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ORIGINAL ARTICLE

Neurocognitive Investigation of *Morinda tinctoria* against Amyloid Beta-Induced Oxidative Insult and Cognitive Impairment in Albino Mice: A Phytotherapeutic Approach

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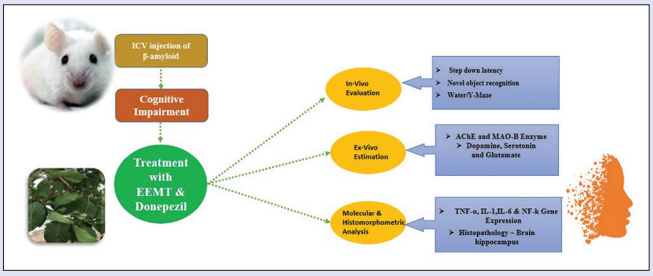
ABSTRACT

Background: Alzheimer's is a progressive neurodegenerative disorder characterized by cognitive declination associated with several clinicopathological changes. Due to consistent drawback in the conventional therapy, now the attentions toward herbal therapeutics have been increased in recent times. *Morinda tinctoria* Roxb (MT) is a novel medicinal herb reported with versatile therapeutic activity. Still now, there is no proper scientific data available to confirm its efficacy against neurodegeneration. **Objectives:** The objective of the present research is to investigate the neurocognitive enhancing potential of MT against amyloid beta (Aβ) (25–35)-induced memory dysfunction in mice. **Materials and Methods:** Animals were pretreated with ethanol extract of MT (EEMT) and standard donepezil followed by single intracerebro ventricular injection of Aβ25–35. Estimation of antioxidant enzymes, metabolic enzymes, and neurotransmitters was performed along with quantification of inflammatory cytokines using quantitative polymerase chain reaction method. **Results:** The outcome of the study clearly indicates that treatment with EEMT (200 and 400 mg/kg) and donepezil (5 mg/kg) had significantly ameliorated the cognitive stress induced by Aβ25–35, which was evident from the data obtained from memory and learning task. There was a sequential decrease in pro-inflammatory cytokines such as interleukin (IL)-6, tumor necrosis factor, IL-1, and nuclear factor-κ level in EEMT and donepezil treated groups. Histological finding of the samples belonging to EEMT and donepezil treated groups reveals the presence of dense network of pyramidal cell in CA layers with increased number of granular cells aligned on the dentate gyrus zone of the hippocampi. **Conclusion:** Neurotherapeutics from the herbal origin advocates the potential therapeutic efficacy against Alzheimer's and may become novel entity in the clinical management of neurodegenerative diseases.

Key words: Alzheimer's, amyloid beta, inflammation, *Morinda tinctoria*, neurocognitive, neurodegeneration

SUMMARY

- Alzheimer's is a complex disease interrelated with multiple molecular mechanisms involved in its pathogenesis.
- Usage of conventional drugs that act by single mechanism may not be therapeutically beneficial in treating Alzheimer's
- Supplementation of herbal antioxidants surely uplifts the level of these biologically active enzymes and shall act as a better therapeutic moiety in managing the stress induced neurodegeneration
- The herb *Morinda tinctoria* may have wider therapeutic opportunity and shall better considered as potential lead in the management of AD



Abbreviations used: MT: *Morinda tinctoria* Roxb; ICV: Intracerebro ventricular; EEMT: Ethanol extract of *Morinda tinctoria*; Aβ: Amyloid beta (25–35); IL-6: Interleukin 6; TNF-α: Tumor necrosis factor-α; IL-1: Interleukin-1; NF-κ: Nuclear factor-κ; PCR: Polymerase chain reaction; Q-PCR: Quantitative polymerase chain reaction; CA: Cornu amonis; DG: Dentate gyrus; AD: Alzheimer's disease; CD4: T lymphocytes; ACh: Acetylcholine; ACh: Acetylcholinesterase enzyme; FDA: Food and Drug Administration; CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals; IAEC: Institutional Animal Ethics Committee; NIS: National Institute of Siddha; AYUSH: Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homoeopathy; Govt: Government; SDL: Step-down Latency; STM: short-term memory; LTM: Long-term memory; ANOVA: One-way analysis of variance; SE: standard error; P: Probability; ROS: Reactive oxygen species; GR: Glutathione reductase; GPx: Glutathione peroxidase; CAT: Catalase; SOD: Superoxide dismutase; H_2O_2: Hydrogen peroxide; NADP: Nicotinamide adenine dinucleotide phosphate; TBA: Thiobarbituric acid; MAPK: Mitogen-activated protein kinases; CNS: Central nervous system; ACTB: β-actin.

