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Rosmarinic acid-rich ethanolic extract of *Orthosiphon stamineus* ameliorates cognitive and hippocampal long-term potentiation in chronic cerebral hypoperfusion rat model

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Abstract: Chronic cerebral hypoperfusion (CCH) is one of the main causes of vascular dementia caused by the reduced blood flow to the brain. *Orthosiphon stamineus* (OS) is a medicinal herb exhibiting pronounced neuroprotective, anti-oxidant and anti-inflammatory activities due to its high rosmarinic acid content. This study investigated the nootropic effect of OS ethanolic extract on cognitive functions in CCH rats. CCH was developed by permanent bilateral occlusion of the common carotid artery (PBOCCA). Passive avoidance task (PAT) and Morris water maze (MWM) test were conducted to evaluate cognitive functions followed by *in vivo* long-term potentiation (LTP) for assessing neuroplasticity. The rosmarinic acid content of OS ethanolic extract was quantified using a validated HPLC-PDA. Treatment with OS ethanolic extract significantly increased step-through latency in the PAT, decreased escape latency at a low dose of OS extract in the MWM and rescued the LTP impairment at the highest dose in CCH rats. These results strongly support the effectiveness of rosmarinic acid-rich OS extract (5.088 % w/w) in treating pathological vascular dementia caused by CCH.

Keywords: *O. stamineus*; passive avoidance task; Morris-water-maze; cognitive function; chronic cerebral hypoperfused rats

1.0 INTRODUCTION

Alzheimer's disease (AD) and vascular dementia (VD) are the two most prevalent forms of dementia. AD is a chronic and age-related progressive neurodegenerative disorder. However, AD often appears as a pseudo-form of vascular disease, specifically at the early stage when persistent cerebral hypoperfusion escalates cognitive impairment (Damodaran et al., 2014). The most common form of AD is 'sporadic' AD induced by multiple causes such as genetics, diet, lifestyle, chemicals or environment. One of the histopathological hallmarks of sporadic AD is the development of intraneuronal neurofibrillary tangles (NFTs) and extracellular senile plaques associated with neurodegeneration throughout the brain (Ghumatkar et al., 2015).

Chronic cerebral hypoperfusion (CCH) is a major cause of cognitive impairment and a vital determinant of dementia. In most cases, the chronic reduction in cerebral blood flow (CBF) is associated with severe memory disturbance (Xu et al., 2010). Permanent bilateral occlusion of the common carotid arteries (PBOCCA) in rats induces a substantial decrease in CBF and serves as a well-characterized animal model used to examine CCH cognitive magnitudes (Damodaran et al., 2014; 2018; 2019; Yan et al., 2015). These animals display impairment in learning and memory preceded by neuronal degeneration and reduction in microvascular, similar to those developed in a patient with vascular dementia (Farkas et al., 2004). Studies focused on this rat model have been evaluated to be a helpful approach to inhibit, slow or reverse the deterioration of dementia consistent with reduced cerebral blood flow (Jin et al., 2014; Yan et al., 2015).

Orthosiphon stamineus, or 'Cat's Whiskers' is a popular medicinal herb in Southeast Asia. The plant is locally known as 'Misai Kucing' in Malaysia and 'Kumis Kucing' in Indonesia. O. stamineus (OS) leaves are traditionally brewed into a herbal decoction or tea, also known as 'Java Tea'. The OS leaves are known for their use in traditional medicinal systems as a prophylactic and curative agent for urinary stones, diabetes, and hypertension and as a diuretic agent (Ashraf et al., 2018; Pariyani et al., 2015). OS is rich in polyphenols, with at least 20 compounds comprising flavonol glycosides, methylated flavones, and caffeic acid derivatives have been identified (Sumaryono et al., 1991; Yam et al., 2010). Previous studies have shown that these polyphenol compounds were the major contributors to the poly-pharmacological effects of OS, such as anti-oxidant, anti-inflammatory, antidiabetic, antibacterial, neuroprotective and anti-hypertensive activities. Rosmarinic acid, the major polyphenol of OS, has been found to have distinct pharmacological activities. Many studies showed that this phenolic acid class effectively reduces oxidative stress and promotes neuroprotection (Bhullar & Rupasinghe, 2013). In addition, OS extract was shown to restore age-related short-term memory impairment and reduce or minimize the rate of neurodegeneration (George et al., 2015). Besides, other studies utilize animal models chemically induced with scopolamine and streptozotocin to study the nootropic effect of OS extract (Retinasamy et al., 2019;2020). In those studies, OS extract was reported to improve learning and memory in behavioural tasks such as elevated plus maze test, passive avoidance and novel object recognition. Numerous *in vitro* experiments also reported that OS extract protected neuronal cells from oxidative stress induced by H_2O_2 via anti-oxidant mechanisms (Sree et al., 2015). Thus, it is proposed that OS could be used as adjuvant therapy for neurodegenerative diseases.

Although a few studies reported the in vitro and in vivo neuroprotective effects of OS extract, the information regarding its effects on behavior and cognition in CCH rats for treating AD and vascular dementia remains scarce. Therefore, the present study was carried out to investigate further the behavioral impact of OS treatment in rodents assessing spatial efficiency (by Morris Water Maze model) and a passive avoidance task. Lastly, the basal synaptic transmission and assessed hippocampal inhibition of LTP in CA1 area after treatment with OS in CCH rats were also evaluated utilizing *in vivo* recording. To our knowledge, this is the first study to investigate the effect of OS extract in the PBOCCA-induced CCH rat model.

2.0 MATERIALS AND METHODS

2.1 Preparation of Orthosiphon stamineus (OS) ethanolic extract

The 50% ethanolic OS leaf extract was procured from NatureCeuticals Sdn. Bhd., Kedah, Malaysia, as a standardized botanical product- C5OSEW5050ESA OS, or commercially known as Lanctos 75TM (Catalogue No: 931886-P). Under this formulation, rosmarinic acid is enriched as the major active marker compound, which constitutes between 5–8% w/w, and some minor flavonoids (<1%) such as eupatorin 0.6%, sinensetin 0.2% w/w, and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF) 0.05-0.1% w/w. The production of this botanical extract involves extracting OS leaves using a mixture of ethanol and water (1:1, v/v) under an optimized GMP-based procedure and the environment by a DIG-MAZ extractor. The DIG-MAZ

extraction system extracts plant materials in vacuo with a fixed pressure (up to 8 bar or up to 150 psi). The final product was kept in an airtight container until further experimentation.

