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# Neuroprotective Effects of *Ocimum basilicum* L. var. *thyrsiflora on* Scopolamine-Induced Non-Spatial Memory Deficits in Rats

(Kesan Neuropelindung *Ocimum basilicum* L. var. *thyrsiflora* pada Kemerosotan Ingatan Bukan Reruang Aruhan Skopolamina Tikus)

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ABSTRACT

Pharmacological studies indicated that *Ocimum basilicum* L. var. *thyrsiflora* has numerous therapeutic potentials. The aim of this study was to investigate the neuroprotective action of *O. basilicum* leaf extract against scopolamine-induced non-spatial memory deficits in rats and to determine the changes in mRNA expressions of genes implicated in cognition and neuroprotection. *O. basilicum* leaves were extracted with 80% ethanol and verified for the presence of rosmarinic acid using high performance liquid chromatography method. Male Wistar rats were treated orally with either*O. basilicum* or the positive control piracetam for 14 days prior to the injection of 0.5 mg/kg scopolamine on the day of the novel object discrimination (NOD) test. Hippocampi were collected at the end of the test. mRNA expression of nicotinic acetylcholine α7 subunit (NA7), muscarinic M1 receptor (M1), neuronal nitric oxide synthase (nNOS), and 5-hydroxytryptamine receptor 3A (HTR3A) genes in the hippocampi were analyzed using qPCR method. The presence of rosmarinic acid in the plant extract was detected at chromatogram peak of Rt=16.891. NOD test results indicated that the lower dose of *O. basilicum* (200 mg/kg) significantly ($p$<0.05) reversed scopolamine-induced memory deficits in rats similar to the effects of piracetam. In addition, *O. basilicum* at the same dose alleviated the increase in mRNA expressions of the NA7, M1, nNOS, and HTR3A genes induced by scopolamine. The present findings suggest that *O. basilicum* is potentially neuroprotective in preventing memory impairment through alleviation of scopolamine-induced changes in hippocampal mRNA expression implicated in cognition and neuroprotection.

Keywords: Cognition; gene expression; memory; neuroprotection; *Ocimum basilicum*

ABSTRAK

Kajian farmakologi menunjukkan bahawa *Ocimum basilicum* L. var. *thyrsiflora* mempunyai banyak potensi terapeutik. Matlamat kajian ini adalah untuk mengkaji kesan neuropelindung ekstrak daun *O. basilicum* terhadap kemerosotan ingatan bukan reruang aruhan skopolamina pada tikus dan untuk menentukan perubahan dalam ekspresi mRNA gen yang terlibat dalam kognisi dan perlindungan saraf. Daun *O. basilicum* telah diekstrak dengan 80% etanol dan kehadiran asid rosmarinik disahkan menggunakan kaedah kromatografi cecair berprestasi tinggi. Tikus Wistar jantan diberi *O. basilicum* atau *piracetam* sebagai kawalan positif secara oral selama 14 hari sebelum suntikan skopolamina 0.5 mg/kg diberikan pada hari ujian diskriminasi objek novel (NOD). Hipokampus dikumpul pada akhir ujian tersebut. Ekspresi mRNA subunit α7 nikotinik asetilkolin (NA7), reseptor M1 muskarinik (M1), nitrik oksida sintase neuron (nNOS), dan gen reseptor 5-hidroksitriptamina 3A (HTR3A) dalam hippokampus dianalisis menggunakan kaedah qPCR. Kehadiran asid rosmarinik dalam ekstrak tumbuhan dikesan pada puncak kromatogram Rt=16.891. Keputusan

ujian NOD menunjukkan bahawa dos *O. basilicum* yang lebih rendah (200 mg/kg) memulihkan kemerosotan ingatan yang disebabkan oleh skopolamina dengan ketara ($p<0.05$) pada tikus serupa dengan kesan piracetam. Di samping itu, *O. basilicum* pada dos yang sama mengurangkan peningkatan dalam ekspresi mRNA gen NA7, M1, nNOS dan HTR3A yang diaruh oleh skopolamina. Hasil kajian ini menunjukkan bahawa *O. basilicum* mempunyai potensi neuropelindung dalam mencegah kemerosotan ingatan melalui pengurangan kesan yang disebabkan oleh skopolamina dalam ekspresi mRNA hipokampus yang terlibat dalam kognisi dan perlindungan saraf.

Kata kunci: Ekspresi gen; ingatan; kognisi; *Ocimum basilicum*; perlindungan saraf

## Introduction

*Ocimum basilicum* L., or sweet basil, is an herb native to Asia, Africa, North America, and the Mediterranean (Grayer et al. 1996). It belongs to the Lamiaceae family, which grows in several regions throughout the world (Baritaux et al. 1992). *Ocimum basilicum* var. *thyrsiflora* L. is an *O. basilicum* cultivar commonly known as Thai basil or locally known as 'kemangi' in Malaysia. It is generally characterized by its purple stems and flowers (Figure 1). *O. basilicum* is cultivated for culinary use and almost all parts of the plant are used for medicinal treatments. Traditionally, it is used to treat various human ailments, such as cough, ringworm infections, menstrual disorders, headache, fever, and bowel disorders (Vlase et al. 2014). Indeed, *O. basilicum* has a wide range of other pharmacological activities. For instance, it is an antioxidant (Berić et al. 2008; Othman et al. 2021) and also exhibit anti-inflammatory properties (Othman et al. 2021; Rameshrad et al. 2015) and recently considered as a neuroprotective agent (Bora, Arora & Shri 2011; Koutroumanidou et al. 2013; Seyed et al. 2021).

