphol A, staminol A, and orthosiphonone A.

3. Result

3.1. Characterization of O. stamineus Ethanolic Extract. Chromatographic profile of *O. stamineus* ethanolic extract composition and reference compounds are as shown in Figures 1 and 2, respectively. The peaks corresponding to selected seven compounds were identified based on retention time against reference standards, and the UV spectrum. The peaks of ombuin (3,3′,5-trihydroxy-4′,7-dimethoxyfl vone) (0.14%), 3′-hydroxy-4′,5,6,7-tetramethoxyfl vone (0.10%), sinensetin (0.07%), orthosiphol B (0.26%), orthosiphol A (0.67%), staminol A (0.45%), and orthosiphonone A (0.12%) are eluted at retention times 7.675 min, 11.220 min, 15.554 min, 32.391 min, 35.911 min, 37.196 min, and 39.604 min, respectively. The resulting standardized extract is based on the group of marker compounds.

3.2. In Vitro Adenosine A_{2A} Receptor (A2AR) and Adenosinse A_1 Receptor (A1R) Assays. Th ethanolic extract of *O. stamineus* leaves showed signific nt binding activity with 74%

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Table 5: Results of *in vitro* adenosine A_{2A} and adenosine A_1 assays.

Assay	Concentration (μg/mL)	Inhibition (%)	IC_{50} (μg/mL)
Adenosine A_{2A} binding assay	15	17	60.07
	150	74	
Adenosine A_{2A} functional assay antagonist	3	14	51.5
	30	26	
	300	98	
		Increase in cAMP (%)	
Adenosine A_{2A} functional assay agonist	3	−1	—
	30	−3	
	300	−8	
Adenosine A_1 functional assay antagonist activity	3	0	95.1
	30	0	
	300	100	
		Reduction in neurogenic twitch (%)	
Adenosine A_1 functional assay agonist activity	3	5	—
	30	12	
	300	29	

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inhibition of A2AR at a dose of 150 μg/mL and antagonist activity in the A_{2A} functional assay at 300 μg/mL with 98% inhibition of cAMP response induced by NECA (Table 5). The extract showed similar activity in A1R inhibition, with an antagonist activity at 300 μg/mL where the extract displayed 100% inhibition of response induced by cyclohexyladenosine (CHA). Th antagonist activity of the *O. stamineus* leaves ethanolic extract to adenosine A_{2A} and adenosine A_1 receptors suggests the biological activity of *O. stamineus* in an *in vitro* system. The IC_{50} for A2AR binding activity is estimated at 60.07 μg/mL and determined with nonlinear regression analysis by Inplot GraphPad Prism, San Diego, CA, computer program. The IC_{50} for A1R antagonist is 95.1 μg/mL (Figure 3). Th IC_{50} for A2AR antagonist based on the response curve is 51.5 μg/mL (Figure 4). Th Ki value for the A2AR binding assay is calculated using the Cheng-Prusoff equation (1973) and is estimated at 33.72 mM.

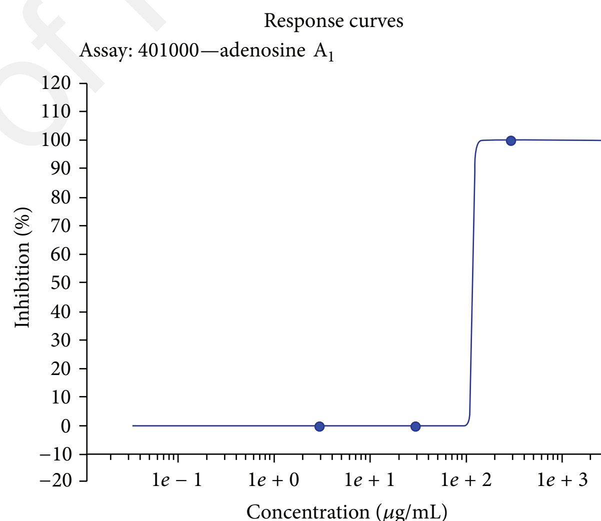

Figure 3: Response curve for adenosine A_1 antagonist assay. *Th IC_{50} of adenosine A_1 antagonist assay for *O. stamineus* ethanolic extract is 95.1 μg/mL.

3.3. Social Recognition Test. In the SRT procedure, SD rats presented a clear impairment of the juvenile recognition ability (recognition index) in comparison to control rats ($p < 0.001$), since control group spent as much time investigating the juvenile rat during the second encounter as they did on the first exposure. Th difference between treated and control groups on juvenile recognition ability is showed with more details in Table 6, with detailed analysis of the investigation time. Th investigatory behaviour of the adult SD rats was concentrated in the fi st 5 min of the juvenile presentation, with a signific nt reduction in the investigation time during the second encounter 120 min later. Th effects of the administration of acute doses of *O. stamineus* extract (200, 300, and 600 mg/kg, p.o., and 60, 120 mg/kg, i.p.), *P. minus* (200 mg/kg, p.o.), *G. biloba* (120 mg/kg, p.o.), donezepil (3 mg/kg, i.p.), and the vehicle (p.o.) in the SD rats social investigation time are given in Table 6. *O. stamineus* extract has shown signific nt ($p < 0.001$) change in RI compared to vehicle control at an oral dose of 300 mg/kg and 600 mg/kg, respectively. It also exerted significant ($p < 0.001$) change in RI at a dose of 120 mg/kg i.p. compared to vehicle control. However, 200 mg/kg oral and 60 mg/kg i.p. dose remained insignific nt for *O. stamineus* extract. The reduction in inflexi n was further confi med with signific nt ($p < 0.05$, $p < 0.001$, and $p < 0.05$) change in activity before (C1) and after (C2) treatment for *O. stamineus* extract group, at oral doses of 300 mg/kg, 600 mg/kg, and 120 mg/kg i.p., respectively (Table 4, C1 versus C2 signific nce). The extract

Table 6: Effect of *O. stamineus* (BT 00119), *P. minus* (PM 00012), *G. biloba* (GBE 00110), and donepezil on recognition index with respect to duration of interactions in social recognition test in the SD rats.

Treatment (mg/kg) p.o./i.p., immediately aft r C1	Route	Investigation duration (seconds) First contact (C1)		Second contact (C2) 120 min after C1		C2 versus C1	Recognition index (C2/C1)	
		Mean ± SEM	p value	Mean ± SEM	p value	p value	Mean ± SEM	p value
Vehicle	p.o.	22.00 ± 11.59	—	25.00 ± 13.0	—	0.1777	1.107 ± 0.20	—
BT 00119 (200)	p.o.	28.67 ± 5.36	0.3146	14.33 ± 8.51	0.2656	0.1273	0.5033 ± 0.24	0.0661
BT 00119 (300)	p.o.	57.33 ± 7.53[a*]	0.0315	14.33 ± 2.90	0.2348	0.0119[b*]	0.2400 ± 0.02	0.0068[c***]
BT 00119 (600)	p.o.	71.00 ± 7.81[a*]	0.0124	8.333 ± 1.85	0.1374	0.0051[b***]	0.1133 ± 0.017	0.0042[c***]
PME 00012 (200)	p.o.	48.67 ± 6.88	0.0595	31.00 ± 5.68	0.3475	0.1419	0.6833 ± 0.18	0.0986
GBE 00110 (120)	p.o.	52.67 ± 5.69[a*]	0.0382	33.67 ± 2.84	0.2759	0.0775	0.6600 ± 0.11	0.0651
Donepezil (3)	i.p.	30.67 ± 1.20	0.2492	18.33 ± 4.33	0.3266	0.0621	0.5500 ± 0.11	0.0593
BT 00119 (60)	i.p.	26.67 ± 4.80	0.3644	8.667 ± 5.23	0.1550	0.1801	0.3867 ± 0.28	0.0539
BT 00119 (120)	i.p.	51.00 ± 2.51[a*]	0.0354	14.00 ± 2.51	0.2272	0.0136[b*]	0.2733 ± 0.05	0.0086[c***]

p.o. = per oral, i.p. = intraperitoneal, and SEM = standard error mean.
[a*]$p < 0.05$ indicates the signific nce of firs contact in comparison with vehicle control for all groups.
[b*]$p < 0.05$ and [b***]$p < 0.001$ indicate the signific nce in comparing the change in activity before and after treatment.
[c**]$p < 0.01$ and [c***]$p < 0.001$ indicate the signific nt changes of RI when compared with vehicle control.

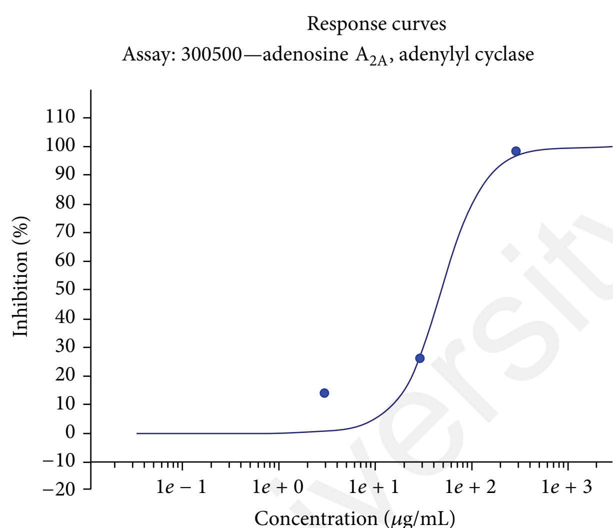

Figure 4: Response curve for adenosine A_{2A} antagonist assay. *Th IC_{50} of adenosine A_{2A} antagonist assay for *O. stamineus* ethanolic extract is 51.5μg/mL.

of *P. minus* and *G. biloba* has shown small change in inflexi n; however it remained insignificant for RI compared to vehicle, at an oral dose of 200 mg/kg and 120 mg/kg. The standard drug donepezil dosed at 3 mg/kg i.p. has shown change in inflexi n but no signific nt change in RI as compared to vehicle control.

4. Discussion

Th chemical constituents and the A2AR binding activity of *O. stamineus* extract have demonstrated that, with a single treatment of *O. stamineus* leaves extract after C1, the time spent in scrutinizing the same partner at a second meeting, 120 min later, is shortened. Th extract-induced reduction of the exploration time can be attributed to learning of the specifi information of the partner retained from the first meeting that reduced the need for new information. Th assumption that specific attributes of a particular partner were remembered is strengthened by the significant RI ($p < 0.001$) seen with the test extract. The ethanolic extract of *O. stamineus* leaves showed signific nt activity with 74% inhibition of A2AR at a dose of 150 μg/mL. Therefore, the study suggests the possible binding of the *O. stamineus* extract to A2AR, attributing the social recognition task with this biological activity.

Th present results demonstrate that the SD rats present a signific nt impairment of short-term social memory in SRT in the vehicle group as the RI of more than 1 signifie no improvement in recognition (RI should be <1). In fact, a longer time to recognize juvenile rat (C2) was observed for the vehicle group. The findings also suggest the involvement of the adenosine receptors in this response, since the acute administration of *O. stamineus* leaves extract reversed this social memory defic t in SD rats. Several studies have demonstrated that the selective blockade of adenosine A_1 and adenosine A_{2A} receptors facilitates learning and memory in rodents models [22, 34]. Th Ki value of the extract in this experiment was 33.72 mM. Caffeine and theophylline, another naturally occurring xanthine mainly found in tea, are nonselective AR antagonists. Thei stimulating properties are associated with micromolar range affinities for the A2AR. Although caffeine and theophylline have similar *in vitro* affinities for the A_{2A} receptor, caffeine has a higher stimulating effect due to a higher brain unbound fraction wt a Ki value of 23400 nM [35]. Th ugh caffeine derivatives possess stronger A2AR binding activities, the multicompounds that exist in *Orthosiphon stamineus* extract including flavonoids may have affected memory more than one way possibly also via other receptors such as the inhibition of acetylcholinesterase,

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thereby enhancing cognition [36]. Furthermore, the extract tested in this study also possessed additional A1R antagonist activity, as previously reported in *O. stamineus* [17]. From the SRT, promnesic property of *O. stamineus* leaves extract was observed in SD rats at dose dependent manner of 300 mg/kg and 600 mg/kg p.o. and 60 mg/kg and 120 mg/kg i.p. Thi study revealed that the *O. stamineus* leaves extract (300 and 600 mg/kg, p.o.; 60 and 120 mg/kg i.p.) exerted signific nt activity when compared to standard donepezil (3 mg/kg i.p.) statistically reaching signific nce in all except at 60 mg/kg i.p. where RI was only almost significant ($p < 0.0539$). Th *O. stamineus* leaves extract appears to prevent the amnesic effect of the long delay (120 minutes) where such a preventive effect may be deduced as promnesic. The extract at 120mg/kg i.p. was comparable to 300 mg/kg and 600 mg/kg orally dosed in RI, signifying greater bioavailability when administered i.p. at only one-third of the oral dosage.

As for *G. biloba* extract, a 120 mg/kg oral dose failed to demonstrate signific nt activity in this study but in another study, a single i.p. injection of *G. biloba* extract at 120 mg/kg dose demonstrated improvement in recognition performances in young rats in a similar olfactory animal model study [32]. This may be due to *G. biloba* having a lower bioavailability when administered through nonintravenous route (as observed in this study). The herb *P. minus* on the other hand has been shown to possess antiacetylcholinesterase activity in a recent study [27] although in the current study improvement in recognition index was not signifi ant. Th promnesic effects are probably more apparent in a model that tests attention rather than learning and memory from olfactory cues. This is in parallel to findi gs by Blokland [37] where it was suggested that the role of acetylcholine in learning and memory processes was still not conclusive, rendering its role more important in attention processes. A different animal model such as Barnes maze that tests spatial learning and memory instead of memory by olfactory and social cues, such as in this study, may have been a better model to investigate *P. minus* [27].

Based on the mean of RI for the extracts given i.p., donepezil fared better (in terms of lower mean) than the plant extract of *G. biloba* at their tested dosage though the route of administration was different. A2AR activity has never been known for *G. biloba* though anticholinesterase activity has been reported [25] and *in vivo* memory improvement has been documented for *G. biloba* extracts [26]. Donepezil, a reversible inhibitor of cholinesterase which is clinically used for treatment of dementia, showed slightly weak (RI at $p < 0.0593$) cognition enhancing properties at 3 mg/kg i.p. The effect of 3 mg/kg donepezil is similar to 60 mg/kg i.p. of *O. stamineus* extract based on the mean at C1 and having changes to RI aft r treatment at $p < 0.0593$ and $p < 0.0539$, respectively. Thi may be due to it being a popular acetylcholinesterase inhibitor for memory impairment which is age-related and long term such as Alzheimer and dementia. Th short reaction time (120 minutes) and the use of adult but not aged rats to impart a signific nt effect in the RI may have contributed to the weaker response in the Donepezil group.

Neurodegenerative disease can be the result of neuronal cell death caused by oxidative stress, apoptosis, and inflammation. Apart from A2AR activity, alcohol extracts of *O. stamineus* leaves may possess other biological activities that are neuroprotective. They have been reported to possess antiapoptotic effects in a H_2O_2 (a potent free radical) induced cell apoptosis [5]. The antioxidant properties of *O. stamineus* in addition may play a positive role in the prevention of neurogeneration caused by damaging free radicals [7]. The *O. stamineus* is known to contain several classes of bioactive compounds such as flavonoids, diterpenes, triterpenes, saponins, sterols organic acids, caffeic acids derivatives, chromenes, and oleanic and ursolic acid, known for [12–16]. Flavonoids have been shown to possess antioxidative and anti-infl mmatory effects that suggest neuroprotective property [36]. Oleanic acid which has been isolated from *O. stamineus* has been reported to protect against neuronal death induced by beta-amyloid in cultured rat cortical neurons and improve beta-amyloid induced memory defic t in mice [38]. Ursolic acid reduced the production of proinflammatory cytokines and neurotoxic reactive oxygen species, thus possibly leading to an additional neuroprotective effect [39].

Caffeine is another example of adenosine A_{2A} receptor antagonist that modulates the release of different neurotransmitters in the olfactory bulb of rodents [40] known to play a role in social olfactory recognition [41]. It appears to be that *O. stamineus* extract behaves similarly to caffeine in improving short-term memory and alertness. *O. stamineus*, however, does not contain caffeine but is rich in terpenoids and flavonoids. Terpenoids from natural products such as *G. biloba* and Asian ginseng (*Panax ginseng*) are currently being investigated as potential therapeutics in Alzheimer's disease, already showing some promise [42]. Terpenoids were identifi d in the *O. stamineus* extract used, that is, orthosiphol B and orthosiphol F, staminol A, and orthosiphonone A, which makes this extract a potential candidate for further investigation in the area of cognition disorder.

5. Conclusion

Our findi gs suggest that the propriety standardized ethanolic extract of *O. stamineus* may reverse age-related defic ts in short-term social memory and can be considered to prevent or decrease the rate of neurodegeneration. Th further investigation of not only adenosinergic but also other neurotransmitters in producing improvements in cognition should be evaluated in the future. The involvement of A_1 and A_{2A} blockade in the social memory defic t can be further clarifie in their role in the *Orthosiphon stamineus* effects along with selective A_1 and A_{2A} antagonist assayed in a social recognition tests for confirmation of target.

Conflict of Interests

The authors declare that Annie George, Sasikala Chinnappan, and Hoi Jin Wong are employees of Biotropics Berhad Malaysia which funded this study. Biotropics

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Malaysia Berhad has filed a patent based on this discovery (WO 2011/078652 A1). Yogendra Choudhary, Vandana Kotak Choudhary, and Praveen Bommu have no conflict of interests in this study.

Authors' Contribution

Annie George and Sasikala Chinnappan were responsible for the conception of the study and participated in the *in vivo* design and worked on drafting of the paper. Vandana Kotak Choudhary carried out the monitoring and coordination of the study, Yogendra Choudhary interpreted the data and participated in literature search and drafti g of the paper. Praveen Bommu carried out the experimental work and collected and interpreted the data. Hoi Jin Wong worked on the standardisation of the herbal extract used in this study. All authors read and approved the fin l paper. All authors contributed equally to this work.

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3.3 Publication 3

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3.3.1 Contribution of co-authors

Name	**Contribution**
Annie George	Conceived of the study, the *in vitro* assays, animal study design, and interpretation of data and writing of the manuscript.
Sasikala Chinnappan	Involved in the selection of animal study design, interpretation of data and contributed to manuscript.
Meena Chintamaneni	Assisted in animal study and interpreted data.
Vandana Kotak	Monitoring and coordinating of animal study.
Yogendra Choudhary	Contributed to interpretation of data and manuscript.
Thomas Kueper	Assisted in the *in vitro* enzyme assay.
Ammu K Radhakrishnan	Edited the manuscript.

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RESEARCH ARTICLE Open Access

Anti-inflammatory effects of *Polygonum minus (Huds)* extract (Lineminus™) in in-vitro enzyme assays and carrageenan induced paw edema

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Abstract

Background: The study was aimed to evaluate the anti-inflammatory activity of ethanolic and aqueous extracts of *Polygonum minus* (Huds) using in vitro and in vivo approaches.

Methods: The in vitro tests used to evaluate ethanolic extract are cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), lipooxygenase (5-LOX), secretory phospholipase-A2 (sPLA2) inhibition assay whilst the in-vivo effect was measured by the ability of aqueous extracts to reduce paw edema induced by λ-carrageenan, in rats.

