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In vitro anti-cholinesterase activity and *in vivo* screening of *Coccoloba uvifera*, *Mimusops elengi* and *Syzygium aqueum* extracts on learning and memory function of chronic cerebral hypoperfusion rat

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Abstract: Vascular dementia (VaD), is one of the most common types of dementia in the ageing population, initiated by chronic cerebral hypoperfusion (CCH). At present, effective therapeutic approaches to cure VaD are still missing. Cholinergic system dysfunction in the central nervous system (CNS) has been recognised as one of the main reasons for learning and memory impairment in VaD patients. Therefore, medications that restore the level of acetylcholine (ACh) neurotransmitter by inhibiting cholinesterase activity were proposed as a potential candidate to treat VaD patients. Permanent occlusion of bilateral common carotid arteries (POBCCA) surgery method was performed to develop CCH model in rats. The present study evaluated the anti-cholinesterase activity of three Malaysian plant methanol leaf extracts in vitro and further validated its cognitive-enhancing effects *in vivo* using POBCCA rats. The selected plant extracts were *Coccoloba uvifera* (stems), *Mimusops elengi* (leaves) and *Syzygium aqueum* (leaves). The *in vitro* anti-cholinesterase activities of these plants were determined using Ellman's method. The effects of selected plant extracts (100 and 200 mg/kg, p.o.) on learning and memory functions were evaluated using a series of behavioural tests. All the selected plant extracts exhibited good anti-acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activities *in vitro*, with IC_{50} ranging from 3.67 to 16.04 and 5.6 to 13.95 µg/mL, respectively. Extracts of *S. aqueum* (200 mg/kg) improve both short- and long-term recognition memories, whereas *M. elengi* and *S. aqueum* (200 mg/kg) extracts improve spatial learning. None of the extracts impaired motor and exploratory functions in POBCCA rats. In conclusion, methanol extracts of *C. uvifera*, *M. elengi* and *S. aqueum* showed good anti-cholinesterase activity *in vitro*. However, only *M. elengi* and *S. aqueum* improve learning and memory function in POBCCA rats.

Keywords: Chronic cerebral hypoperfusion; vascular dementia; anti-cholinesterase; *Mimusops elengi*; *Syzygium aqueum*

1.0 INTRODUCTION

Dementia refers to a clinical syndrome characterised by progressive deterioration of cognitive functions, which results in patients' inability to carry out daily activities and live independently (Duong *et al.*, 2017). Dementia remains one of the greatest global public health challenges facing by society regarding economic costs and social burdens since most patients need long-term treatment at their home or nursing home (Prince *et al.*, 2013). Vascular dementia (VaD) is the second most common type of dementia after Alzheimer's disease (AD), caused by insufficient blood flow to the central nervous system (CNS) (Lobo *et al.*, 2000). VaD is a heterogeneous disorder and the patients often possess different clinical presentation and cognitive profiles depending on the origin and type of vascular occlusion, arterial territories network, haemorrhage, and vessel size. VaD is classified into different subtypes such as cortical, subcortical, strategic infarct and mixed dementia (Kurz, 2001; Román, 2002; Jellinger, 2013; Khan *et al.*, 2016). Thus, defining pure VaD is challenging in practice. The risk factors of VaD include stroke, cardiovascular, hypertension, diabetes, obesity, smoking and depression (Vijayan & Reddy, 2016). To date, there is no medication approved for VaD treatment (Pantoni, 2004). This is due to fewer clinical trials being conducted for VaD compared to AD. Cholinergic signalling in the CNS plays an essential role in cognitive processing such as attention, memory and motivation (Ballinger *et al.*, 2016). Patients with possible VaD showed cognitive improvement after treated with cholinesterase inhibitors such as donepezil, galantamine and rivastigmine during clinical studies (Noufi *et al.*, 2019). Therefore, medications that target the cholinergic system with anti-cholinesterase activity have been proposed as potential therapeutic strategies to improve the cognitive function of VaD patients.

Medicinal plants have attracted much attention in recent years as potential new therapeutic agents for neurological disorders. Medicinal plants are rich in secondary metabolites and oils with therapeutic values (Panda & Jhanji, 2020). Hence, exploring this abundantly available natural resource may lead to discovering potential agents with the anti-cholinesterase activity that can efficiently improve cognitive function in dementia patients with fewer side effects. The selection of plant extracts in the current study was based on a previous *in vitro* screening study of 117 plant extracts in which the selected plant extracts showed the highest cholinesterase inhibition activity (Amir Rawa *et al.*, 2019). Permanent occlusion of bilateral common carotid arteries (POBCCA) in rats is a widely utilised chronic cerebral hypoperfusion (CCH) model that closely resembles human VaD conditions (Azam *et al.*, 2018). POBCCA reduces cerebral blood flow (CBF) and causes progressive neuronal degeneration, cholinergic dysfunction, learning and memory impairment or cognition deficits in the rat (Kitamura *et al.*, 2012; Amenta *et al.*, 2002). This model is useful for investigating the pathophysiology of CCH and discovering drugs with potential therapeutic values for VaD (Institoris *et al.*, 2007). The current study was conducted to validate the *in vitro* anti-cholinesterase activity of 3 different plant extracts, *C. uvifera*, *M. elengi* and *S. aqueum* and further evaluate the effect of these extracts on the cognitive function of POBCCA rats using a series of behavioural tests.

2.0 MATERIALS AND METHODS

2.1 Chemical Materials

Methanol, physostigmine, dimethyl sulphoxide (DMSO), sodium phosphate buffer (pH 7.5), 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), acethylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI), acetylcholinesterase (AChE), butyrylcholinesterase (BuChE), ketamine and xylazine.

2.2 Plant materials

The raw materials of *C. uvifera, M. elengi* and *S. aqueum* were collected from its natural habitat, Penang Island, Malaysia. The authenticity of the plants was verified by a botanist, Dr Rahmad Zakaria, from Herbarium unit, Universiti Sains Malaysia, and voucher specimens were deposited at the herbarium unit, School of Biological Sciences, Universiti Sains Malaysia, Malaysia. Voucher specimen number: *C. uvifera* (11830), *M. elengi* (11829) and *S. aqueum* (11832).