2.2 Quality control of OS ethanolic extract

2.2.1 Reagents and Chemicals

The reference standards: rosmarinic acid (purity≥98%), sinensetin (purity≥98%), eupatorin (purity≥98%), 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (purity≥98%) were purchased from Sigma Aldrich (USA). Ethanol (analytical grade), methanol (HPLC grade), acetonitrile (HPLC grade) and formic acid (98–100%) were purchased from Merck (Germany). Deionized water (18.2 MΩ) was used for HPLC analysis.

2.2.2 Chromatographic conditions

Utilizing HPLC fingerprint analysis, the extract was screened for several marker compounds, namely sinensetin (SIN), eupatorin (EUP), 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone (TMF) and rosmarinic acid (RA) according to the study reported by Saidan et al. (2015a). A simple and selective HPLC method was developed and validated for profiling OS ethanolic extract. The HPLC analysis was performed on an Agilent 1200 series HPLC system coupled to a photodiode array detector (Agilent, CA, USA). Briefly, a solution of lyophilized OS extract was prepared at 1000 µg/mL in methanol and centrifuged to remove the precipitate. The chromatographic separation was achieved on a Zorbax Eclipse Plus C18 column, 4.6 x 150 mm, 3.5 µm) (Agilent, CA, USA) at an adjusted temperature of 35 °C. The total run time of the separation was 30 min, and the injection volume was 10 µL. The mobile phase used for the separation was a mixture of 0.1% formic acid in water (pH 2.7) (A) and acetonitrile (B), running at a gradient method with a flow rate of 0.75 mL/min. The gradient method started with 80% A and 20% B (hold for 2 min), then slowly increased to 0 % A and 100 % B (at 20 min) as described in **Table 1**.

Table 1. Programmed gradient HPLC method

Time	Flow rate (mL/min)	Solvent ratio	
		A (0.1% formic acid)	B (Acetonitrile)
2	0.75	80	20
7	0.75	70	30
15	0.75	50	50
25	0.75	0	100
30	0.75	80	20

Detection (RA, TMF, EUP and SIN) and quantification of RA were conducted using an Agilent photodiode array detector at λmax of 330 nm. Each marker was identified by comparing the HPLC retention time and UV spectrum of the analyte with that of the reference standard. A peak purity test was performed using ChemStation LC3D software to ensure the method selectivity for reliable quantification of RA.

2.2.3 Preparation of stock solution and method validation

A 1000 µg/mL stock solution of rosmarinic acid (RA) standard was prepared in methanol and then diluted into a series of working standard solutions of 7.81, 15.625, 31.25, 62.5, 125, 250, and 500 µg/mL. A calibration curve was constructed by plotting the peak area against its respective concentration on each analysis day. Accuracy (% recovery) was evaluated by spiking three different concentrations of RA standard (high spike: 125 µg/mL, medium spike: 62.5 µg/mL and low spike: 31.25 µg/mL) into a diluted OS ethanolic extract (500 µg/mL). For intra- and inter-day precision, three concentrations (high, medium and low) of RA standard were analyzed in triplicates and expressed as relative standard deviation, % RSD.

2.3 Animals

Animal (Sprague Dawley) rats were acquired weighing 200 – 300 g from the Animal Research and Service Center (ARASC). The rats were housed 5 per cage on a regular 12:12 light/dark cycle with lights on at 7:00 am and were maintained at room temperature (25±2°C). Food and water were given ad libitum. The experimental protocols were authorized and performed based on the approval of the Animal Ethics Committee Universiti Sains Malaysia (USM/Animal Ethics Approval/2015 (677)).

2.4 Surgery

The PBOCCA occlusion surgery was performed as defined in (Damodaran et al., 2014; 2018; 2019). In short, intraperitoneal injection of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (10 mg/kg) anesthetized all rats. For PBOCCA surgery, the typical carotid arteries were revealed by a ventral midline incision, whereby the common carotid arteries were then gently isolated from their sheaths and vagus nerves and permanently tied to a 5/0 silk suture about 8–10 mm below the origin of the main carotid artery. The depth of anaesthesia was assessed by the presence and absence of the pedal withdrawal reflex while pinching the hind paws of the animals. The animal was placed on a heating pad to maintain body temperature

throughout anesthesia. Finally, after surgery, the rats were given 1 ml saline and placed in warm, clean, dry, quiet environments away from other animals in an individual cage. Antiseptic was applied to the stitches area to prevent any infection. Rats were continuously monitored until they maintained upright posture and walked normally around the cage. All rats were left 2 weeks before conducting behavioural tests for recovery from surgical procedures to avoid any compromised motor functions and exploratory behaviour in rats due to pain or distress.

2.5 Drug administration and experiment design

The rats were randomly divided into five groups two weeks after surgery: Sham rats treated with vehicle (n = 6); PBOCCA rats treated with vehicle (n = 6); PBOCCA rats treated with 100, 200, and 400 mg/kg OS ethanolic extract (n = 6) respectively. OS ethanolic extract was dissolved in the vehicle before administration to PBOCCA rats via oral gavage to the rats of respective groups in a volume of 10.0 mL/kg per body weight one-hour post behavioral test. However, during the LTP electrophysiology study, different groups of animals were treated 1 h before recording. The experimental design is stated in **Figure 1**.

2.6 Passive Avoidance Test

The passive avoidance system consists of two segments: a lightbox (plexiglass) and a dark box (black) separated by a guillotine door (88 cm), all of the same scale (20x20x20x40 cm each). The lighted box was well-lit with a lamp (60 W) above the equipment and the floor was composed of 2 mm diameter and 8 mm gaps of stainless-steel grids. In the dark compartment, the grid floor delivered an unpredictable electrical shock (50 Hz, 10 s, 0.5 mA intensity) by an integrated stimulator. An acquisition trial was carried out 2 weeks after the PBOCCA surgery. The rat was permitted to fully explore each compartment for 3 mins during the acquisition experiment. Every rat was first placed in the light compartment during the training session, whereby the door between the compartments was opened 10 s later, and the latency to reach the dark compartment with all four paws was measured. When the rat with all four paws reached the dark compartment, the door was closed, and the 0.5 mA foot shock was delivered for 10 s. Then, the rat was withdrawn and placed back into its cage. A retention test was then performed 24 h after the training session, whereby it was administered similarly to the training session except that the shock in the dark compartment was not delivered to the grid surface. The outcome of this behaviour test was the latency period to reach the dark compartment. The retention trial was completed once the rat entered the dark compartment. If, within 300 s, the rat refused to enter the dark compartment, the retention trial was completed, and the step-through latency time was recorded as 300 s.