Results: The ethanolic extract inhibited the activities of 5-LOX and COX-1($p < 0.05$) whilst the inhibitory effect on COX-2 was only moderate. A marked inhibition of 5-LOX was observed at 30 μg/ ml. The extract did not inhibit the activity of sPLA2. The ability of the ethanolic extracts of *Polygonum minus* to inhibit both 5-LOX and COX, prompted a study to evaluate the effects of using an aqueous extract of *Polygonum minus* (Lineminus^{TM}); as this would be more suitable for future clinical testing. The anti-inhibitory activity of the aqueous extract from this plant was evaluated using a rat model where inflammation was induced in the paws by injection of λ-carrageenan. The aqueous extracts from *Polygonum minus* administered at doses of 100 and 300 mg/kg body weight (b.w.), significantly ($p < 0.01$) reduced paw edema induced by λ-carrageenan in the experimental model, at 4 h compared to the vehicle control. Furthermore, administration of 100 mg/kg b.w. or 300 mg/kg b.w. completely reduced inflammation of the paw 4 h after injection.

Conclusion: These findings suggest that aqueous extract of *Polygonum minus* possesses potent anti-inflammatory activities.

Keywords: Inflammation, *Polygonum minus* (Huds), Paw edema, Cyclooxygenase, Lipooxygenase, Secretory phospholipase-A2

Background

Inflammatory reaction, typically characterized by redness, swelling, heat, and pain is one of the most important host defense mechanisms against invading pathogens. However, persistent or over-inflammation can result in tissue damage and possibly failure of vital organs. In a number of pathological conditions, acute and/or chronic inflammation can lead to acute or chronic inflammatory diseases such as septic shock, rheumatoid arthritis, gastritis, and atherosclerosis [1,2]. Over a period of time, inflammation due to trauma, genetic predisposition, stress and age can cause damage to cells of the body, thus releasing various membrane components that can activate the inflammatory process. The phospholipids liberated from the lipid bilayer membrane can be converted to arachidonic acid (AA) by the enzyme phospholipase A2 (PLA2). Arachidonic acid produced plays an important role in many metabolic pathways and is useful when produced in moderation. However in cases of severe inflammation such as joint damage, the AA is produced in excess [3]. Excess AA is converted by the cyclooxygenase (COX) and lipooxygenase (LOX) pathways into powerful

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inflammatory substances such as prostaglandins (PG) and leukotrienes (LT), respectively [4,5].

In the past few years, there has been concerted research focusing on the LOX pathway as controlling this pathway can play an important role in the relief of joint pain. The LOX pathway is a parallel inflammatory pathway to the COX pathway, where the AA produced is converted to LT, one of the strongest chemotactic agent produced in the body [6,7]. If left unregulated, these inflammatory pathways can cause joint damage. Cyclooxygenase-1(COX-1) and 5-lipooxygenase (5-LOX) are key enzymes involved in the formation of pro-inflammatory mediators such as eicosanoids from AA [8]. The COX-1 is constitutively expressed in many tissues and PGs produced by the action of COX-1 mediate housekeeping functions such as cytoprotection of gastric mucosa, regulation of renal blood flow and platelet aggregation, mostly through formation of eicosanoids from AA [9]. The 5-LOX enzyme plays a key role in the metabolism of AA to produce leukotrienes. Several studies suggest that there is a link between 5-LOX and carcinogenesis due to inflammation in human and animals [10]. Leukotrienes also play crucial roles as mediators in allergy and inflammation [11]. In addition, these pro-inflammatory mediators are also linked to some of the pathophysiological conditions of the brain such as cerebral ischemia, brain edema and brain tumors due to increased permeability of the blood–brain barrier (BBB) [12]. The 5-LOX enzyme is expressed in human brain tumors [10], hence it may play a role in inducing brain edema which causes brain tumor.

In the recent years, the use of herbal remedies for the treatment of inflammatory disease has been gaining momentum [13]. There has been some concern over the use of COX-2 inhibitors ie rofecoxib and valdecoxib for therapeutic interventions, causing some of these therapeutic products to be either withdrawn or made to carry a warning by the Food and Drug Authority (FDA) of the USA [14,15]. Due to risk of cardiovascular and skin related toxicities, rofecoxib and valdecoxib were withdrawn from the market in September 2004 and March 2005 respectively [16]. On the other hand, inhibitors of the 5-LOX enzyme that are of herbal origin are reported to offer significant relief and do not appear to have any adverse effects. Therefore, 5-LOX inhibitors of plant origin are gradually becoming the preferred choice of treatment for some of the diseases due to chronic inflammation [17,18].

Polygonum minus [(Huds) (Polygonaceae)] is a small herbaceous plant commonly known in Malaysia as Kesum [19]. This plant is a small, annual, slender, glabrous and erect to ascending herb, with tall or long branches [19]. This plant has a sweet and pleasant aroma and is commonly used by Malaysians as a flavoring ingredient [20] in Malaysian local dishes such as Laksa, Nasi Kerabu and Nasi Ulam [21]. Traditionally, this plant has been used to treat digestive disorders and dandruff. It is also used in the perfume industry because of its volatile oil [22,23]. It has proven to be a potent natural source of antioxidants and there are several reports that claim that it has a high level of free radical scavenging activity and reducing power [24]. Flavonoids have been reported in *Polygonum minus* [25] and these phenolic compounds have been associated with anti-inflammatory effects [26], but none reports anti-inflammatory effects of *Polygonum minus*.

Meanwhile, safety studies such as acute and subacute toxicity of aqueous extract of *Polygonum minus* (biotropics®PM101), in Wistar rats has shown that the no-observed-adverse-effect-level (NOAEL) following oral administration for 28 days, to be more than 1000 mg/kg body weight [27]. The aqueous, ether and ethanol extracts of *Polygonum minus* have also been extensively studied for their phenolic content, anti-oxidant and cytoprotective activity [28], hence this study is to investigate whether *Polygonum minus* extracts possess anti-inflammatory activity as a result of flavonoids content and antioxidant property.

In the present study, we investigated the anti-inflammatory activities of extracts obtained from the aerial parts consisting stem and leaves of *Polygonum minus* using in vitro and in vivo approaches.

Methods

Chemicals and drugs

The chemicals λ-carrageenan, diclofenac sodium and carboxy methyl cellulose (CMC) were purchased from Sigma-Aldrich Chemical Co. in Mumbai, India. Cyclooxygenase (COX) Inhibitory Screening Assay Kit, Lipoxygenase (5-LOX) Inhibitor Screening Assay Kit, Secretory Phospholipase A2 (sPLA2) (Type V) Inhibitor Screening Assay Kit, Diclofenac, Nordihydroguaiaretic acid (NGDA) and Arachidonic Thioester Phosphatidylcholine (TEPC) were procured from Cayman Chemical Company (Tallinn, Estonia).

Plant material

Polygonum minus was procured from Biotropics Malaysia Berhad, Malaysia. The plant material was identified on the basis of exomorphic characters and review of literature by a Taxonomist from Institute Bio Science, University Putra Malaysia (UPM). The voucher specimen of the plant (SK 2077/12) was deposited in the Herbarium, Institute Bioscience UPM of Malaysia. The aerial parts comprising the stem and leaves were extracted using different extraction techniques to produce ethanol extract for in-vitro assays and aqueous extract for in-vivo assay.

Extract preparation for in-vitro assays

The aerial parts were dried by oven drying at a temperature of 40°C for 48 hours and shredded to 2 to

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5 cm in size. Then, 100 g of the milled plant material were subjected to an organic extraction with 750 ml 90%:10% (v/v) ethanol: water mixture with aid of sonication at maximum temperature of 40°C for 30 minutes. The solvents were reduced by evaporation on a rotary evaporator at reduced pressure (15 mbar) to approximately 50 ml. The remaining mixture was added with water to a final volume of 100 ml. The mixture was extracted two times in liquid/liquid separation with 150 ml heptane to remove the lipophilic fraction, followed by three fold extraction in liquid/liquid separation with 150 ml ethyl-acetate to obtain polar and semi-polar fractions. The ethyl acetate phase was evaporated to dryness. After which, 50 mg of ethylacetate extract was dissolved in 100 μl methanol and applied on SPE column (RP-18), which was equilibrated with water and further eluted with 3 ml of 20%, 40% and 70% acetonitrile. The fractions that were eluted with 20%, 40% and 70% acetonitrile were collected, combined and evaporated to dryness.

Extract preparation for in-vivo anti-inflammatory activity

Aqueous extract was prepared for in vivo testing to mimic as close as possible the human consumption of the stems and leaves in cooking and traditional application. Hence, the aerial parts (comprising stem and leaves) were dried by oven drying at a temperature of 40°C for 48 hours and shredded to 2 to 5 cm in size. A total of 100 g dried aerial parts were then subjected to percolation using 1000 ml of purified water and extracted at a temperature of about 80°C. The extract was further filtered, concentrated using rotary evaporator with the water bath temperature at 65°C and freeze-dried. The extract was a standardised propriety extract with quercetin-3-glucuronide (0.59%) and quercitrin (0.27%) as marker compounds and trademarked as Lineminus™ [29]. The dried crude extract was dissolved in 0.5% CMC (carboxymethyl cellulose) solution to produce two test doses of 100 mg/kg and 300 mg/kg respectively prior to pharmacological testing.

Enzyme assays COX-1 & COX-2, 5-LOX and sPLA2

The enzyme assays are part of a preliminary screening program for anti-inflammatory activities to justify further in vivo test in the area of inflammation. Hence the ability of *Polygonum minus* extract to inhibit COX-1 & COX-2, 5-LOX and sPLA2 were evaluated.

Cyclooxygenase (COX-1 and COX-2) inhibition assay

The extract was dissolved in 100% DMSO to prepare a stock concentration of 10 mg/ml. The extract was tested in triplicates at 30 and 100 μg/ml using a commercial COX inhibitory screening assay kit as recommended by the manufacturer (Cayman test kit-560131, Cayman Chemical Company). The COX inhibitor screening assay directly measures the amount of Prostaglandin2α produced in the cyclooxygenase reaction. Diclofenac (MW = 296.14) was run as the positive control for inhibition of COX-1 and COX-2. A volume of 10 μl each of test extract and vehicle were diluted to 20 μl with 0.1 M Tris–HCl pH 8.0 and pre-incubated with the enzyme at 37°C for 15 minutes prior to the addition of AA. The reaction is initiated by addition of 10 μl 10 mM AA and the tube was incubated at 37°C for another 2 minutes. Reaction was terminated by addition of 50 μl 1 N HCl and saturated stannous chloride. Assays were performed using 100 units of ovine COX-1 and human recombinant COX-2. An aliquot is removed and the prostanoid produced is quantified spectrophotometrically via enzyme immunoassay (EIA).

Lipooxygenase (5-LOX) inhibition assay

The extract was tested in triplicates at 30 and 100 μg/ml using the LOX inhibitor screening assay kit using the protocol recommended by the manufacturer (Cayman test kit-766700, Cayman Chemical Company). This assay measures the hydroperoxides generated from incubating a 5-LOX enzyme with its substrate, AA. Nordihydroguaiaretic acid (MW = 302.36) was used as the positive control. A volume of 10 μl each of test extracts and vehicle were pre-incubated with 90 μl 5-LOX enzyme in a 96-well plate. The reaction was initiated by addition of 10 μl 1 mM AA and the plate was shaken for 5 minutes. Then, 100 μl of chromogen from the test kit was added to stop enzymic reaction and for color development. The plate was placed on a shaker for another five minutes and absorbance at 490 nm was measured using microplate reader.

Secretory Phospholipase A_2 (sPLA2) inhibition assay

The extract was tested in triplicates at 30 and 100 μg/ml using the sPLA2 (Type V) inhibitor screening assay kit as recommended by the manufacturer (Cayman test kit-10004883, Cayman Chemical Company). This assay kit contains a human recombinant Type V sPLA2 and reaction mixtures. Arachidonic thioester phosphatidylcholine was used as the positive control. A total of 10 μl each of test extract and vehicle were pre-incubated with 25 mM Tris–HCl buffer pH 7.5 containing 10 μl enzyme in a 96-well plate. The reaction was initiated by addition of 200 μl 1.66 mM Diheptanoylthio-PC and the plate was shaken for 30 seconds and incubated at 25°C for 15 minutes. Further, 10 μl 5,5′-dithio-bis-(2- nitrobenzoic acid) (DTNB) was added to stop the enzymatic reaction and to allow for color development. The plate was placed on a shaker for one minute to mix and absorbance was measured at 405 nm using plate reader.

Experimental animals

Thirty two male and female Wistar albino (WA) rats aged between 8–10 weeks and weighing between 200–245 g

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were allowed to acclimatize into the standard laboratory conditions. The rats were placed in a room with controlled temperature (22 ± 1°C), relative humidity (54 – 68%) and with 12 h light/12 h dark cycles for one week before they were used in the study. Animals were provided with Nutrilab rodent diet (M/s Provimi Animal Nutrition Pvt. Ltd. India) and aquaguard water ad libitum. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of SPTM, India [vide approval No. BIO/IAEC/479, dated September 03, 2012]. The study was conducted in accordance with the recommendation of the IAEC as well as the "Purpose of Control and Supervision of Experiments on Animals (CPCSEA)" guidelines for laboratory animal facility published in the Gazette of India, December 15th 1998.

Carrageenan-induced paw edema animal model

The test was conducted according to method based on previous published study [30]. Briefly, 0.1 ml carrageenan was injected into the sub-plantar region of the left hind-paw of each rat. The volume (ml) of induced edema was measured using a digital plethysmometer (IITC, USA). The rat's foot pad became edematous soon after the injection of carrageenan. The rats were then randomly divided into four groups of eight (n = 8) rats each. The rats in the four experimental groups were fed with the various test agents 30 min after the carrageenan injection; (i) vehicle control (CMC 0.5%); (ii) positive control [diclofenac sodium (10 mg/kg)]; (iii) *Polygonum minus* aqueous extract (100 mg/kg) and (iv) *Polygonum minus* aqueous extract (300 mg/kg); respectively. The paw volume of each rat was measured at different time intervals (0, 2, 4, and 6 h) after carrageenan injection using this instrument. The percentage inhibition was calculated using the formula 100 (1-Vt/Vc), where Vc was the mean edema volume observed in the control group whilst Vt was the edema volume measured in the test groups [31]. All data of each group were expressed as mean ± SEM (n = 8). Statistical analyses were performed with 'Graph pad prism v4' software and were carried out using one way ANOVA followed by Dunnet post-hoc test. Statistical significance was set at $p \leq 0.05$.

Results

Effect of Polygonum minus extract on in-vitro anti-inflammatory assays

Polygonum minus ethanolic extract showed dose-dependent inhibition of COX-1 and COX-2 with 100 and 25% inhibition respectively at 100 μg/ml (Table 1). Inhibition of 5-LOX at both tested doses (30 and 100 μg/ml) was 100%. The ethanolic extract from *Polygonum minus* did not show any inhibitory activity on the sPLA2 enzyme at both the concentrations (30 and 100 μg/ml) tested. The ethanolic extract from *Polygonum minus* inhibited COX-1, COX-2 and 5-LOX enzymatic activities.

Carrageenan-induced paw edema animal model

Injection of λ-carrageenan into the sub-plantar region of the left hind-paw rapidly induced paw edema. A significant ($p < 0.01$) reduction of paw edema volume was observed in the rats that were fed with the aqueous extract from *Polygonum minus* (100 and 300 mg/kg b.w.) after 4 h compared to the rats that were fed with the vehicle control (Table 2). These findings indicate that the aqueous extract of *Polygonum minus* posses potent anti-inflammatory properties. These findings show that the aqueous extracts (100 and 300 mg/kg b.w.) of *Polygonum minus* inhibited the inflammatory processes induced by the injection of λ-carrageenan in the paw of rats after 4 h of oral administration.

Discussion

Dual 5-LOX/COX inhibitors are potential new drugs to treat inflammation. These agents act by blocking the formation of both PG and LT but appear to have no measurable effects on the formation of lipoxin. These types of inhibitors of inflammation with dual functions help to avoid some of the disadvantages of selective COX-2 inhibitors [32].

Leukeotrienes (LTs) also play a major part in the inflammatory process [33]. These compounds are synthesized via the lipooxygenase pathway with the help of the 5-LOX enzyme. The present study shows that *Polygonum minus* extract has anti-inflammatory effects that can inhibit both the COX-2 and 5-LOX enzymes. The *Polygonum minus* has been reported to be rich with flavonoids [34,35]. Flavonoids can interrupt the oxidative generation of AA from phospholipids and reduce the downstream production of inflammatory metabolites from AA metabolism, oxidative damage, and induction of inducible inflammatory pathways due to their potent antioxidant capacity [36]. Based on a published paper [37] it was found that a wide variety of flavonoids modulate the activities of AA metabolizing enzymes such as PLA2, COX, and 5-LOX. The *Polygonum minus* aqueous extract used in this study was reported to possess high antioxidant content demonstrated by the ORAC score of between 16,000 to 35,000 μmol TE/g [29,38]. Other studies showed that flavonoids with antioxidant capacity could reduce the cellular conversion of AA to MDA (malondialdehyde) in patients suffering from chronic inflammation [39]. Hence, it is possible that the high antioxidant capacity and flavonoids content of the extract used, have contributed to the anti-inflammatory effects seen in in vitro testing.

In the present study the expression of reactive oxygen species (ROS), TNF-α and nuclear factor (NF)-κBprotein

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Table 1 Effect of *Polygonum minus* extract on COX-1 & COX-2, 5-LOX and sPLA2 inhibition

Receptor/enzyme	*Source*	*Substrate*	*% Inhibition of standard*		*% Inhibition of Polygonum minus at doses*	
			Standard	*% of inhibition*	*30 μg/ml*	*100 μg/ml*
COX-1	Ovine platelets	Arachidonic acid	Diclofenac (1 μM)	100	30	100
COX-2	Human recombinant	Arachidonic acid	Diclofenac (1 μM)	100	00	25
5-LOX	Human recombinant Lipooxygenase	Arachidonic acid	NGDA (100 μM)	100	100	100
sPLA2	Human recombinant Type V sPLA2	Diheptanoyl thio-PC	TEPC (20 μM)	90	00	00

were not evaluated but could be expected by the well-established antioxidant property of *Polygonum minus*, the current study that evaluated its anti-inflammatory activity and when comparing to the closely related *Polygonum hydropiper*, also known as laksa leaf in Singapore. Anti-inflammatory effects in vitro and in vivo for *Polygonum hydropiper* were reported, demonstrated by the inhibition of mRNA expression of pro-inflammatory genes such as COX-2, TNF-α, nuclear factor (NF)-κB and PG [40]. A parallel to this relative of *Polygonum minus* was drawn due to molecular systematic studies of the two showing 100% similarity using molecular genetic marker derived from four accessions of *Polygonum minus* [41]. These results suggest that *Polygonum minus* extract may have also inhibited formation of LTs from 5-LOX and may prevent accumulation of these key inflammatory factors, which contribute to tissue damage through a putative 5-LOX shunt seen with NSAIDs. The current experiments demonstrate that, unlike NSAIDs, *Polygonum minus* extract does not only inhibit COX metabolism of AA to PGs rather it acts via inhibition of both COX and 5-LOX enzyme activity. Other well researched flavonoids, such as green tea catechins and quercitin have been shown to inhibit sPLA2 thus modulating the generation of AA from membrane phospholipids [42,43]. The *Polygonum minus* extract in the tested doses did not inhibit PLA2 activity in this study. The result suggests that *Polygonum minus* extract does not have the ability to modulate the generation of AA from membrane phospholipids produced by the destruction of tissue. Instead, it appears that *Polygonum minus* extract might possess potent active principles, which inhibits 5-LOX enzyme. With this point of view, the anti-inflammatory activity of *Polygonum minus* extract was evaluated using an established rat model to study inflammation. This rat model is widely used to screen the ability of new anti-inflammatory agents to reduce local edema induced in the rat paw by injection of an irritant agent [44]. Carrageenan induced edema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The early phase (1–2 h) of the carrageenan model is mainly mediated by histamine, serotonin [45]. Flavonoids including quercetin as identified in the aqueous extract used in this study, have been reported to possess inhibitory effect on histamine release in mast cells which is an anti-inflammatory reaction [46]. The second phase of the carrageenan model is related to the release of prostaglandins and bradykinins [45,47]. The *Polygonum minus* aqueous extract showed significant anti-inflammatory effect in λ-carrageenan-induced rat paw edema around 4 hours after administration of carrageenan. The maximum inhibition takes place around 4 hours during which these anti-inflammatory mediators are expected to be

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Table 2 Effect of *Polygonum minus* extract on paw volume and percentage inhibition in carrageenan induced paw edema

Groups & Dose	*Mean paw volume ± SEM (mL) (% Inhibition[a])*			
	0 hr	*2 hr*	*4 hr*	*6 hr*
Vehicle control (0.5% CMC) 0 mg/kg b.w.	1.35 ± 0.03	1.46 ± 0.03	1.50 ± 0.03	1.42 ± 0.04
Standard (diclofenac)	1.38 ± 0.04	1.50 ± 0.02	1.37 ± 0.03*	1.41 ± 0.04
10 mg/kg b.w.	(−2.22%)	(−2.74%)	(8.66%)	(0.70%)
Polygonum minus aqueous extract	1.33 ± 0.02	1.47 ± 0.04	1.32 ± 0.03**	1.40 ± 0.05
100 mg/kg b.w.	(1.48%)	(−0.68%)	(12.00%)	(1.41%)
Polygonum minus aqueous extract	1.38 ± 0.03	1.42 ± 0.02	1.34 ± 0.02**	1.35 ± 0.04
300 mg/kg b.w.	(−2.22%)	(2.74%)	(10.67%)	(4.93%)

Values are expressed as mean ± SEM, n = 8 (*4Male + 4Female*); (−) = No inhibition.
[a]Inhibition is reported as a percentage compared to control.
*Vehicle control vs standard (p < 0.05).
**Vehicle control vs *Polygonum minus* (100 mg/kg) / *Polygonum minus* (300 mg/kg) (p < 0.01).

released. An anti-inflammatory effect was also observed in vivo in another closely related plant species, *Polygonum hydropiper*, which is also known as Kesum in Malay [40].