2.3 Plant extraction

The collected plant materials were air-dried until a constant weight was obtained. The dried plant materials were ground using a mechanical grinder into fine powder. Approximately 800 g of each ground plant material powder were soaked in 8 L of methanol (1:10 W/V) in respective beakers. The mixture was sealed tightly and left at room temperature for three days. The mixture was regularly stirred using a glass rod each day. The mixture of the respective plant materials in methanol was filtered with filter paper (Whatman No. 1) using a filter funnel to separate marc from the micelle. The filtered micelle was evaporated at 40 °C under reduced pressure using a rotary evaporator to remove the solvent in the micelle, and the resulting concentrated crude extract was collected. The collected

marc was extracted twice again with the same volume of methanol to ensure all the bio-compounds fully extracted from the plant material. The evaporated crude extract was kept in laminar flow for 3 days to remove traces of methanol. The crude extract was then subjected to a freeze-drying process using a freeze dryer and stored at -80 °C for further use.

2.4 *In vitro* anti-cholinesterase assay

The plant extracts were evaluated for their potential *in vitro* anti-cholinesterase activity in triplicate based on Ellman's method described previously with slight modifications (Amir Rawa *et al.*, 2019). Physostigmine was used as the positive control. Stock solutions of plant extracts were prepared in 100% DMSO. In a 96-well microplate, 140 µL of 0.1 M sodium phosphate buffer (pH 7.5), 20 µL of samples, and a 0.09 unit/mL AChE enzyme were added. After 15 min of pre-incubation at 25 °C, 10 µL of 10 mM DTNB and 14 mM ATCI each was added to give a final reaction volume of 200 µL. The microplate was shaken for 10 s, and the absorbance readings on each well were measured immediately for 30 min at 412 nm using a microplate spectrophotometer (Thermo Scientific, USA). Anti-BuChE assay was performed based on the same procedure as described before, using BuChE and BTCI. The final absorbance of each tested sample was corrected by subtracting the absorbance of their respective blank without enzyme addition. The inhibition percentage was calculated using the following formula:

$$\text{Percentage inhibition} = \frac{\text{Absorbance of control - Absorbance of tested sample}}{\text{Absorbance of control}} \times 100\%$$

2.5 Animals

All experimental procedures involving animals were reviewed and approved by the Animal Ethics Committee, Universiti Sains Malaysia (USM/Animal Ethics Approval/2016/(103)(791). Male Sprague Dawley weighing 250–300 g was obtained from Animal Service and Research Centre, Universiti Sains Malaysia. Animals were kept in the animal transit room for a week for acclimatisation before the animals were subjected to POBCCA surgery. The animal room was regulated with a controlled temperature (±24 °C) and a standard 12 h light and 12 h dark cycle with the lights turned on at 7.00 a.m. Animals could access food and water ad libitum.

2.6 POBCCA surgical procedure

POBCCA surgery was performed to induce CCH in rats, according to Damodaran *et al*. (2014). Rats were fasted for at least 8 hours before surgery. The rats were anaesthetised with a combined intraperitoneal injection of 80 mg/kg ketamine (Ilium, Australia) and 10 mg/kg xylazine (Ilium, Australia) with a volume of 1.0 mL/kg rats. CCH was induced in rats by making a ventral midline incision at the neck to expose and isolate the common carotid arteries from their connective tissues and vagus nerves. Then, the carotid arteries were permanently doubly ligated with a 6/0 silk suture approximately 8 to 10 mm below the origin of the external carotid artery. The incision was stitched using silk suture, and wound gel (Octenisept, Schulke, Germany) was applied to the incision area to prevent wound infection. The body temperature of the rat was maintained at 37 °C with a thermal blanket throughout the surgery procedure. The control rats were subjected to Sham-operation in which the rats undergo the same procedure without ligating the common carotid arteries. The rats were kept individually in a single cage to recover for 14 days in an isolated room maintained at a temperature of ±25 °C. The sutured area was cleaned daily, and wound gel was applied to avoid infection. The body weight, food and water intake were monitored daily. After 2 weeks, the rats were subjected to behavioural tasks.

2.7 Drug administration and experiment design

The rats were randomly divided into eight treatment groups two weeks after surgery: Sham rats treated with vehicle (distilled water (dH2O)) (n=6); POBCCA rats treated with vehicle (dH2O) (n=6); POBCCA rats treated with 100 and 200 mg/kg *C. uvifera* (n=6) respectively; POBCCA rats treated with 100 and 200 mg/kg *M. elengi* (n=6) respectively; POBCCA rats treated with 100 and 200 mg/kg *S. aqueum* (n=6) respectively. The plant crude extracts were dissolved in dH2O and sonicated in an ultrasonic bath until fully dissolved. The plant extracts and dH2O were administrated via oral gavage to the rats of respective groups in a volume of 10.0 mL/kg per body weight one hour before conducting the behavioural test. During automated open field test (AOFT) plant extracts or dH2O were administrated before the testing session, while during the novel object recognition (NOR) test, the treatment was done before the familiarisation phase. Animals were treated with either plant extracts or dH2O before training and probe trial during the Morris water maze (MWM) test.