The neuroprotective effect of *Ocimum basilicum* leaf extract has been studied in several *in vivo* and *in vitro* models of neurodegeneration such as cerebral injury induced by transient bilateral common carotid artery occlusion, chemically-induced memory deficits, and chemically-induced oxidative stress (Mohd-Zahid et al. 2018; Sarahroodi et al. 2012; Singh et al. 2018, 2016). A previous study by Singh et al. (2018) demonstrated a marked improvement in long-term memory and motor coordination as a result of pretreatment with *O. basilicum* following a cerebral infarct in mice. In a different *in vivo* study, hydroalcoholic extract of *O. basilicum* significantly ($P<0.05$) increased memory retention and increased memory retrieval in a passive avoidance test (Sarahroodi et al. 2012). In addition, *O. basilicum* pre-treatment reversed memory deficits induced by scopolamine in mice, evident by significant ($p<0.05$) decrease in the transfer latency time and increase in step down latency in elevated plus maze and passive shock avoidance task, respectively (Singh et al. 2016). The extract also significantly reduced the brain acetylcholinerasterase (AChE) activity and oxidative stress. Anatomical changes such as increased pyramidal cells in hippocampal and cortical regions were observed (Singh et al. 2016).

FIGURE 1. *Ocimum basilicum* var. *thyrsiflora* L., also known as Thai basil or 'kemangi'

A few *in vitro* studies investigated the role of *O. basilicum* in neuroprotection against oxidative damage in neuronal cells (Mohd-Zahid et al. 2018; Singh et al. 2016). *O. basilicum* extract showed marked free radical scavenging, reducing power and AChE inhibition activities (Singh et al. 2016). Similarly, pretreatment with *O. basilicum* extract protected against $H_2O_2$-induced cell death by increasing cell viability, reducing apoptosis and decreased the markers of oxidative stress in SK-N-SH neuroblastoma cells (Mohd-Zahid et al. 2018). Taken together with phytochemical studies of the plant, these *in vivo* and *in vitro* studies concluded that the neuroprotective and memory enhancing effects of *O. basilicum* are due to its antioxidant activity which may be attributed to the presence of polyphenols, flavonoids and essential oil (Al-Snafi 2021a, 2021b; Mohd-Zahid et al. 2018; Singh et al. 2016).

Our previous study demonstrated that *O. basilicum* extract showed *in vitro* neuroprotective activities by lowering oxidative damage caused by $H_2O_2$-induced reactive oxygen species (ROS) through intracellular superoxide dismutase (SOD) restoration (Mohd-Zahid et al. 2018). These findings are comparable to rosmarinic acid treatment, a natural polyphenol which has been considered as one of the major bioactive compounds found in *O. basilicum* that exerts various biological activities including neuroprotection (Jayasinghe et al. 2003; Srivastava et al. 2014; Mohd-Zahid et al. 2018). Previous studies have been demonstrated that rosmarinic acid ameliorated scopolamine-induced learning and memory deficits in rats (Hasanein & Mahtaj 2015).

It is well known that neurotransmitter acetylcholine (ACh) is involved in learning and memory processes (Deutsch 1971). Meanwhile, piracetam, a cyclic derivative of γ-aminobutyric acid (GABA) showed protective effects on brain function by increasing the ACh formation (Müller, Eckert & Eckert 1999). A report by Warburton et al. (2003) demonstrated that cholinergic neurotransmission within the perirhinal cortex of the temporal lobe is essential for recognition, memory and synaptic plasticity, which has the ability to establish the synaptic connection between two neurons to increase or decrease the neuronal network. On the other hand, scopolamine, which is an anticholinergic agent specific to muscarinic receptors has been shown to cause recognition memory deficits, which is characterized by the absence of explorative behavior toward novel objects (Bartolini, Casamenti & Pepeu 1995; Bertaina-Anglade et al. 2006; Besheer, Short & Bevins 2001; Ennanceur & Meliani 1992a). Scopolamine causes deficits in sensory memory by antagonizing central cholinergic function. It is an anti-muscarinic agent that binds competitively to M1 receptor (Pitsikas et al. 2003). There are currently no available data on the neuroprotective effects of *Ocimum basilicum* var. *thyrsiflora* on memory deficits in rats. In addition, whether *O. basilicum* improves memory and cognition by directly competing with scopolamine binding on the M1 receptor or exerts its pharmacological activity by regulating other receptors or proteins is not known. Memory and cognition are regulated by a range of neurotransmitters, including ACh, serotonin, GABA, and norepinephrine (Sheffler, Reddy & Pillarisetty 2021).

The protective potential of *Ocimum basilicum* var. *thyrsiflora* L. leaf extract in reversal of scopolamine-induced memory deficits in rats was investigated using non-spatial memory test after 14 days of treatment with the extract. Similarly, piracetam, a nootropic agent known to have neuroprotective effects against scopolamine-induced memory deficits (Chidamabaram et al. 2015), was used as a positive control. Expression of genes representing different classes of neurotransmitters involved in memory and cognition were also measured to identify potential implicated molecular pathways. The presence of rosmarinic acid in the leaf extract was identified using high performance liquid chromatography (HPLC) method.

## Materials and Methods

### Plant materials and preparation for *O. basilicum* leaf extract

*O. basilicum* crude extract was prepared according to previously published protocol (Mohd-Zahid et al. 2018). *Ocimum basilicum* var. *thyrsiflora* L. plants were purchased from a local plantation in Gombak, Kuala Lumpur. A voucher specimen (HF100) was deposited at the Herbarium Universiti Kebangsaan Malaysia (UKM), Bangi. Briefly, the leaves were dried and ground into fine powder form. The powder was then macerated in 80% ethanol, filtered and concentrated using a rotary evaporator under reduced pressure. The extract was freeze-dried to obtain the crude extract and stored at 4 °C in an amber glass bottle until further use. The percentage yield for crude extract was 14.90%. The *O. basilicum* extract was dissolved in 1.5% Tween-20 as a vehicle before oral administration.