Conclusion

These studies provide evidence to show that the *Polygonum minus* exert an anti-inflammatory effect by inhibiting both COX and 5-LOX activity, which was studied using an in-vitro model and further qualified in vivo.

Abbreviations

COX-1: Cyclooxygenase-1; COX-2: Cyclooxygenase-2; 5-LOX: lipooxygenase; sPLA2: Secretory phospholipase-A2; B.W.: Body weight; AA: Arachidonic acid; PG: Prostaglandins; LT: Leukotrienes; BBB: Blood–brain barrier; FDA: Food and Drug Authority; CMC: Carboxy methyl cellulose; NGDA: Nordihydroguaiaretic acid; TEPC: Thioester Phosphatidylcholine; UPM: University Putra Malaysia; EIA: Enzyme immunoassay; DMSO: Dimethyl sulfoxide; DTNB: 5,5′-dithio-bis-(2 nitrobenzoic acid); WA: Wistar albino; IAEC: Institutional Animal Ethics Committee; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals; PGH2: prostaglandin H2; FLAP: 5-LOX activating-protein; ROS: Reactive oxygen species; MDA: malondialdehyde; PLA2: Phospholipase A2 activity.

Competing interests

We declare that Annie George and Sasikala Chinnappan are employees of Biotropics Berhad Malaysia who funded this study and the article processing fee. Yogendra Choudhary, Vandana Kotak C, Meena Chintamaneni, Thomas Kueper and Ammu K Radhakrishnan declare they have no conflict of interest in this study. The findings of the study have been applied for patent by Biotropics Malaysia Bhd.

Authors' contributions

AG and SC conceived of the study, its design, coordination and drafting of the manuscript, MC carried out the experimental work, collected and interpreted the data, VKC carried out the monitoring and coordination of the study, YC interpreted the data, participated in literature search and drafting of the manuscript, TK carried out the in vitro experimental work, collected and interpreted the data, AKR participated in literature search, review and drafting of the manuscript. All authors read and approved the final manuscript.

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3.4 Publication 4

Udani, J. K., **George, A.**, Musthapa, M., Pakdaman, M. N., & Abas, A. (2014). Effects of a proprietary freeze-dried water extract of *Eurycoma longifolia* (Physta) and *Polygonum minus* on sexual performance and well-being in men: A randomized, double-blind, placebo-controlled study. *Evidence-Based Complementary and Alternative Medicine*, *2014,* ID179529.

3.4.1 Contribution of co-authors

Name	**Contribution**
Jay K. Udani	Medical doctor and principal investigator of the clinical study and contributed to the manuscript.
Annie George	Involved in the clinical study design and protocol; analysis and writing of manuscript.
Mufiza Musthapa	Assisted in animal study and interpreted data.
Michael N. Pakdaman	Involved in manuscript.
Azreena Abas	Involved in coordinating the study.

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Research Article

Effects of a Proprietary Freeze-Dried Water Extract of *Eurycoma longifolia* (Physta) and *Polygonum minus* on Sexual Performance and Well-Being in Men: A Randomized, Double-Blind, Placebo-Controlled Study

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Background. Physta is a proprietary product containing a freeze-dried water extract of *Eurycoma longifolia* (tongkat ali), which is traditionally used as an energy enhancer and aphrodisiac. We aim to evaluate a 300 mg combination of Physta and *Polygonum minus*, an antioxidant, with regard to sexual performance and well-being in men. *Methods*. Men that aged 40–65 years were screened for this 12-week randomized, double-blind, placebo-controlled, parallel-group study. Outcome measures included validated questionnaires that aimed to evaluate erectile function, satisfaction with intervention, sexual intercourse performance, erectile hardness, mood, and overall quality of life. *Results*. 12 subjects in the active group and 14 in the placebo group completed the study. Signific nt improvements were noted in scores for the Sexual Intercourse Attempt diary, Erection Hardness Scale, Sexual Health Inventory of Men, and Aging Male Symptom scale ($P < 0.05$ for all). Th ee adverse events were reported in the active group and four in the placebo group, none of which were attributed to study product. Laboratory evaluations, including liver and kidney function testing, showed no clinically signific nt abnormality. *Conclusion*. Supplementation for twelve weeks with *Polygonum minus* and the proprietary *Eurycoma longifolia* extract, Physta, was well tolerated and more effective than placebo in enhancing sexual performance in healthy volunteers.

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1. Introduction

Erectile dysfunction (ED) occurs as a result of a lack of blood fl w to the penis, impairing the ability to achieve or maintain an erection suitable for sexual intercourse [1]. Thi condition affects approximately 30 million men in the United States and approximately 52% of men aged 40–70 years worldwide [1]. While the incidence of erectile dysfunction increases with age, men at all ages are at risk. Risk factors for ED include heart disease, hypertension, hyperlipidemia, diabetes, prostate disease, depression, stress, anxiety, smoking, and prescription/recreational drug use.

Testosterone is a steroid hormone that plays a key role in development and maintenance of the male reproductive system. As men age, the endogenous production of testosterone by the Leydig cells tends to decrease at a rate of 1.6% per year [2]. Decreased levels of testosterone, including levels that are still within the normal range, have been linked to erectile dysfunction, particularly in older men [2–4].

Th most popular prescription medications aimed at treating ED today are classified as cGMP-specific phosphodiesterase type 5 (PDE5) inhibitors, which act by regulating blood fl w to the penis. Examples of such medications include sildenafil (Viagra), tadalafil (Cialis), and vardenafil

(Levitra) [5–7]. However, side effects from these medications as well as their interactions with vasodilator therapy have left many men to turn to more natural options for management of their sexual health [8–10].

Eurycoma longifolia (Physta) is a tree native to the jungles of Malaysia, Thailand, and Indonesia and is believed to enhance sexual performance. Th root of *Eurycoma longifolia* has been promoted as a tonic, energy enhancer, and aphrodisiac [11–13]. An unpublished *in vitro* study demonstrated a fourfold increase in testosterone levels among human testicular cells when exposed to *Eurycoma longifolia*. *Eurycoma longifolia* has been found to facilitate conversion of pregnenolone to progesterone, cortisol, 5-dehydroepiandrosterone (DHEA), and testosterone in rabbit corpus cavernosum tissues [14]. An animal study has shown that consumption of *Eurycoma longifolia* in sexually sluggish and impotent male rats can lead to increased serum testosterone levels, reduced ejaculation latencies, and an increased likelihood of mounting and ejaculating [15]. Toxicity evaluation on *Eurycoma longifolia* has been performed in humans and the acceptable daily intake was determined to be 1.2 g per day for an adult male [16].

Polygonum minus is an aromatic plant originating from southeast Asia, belonging to the family Polygonaceae. It has fl vonoid properties and is widely used as a food additive [17]. It has been described to have antioxidant activity, antimicrobial activity, and antiulcer activity [18]. The e are no known interactions between *Eurycoma longifolia* and *Polygonum minus*.

Validated measures of male sexual health aim to measure subjective satisfaction, penile hardness, frequency and quality of sexual intercourse attempts, and overall physical activity. The erectile dysfunction inventory for treatment satisfaction (EDITS) questionnaire is a 5-point scale ranging from 0 to 4 [19]. This scale is used to evaluate change over time in response to treatment. Th sexual intercourse attempt (SIA) diary consists of 10 yes/no questions evaluating erectile function in healthy subjects. Th EHS is a four-point scale used to evaluate changes in hardness by rating hardness during each sexual intercourse attempt on a scale of 1 to 4. A higher score indicates increased satisfaction with erectile hardness [7]. Th sexual health inventory for men (SHIM) questionnaire is a six-point scale aimed at identifying the degree of erectile function, with a score of 21 or below indicating erectile dysfunction [6]. The aging male symptom score (AMS) is a six-point Likert scale ranging from 0 to 5 measuring satisfaction with overall physical function. A higher score indicates worse function [20]. Th index of erectile function (IIEF-5) is a six-point scale that aims to evaluate the effect of erection problems on an individual's sex life over the previous four weeks [21]. Th self-esteem and relationship (SEAR) questionnaire is a patient-reported measure of psychosocial variables that uses a six-point Likert scale ranging from 0 to 5 to evaluate confidenc , self-esteem, and the quality of sexual relationships [5]. Th beck depression inventory (BDI) and beck anxiety inventory (BAI) score individual items on a four-point scale (0–3) to subjectively evaluate the degrees of depression and anxiety, respectively [22, 23].

Thi randomized, double-blind, and placebo-controlled pilot study will provide valuable data with regard to the proposed effects of the *Eurycoma longifolia* containing proprietary product, Physta, in improving sexual performance, quality of life, and well-being.

2. Methods

2.1. Investigational Product. Th investigational product for this study was a combination of *Eurycoma longifolia* and *Polygonum minus* extracts. *Eurycoma longifolia* was prepared as Physta, a proprietary freeze-dried water extract of *Eurycoma longifolia* root with a drug-extract ratio (DER) of 1: 25. *Polygonum minus* leaves were prepared as a water extract with a DER of 1: 10. The study product tablets were produced under continuous quality of good manufacturing process requirements by Biotropics Malaysia (Berhad, Kuala Lumpur, Malaysia). Th placebo was prepared as a sensory-identical tablet composed of α-lactose-monohydrate, microcrystalline cellulose, and magnesium stearate. Th active product contained 200 mg of *Eurycoma longifolia* and 100 mg of *Polygonum minus*. Dosage instructions for each product were to take one tablet per day.

2.2. Subjects. We aimed to enroll healthy male participants between the ages of 40 and 65 years. Subjects were recruited from the community by methods including advertisements and recruitment databases. Phone screening was performed prior to scheduling an inclinic screening visit. Participants were required to be in a stable heterosexual relationship for at least six months. Both partners had to agree to attempt intercourse at least once a week, on average, during the study. Exclusion criteria are outlined in Table 1.

2.3. Study Design. We aimed to enroll 30 men in this randomized, double-blind, and placebo-controlled parallel-design study. Institutional Review Board (IRB) approval was obtained (Copernicus Group IRB, Cary, NC, USA) prior to the initiation of any study-related activities. Simple randomization was prepared using a computer program based on the atmospheric noise method and sequential assignment was used to determine group allocation [24]. Subjects, clinical staff data management staff and statistical analysis staff were unaware of the study group. The study was conducted at the Staywell Research clinical research site located in Northridge, California.

An inclinic screening visit was performed 2 weeks prior to the baseline visit. At screening, inclusion and exclusion criteria were reviewed, vital signs were measured, and a generalized history and physical examination were performed. Labs performed at screening included safety labs (CBC, CMP, and urinary analysis) as well as serum total and free testosterone. Questionnaires were administered to subjects to confi m eligibility. The e included the BPH Symptom Score, the International Index of Erectile Function (IIEF), the Sexual Health Inventory for Men (SHIM) Questionnaire, the Beck Depression Index, the Beck Anxiety Index, and the Alcohol/Compliance Questionnaire.

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Table 1: Inclusion criteria, exclusion criteria, and study controls.

Inclusion criteria
Male
Age between 40 and 65 years
In a stable heterosexual relationship for at least 6 months
Testosterone levels ≤450 ng/dL
Index of erectile dysfunction scores of 17–25
Exclusion criteria
History of prostate cancer
Elevated prostate-specifi antigen (PSA)
Benign prostate hypertrophy (BPH) scores ≥40
Penile anatomical abnormalities
Premature ejaculation
Cardiovascular disease
Resting hypotension (resting systolic blood pressure <90 mmHg)
Resting hypertension (resting systolic blood pressure >170 mmHg or diastolic pressure >110mmHg)
Primary hypoactive sexual desire
Abnormal prostate exam during the screening visit
Study controls
Both partners had to agree to attempt intercourse at least once per week on average during the study

Subjects who passed screening were randomized on visit 2 (week 0). Baseline endpoint data was collected and product was dispensed. Subjects returned for two more visits at week 6 and week 12, where completed diaries and any remaining study product were returned, labs were drawn, and a series of scales and questionnaires were administered. Sexual intercourse diaries were completed after each sexual intercourse attempt. Safety labs were repeated at the conclusion of the study at week 12.

Throughout the course of the study, subjects were instructed not to alter their current diet and exercise habits. Th Bodymedia Fit System Armband was used to collect activity data. A breakdown of all study evaluations and procedures is demonstrated in Table 2.

2.4. Endpoints. Th primary objective of this study was to compare the proprietary tongkat ali/*Polygonum minus* combination to placebo with regard to sexual performance. Measured endpoints for this objective included the following series of subjective surveys: the EDITS questionnaire, the SIA diary, the EHS scale, the SIA logs, the EHS scale, the SHIM questionnaire, the AMS score, and the IIEF-5.

For the SIA diary, "sexual activity" was defin d as partial penile entry into the partner's vagina, while "sexual intercourse" was defin d as penetration of the entire penile shaft into the partner's vagina. "Ejaculation" was defin d as the ejection of semen from the penis. "Foreplay" consists of kissing, hugging, touching, intimacy, masturbation, and/or oral sex.

Th secondary objective was to compare the proprietary tongkat ali/*Polygonum minus* combination to placebo on quality of life endpoints. This quality of life endpoints included the SEAR Questionnaire, the Beck Depression Index, and the Beck Anxiety Index. A summary of all the scales and questionnaires used in this study is summarized in Table 3.

Th tertiary objective of this study was to compare a proprietary tongkat ali/*Polygonum minus* combination to placebo on serum testosterone levels (total and free). The normal ranges for total testosterone are 350–1.200 ng/dL and 5.4–12.3ng/dL for free testosterone. Analysis involved immunoassay for total testosterone and radioimmunoassay (RIA) for the free testosterone. Laboratory testing was performed by Primex Laboratories (Van Nuys, CA).

Additional endpoints included height, weight, waist circumference, hip circumference, body fat percentage, and armband data. Safety endpoints included complete blood count, comprehensive metabolic panel, urinary analysis, at week 6 and week 12, and adverse event monitoring.

2.5. Statistics. Completers analysis was performed, including only those who completed all study visits toward the final data set. Paired sample t-tests were used within subject means comparisons and independent sample t-tests between group comparisons (placebo versus active group). Statistical analyses were performed using SPSS Base System ver. 18 (IBM SPSS Inc., Chicago, IL, USA). Signific nce was indicated at $P < 0.05$.

3. Results

62 subjects were screened and 30 were randomized to either placebo ($n = 15$) or product ($n = 15$). Four subjects terminated the study early due to relocation, uncontrolled bowel movement, loss to follow-up, or voluntary subject withdrawal. The complete attrition chart is demonstrated in Figure 1.

Satisfaction with the study product, as measured by the EDITS Questionnaire, showed no signific nt difference between the active group and placebo group at six or 12 weeks ($P > 0.05$). However, within-group analysis of the active product group demonstrated a signific nt increase in satisfaction when comparing six to 12 weeks ($P = 0.027$). Analysis of the placebo group showed no signific nt change (Table 4).

Analysis of the SIA questionnaire revealed a significant change from baseline to 12 weeks in the active group as compared to the placebo group in 7 of the 11 scales. At 12 weeks, a signific nt improvement was noted in the ability to insert the entire shaft into the partner's vagina, the overall satisfaction with the sexual experience, and the overall score for the erection during the SIA. Subjects also noted a significant increase in the elapsed time from erection perceived hard enough for penetration to withdrawal from partners vagina (7.47 minutes at baseline to 19.56 minutes at 12 weeks, $P < 0.05$). No signific nt changes were noted in the placebo group with respect to these variables (Table 5).

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Table 2: Visit details.

	V1 Screening visit Week 2	V1.5	V2 Baseline Visit Week 0	V3 Week 6	V4 Week 12
Protocol activity					
Informed consent	x		—	—	—
Inclusion/exclusion	x		—	—	—
Medical/medication history	x		—	—	—
Intercurrent medical issues/AE review	—		x	x	x
Review concomitant therapies	x		x	x	x
Physical examination (including prostate exam)	x		—	—	x
Randomization	—		x	—	—
Vital signs	x		x	x	x
Anthropomorphic measures	x		—	x	x
BIA	—		x	x	x
Dispense bodymedia armband	—		x	x	x
Dispense subject diaries	x	Randomization assignment and paperwork	x	x	—
Collect subject diaries	—		x	x	x
Dispense product	—		x	x	—
Pill count/compliance assessment	—		—	x	x
Administer scales and questionnaires					
Benign prostate hyperplasia (BPH) symptom score	x		—	—	—
Index of erectile function (IIEF-5)	x		x	x	x
Erectile dysfunction inventory for treatment satisfaction (EDITS) questionnaire	—		—	x	x
Sexual health inventory questionnaire	x		—	—	—
Aging males symptom score (AMS)	—		x	x	x
Self-esteem and relationship questionnaire (SEAR)	—		x	x	x
Beck depression index	x		x	x	x
Beck anxiety index	x		x	x	x
Alcohol/compliance questionnaire	x		—	—	—
Laboratory					
Total + free testosterone	x		—	x	x
CBC	x		—	—	x
CMP	x		—	—	x
UA	x		—	—	x

Only two questions on the SIA questionnaire were noted to have a significant improvement among the placebo group from baseline to 12 weeks. The e included the frequency of ejaculating while still inside the partner and the overall satisfaction with the hardness of erection. These responses also showed signific nt improvement in the active group (Table 5).

Th mean baseline scores on the EHS were 2.54 for the active group and 2.14 for the placebo group. Among the active group, a statistically signific nt improvement in EHS score was noted at 6 and at 12 weeks when compared to baseline ($P < 0.05$). No signific nt changes were noted in the placebo group. The SHIM score demonstrated a significant improvement from baseline to week 12 in the active group only ($P < 0.005$). Th 12-week SHIM score of 19.85 was also significantly higher than the placebo group, which was 14.29 ($P < 0.005$). Analysis of the AMS scale at 12 weeks showed signific nt improvement from baseline among the

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Table 3: Scales and ranges.