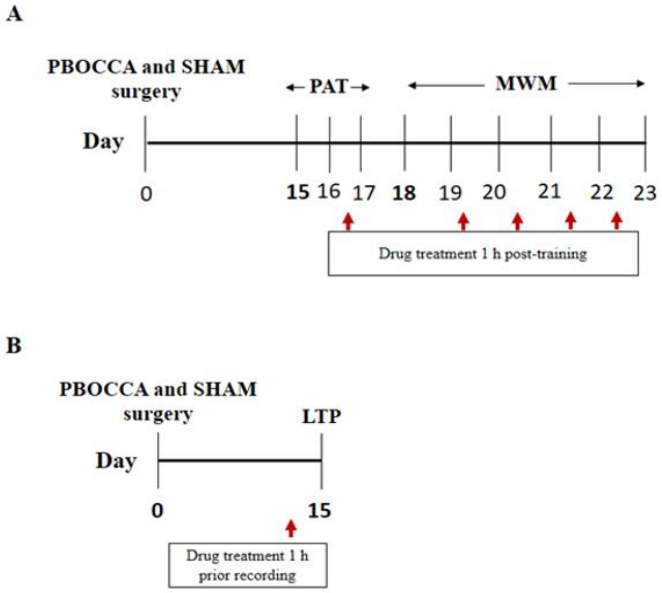

Figure 1. Experimental design for OS extract treatment in a chronic cerebral hypoperfusion rat model. The PBOCCA and sham surgery was conducted on day 0. All behavioral tasks were conducted after 14 days post-surgery; **(A)** Represent the timeline of the behavioral tasks and the treatments conducted on sham and PBOCCA-treated groups. For passive avoidance tasks (PAT), the task was started with habituation day 15, followed by training. The treatments of all extracts were given after 1 h post-training on day 16, and a retention test was conducted on day 17; for Morris water maze (MWM) tasks, habituation was started on day 18, and training was conducted for 4 days from day 19 to 22 followed by probe trial on day 23. Finally, during MWM, the treatment was given after 1 h post-training. **(B)** In a separate experiment, the long-term potentiation (LTP) experiment was conducted on 14 days post-surgery, and the treatment was given before LTP surgery. Both studies were conducted on different animals.

2.7 Morris Water Maze

The Morris water maze has a circular black pool (160 cm diameter, 70 cm water depth). The pool was placed in a large test room with different visual indicators. In addition, the pool was filled with water to a depth of 39 cm and held at 25 ± 1 °C, then turned opaque using white paint. The pool was split into four quadrants, with a platform (10 cm diameter), placed in one quadrant 2 cm below the water surface in a fixed location. The rats were allowed a pre-training session a day before the formal training, where they could swim freely in the pool for 60 s without an escape platform. There was

routine testing during the formal testing consisting of four trials whereby the rat was placed in the water from four separate starting points during the trials, and the latency of escaping the platform was tracked. This training was performed for four consecutive days in which each rat was given a maximum of 60 s to locate the platform and climb onto it. The failure to complete the task within the 60 s resulted in the trial being stopped and a maximum score of 60 s given. The rat was then directed by hand to the hidden platform and allowed to stay on the platform for 10 s before being withdrawn from the water. A probe trial was performed on the 5th day after training in which the rat was released in the pool without the escape platform for 60 s. The time spent in the target quadrant (previous location of the platform) was recorded.

2.8 In vivo electrophysiology

A new batch of animals were used to conduct electrophysiological procedures. The rats were intraperitoneally anesthetized with urethane (Sigma; 2.0 g/kg, administered as four doses of 0.5 g/kg each, about 20 mins apart; 0.5 g/kg supplements were administered if necessary) before being put in the stereotaxic frame. Local analgesic Xylocaine (5 mg/kg; AstraZeneca, Australia) was subcutaneously injected over the skull to the surface of the incision. The rats were put on the stereotaxic frame, and the ear bar was set in the right and left ears of the rat's auditory canal. A homeothermic blanket (Harvard Apparatus) maintained the rat's body temperature at 36 ± 1 ºC. After that, a minor incision was made on the scalp, and the connective tissue adhered to the skull was scraped and cleaned. 30 % hydrogen peroxide (Sigma) was used to remove the tissue so the bregma and lambda became visible. Four small holes were drilled on the skull overlying the hippocampus CA1 (AP: -4.2. ML: +3, V: -3), CA3 (AP: -4.2, ML: -3, V: -4) and two other small holes above the frontal region for the placement of ground and reference electrodes. The ventrally lowered recording electrode (Perfluoroalkoxy [PFA] Insulated Steel Tube, A-M Devices, USA) was used to stimulate the CA3 area with a bipolar stimulating electrode (SNE 100, MicroProbes, USA). In addition to CA3 stimulation, the final ventral location of the stimulating electrode CA3 and the recording electrode is adjusted to yield optimum field excitatory postsynaptic potential (fEPSP) amplitude in CA1. The stimulation electrode was linked to a stimulus isolator with constant current output (ML 180 Stimulus Isolator, ADInstruments, Australia). The recording, ground, and reference electrodes were linked to an amplifier (Model 1800, A-M Systems Inc., Sequim, WA; half-amplitude filters set at 0.1 Hz to 500 Hz), which digitized at 100 Hz and processed the registered signal for offline processing (PowerLab 16/s System, ADInstruments, Australia).

The strength needed for each rat to evoke the maximum amplitude of fEPSP was calculated by input-output curves. The stimulus was given with increased intensities (0.1 to 1.0 mA) with intervals of 0.1 mA. For the remainder of the experiment, the stimulus intensity eliciting 50-60% of maximal fEPSP amplitude was used in the following experiment. A baseline of the fEPSP was recorded for LTP experiments over 60 mins. Baseline monitoring was considered stable when the amplitude of fEPSP stayed within 90-100% of the average amplitude of fEPSP over the baseline duration of 60 mins. After establishing a steady baseline, theta-burst stimulation (TBS) was applied as a train of ten bursts (each burst consists of 5 pulses at 100 Hz), with bursts repeated every 200 ms for a single session. Single-pulse stimulation of the CA3 resumed every 30 s for a recording time of 3 h after the TBS.

2.10 Statistics

Data were represented as the means ± SEM. The normality and lognormality were tested by the Shapiro-Wilk test. Passive avoidance task and probe trial were analyzed using one-way ANOVA followed by Bonferroni post hoc test. Spatial learning during Morris water maze task training was analyzed using a two-way repeated measure ANOVA followed by the Bonferroni post hoc test. During the electrophysiological study, input-output curve data and time-course changes in fEPSPs amplitude after TBS were analyzed using a two-way repeated measure ANOVA, followed by Bonferroni post hoc test. Data for the mean of the fEPSP amplitude for the last 60 min of 3 h LTP recording was analyzed using one-way ANOVA followed by Bonferroni post hoc test. Probability values of less than 5% ($p<0.05$) were considered significant. GraphPad Prism software (version 7) was used to perform all statistical analyses.

