Anti-Acetylcholinesterase, Anti-Inflammatory and Anti-Oxidant Activities of Raw-Extract Centella Asiatica (RECA) on Lipopolysaccharide (LPS)-Induced Neuroinflammation Sprague Dawley Rats

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Abstract

The present study was designed to investigate the potential of raw-extract of Centella asiatica (RECA) in suppressing acetylcholinesterase (AChE), inflammations and oxidative stress via induction of lipopolysaccharide (LPS) on animal model of Sprague Dawley rats. Centella asiatica is a plant that has been used as a traditional herbal remedy for the management of several diseases, including memory improvement, treatment of mental fatigue and wound healing. Pre-treatment with RECA in vitro significantly reduced the AChE activity in a concentration-dependent manner with I50 value of 57.47 ± 13.55 µg/ml. Interestingly, this result was parallel with in vivo studies. Moreover, the level of pro-inflammatory cytokines and oxidative stress were significantly reduced by RECA in dose-dependent manner. Overall, our findings clearly dictate the potential of RECA as AChE inhibitor as well anti-inflammatory and anti-oxidant agents.

Keywords: Acetylcholinesterase inhibition; anti-inflammatory; anti-oxidant; lipopolysaccharide (LPS); raw-extract Centella asiatica (RECA).

1. Introduction

Alzheimer’s disease (AD) is a progressive, debilitating and heterogeneous neurological disorder, which is characterized clinically by memory loss, impaired cognitive functions and inappropriate behaviour that serious enough to impose severe strains on the social welfare systems. The etiopathogenesis of this disease is multifactorial and recent evidence demonstrates that the deficits in central cholinergic neurotransmission in the brain, inflammatory injury and induction of oxidative stress also interrelated on progression of AD [1, 2]. Primarily, it is believed that the memory impairment in patients with dementia occurs due to the excessive amount of acetylcholinesterase present in the brain. This enzyme when in excess, could further leads to decrease the concentration of neurotransmitter such as acetylcholine that will eventually result in deficit transmission of information in AD patients [3]. Consequently, most of the drugs in prevention of AD are working as acetylcholinesterase inhibitors such as tacrine, rivastigmine, donepezil and galantamine [4].

Inflammation is defined as part of complex biological response of normal host to foreign and harmful stimuli. However, it may potentially lead to neuronal dysfunction and eventually death due to prolong presence of inflammatory agents, namely, viruses, antigens, exotoxins and endotoxins that stimulate the activation of inflammatory cytokines [5], [6]. On the other hand, oxidative stress is one of the prime causes that can lead to neuronal apoptosis and promote inflammation due to the over expression and accumulation of reactive oxygen species (ROS). It is essentially reflected as a condition in which disequilibrium state occurs when the production of ROS is over capacity of internal defense mechanisms [7]-[9]. Recent studies emphasized that the interrelation of oxidative stress and chronic inflammation has been implicated in various chronic diseases, for instance, neurodegenerative diseases of the central nervous system [10], [11]. Neural cells are highly vulnerable to oxidative stress mediated neurodegeneration due to two critical aspects; high metabolism and restricted cell renewal [12], [13].

The development of strategies for reducing inflammation and oxidation level could open up new window into effective treatments for these diseases. By keeping this as a key factor, some herbal products containing biological active molecules could participate as a substitute for allopathic medicine [14]. Phytopharmaceuticals are gaining considerable public and scientific attention as modern and traditional system of medicine owing to their wide therapeutic potential, superior safety and consumer acceptability [15]. Various natural compounds, namely, triterpenes, lignans, flavonoids, and alkaloids have been reported to exert diverse beneficial pharmacological activities, such as anticholinesterase, anti-inflammatory, antioxidant and anti-amyloidogenic [16]. On top of that, some compounds, such as curcumin, melatonin,
green tea, vitamin C, and aged garlic extract yielded flourishing results when used in patients with AD [17]–[19]. 

