Evaluation of neuroprotective potential of methanolic extract of *Hemidesmus indicus* extract in Aβ (1-42) induced rats

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ABSTRACT

Excessive cerebral deposition of amyloid-beta (Aβ) peptides with 40-42 amino acids are the major neuropathologic feature of Alzheimer's Disease (AD), accompanied by a progressive and functional decline in cognition. With the failing attempts in the development of new pharmacological intervention and due to suboptimal results from the existing therapies available for the treatment of AD, there is a constant hunt for a new therapeutic alternative to address this severe neurodegenerative disease. The present study aimed to investigate the neuroprotective effect of *Hemidesmus indicus* extract in Aβ (1-42) infused model of AD. Sporadic model of AD was achieved by intracerebroventricular (i.c.v) injection of Aβ (1-42) peptide in Wistar rats, followed by treatment with methanolic extract of *H. indicus* (MEHI) at 100 and 200 mg/kg for 28 days. Locomotor activity, Radial arm Maze task and Passive avoidance test were used for the assessment of neurobehavioral deficits. After completion of 28 days treatment protocol, animals were euthanized and brains were collected for neurochemical analysis. Reversal of cognitive impairment was observed by MEHI on Aβ (1-42) rats, as evidenced by improved spatial memory learning. Furthermore, MEHI attenuated Aβ (1-42) induced oxidative stress and inhibited acetylcholine esterase (AChE) activity. Collectively, these findings exhibited neuroprotective activity of MEHI by ameliorating Aβ (1-42) mediated neuronal damage, thereby can stand as a potential disease-modifying therapeutic for curbing AD pathology.

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INTRODUCTION

Alzheimer's Disease (AD) is considered to be common, fatal and progressive degenerative brain disease with complexed underlying pathology. Common characteristics include memory loss, confusion, difficulty in decision-making, and behavioural changes (Chapman *et al.*, 2018). Complexity and uncertainty in the pathogenesis of AD have made the establishment of anti-AD drugs more challenging. However, multiple hypothesis regarding the AD pathophysiology were suggested in the past decades, where the appearance of senile

plaques and intraneuronal neurofibrillary tangles were found at molecular levels (Souza *et al.*, 2016). Other proposed hypothesis includes oxidative stress and cholinergic hypothesis (Lian *et al.*, 2017).

Recently scientist has shown their keen interest in designing drug candidates which might perform on multiple targets. Although several clinical trials were previously conducted in search of novel therapeutics, all failed as they were found to be less efficacious (Rahman *et al.*, 2019). Thereby, disease-modifying treatment for AD is much needed which can efficiently delay neuropathological manifestations and can produce an improvement in clinical signs and symptoms.

Acetylcholine (ACh) level restoration by inhibiting acetylcholine esterase (AChE) enzyme is the worldwide accepted present-day remedy. It was earlier reported that in addition to improving ACh levels, AChE inhibitors found to alleviate the cognitive impairment in AD patient by delaying the formation of Aβ plaque and thus act as a disease-modifying agent (Uddin *et al.*, 2020). Hence there is increasing demand to screen more of extracts or compounds from the herbal origin for AChE inhibition property which can act as a potential molecule for the treatment of AD in future.

With increasing evidence of a wider range of safety and fewer side effects, an herbal or alternative system of medicines has gained its popularity over the decades which encouraged the development of the therapeutics from the natural sources (Wu *et al.*, 2019; Briffa *et al.*, 2017). Notably, in search of effective disease-modifying treatment for AD, a surge of demand was observed to identify traditional herbal preparations or plant-derived small molecules with promising neuroprotective activities (Cragg and Newman, 2013). *Hemidesmus indicus,* belonging to the family Apocynaceae, commonly known as Anantmool or Sariva, is indigenous plant of India and is a familiar drug in the Ayurveda. Known for numerous health profitable activities, *H. indicus* has been blessed with anti-asthmatic, antibacterial, anti-hyperglycemic, anti-atherogenic, antinoniceptive, anti-spasmodic, antipyretic, anti-hepatotoxic, antidiarrhoeal, immunopotentiating, anti-epileptic (Nadkarni, 1976), anti-oxidant, anti-thrombotic (Mary *et al.*, 2003), anti-inflammatory, antiulcerogenic (Anoop and Jegadeesan, 2003) and antidotes (Alam and Gomes, 1998) properties. Moreover, it was also reported to be used for the management of leucorrhoea, blood disorders and bronchitis (Nadkarni, 1976). From ancient times, *H. indicus* has been used in more than fifty formulations such as Saribadyasavam, Aravindasavam,