3.0 RESULTS

3.1 Determination of rosmarinic acid content in the prepared OS ethanolic extract

3.1.1 Chemical profile of OS ethanolic extract

By using continuous stirring counter current technology, air-dried OS leaves powder was extracted with 50% ethanol. The obtained extract was evaporated in vacuo and then lyophilized to obtain 9.8 g of dried powder (extraction yield = 9.8 %). Thleafared OS leaf extract was then subjected to HPLC analysis at a fixed concentration of 1000 µg/mL using a simple and selective HPLC method. HPLC chromatogram for a representative OS

leaves extract (50% ethanolic extract) and the reference standards (RA, TMF, SIN, EUP) are given in **Figure 2**. The chromatographic peak of each marker in the extract was identified by matching its retention time and UV spectrum with the respective reference standard. In this study, rosmarinic acid (RA), a caffeic acid derivative, was found abundantly in the prepared OS ethanolic extract, while the methylated flavones: 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF; retention time, 16.3 min), sinensetin (SIN; retention time, 18.84 min) and eupatorin (EUP; retention time, 19.31 min) were present in trace amount and were not quantified.

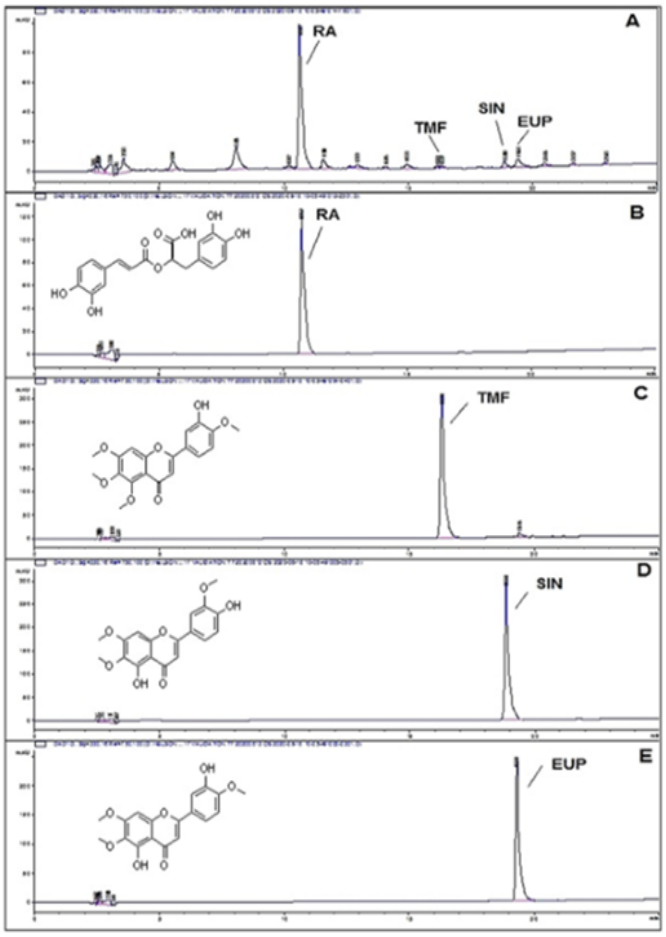

Figure 2. A representative HPLC chromatogram of **(A)** ethanolic extract of OS; **(B)** Rosmarinic acid (RA); **(C)** 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (TMF); **(D)** Sinensetin (SIN); **(E)** Eupatorin (EUP).

3.1.2 Validation of the developed HPLC-PDA method

The developed HPLC analytical method was validated for quantifying rosmarinic acid (RA), the major phenolic marker in the 50% ethanolic extract of OS leaves. The chromatographic peak of RA in the tested sample was identified at the retention time of 10.74 min, which was similar to RA standard. The developed method was selective due to its ability to separate RA from other detected compounds/ interferences, as indicated by the UV profiles in **Figure 3**. A linear calibration curve of the RA standard was achieved between 7.83 and 500 µg/mL with an equation of y = 17.768× + 132.07 and a correlation coefficient, R2 = 0.9998. RA recovery rate (%) ranged from 100.28 to 103.62% in all the spiked samples, indicating the method is accurate. The intra- and inter-day precision values (% RSD) ranged from 1.42 to 2.56% (< 5%), suggesting the developed analytical method is reproducible.

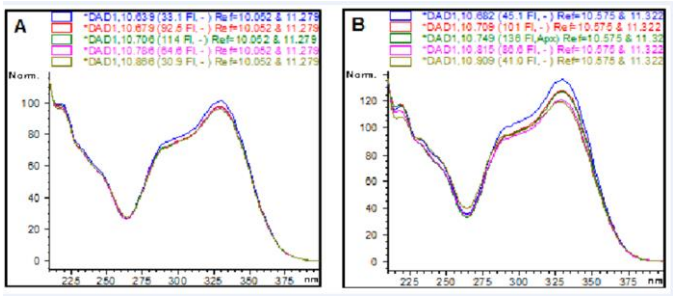

Figure 3. (A) UV spectrum of detected rosmarinic acid in the sample; (B) rosmarinic acid standard at retention time 10.74 min.

3.1.3 Rosmarinic acid content

The RA content in the prepared OS ethanolic extract (1 mg/mL) was 50.88 ± 3.02 µg/mL or equivalent to 5.088 % (50.88 mg/g) of the total extract. The result indicated that OS ethanolic extract used in this study was enriched with rosmaniric acid.

3.2 Effects of OS ethanolic extract on passive avoidance task

Figure 4 indicates the findings obtained from the PAT where the memory performance of the three doses of OS extracts (100, 200 and 400 mg/kg) was evaluated 24 hours after OS administration in the consolidation phase of post-training. During the post 24 hours retention trial, memory performance was determined by assessing the latency to reach the dark (shock-paired) compartment. OS dose-dependently enhanced memory consolidation in a PAT to a similar degree as a sham ($F_{4,25} = 21.44$, $P<0.0001$ versus PBOCCA vehicle). The PBOCCA vehicle significantly exhibits impaired memory consolidation in PAT ($P<0.0001$).