### CONFIRMATION OF ROSMARINIC ACID PRESENCE IN THE EXTRACT USING HPLC

Chromatographic analysis was performed using HPLC Quaternary Gradient Module (Waters Co., Milford, MA, USA) equipped with a photodiode array detector (Waters Co., Milford, MA, USA) and Empower 2 software. Separations were carried out on a SunFire C18 (5 µm, 4.6 × 50 mm) column. The separation was conducted according to Srivastava et al. (2014). In summary, deionized water+0.1% orthophosporic acid and methanol (HPLC grade)+0.1% orthophosporic acid were used as mobile phase A and mobile phase B, respectively, by following the gradient program as follows: 0-2 min, 0% B (isocratic), 2-5 min, 40% B (linear gradient), 5-10 min, 50% B (linear gradient), 10-18 min, 50% B (isocratic), 18-23 min, 40% B (decreasing gradient), 23-25 min, 0% B (equilibration). The injection volume was 20 µL and the flow rate of the elution was 1.0 mL/min. The column was maintained at 25 °C throughout the analysis. The wavelength for detection was set at 280 nm.

### ANIMALS

Seventy (n=70) male Wistar rats weighing between 150 and 200 g were purchased from Taconic Breeding Centre, Singapore. The rats were randomly housed in plastic cages with five rats in each cage. The animals were housed at 28 ± 1 °C under a standard 12-hour/12-hour light/dark cycle, and had *ad libitum* access to normal laboratory food and water. All procedures were carried out based on the protocol approved by the UKM Animal Ethics Committee (FF/2014/NORAZRINA/21-MAY/585-JUNE-2014-JUNE-2016).

### STUDY DESIGN

The rats were divided into seven groups (n=10 each) and given the following treatment: vehicle control group which received oral solution of 1.5% Tween-20; scopolamine 0.5 mg/kg given intravenously, piracetam 250 mg/kg given orally, scopolamine+piracetam, scopolamine+*O. basilicum* 200 mg/kg, scopolamine+*O. basilicum* 400 mg/kg, and *O. basilicum* 400 mg/kg. The groups that received *O. basilicum* and piracetam were treated for 14 days by oral gavage and intraperitoneally, respectively, before scopolamine was injected 30 minutes before the behavioral test on day 15. Scopolamine hydrobromide trihydrate and piracetam were purchased from Sigma-Aldrich, France. A previous study reported the effect of daily administration of *O. basilicum* for 14 days which significantly ameliorated the oxidative damage induced by monosodium glutamate by increasing the time spent in the preferred arm in the T-maze test (Badiana et al. 2021). *O. basilicum* has also been shown to increase memory retention and retrieval of mice optimally at 400 mg/kg by using passive avoidance apparatus (Sarahroodi et al. 2012). Taken together, these findings formed the basis of dose selection and duration of treatment for the present study.

### BEHAVIORAL TEST

Novel object discrimination (NOD) test was conducted as described by Azmi et al. (2006) with a minor modification. In brief, the animals were habituated to the testing arena one day before the behavioral test. On the test day, animals were subjected to additional habituation for 3 minutes followed by another 1-minute break wherein the animals were free to explore their own cages. The animals then began the first trial (acquisition phase). At this time, the animals were permitted to explore two similar objects (A1 and A2) for 3 minutes. This was followed by a 1-minute inter-trial interval, which was spent in the animals' respective home cages while the experimenter cleaned the cage using 10% ethanol. In the second trial (choice phase), one of the objects presented in the first trial was replaced with a novel object (different shape and different color, B) and the other familiar object (A3) was reintroduced into the arena. The animals were then allowed to explore the novel and familiar objects for another 3 minutes in the testing arena.

Exploration of an object was defined as the rat directing its nose toward an object at a distance of ≤ 2 cm while sniffing or touching the object. Sitting on or beside, leaning against, or biting the objects were not considered as exploration. The time spent by each rat exploring each object was scored manually using a recorded video. The positions of the familiar and novel objects were counterbalanced and randomly permutated during the acquisition and choice trials to reduce object and place preference effects (Ennaceur & Meliani 1992b). All behavioral testing was conducted during the light cycle. At the end of the NOD test, the rats were sacrificed by decapitation and the whole brain was removed. The hippocampi were dissected and subjected to quantitative polymerase chain reaction (qPCR) analysis.

### RNA EXTRACTION AND REVERSE TRANSCRIPTION

One mL QIAzol Lysis Reagent (Qiagen Sciences, Maryland USA) was added to approximately 50 mg of hippocampal tissue. Tissues were homogenized using

a compatible Tissue Ruptor Disposable Probes (Qiagen GmBH). RNeasy Lipid Tissue Mini Kit (Qiagen) was used to perform the downstream RNA extraction following the manufacturer's protocol. Quantification of total RNA samples was carried out using NanoQuant, which also indicated that the extracted samples were of good purity. RNA samples were sent to Malaysia Genome Institute for RNA integrity check using Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). All samples showed good quality of the intact RNA samples based on the acquired RNA integrity number (RIN). cDNA was reverse-transcribed using iScript RT Supermix (Bio-Rad) from 1 µg RNA in 20 µL reactions. No-reverse-transcriptase control reactions were also performed for each sample.