Dhanwantharam thailam, Asokaghirtam, Narayana thailam etc. However, studies that can define the role of *H. indicus* on neuroprotection is very limited and hence required to be explored (Sivarajan and Balachandran, 1994).

Based on the aforementioned medicinal importance, the current study is designed to monitor the ability of methanolic extracts of *H. indicus* (MEHI) roots to reverse the neurogenesis-induced damage by evaluating its antioxidant and AChE inhibitory potential in Aβ (1–42) infused rat model of AD.

MATERIALS AND METHODS

Drugs and chemicals

Aβ (1–42) peptides were obtained from Sigma-Aldrich, USA. All chemicals and reagents purchased were of analytical grade only.

Animal procurement

250-300 g of adult male Wistar rats, aged three months, were provided by JSS College of Pharmacy, Ooty, Tamilnadu, India. Animals were housed six per cage and were kept in polypropylene cages in a climate-controlled laboratory condition (22-24°C; 45–65% humidity) with free access to sufficient pellet diet and water *ad libitum*. All protocols were approved by the Institutional Animal Ethics Committee (IAEC) (JSSCP/IAEC/PhD/Pharmacy Practice /01/2018-19) and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council (US), 2011).

Preparation of Plant extraction

Dried roots of *H. indicus* was acquired from local markets of Nilgiris district, India and was authenticated by Dr S. Rajan, field botanist, Central Council for Research in Homeopathy, Department of AYUSH, The Nilgiris, Tamilnadu, India. The dried roots were powdered and were soaked in a conical flask with 90% methanol for two days, followed by filtration with Whatman filter paper. Later on, the extract was concentrated under high pressure and the residue was lyophilized, yielding MEHI which was further protected from light and stored at 4°C for further use.

Surgical procedure

Surgical procedures were conducted by using stereotaxic apparatus where all the animals were weighed and anesthetized with ketamine hydrochloride (91 mg/kg, i.p.) and xylazine (9.1 mg/kg, i.p.) and fixed into the apparatus. 2 μl of aCSF containing 10 μg of Aβ (1–42) was subsequently injected into the burr hole above

the bilateral hippocampal coordinates (anterior-posterior (AP) = −3.5 mm, medial-lateral (ML) = ± 2.0 mm from the bregma and dorsal-ventral (DV) = 2.7 mm from the skull surface) after midline sagittal incision (Rahman *et al.*, 2019).

Experimental protocol

All animals were randomly allocated into four groups (n=6): Sham-operated (SO) group, Aβ (1–42) infused group, Aβ (1–42) + MEHI (100 mg/kg), Aβ (1–42) + MEHI (200 mg/kg) group. SO group was infused with two μl of aCSF bilaterally while animals in all other groups received bilateral i.c.v. Injection of Aβ (1–42) peptides dissolved in aCSF in a concentration of 10 μg/2μl. After five days from Aβ (1–42) peptides infusion, animals from treatment groups were orally treated with 100 mg/kg/d and 200 mg/kg/d of MEHI for a period of 28 days (Figure 1).

Behavioural, cognitive assessments

With the help of actophotometer, animals were evaluated for their locomotor behaviour by analysing their basal activity score (Reddy and Kulkarni, 1998). Additionally, radial arm maze (RAM) and passive avoidance test were conducted in order to assess learning and memory trails in rats by measuring reference and working memory errors and transfer latency time respectively (Sehgal *et al.,* 2012; Barkur and Bairy, 2015).