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INTRODUCTION

Alzheimer's is a complex disease interrelated with multiple molecular mechanisms involved in its pathogenesis. Alzheimer's disease (AD) has become the most common cause of dementia throughout the globe. Around 47 million cases of dementia have been reported in the year 2015, and it is expected to be double by 2030.[1] Failure of

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metabolic protein clearance leads to deposition of beta-amyloid (Aβ) and hyperphosphorylated tau on the core functional areas of the brain, which propagates certain traumatic biochemical events that trigger the incidence of oxidative stress and inflammation.[2,3]

Activation of brain microglia, astrocytes, and CD4+T cell by the dense and diffused amyloid deposits kick starts the episodes of neuroinflammation.[4] Chronic inflammation plays a vital role in neurodegeneration, and the real factorial truth is neurons once degenerated are unable to rejuvenate under any circumstance.[5] Implication of oxidative stress on mitochondrial phosphorylation attributes to increased production of free radicals. These unstable radicals quench the electron-dense neural tissues, which results in apoptosis and cell death. Normally, brain manages this stressful condition by eliciting antioxidant enzyme cascade mechanism, but in the event of AD, there was a severe declination in the defensive scavenging enzymes which accelerates the level of lipid peroxidation and also fluctuates the neural signaling. Dysregulation in signal transduction further aggravates the pro-inflammatory responses and provokes the release of inflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1β, and IL-6.[6]

Brain controls the neurological rhythm of memory and learning through several neurotransmitters on which the most potential one is acetylcholine (ACh). ACh primarily mediates new memory formation, storage, and retrieval based on the inputs transmitted via hippocampi.[7] Acetylcholinesterase enzyme (AChE) plays a very crucial role in metabolizing acetylcholine; hyperactive AChEs around the amyloid plaques were observed and documented in earlier *in vitro* research investigations. AChE tends to form a stable complex with amyloid protein and alters the assembly of fibrils, which in turn precipitates its neurotoxicity.[8] Preclinical evidence have strongly correlates that Aβ promotes the expression of AChE, thereby depleting the level of ACh.[9] AChE inhibitors are the class of Food and Drug Administration-approved drugs (donepezil, rivastigmine, and galantamine), which are currently used for the clinical management of AD.[10] These drugs provide only symptomatic relief, whereas upon regular usage, they impart some potential adverse effects which include bradycardia, fainting, headache, loss of appetite, constipation, confusion, and dizziness.[11]

Usage of drugs that act by single mechanism may not be beneficial in managing AD, hence there is a potential need of alternative therapeutics preferably from herbal origin that can be effective in combating the symptoms of AD. The prominence on the use of medicinal plants had hitherto been placed on the treatment rather than prevention of diseases.[12] One such novel Indian medicinal herb is *M. tinctoria* Roxb, which belongs to the family of Rubiaceae. It was evident through literatures that this herb has numerous phytocomponents with versatile pharmacological activity. Reviews strongly suggested that the leaf and fruit extracts of MTR possess wound-healing activity.[13] Further, leaves of MTR possess significant beneficial activity such as febrifuge,[14] antimicrobial,[15] anticonvulsant,[16] Anti-ulcer,[17] anti-diabetic,[18] and antioxidant activities.[19] Still now, there is no proper scientific data available to confirm its efficacy against neurodegeneration. Hence, the main aim of the present investigation is to evaluate the neurocognitive potential of *M. tinctoria* against Aβ (25–35)-induced inflammation and oxidative stress in mice.