### qPCR

Real-time quantitative PCR was performed using an Eppendorf Master cycler RealPlex4 (Eppendorf) instrument. Reactions were prepared in 10 µL total volume. Each reaction contained 2x SsoAdvanced™ Universal SYBR® Green Super mix (Bio-Rad), 200 nM of forward and reverse primers each, nuclease-free water, and 1 µL cDNA from the reverse transcriptions as template. All primers were synthesized by Integrated DNA Technologies. The details of these primers are provided in Table 1. The thermal cycles were performed based on the manufacturer's protocol. Briefly, the reaction comprised 1 cycle at 95 °C for 30 seconds for DNA polymerase activation and initial DNA denaturation, followed by 35 cycles of amplification at 95 °C for 10 seconds (denaturation) and 60 °C for 60 seconds (annealing/extension). Melting point analyses were included in every PCR run. The specificity of the product was checked and confirmed using a single melt peak. No-template and no-reverse transcriptase controls were also included to out rule mRNA expression arising from DNA contamination.

TABLE 1. Details of the primers used for qPCR analysis

| Gene symbol & ID | Primer sequences | Primer length (nt) | Product size (bp) | References |
|---|---|---|---|---|
| CHRNA7 (NM_012832.3) | F: AACTGGTGTGCATGGTTTCTGCGC<br>R: GATCTTGGCCAGGTCGGGGTCCC | 24<br>24 | 300 | (Yamamoto et al. 2011) |
| CHRM1 (NM_080773.1) | F: AGCAGCTCAGAGAGGTCACAGCCA<br>R: GGGCCTCTTGACTGTATT TGGGGA | 24<br>24 | 273 | (Yan, Flores-Hernandez & Surmeier 2001) |
| nNOS (NM_052799.1) | F: AATGGAGACCCCCCTGAGAAC<br>R: TCCAGGAGGGTGTCCACCGC | 21<br>20 | 383 | (Kumagai et al. 2004) |
| HTR3A (NM024394.2) | F: AGCCTTGACATCTATAACTTCC<br>R: TCCGACCTCACTTCTTCTG | 22<br>19 | 125 | (Rahman et al. 2009) |
| HTR3B (NM_022189.1) | F: CGGCACAGTGAGCAAGAACG<br>R: AGTGGCACAGGATGGCGAGC | 20<br>20 | 244 | (Sudweeks, Hooft & Yakel 2002) |
| β-Actin (NM_031144.3) | F: TGGAGAAGAGCTATGAGCTGCCTG<br>R: GTGCCACCAGACAGCACTGTGTTG | 24<br>24 | 202 | (Kumagai et al. 2004) |
| GAPDH (NM_017008.4) | F: CCCCCAATGTATCCGTTGTG<br>R: TAGCCCAGGATGCCCTTTAGT | 20<br>21 | 118 | (Piller, Decosterd & Suter 2013) |
| cycA (NM_017101.1) | F: TATCTGCACTGCCAAGACTGAGTG<br>R: CTTCTTGCTGGTCTTGCCATTCC | 24<br>23 | 127 | (Moura et al. 2014) |

### qPCR DATA ANALYSIS

Each qPCR reaction was run in triplicate, and the mean Ct values were used for downstream calculations and analysis (Ct is the crossing point threshold of the sample for the amplified genes). Standard curves were generated for each set of primers and slope values were obtained from the linear regressions. Reaction efficiencies were calculated using the formula below (Ramakers et al. 2003):

$$\text{Efficiency} = E = 10^{-1/\text{slope}}.$$

The efficiency values for each set of primers were nicotinic acetylcholine α7 subunit (NA7) (2.163), muscarinic M1 receptor (M1) (2.119), neuronal nitric oxide synthase (nNOS) (2.259), 5-hydroxytryptamine receptor 3A (HTR3A) (2.082), 5-hydroxytryptamine receptor 3B (HTR3B) (2.728), β-actin (2.093), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (2.077), and cyclinA (cycA) (2.063). Subsequently, the relative mRNA expression ratio was calculated using the following formula:

$$\text{Ratio} = (\text{Efficiency}_{\text{target}})^{\Delta\text{Ct target(control-sample)}} / (\text{Efficiency}_{\text{ref}})^{\Delta\text{Ct ref(control-sample)}},$$

where target genes were normalized to GAPDH as the reference gene, and vehicle-treated specimens were used as controls.

### STATISTICAL ANALYSIS

The parameter measured in the behavioral experiment was the time spent exploring each object during acquisition (A1 and A2) and choice (A3 and B) trials. Data were analyzed using Prism software version 5, and the results obtained are expressed as means ± standard errors of the mean (SEMs). Paired student's t-tests (two-tailed distribution) was utilized to analyze the statistical difference between the time spent exploring the two identical objects (A1 and A2) or the familiar and novel objects (A3 and B). The genetic data were analyzed using one-way ANOVA followed by Dunnett's *post hoc* multiple comparisons. Statistical significance was set at a probability level of $p < 0.05$ for all data.

## RESULTS AND DISCUSSION

The present study evaluated the neuroprotective potential of *O. basilicum* leaf extract against non-spatial memory deficits induced by scopolamine and its influence on the potential receptor-expressing genes involved in memory processes and neuroprotection.

The presence of an active compound rosmarinic acid in the *O. basilicum* hydroethanolic extract was confirmed using HPLC (Figure 2(A)) in accordance with our previous report (Mohd-Zahid et al. 2018). The peak was detected at similar retention time as rosmarinic acid standard compound (Figure 2(B)) which was Rt=16.82 min. Rosmarinic acid content was the highest in the ethanolic fraction of *O. basilicum* and the hexane fraction showed the least amount suggesting that this compound is effectively extracted in polar solvents (Al-Snafi 2021a; Mohd-Zahid et al. 2018). Several bioactive compounds have been identified and associated with neuroprotective activities of *O. basilicum* include apigenin, luteolin, anthocyanins and eugenol (Seyed et al. 2021).