Scales and scoring	Range	Explanation
EDITS	0–4	Satisfaction with current treatment or intervention
SIA	Yes/No	Subjective rating of erectile function
EHS	1–4	Rate current level of hardness during intercourse
SHIM	0–21	Used in assessment of erectile dysfunction
AMS	0–5	Satisfaction with overall physical function
IIEF-5	0–5	Erection problems in sex life over 4-week interval
SEAR	0–5	Questionnaire on psychosocial variables
BDI	0–3	Evaluates degree of depression
BAI	0–3	Evaluates degree of anxiety

EDITS: Erectile Dysfunction Inventory for Treatment Satisfaction.
SIA: Sexual Intercourse Attempts.
EHS: Erection Hardness Scale.
SHIM: Sexual Health Inventory Questionnaire for Men.
AMS: Aging Male Symptom Score.
IIEF-5: Index of Erectile Function.
BDI: Beck Depression Inventory.
BAI: Beck Anxiety Inventory.

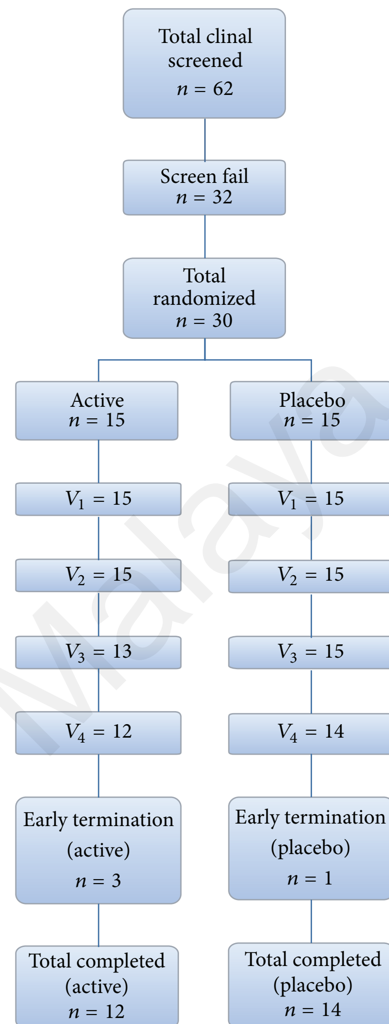

Figure 1: Attrition chart.

active group only. Thi 12-week score in the active group (20.85) was also signific ntly improved from the placebo group (26.0, $P = 0.037$).

Analysis of the IIEF-5 responses showed no signific nt difference between the groups or when compared to baseline.

With respect to the SEAR questionnaire, overall relationship satisfaction decreased in both groups and was signific ntly lower in the active group at six and 12 weeks ($P = 0.012$ at 12 weeks). The decrease from baseline was signifi ant at both six and 12 weeks in the active group and was not significant in the placebo group. Sexual relationship satisfaction was signific ntly lower in the active group compared to the placebo group at baseline and at 12 weeks ($P < 0.0001$). Th reduction from baseline was signific nt for the placebo group at six weeks only. Self-esteem scores showed no signific nt change in either group.

Analysis of the BDI and BAI showed no signific nt difference between active and placebo groups and no significant change from baseline over time.

Total testosterone levels increased from a baseline value of 359.2 ng/dL in the active group and 308.5 ng/dL in the placebo group to 396.4 ng/dL and 32.7 ng/dL at 12 weeks, respectively ($P < 0.005$ for active and $P < 0.05$ for placebo). Free testosterone levels also demonstrated a statistically significant decline in both groups ($P < 0.05$) (Table 6).

Armband data did not show any signific nt differences between groups regarding energy expenditure or physical duration. There were no signific nt differences in vital signs between groups or compared with baseline.

There were no significant changes in weight from baseline in either group and no signific nt difference between groups with regard to weight measurement. There were no significant changes in waist measurement from baseline in either group; however, the waist measurements in the active group were significantly lower than those of the placebo group at week 6 ($P = 0.032$) and week 12 ($P = 0.016$). Th hip measurements did not demonstrate any change from baseline in either active or placebo group; however, hip measurements in the active group were significantly lower than those of the placebo group at week 12 ($P = 0.015$).

With regard to the safety profile, we noted no clinically significant changes in any of the laboratory parameters throughout the study. Our study shows no signific nt changes from baseline or against placebo in relevant liver and kidney lab values, including albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, bilirubin, blood urea nitrogen (BUN), creatinine, or calculated glomerular filtration rate (GFR) (Table 7). No adverse events were attributed to the test product. No serious adverse events were reported.

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Table 4: Effect of treatment on various sexual dysfunction endpoints.

	Baseline (mean ± SE)		6 weeks (mean ± SE)		12 weeks (mean ± SE)	
	Active	Placebo	Active	Placebo	Active	Placebo
Erectile Dysfunction Inventory of Treatment Satisfaction (EDITS)	Not possible to score	Not possible to score	52.56 ± 6.80	68.59 ± 8.03	74.68 ± 8.98	78.53 ± 9.89
Erection Hardness Scale (EHS)	2.54 ± 0.22	2.14 ± 0.23	2.95 ± 0.22[d]	2.68 ± 0.15	3.54 ± 0.11[b,c]	2.87 ± 0.27
Sexual Health Inventory for Men (SHIM)	15.77 ± 1.32	12.36 ± 1.45	16.92 ± 1.46	14.21 ± 1.56	19.85 ± 1.21[a,c]	14.29 ± 1.81
Aging Males Symptom (AMS) scale	25.85 ± 2.02	29.43 ± 2.25	23.31 ± 1.54[d]	24.71 ± 1.79[c]	20.85 ± 1.10[b,d]	26.00 ± 2.78

[a] $P < 0.005$ versus placebo at this time point.
[b] $P < 0.05$ versus placebo at this time point.
[c] $P < 0.005$ versus baseline in the treatment group.
[d] $P < 0.05$ versus baseline in the treatment group.

Table 5: Effect of treatment on sexual intercourse assessment (SIA).

SIA questions	Baseline (mean ± SE)		6 weeks (mean ± SE)		12 weeks (mean ± SE)	
	Active	Placebo	Active	Placebo	Active	Placebo
Was this the fi st attempt at sexual intercourse on this day?	1.00 ± 0.00[a]	0.97 ± 0.026	0.98 ± 0.023	0.98 ± 0.017	0.96 ± 0.038	0.99 ± 0.009
Was foreplay or sexual activity initiated with the goal of sexual intercourse?	1.00 ± 0.00[a]	0.95 ± 0.04	0.96 ± 0.03	0.95 ± 0.03	0.95 ± 0.05	0.98 ± 0.02
Did foreplay precede the attempted intercourse?	1.00 ± 0.00[a]	0.97 ± 0.02	0.97 ± 0.03	0.95 ± 0.03	0.93 ± 0.05[a]	0.98 ± 0.02
Were you able to achieve at least some erection?	1.00 ± 0.00[a]	0.95 ± 0.05	0.98 ± 0.02	0.95 ± 0.02	1.00 ± 0.00[b]	0.97 ± 0.02
Were you able to insert your entire penile shaft into your partner's vagina?	0.71 ± 0.11	0.42 ± 0.13	0.84 ± 0.07	0.64 ± 0.09	0.96 ± 0.02[b,c]	0.62 ± 0.13
Did your erection last long enough for you to have successful intercourse?	0.44 ± 0.12	0.35 ± 0.12	0.67 ± 0.09[c]	0.54 ± 0.10	0.89 ± 0.04[b]	0.60 ± 0.13
Elapsed time from erection perceived hard enough for penetration to withdrawal from your partner's vagina (in minutes)?	7.47 ± 2.06	7.06 ± 1.82	11.81 ± 2.65	7.27 ± 1.67	19.56 ± 3.93[c]	12.28 ± 3.17
Did you ejaculate while still in your partner?	0.29 ± 0.11	0.33 ± 0.12	0.52 ± 0.10[c]	0.53 ± 0.12	0.62 ± 0.11[c]	0.65 ± 0.12[c]
Overall, were you satisfie with the hardness of your erection?	0.28 ± 0.11[b]	0.06 ± 0.03	0.55 ± 0.10[c]	0.24 ± 0.09	0.70 ± 0.09[a,d]	0.35 ± 0.13[c]
Overall, were you satisfie with this sexual experience?	0.33 ± 0.10	0.33 ± 0.13	0.61 ± 0.10[c]	0.44 ± 0.11	0.87 ± 0.06[b,e]	0.52 ± 0.14
Please rate the range of your erection during this sexual intercourse attempt.	2.54 ± 0.22	2.14 ± 0.23	2.95 ± 0.22[c]	2.68 ± 0.15	3.54 ± 0.11[a,d]	2.87 ± 0.27

[a] $P < 0.05$ versus placebo at this time point.
[b] $P < 0.0005$ versus placebo at this time point.
[c] $P < 0.05$ versus baseline in the treatment group.
[d] $P < 0.005$ versus baseline in the treatment group.
[e] $P < 0.0005$ versus baseline in the treatment group.

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Table 6: Effect of treatment on testosterone levels.

	Baseline (mean ± SE)		6 weeks (mean ± SE)		12 weeks (mean ± SE)	
	Active	Placebo	Active	Placebo	Active	Placebo
Total testosterone	359.23 ± 27.09	308.47 ± 23.70	396.54 ± 36.41[a]	334.33 ± 27.86[a]	396.46 ± 47.26[a]	321.67 ± 27.51[b]
Free testosterone	10.73 ± 1.12	10.43 ± 0.72	10.14 ± 1.08[b]	8.34 ± 0.57	8.55 ± 1.07[b]	7.33 ± 0.82[b]

[a] $P \leq 0.005$ versus baseline in the treatment group.
[b] $P \leq 0.05$ versus baseline in the treatment group.

Table 7: Safety lab values.

		Baseline	12 weeks	Difference	Signific nce
Albumin	Active	4.4 ± 0.2	4.2 ± 0.30	−0.20	$P = 0.351$
	Placebo	4.29 ± 0.018	4.3 ± 0.05	0.01	
AST	Active	22.33 ± 5.55	20.92 ± 4.41	−1.40	$P = 0.406$
	Placebo	22.64 ± 8.61	21.71 ± 6.10	−0.90	
ALT	Active	21.87 ± 9.26	22.31 ± 9.97	0.44	$P = 0.243$
	Placebo	22.29 ± 12.57	23.71 ± 8.71	1.42	
Alkaline phosphatase	Active	77.73 ± 19.19	68.85 ± 19.0	−8.90	$P = 0.573$
	Placebo	75.00 ± 15.85	70.43 ± 19.84	−4.60	
Total bilirubin	Active	0.78 ± 0.44	0.78 ± 0.63	0.00	$P = 0.271$
	Placebo	0.73 ± 0.18	0.66 ± 0.24	−0.10	
BUN	Active	16.33 ± 4.82	17.23 ± 3.63	0.90	$P = 0.668$
	Placebo	16.86 ± 4.74	17.57 ± 5.37	0.71	
Creatinine	Active	1.08 ± 0.13	1.08 ± 0.13	0.00	$P = 0.267$
	Placebo	1.09 ± 0.16	1.05 ± 0.23	−0.00	
Estimated GFR	Active	77.0 ± 11.7	78.08 ± 10.3	1.08	$P = 0.296$
	Placebo	77.21 ± 15.2	82.21 ± 20.2	5.00	

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4. Discussion

Eurycoma longifolia has been historically used as an aphrodisiac in Asian countries for many years. In this study, a proprietary freeze-dried water extract of *Eurycoma longifolia,* Physta, was investigated for its effect on sexual performance, satisfaction, and well-being in subjects who desired improved sexual performance. Our study demonstrates signific ntly improved subjective libido scores as well as improved sexual performance when compared to placebo. The results also suggested a potential benefit to anthropometric measures including waist and hip measurements.

A recent study by Ismail et al. enrolled 109 men between 30 and 55 years of age in a randomized, double-blind, and placebo-controlled study on *Eurycoma longifolia* and sexual well-being. They found a signific nt improvement in SF-36 scores, a scale evaluating overall physical functioning. Th y also noted higher scores in overall erectile function per the SIA as well as improved sexual libido. Their finding of decreased fat mass in subjects with BMI > 25 kg/m^2 corroborates with our findings of decreased waist and hip circumference, suggesting a role of *Eurycoma longifolia* in weight loss [25]. A recent open-label study on subjects with late-onset hypogonadism used a water soluble extract of *Eurycoma longifolia* and demonstrated improved AMS score and increased serum testosterone levels [26].

The potential mechanism of action for *Eurycoma longifolia* as an aphrodisiac is not yet clear. *Eurycoma longifolia* is rich in compounds such as eurycomaoside, eurycolactone, eurycomalactone, eurycomanone, and pasakbumin-B, which may play a role in its aphrodisiac properties [27]. *Eurycoma longifolia* is oft n referred to as an adaptogen, an agent that may rejuvenate the body through restoration [14, 28–30].

Several animal studies have shown that *Eurycoma longifolia* intake resulted in increased sexual interest and arousal [15, 31–37]. Among *Andrographis paniculata* induced infertile rats, *Eurycoma longifolia* was found to increase serum testosterone as well as sperm count, motility, and morphology [15, 38]. Our study revealed an increase in total testosterone by 10.36% in the treatment group and 4.28% in the placebo group. Th findi g of increased levels of total testosterone and decreased levels of free testosterone poses many questions regarding testosterone metabolism. *Eurycoma longifolia* may play a role in increasing production of sex-hormone binding globulin. Alternatively, supplementation with *Eurycoma longifolia* may lead to increased metabolism and breakdown of free testosterone. Studies supporting this hypothesis have shown that abstinence appears to increase testosterone levels [39, 40]. As a higher standard deviation was observed in the treatment group after 12 weeks of supplementation, considering the adaptogenic nature of the herb, it may be postulated that the herb was most effective in subjects with lower baseline levels of testosterone [26, 29, 30].

In our study, satisfaction scores did not improve in either group. This could be related to increased anxiety related to

the testing atmosphere or due to unrealistic expectations of the study product. Thus while the SIA questionnaire demonstrated signific nt improvements in sexual performance, if these improvements did not meet the subject's expectations, then the subject may note lower satisfaction scores.

With regard to product toxicity, animal studies on toxicity showed no toxicity effects of *Eurycoma longifolia* on Wistar rats in acute (2000 mg/kg), subacute (250, 500, and 1000 mg/kg over 28 days), or chronic (250, 500, and 1000 mg/kg over 90 days) dosing periods. No mortality or changes in physiological activities were noted [41]. Over our study period of 12 weeks (84 days), no adverse events occurred that were attributable to study product. Vital sign measurements and routine blood work, including complete blood count (CBC) and complete metabolic profile (CMP), also showed no significant change. Additionally, this is the fi st clinical trial to evaluate the safety of the *Polygonum minus* extract, and from our study we fin that *Polygonum minus* is safe to consume in the study population represented here. No adverse interactions were noted from the combination of *Eurycoma longifolia* and *Polygonum minus*.

A common safety concern among those concerns considering consumption of a natural product is liver and kidney toxicity. Our study shows no signific nt changes from baseline or against placebo in relevant liver and kidney lab values, including albumin, AST, ALT, alkaline phosphatase, bilirubin, BUN, creatinine, and calculated GFR (Table 7).

A major limitation of this study involves the nature of the endpoints being measured. Requiring data collection with regard to sexual performance compromises the goal of inducing a physiologic state that "by its inner nature, is deeply linked with issues of intimacy, privacy, and sexual arousal" [42]. Requiring individuals to think about and record their degree of hardness interferes with the intimate environment in which individuals commonly achieve and maintain erections. Testing in this manner introduces confounders related to anxiety and unfamiliarity [43]. Additionally, participants in this study represent a nondiseased (healthy) population. The e individuals are not particularly familiar with a clinical environment and may be uncomfortable sharing intimate details regarding their sex life. Th act of discussing and fi ling out surveys regarding desire, performance, and satisfaction may lead to laboratory-induced inhibition and decreased sexual drive [42, 44].

In conclusion, Physta signific ntly improved sexual performance across several clinically important parameters after 12 weeks when compared to placebo. In addition, the product was well tolerated, with excellent safety profiles (identical to placebo) at the administered dose of 300 mg daily for 12 weeks. However, the study product was not able to improve satisfaction with sexual performance. The results of this study suggest that this product may be of interest to generally healthy middle-aged men who desire improved sexual performance and health. Further research is still needed in order to confirm the mechanism of action in human males. Also, there is a need for further research in younger and older men, in men with cardiovascular disease and diabetes, and in individuals using medications to treat ED and other disorders.

Disclosure

Medicus Research has ongoing research support grants from Biotropics Malaysia (Berhad, Malaysia). J. K. Udani has provided consulting services to Biotropics. A. A. George, M. Musthapa, and A. Abas represent Biotropics Science and Clinical Trials. Medicus Research does not endorse any brand or product.

Conflict of Interests

Jay K. Udani and Michael N. Pakdaman are employed by Medicus Research. Medicus Research received research funding for this study from Biotropics Malaysia (Berhad, Kuala Lumpur, Malaysia). Annie George, Mufiz Musthapa, and Azreena Abas are employees of Biotropics Malaysia. Michael N. Pakdaman, Jay K. Udani, and Medicus Research do not endorse any brand or product. Medicus Research does not have any fin ncial interests with any supplement manufacturer or distributor.

Authors' Contribution

J. K. Udani was the Principal Investigator. A. A. George, M. Musthapa, and A. Abas contributed to writing of the paper.

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3.5 Publication 5

George, A., Suzuki, N.,Abas, A. B., Mohri, K., Utsuyama, M., Hirokawa, K., & Takara, T. (2016). Immunomodulation in middle-aged humans via the ingestion of Physta® standardized root water extract of *Eurycoma longifolia* Jack-A randomized, double-blind, placebo-controlled, parallel. *Phytotheraphy Research*, *30*(4), 627-635.

3.5.1 Contribution of co-authors

Name	**Contribution**
Annie George	Involved in the development of clinical study design and protocol, analysis of data and manuscript writing.
Naoko Suzuki	Involved in design of clinical study protocol, performed the clinical measurements, analyzed samples, statistics and involved in manuscript.
Azreena Binti Abas	Involved in design of clinical study protocol.
Kiminori Mohri	Involved in design of clinical study protocol and manuscript.
Masanori Utsuyama	Involved in the clinical measurements, analyzed samples and involved in manuscript.
Katsuiku Hirokawa	Involved in the clinical measurements, analyzed samples and involved in manuscript.
Tsuyoshi Takara	Involved in design of clinical study protocol, clinical measurements, analyzed samples and involved in manuscript.

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Immunomodulation in Middle-Aged Humans Via the Ingestion of Physta® Standardized Root Water Extract of *Eurycoma longifolia* Jack—A Randomized, Double-Blind, Placebo-Controlled, Parallel Study

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This study was aimed to investigate the capacity of a standardized root water extract of *Eurycoma longifolia* (Tongkat Ali, TA), Physta® to modulate human immunity in a middle-aged Japanese population. This randomized, double-blind, placebo-controlled, parallel study was conducted for 4 weeks. Eighty-four of 126 subjects had relatively lower scores according to Scoring of Immunological Vigor (SIV) screening. Subjects were instructed to ingest either 200 mg/day of TA or rice powder as a placebo for 4 weeks [TA and Placebo (P) groups] and to visit a clinic in Tokyo twice (weeks 0 and 4). SIV, immunological grade, immunological age, and other immune parameters were measured. Eighty-three subjects completed the study; 40 in the TA group and 41 in the P group were statistically analyzed, whereas two were excluded from the analyses. At week 4, the SIV and immunological grade were significantly higher in the TA group than those in P group ($p < 0.05$). The numbers of total, naïve, and CD4$^+$ T cells were also higher in the TA group than those in P group ($p < 0.05$). No severe adverse events were observed. The results suggest that ingestion of the root water extract of TA (Physta®) enhances comprehensive immunity in both middle-aged men and women. This study is registered in UMIN-CTR (UMIN000011753). Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: Tongkat Ali; eurycomanone; comprehensive immunity; Scoring of Immunological Vigor.

