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THE NIGELLA SATIVA (L.) SEEDS EXTRACT TREATMENT ON THE HIPPOCAMPAL GFAP IN 2VO-INDUCED CHRONIC NEUROINFLAMMATION

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ABSTRACT

Neuroinflammation has been closely linked to neurodegeneration that leads to dysfunction of memory and learning where glial fibrillary acidic protein plays important role within hippocampus. In experimental neurodegeneration achieved by two-vessel occlusion (2VO) intervention, Nigella sativa (L.) seeds extract (NSSE) has shown to have anti-neuroinflammatory and antioxidant properties. Toxic free radicals formation and prevention of neuroinflammatory mediators are of two possible mechanisms. The current study aimed to investigate the anti-neuroinflammatory effect of Nigella sativa and the role of GFAP as a relevant biomarker of neuroinflammation. The relative normalized hippocampal GFAP mRNA expressions (ΔΔCq) of Nigella sativa (L.) seeds extract treatment (100mg/kg/day orally) was compared with that of healthy control (HC) and untreated 2VO groups (n=6) of rats. The brain hippocampal tissues were humanely collected at the end of 10th treatment week and preserved in Allprotect™ reagent at –80°C. Total RNA was extracted and purified by phenol/chloroform method using kits, and reverse transcribed into cDNA and relatively quantified as per ΔΔCq. The NSSE treatment showed significantly ($P<0.001$) different relative GFAP mRNA expression in the treated group as compared to that of untreated 2VO whilst it was insignificantly ($P>0.5$) different to that of HC. Prolonged or daily treatment with NSSE may possess moderate anti-neuroprotective activity within hippocampus.

Keywords: Nigella sativa, neuroinflammation; two-vessel occlusion; glial fibrillary acidic protein; hippocampus; reverse transcription quantitative PCR (RT-qPCR)

INTRODUCTION

Neuroinflammation occurs in almost all types of neurodegenerative diseases varying to unique pathology and symptoms having specific triggers of neuronal damage whereas each environmental toxin or genetic mutation is specific for a selected neurodegeneration (Block *et al*., 2007). The glial fibrillary acidic protein (GFAP) that controls the up or down regulation of astrocytes (astroglia collectively) have been closely linked to neuroinflammation and considered as a relevant biomarker for astrogliosis (also known as astrocytic hypertrophy) after any peripheral nerve injury while its process in the pathogenesis of neurodegeneration and cognitive impairments are associated to Alzheimer's disease (Garrison *et al*., 1991; Hauss-Wegrzyniak *et al*., 1998). Anatomically, it is the major constituent of glial intermediate filaments (IF) protein found mainly in differentiated fibrous and protoplasmic astrocytes of the brain and spinal cord of the central nervous system. The

