NEUROPROTECTIVE PROPERTIES OF *NIGELLA SATIVA* (L.) SEEDS AND *MURRAYA KOENIGII* (L.) SPRENG LEAVES EXTRACTS IN EXPERIMENTAL ANIMAL MODELS

BY

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ABSTRACT

The anti-oxidant properties of both *M. koenigii* leaves and *N. sativa* seeds extracts have been associated with many of their pharmacological activities including neuroprotective potentials in experimental animal models. The purpose of the current study was to analyze the anti-oxidant properties and assess neuroprotective effects of the extracts in zebrafish and rat models. The solubility and thin layer chromatographic (TLC) techniques have been used as classical methods for physicochemical characterization. Experimental neuro-excitotoxicity was induced by $AlCl_3$ (20 µg/mL) and MSG (475 µg/mL) in zebrafish embryos and larvae models through immersion technique while neuroinflammation by two-vessel occlusion (2VO) in healthy male Sprague Dawley rats. It was confirmed that *N. sativa* oil (NSO) and water soluble extract (WSE) of *N. sativa* seeds have different physicochemical properties while WSE has exhibited similar R_f value of 0.95 to that of both *Tualang* and *Kelulut* honeys. The presence of thymoquinone (TQ) in NSO was confirmed at (R_f = 0.86) compared to the standard TQ. *M. koenigii* leaves extract (MKLE) has showed the most potent anti-oxidant property with (IC_{50}=7.63 µg/mL) followed by WSE (IC_{50}= 33.32 µg/mL), NSO alone (IC_{50}= 73.67 µg/mL) and NSO + WSE (IC_{50}= 78.22 µg/mL) respectively against 1, 1-diphenyl-2-hydrazyl (DPPH). Both NSO (0.125 µg/mL) and WSE (80 µg/µmL) have shown to protect the deformities of neurotoxicity significantly ($P < 0.05$) in $AlCl_3$-induced neurotoxic zebrafish embryo model only after 48 hours of post-induction (hpi). In addition, WSE has also exhibited to protect the deformities of excitotoxicity in both of MSG-induced embryos (50 µg/mL) and larvae (80 µg/mL) models significantly ($P < 0.05$) compared to that of MSG (475 µg/mL) after 48 hpi. 24 healthy adult male Sprague Dawley rats were randomly divided into four groups (n=6); Healthy Control (HC); 2VO-untreated (2VO); 2VO+NSO treated (NSO) and 2VO+MKLE treated (MKLE). The NSO (100%, 1 mL/kg of b.w) and MKLE (50 mg/kg/day orally) groups were pre-treated for 10 days prior to 2VO surgery and continued until all animals were sacrificed at the end of 10^{th} postoperative week. Total RNA was extracted, purified and relatively quantified as per relative normalized gene expression (ΔΔCq) of two-step RT-qPCR assay with pre-designed QuantiTect® primers. There were significant ($P<0.01$) folds of difference in GFAP *m*RNA expression of NSO and HC groups as compared to that of untreated 2VO while there was no significant *(*$P > 0.05$) of GFAP *m*RNA expressions for NSO vs. HC and MKLE vs. 2VO. Conversely, GFAP *m*RNA expression for MKLE was significantly ($P < 0.05$) different from NSO group. There was a significantly ($P < 0.05$) down-regulated MAP2 *m*RNA expression in both 2VO and NSO groups as compared to that of HC. Yet, the MAP2 *m*RNA expressions in both NSO and MKLE treated groups were not significantly different ($P > 0.05$) to that of 2VO untreated. The overall findings suggest that MKLE could have mild neuroprotective potential via glutamate receptors only while *N.sativa* seeds extract could have superior neuroprotective activity via both of glutamate and MI muscarinic acetylcholine receptors. It is proposed that zebrafish embryo model of 24 hpf developed in this study could be used as a reliable tool to investigate neuroprotective potentials of any other crude extract or leading anti-AD drug in neurobehavioral sciences.