INTRODUCTION

The tree *Eurycoma longifolia* Jack, classified as Simaroubaceae of Sapindales and also known as Tongkat Ali (TA), originally comes from Southeast Asia, such as Malaysia, Vietnam, Java, and Thailand (Bhat and Karim, 2010). TA includes a large number of compounds such as eurycomanone, glycosaponin, and polysaccharides, etc. (Kuo *et al.,* 2003). The roots of TA have traditionally been used as a tonic, energy enhancer, and aphrodisiac (Gimlette and Thomson, 1977; Ismail *et al.,* 1999; Jagananth and Ng, 2000; Bhat and Karim, 2010). Subsequent scientific researches into TA supplementation revealed testosterone hormone modulation, especially in hypogonadic men, increased muscle strength and size, recovery from fatigue, and improved moods (Tambi *et al.,* 2011; Hamzah and Yusof, 2003; Talbott *et al.,* 2006; Talbott *et al.,* 2013). Previous *in vitro* studies have revealed the anticancer effects of TA-derived quassinoids, including eurycomanone, against colon, breast, lung, and skin cancers (Tada *et al.,* 1991; Zakaria *et al.*, 2009; Wong *et al.,* 2012). In addition, extracted TA has antioxidative effects (Varghese *et al.,* 2013). These cancer-suppressing and antioxidative effects of TA suggest that its ingestion enhances immune functions in humans.

The immune system protects the host from infections by pathogenic organisms (e.g. bacteria and viruses) and cancer growth. Humans possess both innate and adaptive immunity (Murphy, 2014). The innate immunity serves as a defense system against pathogens and cancers, beginning at the first infection or occurrence in the human body. In contrast, adaptive or acquired immunity results in the formation and storage of immune memory of a pathogen following infection for subsequent possible occurrence of the same infection (Murphy, 2014). Immunological functions

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decrease with factors such as aging, stress, and infection, rendering the host vulnerable to infectious diseases. However, a comprehensive evaluation of human immunity has remained difficult, because many immune determinants are associated with the defense system depending on the situation and are evaluated as a whole, rather than individually (Hirokawa *et al.,* 2009).

A patented immune evaluation method, the Scoring of Immunological Vigor (SIV), was proposed by Utsuyama *et al.* (2009) and Hirokawa *et al.* (2009). The use of SIV can objectively evaluate human's immunity with eight immune parameters which are easily affected by aging, stress and illness. SIV is the sum of eight immunological functional scores with three-point grades: numbers of total T cells, naïve T cells and $CD8^+CD28^+$ T cells, NK cells, and B cells; ratios of $CD4^+/CD8^+$ T cells ($CD4^{+/}CD8^+$) and naïve/memory T cells; and the T cell proliferative index (TCPI). SIV was derived from a database of 300 Japanese people (Utsuyama *et al.,* 2009) and currently from a larger database. The values of these immune parameters were standardized by assigning scores of 3 (high), 2 (moderate), or 1 (low) (Utsuyama *et al.,* 2009). SIV has already been used to evaluate the immunomodulating influences of supplements and fruits in a Japanese population (Fujii *et al.,* 2011; Suzuki *et al.,* 2012). As SIV is calculated using multiple factors, it can describe 'comprehensive immunity' by considering eight parameters and not interpreting each immune function separately. In addition, it can assess the extent of age-related decreases in immunological functions. SIV was classified to five immunological grades according to the total score; (Grade V: sufficiently high zone; Grade IV: safety zone; Grade III: observation zone; Grade II: warning zone; Grade I: critical zone). The observation zone shows mean level of the SIV, but should be required to move up into the higher zone. The warning zone indicates that the level of SIV is not enough to keep health and significant effort is needed to improve SIV. The critical zone indicates that the susceptibility to infections is so high that the individual might easily get illness (Utsuyama *et al.,* 2009). Immunological age is another easily understood immune parameter. This parameter is calculated based on the relationship between age and the TCPI, which is derived from the T cell proliferation activity and number (Utsuyama *et al.,* 2009).

The objective of this study was to evaluate whether the ingestion of a propriety water extract of TA root would comprehensively enhance immune functions. This trial was designed as a randomized, double-blind, placebo-controlled, parallel study of Japanese adults aged 40–59 years. Participants were instructed to ingest either 200 mg of the water extract of TA or rice powder as a placebo for 4 weeks, and they visited the clinic twice (weeks 0 and 4) where their SIV and other immunological factors were measured.

MATERIALS AND METHODS

Subject recruitment. This study was approved by the ethics committee of Seishinkai Medical Association Inc., Takara Medical Clinic (Tokyo, Japan) prior to initiation and took place at Takara Medical Clinic from August to December 2013. Subjects were recruited using the recruitment site Go106 (http://www.monitor-touroku.jp/). All subjects provided and signed the informed consent before undergoing screening procedures.

Subjects were between 40 and 59 years old and were required to fulfill the following inclusion criteria: good health and answered 'Yes' to the screening question of 'Do you usually feel fatigued?' The exclusion criteria for the study were as follows: (i) any previous medical history of heart failure and cardiac infarction; (ii) treatment for one or more of the following diseases: atrial fibrillation, cardiac arrhythmia, hepatic disorder, renal disorder, cerebrovascular disorder, rheumatism, dyslipidemia, hypertension, or other chronic disease; (iii) use of medicines, herbal medicines, or dietary supplements within 30 days before providing informed consent; (iv) any allergies; (v) pregnancy, lactation, or plans to become pregnant during the trial period; (vi) pollinosis; (vii) current smoking history; (viii) enrollment in any other clinical trials within 3 months before providing informed consent; and (ix) investigator's determination of unsuitability for trial participation. The clinical investigation was conducted in accordance with the Declaration of Helsinki. The study has been registered on UMIN-CTR (http://www.umin.ac.jp/, UMIN000011753).

Study design. A randomized, placebo-controlled, double-blind, parallel group, single-centered study was conducted for 4 weeks. Subjects visited the clinic twice: once before starting treatment (week 0) and once at 4 weeks after ingestion (week 4). After fulfilling all inclusion and exclusion criteria, subjects with comparatively lower SIVs (see details in 'Outcome Measurements') at week 0 were recruited from among the 126 screened individuals. Recruited subjects were randomized to either the TA group (42 subjects) or Placebo (P) group (42 subjects).

Intervention. TA capsules included 200 mg of a standardized *E. longifolia* water-soluble TA root extract (Physta®) and 30 mg of fatty acid sucrose esters in each hard gelatin capsule. P capsules each included 200 mg of rice powder instead of Physta®. The Physta® TA extract, which was standardized to contain 0.8%–1.5% eurycomanone, >40% glycosaponin, >30% polysaccharide, and >22% protein, was supplied by Biotropics Malaysia Berhad (Shah Alam, Selangor, Malaysia). Both TA and P capsules contained polysaccharides and were manufactured under Good Manufacturing Practices (GMPs) in Japan. Subjects were instructed to take one capsule of TA or P each day.

Subjects were instructed to maintain their regular lifestyle until week-4 visit, including dietary and drinking habits.

Randomization. Stratified randomization sequences were created with computer-generated random numbers using the Statlight #11 program (Yukms Co., Ltd., Tokyo, Japan) on Microsoft Excel 2007 (Microsoft Japan Co., Ltd., Tokyo, Japan). The program allocated subjects based on age (40s/50s) and sex (male/female) into four groups; 40s male, 50s male, 40s female, and 50s female. Sex and age demographic stratifications were set and crossed. Finally,

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the results of these four randomizations were combined and assigned as the final randomization sequence for this trial. Allocation and enrollment were performed by the staff of ORTHOMEDICO Inc., who did not perform any analyses or clinical procedures. The allocation information was disclosed to the investigator, subjects, and a statistician after all measurements were completed.

Outcome measurements. The primary endpoints of this study were the following immune parameters: numbers of neutrophil, lymphocyte, total T cells ($CD3^+$ cells), $CD4^+$ T cells, $CD8^+$ T cells, $CD8^+CD28^+$ T cells, naïve T cells ($CD4^+CD45^+$ cells), memory T cells ($CD45^+CDRO^+$ cells), B cells ($CD20^+$ cells), and NK cells ($CD16^+CD56^+$ cells); ratios of $CD4^+/CD8^+$ T cells and naïve/memory T cells; T cell proliferative activity, TCPI, immunological age, T lymphocyte age, immunological grade and SIV. TCPI was calculated by the following equation:

$$\text{TCPI} = \text{T cell proliferative activity} \times \left(\text{T cell number per mm}^3/1000\right).$$

Immunological age was determined from the average and standard deviation of the pre-immunological age. The pre-immunological age was calculated by the following regression equation of the TCPI and age:

$$\text{Immunological age} = (2.535 - \text{TCPI})/0.017.$$

T lymphocyte age was calculated by the equation between chronological age and $CD8^+CD28^+$ T cell count. SIV, immunological grade, immunological age, and T lymphocyte age comprised the comprehensive immune functions. All measurements and calculations of the above immunological parameters were outsourced to the Institute of Health and Life Science (Tokyo, Japan) (Hirokawa *et al.,* 2009; Utsuyama *et al.,* 2009).

An analysis of moods was performed as a secondary endpoint by evaluating the Profile of Mood States (POMSs, Japanese, brief version) (Yokoyama *et al.,* 1990), which measures six factors including tension/anxiety, depression, anger/hostility, vigor, fatigue, and confusion. Another secondary endpoint was safety (items for safety are described in Table 3 and Supplementary Tables 1 and 2). Blood measurements of 18 hematologic and 31 biochemical parameters were outsourced to Mitsubishi Chemical Medience Co., Ltd. (currently LSI Medience Co., Ltd., Tokyo, Japan). Ten urine and seven somatometric and sphygmomanometric parameters were examined at Takara Medical Clinic.

Sample size estimation and statistical analysis. A sufficient sample size of 42 subjects per group was determined through a power analysis of previous data used to evaluate SIVs (Fujii *et al.,* 2011; Suzuki *et al.,* 2012; unpublished data), with the following settings: statistical power of 80%, significance level of $p < 0.05$, and mean difference in the change in SIV between the two groups of two-thirds the standard deviation (SD), with an allocation ratio of 1:1 and the assumption of an approximate dropout rate of 20% per group. This estimation was performed using the EZR package ver. 1.11 (Kanda, 2013) on R 2.13.0 (2005; R Development Core Team, Vienna, Austria).

Baseline demographics were summarized as means and SDs; age, SIV, immunological age, immunological grade, and T-lymphocyte age were analyzed using Student's *t*-test, whereas the proportions of male subjects and those in their 40s were analyzed using the χ^2 test. The treatment effect (TA versus P) was evaluated for each endpoint using an analysis of covariance model adjusted for each week 0 value, sex, and age. A paired *t*-test was used to compare the weeks 0 and 4 values within groups. All statistical analyses were performed using PASW Statistics 18 (IBM Japan, Ltd., Tokyo, Japan).

RESULTS

Demographics

Subjects were recruited from August 21 through October 18, 2013; initial ingestion ranged from November 4 through 12, 2013 (depending on the subject visits), to week 4 visits from December 2 through 10, 2013. Fig. 1 shows the flow chart of subjects in this study. Eighty-four subjects completed the trial. One subject in the TA group was considered as a dropout by the investigator because this individual had begun vigorous exercise regimen after the week 0 visit. Two participants were excluded from the statistical analyses because one caught a cold during the trial period, which might have caused immune bias, and the other ingested less than 90% of the capsules to be consumed. All of the other 81 participants ingested 90% or more capsules during the study period and did not eat or drink much more than they were already used to, during the study as they were instructed during the informed consent that they were required to continue with the regular lifestyle and not to make severe changes to diet and lifestyle.

The final numbers of subjects in the TA and P groups included in the analyses were 40 and 41, respectively (Table 1). The ratios of male subjects in the TA and P groups were 0.50 and 0.51, and those of subjects in the 40s were 0.58 and 0.61, respectively. The mean ages in the TA and P groups were 48.7 ± 5.3 and 48.1 ± 5.5 years old, respectively. The SIVs in the TA and P groups were 17.9 ± 1.8 and 18.0 ± 1.8 points, respectively. There were no statistically significant differences between the TA and P groups at week 0 (Table 1).

Immunological functions

Immunological outcomes are described in Table 2. A significant difference in SIV was observed between the TA and P groups at week 4 (Fig. 2a, 18.80 ± 2.41 in TA versus 17.95 ± 2.40 in P, $p < 0.05$). In the TA group, SIV significantly increased over the baseline ($p < 0.01$), whereas no significant change in this parameter was observed in the P group after 4 weeks of ingestion. Of the eight subscores in the SIV, the total T cells, TCPI, and naïve/memory T cell ratio increased at week 4 in the TA group, whereas all parameters remained nearly unchanged after 4 weeks of ingestion in the P group (Fig. 3). Moreover, the immunological grade differed significantly between the TA and P groups at week 4 (Fig. 2b, 3.05 ± 0.78 in the TA group versus 2.83 ± 0.54 in the P group, $p < 0.05$). As with SIV, the

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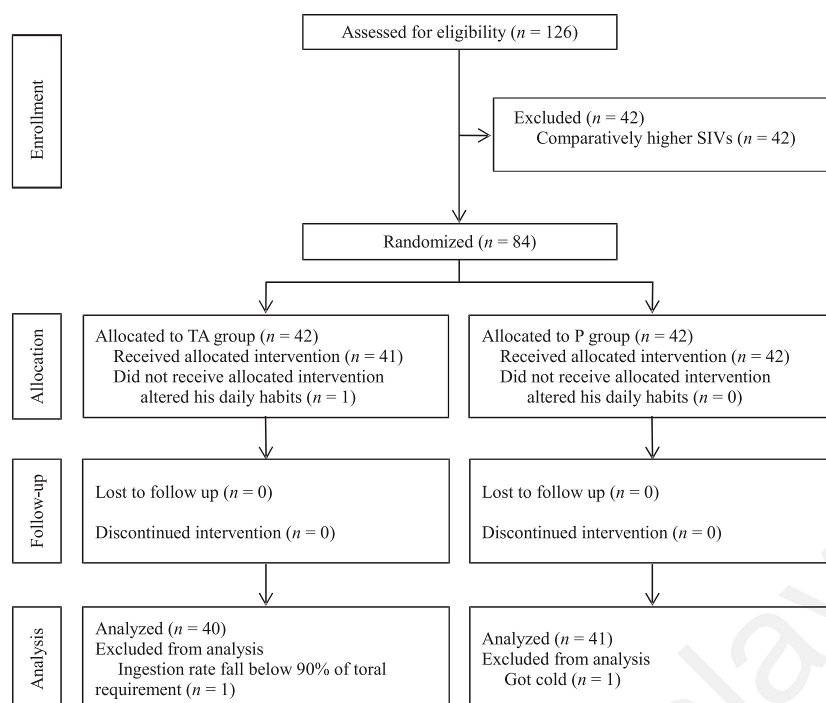

Figure 1. Subject flowchart. Eighty-four of the 126 subjects who participated in the week 0 examination were randomized; 40 in the TA group and 41 in the P group were included as the intention-to-treat population in the final analysis. TA, Tongkat Ali.

Table 1. Demographic information at week 0

	TA	*P*	Significance
Total number	40	41	
Sex ratio (men)	0.5	0.51	1
Ratio aged in 40s	0.58	0.61	0.93
Age	48.7 ± 5.3	48.1 ± 5.5	0.66
SIV	17.9 ± 1.8	18.0 ± 1.8	0.85
Immunological age	54.1 ± 6.8	54.4 ± 7.2	0.83
Immunological grade	2.7 ± 0.5	2.8 ± 0.4	0.42
T-lymphocyte age	51.3 ± 8.2	49.8 ± 8.0	0.41

Age, SIV, immunological age, immunological grade, and T lymphocyte age are shown as means and standard deviations.

immunological grade only increased significantly in the TA group after 4 weeks of ingestion ($p < 0.01$). The immunological grade changed from the warning (Grade II) to the observation zone (Grade III) in the TA group; on the other hand, the one was maintained as the warning zone (Grade II) in the P group. No significant differences in immunological age and T lymphocyte age were observed between the TA and P groups at week 4, although the immunological age significantly decreased in the TA group after 4 weeks of ingestion ($p < 0.001$). A between-group comparison of the week 4 values indicated four other parameters with significance differences: lymphocytes ($p < 0.05$), total T cells ($p < 0.05$), $CD4^+$ T cells ($p < 0.01$), and naïve T cells ($p < 0.05$) (Table 2; Fig. 2c–e).

Analysis of mood by POMS

There was no significant difference between the TA and P groups at week 4, although the tension/anxiety domain score of the TA group was nearly significantly lower than that of the P group ($p = 0.054$). All POMS items changed significantly between weeks 0 and 4 in both the TA and P groups.

Safety and adverse events

Blood and biochemical analyses (Table 3 and Supplementary Table 1), urinalysis, somatometry, and sphygmomanometry (Supplementary Table 2) were performed to evaluate the influence of TA on safety. Although some blood, biochemical, and physical analysis items indicated significant changes between weeks 0 and 4 in both the TA and P groups, all changes were clinically insignificant.

Eight subjects (1 male and 3 females in each group) reported a total of 29 adverse events (AEs) during the ingestion period (Supplementary Table 3), all of which were judged as mild by the investigator. Ten and 19 AEs were reported during 1136 total days in the TA group and 1151 total days in the P group, respectively, and the frequency of reported AEs did not statistically differ between the TA and P groups (odds ratio, 0.529; 95% confidence interval, 0.245–1.14).

DISCUSSION

The present study demonstrated that the ingestion of a standardized *E. longifolia* root water extract, Physta®, improved various immunological parameters such as the total T cells, $CD4^+$ T cells, and naïve T cell numbers, as shown in the results, and improved comprehensive immune functions as demonstrated by the SIV and immunological grade (Table 2; Figs. 2 and 3). The ingestion of a water extract of TA did not lead to a significant difference in POMS, which measures parameters related to subjects' moods, when compared with the ingestion of placebo, although improvements in the

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Table 2. Outcomes of immunological parameters at weeks 0 and 4

Item		Group	Week 0	Week 4		
Neutrophil		TA	3188.33 ± 1307.64	3154.85 ± 1166.19		
		P	3147.46 ± 1025.83	3317.32 ± 1152.76		
Lymphocyte		TA	1497.40 ± 319.53	1511.98 ± 402.08		#
		P	1517.37 ± 379.79	1403.07 ± 404.20	*	
T cell	/μl	TA	1075.03 ± 227.69	1144.58 ± 319.72	*	#
		P	1082.71 ± 275.54	1031.22 ± 285.61		
$CD4^+$ T cell	/μl	TA	661.68 ± 154.63	741.65 ± 245.46	**	##
		P	658.29 ± 170.63	644.32 ± 187.90		
$CD8^+$ T cell	/μl	TA	395.75 ± 136.77	368.58 ± 132.02	*	
		P	429.59 ± 167.67	362.59 ± 147.77	***	
CD4/CD8 ratio	—	TA	1.85 ± 0.73	2.21 ± 1.01	***	
		P	1.73 ± 0.74	2.07 ± 1.00	***	
Naïve T cell	/μl	TA	231.80 ± 89.96	296.53 ± 142.07	***	#
		P	226.10 ± 71.96	244.29 ± 94.88		
Memory T cell	/μl	TA	429.88 ± 120.82	445.15 ± 158.25		
		P	432.20 ± 151.14	400.02 ± 153.05		
Naive/memory ratio	—	TA	0.58 ± 0.25	0.70 ± 0.32	***	
		P	0.59 ± 0.27	0.70 ± 0.38	**	
$CD8^+CD28^+$ T cell	/μl	TA	247.03 ± 94.41	239.88 ± 87.16		
		P	264.41 ± 102.49	240.59 ± 93.95	*	
B cell	/μl	TA	200.08 ± 91.59	150.85 ± 75.27	***	
		P	213.95 ± 112.89	161.54 ± 100.93	***	
NK cell	/μl	TA	153.15 ± 78.10	155.53 ± 71.54		
		P	156.22 ± 81.79	144.34 ± 78.22		
T cell proliferative index	—	TA	1.52 ± 0.23	1.72 ± 0.22	***	
		P	1.45 ± 0.26	1.70 ± 0.21	***	
T cell proliferative activity	—	TA	1.64 ± 0.45	1.96 ± 0.58	***	
		P	1.57 ± 0.49	1.75 ± 0.52	*	
Immunological age	Years	TA	54.09 ± 6.79	50.43 ± 8.54	***	
		P	54.43 ± 7.22	52.45 ± 7.80	*	
T lymphocyte age	Years	TA	51.30 ± 8.19	51.90 ± 7.50		
		P	49.82 ± 8.01	51.28 ± 7.56	*	
SIV	—	TA	17.93 ± 1.80	18.80 ± 2.41	**	#
		P	18.00 ± 1.76	17.95 ± 2.40		
Immunological grade	—	TA	2.70 ± 0.46	3.05 ± 0.78	**	#
		P	2.78 ± 0.42	2.83 ± 0.54		

*$p < 0.05$;
**$p < 0.01$;
***$p < 0.001$ between weeks 0 and 4 within the TA or P group.
#$p < 0.05$;
##$p < 0.001$ between the TA and P groups at week 4. P, placebo; TA, Tongkat Ali; SIV, Scoring of Immunological Vigor.

anxiety/tension domain within the TA group reached near significance ($p < 0.054$).