The NOD task is useful in studying short-term, intermediate-term, and long-term memory (Dodart, Mathis & Ungerer 1997; Ennanceur & Delacour 1988). The type of memory assessed is determined through the management of the retention interval, i.e., the amount of time animals must maintain memory of the sample objects presented during the familiarization stage before the test period, when one of the familiar objects is replaced by a novel object (Seifhosseini et al. 2011; Taglialatela et al. 2009). Scopolamine, an antimuscarinic agent, administered in rats has been shown to impair cognitive performance in the NOD task involving learning and memory (Azmi et al. 2006; Kwon et al. 2010; Lu et al. 2018). This suggests that intact cholinergic neurotransmission is required to perform this task (Bartolini, Casamenti & Pepeu 1995; Bertaina-Anglade et al. 2006; Besheer, Short & Bevins 2001; Ennanceur & Meliani 1992a). Piracetam (250 mg/kg) was used as a positive control in this study for reversing scopolamine-induced memory deficits (Chidamabaram et al. 2015). Piracetam is a cyclic derivative of GABA and has a protective effect on brain function against externally applied brain insults, including hypoxia, electroconvulsive treatment, and barbiturate intoxication in experimental animals (Winblad 2005). It increases high-affinity choline uptake, a process that occurs in cholinergic nerve endings and increases ACh formation (Müller, Eckert & Eckert 1999).

Here we report the potential protective effects of *O. basilicum* against scopolamine-induced deficits in non-spatial working memory. Exploration activity of the animals in acquisition trial was measured by scoring the time spent exploring two identical objects (A1 and A2). Paired student's t-tests did not indicate significant

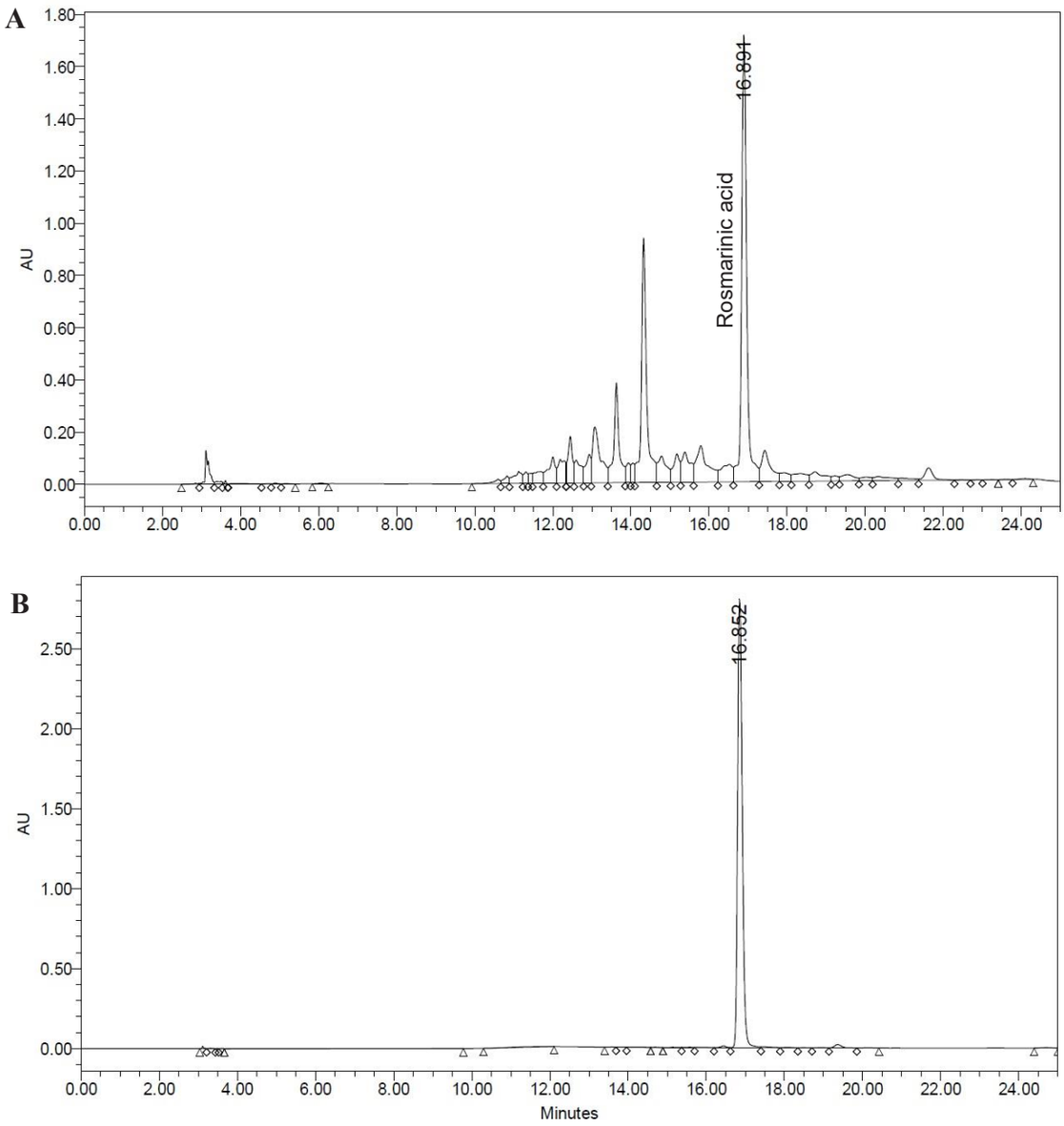


FIGURE 2. (A) *O. basilicum* crude extract showed the presence of rosmarinic acid at Rt=16.891 as compared to (B) the chromatogram of standard biomarker compound, rosmarinic acid which showed a peak at Rt=16.852

differences ($p > 0.05$) in the amount of time spent to explore the two identical objects between the treatment groups (Figure 3(A)). Thus, the rats did not have a preference for either of the similar objects explored in the acquisition trial.