خلاصة البحث

لقد ترافقت الخواص المضادة للاكسدة لكل من نبتة الكاري (*M. koenigii*) والحبة السوداء (*N. sativa*) مع العديد من التاثيرات الدوائية مثل الوقاية العصبية في نماذج حيوانات التجارب. يهدف هذا البحث الى دراسة التركيب الفيزيوكيميائي وتحديد الخواص المضادة للاكسدة وكذلك خواص الوقائية العصبية في كل من الجرذان وأسماك الزيبرا. تعتبر طريقة الإذابة وتقنية الكروماتوغرافيا على الطبقة الرقيقة (TLC) من الطرق التقليدية المستخدمة في التوصيف الفيزيوكيميائي. تم إحداث السمية الخلوية العصبية بتعريض أجنة اسماك الزيبرا لمحلول كلوريد الألومنيوم ($AlCl_3$) بتركيز 20 جزء من المليون وغلوتامات أحادية الصوديوم (MSG) بتركيز 475 جزء من مليون، بينما يتم احداث الالتهاب العصبي لدى ذكور جرذان السبراغ داولي بعمل عقد مزدوج دائمي (2VO). لقد أثبتت التجربة وجود اختلاف في التركيب الفيزيوكيميائي لكل من زيت الحبة السوداء (NSO) وخلاصة بذور الحبة السوداء المنحلة في الماء (WSE) والمستخلصة باستخدام نفس المحلول (الميثانول 98%) بدون أي تجزئة، بينما أظهرت WSE قيمة R_f مشابهة لكل من خلاصات عسل التوالانغ (*Tualang*) وعسل الكلولوت (*Kelulut*). تم إثبات وجود الثيموكوينون (TQ) في NSO (R_f = 0.86) مقارنة بالثيموكوينون المعياري. أظهرت مستخلصات أوراق الكاري (MKLE) أقوى نشاط مضاد للأكسدة (IC_{50} = 7.63 مكروغرام/مل)، تلتها WSE (IC_{50} = 33.32 مكروغرام/مل)، ثم NSO منفردة (IC_{50} = 73.67 مكروغرام/مل)، ثم NSO وWSE (IC_{50} = 78.22 مكروغرام/مل) كلا ضد الجذر الحر الثابت (DPPH). أظهرت كلا من WSE بتركيز 80 مكروغرام و NSO بتركيز 0.125 مكروغرام القدرة على ايقاف سوء التشكل الناتج عن السمية العصبية المحدثة بكلوريد الألمونيوم في اسماك الزيبرا فقط بعد 48 ساعة من الإحداث، بينما أبدت WSE القدرة على ايقاف سوء التشكل في كل من الأجنة واليرقات بعد احداث السمية العصبية بغلوتامات أحادية الصوديوم مقارنة مع WSEمع جرعات 50 و80 جزء من المليون بعد 48 ساعة. أستخدم في هذه التجربة 24 جرذا من نوع سبراوغ داولي، حيث قسمت الى 4 مجموعات كالتالي: مجموعة مرجعية (HC)، مجموعة جرذان غير معالجة بـ 2VO (2VO)، مجموعة جرذان معالجة بـ 2VO و NSO (NSO)، مجموعة جرذان معالجة بـ 2VO و MKLE (MKLE). عولجت مجموعتي NSO (100%، 1 مل/كغ) و MKLE (50 مغ/كغ/يوم) فمويا عشرة أيام قبل جراحة 2VO ، واستمر العلاج لعشر أسابيع قبل قتلها. تم بعد ذلك جمع عينات الحمض النووي الريبوزي (RNA) وتنقيته وثم معايرته بطريقة النسخ العكسي للحمض النووي الريبي (DNA) كتعبير جين معتدل مرتبط ($\Delta\Delta Cq$) من مرحلتين (RT-qPCR) مع اعدادت (QuantiTect®) المجهزة مسبقا. لقد لوحظت اختلافات مهمة ($P<0.01$) في تعبير GFAP mRNA عند مجموعتي HC و NSO مقارنة مع مجموعة 2VO ، بينما لم يكن هناك اختلاف مهم احصائيا ($P< 0.05$) في تعبير GFAP mRNA بين مجموعتي NSO وCE وكذلك بين مجموعتي MKLE و 2VO. لقد لوحظ اختلاف مهم احصائيا في تعبير GFAP mRNA عند مجموعة MKLE بالمقارنة مع مجموعة NSO بعد 10 اسابيع من المعالجة. وقد لوحظ ايضا انخفاض مهم احصائيا في تعبير MAP2 mRNA ($P<0.05$) عند كلا مجموعتي 2VO وNSO بالمقارنة مع مجموعة CE، بينما لم يكن الاختلاف مهما احصائيا تعبير MAP2 mRNA بين مجموعتي NSOو MKLE المعالجة ($P>0.05$) مقارنة بالمجموعة الغير معالجة بـ 2VO. تشير النتائج النهائية أن MKLE يمتاز بوقاية عصبية خفيفة التي من الممكن أن تكون عن طريق مستقبلات الغلوتامات فقط، بينما من الممكن أن تؤثر مستخلصات الحبة السوداء عن طريق مستقبلات الغلوتامات ومستقبلات الأسيتيلكولين الموسكارينية MI والتي تمتاز بفعالية الوقاية العصبية الاعلى. من المقترح أيضا أن نموذج اسماك الزيبرا (24 ساعة بعد التخصيب) الذي طورناه في هذه الدراسة لتكون نماذج مثالية ومفضلة أكثر من أي نماذج أخرى للتحقيق في الخواص المحتملة لمستخلصات أخرى، أو دراسة العقاقير المضادة لمرض الزهايمر في مجال العلوم السلوكية العصبية.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Pharmaceutical Sciences (Pharmacology).