Immunomodulating effects of TA

This study targeted the middle-aged (40–59 years old) population. It is well known that immune responses decrease in their activity with age (Salam *et al.,* 2013). For instance, Hirokawa *et al.* (2009) clearly reported age-dependent immune dysfunction using the same immune parameters used in this study. Our target population of 40s and 50s are then the population which begin to experience the decline in immune functions and would therefore be potential candidates to investigate for immune improving and restorative properties of intervention.

The numbers of total and naïve T cells were significantly greater in the TA group than those in the P group at week 4 (Table 2; Fig. 2). An increase in T cell numbers represents improved adaptive, or cell-mediated, immunity. Naïve T cells normally undergo thymic involution as a result of aging, causing a decrease T cell repertoire (Salam *et al.,* 2013). In fact, immune system efficiency is said to decline with age through a process known as immunosenescence. A contributing factor to this process is a reduction in the number of naïve T cells (Hawkley and Cacioppo, 2004). However, the treatment with the TA water extract in this study was able to increase the numbers of both total and naïve T cells and lymphocytes. The TA extract might affect immunity through one of two processes. First, the standardized TA water extract, Physta® contains a 4.3-kDa peptide (Sambandan *et al.,* 2006; Asiah *et al.,* 2007) (which might also contribute to T cell activation), as small peptides are known to stimulate T cells (Watts *et al.,* 1985). Second, glucocorticoids such as cortisol, which is also known as a stress hormone, promote immunosenescence by inducing a shift from a $CD4^+$ to $CD8^+$ dominant pattern of immunity (Hawkley

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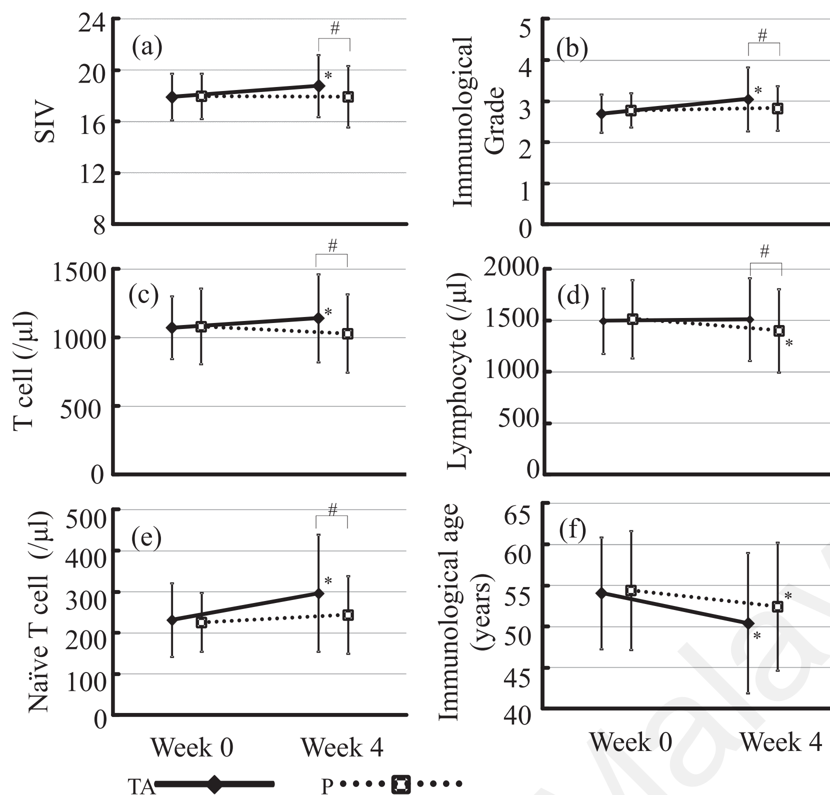

Figure 2. Immunological parameters with differences between and within groups. Six parameters [(a) SIV, (b) immunological age, (c) number of total T cells, (d) number of lymphocytes, (e) number of naive T cells, and (f) immunological grade] showed significant differences. $^{\#}p < 0.05$ between TA and P groups at week 4. $^{*}p < 0.05$ between weeks 0 and 4 within the TA or P group. Solid lines and rhombuses (♦) indicate the TA group and dashed lines and open squares (□) indicate the P group. SIV, Scoring of Immunological Vigor.

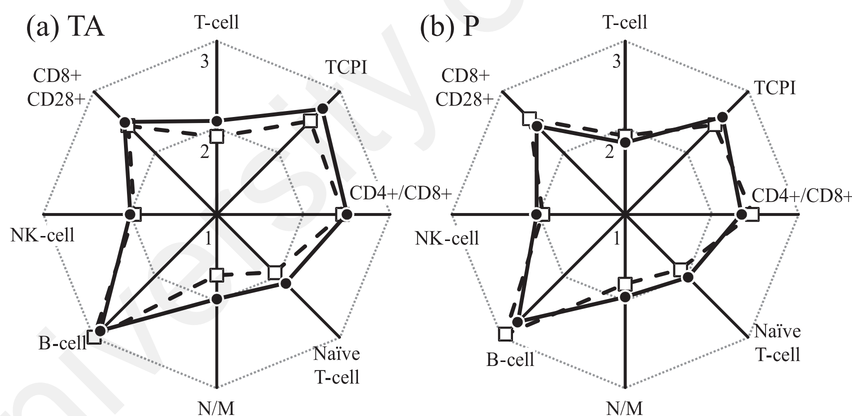

Figure 3. Eight immunological subscores in (a) TA and (b) P groups. Eight immunological subscores in the (a) TA and (b) P groups. Subscores ranged from 1 to 3. Open squares (□) with dashed lines express the mean subscores at week 0, and solid circles (•) with solid lines indicate the mean subscores at week 4. N/M, naive/memory T-cell ratio; TA, TA, Tongkat Ali; TCPI, T-cell proliferative index.

and Cacioppo, 2004). However, with TA supplementation, this dominance may have been reversed. The number of $CD4^+$ T cells in the TA group was significantly higher than that in the P group at week-4. In a report by Talbott *et al.* (2013), with a 4-week supplementation of a TA water extract, the stressed population had reduced cortisol levels and reported fewer symptoms of stress. As a result, the higher number of T cells in this study may have occurred as an indirect effect from possibly lowered cortisol levels. As with Talbott *et al.* (2013) study, an almost significant improvement in the tension/anxiety domain with TA supplementation was also observed.

According to Buford and Willoughby's review (2008), dehydroepiandrosterone (DHEA) opposes the action of the glucocorticoid cortisol, and acts in an immunomodulatory manner by shifting from a $CD8^+$ dominant pattern to $CD4^+$ dominance. This is in alignment with another study where an increase in $CD4^+$ T cells was observed with DHEA supplementation in a mouse model of *Mycobacterium tuberculosis* (Hernandez-Pando *et al.*, 1998). Water extract of TA has been found to increase DHEA levels in humans (Tambi, 2007). DHEA production gradually declines after adolescence while cortisol levels remain unaltered, creating an imbalance between DHEA and cortisol caused during aging. It is suggested that TA supplementation may have led to an increase in the levels of DHEA, thus affecting $CD4^+$ T and naïve T cell levels.

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Table 3. Biochemical analyses of safety parameters

Item	Reference value	Group	Week 0	Week 4		
AST	10–40 IU/L/37 °C	TA	19.18 ± 4.48	19.85 ± 4.50		
		P	19.12 ± 5.25	23.15 ± 13.22	**	
ALT	5–45 IU/L/37 °C	TA	16.28 ± 6.64	16.93 ± 6.97		
		P	17.80 ± 11.24	22.39 ± 25.02		
γ-GTP	M: −80 IU/L/37 °C F: −30 IU/L/37 °C	TA	22.93 ± 15.69	22.60 ± 13.32		
		P	27.78 ± 27.27	28.59 ± 28.30		
ALP	100–325 IU/L/37 °C	TA	183.48 ± 55.67	185.00 ± 54.25		
		P	187.12 ± 69.29	193.41 ± 71.22	*	
LDH	120–240 IU/L/37 °C	TA	193.75 ± 24.76	187.20 ± 27.90	*	
		P	194.37 ± 26.28	188.37 ± 26.27	*	
LAP	37–61 IU/L/37 °C	TA	50.80 ± 7.86	49.00 ± 7.74	***	
		P	53.56 ± 10.36	52.32 ± 11.65	**	
Total bilirubin	0.2–1.2 mg/dL	TA	0.82 ± 0.24	0.76 ± 0.20		
		P	0.81 ± 0.23	0.82 ± 0.24		
Direct bilirubin	0.0–0.2 mg/dL	TA	0.10 ± 0.05	0.09 ± 0.05		
		P	0.09 ± 0.04	0.09 ± 0.04		
Indirect bilirubin	0.2–1.0 mg/dL	TA	0.72 ± 0.21	0.68 ± 0.16		
		P	0.72 ± 0.20	0.73 ± 0.23		
Cholinesterase	200–452 IU/L/37 °C	TA	315.18 ± 63.73	325.73 ± 69.01	**	
		P	305.27 ± 55.72	315.15 ± 63.59	*	
ZTT	2.0–12.0 U	TA	7.24 ± 2.76	7.42 ± 2.78		
		P	6.52 ± 2.59	6.96 ± 3.10	*	
Total protein	6.7–8.3 g/dL	TA	7.03 ± 0.27	7.07 ± 0.35		
		P	7.11 ± 0.33	7.14 ± 0.33		
Urea nitrogen	8.0–20.0 mg/dL	TA	12.43 ± 3.12	12.13 ± 3.01		
		P	12.36 ± 3.23	12.40 ± 3.46		
Creatinine	0.47–0.79 mg/dL	TA	0.74 ± 0.16	0.70 ± 0.14	***	
		P	0.73 ± 0.13	0.72 ± 0.15		
Uric acid	M: 3.8–7.0 mg/dL F: 2.5–7.0 mg/dL	TA	4.91 ± 1.22	4.87 ± 1.29		
		P	4.81 ± 1.34	4.79 ± 1.34		
CK	40–150 IU/L/37 °C	TA	121.50 ± 59.18	108.93 ± 34.18	***	
		P	93.98 ± 36.46	117.61 ± 60.12		
K	3.5–5.0 mEq/L	TA	3.86 ± 0.30	4.07 ± 0.38	***	
		P	3.84 ± 0.26	4.09 ± 0.37	***	
Cl	98–108 mEq/L	TA	102.75 ± 1.71	103.23 ± 1.61	***	#
		P	101.95 ± 2.00	102.44 ± 1.90		
Na	137–147 mEq/L	TA	141.48 ± 1.77	141.00 ± 1.52		
		P	141.44 ± 2.15	140.88 ± 1.44		
Ca	8.4–10.4 mg/dL	TA	9.45 ± 0.44	9.45 ± 0.36		
		P	9.52 ± 0.29	9.49 ± 0.29		
Inorganic P	2.5–4.5 mg/dL	TA	4.05 ± 0.75	3.25 ± 0.46	***	
		P	4.10 ± 0.82	3.19 ± 0.43	***	
Fe	M: 50–200 mg/dL F: 40–180 mg/dL	TA	95.28 ± 39.99	98.13 ± 41.91		
		P	98.39 ± 32.23	97.00 ± 34.64		
Amylase	40–122 IU/L/37 °C	TA	67.35 ± 21.45	70.33 ± 20.75		
		P	69.83 ± 17.83	79.88 ± 49.34		
Total cholesterol	120–219 mg/dL	TA	201.28 ± 29.99	211.23 ± 32.56	**	
		P	201.29 ± 31.51	206.17 ± 33.15		
HDL cholesterol	40–95 mg/dL	TA	68.50 ± 17.52	72.88 ± 20.55	***	
		P	68.66 ± 16.88	69.02 ± 17.77		
LDL cholesterol	65–139 mg/dL	TA	114.00 ± 24.42	119.43 ± 30.34		
		P	113.27 ± 27.40	117.22 ± 31.04		
Triglyceride	30–149 mg/dL	TA	86.90 ± 60.97	80.28 ± 42.63		
		P	88.88 ± 46.82	87.29 ± 42.86		
Free fatty acid	0.10–0.90 mEq/L	TA	0.78 ± 0.22	0.60 ± 0.24	***	
		P	0.76 ± 0.23	0.68 ± 0.29		
Glucose	70–109 mg/dL	TA	82.68 ± 5.79	82.60 ± 10.27		
		P	84.90 ± 8.00	83.93 ± 8.59		
HbA1c	4.6%–6.2%	TA	5.39 ± 0.30	5.38 ± 0.29		
		P	5.33 ± 0.23	5.35 ± 0.26		
Glycoalbumin	12.3%–16.5%	TA	14.30 ± 1.24	14.16 ± 1.24	*	
		P	14.19 ± 1.37	14.04 ± 1.22	*	

*$p < 0.05$;
**$p < 0.01$;
***$p < 0.001$ between weeks 0 and 4 within the TA or P group;
#$p < 0.05$ between the TA and P groups at week 4. P, placebo; TA, Tongkat Ali.

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In this study, TA supplementation caused significant increases in immunological age in the TA group (Table 2). Specifically, the TA group had a younger immunological age (by 4 years) after a 4-week TA supplementation. Other anti-aging properties of TA, such as improvements in hormonal balance, strength, quality of life, and sexual health, have also been previously reported (Tambi *et al.,* 2011; Henkel *et al.,* 2014). The TA group in the present study also had significantly higher numbers of lymphocytes relative to the P group after the 4-week supplementation period. In the *in vitro* DHEA treatment on peripheral blood leukocytes obtained from older donors (≥65 years old), increased leukocytic RACK-1 expression and lymphocyte proliferation were observed. This suggests the further role of this hormone in the modulation of RACK-1 expression and immune functions (Corsini *et al.,* 2005).

In a recent study by Muhamad *et al.* (2015), the TA water extract was evaluated in subjects for endurance running and immune functions. Subjects on TA had improved immune functions characterized by the increase in NK cells. Natural killer cell is an immune cell that is very important in defense against viral infection. It is one of the lymphocytes and a component of the innate immune system [31]. In this study, there was an increasing trend in NK cells in the TA group and the reverse in the P group between week 0 and week 4. TA water extract (Physta®) also contains >30% polysaccharide and >40% glycosaponins. Polysaccharides from medicinal plants have been reported to improve NK cell levels (Nair *et al.,* 2004).

There were no significant differences between the both groups for naïve/memory ratio and T cell proliferative index, even though naïve and total T cells significantly increased in the TA group. In fact the T cell proliferative activity was strongly significantly higher in the TA group ($p < 0.001$) compared to that of the P group ($p < 0.05$). This could be because of the homeostasis balance in the body and short time of supplementation (1 month) in a small sample size which did not allow for drastic in between group differences in these areas. With the activation of adaptive immunity, the immunological age got younger by 4 years in the TA group and the overall significant improvement in SIV and immunological grade which were derived from these subscores.

Possible mechanism of immune improvement via ingestion of TA

The antioxidative effects of eurycomanone, a compound in the extracted TA, (Varghese *et al.,* 2013) might have also caused improvements in immunity (Hirokawa *et al.,* 2009). In addition to aging, oxidative stress plays an important role in the progression of immunological decline, and antioxidant compounds can enhance antioxidant-producing enzymes (Hirokawa *et al.,* 2009) to slow the decline. In almost all living systems, cells require adequate levels of antioxidant defense to avoid the harmful effects of excessive reactive oxygen species productions and to prevent immune cell damages.

Safety

There were no severe AEs reported by the subjects or observed through clinical parameters (Table 3 and Supplementary Tables 1–3). The AEs were mild in the both groups and not related to the products. Based on the biochemical results which had some statistical differences between both the groups, these were not clinically significant as the changes were all within normal levels. Serum lipids such as HDL cholesterol and triglycerides were improved in the TA group although it was not significant in triglycerides. The mechanism is not well understood though this phenomenon have been reported in testosterone treated hypogonadic subjects (Tambi *et al*., 2011; Yassin *et al.,* 2014). A 200 mg/day oral dose of a water extract of TA is considered as safe under the conditions of this study.

Limitations and foresight

The current study was conducted, performed, and measured with a primary focus on acquired immunity, as determined by total T cells and T cell subsets. Further studies should be performed to investigate the effects of the TA water extract on other components of immunity, such as innate immunity, intestinal immunity, and cytokine and/or chemokine production. This study recruited only middle-aged subjects, and it would be useful to evaluate the effects of TA in younger subjects in order to investigate the effect of TA on other age groups. Furthermore, another inclusion criterion such as allergic subjects could also be included to investigate the immunomodulatory effect of TA. This study was conducted for 4 weeks as previous studies on immunity using other food ingredient showed immune improvements after 4-week ingestion (Suzuki *et al*., 2012, unpublished data). A longer intake should help in the understanding of TA's immunomodulating activity in the long term.

CONCLUSION

This clinical study was aimed to evaluate whether the comprehensive immune functions of middle-aged men and women with comparatively lower levels of immunity would be improved by the ingestion of a 200 mg/day dose of the water extract of TA for 4 weeks. In conclusion, comprehensive immune measurements (SIV and Immunological grade) revealed significant improvement following TA ingestion. Immunological grade in the TA group was improved from the warning zone (Grade II), which is required to improve the immunity, to the observation zone (Grade III). According to these findings, intake of the TA might contribute immunological improvement, which results in maintenance and improvement of health. Our findings indicate for the first time the immune-related improvements following the ingestion of the propriety water extract of TA (Physta®).

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AUTHORS' CONTRIBUTION

AG, NS, ABA, KM, and TT designed the study. NS, MU, KH, and TT performed the clinical measurements and analyzed samples. NS statistically analyzed the data. All authors discussed and wrote the manuscript.

Conflicts of Interest

AG and ABA are employed by Biotropics Malaysia Bhd, sponsor of the study. NS is an employee of ORTHOMEDICO Inc. KM (Ph.D.) is an Emeritus Professor of Meiji Pharmaceutical University. MU (Ph.D.) and KH (M.D., Ph.D.) are staff in Tokyo Medical and Dental University and also belong to a contract research organization for immune measurements. TT (M.D.) is the Principal Investigator.