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first report on astrogliosis (i.e., reactive astrocytes or hyperactivity of astroglia) has been introduced by Garrison and his co-workers in 1990 and later on it has been recognized to be an up-regulated marker in neuroinflammation (Azzubaidi *et al*., 2012; Garrison, *et al*., 1991). Glial cells (i.e., astrocytes) have been known as inflammatory mediators of neurodegenerative disease and involve in providing glia-neuron contact, maintenance of ionic homeostasis, buffering of excess neurotransmitters, secreting neurotrophic factors and serving as critical component of the blood-brain barrier (Block *et al*., 2007; Pekny *et al*., 2013). GFAP becomes activated in response to immunologic (infiltration of leucocytes from residential cells) challenges or brain injuries that cause restriction of blood flow and deprivation of oxygen supply to hippocampus (Aloisi, 1999; Tacconi, 1998). It has been reported that activated GFAP (i.e., hypertrophic astrocytes or astrogliosis) forms glial scars that hinder axonal regeneration while there is a clear relationship between astroglia and microtubule associated protein (MAP) or microglia in both resting and activated conditions (Kahn, 1995; Rezaie, 2002). Microtubule associated protein 2 (MAP2) has been considered as an important bio-marker linked to neuroprotection in response to neuronal damage or any kind of central nervous system injury (Harukuni & Bhardwaj, 2006; Park *et al*., 2012). Microglia were first described as a unique cell type differing in morphology from other glia and neurons, comprising approximately 12% of the brain (del Rio-Hortega, 1932). MAP2 has been found to be down-regulated in experimental neurodegeneration of two-vessel occluded (2VO) rats (Azzubaidi *et al*., 2012; Block *et al*., 2007; McGeer *et al*., 2003). Microglial response to neuronal damage has been considered as a long-lived and self-propelling condition (Gao, 2003; Huh *et al*., 2003). The neurotoxic response of microglia to chronic restriction of blood flow to hippocampus is a critical component of microglia-mediated neurotoxicity across multiple neurodegenerative diseases (Wenk, 2003). The neurotoxic activation of MAP2 or microglia in response to neuronal injury is commonly known as reactive microgliosis. Microglia consistently produces reactive oxygen species (ROS) when activated by multiple pro-inflammatory triggers, endogenous protein toxins (β-amyloid peptide, α-synuclein) and neuronal injury. It has been reported that MAP2 mRNA expression plays a crucial role in neuronal plasticity and synaptic intact (Yang *et al*., 2007) while GFAP mRNA in neuroinflammation. It was also believed that these two proteins are sensitive to experimental neurodegeneration attained by (2VO), a surgical method that needs ligature of both left and right carotid arteries permanently (Ábrahám & Lázár, 2000; Azzubaidi *et al*., 2012; Farkas *et al*., 2007; Saxena *et al*., 2011). This procedure has been widely used and recognized for its effective induction of neurodegeneration in animal models (Farkas & Luiten, 2001; Fujishima *et al*., 1976). Both of the proteins are equally vital to maintain hippocampus of the brain through cellular structure, synaptic plasticity and branching, axonal and dendritic functions (Munro *et al*., 2001; Ohashi *et al*., 2002; Taketo & Yoshioka, 2000; Yamazaki *et al*., 2002).

Murraya koenigii (curry) leaves that have been using as a spice in many Asian cuisines for centuries (Gupta *et al*., 2011; Jain *et al*., 2012). Many relevant scientific studies have reported that it has free radical scavenging and anti-amnesic effects inhibiting brain cholinesterase activity (Mani *et al*., 2013; Tembhurne & Sakarkar, 2010; Tembhurne & Sakarkar, 2011; Vasudevan & Parle, 2009). Several bioactive compounds have been isolated from different morphological parts of this plant. The most studied phytoconstituents go to its carbazole alkaloids (e.g. mahanine, mahanimbine and murrayafoline) and essential oils of the leaves (Gupta *et al*., 2011; Ramsewak, 1999).

The reverse transcription quantitative PCR (RT-qPCR) is one of the most distinguished techniques for single cell gene regulation studies in terms of accuracy, sensitivity, reproducibility and fast results. This technology has been established as the gold standard for medium throughput gene expression analysis (Derveaux, 2009).

EXPERIMENTAL SECTION

Animals and treatments

18 male adult SD rats (250-350gm) were randomly divided into three groups (n=6); Healthy Control (HC), 2VO untreated (2VO) and 2VO + MKLE treated (MKLE) after one week of acclimatization. The (MKLE) group was pre-treated (50mg/kg/day) for 10 successive days and it was continued until all the animals were sacrificed at the end of 10th postoperative week. The animals had free access to standard laboratory food and water ad libitum, and two animals were housed in each cage under (12 hr) light-dark cycle. The care for laboratory animals was taken as per guidelines of the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication) as well as the guidelines and recommendations of IIUM ethical committee and Malaysian National Animal Welfare Foundation.

Extract preparation and administration

About 10kg of fresh and matured M. koenigii leaves were collected from a local supplier in Kuala Lumpur after being authenticated and documented with a voucher specimen (PIIUM211) by Dr. Norazian Binti Mohd Hasan, a phytochemist and botanist from Faculty of

Pharmacy, IIUM. The leaves were shade-dried at room temperature (25°C± 1°C) for 14 days and undergone for crushing in electric blender to form a coarse powder. The sample was then subjected to extraction with 95% methanol using Soxhlet's extractor and defatted using petroleum ether (60-80ºC) (Juvekar, 2006). The extract was dried using RotaVapor-210 (Buchi, Switzerland) at slow evaporation process at very low pressure and temperature to make it solvent-free. A dry and clean extract of 200g by weight as a dark brown residue was yielded. The yield of MKLE was 20g/kg (w/w) of dry curry leaves. The extract was then stored at 2-8°C for forced oral treatment.