3.3 Effects of OS ethanolic extract on Morris water maze

Figure 5A demonstrates the effects of the post-training administration of 100, 200 and 400 mg/kg OS extracts during the MWM test acquisition testing on the PBOCCA

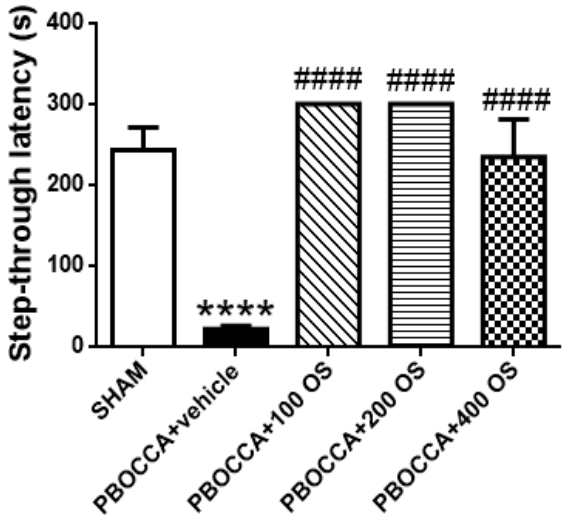

Figure 4. The effects of 100, 200 and 400mg/kg OS extract on step-through latencies during consolidation (administered post-trial) of a passive avoidance task in rats. Data are expressed as mean ± SEM n = 6. **** p<0.0001 vs. SHAM and #### p<0.0001 vs. PBOCCA+VHL, analyzed by one-way ANOVA followed by Bonferroni post hoc test.

treated with vehicle-induced spatial learning and reference memory deficit. Two-way repeated ANOVA analysis shows a statistically significant between the treatment (F4,92 = 5.711, P=0.0004) and days (F3,92 = 9.818, P<0.0001) but not with the interaction between the groups (F12,92 = 0.6361, P=0.8063). Vehicle-treated PBOCCA (VHL) demonstrated longer escape latency to reach the hidden platform than the sham, with a significant rate on day 3 (p=0.0043). The low dose of 100 mg/kg OS extracts treated PBOCCA rats displayed significant improvement of spatial memory during the day 3 (P = 0.0172) training compared to vehicle-treated PBOCCA rats. The result indicates that repeated administration of low dose 100 mg/kg OS extracts is efficient in improving learning and memory compared to higher doses 200 and 400 mg/kg.

The efficiency of the probe trial was assessed by evaluating the effect of the OS extracts of 100, 200 and 400mg/kg on the duration spent in the target quadrant **(Figure 5B)**. This experiment was conducted following acquisition training and reflect a spatial bias of animals toward the previous location of the hidden platform. The pool was separated into four quadrants of equally, a chance level of performance i.e. the percent of time swam in the earlier target quadrant would generally approximate 25%. As specified in Fig 5B, there were no significant effects on the percentage of time spent in the previous target quadrant (F4,18 = 0.08482, P=0.9860).

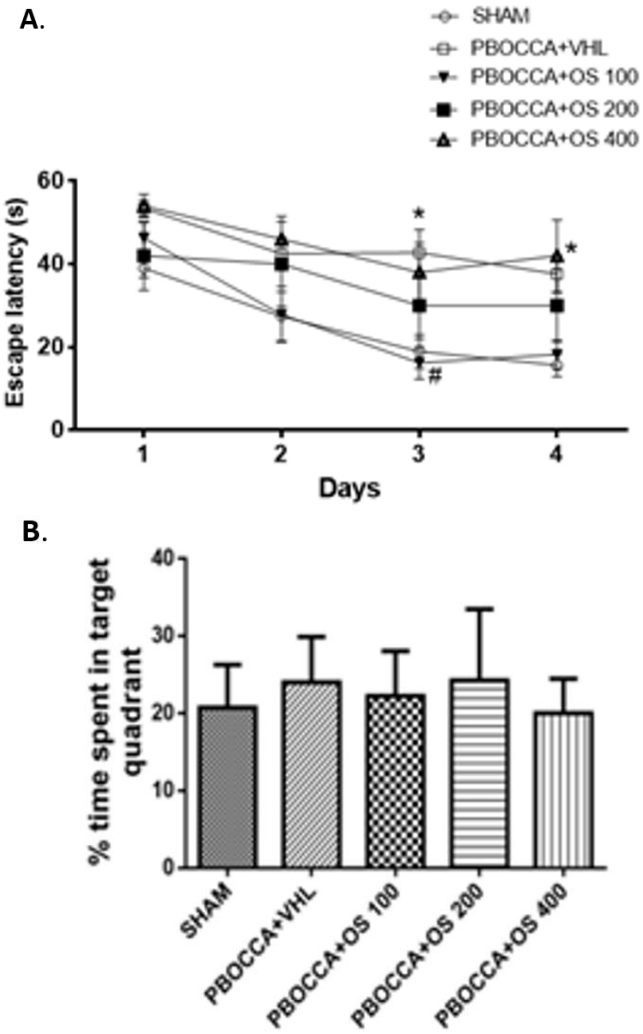

Figure 5. (A) The effects of 100, 200 and 400mg/kg OS extract administration on Morris water maze test. The escape latency to the hidden platform and learning patterns of animals during days 1 – 4 of training sessions. (B) The effects of 100, 200 and 400mg/kg OS extract administration on the percentage time spent in the target quadrant during probe trial of the Morris water maze test. Data are represented as mean ±S.E.M. n=6. * p<0.05 vs. SHAM, analyzed by two-way repeated measure ANOVA followed by Bonferroni post hoc test.

3.4 Effects of OS ethanolic extract on *in vivo* electrophysiology

The characteristics of basal synaptic transmission in the CA1 region of the hippocampus after treatment with the OS extracts were evaluated using the input-output curve. The input-output curve relationship between the electrical stimulation at the CA3 region and the resulting amplitude of the fEPSP recorded in the CA1 region of the hippocampus are shown in **Figure 6A**. In PBOCCA,