**Centella asiatica** (*C. asiatica*) is a psychoactive medicinal plant belonging to the family of Apiaceae (Umbelliferae) which is locally known as pegaga in Malay. It is a type of herb that commonly eaten raw as a salad, especially among the Malay communities in Malaysia. Various uses are claimed for the plant, which has been used for centuries in Ayurvedic and Chinese traditional medicines for memory improvement, treatment of mental fatigue, wound healing, asthma and toxic fever [20]–[22]. Moreover, it also has been shown to possess cholingeric, antioxidiant, anti-inflammatory and anticancer activities [23]–[25]. The most prominent group of active compounds isolated from this plant is triterpenoid glycosides including asiatic acid, madecassic acid, asiaticoside and madecassoside; which are believed to be responsible for its wide therapeutic. Other minor saponins are centelloside, bramoside, braminoside and centelloasponines B, C and D [26], [27]. Nevertheless, its exact mechanism of action in the treatment and management of neurodisorders has not been clearly understood [28].

To this regard, this study is mainly devoted to finely evaluate the effect of *C. asiatica* extract designated as raw-extract Centella asiatica (RECA), in ameliorating the AChE, neuroinflammation and oxidative stress on lipopolysaccharides-induced Sprague Dawley rats’ model.

2. **Material and Method**

2.1. The Collection of Plant Material

Raw materials were obtained from Herbagus Trading, Pulau Pinang, Malaysia. Voucher specimen (CA-K017) was prepared and deposited in the Atta-Ur-Rahman Institute for Natural Product Discovery, UiTM Puncak Alam for future reference.

2.2. Preparation of Raw-Extract Centella asiatica (RECA)

The whole plant was washed, cleaned and oven dried at 40°C. A total of 50 kg powdered pegaga (*C. asiatica*) leaves was extracted in five batches. In each batch, 10 kg of pegaga powder was extracted with 95% denaturated ethanol (60 L ethanol + 40 L deionized water) for 8 hours at temperature 60°C. A total of 14.8 L of concentrated liquid extract was obtained and was subjected to freeze drying process to give a total of 7.96 kg of extract (15.92% yield). This extract was designated as raw-extract *Centella asiatica* (RECA).

2.3. Cell culture

Human neuroblastoma cells, SH-SY5Y, was obtained from the American Type Culture Collection (ATCC no. CRL-2266) and cultured in the mixture of Minimum Essential Medium (MEM) Eagle with Earle’s salt and F12-K medium in 1:1 ratio supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified incubator maintained at 37°C under 5% CO2/95% air. The medium was changed every 2 days and subculture was performed when cells reached 80% confluent. Cell numbers were assessed using a haemocytometer.

2.4. Cell Viability Determination Using MTT assay

The cells were seeded into 96-well plates with 10 × 10³ cells/well. For the study of dose-dependent effects of RECA toxicity, the cells were treated with various concentrations of medium supplemented with RECA (3.91–1000 μg/ml) and incubated for 24 and 48 h. A solution of the MTT reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, (5 mg/ml PBS) was added to SH-SY5Y cells in each well and incubated for 3 h. Resulting formazan was solubilized with 100 μl dimethyl sulfoxide (DMSO). The plate was shaken for 15 min and absorbance determined at 540 nm using a microplate reader.

2.5. Measurement of AChE Activity In vitro

Cells were plated at a density of 5×10⁴ cells per well in 6-well. SH-SYSY cells were induced to differentiate into neuronal cells by all-trans-retinoic acid (ATRA; 10 μM). Morphological changes were monitored as indicators of differentiation. After differentiation, culture media was removed and cells incubated with various concentration of RECA (3.91–1000 μg/ml) for next 24 h. Eserine was used as reference drug. Cells supernatants were aliquoted into 96-well microplates and assayed for acetylcholinesterase activity via QuantChrom™ Acetylcholinesterase Assay kit (BioAssay Systems, Hayward, CA) according to the manufacturer’s instructions.

2.6. Experimental Animals

Adult male Sprague-Dawley rats, weighing approximately 200–250 g were procured from Laboratory Animal Facility and Management Faculty of Pharmacy (LAFAM), UiTM Puncak Alam, Selangor. Rats were housed in Individually Ventilated Cages (IVC) cage and maintained under standard laboratory conditions that are automatically kept at 21–25°C and relatively humidity at 45-65 % with a controlled light-dark cycle. All rats had free access to standard laboratory food and tap water ad libitum. Rats were housed in groups and acclimatized for at least 7 days before using them for experiments. Experiments were carried out between 0800 h to 1800 h. The protocol was approved by the Committee on Animal Research and Ethics of the Universiti Teknologi MARA (UiTM) [Reference No: 600-FF (PS. 17/21); dated 28 August 2017]. The laboratory animals were handled and managed in accordance to the Guide for the Care and Use of Laboratory Animal (National Research Council 1996).