The extract was dissolved in a vegetable oil before it was given using a gavage (cannula) at a daily dose of 50mg/kg of body weight having a concentration of 1mg/0.02ml (extract/vehicle) while (n=6) with an average weight of 300gm. The pre-treatment was conducted with the same dose of the extract for 10 successive days before 2VO surgery was conducted.

Two-vessel occlusion (2VO) surgery

A small ventral mid-line incision of 2cm in length was made just above the sternal bone of the neck area to expose both the left and right common carotid arteries under aseptic conditions after pre-anaesthetic preparation of atropine sulphate (0.1mg/kg) was given intramuscularly to prevent respiratory distress. As general anaesthesia, a combination of ketamine and xylazine injection of (90mg/kg and 0.1mg/kg) respectively was given intraperitoneally as earlier mentioned (Azzubaidi, et al., 2012). Arteries were isolated with gentle tweezing of neck muscles from each of the carotid sheath and vagus nerve for proper identification before making ligatures using silk suture (5-0 size) just below the carotid bifurcation into internal and external carotid arteries (Sivilia et al., 2009).

With the exception of occlusion of carotid arteries, surgical procedure in healthy control animals was as similar as those of 2VO. After surgery, the rats were closely monitored at their respective cages for the first 24 hours during post-operative recovery while the temperature was maintained at 37°C with a heating lamp to avoid from incidental hypothermia until complete recovery from the general anaesthesia. Following recovery, a single intraperitoneal injection of buprenorphine (0.05mg/kg) was given to reduce neck muscles pain efficiently as it was not commenced to provide water immediately (Azzubaidi, *et al.,* 2012).

Collection and storage of hippocampal tissues

All the animals were humanely euthanized using over-dosed diethyl ether inhalation at the end of the 10th postoperative week. The hippocampal tissue from each brain sample was identified, dissected to collect quickly on ice-cold stainless steel tray with ice-cold surgical forceps to prevent physicochemical degradation of the tissues as it is reported that lipid tissues are highly susceptible to surrounding temperature. The room temperature and aseptic conditions were maintained for the whole process. The samples were then immediately stored in Allprotect™ tissue reagent (Qiagen, Germany) at – 20°C until further processing.

Total RNA extraction

Prior to tissue disruption and homogenization, the weight of all 18 individual hippocampal tissues were taken to quantify the starting material which was 26 mg by average weight and the variation was less than ±10%. Total RNAs from hippocampal tissues were then extracted by QIAzol® Lysis reagent (450-600µl) using TissueRuptor™ attached with disposable probes according to manufacturer's protocol (RNeasy Lipid Tissue Mini Kit, Qiagen, Germany). The homogenate formed was kept on bench-top for 5-7 min at 18°C to disperse the foam before adding 200µl of chloroform followed by vortexing for 15s. The lysate was again kept for 3 min on the bench-top at room temperature. Then, it was subjected to centrifugation at 12,000 x g for 15 min at 4°C. The clear aqueous phase (i.e., the uppermost layer) of 600µl was transferred into a new collection tube (2ml) in which 600µl of 70% ethanol (supplied) was added and mixed thoroughly by pipetting up and down followed by vortexing for seconds. 700µl of the sample was transferred again into RNeasy Mini Spin column placed in a 2ml collection tube and centrifuged at 8,000 x g for 15s at room temperature.

After discarding the flow-through, 700µl of buffer RW1 was added to the spin column and centrifuged at 8,000 x g for 15s at room temperature. Again, the flow-through was discarded before adding 500µl of Buffer RPE to the spin column and centrifuged at 8000 x g for 15s at room temperature. The flow-through was discarded; again 500µl of Buffer RPE was added to the column before centrifugation at 8,000 x g for 2 min.