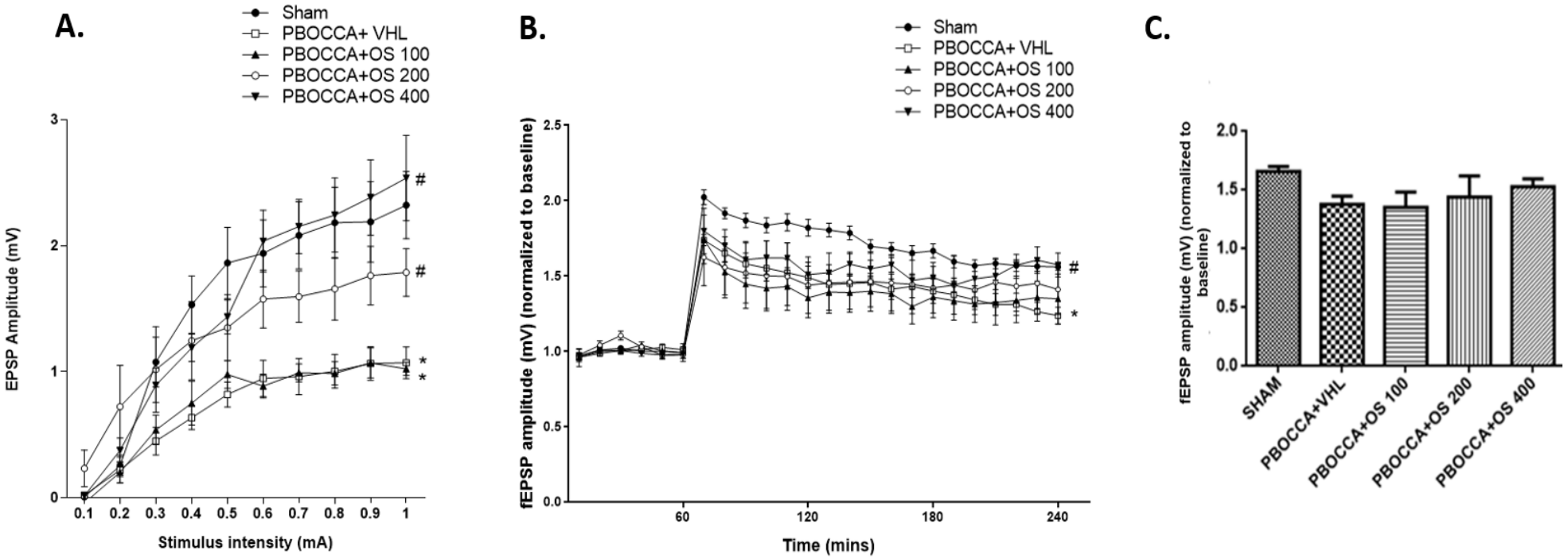

Figure 6. Effects of 100, 200 and 400mg/kg OS extract on LTP in the CA1 hippocampus. **(A)** Input-output relationship. **(B)** Time course changes in fEPSP amplitude are expressed as normalized fEPSP over a 10 min interval with the mean baseline value. **(C)** The mean of fEPSP amplitude for the last 60 min of 3h LTP recording following TBS. Data are represented as mean ± SEM n= 6 rats. * $p<0.001$ vs. SHAM and # $p<0.001$ vs. PBOCCA+VHL, analyzed using a two-way repeated measure ANOVA, followed by Bonferroni post hoc test.

animals treated with vehicle and 100mg/kg OS demonstrated significant suppression of the fEPSP amplitude compared to sham (sham vs PBOCCA+VHL, p=0.0022; sham vs PBOCCA+100mg/kg OS, p=0.0023). The change could be the result of the persistent loss of hippocampal CA1 neurons, a decrease in stimulated glutamate release, or both. However, there is some improvement of PBOCCA synaptic transmission after treatment with 200 and 400mg/kg OS (p<0.0001 and p=0.0056, respectively in comparison with PBOCCA+VHL).

LTP was assessed after baseline recordings for 60 mins and recorded for 3 hours after TBS. The initial magnitude of potentiation after TBS was much lower in treated PBOCCA than in sham. All PBOCCA treatments showed depression of the fEPSP amplitude throughout 3 hours of recording (p<0.0001 when compared to sham) **(Figure 6B**). Two-way repeated ANOVA analysis demonstrated a statistically significant between the treatment ($F_{4,504}$ = 29.75, P<0.0001) and time ($F_{23,504}$ = 39.59, P<0.0001) but not with the interaction between the groups ($F_{92,504}$= 0.772, P=0.9316). At this cellular level of the hippocampus, all treated-PBOCCA with OS did not show any significant enhancement of the LTP (P>0.05) except for 400mg/kg OS (p=0.0014)**(Figure 6C)**. Hence, a single administration of the high dose of OS can enhance LTP after 2 hours of recordings.

4.0 DISCUSSION

Permanent bilateral occlusion of the common carotid arteries (PBOCCA) of rats was found to be capable of mimicking the chronic cerebral hypoperfusion disorder and is thus considered a model to understand the pathophysiology of learning and memory impairments related to cerebral circulation disorder as well as for assessing therapeutic potential and mechanism of putative anti-Alzheimer drugs (Damodaran et al., 2014; Farkas et al., 2007; Liu et al., 2007). Following the PBOCCA surgery, the cerebral blood volume was found to decrease by 25–50%, declining slowly over a week, with its results lasting over many months, similar to the reduced cerebral blood flow reported with aging and dementia (Farkas et al., 2007; Ni et al., 1994). We examine the cognitive impairment and the pathophysiological transition after 2 weeks of occlusion of carotid arteries. Previous studies have shown that persistent cerebral hypoperfusion after PBOCCA surgery-induced spatial learning and memory deficits (Cechetti et al., 2012; Damodaran et al., 2014; Vicente et al., 2009; Xi et al., 2014;) and non-spatial memory dysfunction (Sarti et al., 2002).

Nevertheless, the main underlying mechanisms are poorly understood despite knowing that chronic cerebral hypoperfusion causes cognitive deficits. Yet, Ahad et al. 2020 have identified oxidative stress, inflammation, cholinergic dysfunction and glutamate-mediated excitotoxicity as the pathophysiology of cerebral ischemia that contributes to neuronal cell death and cognitive dysfunction. According to Kim and colleagues, excessive free radical formation during CCH, including superoxide anions (O_2-), hydroxyl radicals (-OH), and hydrogen peroxide (H_2O_2) contributes to oxidative stress. It causes lipid, protein, and DNA malfunction, contributing to cellular damage and death (Kim et al., 2015). Aging has been reported to induce accelerated oxidative damage through natural aging and Alzheimer's disease in the brains of older adults (Rabiei et al., 2014).

Previous studies suggested inflammation triggers blood-brain barrier (BBB) impairment during CCH. CCH-induced necrotic cells release endogenous molecules, which can act as damage-associated molecular patterns and evoke inflammatory responses. These patterns may be directly or indirectly involved in BBB impairment. It was postulated that CCH-induced BBB permeability promotes the extravasation of macromolecules, including immune cells, into CCH brains. This activates astrogliosis and the release of inflammatory cytokines that could directly damage microvascular endothelial cells, leading eventually to BBB impairment (Hei et al., 2018). Cholinergic signalling is directly related to cognitive functions, including memory and learning. Cholinergic indicators such as enzymatic activity such as acetylcholinesterase, butrylcholinesterase, and choline acetyltransferase, and acetylcholine level can be utilized to study cholinergic function (Kumaran et al., 2022). Many investigations of cholinergic impairment in cerebral ischemia associated with vascular dementia have revealed a significant decline in cholinergic markers.