Brain homogenate preparation

After completion of 28 days treatment protocol, all the animals were euthanized, decapitated and the brains were extracted for biochemical assessment. After centrifugation of homogenates at 1000 rpm at 4°C for 3 min, the supernatant was separated and was stored until further use.

Biochemical assessment

Determination of hippocampal AChE level

In order to assess AChE activity in rat hippocampus, Ellman's method was used (Ellman *et al.*, 1961). Absorbance of the assay mixture consisted of 0.05 ml of supernatant, 3 ml of 0.01M sodium phosphate buffer (pH 8), 0.1 ml of 0.2 mM 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent), and 0.1 ml of acetylthiocholine iodide was measured at 412 nm. The enzyme activity was calculated using an extinction coefficient of $13.6 \text{mM}^{-1} \text{cm}^{-1}$ and was expressed in micromoles of acetylthiocholine iodide hydrolysed per min per g of protein.

Determination of hippocampal antioxidant

Estimation of superoxide dismutase (SOD)

SOD activity in brain regions was assayed by the method suggested by Kakkar *et al.* (1984). Reaction mixture (1.2 ml of 0.052 M sodium pyrophosphate buffer (pH 8.3), 0.1 ml of 186 μM phenazonium methosulphate, 0.3 ml of 300 μM nitroblue tetrazolium, 0.1 ml brain homogenate, 0.2 ml of 780 μM NADH) was incubated for 90 sec at 30°C followed by addition of 0.1ml of glacial acetic acid to stop the reaction. To this mixture, 4.0 ml of n-butanol was added and centrifuged at 4000 rpm for 10 min. The absorbance of the organic layer was read at 560 nm.

Estimation of catalase (CAT) activity

CAT activity was measured using a spectrophotometric procedure according to the method of Aebi (1984). 0.1 ml of brain homogenate and 1.9 ml of 50 mM phosphate buffer was mixed and incubated at 25 ºC for 30 min. To initiate the reaction, 1.0 ml of 30 mM H_2O_2 was added to the mixture and absorption was taken for 3 min at 240 nm at an interval of 30 sec.

Estimation of reduced glutathione (GSH) activity

Ellman method was used to estimate GSH level in the brain (Ellman, 1959). The equal quantity of brain homogenate and 10 % trichloroacetic acid were mixed and centrifuged at 10000 rpm for 15 min. 0.5 ml of supernatant was then separated and to this 1.5 ml of 0.2 M tris buffer (pH 8.2), and 0.1 ml of 0.01 M DTNB was added and made up to the volume of 5 ml with 2 % SDS solution. The absorbance of samples was noted at 412 nm.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 8.0.2 (263) software. All data were expressed as mean ± standard deviation (SD). Two-way analysis of variance (ANOVA) was performed to analyse group difference in the behavioural cognitive assessment. All other parameters were measured using one-way ANOVA followed by Bonferroni's post-hoc test. Values were considered to be significant when $P < 0.05$.