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CHAPTER 4: DISCUSSION

The aim of this research was to highlight the use of selected plants of Malaysia that has been used traditionally for various indications, in an evidence based strategy for healthy ageing. In this chapter, the scientific evidence demonstrating the healthy ageing properties of the plants and the hypothesis that the healthy ageing properties of the plants may be due to its' antioxidant properties are discussed. Other healthy ageing properties of the three selected plants ie. *Polygonum minus*, *Orthosiphon stamineus* and *Eurycoma longifolia* are also further discussed in the area of cognition, inflammation, immunity and an improvement in the quality of life to draw parallels between these plants.

4.1 The neuroprotective benefits of *P. minus* and *O. stamineus*

In this research, the *P. minus* investigated was found to possess neuroprotective benefits. The extract of *P. minus* was able to permeate the living erythrocytes cells thus protecting it from oxidative stress assault demonstrated in the e-CAP assay. The relevance of this plant extract in a biological system was further demonstrated in the improvement of learning and memory *in vivo*. The study 2 demonstrated additionally that the ethanolic extract of *O. stamineus* has pharmacologically active A2A receptor antagonist activity (George *et al*., 2015). The *O. stamineus* ethanolic extract improved the memory of rats treated with the extract. Hence, there appears to be a link between antioxidant activity, adenosine receptor binding and neuroprotection which was demonstrated in this study.

The challenge in flavonoids and phytochemicals in affecting neurons in the brain is to surpass the blood brain barrier. Study 1 suggests that the compounds in the extract

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of *P. minus* may be able to permeate and protect the cells from within, against free radical damage. Some flavonoids in fact, have been reported to cross blood brain barrier *in vitro* (Shuter *et al*., 1990). Scopolamine memory impairments used in the Barnes Maze animal model of this study have also been associated with brain oxidative stress (El-Sherbiny *et al*., 2003). Scopolamine has been shown to trigger the induction of reactive oxygen species and cause free radical injuries (Fan *et al*., 2005). Herbal extracts with high antioxidant activity have been reported to scavenge free radicals and prevent scopolamine induced lipid peroxidation (Kim *et al.,* 2008). The *P. minus* extract has been reported to possess up to 98.3% of lipid peroxidation inhibitory activities (Vimala *et al*., 2011). Through the extracts' antioxidant properties which could permeate cells, it was able to restore memory from the damaging effects of scopolamine. In addition, the standardized extract of *P. minus* contains glucoronide-3-quercetin which has been reported to reduce the formation of β-amyloids plaques in the brain which causes Alzheimers (Ho *et al*., 2013).

In other studies, the extract of the herb Convolvulus showed significant improvement in learning in a similar Morris water maze animal model of scopolamine induced deficit in memory (Bihaqi *et al*., 2011). The extract was also found to inhibit acetylcholinesterase activity in the cortex and hippocampal subregion and increase the antioxidant enzyme gluthathione reductase, SOD and gluthathione (GSH). Though the gluthathione reductase, SOD and GSH was not evaluated in this study, *P. minus* was reported to increase SOD levels in a separate study in rats induced with gastric lesions (ulcer), in response to gastrointestinal inflammation (Qader *et al*., 2012). These combination of factors probably led to its' memory restoration and improvements.

The clinical trial using a polyherbal mixture containing *P. minus*, saw an improvement from baseline for executive functioning, cognitive flexibility, reaction time, and working memory demonstrated within 4 hours of ingestion (Udani *et al.*,

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2013). The polyherbal mixture exhibited a neurostimulatory effect. When *P. minus* was used as a monoherbal product in a placebo controlled randomized, six weeks trial, an improvement in attention, mood, short-term memory and the quality of life especially in socializing (which improves when depression secedes) were demonstrated (Yahya *et al*., 2017). This provides evidence of the highly antioxidant, potentially multi-targeted activity of the herbal extract against neurodegeneration.

Another receptor targeted in the screening for pro-cognitive effects are the adenosine receptors. The bioactive compounds including highly oxygenated isopimarane-type diterpenes, orthosiphols A-E, monoterpenes, triterpenes, saponins, flavonoids, hexoses, organic acids, rosmarinic acid, chromene and myoinositol in *O. stamineus* have been reported (Olah *et al.*, 2004). The polyphenols are the predominant constituents in the plant, which have been reported to be effective in reducing oxidative stress by inhibiting lipid peroxidation in biological systems (Hollman & Katan, 2009).

It appears in Study 2 that *O. stamineus* extract behaves similarly to caffeine in improving short-term memory and alertness in a dose dependent manner. *O. stamineus*, however, does not contain caffeine but is rich in terpenoids and flavonoids (Olah *et al.*, 2004). Terpenoids from natural products such as *Ginkgo biloba* and Asian ginseng (Panax ginseng) are currently being investigated as potential therapeutics in Alzheimer's disease (Yoo & Park, 2012). The terpenoids orthosiphol B and orthosiphol F, staminol A, and orthosiphonone A were identified in the *O. stamineus* extract used, which makes this extract a potential candidate for further investigation in the area of cognition disorder. Meanwhile, the *O. stamineus* has been reported to possess antioxidant activity with orthosiphol A, B, D, H, K, M, N, O, X, Y, staminol A, neoorthosiphol B, staminols C and D, orthosiphonones C and D and 14-deoxo-14-*O*-acetylorthosiphol Y, nororthosiphonolide A, orthosiphonone A, secoorthosiphols B and C and 3-*O*-deacetylorthosiphol I showing stronger antioxidative activity than NG-

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monomethyl-L-arginine as positive control (Akowuah *et al*., 2004). The compound 2-*O*-deacetylorthosiphonone A showed the most potent activity, with an IC_{50} value of 35.0 μM. The standardized extract used in this study consisted of biomarkers orthosiphol A, B and orthosiphonone A and staminol A all of which were shown to possess superior antioxidant activity (Akowuah *et al*., 2004). In another study, a methanolic fraction of *O. stamineus* demonstrated neuprotective activities demonstrated in a study of human neuroblastoma cell line SH-SY5Y which were induced with oxidative stress using H_2O_2 (Sree *et al*., 2015). The methanolic fraction of *O. stamineus* pretreatment, dose dependently prevented cell death by H_2O_2 treatment. The extract at 1000 µg/ml almost neutralized H_2O_2 induced oxidative stress (95.3 ± 3.6, $p < 0.01$) demonstrating its effectiveness in preventing oxidative stress. Additionally, the extract enhanced expression of genes for antioxidant enzymes CAT, SOD and GPx respectively ($p < 0.01$) in SH-SY5Y neuroblastoma cells. The cells treated with H_2O_2 decreased the integrity of mitochondrial membrane, however cells pretreated with *O. stamineus* fraction prevented the loss of mitochondrial membrane integrity. The methanolic fraction of *O. stamineus* can attenuate the H_2O_2 induced oxidative stress by improving the antioxidant status, cell viability, ROS formation, mitochondrial membrane integrity and regulation of gene expression. It is possible that the extract used in this research had these effects in the animal study tested. The role of A1 receptors has been studied in a model using human neural cells (neuroblastoma SH-SY5Y cells) that naturally express A1 receptors (Angulo *et al*., 2003). The activation of A1 receptors has been found in degenerating neurons Aβ plaques in the hippocampus and frontal cortex of AD. The positive involvement of A1 receptors in the neurodegenerative structures of AD suggest that A1 receptors may play a role in the pathogenesis of AD and an antagonist activity of the said receptor may prevent further degeneration. As these receptors are found in neuroblastoma, the antioxidant protection of these cells together with adenosine A1 and

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A2A receptors antagonist activity could have contributed to the memory enhancing effects of *O. stamineus* in this study. The *in vivo* neuroprotective effect of a flavonoid rich *O. stamineus* extract was also found to reverse scopolamine induced learning and memory dysfunction demonstrated in another animal model for memory and learning ie. the novel object recognition test and an elevated plus maze test (Retinasamy *et al*., 2016). In the study, rats were similarly treated once daily with *O. stamineus* extract and donepezil (1 mg/kg) as positive control by oral route for 19 days in a scopolamine induced cognitive deficit.

Apart from neuroprotective properties, the *O. stamineus* has also been reported to possess other degeneration preventing effects. Anti-inflammatory effects by inhibiting carrageenan-induced hind paw oedema in mice (Masuda *et al*., 1992) and hepatoprotective effect caused by antioxidant and free radical scavenging properties has been reported (Yam *et al*., 2009). Another study reported that methanol extract of leaves at a dose of 200 mg/kg showed hepatoprotective activity on paracetamol-induced rats. Further, they proposed that these properties were due to the ability of the plant extract in preventing the depletion of the tissue gluthathione antioxidant enzyme level (Maheswari *et al*., 2008). Immunomodulatory effects were also reported for *O. stamineus* (Kong *et al*., 2014).

The *O. stamineus* is also used traditionally for the treatment of hypertension and qualified scientifically. An *O. stamineus* extract, exerted a mild but significant reduction of systolic, diastolic and pulse pressure reduction and in metabolic syndrome control in hypertensive dyslipidaemic subjects (Cicero *et al*., 2012). Meanwhile, a recent review article, suggests that oxidative stress could be a key player in the pathogenesis of hypertension (Baradaran *et al*., 2014). A reduction in superoxide dismutase and glutathione peroxidase activity has been observed in newly diagnosed and untreated hypertensive subjects, which are inversely correlated with blood pressure (Redon *et al*.,

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2003). Hydrogen peroxide production is also higher in hypertensive subjects (Pedro-Botet *et al*., 2000). Furthermore, hypertensive patients have higher lipid hydroperoxide production (Miller *et al*., 1998). These scientific evidence is possibly attributed to its' antioxidant property. The *O. stamineus* is therefore a potential candidate in attenuating the age related onset of degeneration in cognition, inflammation, immunomodulation and cardiovascular health. While there are clinical studies of the herb in the indication of hypertension, more in the area of cognition, inflammation and immunomodulatory effects thus should be explored in the future due to the other potentially disease alleviating properties of the plant.

4.2 The anti-inflammatory affects of *P. minus* for healthy ageing

The anti-inflammatory effect of *P. minus* was demonstrated via inhibition of 5-lipooxygenase and 2-cyclooxygenase enzymes in Study 3. The anti-inflammatory effect was further characterized by a significant reduction of paw oedema in an animal model. The *P. minus* have also other benefits probably associated with its' antioxidative effects. The *P. minus* have also been reported *in vivo* to possess immune enhancing effects by increasing the rate of phagocytosis (George *et al*., 2014). Further a methanolic extract of *P.minus* was reported to have significant hepatoprotective activity in a dose dependent manner in *in vivo* models with CCL_4 and paracetamol induced oxidative stress on liver (Christapher *et al*., 2016). The author went on to propose the inclusion of *P. minus* leaves in food as it may help to counteract different types of chemically-induced and oxidative stressed cell damage. The anti-inflammatory effect of *P. minus* in this research further justifies this proposal. Free radicals are generated by the metabolism of arachidonic acid mediated by both cyclooxygenases and lipoxygenases (Jyothi *et al*., 2016). The cyclooxygenase enzyme incorporates oxygen into arachidonic acid, converting it to hydroperoxy endoperoxide prostaglandin G2 (PGG_2). The

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hydroperoxidase reduces hydroperoxides such as PGG_2 to Prostaglandin H_2 which is the precursor to prostaglandins. The free radical O_2^-, is released by the hydroperoxidase activity of this enzyme in the presence of NADH and NADP. Metabolism of arachidonic acid by 5-lipoxygenase leading to the formation of leukotrienes also produce O_2^- in the presence of either NADH or NADPH which leads to inflammation (Lotzer *et al*., 2005).

Flavonoids are known to interrupt the oxidative generation of AA from phospholipids and reduce the downstream production of inflammatory metabolites from AA metabolism, oxidative damage, and induction of inflammatory pathways due to their potent antioxidant capacity (Messina *et al*., 2009). A wide variety of flavonoids modulate the activities of AA metabolizing enzymes such as PLA2, COX, and 5-LOX (Kim *et al*., 2008). The *P. minus* aqueous extract used in this study was reported to possess high antioxidant content demonstrated by the ORAC score of between 16,000 to 35,000 μmol TE/g in Study 1 (George *et al*., 2014). Other studies showed that flavonoids with antioxidant capacity could reduce the cellular conversion of AA to MDA (malondialdehyde) in patients suffering from chronic inflammation (Altavilla *et al*., 2009). Hence, it is possible that the high antioxidant capacity and flavonoids content of the extract used, have contributed to the anti-inflammatory effects seen *in vitro* and *in vivo*. The inhibition of mRNA expression of pro-inflammatory genes such as COX-2, TNF-α, nuclear factor (NF)-κB and PG has been reported elsewhere with *P. minus* (Yang *et al*., 2012).

4.3 Improved hormonal balance with medicinal plants

The effect of oxidative stress on hormonal balance is further discussed. In Study 4, the testosterone levels improved with *E. longifolia* supplementation which was accompanied by an improvement in sexual function and quality of life. In an *in vivo*

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study, the root extract of *Jurenia dolomiaea* restored the CCl_4 induced oxidative stress in rats by restoring depleted antioxidant enzymes and testosterone levels in the testes (Shah & Khan, 2017). The leydig cells exposed to oxidative stress exhibit reduced activities of antioxidant enzymes (e.g., catalase, superoxide dismutase, and glutathione peroxidase, reduced intracellular glutathione content, increased secretion of the malondialdehyde:a product of lipid peroxidation (Cao *et al*., 2004; Chen *et al*., 2009; Chen *et al*., 2010), increased oxidative modification of DNA (Beattie *et al*., 2013) and reductions in the mitochondrial membrane potential required for testosterone synthesis (Allen *et al*., 2006; Aitken & Roman, 2008). Oxidatively damaged Leydig cells are less sensitive to Lutein Hormone (LH), with fewer LH receptors expressed per cell, leading to reduced activities of several enzymes of the testosterone biosynthetic pathway (CYP11A1, 3β-HSD, CYP17A1 hydroxylase, CYP17A1 lyase, 17β-HSD), and inhibition of testosterone synthesis (Cao *et al*., 2004; Chen *et al*., 2009; Chen *et al*., 2010; Aitken & Roman, 2008; Zhou *et al*., 2013; Othman *et al*., 2012). The *E. longifolia* on the other hand, has been shown to increase testosterone levels in Leydig cells via the upregulation of CYP17 lyase enzyme in the testosterone biosynthesis pathway (Aminuddin, 2003), thus increasing testosterone production, hence ameliorating the negative impact of oxidative stress. In addition, Mancini *et al*., (2008) demonstrated in hypogonadic subjects that there was a link between decreased levels of plasma coenzyme Q10 (CoQ10), a lipidic antioxidant with decreased testosterone levels. When the subjects were treated with testosterone, the coenzyme Q10 (CoQ10) levels increased along with total antioxidant capacity. Clinically, *E. longifolia* supplementation has improved testosterone levels in hypogonadic subjects while improving the symptoms of ageing significantly (Tambi *et al*., 2012). Testosterone levels are known to decline with age with as much as 50% between ages 20 and 80

(Hermann & Berger, 1999). A supplementation with this plant would therefore be a good alternative to hormonal therapy for healthy ageing.

In a review by Epel *et al.* (2009), stress induces high cortisol, glucose, and insulin, and low androgens and growth hormones leading to oxidative stress and systemic inflammation, which in turn impair cell ageing processes. Accumulations of lipofuscin and amyloid contribute significantly to a decline in numerous physiological functions such as bone density, muscle strength, and libido (Schubert & Jockenhovel, 2005). Lipofuscin, also known as the "age pigment," is a marker of cell senescence (Terman *et al*., 2006). It cannot be degraded but accumulates during the lifespan of a cell. It is possible that lipofuscin formation is governed by ROS, mainly hydrogen peroxide, and is largely eliminated by catalase and glutathione peroxidase (Terman *et al*., 2006). Decreases in testosterone levels with ageing (Hermann & Berger, 1999) may reflect ROS elevations, with resulting accumulation of amyloid and lipofuscin around Leydig cells, resulting in poorer testosterone production. Ultimately, the reproduction and male fertility is also affected. Although the cause of decreasing sperm parameters is not completely clear, it has been suggested due to increased ROS production (Cocuzza *et al*., 2008; Agarwal *et al*., 2014) and is a probable cause of idiopathic male infertility. The amelioration of oxidative stress with *E. longifolia* supplementation may have contributed to the positive effects in two clinical studies which had demonstrated the improvement in sperm profiles (ie. higher number of normal compared to damaged sperms, higher volume of semen (Ismail *et al*., 2012; Tambi *et al*., 2012) and fertility in males with idiopathic infertility (Tambi *et al*., 2010).

The *Eurycoma longifolia* was clinically shown not only to boost immunity but also reduce cortisol and stress related profile of mood (Talbott *et al.*, 2013; Ang & Cheang, 1999). The corresponding quality of life improved (Ismail *et al*., 2012; Tambi *et al.*, 2012; Udani *et al*., 2014). Hormonal levels of testosterone which reduces with

age, also improved to healthy levels with its' supplementation (Tambi *et al*., 2012: Henkel *et al*., 2014). True to being an antioxidant (Christapher *et al*., 2013), *E. longifolia* has reported other benefits such as anti-inflammatory activity (Han *et. al.,* 2016). In a carrageenan-induced oedema in mice model, *E. longifolia* showed an anti-inflammatory effect comparable to that of diclofenac. Further *in vitro* molecular study using macrophage cells revealed that *E. longifolia* suppressed NF-κB translocation to the nucleus, leading to the inactivation of the NF-κB signaling pathway and reduction in the expression of cyclooxygenase-2 and inducible nitric oxide synthase. These results exhibited the beneficial effects of *E. longifolia* for alleviating pain and inflammation, which were exerted through inactivation of the NF-κB signaling pathway. Sharp decreases in blood levels of testosterone and luteinizing hormone has been associated with inflammation (O'Bryan *et al*., 2000; Sarkar *et al*., 2010). The anti-inflammatory effects of *E. longifolia* may have been attributed to the testosterone hormone restorative nature of the plant. The *E. longifolia* have demonstrated many anti-ageing characteristics including hormonal balance, an improvement in ageing symptom, immunomodulation and anti-inflammation in past research and the current one, positioning this plant as well as a potential candidate for healthy ageing.

4.4 Medicinal plants for improved quality of life

In Study 4, the improvement of the quality of life measured by the Sexual Health Inventory of Men and the Ageing Male Symptom scale was accompanied by an increase in testosterone levels with *P. minus* (100 mg) and *E. longifolia* (200 mg) supplementation.. In the case of *P. minus*, the supplementation with the herb, improved socialization (social functioning domain within Quality of Life questionnaire) and reduction of tension, anger and depression (Udani, 2013; Yahya *et al*., 2017). Cognitive function is considered to have more influence on QoL scores than gender or age

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(Konagaya *et al.*, 2009). In another study that evaluated the effect of cognition with the quality of life, the total score for QoL was significantly higher for those who saw their family members and relatives frequently rather than rarely, who were always busy with social activities rather than rarely or never, who had more years of education, and who were frequently exercising (Ozge, 2015). Improved socialization with *P. minus* supplementation improved mental health. In an enrichment programme in the elderly, DHEA, testosterone, estradiol, and growth hormone levels increased, while significantly attenuating decreases in height, likely indicating less bone loss (Arnetz *et al.*, 1983). In fact *E. longifolia* has also been reported to improve bone density possibly preventing testosterone deficiency associated osteoporosis as a result of ageing (Thu *et al*., 2017). It is plausible that *E. longifolia* improves the quality of life associated with changes in immune, hormonal, physical and mental states of the subjects. Improvement in mood evaluated by the POMS was observed with *E. longifolia* (Talbott *et al*., 2013) and *P. minus* (Yahya *et al*., 2017). A similar improvement in quality of life using the SF-36 quality of life questionnaire was observed with *O. stamineus* supplementation (Ardiyanto *et al*., 2016.)