2.7. Vehicle

Rivastigmine (5 mg/kg) and LPS (serotype 055:B5, Sigma, St. Louis, MO, USA) were diluted in normal saline. RECA was suspended in normal saline to obtain concentration of 250, 300 and 350 mg/kg. RECA and rivastigmine were administered orally. LPS was injected intraperitoneally.

2.8. Drug Administration

The rats were randomized into 6 groups (n=6 rats per group) (Table 1.). Control and LPS groups were administered with normal saline while rivastigmine (5 mg/kg) acted as reference drug group. RECA was administered orally at the concentrations of 250, 300 and 350 mg/kg. After 10 days of RECA oral administration, except the control group, rats were injected with LPS (250 μg/kg) in order to introduce inflammation.

<table>
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<tr>
<th>Animal Group</th>
<th>Treatment</th>
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<tr>
<td>(i)</td>
<td>Vehicle for RECA</td>
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<td>(ii)</td>
<td>RECA (250 mg/kg, PO) + LPS (250 μg/kg, ip)</td>
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<td>RECA (300 mg/kg, PO) + LPS (250 μg/kg, ip)</td>
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<td>(v)</td>
<td>LPS (250 μg/kg, ip) + vehicle for RECA</td>
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<td>(vi)</td>
<td>Rivastigmine (5 mg/kg, PO) + vehicle for RECA</td>
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2.9. Collection of Brain Samples

The rats were sacrificed by cervical dislocation under light anesthesia. Immediately after decapitation, the whole brain was carefully removed from the skull, washed and homogenized in ice-
cule in viable cells. It was observed that cell viability increased by the presence of mitochondrial dehydrogenase activity of the resulting intracellular purple formazan formed from reduction of TNB (yellow colour formed) is directly proportional to DTNB was added into 50 µL samples in a 96-well plate. The production of TNB in a 96-well plate. The intensity of the colour change was measured at 412 nm of the 2nd and 10th min. The activity of this enzyme was determined by calculating the difference of absorbance between the 2nd and the 10th min in comparison to the standard.

2.10.3. Estimation of Reduced Glutathione (GSH) Level

GSH was estimated by using the Cayman’s GSH Assay (Cayman Chemical, Ann Arbor, MI) in accordance with the manufacturer’s protocol. Briefly, 150 µL freshly prepared assay cocktail containing MES buffer, cofactor mixture, enzyme mixture, water and DTNB was added into 50 µL samples in a 96-well plate. The production of TNB (yellow colour formed) is directly proportional to the concentration of GSH in sample. The absorbance was measured at 450 nm using microplate reader.

2.11. Statistical Analysis

For statistical analysis, each of the experimental values was compared with its corresponding control. Results were expressed as the mean ± standard error of the mean (SEM). Mean differences among groups were evaluated by One-Way Analysis of Variance (ANOVA) using the Graphpad Prism 6.0 software (SAS Institute, NC, USA). Post-hoc comparisons between groups were made using Tukey’s test. At least, at the level of p less than 0.05, the results were considered as statistically significant.

3. Results

3.1 Effect of RECA on Viability of SH-SY5Y Cells

The cytotoxic effect of RECA at concentration ranging from 3.91 – 1000 µg/mL was investigated on neuroblastoma cells (SH-SY5Y) by using MTT assay. Effect of RECA on SH-SY5Y cell line was evaluated after 24 h and 48 h (Fig. 1 (a) and (b)) of cell exposure to the extract. Percentage cell viability was calculated by measuring the resulting intracellular purple formazan formed from reduction of MTT solution by the presence of mitochondrial dehydrogenase in viable cells. It was observed that cell viability increased with the increase in concentrations of RECA. However, the cell viability was slightly lowered at 48 h as compared to 24 h of exposure. RECA at the highest concentration of 1000 µg/mL showed maximal cell viability at both hours of exposure (**p ≤ 0.001 and ***p ≤ 0.0001). Since the concentration of RECA up to 1000 µg/mL did not exhibit cytotoxicity against SH-SY5Y cell line, thus these concentrations were further used for measuring the AChE activity in vitro.