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AChE	Acetyl cholinesterase enzyme
ADAD	Autosomal dominant Alzheimer's disease
$AlCl_3$	Aluminium chloride
AF	Arterial fibrillation
Al-20	Aluminium chloride (20 µg/mL) / Co-treated $AlCl_3$
ALS	Amyotrophic lateral sclerosis
AMV	Avian myeloblastoma leukemia virus
ARMD	Age-related macular degeneration
AP	Awkward position (zebrafish)
APP	Amyloid precursor protein
APOE	Apolipoprotein E
ATP	Adenosine triphosphate
Avg.	Average
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
BH	Black honey (Tualang Honey)
BM	Body movement
BSC	Biosafety cabinet
bp	Base pair
b.w	Body weight
CA	Cardiac arrhythmia
CA1	Central amygdala (hippocampus)/ cortical area 1 (pyramidal cells)
CBF	Cerebral blood flow
CCA	Common carotid artery (-ies)
CCH	Chronic cerebral hypoperfusion
CGRP	Calcitonin gene related peptide
CHF	Congestive heart failure
CMA	Chymase gene / Heart Chymase
CNS	Central nervous system
Conc.	Concentration
CRGP	Calcitonin Gene Related Peptide
CRPF	Cortical renal plasma flow
CRS	Chinese Restaurant Syndrome
CVD	Cardiovascular disease
CLU	Clusterin
CR1	Chicken Repeat 1
D.log	Displaced log along the slope Difference
DCM	Dichloromethane
DF	Deformed face or Doggy face (zebrafish)
Dif.	Difference
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide

DO	Dissolved oxygen
DPPH	Diphenyl-2-picryl-hydrazyl
dpf	Days of post-fertilization
DS	Down's syndrome
ds	Double strand
DZ	Drowsiness
ELT	Escape latency time
EOFAD	Early onset Familial Alzheimer's disease
EP	Escape platform
EPSP	Excitatory synaptic potential
Eqs	Equations
EtBr	Ethidium bromide
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Gas chromatography
GFAP	Glial fibrillary acidic protein
GFR	Glomerular filtration rate
GMO	Genetically modified organism
GRP	Glial restricted precursor cells
GOI	Gene of interest
GSH-Px	Glutathione peroxidase
HA	Hyperactive
HC	Healthy control
HCl	Hydrogen chloride
HD	Hungtington's disease
HPLC	High performance liquid chromatography
HPRT1	Hypoxanthine phosphoribosyltransferease 1
hpf	Hours of post-fertilization
hpi (HPI)	Hours of post-induction
HIC	Head in chorion
ICRACU	Integrated Centre for Research Animal Care and Use
IC50	50% inhibitory concentration
IP	Intraperitoneal
IRI	Ischemia-reperfusion injury
LBs	Lewy bodies
LNs	Lewy neuritis
MAP2	Microtubule associated protein 2
MCI	Mild cognitive impairment
MDA	Malondialdehyde
ME	Mother emulsion
MKLE	Murraya koenigii leaves extract / MKLE treated group
MKL	Murraya koenigii leaves
MMLV	Monoley murine leukemia virus
MWM	Morris water maze
NFT	Neurofibrillary tangles
ng	Nanogram
NMDA	N-methyl-D-aspartate receptor
MSG	Monosodium glutamate
NC	Normal control
NO	Nitric oxide

NSAIDs	Non-steroidal anti-inflammatory drugs
NSO	Nigella sativa seeds extract / NSO treated group
NSS	Nigella sativa seeds
NSO	Nigella sativa oil
NTC	No template control (Blank well)
NTP	National Toxicological Program (USA)
NO	Nitric oxide
OD	Optical density
PD	Parkinson's disease
PE	Primary emulsion
PICALM	Phosphatidylinositol Clathrin Assembly Lymphoid-Myeloid Leukemia
RT-qPCR	Reverse transcriptase quantitative-polymerase chain reaction
RNS	Reactive oxygen species
RO	Reverse osmosis
ROS	Reactive oxygen species
S	Stock
SBH	Stingless bee honey (Kelulut honey)
SD	Sprague Dawley rats
SE	Secondary emulsion
SFE	Supercritical Fluid Extraction
SORL1	Sortilin-related receptor, L (DLR Class) A
ss	Single strand
T	Tyrosine
TE	Thrombotic episode or Tertiary emulsion
TLC	Thin layer chromatography
TM	Tail movement
TQ	Thymoquinine
TREM2	Triggering Receptor Expressed on Myeloid Cells 2
Te	End temperature
Tm	Melt temperature
T0	Initial temperature or Begin temperature
T80	Tween 80
VD	Vascular dementia
VEGF-A	Vascular endothelial growth factor A
2VO	Two-vessel occlusion / 2VO untreated group
Vol.	Volume
W	Working
WM	White matter
WSE	Water soluble extract of *N.sativa* seeds

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Nigella sativa L. (*N.sativa*) is an annual herbaceous flowering plant belonging to *Ranunculaceae* family widely grown in the Mediterranean countries, Western Asia, Middle East, and Eastern Europe. The preventive and relieving effects of *N.sativa* seeds have been attributed to its prominent phytoconstituents such nigellicine, nigellidine, TQ, dithymoquinone, thymol and carvacrol (Ahmad et al., 2013). The essential oil of *N.sativa* seeds has been reported to contain various pharmacologically active constituents including TQ (30-48%), thymol, thymohydroquinone, dithymoquinone, p-cymene (7-15%), carvacrol (6-12%), sesquiterpene longifolene (1-8%), 4-terpineol (2-7%), *t*-anethol (1-4%) and α-pinene (Houghton et al., 1995; Ahmad et al., 2013).