Similarly, the exploratory activity of the rats during the choice trial was determined by assessing the length of time spent exploring either the familiar object (A3) or the novel object (B). Paired student's t-test indicated that there were significant differences ($p < 0.05$) in the time spent exploring the novel vs. the familiar object in the groups treated with vehicle, piracetam, piracetam+scopolamine, and *O. basilicum* 200 mg/kg + scopolamine (Figure 3(B)). Paired student's t-test did

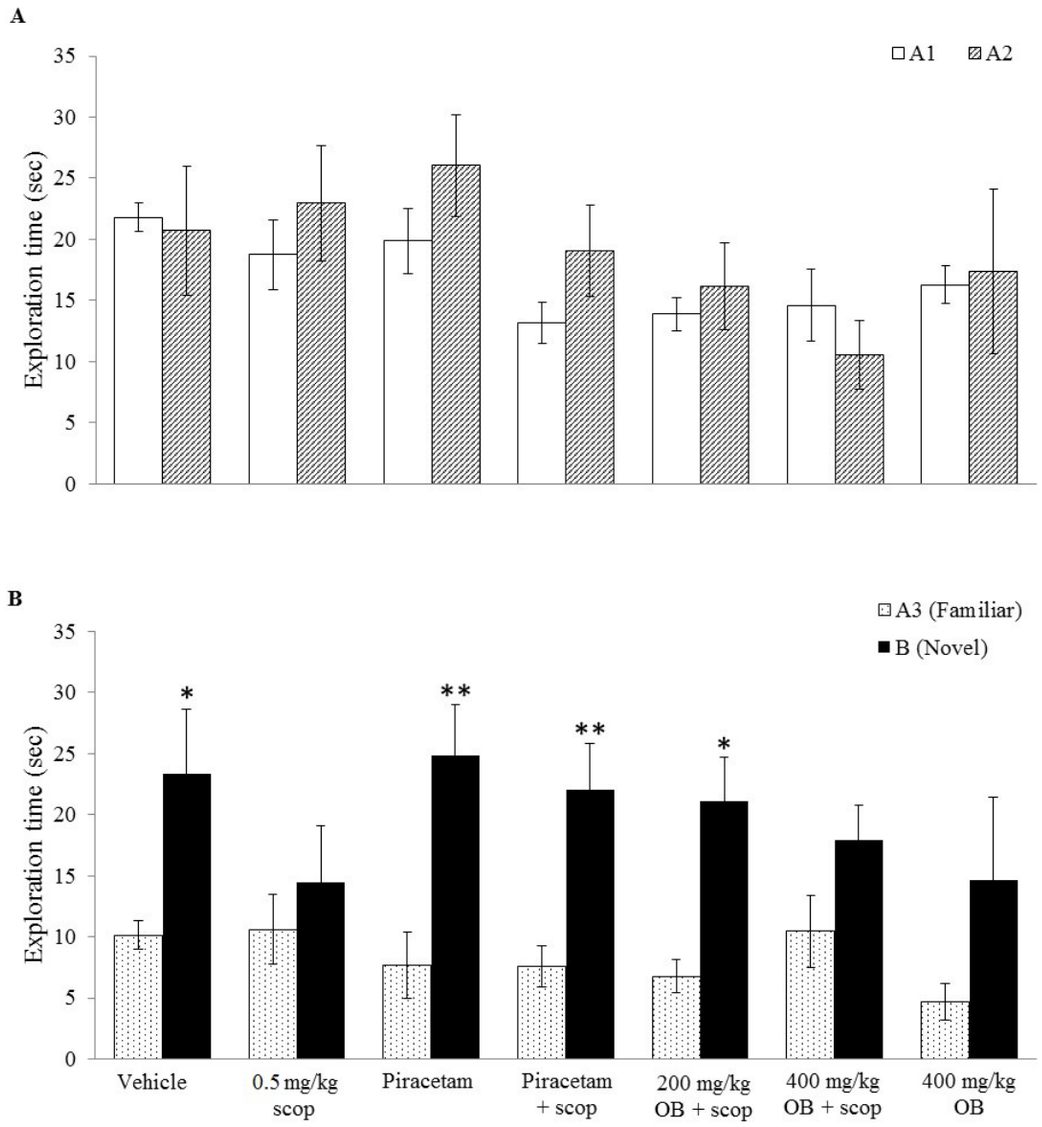


FIGURE 3. (A) The exploration time for both identical objects A1 and A2 during the acquisition phase, and (B) the exploration time for familiar object A3 and novel object B during the choice phase. Data are presented as means in seconds (± standard errors of the mean) with n = 10 in each treatment group. * $p \leq 0.05$, ** $p \leq 0.01$ vs. A3. Scop: scopolamine; OB: *O. basilicum* extract

not indicate any significant differences ($p > 0.05$) in rats treated with scopolamine alone, *O. basilicum* 400 mg/kg + scopolamine, and *O. basilicum* alone. This indicates that the rats lost their recognition memory, which is used to discriminate between the familiar and novel objects during the choice trial.