4.5 Improved immunological balance with medicinal plants for healthy ageing

In Study 5 by George *et al*. (2016), *E. longifolia* supplementation caused significant decrease in immunological age in the *E. longifolia* group. The study which evaluated *E. longifolia* for immunomodulation in the middle-aged (40–59 years old) and stressed population, (since immune responses are known to decrease in their activity with age and stress) (Salam *et al.,* 2013), saw the numbers of total and naïve T cells and overall SIV significantly increase in the *E. longifolia* group representing an improved adaptive, or cell-mediated immunity. There was an increasing trend in NK cells in the *E. longifolia* group and the reverse in the placebo group between week-0 and week-4.

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The efficiency of the immune system declines with age through a process known as immunosenescence (Gruver *et al*., 2007) and antioxidant compounds can enhance antioxidant producing enzymes to slow the decline (Hirokawa *et al*., 2009). In almost all living systems, cells require adequate levels of antioxidant defence to avoid the harmful effects of excessive reactive oxygen species productions and to prevent immune cell damages. Naïve T cells normally undergo thymic involution as a result of ageing, causing a decrease in T cells (Salam *et al*., 2013). However, the treatment with the *E. longifolia* was able to increase the numbers of both total and naïve T cells and lymphocytes. The *E. longifolia* extract may have affected immunity through one of two processes. Firstly, by the content of a 4.3-kDa peptide found in *E. longifolia* water extract (Sambandan *et al*., 2006; Asiah *et al*., 2007) which may have contributed to T cell activation, as small peptides are known to stimulate T cells (Watts *et al*., 1985). Secondly, glucocorticoids such as cortisol, which is also known as a stress hormone, promote immunosenescence by inducing a shift from a CD4+ to CD8+ dominant pattern of immunity (Hawkley & Cacioppo, 2004). However, with *E. longifolia* supplementation, this dominance was reversed as the number of CD4+ T cells in the *E. longifolia* group was significantly higher than that in the placebo group. In a study by Talbott *et al.* (2013), supplementation of an *E. longifolia* water extract, had reduced cortisol levels resulting in fewer symptoms of stress. The higher number of T cells in this study may have occurred as an indirect effect from possibly lowered cortisol levels. According to a review by Buford and Willoughby (2008), dehydroepiandrosterone (DHEA), the precursor to testosterone opposes the action of the glucocorticoid cortisol, and acts in an immunomodulatory manner by shifting from a CD8+ dominant pattern to CD4+ dominance. This is in alignment with another study where an increase in CD4+ T cells was observed with DHEA supplementation in a mouse model of *Mycobacterium tuberculosis* (Hernandez-Pando *et al*., 1998). Water extract of *E. longifolia* has been

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found to increase DHEA levels in humans (Tambi & Kadir, 2007; Henkel *et al*., 2014). The production of DHEA gradually declines after adolescence while cortisol levels remain unaltered. An imbalance between DHEA and cortisol caused by ageing can occur. It is suggested that *E. longifolia* supplementation may have led to an increase in the levels of DHEA, thus affecting CD4+ T and naïve T cell levels that led to an overall younger immunological age. There was also a reducing trend in neutrophil in the *E. longifolia* group and an increasing trend in the placebo group in the immunomodulatory study of George *et al*. (2016)-Study 5. It is noted that stress enhances neutrophilia and neutrophil counts (Nishitani & Sakakibara, 2014) but the supplementation with *E. longifolia* prevented the increase in neutrophils. Plant extracts are known to regulate neutrophils (Vicariotto, 2014).

Other anti-ageing properties of *E. longifolia*, such as improvements in hormonal balance, strength, quality of life, and sexual health, have also been previously reported (Tambi *et al*., 2012; Henkel *et al*., 2014; Udani *et al*., 2014). The *E. longifolia* group in Study 5 also had significantly higher numbers of lymphocytes relative to the placebo group after the supplementation. The similar trend was observed in the *in vitro* study, whereby DHEA, in a treatment on peripheral blood leukocytes obtained from older donors (≥65 years old), increased leukocytic RACK-1 expression and lymphocyte proliferation (Corsini *et al.,* 2005). Additionally in another study by Muhamad *et al*. (2015), which evaluated the *E. longifolia* water extract in endurance running and immune functions in recreational athletes, subjects on *E. longifolia* had improved immune functions characterised by the increase in NK cells. Natural killer cell is an immune cell that is very important in defence against viral infection. It is one of the lymphocytes and a component of the innate immune system. This suggests the further role of this hormone in the modulation of immune functions.

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The overall significant improvement in SIV and immunological grade which were derived from these sub-scores of immune functions, may likely also be caused via the antioxidant property of *E. longifolia*. The antioxidative effects of eurycomanone, a compound in *E. longifolia* (Christapher *et al*., 2013) might have also led to improvements in immunity (Hirokawa *et al.,* 2009).

4.6 Limitations of the studies

In the *in vivo* models that investigated the effects of *P. minus* and *O. stamineus* on memory, an investigation on the antioxidant enzymes in the serum of the animals would have provided information linking the antioxidant effects of the plant with the antioxidant levels in the blood and memory improvement. In the absence of antioxidant enzymes data of the animal serum, in the current research, the antioxidant properties of the plant was proven *ex vivo* and pharmacological mechanism determined (A1R, A2AR and acetylcholinesterase inhibitory activities) as the mechanisms of action for the improvements in memory. In the *in vivo* study investigating *O. stamineus*, adult male rats were used to demonstrate memory improving effects of the extract in a scopolamine induced memory impairment model. It would be an added advantage to observe the effect of the extract using aged rats where several underlying physiological changes could contribute to a less than optimal cognitive function. The effect of the extract in aged rats should definitely be explored in the future with the proven proof of concept in this study.

While anti-inflammatory effects was observed *in vivo* with the use of *P. minus* with the inhibition of pro-inflammatory enzymes lypoxygenase and cyclooxygenase, the investigations of inflammatory cytokines would have further shed light on the extent of the mechanism of action of *P. minus* in reducing inflammation. Currently, only the immunomodulatory effect of the same extract through increased phagocytosis has been

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reported (George *et al*., 2014). The analysis of serum levels of enzymes lypoxygenase and cyclooxygenase would have provided important information and a more direct link between the inhibition of the enzymes to the reduction of inflammation in the paw oedema model and could be explored in the future along with investigations for other inflammatory cytokines.

In the clinical studies (Studies 4 and 5), few of the between group parameters did not reach statistical significance. As the clinical studies are conducted in healthy subjects, subtle differences between groups may be difficult to distinguish. These limitations and small sampling size (Study 4) could contribute to lack in statistical significance. In Study 5 on mood in stressed subjects, the cortisol levels which perhaps may have provided valuable information to understand the efficacy of *E. longifolia* on hormones, was not evaluated. Though the effect of *E. longifolia* in reducing cortisol levels have been reported before (Talbott *et al*., 2013), the evaluation of this hormone in this study would help in understanding the association between stress, *E. longifolia*, cortisol and individual immune parameters.

Dietary antioxidants such as vitamin C, vitamin E and carotenoids also play an important role in prevention and reduction of oxidative stress (Nimse & Pal, 2015). Epidemiologic studies have shown a negative correlation between regular consumption of fruits and vegetables and the prevalence of some degenerative diseases (Da Costa *et al*., 2012). There is literature that supports multivitamins or multinutrient supplementation to have cognitive and/or mood benefits across the adult life span (Schlebusch *et al*., 2000; Harris *et al*., 2011; Harris *et al*., 2012).

4.7 Future directions and potential applications of the studies

Future studies using a combination of vitamins and minerals with antioxidant herbs should be evaluated for healthy ageing benefits. The limitations in the knowledge

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of the mechanism of action should be addressed in the future as clear benefits of the plants have been proven in this research.

The extract from these plants can be formulated into capsules and drinks to provide functional physical benefits among the elderly. In the case of *E. longifolia*, the healthy modulation of testosterone levels can help in the management of muscle strength and form to prevent early onset of sarcopenia. Healthy hormonal levels are also important in preventing testosterone deficient associated bone loss leading to osteoporosis. A healthy musculature and form will ensure better of quality of life from independent living. A combination of these herbs to provide physical and mental well-being is worth looking into, since clear potential prevention of mental decline through alterations at enzyme and receptor levels in the brain, namely adenosine receptors and acetylcholinesterase were observed with *O. stamineus* and *P. minus*. The anti-inflammatory effects of *P. minus* could lend some much needed natural remedy to osteoarthritis which is characterized by joint stiffness and immobility due to joint degeneration and inflammation.

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CHAPTER 6: CONCLUSION

The increased longevity or life expectancy is often accompanied by less favourable circumstances of high living standards, loss of mobility and poor health which lead to a poor quality of life, hence the urgent need to manage ageing healthily. This research establishes scientific approaches in qualifying medicinal plants for healthy ageing whereby the selected Malaysian plants are able to protect livings cells against oxidative stress, improve cognition, immunity, hormonal balance, quality of life, prevent degeneration of liver functions and possess anti-inflammatory properties.

In Study 1, the cell-based antioxidant protection in erythrocytes (CAP-e) afforded by *P. minus* extract demonstrates antioxidant protection from within the cell proving the extracts' permeability into living cells. The effectiveness of natural products is often plagued by poor cell permeability such as in the case of blood brain barrier in ameliorating diseases. Hence, this finding is a positive outcome with cell protection from within. Scopolamine, generally used to induce memory impairment *in vivo,* is able to trigger the induction of reactive oxygen species and causes free radical damage via lipid peroxidation. Evidently, *P. minus* which is known to promote antioxidant enzyme production *in vivo,* is able to inhibit this lipid peroxidation. In addition, the extract possesses acetycholinesterase inhibitory activity, further providing neuroprotective effect demonstrated *in vivo*. The composition of the standardized *P. minus* extract also contains glucoronide-3-quercetin which is linked with the prevention of Alzheimers. The extract is subsequently able to exert its' effect clinically by improving memory, attention and mood in subjects who are predisposed to memory loss.

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Likewise the *O. stamineus* which is high in antioxidants exhibit *in vitro* binding and antagonistic activity for A1R and A2AR as bioactivity target sites in the restoration of memory (Study 2). These subsequently improve learning and memory *in vivo.* These properties of the herb are beneficial since in an ageing population, learning and retaining new information become more challenging. The *O. stamineus* is also known to be able to enhance the expression of genes for antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidise in oxidatively stressed SH-SY5Y neuroblastoma. The pharmacological property which promotes cognition, known antioxidant protection of neurological cells and the *in vivo* manifestation of improved memory has proven this herb against neurodegeneration. Its' wider anti-ageing benefit through its' traditional and scientific use in maintaining blood pressure, which can have a cardiovascular benefit; anti-inflammatory and immunomodulatory properties, places this herb as another candidate for healthy ageing.

In Study 3, the *P. minus* extract demonstrates inhibitory effects to both cyclooxygenases and lipoxygenases potentially reducing/preventing the production of inflammatory metabolites from arachidonic acid metabolism, an important inflammatory pathway. Both cyclooxygenases and lipoxygenases are enzymes within this pathway and inhibiting them would reduce the inflammatory response. This was confirmed in the reduction of paw oedema in the carrageenan induced paw oedema of a rat model. This extract may be able to alleviate ageing related disease such as rheumatoid arthritis through its' anti-inflammatory properties which could improve a persons' mobility and hence quality of life. This research has proven that *P. minus* is able to improve both neurological health and inflammation. This herb has also other benefits such as immunomodulatory and hepatoprotective activity. Hence, *P. minus* leaves is a candidate for healthy ageing to be included in food and to counteract oxidative stressed cell damage.

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Apart from improving cognition, this research has shown other benefits as a result of medicinal plant supplementation. There are overwhelming evidences to support the root of *E. longifolia* of having beneficial health properties through the modulation of the testosterone hormone in alleviating symptoms of ageing, increasing physical strength and energy, improving fertility and sexual health. It is also well known that testosterone reduces with age, stress, sedentary lifestyle and environmental factors. Antioxidants are able to prevent excessive oxidative stress in Leydig cells which causes cell damage making it less sensitive to Luteinizing Hormone (LH). By having fewer LH receptors expressed per cell, reduced activities of the enzymes in the testosterone biosynthetic pathway occurs, leading to non-optimal levels of the hormone. Other than hormonal balance, nutrition, lifestyle, physical and mental activities contribute to healthy ageing. When *P. minus* was supplemented together with *E. longifolia*, the erectile function, testosterone levels and the symptoms of ageing in men with mild erectile dysfunction, improved significantly (Study 4).

The current research also demonstrates the immunomodulatory effects of *E. longifolia* and this is related to the plants' hormone modulating ability. This is proven clinically when supplemented for one month with *E. longifolia.* Immunosenescense of the ageing population showed improvement with higher Scores of Immunological Vigour (SIV) and Immunological Grade (IG), leading to a younger immunological age (Study 5). Amelioration from oxidative stress through *E. longifolia* supplementation may have played an important role in the progression of immunological decline. The ability of *E. longifolia* to increase testosterone and reduce cortisol levels may have caused the shift towards increased CD4+ T cells, improving immunological health. In addition, the increase in lymphocytes and reduction in neutrophils, further improved the immune status of the subjects consistent with immunomodulatory effects.

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This research is able to demonstrate the beneficial effects of selected medicinal plants as an alternative and complementary therapy in age related degeneration from oxidative stress. Neuroprotective, anti-inflammatory, hormonal balance and immunomodulatory effects which eventually improve the quality of life was evidenced in this programme. Hence, *P. minus*, *O. stamineus* and *E. longifolia* are potential candidates scientifically substantiated for preventive healthcare towards healthy ageing.

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LIST OF PUBLICATIONS AND PAPER PRESENTED

PUBLICATIONS

1. Imam, M. U., Ismail, M., Chinnappan, S. M., & **George, A.** (2017). *Eurycoma longifolia* Jack water extract (Physta®) and its 4.3Kilodalton peptide increase insulin secretion from pancreatic cells and enhance glucose uptake in insulin-sensitive cells, in vitro. *European Journal of Scientific Research*, *147*(2), 177-187. **- Appendix A.**

2. **George, A.,** Suzuki, N., Abas, A. B., Mohri, K., Utsuyama, M., Hirokawa, K., & Takara, T. Immunomodulation in middle-aged humans via the ingestion of Physta® standardized root water extract of *Eurycoma longifolia* Jack—A randomized, double-blind, placebo-controlled, parallel. *Phytotheraphy Research*, *30*(4), 627-635.

3. **George, A**., Chinnappan, S., Choudhary, Y., Choudhary, V. K., Bommu, P. & Wong, H. J. (2015). Effects of a proprietary standardized *Orthosiphon stamineus* ethanolic leaf extract on enhancing memory in Sprague Dawleyrats possibly via blockade of adenosine A2A receptors. *Evidence-Based Complementary and Alternative Medicine*, *2015,* ID375837.

4. **George, A**., Chinnappan, S., Chintamaneni, M., Bommu, V. K., Choudhary, Y. Kueper, T., & Radhakrishnan, A. K. (2014). Anti-inflammatory effects of *Polygonum minus* Huds. extract (Lineminus™) in *in-vitro* enzyme assays and carrageenan induced paw oedema. *BMC Complementary and Alternative Medicine, 14*, 355.

5. **George, A.**, Ng, C. P., O'Callaghan, M. Jensen, G. S., & Wong, H. J. (2014). *In vitro* and *ex vivo* cellular antioxidant protection and cognitive enhancing effects of an extract of *Polygonum minus* Huds. (Lineminus™) demonstrated in a Barnes Maze animal model for memory and learning. *BMC Complementary and Alternative Medicine, 14*, 161.

6. **George, A.**, Chinnappan, S., Choudhary, Y., Bommu, P., &Sridhar, M. (2014). Immunomodulatory activity of an aqueous extract of *Polygonum minus* Huds. on Swiss albino mice using carbon clearance assay. *Asian Pacific Journal of Tropical Disease, 4*(5), 398-400. - **Appendix B.**

7. Udani, J. K., **George, A**., Musthapa, M., Pakdaman, M. N., & Abas, A. (2014). Effects of a proprietary freeze-dried water extract of *Eurycoma longifolia* (Physta) and *Polygonum minus* on sexual performance and well-being in men: A randomized, double-blind, placebo-controlled study. *Evidence-Based Complementary and Alternative Medicine*, *2014,* ID179529.

8. Norhayati, M. N., **George, A**., Hazlina, N. H., Azidah, A. K., Idiana, H. I., Law, K. S., Bahari, I. S., Zahiruddin, W. M., Liske, E., & Azreena, A. (2014). Efficacy and safety of *Labisia pumila var alata* water extract among pre- and postmenopausal women. *Journal of Medicinal Food*, *17*(8), 929-938. - **Appendix C.**

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9. Chen, C. K., Mohamad, W. M., Ooi, F. K., Ismail, S. B., Abdullah, M. R., & **George, A.** (2014). Supplementation of *Eurycoma longifolia* Jack extract for 6 weeks does not affect urinary testosterone: epitestosterone ratio, liver and renal functions in male recreational athletes. *International Journal of Preventive Medicine, 5*(6), 728-733. **- Appendix D.**

10. **George, A**., & Henkel, R. (2014). Phytoandrogenic properties of *Eurycoma longifolia* as natural alternative to testosterone replacement therapy. *Andrologia*, *46*(7), 708-721. - **Appendix E.**

11. **George, A.,** Liske, E., Chen, C. H., & Ismail, S. B. (2013). The *Eurycoma longifolia* freeze dried water extract – Physta® does not change normal ratios of testosterone to epitestosterone in healthy males. *Journal of Sports Medicine and Doping Studies*, *3*(2), ID1000127, 1-6. - **Appendix F.**

12. Talbott, S. M., Talbott, J. A., **George, A.,** & Pugh, M. (2013). Effect of Tongkat Ali on stress hormones and psychological mood state in moderately stressed subjects. *Journal of International Society of Sports Nutrition, 10*(1), 28. - **Appendix G.**

13. Ismail, S. B., Mohammad, W. M. Z. W., **George, A.**, Hussain, A. H. N., Kamal, Z. M. M., & Liske, E. (2012). Randomized clinical trial on the use of PHYSTA freeze-dried water extract of *Eurycoma longifolia* for the improvement of quality of life and sexual well-being in men. *Evidence-Based Complementary and Alternative Medicine, 2012*. ID429268, 1-7. - **Appendix H.**

14. Okujo, N., Iinuma, H., **George, A.**, Khor, S. M., Tan, L. L., Ng S. T., …. Kondo, S. (2007). Bispolides, novel 20-membered ring macrodiolide antibiotics from Microbispora. *Journal of Antibiotics, 60*(3), 216-219. - **Appendix I.**

PRESENTATIONS

1. **George, A.** (2016). Oral paper on Physta Tongkat Ali root for healthy ageing, presented at the Vitafoods Europe Conference, 10-11 May, 2016, Palexpo, Geneva, Switzerland. **Awarded Industry Success Story in the Healthy Ageing Category**. - **Appendix J.**

2. **George, A.** (2010). Oral paper on traditional and complementary medicine: plants to medicines, presented at the 13th NIH Scientific Meeting, *4th National Conference for Clinical Research*. The Royale Chulan Kuala Lumpur. - **Appendix K.**

3. **George, A.**, Miller, M., Gruenwald, J., Abas, A., Mufiza, M., Evans, M., & Guthrie, N. (2010). A randomized, double-blind, placebo controlled, parallel group trial toinvestigate the safety and efficacy of *Labisia pumila* water extract (BIO LP101) in healthy females. Poster paper presented at the *International Conference of Natural Product*, Penang, Malaysia. **- Appendix L.**

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4. **George A.** (1998). Oral paper presented at survey of potential disease causing organisms associated with *Gracilaria changii* at the *XVIth International Seaweed Symposium*, in Cebu City, Philippines. **Awarded Best Student Paper (Third place). – Appendix M.**

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