The spin column was carefully removed from 2ml tube and placed it back to a new 1.5ml tube and added 30µl of RNase-free water directly to the column membrane and centrifuged at 8000 x g for 1 min after which the spin column was removed and the pure total RNA sample was immediately labelled and stored at -80°C for experimental use. In short, total ribonucleic acid (RNA) from 18 samples having an average weight of 26 mg per tissue was extracted by acid guanidium thiocyanate-phenol-chloroform method involving photophobic QIAzol lysis

reagent as described in RNeasy® Lipid Tissue Handbook (Qiagen, Germany).

RNA quality and Integrity

As the sample degradation mainly depends on collection method, the storage and preservation of tissues, here, we preserved the samples with Allprotect™ tissue reagent (Qiagen, Germany) at -30ºC for less than six months when they were harvested. The purity and quality of RNA samples by ratio of OD260/280 (≥1.8 to 2.0) and quantity in ng/µl were determined by UV/VIS spectrophotometric method. As the integrity of total RNA could not be traced by UV/VIS method of 260nm (specific for nucleic acid) and 280nm (specific for proteins), the high innovative lab-on-chip technology of Experion® (Bio-Rad, USA) (Fleige & Pfaffl, 2006; Fleige et al., 2006) was used for the investigation of ribosomal 28S/18S ratio that plays an important role in determining the level of sample degradation. Only RNA samples with an A260nm/A280nm absorption ratio ≥ 1.8 having RNA integrity (RQI) values between 7.5 and 10 were subjected to downstream RT-qPCR assay.

Reverse transcription (RT) and reverse transcript quantitative PCR (RT-qPCR)

After extraction, quantification and integrity of total RNA, 5µl (5ng/µl) of RNA was reversed by 4 µl of 5x iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA) that contains deoxynucleotide triphosphate (dNTP), oligo (dT), Moloney murine leukemia virus-reverse transcriptase MMLV-RT (RNaseH+), RNase inhibitor, random primer mix, gene-specific primer enhancer, buffer, MgCl2 and stabilizers where RNase-free water of 11µl made the final volume of 20µl in each reaction. The reaction conditions for RT by Mastercycler® Pro Thermal cycler (Eppendorf®) were set according to manufacturer's protocol (iScript™, Bio-Rad). Quantitation of cDNA was determined by UV absorbance at 260nm and 280nm by BioPhotometer® Plus with Hellma® Tray Cell (Eppendorf®). The standard curve analysis (Fig.1) was observed prior to RT-qPCR assay in six-log concentration by serial dilutions (1:10 to 1:1,000,000) of randomly selected two RNA samples (5ng/µl) from each group. The two-step reverse transcription quantitative PCR (RT-qPCR) was performed at pre-determined annealing temperature of 59 ºC by (10µl/well) of SsoFast™ EvaGreen® (SYBR Green I) based detection using 10x pre-designed QuantiTect™ primers (Qiagen, Germany) (2µl/well) of two controls (18S rRNA & Actin B) and targets (MAP2 and GFAP) in technical replicates in a low-profile 96-well plate of 20µl reaction that included 3µl of RNase-free water per well while NTC or No-RT was used RNase-free water alone. The melt curve analysis (Fig.2 & 3) at P-value 0.01 and regulation thresholds 4.00 were also respectively done by CFX®96 Real-Time System (Bio-Rad, Hercules, CA, USA). The intergroup comparison (Fig.4) was done based on the mean relative normalized gene expression (ΔΔCq) levels where Cq is the threshold quantity at ±1 SEM as earlier mentioned method of (Schmittgen & Livak, 2008). The Minimum Information for publication of quantitative real-time PCR experiments (MIQE) guidelines has been strictly maintained (Bustin *et al*., 2009).

Statistical Analysis

Results expressed are the relative normalized gene expression (ΔΔCq) as mean ±SEM. Statistical analysis of the data was done by independent, unpaired student t-test at 95% confidence interval (CI) using GrapPad® Prism. The p values less than 0.05 were considered statistically significant and the method applied for comparison was as of (Schmittgen & Livak, 2008). The expression of each targeted gene was normalized relative to the internal (endogenous) control transcripts (Schefe *et al*., 2006).