2.8 Automated open field test

The automated open-field test was performed as described by Tiang *et al*. (2020) to evaluate the motor and exploratory function of rats using an automated open-field apparatus (Panlab Infrared Actimeter, Spain). The apparatus is a square arena enclosed by perspex

walls (20 cm in height, 45 cm in length and 45 cm in width), with the floor of the apparatus divided into 5 zones. The detection unit consists of a 45 × 45 cm InfraRed frame with a total of 32 IR beams (16 on the x axis and 16 on y-axis spaced 2.5 cm apart) located on the sides of the apparatus. AOFT consists of 2 sessions, habituation and testing. During habituation, the rat was released at the centre of the arena of the open field apparatus for 10 min. The rat was then returned to the cage, and the apparatus was cleaned with 70 % ethanol to remove any odour cues from the previous animal before releasing the next rat. The testing session was conducted the next day, and the rat was placed at the centre of the arena in the open-field apparatus and allowed to move freely in the open field apparatus for 20 min. The experiment was carried out in an adequately but dimly lit room to allow rats to see and explore their surrounding environment while avoiding stress from bright lights. The movement of the rat was recorded using the software (Actitrack, Panlab, Spain) and analysed offline. Three parameters were measured in this AOFT: (i) locomotor activity, (ii) mean velocity and (iii) total distance travelled.

2.9 Novel object recognition (NOR) test

The NOR test was conducted as outlined by Antunes & Biala (2012) to assess short-term and long-term recognition memory. The experiment was conducted in a dim red-light illuminated condition using a black Plexiglas box with the following dimensions: 45 cm × 45 cm × 45 cm (Height × Width × Length). A camera (Sony HDR-XR260, USA) was placed directly above the arena to monitor the experiment. The test consists of 3 phases: habituation, familiarisation, and test phase. During the habituation phase, the rat was released into an empty arena facing the wall of the box and allowed to explore the arena for 5 min before returning to the cage. All the animals were subjected to the same procedure. The apparatus was cleaned with 70% ethanol between each trial interval to eliminate any olfactory clues that might have been left behind during the previous trial. On the next day, the familiarisation phase was conducted using the same procedure. However, this time, the rat was released into the arena consisting of 2 identical objects placed in two corners of the arena, equidistant from each other. The rat was allowed to explore the objects for 5 min. To evaluate the short-term recognition memory in rats, the test phase was conducted 2 h after the familiarisation phase in which the rats was released into the arena consisting of 1 familiar object and 1 novel object for 5 min to explore each object. The position of the familiar and novel object was interchanged from left to right in order to prevent bias for a particular location. The long-term recognition memory was assessed in rats using the same procedure for each phase, except the test phase was conducted 24 h after the familiarisation phase.

Exploration was defined as the animal sniffing the object within 2 cm. Climbing or sitting on and biting an object was not considered as exploration. Rats with intact recognition memory would spend more time exploring novel object as compared to a familiar object. To evaluate recognition memory in rats, the following parameters: time (s) spent exploring the novel object (Tn), familiar object (Tf), and total time (s) spent exploring both objects (Tf+Tn) were measured during the test phase. Recognition index (RI) refers to the time spent exploring the novel object relative to the total object exploration. The RI was determined using the following equation, RI=((Tn)/(Tf +Tn)). Rats that spend less than 20 seconds exploring both objects were excluded.

2.10 Morris water maze test

The MWM test was commonly used to investigate spatial learning and reference memory in animal models. This test was conducted as described by Vorhees & Williams (2006). The apparatus consists of a circular pool with non-reflective interior surfaces (180 cm in diameter and 70 cm in height) and filled with water at 21±1 °C to a depth of 52 cm. The water was made opaque using non-toxic white paint (Nippon Paint, Japan). The pool was divided into four quadrants of equal area (North, East, South and West). A camera (Logitech C270, Switzerland) was mounted above the middle of the pool with which the motion of the rat was recorded. Since the animals rely on distal visual cues for navigation, the laboratory room's settings remained unchanged throughout the experiment.

MWM test requires 7 days to complete and consists of 4 sessions: habituation, training, probe trial and visible platform test. The test started with habituation, in which the rat was allowed to swim freely in the pool without a platform for 60 s. The rat was released facing the wall of the tank to minimise bias. The duration was monitored using a stopwatch, and at the end of 60 s, the rat was removed from the pool, wiped with a towel, and dried under the heat of the lamp after each trial before returning to the cage. The faeces of the previous rat was removed from the pool using a net before releasing the next rat. Habituation was done to assess any motor disabilities in the rats and familiarise the rats in the water environment.

During the training session, a rat was released into the pool containing a circular transparent platform made from Plexiglas (10 cm diameter and 50 cm high), submerged 2 cm below the water surface to make it invisible in the central part of the west south quadrant. This quadrant was designated as the target quadrant, and the platform's position kept constant all through the experiment. All rats were trained with one block of four, 60 s trials each at the approximately same time each day for 5 consecutive days. The rat was released into the pool from a different starting point on each training trial, and the starting point was changed each day. Once the rat found the hidden platform, it was allowed to stay on it for 15 s. The time is taken for the rat to reach/ find the hidden platform (escape latency) was scored using a stopwatch and recorded using a camera for each trial session. If the rat failed to find the platform within 60 s, it was gently directed to the platform and allowed to remain on the hidden platform for 15 s. These rats were given a score of 60 s.

On the 7th day, each rat completed a 60 s probe trial (one trial) and a visible platform test (2 trials). The platform was removed during the probe trial before releasing the rat into the pool. The movement of the rat in the entire pool and time spend in the targeted quadrant was recorded and analysed using a smart video tracking system (Harvard Apparatus, USA). A visible platform test was performed immediately after the probe trial to detect any visual and motivational impairment in rats. The platform was placed 1 cm above the water surface in the centre of the northeast quadrant to make it visible to the rat. Each rat was released twice into the pool to locate the visible platform. The time taken for the rat to reach the platform was scored using a stopwatch.

2.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 for Windows (GraphPad Software, USA). In vitro assay data were expressed as mean±standard deviation, and *in vivo* data were expressed as mean±standard error (SEM). For AOFT and NOR test, data were analysed using one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test. The escape latency during spatial training in the MWM test was analysed using a two-way repeated measure ANOVA followed by Bonferroni *post hoc* test. During the probe trial of the MWM test, the time of each rat spent in the target quadrant was analysed by one-way ANOVA followed by the Bonferroni *post hoc* test.