The seeds were also reported to possess many non-oily and non-caloric components in trace amounts including pyrazole alkaloids (nigellidine and nigellicine), isoquinoline alkaloids (nigellicimine and nigellicimine-N-oxide), saponin, vitamins (riboflavin, thiamine, niacin, pyridoxine, folic acid and vitamin E), and minerals (potassium, sodium, calcium, phosphorus, magnesium, copper and iron) (Nergiz et al., 1993; Gholamnezhad et al., 2016).

The fixed oil (36-38%) of *N.sativa* seeds has been reported to compose mainly of unsaturated fatty acids including arachidic and eicosadienoic acids (Houghton et al., 1995). TQ has been reported to have potential therapeutic properties such as anti-inflammatory, anti-histaminic, hepatoprotective, anti-oxidant and neuroprotective in

animal models (Hosseinzadeh et al., 2007; Khazdair, 2015). According to (Mohamed et al., 2002), TQ (1 mg/kg, injected into the tail vein) has increased the glutathione level and reduced perivascular inflammation and encephalomyelitis symptoms in rats. It was also reported that TQ (15 mg/kg, i.p injection in mice) treatment has showed 90% preventive and 50% curative effects in chronic relapsing multiple sclerosis (Mohamed et al., 2008).

Murraya koenigii (L.) Spreng (*M.koenigii*) or curry leaves belong to *Rutaceae* family is one of the most well-known ingredients in South and Southeast Asian cuisines including Malaysia. The leave have a little pungently bitter and softly citrus taste. From the leaves, different compounds have been isolated including carbazole alkaloids, volatile oils and many others. Several studies have been carried on its phytochemical screening using different types of solvents for extraction such as petroleum ether, ethyl acetate, chloroform, ethanol, methanol and water (Handral et al., 2012).

It was reported that the leaves contain proteins, carbohydrates, fiber, minerals, carotene, nicotinic acid and vitamin C with high amount of oxalic acid. The leaves were also found to have crystalline glycosides, carbazole alkaloids, koenigin and resin (Handral et al., 2012). Alkaloids such as giriminbine, iso-mahanimbin, koenine, koenigine, koenidine and koenimbine were also found in the leaves (Narasimhan et al., 1975). These compounds were known to exhibit various bioactivities including anti-oxidant and anti-amnesic activities (Mani et al., 2012; Mani et al., 2013). The petroleum ether extract of the leaves pre-treatment (300 and 500 mg/kg) for 15 days has been reported to improve memory and learning in aged mice which was comparable with the effect of standard Piracetam (400 mg/kg) and it was also found that the same dose of petroleum ether extract has remarkably reduced the brain

cholinesterase activity but inferior to that of Doneprezil (0.5 mg/kg) treated mice (Tembhurne, 2010; Handral et al., 2012). Isolated carbazole alkaloids such mahanimbine and koenigine from the leaves have been reported to exhibit high degree of DPPH free radical scavenging activity (Rao et al., 2007). Many pharmacological activities of this plant have been investigated so far where most of the studies have been carried on the leaves using various solvents including methanol (Handral et al., 2012).

Recently, both *N.sativa* or black cumin seeds and *M.koenigii* or curry leaves have been considered as effective natural remedies against neuroinflammation-mediated neurodegeneration, ROS in apoptosis, cerebral ischemia and hypoxia of CCH (Alsaif, 2007). Studies on these two natural herbs have reported them possessing some common bioactivities such as anti-inflammatory, anti-oxidant and anti-amnesic activities (Vasudevan et al., 2009; Tembhurne, 2010).