Fig. 1: Cytotoxicity effect of RECA against SH-SY5Y cell line after 24 h (a) and 48 h (b) of exposure using MTT assay at the concentration ranging from 3.91 to 1000 µg/mL. Results are expressed as the mean ± SEM in triplicate. **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001 compared to negative control (untreated cells) denoted as 100%.

3.2 Effect of RECA on AChE Activity In Vitro

To evaluate the inhibitory effect on AChE activity in vitro, differentiated human neuroblastoma cells (SH-SY5Y) were used in this study. The differentiated cells were exposed to RECA at concentration ranging from 3.91 to 1000 µg/mL for 24 h. Enzyme activity was estimated by Ellman’s method and expressed as one unit of enzyme catalyzes the production of 1 µmole of thiocholine per minute under the assay conditions (pH 7.5 and room temperature). Eserine was used as the standard AChE inhibitor in this study. Based on Fig. 2, differentiated SH-SY5Y cells (RA) showed a significant increased (**p < 0.01) in AChE activity as compared to undifferentiated cells. However, AChE activity in differentiated cells showed significant decrease in a concentration dependent manner when the cells were treated with RECA for 24 h. Interestingly, RECA at the highest concentration (1000 µg/mL) showed significant maximal inhibition (****p ≤ 0.0001) on AChE activity as compared to RA. The IC_{50} values for RECA and eserine were 57.47 ± 13.55 µg/mL and 0.02 ± 0.003 µg/mL, respectively.

Fig. 2: Effect of RECA on acetylcholinesterase activity after 24 hours of exposure at the concentration ranging from 3.91 to 1000 µg/mL. Results are expressed as the mean ± SEM in triplicate, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001 compared to differentiated cells (RA), whereas ***p ≤ 0.01 as compared to untreated cells (NC).
3.3 Effect of RECA on AChE Activity in LPS-induced Rats Model

The effect of increasing concentration of RECA on AChE activity was further evaluated by using LPS-induced Sprague Dawley rats model. Based on Fig. 3, the LPS-induced group showed elevation in AChE activity as compared to control. Treatment of rats with RECA prior to LPS injection could ameliorate LPS-induced enhancement in AChE activity, in dose dependent manner. Rivastigmine group on the other hand, showed reduction in AChE activity to 3309.24 U/L from 5661.30 U/L in the LPS group.

Fig. 3: Effect of RECA on brain acetylcholinesterase activity in LPS-induced rats' model. Results are expressed as the mean ± SEM in triplicate.

3.4 Effect of RECA on TNF-α and PGE2 Expression

The effect of RECA on pro-inflammatory cytokines production is presented in Fig. 4 (a) and (b), TNF-α (916.63 pg/ml) and PGE2 (27.42 pg/ml) were present in control group. However, the basal level of TNF-α and PGE2 was markedly increased (**** p ≤ 0.0001) to 1085.98 pg/ml and 219.28 pg/ml, respectively, by the group induced with LPS alone as compared to the respective control group. Pre-treatment with RECA significantly declined subsequent TNF-α and PGE2 productions in dose dependent manner, as compared to LPS-induced group. The effect of RECA at the dosage of 350 mg/kg was comparable with that of the standard drug, rivastigmine, in ameliorating the TNF-α level.

Fig. 4: Effect of RECA on pro-inflammatory cytokines production. LPS stimulation significantly increased the levels of TNF-α (a); and PGE2 (b), Treatment with RECA decreased the levels of: TNF-α (a); and PGE2 (b) in a concentration dependent manner. Results are expressed as the mean ± SEM in triplicate. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 and **** p ≤ 0.0001 vs. LPS; **** p ≤ 0.0001 vs. Control.