RESULTS

The relative normalized hippocampal GFAP and MAP2 mRNA expressions in 2VO rats treated with MKLE were expressed as mean ± SEM (Figure.1.(d)) of technical replicates in 20µl reaction. The standard curve analysis (Figure.1(a)) for each primer in a six-log concentration prepared by serial dilutions (102 to 107) from a mixture (5µl x 2 samples/group) of two randomly selected intact RNA samples representing each group was run together by conventional PCR method and observed at 59ºC according to (S. Derveaux, 2009). The melt-curve analysis (Fig.1.(b) & (c)) for each targeted gene was normalized and amplified in parallel with the selected control genes (18s rRNA & Actin B) while the inter-run calibrator (IRC) with known concentration of untreated control was maintained accordingly as prescribed below.

In this study, 2VO-induced male SD rats were employed to produce neurodegeneration with subsequent chronic cerebral ischemia and hypoxia which have been closely linked with neuroinflammation and memory deficits including Alzheimer's disease (AD) (De Jong et al., 1999) where hippocampus is the major brain area prone to dysfunctions (De Jong *et al.,* 1999; Farkas, 2002). The GFAP is an intermediate filament (IF) thought to be very specific for astrocytes (also known as astroglia collectively) found mainly in the brain and spinal cord of the central nervous system (Pekny, *et al.,* 2013). It has been directly linked to neuroinflammation and its process in the pathogenesis of neurodegeneration while cognitive impairments are associated with AD and dementia (Hauss-Wegrzyniak, et al., 1998) as well. It was Garrison and his co-workers who were the first to report a

correlation between astrocytes and hypersensitivity to pain after peripheral nerve injury which was considered as an indicator for glial activation (i.e., astrogliosis) (Garrison *et al.*, 1991). It has been widely used as a distinct molecular marker to label astrocytic proliferation that accumulates in reactive astrocytes as a result of neuroinflammation in chronic cerebral hypoperfusion (Juma et al., 2011) in AD. Alike MAP2, GFAP involves in cellular functioning processes such as cell structure and movement, cell communication and the functioning of the blood brain barrier (BBB). The MAP2 is one of the members of tau family protein that interacts with microtubules of the brain which are vital components of cytoskeleton serving as structural components within the cells and involving in many cellular processes including cytokinesis, mitosis and vascular transport (Cohen *et al*., 2013). It is mostly found in dendrites. It also plays important role in maintaining the structure and shape of microtubules to facilitates neuronal plasticity, dendritic remodelling and axonal growth etc., in order to maintain and/ or recover the spatial memory and navigational power within the hippocampus during neuroglial crises (Al-Bassam *et al*., 2002; Dehmelt & Halpain, 2005; Farkas *et al*., 2007; Liu *et al*., 2005). The core objective of the current study was to compare the relative gene expression (ΔΔCq) of GFAP and MAP2 in MKLE treated 2VO male SD rats to that of HC and 2VO untreated as important biomarkers of neuroinflammation and neuroprotection on the progress of neurodegeneration which was experimentally achieved by 2VO surgery that uses permanent bilateral double ligature of common carotid arteries to restrict the cerebral blood flow and

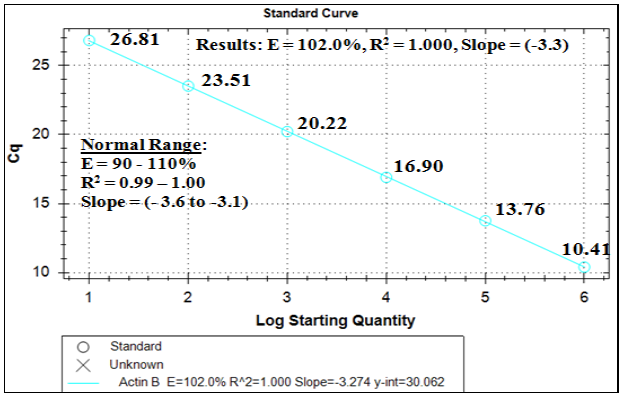

Figure 1(a): Showing the standard curve analysis for one of the control genes (Actin B) in six-log concentration prepared by serial dilutions (1:10 to 1:1,000,000) of two randomly selected pure RNA samples mixture of 5µl (5ng/µl) x 3 groups. The resulted average cycle difference is 2.7 when Cq is the crossing point or quantification cycle run by CFX96® Real-Time System.