Excessive accumulation of glutamate at synapses leads to excitotoxicity. The overactivation of NMDA Receptors via glutamate binding contributes to the excessive influx of Ca^{2+} into neurons. Excess Ca^{2+} influx into the cell causes the activation of harmful phospholipases, endonucleases, and calpains, resulting in cell organelle and membrane damage and, in most cases, necrotic-like cell death during acute cell death (Voslet et al., 2008). Cellular changes, including protein synthesis/folding disruptions, mitochondrial malfunction, and altered cell signaling were reported during delayed cell death (Cross et al., 2010; Green & Llambi, 2015; Akbar et al., 2016).

OS extract have been reported to possess antiapoptotic effects in an H_2O_2 (a potent free radical) induced cell apoptosis (Abdelwahab et al., 2011).

The medicinal significance of OS has been well documented, particularly as regards its anti-oxidant and anti-inflammatory activities (Awale et al., 2003; Arafat et al., 2008). Phytochemical studies revealed OS leaf extracts contain phenolic compounds, including RA, 2,3-dicaffeoyltartaric acid, eupatorin, sinensetin, oleanolic acid, ursolic acid, pentacyclic triterpenes, b-sitosterol and others (Awale et al., 2003; Saidan et al., 2015b; Ashraf et al., 2018; Yehya et al., 2018). Among these active compounds, RA was reported to be the major flavonoid present in the 50% ethanolic extract of OS extract and plays a central role in various pharmacological activities exerted by OS extract including neuroprotection and enhancement of learning and memory (Essa et al., 2012; Fonteles et al., 2016; Taram et al., 2018; Fachel et al., 2019)In agreement to previous studies, **Figure 2** display the findings collected from the chromatographic analysis of the ethanolic OS extract. The chromatographic profile reveals that the OS extract is rich in rosmarinic acid (5% w/w of total extract), which appears to exhibit potent anti-oxidant activity that can avoid any free radical-mediated oxidative harm.

Previous study done by Retinasamy et al. (2019) demonstrated pre-treatment with OS extract were observed to improve learning and memory in scopolamine-induced amnesia model via hippocampal neurogenesis. Cytokines and other inflammatory mediators, including prostaglandin E_2 (PGE_2) and nitric oxide (NO) generated by cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) correspondingly, activate and drive inflammation and hence serve as targets for anti-inflammatory drug development. In vitro study by Laavola et al. (2012) disclosed that the OS extract and its flavonoid-containing OS fraction were found to inhibit iNOS expression as well as NO and PGE_2 production. Eupatorin and sinensetin identified in OS extract inhibited iNOS and COX-2 expression, production of NO and PGE_2, and lipopolysaccharide (LPS)-induced activation of transcription factor signal transducers and activators of transcription 1α (STAT1α). The OS extract and the compounds also inhibited tumor necrosis factor α (TNF-α) production. Furthermore, eupathrin and sinensetin inhibited carrageenan-induced paw inflammation in vivo, suggesting the presence of anti-inflammatory activity in OS extracts. In PBOCCA rats, it was observed that the rates of superoxide, DNA degradation and neuronal death in the hippocampus subfield CA1 were increased. This led to cognitive disorder formation (Choi et al., 2014). RA, the major phenolic constituent of OS ethanolic extract **(Figure 2)** was found to be very efficient in stopping oxidative stress and hydrophilic radicals from modifying the lipid membranes (Fadel et al., 2011). Long-term brain function is often correlated with RA as a natural anti-oxidant found in the extract. Short-term treatment of this compound has demonstrated that prefrontal blood flow is reliably modulated by direct impact on cognitive function and alertness (Kennedy, 2019). Besides that, RA have been reported to inhibit both acetylcholinesterase and butrylcholinesterase in vitro. Inhibition of these enzymes reduce the breakdown of acetylcholine level and restrain the cholinergic function in central nervous system (CNS) (Gülçin et al., 2016). Interestingly in another study, RA was found to activates $GABA_A$ receptors in cerebrocortical synaptosomes of rats to decrease Ca^{2+} influx and CaMKII/synapsin I pathway to inhibit the evoked glutamate release (Wang et al., 2021). Based on these evidences, OS extract rich in RA may counteract the CCH pathophysiology via reducing oxidative stress and neuroinflammation, restrain cholinergic function, decrease glutamate-mediated excitotoxicity and promote neurogenesis to reduce neurodegeneration and improve cognitive function. Therefore, it can be further corroborated that the OS extract may be a potential candidate for potential anti-Alzheimer's drug therapy, but more work is required to further validate the mechanism.

The passive avoidance task is mainly used to evaluate suitable treatment on the three memory levels, typically learning acquisition, memory retention and retrieval period (Eagle et al., 2016; Moosavi et al., 2018; Rabiei et al., 2014). Therefore, the passive avoidance task was used to test the 24-hour memory retention of an aversive incident from which the rat acquires to escape the dark compartment supplied with a moderate electrical foot shock (0.5 mA, 10s) during training (Damodaran et al., 2014). Our studies showed that PBOCCA rats dramatically reduced the ability to retain avoidance memories as opposed to the sham group after PBOCCA surgery. This suggested that the PBOCCA rats had not remembered or regained the memory of the previous traumatic experience of foot shock encountered in the dark compartment, which implied poor retention memory as demonstrated by decreased step-through latency to enter the dark compartment during the experiment. In this aversively motivated task, it can also be concluded that PBOCCA caused rats to have decreased emotional reactivity while reducing their reaction to avoidance. Regardless of the

established essence of the underlying deficit, it is obvious that PBOCCA induces an enduring deterioration of behavior in the expression of an avoidance response to a condition linked to a noxious sensory stimulus (Damodaran et al., 2014). On the other hand, PBOCCA rats treated with three doses of OS extract increased step-through latencies 24 h after training. This finding indicates that the rats treated with OS extract exhibit acquisition of avoidance learning.

On the other hand, the MWM has been routinely used to assess spatial learning and memory deficits in rats (D'Hooge & De Deyn, 2001). PBOCCA rats had impaired spatial learning and memory, presented by longer escape latency and shorter time spent in the target quadrant in the MWM, as compared to the sham-operated rats, according to the study by (Damodaran et al., 2014). Cognitive functions were examined for enhancement in the rats treated with OS extracts. Both 100 and 200 mg/kg of OS extracts have enhanced spatial learning and memory, indicating that this dosage may alleviate impairment in PBOCCA rats. In line with the results, it was observed that the vehicle-treated PBOCCA rats displayed hippocampus-dependent spatial learning and memory deficits as assessed using the MWM test and contextual memory as assessed using the step-through PAT. Interestingly, the low dose of treatment with OS extracts was found to relieve both of these cognitive deficits in PBOCCA rats.