RESULTS AND DISCUSSION

Multifactorial pathogenesis of AD leads to the discoveries of the plant-based molecules which may act as multitargeted drug and could be an efficient therapeutics with a wider range of safety (Upadhyay *et al.*, 2018). Although several plants are being used traditionally since ancient times for treating CNS disorders due to lack of documentation, there is little evidence of their health beneficial properties. *Hemidesmus indicus*, Indian sarsaparilla, is used in Ayurveda as medicine for memory enhancing property since antediluvian times. In the current study, we have validated the neuroprotective effects of MEHI on Aβ (1–42) peptide-induced oxidative
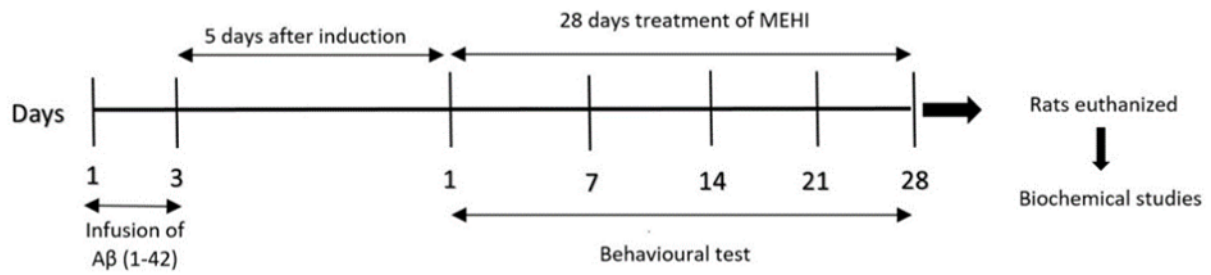
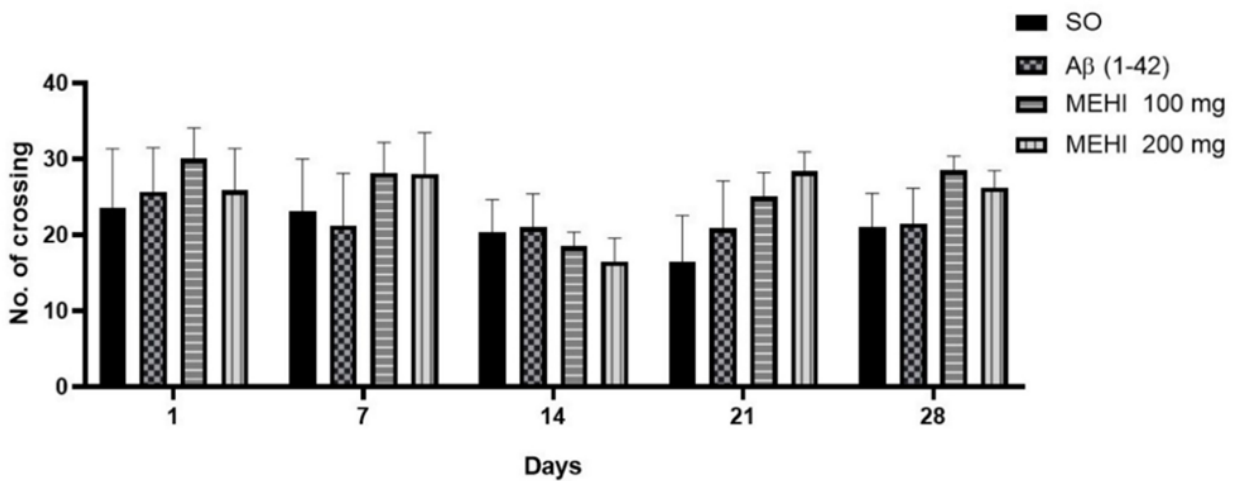**Figure 1: Diagrammatic Scheme of an experimental procedure**