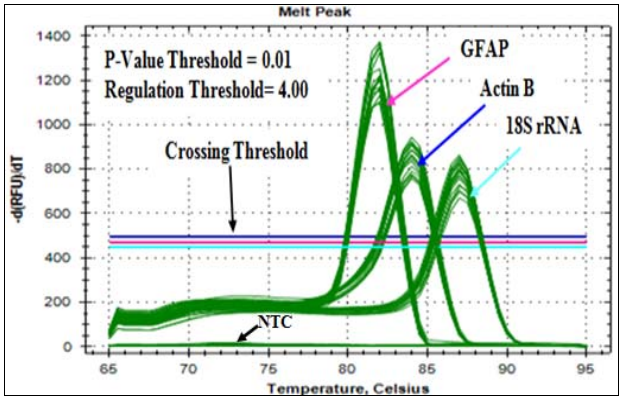

Figure 1(b): The melt curve analysis for targeted GFAP *m*RNA expression of MKLE treatment in respect to the control genes (18S rRNA & Actin B) run by CFX96® Real-Time System.

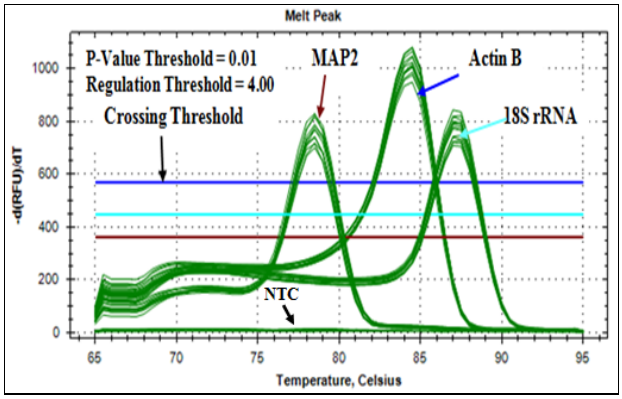

Figure 1(c): The melt curve analysis for targeted MAP2 *m*RNA expression of MKLE treatment in respect to the control genes (18S rRNA & Actin B) run by CFX96® Real-Time System.

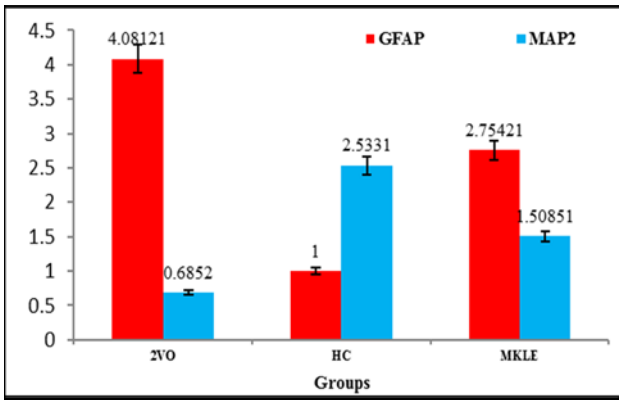

Figure 1(d): The gene expression analysis showing insignificantly different #(*P*>0.01) relative MAP2 *m*RNA expressions (ΔΔCq) ofMKLE treated (50mg/kg/day)