3.0 RESULTS

3.1 *In vitro* anti-cholinesterase activity of plant extracts

The *in vitro* cholinesterase activity assay revealed that all the plant extract have potential anti-cholinesterase activity with IC_{50} values ranging from 3.67 to 16.04 ug/mL and 5.6 to 13.95 ug/mL for AChE and BuChE activities, respectively. Table 1 shows percentage inhibition and IC_{50} of respective plant extracts against AChE and BuChE.

Table 1: Percentage inhibition and IC_{50} of respective plant extracts against AChE and BuChE

Extract	Concentration (µg/mL)	AChE inhibition activity (%)	IC_{50}	BuChE inhibition activity (%)	IC_{50}
Physostigmine	0.25	84.81± 2.061	0.05± 0.002	64.10±3.883	0.12± 0.005
***C. uvifera* stem**	12.5	93.25±2.743	3.67±0.160	83.01±1.843	5.60±0.149
***M. elengi* leaves**	25 .0	85.47±2.467	9.78±0.104	87.88±2.078	11.17±1.222
***S. aqueum* leaves**	50.0	83.80±1.562	16.04±0.687	91.91±1.518	13.95±1.287

3.2 Effect of plant extracts on motor function of POBCCA rats

The effect of plant extracts treatment on locomotor activity, mean velocity and total distance travelled of POBCCA rats were investigated using AOFT (Figure 1). There was no significant difference in locomotor activity, mean velocity, and total distance travelled between POBCCA + vehicle and Sham + vehicle group rats analysed using one-way ANOVA. POBCCA rats treated with plant extracts, respectively, did not show any significant difference in locomotor activity, mean velocity, and total distance travelled compared to control group rats analysed using one-way ANOVA. The results obtained indicate that POBCCA surgery and plant extract treatment, *C. uvifera* stem, *M. elengi* leaves and *S. aqueum* leaves, respectively, did not impair motor function and exploratory activity in POBCCA rats.

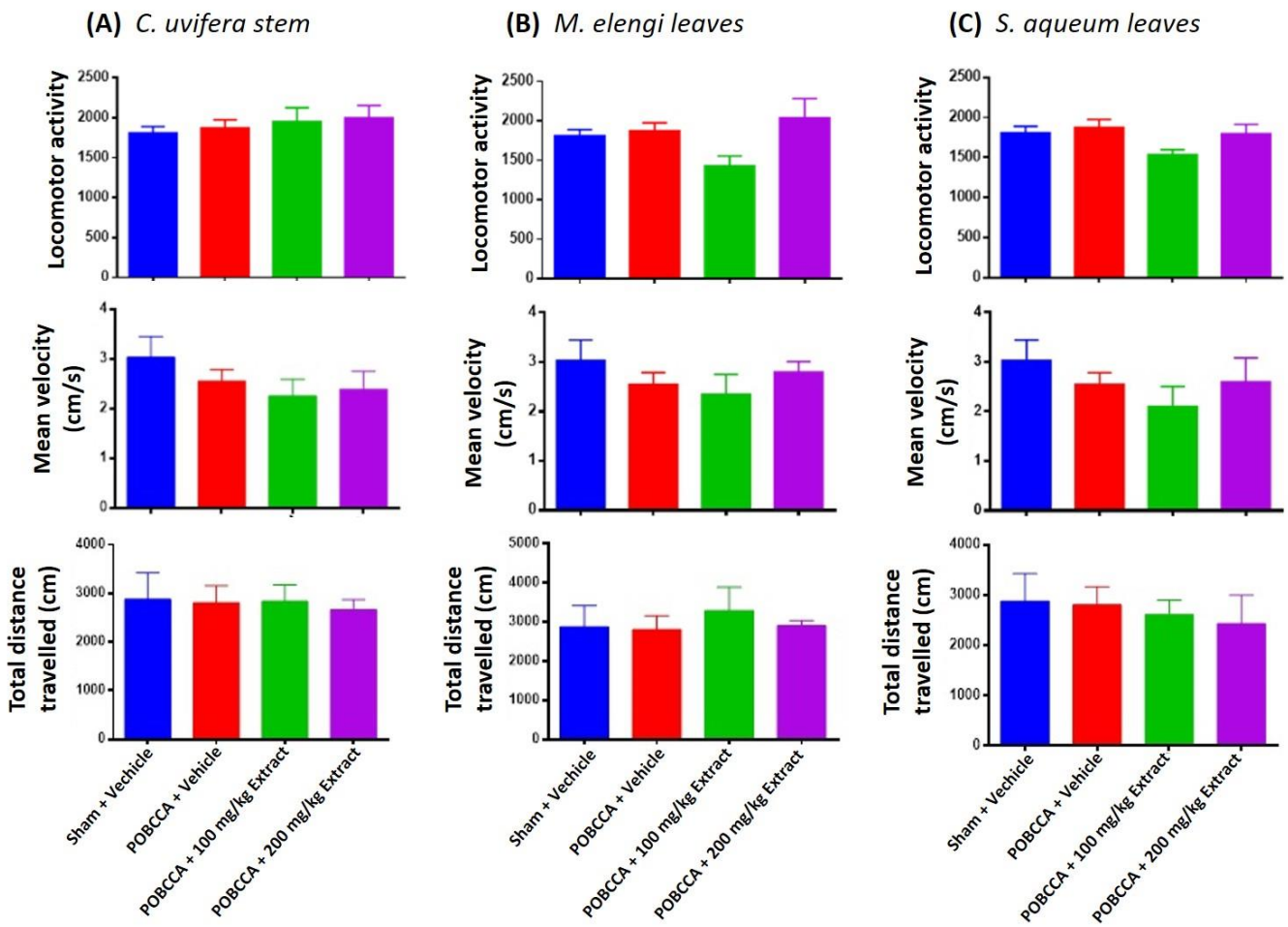

Figure 1: Effect of plant extracts **(A)** *C. uvifera* stem, **(B)** *M. elengi* leaves and **(C)** *S. aqueum* leaves (100 and 200 mg/kg, p.o.) treatment on locomotor activity (top panel), mean velocity (middle panel) and total distance travelled (bottom panel) of POBCCA rats in the AOFT. Data are expressed as the mean±SEM (n=6). There is no significant difference among the group, analysed by one-way ANOVA followed by the Bonferroni *post hoc* test.