Reversal of scopolamine-induced memory impairment was observed at the lower dose of 200 mg/kg. Our results are in agreement with Sarahroodi et al. (2012) which reported that this plant extract improved memory retention and retrieval using a passive avoidance apparatus. The effects observed in this study may be associated with presence of various phytocompounds such as terpenoids, flavonoids and other polyphenols, particularly rosmarinic acid which is found in abundance in various *O. basilicum* extracts

(Al-Snafi 2021a; Mohd-Zahid et al. 2018). In line with this, pretreatment of ethyl acetate extract of *O. basilicum* also attenuated impairments in short-term memory and motor coordination induced by ischemia and reperfusion-induced cerebral damage (Bora, Arora & Shri 2011). Although limited, a number of scientific evidence available on the neuropharmacological effects of *O. basilicum* suggest that this plant may play a protective role against cognitive impairment (Bora, Arora & Shri 2011; Dodart, Mathis & Ungerer 1997; Seyed et al. 2021; Shakeri, Hosseini & Ghorbani 2019). Indeed, *O. sanctum*, which is a plant of the same genus, is used to improve memory. Increasing evidence has substantiated the potential nootropic and neuroprotective actions of this *Ocimum* species, which may be beneficial in the management of neurodegenerative diseases (Giridharan et al. 2011; Joshi & Parle 2006; Yanpallewara et al. 2004).

Four candidate genes were measured to identify possible targets of *O. basilicum* in this study. In this regard, NA7, M1, nNOS, and HTR3A genes were selected based on their involvements in memory processes and neuroprotection, as previously reported (Buhot, Martin & Segu 2000; Paul & Ekambaram 2011). HTR3B gene was excluded from the analysis due to undetectable expression. The differences of mRNA expression levels measured in all treatment groups failed to reach statistical significance. However, scopolamine administration to the rats resulted in an increased trend of hippocampal mRNA expression of the NA7, M1, nNOS, and HTR3A genes (Figure 4). Treatment with piracetam, which was used as a positive control, protected against the scopolamine-induced mRNA expression of NA7, M1, nNOS, and HTR3A. Piracetam treatment resulted in mRNA levels similar to those observed in the vehicle control group.

Treatment with either piracetam (250 mg/kg) or *O. basilicum* (400 mg/kg) alone did not significantly alter the basal levels of NA7, M1, nNOS, or HTR3A mRNA expression. However, treatment with 200 mg/kg of *O. basilicum* resulted in a reduced trend of NA7, M1, nNOS, and HTR3A expression and ameliorated the scopolamine-induced effects. On the other hand, treatment with the higher dose of *O. basilicum* (400 mg/kg) increased NA7, M1, and nNOS mRNA expression levels comparable

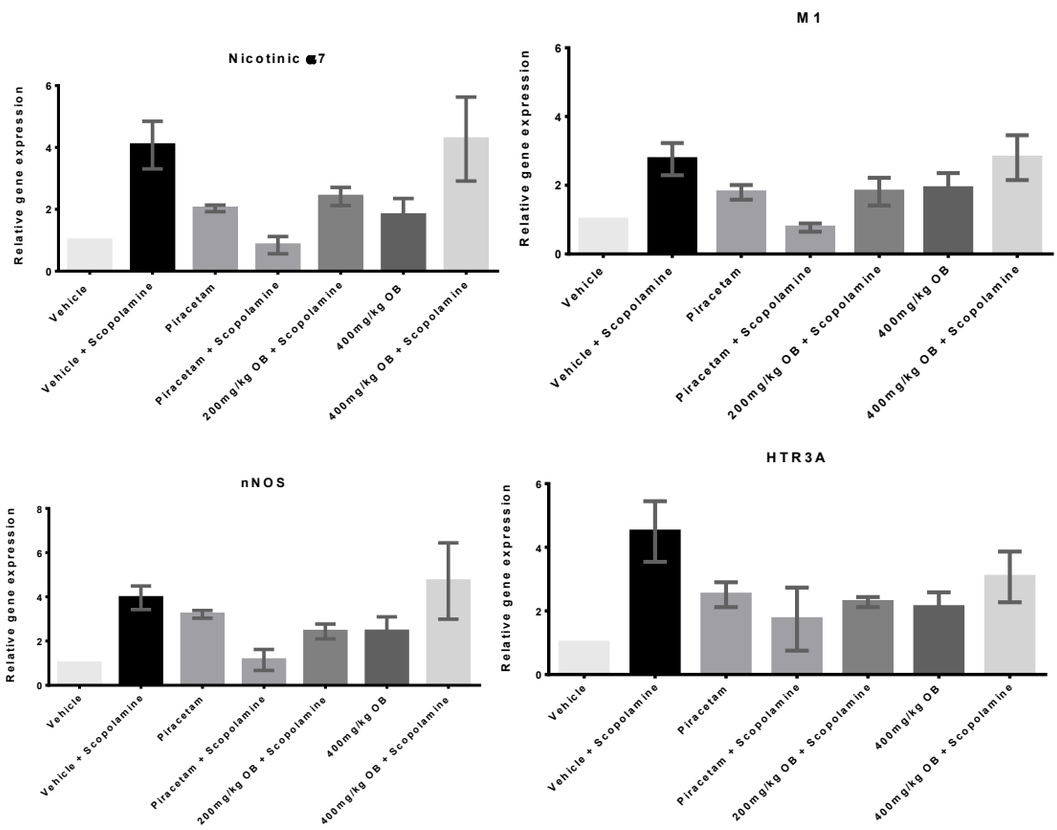


FIGURE 4. Relative mRNA expression of nicotinic acetylcholine α7 subunit (NA7), muscarinic M1 receptor (M1), neuronal nitric oxide synthase (nNOS), and 5-HT3A receptor subunit (HTR3A) in rat hippocampus in the different treatment groups (n = 4 for each group)

similar to scopolamine. These findings are comparable to those of behavioral data in which only the lower dose of *O. basilicum* at 200 mg/kg but not at 400 mg/kg reversed the scopolamine-induced impairment of non-spatial memory. Considering that the dose was two-fold higher, it is possible that at 400 mg/kg, *O. basilicum* aggravated memory impairment induced by scopolamine instead of being neuroprotective.