3.5 Effect of RECA on reduced glutathione (GSH) level

As shown in Fig. 5, GSH level was decreased in the group that was induced with LPS alone. However, pretreatment with RECA showed significant increased level of GSH at the dosage of 300 mg/kg (p ≤ 0.05) and 350 mg/kg (**** p ≤ 0.0001) as compared to LPS-induced group. Lower dose of RECA had no significant effect on GSH level in LPS injected rats group. Meanwhile, the reference drug, rivastigmine, significantly increased (** p ≤ 0.01 and **** p ≤ 0.0001) the production of GSH as compared to LPS-induced and control groups.

Fig. 5: Effect of RECA on GSH production. LPS stimulation reduced the level of GSH. Treatment with RECA increased the level of GSH in a concentration dependent manner. Values are in mean ± SEM in triplicate. * p ≤ 0.05, ** p ≤ 0.01, and **** p ≤ 0.0001 vs. LPS; *** p ≤ 0.01 and **** p ≤ 0.0001 vs. Control.

4. Discussion

The aim of the study at hand is to investigate the dual modes of action of RECA in ameliorating the AChE activity and its possible association in suppressing the elevated expression of pro-inflammatory cytokines and oxidative stress. AD is a chronic neurodegenerative disease that affects mostly among the elderly. Up-regulation of AChE activity during the progression of AD will further lead to degradation on proper level of acetylcholine (ACh) and cholinergic transmission [4], [30]. As such, inhibition of AChE has been a prospective target against several neurological disorders including AD and used for prevention strategies. An extensive research attention has inclined toward phytochemicals as promising therapeutic candidates, consequent to various adverse events cause by the synthetic drugs [4], [31].

Generally, *C. asiatica* or locally known as pegaga in Malaysia, is a traditional medicinal herb that belongs to the Umbelliferae (Apiceae) family. It has been extensively utilized since ancient times especially in Ayurvedic and traditional Chinese medicines as a remedy to treat multiple ailments [32], [33]. The versatility and efficacy of this plant are owing to its primary active constituents apart from prominent amount in flavonoids and terpenoids [34]. As a representative cell model to evaluate the inhibitory effect on AChE activity in *vitro*, differentiation of the human neuroblastoma cell line, SH-SY5Y, by the all-trans retinoic acid (ATRA) was used in this study. The induction of 10 μM RA towards SH-SY5Y cells was successfully differentiated the cells into neuronal type cells. In order to produce more neuron-like properties and maximize basal AChE activity, SH-SY5Y cells are often induced to differentiate by RA [35]. Morphological changes of the cells showed an extensive elongation of neurites as compared to undifferentiated cells. Pre-treatment of differentiated cells with RECA at various concentrations ranging from 3.91 μg/ml up to 1000 μg/ml for 24 h had significantly suppressed the elevated AChE activity by the RA-differentiated cells. Based on the results obtained, the IC50 values for RECA and the reference drug, eserine, were 57.47 ± 13.55 μg/ml and 0.02 ± 0.003 μg/ml, respectively. According to *in vitro* study that was conducted by Orhan et al., it was found that *C. asiatica* extract was capable to inhibit AChE with 50% of inhibition rate at 150 μg/ml concentration [36]. Meanwhile, in a similar study performed by the same researchers, standardized extract which containing 10.78% of total asiaticoside and madecassoside showed approximately 49% inhibition of AChE activity at concentration 200 μg/ml [21]. We then
decided to investigate the effect of RECA in attenuating the AChE activity observed in vitro can occur in vivo. Interestingly, this activity was parallel as in LPS-induced rats' model, as RECA showed moderate inhibitory activity of AChE in dose-dependent manner. This current result is in agreement with the research conducted by Kumar et al. who demonstrated that administration of an aqueous extract of C. asiatica improved performance in the Morris Water Maze and the elevated plus maze in the mouse D-galactose model of brain aging. This improvement was resultant from the normalization of acetylcholinesterase activity in the brains of treated animals [37]. Moreover, a study conducted by Rahman et al. demonstrated that ethanolic extract of C. asiatica could enhance the cholinergic transmission by blocking both AChE and butyrylcholinesterase (BChE) activities [38].

Based on review by Ahmed et al., alkaloids from plants have been discovered as AChE and BChE inhibitors, followed by terpenes, sterols, flavonoids, and glycosides [39]. However, few animal studies have showed that one of the pentacyclic triterpenes in C. asiatica which is asiatic acid, was found to play prominent role in enhancing the cognitive and memory functions [40], [41].