Hippocampal LTP is a surrogate for studying hippocampus-dependent memory directly at the cellular level. Synaptic plasticity is widely studied and is characterized by a long-lasting increase in synaptic strength caused by pre- and postsynaptic activity (Malenka, & Bear, 2004). Here, we are testing the contribution of OS extracts in the basal synaptic transmission and hippocampal synaptic plasticity responsible for spatial learning and memory formation. PBOCCA rats and 100 mg/kg OS extract exhibited a lower input-output curve than the sham, confirming the effect of low dose 100mg/kg OS extract unable to recover the disruption of hippocampal synaptic transmission induced by CCH rats. Interestingly, 200 and 400 mg/kg OS extracts significantly enhanced basal synaptic transmission (Figure 6A). The mechanisms mediating the enhancement of basal synaptic transmission remain to be determined. However, the possible contribution of the enhancement may be related to improving basal synaptic strength of AMPA receptor-mediated transmission at the CA3-CA1 synapse (Damodaran et al., 2019). Increased insertion of AMPA receptors, especially those containing the GluA1 subunit, into the post-synapse, mediates basal synaptic transmission at Schafer collateral-CA1 synapses (Díaz-Alonso et al. 2017; Granger and Nicoll 2013). AMPA receptors promote the influx of positively charged NA^{+} into the postsynaptic cell, causing it to depolarize. If the depolarization is sufficient to remove the Mg^{2+} block on the NMDA receptor, the channel will open and allow Ca^{2+} into the cell. The fast increase in intracellular Ca^{2+} concentration activates many enzymes that particularly drive LTP induction and increase synaptic strength (Kumar, 2011).

The in vivo electrophysiological recordings showed that PBOCCA rats significantly suppressed the potentiation of LTP, similar to our previous findings (Damodaran et al., 2018; Damodaran et al., 2019). Treatment with the highest dose of OS extract (400mg/kg) significantly restored the in vivo hippocampal LTP suppression caused by CCH after 2 hours of induction with TBS. The elevation of the fEPSPs could be due to the changes in the signal transduction pathways linked to activation protein kinases and changes in gene expression and protein synthesis (Bramham & Messaoudi, 2005). These include protein kinases A and C, calcium/calmodulin-dependent protein kinase II extracellular regulated kinase, CAMP-responsive element binding protein 1 (CREB1) and brain-derived neurotrophic factor (BDNF). The hippocampus undergoes many types of synaptic plasticity, including neurogenesis, alteration in the morphology of the cells and changes in synaptic strength to maintain normal function throughout life (Hajjar et al., 2013). The impairment of synaptic plasticity contributes to cognitive dysfunction. Retinasamy et al. (2019) reported OS extract was found to regulate the mRNA expression of CREB1, BDNF, and TRKB genes, and pre-treatment with OS extract was observed to increase immature neurons against scopolamine-inhibited hippocampal neurogenesis, which was confirmed by the DCX-positive stained cells. DCX is a marker of neuroblasts, neuronal precursor cells, and immature neurons. BDNF-TrkB interaction promotes the survival and differentiation of neurons and synaptic plasticity of the CNS (Bathina & Das, 2015; De Vincenti et al., 2019). Previous studies have shown that activation of CREB ameliorates cognitive deficits via the cholinergic system (Kotani et al., 2006; Zhou et al., 2018). So, it can be speculated that OS extract could be a potent treatment for neurodegenerative diseases, and its possible mechanism might be modulating the cholinergic activity via the CREB-BDNF pathway. Besides that, RA was found to alleviate hippocampal neurogenesis and synaptic plasticity in an Aβ1-42-induced mouse model of Alzheimer's disease in another

study done by Mirza et al. (2021). Neuronal proliferation, migration and maturation were significantly increased in the RA-treated groups by increased NeuN and DCX expression. Synaptophysin (the most widely used protein marker of synaptic plasticity) is an integral membrane protein of synaptic vesicles essential for neurotransmission to increase synaptic strength (Kokotos et al., 2019; Thiel, 1993). The significant increase in synaptophysin expression induced by RA in Aβ1-42-treated mice indicates its beneficial effect on neurotransmission. Fonteles et al. (2016) reported neuronal loss suppression, increased synaptophysin expression, and increased BDNF levels *in vivo*. The results obtained from these studies suggested that RA reverses synaptic plasticity dysfunction by promoting neurogenesis and increasing the synaptic strength of hippocampal neurons. In addition, the hippocampal neuroprotective and neurogenesis activity of RA was supported by studies by Khamse et al. (2020) and Ali and Zahid (2020). Suggesting that RA contained in the OS extract plays an important role in hippocampal plasticity. The reason for all of these indications is that RA has a weak blood-brain barrier (BBB) due to its size of 360 Dalton and its hydrophilic characteristics.

The contradictory data between MWM and LTP may be due to the single and cumulative doses of OS extracts administered in rats. Rats were given post-training administration of 100, 200 and 400mg/kg OS extract four times, once daily, during the acquisition training in MWM; meanwhile, one treatment was given during LTP in *in vivo* electrophysiology. Hence, repeated doses of 400mg/kg in MWM may lead to neuronal death and fail to improve spatial memory. One limitation of this study is the lack of the pharmacokinetic, pharmacodynamics and BBB permeability of OS extract and active compounds in the brain of CCH rats. However, studies report the effect of OS extract and RA in the CNS, the *in vivo* models used in those studies were chemically induced compared to the animal model used in this study, which was physically induced for cognitive dysfunction. Besides that, BBB was found to disrupt during CCH condition (Ahad et al., 2019). Therefore, further pharmacodynamics and pharmacokinetic studies, along with data on the permeability of active compounds of OS extract into BBB, are required to validate the direct or indirect nootropic effect of OS in the CNS of CCH rats.

5.0 CONCLUSIONS

In conclusion, the present studies revealed the pathological manipulation using PBOCCA rats as a model in alternating the synaptic strength and the efficacy of OS ethanolic extract (enriched with RA) in mitigating neuronal death in the CA1 subfield of the hippocampus and improving cognitive impairment induced by PBOCCA rats. The cumulative administration of 100 mg/kg of OS extract improves cognitive function in CCH rats compared to the higher doses of 200 and 400mg/kg. However, further work should be undertaken to study the dose effect of OS extract below 100mg/kg to determine the optimum dose of OS extract for improving cognitive function. The link between altered synaptic or cellular mechanisms and behavioral manifestations of memory is not straightforward. Understanding the possible plasticity mechanisms mediated by the ethanolic extract of OS helps to develop a new therapeutic agent that can be used for treating AD and vascular dementia related to cognitive decline.

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