Figure 2: Effect of MEHI on locomotor activity in Aβ(1-42) infused rats. The values were expressed as mean $\pm$ SD (n=6)
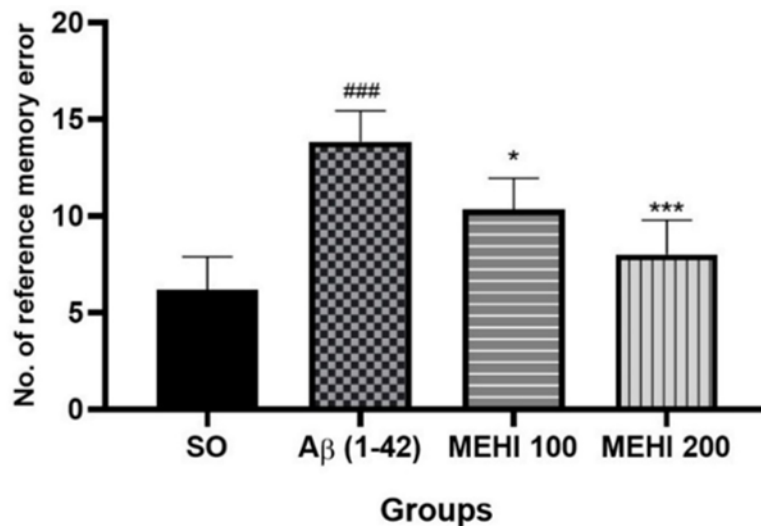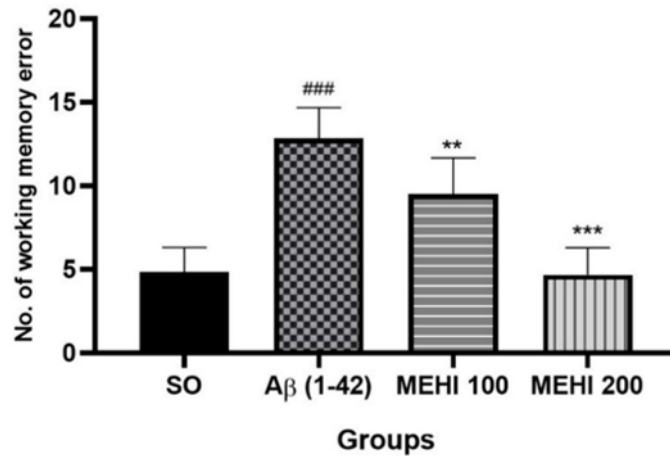

Figure 3: Effect of MEHI on reference memory errors in Aβ (1-42) infused rats. The values were expressed as mean $\pm$ SD (n=6). $^{###}P < 0.001$ vs. SO, $^*P < 0.05$ and $^{***}P < 0.001$ vs. Aβ (1-42) group

Table 1: Effect of MEHI on hippocampal SOD, CAT and GSH level in Aβ (1–42) peptides infused rats.

| Groups	GSH (mM/gm wet tissue)	CAT (μmol/min/g protein)	SOD (units/ min/mg protein)
SO	42.2$\pm$1.45	219$\pm$22.3	3.08$\pm$0.074
Aβ (1–42)	16.5$\pm$1.19###	107$\pm$15.4###	0.0849$\pm$0.391###
Aβ(1–42)+MEHI 100	21.4$\pm$0.62**	169$\pm$24***	1.39$\pm$0.343**
Aβ(1–42)+MEHI 200	31.4$\pm$3.44***	185$\pm$21.9***	2.30$\pm$0.230***

The values were expressed as mean $\pm$ SD (n=6). ###$p < 0.001$ vs. SO, **$p < 0.01$ ***$p < 0.001$ vs. Aβ (1-42) group

Figure 4: Effect of MEHI on working memory errors in Aβ (1-42) infused rats. The values were expressed as mean $\pm$ SD (n=6). ###$P < 0.001$ vs. SO, **$P < 0.01$ and ***$P < 0.001$ vs. Aβ (1-42) group
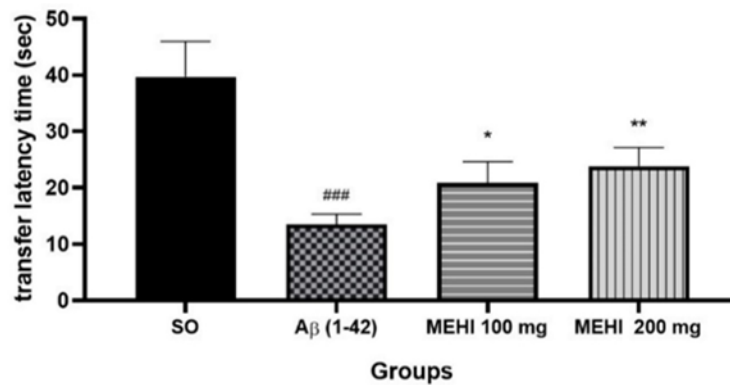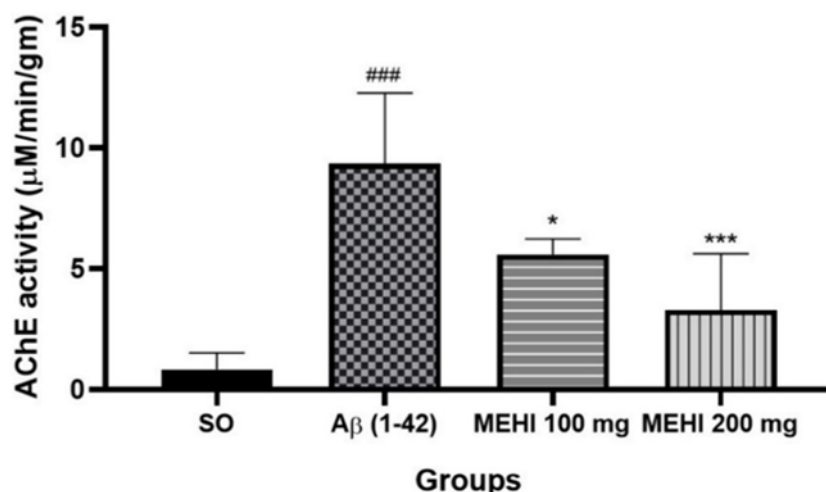