3.3 Effect of plant extracts treatment on short- and long-term recognition memory of POBCCA rats

NOR test was used to evaluate both short- and long-term recognition memory. The recognition index (RI) was calculated for each trial during the test phase to evaluate the preference for the novel object. The effect of respective plant extract on RI during the short- and long-term test phase were presented in Figure 2. POBCCA rats exhibited a significant lower RI value than Sham-operated rats during both the short- (p=0.0288) and long-term test phases (p=0.0137), respectively. Sham-operated rats with intact short- and long-term recognition memory spend more time exploring a novel object than a familiar object. POBCCA rats did not recognise previously explored objects and spent almost equal time exploring both familiar and novel objects during the short and long test phases. This indicates CCH induce impairment of both short- and long-term recognition memories in POBCCA rat.

During the short-term test phase, POBCCA rats treated with *S. aqueum* leaves (200 mg/kg) exhibited significant higher RI value as compared to POBCCA rats treated with vehicle (*S. aqueum* (200 mg/kg): p=0.0173). However, there was no significant difference in the RI value between POBCCA rats treated with *C. uvifera* stem (100 and 200 mg/kg), *M. elengi* leaves (100 and 200 mg/kg) and *S. aqueum* leaves (100 mg/kg) extracts and POBCCA rats treated with vehicle. The rats in these treatment groups spend similar time exploring both familiar and novel object, which indicates the treatment with *C. uvifera* stem (100 and 200 mg/kg), *M. elengi* leaves (100 and 200 mg/kg) and *S. aqueum* leaves (100 mg/kg) extracts did not improve short-term recognition memory in POBCCA rats. During the long-term test phase, the POBCCA rats treated with S. aqueum leaves extract (200 mg/kg) showed significant higher RI than POBCCA rats treated with vehicle (*S. aqueum* 200 mg/kg: p=0.0155).

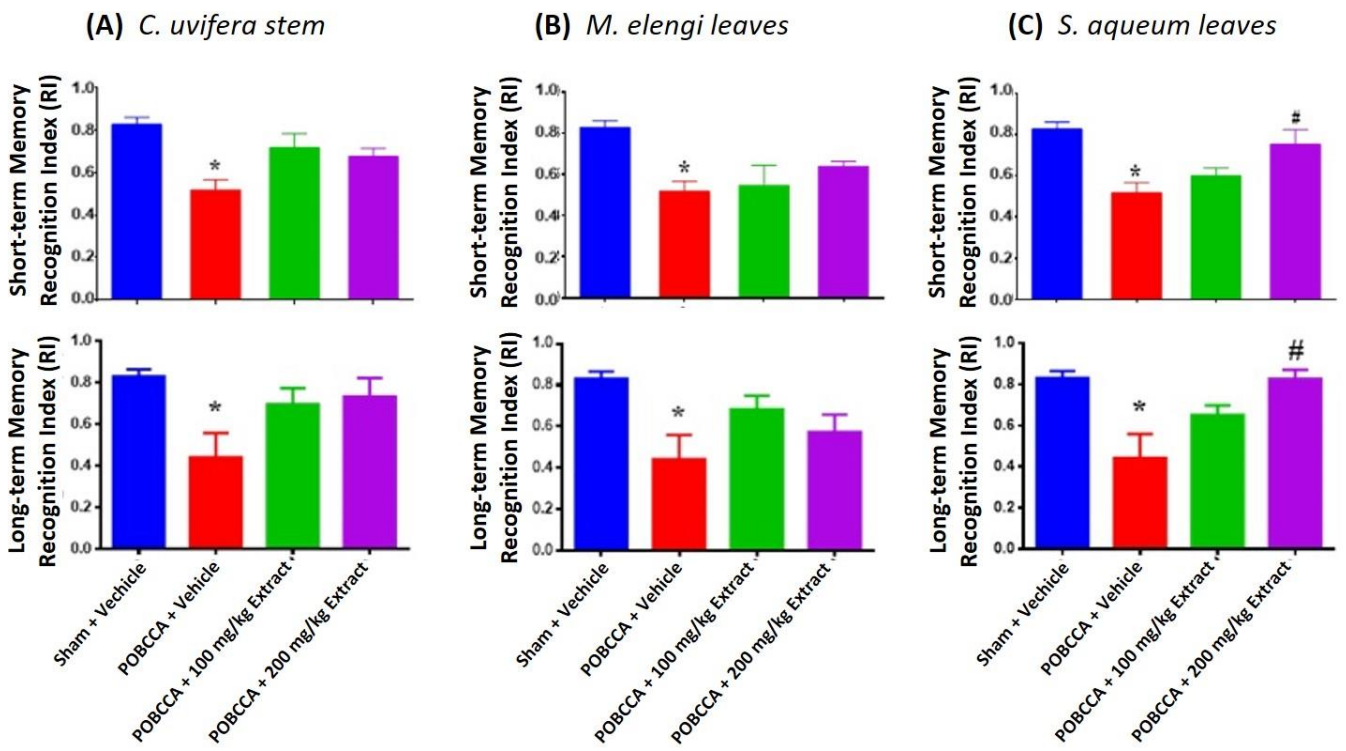

Figure 2: Treatment effect of various plant extract **(A)** *C. uvifera* stem, **(B)** *M. elengi* leaves and **(C)** *S. aqueum* leaves (100 and 200 mg/kg, p.o.), respectively, on POBCCA-induced short-term (top panel) and long-term (bottom panel) recognition memory deficit in the NOR test. Data are expressed as the mean±SEM (n=6), *p<0.05 significantly different from Sham + dH2O whereas #p<0.05 significantly different from POBCCA + dH2O, analysed by one-way ANOVA followed by Bonferroni *post hoc* test.