The up-regulation of neuroinflammation and oxidative stress levels have been implicated to play a critical role in initiation and development of several events in the pathological cascade of AD [42], [43]. In our study, the efficacy of RECA in suppressing the neuroinflammation and oxidative stress was evaluated via LPS-induced Sprague Dawley rats. LPS, a pro-inflammatory bacterial mimenic made from the cell wall of gram-negative bacteria was injected to promote neuroinflammation in the rats' model. Results obtained showed that the group that was induced with LPS alone significantly elevated the pro-inflammatory cytokines, TNF-α and PGE2, as compared to control group. However, the rats that were orally fed with RECA at dosage 250, 300 and 150 mg/kg, showed significant reversed effect towards the production of both cytokines in a dose-dependent manner. A study done by Somchit et al. reported that C. asiatica showed moderate anti-inflammatory property on prostaglandin E1-induced inflammation in a dose-dependent manner, at concentration of 2 mg/kg [44]. This finding was further supported by the in vivo study done by Huang et al., which showed that asiatic acid at concentration of 1.5 and 10 mg/kg capable to reduce the production of inflammatory cytokines on serum level [45]. Therefore, in the present study, the presence of asiatic acid in RECA could possibly reverse the production of cytokines.

Several studies have reported that LPS administration ameliorates antioxidant enzymes activity and regulates the oxidative stress level [46], [47]. The result from the present study showed the decreased in level of reduced glutathione (GSH) in the group that was induced with LPS alone. However, it was observed that RECA used in treatment groups significantly up-regulated the level of GSH. Moreover, it was noted that there was a significant increase in GSH level at the dosage of 350 mg/kg. Xu et al. had reported that treatment with asiaticoside for 7 and 14 days on an MPTP-induced rat model resulted in attenuation of oxidative stress level with increased concentration of GSH. Similar results were also obtained by Xu et al. when tested with another terpenoid of C. asiatica, madecassoside [48], [49]. These findings suggest that higher anti-inflammatory and antioxidant activities exerted by RECA could be due to the presence of the abundant terpenoids, polyphenol, flavonoid, β-carotene, tannin, Vitamin C, and DPPH compounds found in this plant [50].

Despite of vast strategies in delaying the progression of AD, the use of cholinesterase inhibitors (ChEIs) has become the primary therapeutic strategy to date. The availability of ACh at the synaptic level is crucial as ACh is not only plays a pivotal role as neurotransmitter and neuromodulator in the brain, in fact, it is also essentially involves in regulating the immune response through down-regulates the production of inflammatory cytokines that may contribute to the immunopathology associated with AD [51]–[53]. Various studies had documented the anti-inflammatory effects of several AChEIs. In vitro studies done by Eozolin and co-workers showed that PM777, a tetrahydrofuran derivative, which designed as a dual PAF and AChEI suppressed the level of NO, TNFα and ROS in LPS stimulated RAW 264.7 macrophages models [54]. These findings are in accord with study done by Nizri et al. In that report, it is mentioned that AChEIs suppressed lymphocyte proliferation and pro-inflammatory cytokine production, as well as extracellular esterase activity [55]. Other authors demonstrated that physostigmine and neostigmine could decreased the pro- and anti-inflammatory cytokine productions in the cortex and hippocampus of a surgery stress in vivo model. Findings from this work showed a clear correlation between the central nervous system (CNS) and immune systems, and propose a connection between IL-1 β, AChE activity and dysfunction in that cortex and hippocampus [56]. In the light of these observations, it highlights the pivotal role of AChEIs in “cholinergic anti-inflammatory pathway” that could ameliorate the cognitive function decline through down-regulation of inflammation.

5. Conclusion

In summary, the results of the present study demonstrated that RECA exhibited the capability to suppress the AChE, neuroinflammations and oxidative stress level in LPS-induced inflammation rats' model. Reduction in AChE activity with these inflammatory cytokines after RECA administration supports the impression that C. asiatica has a possible relationship between neuroinflammation and cholinergic system in the treatment of neurodegenerative diseases. However, further investigations are required to bring this observation into evidence, as the literature on such synergistic effect particularly for C. asiatica is far too limited to date.

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