Figure 5: Effect of MEHI on acquisition trial in Aβ (1-42) infused rats. The values were expressed as mean $\pm$ SD (n=6). ###$P < 0.001$ vs. SO, *$P < 0.05$ and **$P < 0.01$ vs. Aβ (1-42) group
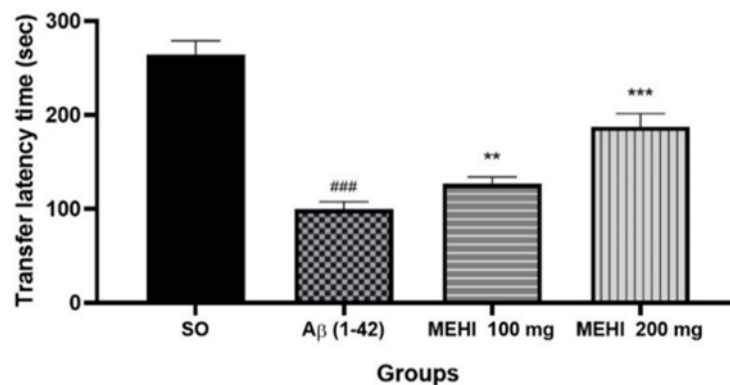
Figure 6: Effect of MEHI onretention trial in Aβ (1-42) infused rats. The values were expressed as mean $\pm$SD (n=6). ###$P < 0.001$vs. SO, **$P < 0.01$ and ***$P < 0.001$ vs. Aβ (1-42) group

Figure 7: Effect of MEHI on hippocampal AChE activity in Aβ(1-42) infused rats. The values were expressed as mean $\pm$ SD (n=6). [###]P < 0.001 vs. SO, *P < 0.05, ***P < 0.001 vs. Aβ (1-42) group

stress and cholinergic dysfunction in Alzheimer's rats.

In our study, we employed Aβ (1-42) infused rat model for the assessment of neurobehavioural deficits in rats by choosing locomotor, RAM and passive avoidance task. Infusion of Aβ (1–42) peptides in the brain successfully deteriorated memory and learning ability in the rats by exhibiting a lower number of correct choices and a higher number of reference ($P < 0.001$) and working ($P < 0.001$) memory errors and also affected passive avoidance task with a significant decrease in transfer latency time ($P < 0.001$) in both acquisition and retention trial when compared to that of SO group. Treatment with both the doses of MEHI showed a significant increase in incorrect choices. However, MEHI 200 mg/kg displayed more effective results with a fewer number of working memory ($P < 0.001$) and reference memory ($P < 0.001$) errors than MEHI 100 mg/kg on comparison with the Aβ (1-42) infused group (Figure 3, Figure 4). Similarly, MEHI 200 mg/kg improved latency time in both trials (acquisition trail ($P = 0.002$) and retention trail ($P < 0.001$)) with better significance than 100 mg/kg (acquisition (P=0.037) and retention trial (P=0.004)) when compared with Aβ (1-42) group. Thus, we can conclude from these observations that MEHI is capable of reversing the memory impairment in rats (Figure 5, Figure 6). Also, none of the doses produced any effect on locomotor activity in rats, indicating the absence of the ruinous effect of MEHI on motor functions of rats (Figure 2). This result is in accordance with the previous report by Shete and Bodhankar (2009) which revealed the absence of deleterious effect of HI on motor activity. Collectively, MEHI demonstrated improvement in learning and memory functions in Aβ (1–42) infused rats.