Treatment of *C. uvifera* and *M. elengi* extract (100 and 200 mg/kg) along with *S. aqueum* extract (100 mg/kg) did not improve long-term recognition memory in POBCCA rats. The results obtained suggest that treatment of *S. aqueum* leaves extract (200 mg/kg) ameliorates short- and long-term recognition memory deficits in POBCCA rats.

3.4 Effect of plant extracts treatment on spatial learning and reference memory of POBCCA rats

The effect of plant extracts on spatial learning and reference memory deficit in POBCCA rats were assessed using MWM test. The escape latency time during spatial training is presented in Figure 3. The percentage time spent in the targeted quadrant during probe trial, the mean swimming speed of rats in the pool during probe trial and escape latency time during visible platform trials are presented in Figure 4.

During spatial training, POBCCA + vehicle rats showed a significantly longer time to reach the hidden platform than Sham-operated rats starting from day 3 until day 5 (day 3: p=0.0020, day 4: p=0.0001 and day 5: p=0.0246). Distinctively, POBCCA rats treated with *S. aqueum* and *M. elengi* leaves extract (200 mg/kg) showed a significant reduction in escape latency time during training starting from day 4 to day 5 compared to POBCCA + vehicle (day 4; *S. aqueum* and *M. elengi* (200 mg/kg): p=0.0041 and p=0.0484 and day 5; *S. aqueum* and *M. elengi* (200 mg/kg): p=0.0024 and p=0.0454). However, POBCCA + *C. uvifera* stem extract (100 and 200 mg/kg) and POBCCA + *S. aqueum* and *M. elengi* leave extracts (100 mg/kg), respectively, showed no significant difference in escape latency time throughout the 5 days training session as compared to POBCCA + vehicle rats.

On day 6, probe trials showed significant less time spend in targeted quadrant by POBCCA + vehicle (p=0.0277) and POBCCA + *C. uvifera* stem extract (100 mg/kg) (p=0.0262) rats compared to Sham-operated rats. POBCCA rats treated with *C. vifera* stem, *M. elengi* leaves, and *S. aqueum* leaves extracts did not show any significant difference in percentage time spend at the targeted quadrant compared to POBCCA + vehicle. The movement of rat analysis using the SMART software system revealed no significant difference in the swimming speed among the POBCCA rats treated with vehicle or respective plant extracts compared to Sham-operated rats. During the visible platform trials, rats in the POBCCA + vehicle and POBCCA + respective plant extract group reached the visible platform with no

significant difference in time compared to Sham-operated rats. The results obtained imply that *M. elengi* and *S. aqueum* leave extracts (200 mg/kg) improve spatial learning in POBCCA rats. However, *C. uvifera* stem extract did not improve spatial learning throughout the 5 days of training.

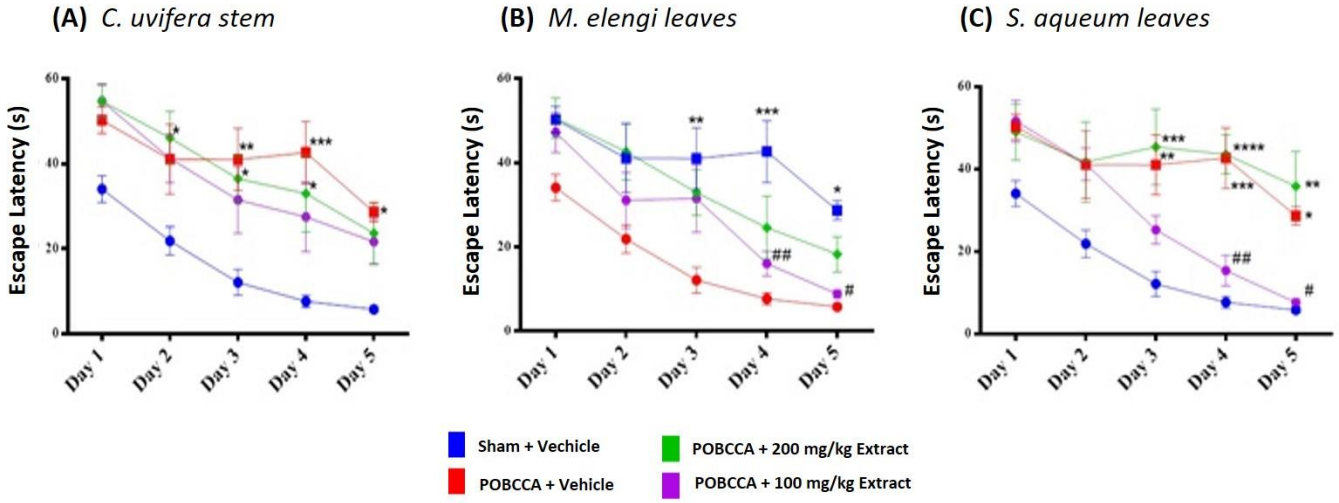

Figure 3: Treatment effect of plant extracts **(A)** *C. uvifera* stem, **(B)** *M. elengi* leaves and **(C)** *S. aqueum* leaves (100 and 200 mg/kg, p.o.) on POBCCA-induced spatial learning deficit in the MWM test. Data are expressed as the mean±SEM (n=6), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 significantly different from Sham + dH2O. $^{\#}$p<0.05, $^{\#\#}$p<0.01 significantly different from POBCCA + dH2O, analyzed by two-way repeated measure ANOVA followed by Bonferroni *post hoc* test.

4.0 DISCUSSION

VaD is caused by the reduced blood supply to the brain due to cerebrovascular disease, leading to a gradual decline in memory and cognitive function (Zlokovic, 2005; Snyder *et al.*, 2015). Although the precise mechanisms involved in VaD remain unclear, cholinergic deficits have been reported in VaD patients' brains (Moretti *et al.*, 2011; Kim *et al.*, 2012). Cholinergic neurons, which are predominately located in the basal forebrain and its axon projections into the hippocampus and cortex, are principally accountable for learning and memory functions (Jia *et al.*, 2004; Okada *et al.*, 2015). Degeneration of basal forebrain neurons has been reported in VaD patients, and lesions in the cholinergic pathways cause decreased ACh releases to the hippocampus and cerebral cortex region, resulting in learning and memory impairment (Wang *et al.*, 2009; Lee *et al.*, 2011). Therefore, targeting the cholinergic system have been suggested as an effective strategy to treat VaD.