Expression levels of the cholinergic NA7 and M1 receptors at the protein level have previously been shown to be significantly increased in scopolamine-treated mice (Falsafi et al. 2012), while an increase expression at the mRNA level has been reported for nNOS (Paul & Ekambaram 2011; Xiao et al. 2014). Cognition-related behavioral tasks have shown that 5-$HT_3$ receptors are involved in scopolamine-induced memory impairment in rodents (Fakhfouri et al. 2019). Scopolamine-induced increase in the expression of HTR3A was reduced following treatment with *O. basilicum* extract and piracetam. It indicates that HTR3A could be involved in mediating the effects of *O. basilicum*. Indeed, the involvement of 5-HT3 in memory processes is evident, for example, the specific antagonist, ondansetron, reduced the number of total working memory errors induced by scopolamine in a dose-dependent manner (Kumar & Kela 2004).

The positive control piracetam is known to be involved in preventing scopolamine-induced memory deficits, as shown by performance in the inhibitory avoidance and object recognition tasks in rats (Chidamabaram et al. 2015; Marisco et al. 2013). It has also been shown to affect memory-related enzyme activity in the hippocampus of scopolamine-treated rats (Marisco et al. 2013). Piracetam also reduced scopolamine-induced increases in nNOS protein and mRNA levels, which corresponded to reductions in error times in the Y-maze test (Marisco et al. 2013; Xiao et al. 2014). To further support this notion, numerous evidences indicate that hippocampus-dependent learning is associated with an increase in hippocampal ACh levels (Haam & Yakel 2017; Seifhosseini et al. 2011), and that anticholinergic drug, such as scopolamine, impair learning and memory functions (Dodart, Mathis & Ungerer 1997; Lu et al. 2018). Indeed, our present findings on target genes expression following scopolamine and piracetam treatment are in accordance with previous studies (Fakhfouri et al. 2019; Falsafi et al. 2012; Paul & Ekambaram 2011; Xiao et al. 2014). The lower dose of *O. basilicum* (200 mg/kg) reduced the effects of scopolamine on target genes expression. However, the effects were relatively lower to that of piracetam in normalizing mRNA expression. It is interesting to consider the possible correlations between memory improvement induced by the low dose of *O. basilicum* (200 mg/kg) and reduced expression of NA7, M1, and nNOS genes. It was previously reported that nNOS activity in mouse brain increases following scopolamine treatment in parallel with an increase in its mRNA expression (Xiao et al. 2014) which were indicative of neuroprotective roles. The observed trend of reduced nNOS mRNA expression with *O. basilicum* was suggestive of its neuroprotective role.

Activation of M1 and NA7 receptors produces pro-cognitive effects (Wallace et al. 2011), whereby the disruptive effect of scopolamine appeared to increase their expression, potentially to compensate the scopolamine-induced memory deficit. The muscarinic M1 receptor which is the binding target of scopolamine, is expressed abundantly in hippocampus (Bradley et al. 2017; Levey 1996) and has received much attention as a potential target for cognitive deficit disorders. Its mechanism of action via glutamatergic signaling is associated to NMDA receptor activation (Dennis et al. 2016). Meanwhile, NA7 receptor mediates hippocampal-dependent learning and memory via glutamatergic synaptic transmission involving PKA- and cAMP- dependent pathways (Cheng & Yakel 2015, 2014). Interestingly, the serotonergic 5-HT3 receptor, an ion channel which belongs to the same family of nicotinic ACh receptor has been found to co-express and cross-regulate with NA7 (Gee et al. 2007; Huang et al. 2014) and was also implicated in scopolamine-induced effects (Barnes et al. 1990; Carli et al. 1997; Lochner & Thompson 2016). The fact that 5-HT3 receptor is significantly expressed in hippocampus (Harrell & Allan 2003) underlies the investigations of its role in learning and memory where cumulative evidences have shown that 5-HT3 receptor antagonists exert pro-cognitive and neuroprotective effects including in Alzheimer's disease models (Mohamed et al. 2021; Spilman et al. 2014). However, specific mechanism of 5-HT3 receptor in hippocampal-dependent cognition is not yet well defined but general serotonergic regulations on memory which also involve other 5-HT receptor subtypes correlate high 5-HT levels to reduced cognitive performance (Riedel et al. 2002; Wallace et al. 2011).

Additionally, nitric oxide (NO) which is represented in this study by the expression level of its synthetizing enzyme, nNOS, exerts its cognitive enhancement effect by activating cyclic guanosine monophosphate (cGMP) pathway and has been shown to induce long-term

potentiation in hippocampus (Harooni et al. 2009; Paul & Ekambaram 2011) while nNOS has also been reported for its cognitive neuroprotection role (Suo & Wang 2021). It is widely understood that complexity of memory and its protection is underlined by a concerted cross-regulation of different neurotransmitter signaling pathways as well as neuroinflammation (Falsafi et al. 2012; Konar et al. 2019). In the present study, we explored on different representative pathways in memory for its potential involvement with *O. basilicum* effects however similar trends of gene expression changes were observed among the candidate genes. The present study has investigated only the changes in gene expression of neurotransmitter receptors and nNOS but further studies are warranted to determine changes of neurotransmitter levels in the brain. Indeed, neither *O. basilicum* nor rosmarinic acid has been reported for their effects on the selected genes or neurotransmitter levels therefore to postulate the mechanism of their neuroprotective effects observed in the presence study would require future investigation.

## Conclusions

In summary, the lower dose of *O. basilicum* extract (200 mg/kg) reversed scopolamine-induced memory deficits in rats similar to the effects of the positive control, piracetam. The lower dose of *O. basilicum* also alleviated scopolamine-induced increase in mRNA expression of NA7, M1, nNOS, and HTR3A genes. These findings point to a notion that *O. basilicum* has a potential use in prevention of memory impairment by altering the expression of genes involved in memory processes and neuroprotection.

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