Restoration of ACh level in the cortex, including hippocampus regions, can improve the cognitive functions in AD patients by inhibition of major form of cholinesterase enzymes, i.e. AChE. Thus, AChE inhibitors have been a popular target for researchers in an effort to discover new AD drugs (Holden and Kelly, 2002). However, side effects associated with AChE inhibitors encouraged scientific investigation for the identification of potent AChE inhibitors from plant source (Mehta et al., 2012). From our explorative study, we found enhanced AChE activity in the hippocampal tissues of Aβ (1-42) group ($P < 0.001$) when compared to the SO group. MEHI enhanced nootropic effect by reducing the cholinergic deficits produced by Aβ (1-42) infusion in the rat brain. Both 100 mg/kg ($P = 0.017$) and 200 mg/kg ($P < 0.001$) of MEHI showed significant decrease in AChE activity on comparison with the Aβ (1-42) infused group (Figure 7). Hence, we can convey that methanol extract of *H. indicus* has considerable AChE inhibitory potential against Aβ (1–42) infused AD model and dose-dependently inhibited the intracellular AChE activity in the rat brain. Our results are inconsistent with the previous findings by researchers, which reported that different extracts of *H. indicus* have successfully inhibited AChE activity (Kundu and Mitra, 2013; Penumala et al., 2018).

Free radicle generation is a very evident feature in AD pathogenesis. These are responsible for the oxidation of cell lipids and DNA damage and creates a neurotoxic environment for plaque accumulation and induces cell necrosis causing neuronal death, which is a major hallmark of AD (Feng and Wang, 2012). Oxidative stress thus generated impairs the functions of various antioxidant enzymes involved in the maintenance of ion homeostasis (Alzoubi

et al., 2013; Serrano and Klann, 2004). Significant decrease in the levels of antioxidant enzymes such as SOD (P <0.001), GSH (P < 0.001) and CAT (P < 0.001) confirms the oxidative damage in Aβ (1-42) infused group when compared to SO. At 100 mg/kg, MEHI significantly improved the antioxidant levels i.e. SOD (P = 0.022), GSH (P = 0.002) and CAT (P < 0.001), comparable with the Aβ (1–42) group. Nevertheless, MEHI at 200 mg/kg also counteracted this decline in the antioxidant enzymes significantly and elevated the levels of the SOD (P < 0.001), GSH (P < 0.001) and CAT (P < 0.001) to normal with more satisfactory results when compared with Aβ (1-42) infused rats (Table 1). This antioxidant potential of methanol extract is in correlation with its AChE activity, thereby, indicates that inhibition of radical generation might have a protective effect on cholinergic neurons. Presence of adequate quantity of terpenoids, flavonoids and phenolic compounds in the methanol extract as reported from earlier studies, could be a probable reason behind the neuroprotective properties of *H. indicus* (Ashalatha *et al.*, 2019).

CONCLUSION

In conclusion, our findings suggest, MEHI upon oral treatment with 100 mg/kg and 200 mg/kg doses, exerted a protective effect against Aβ (1-42) peptides induced memory impairment, oxidative stress and cholinergic hypofunction in a rat model. However, further investigation is needed to elucidate the impact of the MEHI on other neurochemicals and molecular pathways involved in AD pathology which can provide novel insight into the underlying mechanism of protection on age-related neurological disorders.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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