Although *in vitro* techniques may be useful as a preliminary screening method to identify promising medications, the outcome of these studies may not be reproducible in an *in vivo* model as the latter owns a more complex system (Atanasov *et al.*, 2015). In addition, to determine the effect of drugs on behaviour (e.g., cognitive function), a suitable *in vivo* model is required. Hence, this study was conducted to investigate 3 potential plant extracts with *in vitro* anti-cholinesterase activity on cognitive function in the CCH rat model. POBCCA rat model used in this study was established by permanent ligation of both common carotid arteries, resulting in a significant reduction of CBF and display learning and memory impairment, neuronal damage, and biochemical changes resembling the conditions that occurred in VaD patients (Farkas *et al.*, 2007). Over the years, the POBCCA rat model provides a valuable approach to investigate the pathophysiology of chronic cerebrovascular disorders and to screen for potential agents with therapeutic value to treat VaD (Institoris *et al.*, 2007).

The effect of *C. uvifera*, *M. elengi* and *S. aqueum* extracts on motor, learning and memory function of POBCCA rats were evaluated in this study. Among the selected plant, *M elengi* and *S. aqueum* are common plants, while *C. uvifera* is an underutilised plant (Kumaran *et al.*, 2019). No previous studies reported on the nootropic activity of the selected plant extracts except for the methanol extract of *M. elengi* leaves, which were reported to enhance memory in ageing and chemically induced dementia mice model (Joshi & Parle, 2012). Hence, this was the first study to report the effect

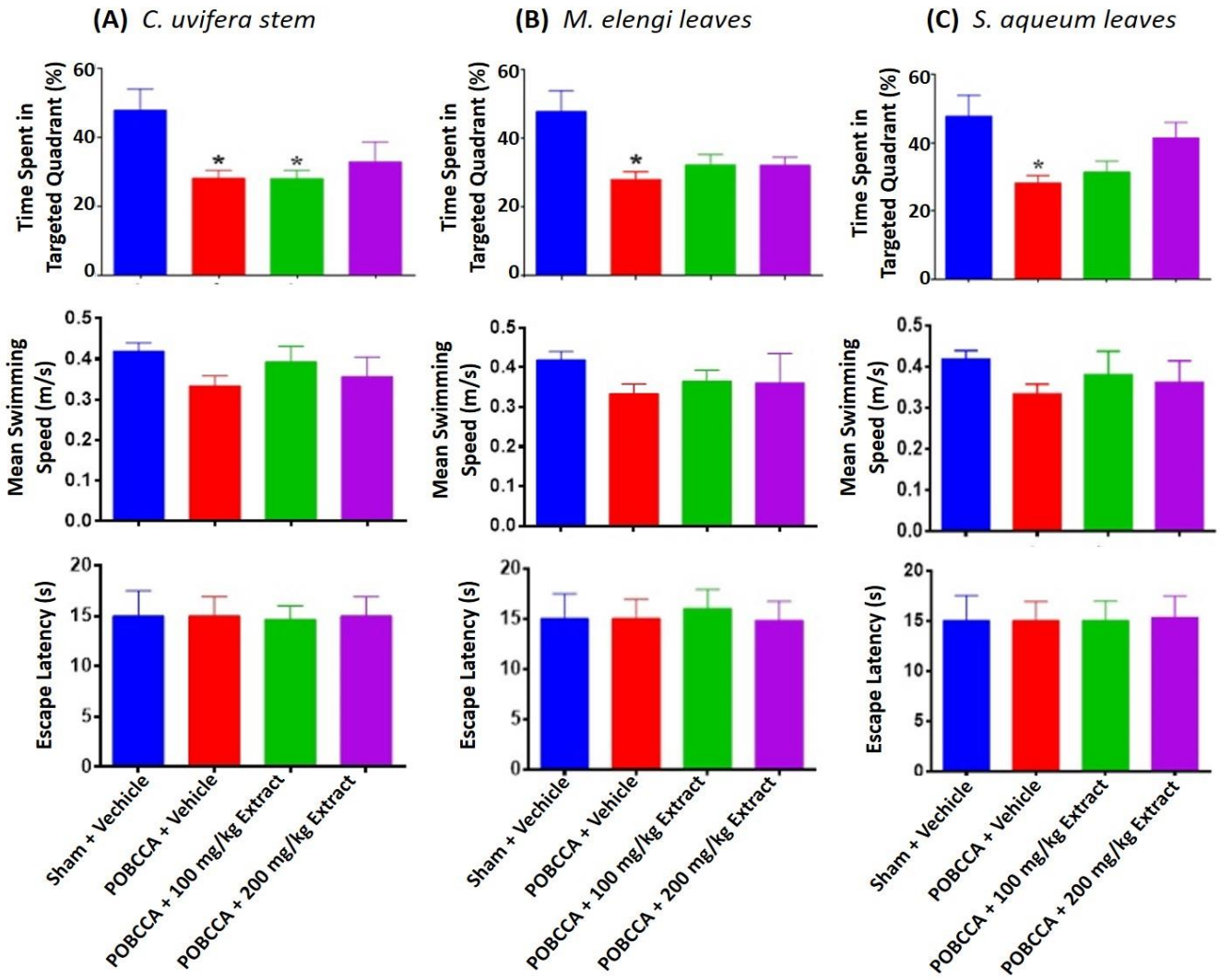

Figure 4: Effect of various plant extract **(A)** *C. uvifera* stem, **(B)** *M. elengi* leaves and **(C)** *S. aqueum* leaves (100 and 200 mg/kg, p.o.) treatment on reference memory deficit (top panel), swimming speed (middle panel) and escape latency time (bottom panel; during visible platform trials) of POBCCA-induced in the MWM test. Data are expressed as the mean±SEM (n=6), *p<0.05 significantly different from Sham + dH2O, analysed by one-way ANOVA followed by Bonferroni *post hoc* test.