Inflammation in central nervous system (CNS) is a key factor in neurodegenerative diseases including Alzheimer's disease (AD). Many relevant scientific studies suggested that neuroglial cells (i.e., astroglia and microglia) play critical role in inflammation-mediated neurodegeneration which could experimentally be achieved by two-vessel occlusion (2VO) in murine models of AD (Farkas et al., 2007; Choi et al., 2011). A series of experiments with chronic cerebral hypoperfusion (CCH) in rat and gerbil models had been started in 1989 and continued until now. The glucose-oxygen levels can easily be manipulated or compromised physiologically by altering the hemodynamic status of cerebral blood flow (CBF) using a rat model that would assume some clinical relevance. CBF could be influenced by manipulating one or more of the three parameters: (1) age of rat, (2) duration of CCH and (3) severity of CCH (Ni et al., 1994; De la Torre, 2000). The severity depends on the supply of

glucose and oxygen to the brain and the duration could be maintained for 1 to 52 weeks while both young and/or aged rats could be used. However, neither 2VO nor 3-VO was sufficient to elicit any sensory-motor deficits or cardio-pulmonary problems in these animals during the period of observation (De la Torre et al., 1993). The 2VO model is easier to perform and less-intrusive surgical intervention compared to that of four-vessel occlusion which increases the risk of extraneous factors confounding the response to the ischemic injury while reduces the scope for recovery experiments (McBean et al., 1998).

Previous studies have showed that microscopic changes of a brain were usually observed after 2VO consisting visuo-spatial memory impairment, hippocampal gliosis (astrogliosis/ microgliosis), mean hippocampal CBF reduction of 32%, loss of microtubule associated protein 2 (MAP2) in the apical dendrites of CA1 (a marker of protein synthesis and pre-synaptic activity), cytochrome oxidase decline in CA1 and posterior parietal cortex (a marker of neuronal energy activity), increased hemeoxygenase-1 expression (a marker of oxidative stress), and extracellular deposits of amyloid precursor protein (APP) which is localized to neuronal cell membranes and concentrated in synapses of neurons .

With the help of the 2VO models, elucidation of the causal and sequential interactions of neurodegeneration, chronic cerebral ischemia and/or hypoxia, neuronal injury and memory deficits could be evaluated. The initiating role of chronic cerebral ischemia in neural damage to the hippocampus, the cerebral cortex, the white matter (WM) areas and the visual system has been demonstrated (Bouma et al., 1991; Farkas et al., 2005).

The 2VO model has been applied successfully by scientists for other research fields, like ischemic WM injury and ischemic eye diseases by the time association of

decreased CBF, particularly in the temporal and parietal cortices, with AD has been firmly established (Matsuda, 2001; Farkas et al., 2007).

Moreover, the relationship between regional protein synthesis in the brain and regional CBF has been shown to be closely linked to AD (Kalia, 2005; Girouard et al., 2006). When blood flow in CNS reduces to 60% of the total flow, protein synthesis is practically suppressed (Xie et al., 1989).

In rodents, permanent ligation of the common carotid arteries or 2VO induces not only morphological abnormalities in hippocampal cells (i.e., microglia and astroglia or neuroglia) but also quantifiable cell loss within 7 months of blood flow reduction (De la Torre et al., 1992; Pappas et al., 1996). The loss of neuronal cell bodies and synaptic contacts are the most obvious signs of neurodegeneration in 2VO models (Ohtaki et al., 2006; Farkas et al., 2007).

In resting condition, microglias monitor the health of neurons cautiously and have strong desire to alleviate the suffering. When the brain is being injured physically, chemically or infected, glial cells become activated and secrete a variety of inflammatory mediators and neurotoxic factors that cause neuronal death (Boje et al., 1992; Chao et al., 1992).

Chronic neuroinflammation, cerebral ischemia and hypoxia with elevated pro-inflammatory cytokines are closely associated with neurodegenerative diseases including AD, Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), taupathies, and age-related macular degeneration (ARMD). Neuroglial crises with chronic neuroinflammation is the starting point for elevated levels of a wide range of potentially neurotoxic molecules such as pro-inflammatory cytokines, proteinases and reactive oxygen species (ROS) (Boje et al., 1992; Jeohn et al., 1998). Several methods have been developed gradually to identify the activated