oxygen supply that resulted in ultimate brain ischemia and hypoxia (Azzubaidi, *et al*., 2012; Farkas *et al*., 2007; Lemos *et al*., 2013; Saxena *et al*., 2011; Zhou *et al*., 2013). Previous studies on 2VO-induced neurodegeneration in vivo reported that GFAP was found to up-regulated indicating 'astrogliosis or hypertrophy' which has been considered as a late consequence of neuroinflammation due to chronic cerebral hypoperfusion taken place in hippocampus (Farkas *et al*., 2007) whilst down-regulated MAP2 was found as the result of neuronal loss and/or damage in 2VO rats after chronic cerebral ischemia and hypoxia (Annaházi *et al*., 2007; Farkas *et al*., 2007). Murraya koenigii (Linn.) Spreng leaves also commonly known as curry leaves in English or kari patta in Hindi (Family: Rutaceae) that represents more than 150 genera and 1,600 species (Jain *et al*., 2012) is a popular flavouring agent or spice in many South and Southeast Asian countries including Malaysia while it had records of various folkloric uses for therapeutic values (Ajay *et al*., 2011; Bhandari, 2012). A great number of relevant scientific studies have been reported on efficacy of the curry leaves with different forms of extract for many types of diseases whereas few of the recent studies stressed on bioactive carbazole alkaloids (e.g., mahanine, mahanimbine, mahaminbicine & murrayafoline etc.) from this plant due to its wide range of curative properties (Kumar *et al*., 2010; Mandal, 2010; Ramsewak, 1999; Rao, 2011; Wang, 2002) such as anti-oxidant, anti-cholinesterase and anti-amnesic (Mani *et al*., 2013; Tembhurne & Sakarkar, 2010; Vasudevan & Parle, 2009), anti-inflammatory and anti-cancer (Gupta *et al*., 2010; Mathur, 2011; Muthumani *et al*., 2009) and anti-diabetic (Arulselvan *et al*., 2006; Yadav, 2002) etc. Nevertheless, this is the first study on crude methanolic extract of the curry leaves to report their anti-neuroinflammatory and neuroprotective effects explored via quantitative gene expression analysis of regulation of these two markers of neuroglial crises and neuronal wellbeing after chronic cerebral ischemia and hypoxia of 10 weeks duration. The results of the current study agreed with the previous reports made by (Mani *et al*., 2013; Muthumani *et al*., 2009; Vasudevan & Parle, 2009) for anti-amnesic, anti-oxidant and anti-inflammatory activities of curry leaves respectively. The results of the current study (Fig.1. d) showed that there was significantly different ($P<0.001$) up-regulated GFAP mRNA expression in 2VO untreated group as compared to that of HC group indicating astrogliosis due to neuroglial activation even after 10 weeks of 2VO surgery while significantly different ($P<0.001$) down-regulated MAP2 mRNA expression in 2VO as compared to that of HC group exhibiting neuronal damage due to apoptotic neurons. On the other hand, MKLE treatment showed significant difference ($P<0.05$) of GFAP mRNA expression as compared to that of 2VO while it was not insignificantly different to that of HC group showing moderate anti-neuroinflammatory activity on chronic cerebral ischemia and hypoxia of 2VO treated rats. And the MAP2 mRNA expression in MKLE treated 2VO group was insignificantly ($P=0.23$) down-regulated as compared to that of 2VO whilst it was significantly less ($P<0.001$) than that of HC group.

CONCLUSIONS

By relative normalized gene expression ($\Delta\Delta Cq$) analysis of the two selected markers (GFAP & MAP2) of neuroglial crises and wellbeing of the brain hippocampal tissues studied (Fig.1. d), it could be concluded that Murraya koenigii (L.) Spreng leaves extract of methanol treated as 50mg/kg/day b.w for 10 weeks in 2VO rats could have very slight or no anti-neuroinflammatory activity while it could have moderate to high neuroprotective activity in experimental neurodegeneration caused by chronic cerebral ischemia and hypoxia including neuroinflammation depending on various factors such as duration of study or treatment, dose, dose interval, type of extract and extraction procedure. The possible mechanism behind this neuroprotective activity of MKLE was due to either free radical scavenging or prevention of formation of toxic-free radicals within hippocampal neurons. But, MKLE treatment has failed to provide or restore neuronal plasticity, dendritic remodelling and axonal outgrowth sufficiently via prevention of MAP2 from gradual down-regulation after chronic cerebral ischemia and hypoxia induced by 2VO surgery. Further studies could be recommended using different solvent and extraction protocols.

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