of all selected plant extracts on the cognitive functions of POBCCA rats. No pharmacological activities have been reported on *C. uvifera* stem extract. Whereas, *M. elengi* leaves extract has been reported to have potential anti-helminthic (Jana *et al.*, 2010), analgesic (Karmakar *et al.*, 2011), anti-tyrosinase (Narayanaswamy *et al.*, 2011), anti-atherosclerotic (Satishchandra & Sumithra, 2011), anti-inflammatory (Khatri *et al.*, 2014), anti-hyperglycemic and anti-hyperlipidemic activity (Zahid *et al.*, 2012). *S. aqueum* leaves extract was reported to possess anti-inflammatory, anti-nociceptive, hepatoprotective activity (Osman *et al.*, 2009; Sobeh *et al.*, 2018), anti-diabetic (Palanisamy & Manaharan, 2015), anti-tyrosinase, lipolytic and anti-cellulite activity (Palanisamy *et al.*, 2011).

The performance of the subject in behavioural task relies on motor function. AOFT was conducted to determine if POBCCA surgery or treatment of respective plant extracts affects the motor function of the rats. A time-course study done by Damodaran *et al*. (2014) showed POBCCA procedure did not impair motor function over 4 weeks after surgery. The results obtained in this study were similar to the previous finding. POBCCA did not impair the motor function and exploratory behaviour of rats. Similarly, treatment of *C. uvifera*, *M. elengi* and *S. aqueum* extracts did not affect motor function and exploratory behaviour of rats. The locomotor activity, total velocity and total distance travelled of POBCCA rats treated with these plant extracts were similar to Sham-operated rats. This indicates that POBCCA and selected plant extract

treatment did not influence the motor function of rats during the behavioural task. Therefore the performance of the rats in cognitive tasks was solely dependent on the learning and memory capacity of the rats.

In mammals, non-spatial and spatial memories formation relies upon the hippocampus and associated medial temporal lobe (MTL) structures (Cohen *et al.,* 2013). NOR test was used to evaluate non-spatial memory in POBCCA rats. The novelty preference concept is based on rodent's natural preference for novelty and their ability to remember previously encountered objects, which are closely related to the conditions in which human recognition memory is evaluated. On the other hand, the MWM test was utilised to investigate spatial memory. This test relies on the ability of the rodent to remember the environmental cues and find a hidden platform to escape from the water. Spatial memory supports humans and other mobile animals to navigate objects and places in the environment. In the present study, the results obtained from behavioural tasks indicate that both the non-spatial (short- and long-term) and spatial memories function were impaired after CCH in rats. These observations are consistent with previous studies in which POBCCA rats demonstrated a deficit in non-spatial and spatial memories (Hu *et al.,* 2017; Sohn *et al.,* 2019; Yao *et al.,* 2019; Qu *et al.,* 2020). The hippocampus plays a vital role in encoding, and newly acquired information is temporarily stored as STM within the hippocampus (Macdonald *et al.,* 2013) before being transmitted to the cerebral cortex for long-term storage (Ivanco & Racine, 2000; Williams *et al.,* 2004; Mehta, 2018). CCH induced neuronal cell death in the hippocampus, and cerebral cortex have been pointed out to disrupt CNS network signalling (e.g., glutamatergic and cholinergic) and leads to learning and memory dysfunction in POBCCA rats (Saxena *et al.,* 2015; Ahad *et al.,* 2020).

Interestingly, treatment of *S. aqueum* extract significantly ameliorates short- and long-term recognition memory in POBBCA rats. In contrast, POBCCA rats treated with *C. uvifera* and *M. elengi* extracts showed no significant increase in recognition index during short- and long-term memory tests compared to POBCCA rats. POBCCA rats treated with *M. elengi* and *S. aqueum* extracts showed significant improvement in spatial learning during training. However, both plant extracts did not improve reference memory during the probe trial. Conversely, treatment of *C. uvifera* extract did not improve both the spatial and reference memories in POBCCA rats. Rats in all groups demonstrated similar mean swimming speeds during probe trials and escape latency time during visible platform tests. This indicates that the results obtained from the spatial training and reference memory during probe trials were not affected by any motor function during swimming, lack of motivation and vision impairment in POBCCA rats. The nootropic effect of *M. elengi* and *S. aqueum* extract may be due to the restoration of the cholinergic system via cholinesterase inhibition in CNS of POBCCA rats. However, further molecular studies should be performed to validate the anti-cholinesterase activity of these two plants. The AChE and BuChE activity in CNS of POBCCA rats should be determined using Elman's method along with quantification of acetylcholine level using liquid chromatography-mass spectrometry or enzyme-linked immunosorbent assay.

5.0 CONCLUSIONS

The current study investigates the anti-cholinesterase activity of *C. uvifera* stem, *M. elengi* leaves, and *S. aqueum* leaves extracts *in vitro* and their effect on the cognitive function of CCH-induced rats. All plant extracts exhibited good anti-cholinesterase activity *in vitro*. Leaves extracts of *M. elengi* and *S. aqueum* have improved learning and memory deficits in CCH rats by enhancing spatial learning and memory. Additionally, *S. aqueum* extract improves non-spatial short- and long-term recognition memory in CCH rats. However, *C. uvifera* extract did not improve learning and memory in CCH rats. Furthermore, oral administration of all extracts did not affect basic motor function and exploratory behaviour of CCH rats in the AOFT. The nootropic effect of *M. elengi* and *S. aqueum* leaves extract requires further molecular investigations to confirm the anti-cholinesterase activity of these plants. In conclusion, the outcome of this study provided scientific evidence to support the therapeutic potential of *M. elengi* and *S. aqueum* leaves in the treatment of VaD.

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