MATERIALS AND METHODS

Experimental animal

Healthy Swiss albino male mice weighing between 20 and 25 g obtained from laboratory animal house facility of C. L. Baid Metha College of Pharmacy, Chennai, Tamil Nadu, India, were used for the present study. The animals were maintained under standard recommended condition in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals guideline for care and management of lab animals. The animals were maintained at 25°C ± 2°C, 12 h light/dark cycle, and given standard pellet diet (Sai feeds, Bengaluru, Karnataka, India) and water provided *ad libitum*. The animals were acclimatized to the laboratory condition 2 weeks prior to the start of the study. All the animals were housed in polypropylene cages using sterilized paddy husk bedding. Principles of animal handling were strictly adhered to, and the handling of animals was made under the direct supervision of animal ethics committee of the institute. The experimental protocol was approved by the Institutional Animal Ethics Committee of C. L. Baid Metha College of Pharmacy, Chennai, Tamil Nadu, India.

Collection and authentication of plant material

The leaf part of the herb *M. tinctoria* Roxb (MT) known by its name Nunaa (syn) attracts potential therapeutic focus due to the presence of versatile bioactive components. Herb MT belonging to *Rubiaceae* family, were collected from Kancheepuram district of Tamil Nadu during December. The plant material was identified and pharmacognostically validated by an authorized botanist at the medicinal botany department, National Institute of Siddha, Department of Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homoeopathy (Government of India), Chennai 600047, India, under the Ministry of Health and Family Welfare, Government of India. The sample specimen of the plant material was archived in the School of Pharmacology and Toxicology, C.L. Baid Metha Pharmacy College, Chennai 600097, Tamil Nadu, India.

Extraction methodology

Harvested leaf part of the herb MT was thoroughly cleaned under tap water followed by surface sterilization to rule out possible plant pathogens. The sample was subjected to dry (shade dry) under optimal room temperature to preserve the nature and functionality of the phytocomponents. About 1000 g of the sufficiently dried leaf material of MT was pulverized to get coarse consistency followed by extraction using Soxhlet hot continuous extraction procedure. Extracts thus obtained were synonymous to the ethanol extract of *M. tinctoria* (EEMT) and were subjected to vacuum filtration to get the final yield of 24.2% w/v.

Cerebroventricular injection of fragment protein amyloid beta (25–35) and drug treatments

Cognitive impairment in mice was induced by intracerebroventricular (ICV) injection of protein fragment (Aβ 25–35) by rightly tracing the mid-bregma anatomical location on the skull. All the experimental animals were successively challenged with cerebral injection of amyloid protein fragment by using Hamilton microsyringe with the maximum volume of 10 µL.[20]

Experimental protocol

As per the experimental protocol, the animals were specifically allocated to five groups, which included one control and four treatment groups, with eight mice in each group:

- Group I: Animals injected with phosphate-buffered saline (10 µl), ICV
- Group II: Animals injected with Aβ peptide (10 µl) by ICV
- Group III: Pretreated animals injected with Aβ peptide (10 µl) by ICV on the 21st day and treatment continued with EEMT 200 mg/kg, p.o. till the 28th day

- Group IV: Pretreated animals injected with Aβ peptide (10 µl) by ICV on the 21st day and treatment continued with EEMT 400 mg/kg, p.o. till the 28th day
- Group V: Pretreated animals injected with Aβ peptide (10 µl) by ICV on the 21st day and treatment continued with donepezil (5 mg/kg), p.o. till the 28th day.

In vivo cognitive behavioral evaluation

In vivo neurocognitive behavioral evaluations were carried out with the following memory and learning task that includes conditioned passive avoidance for ascertaining the level of short- and long-term memory,[21] novel object recognition test (memory retention),[22] Y-maze (behavioral alteration),[23] and working memory task using water-maze test apparatus.[24]

Assessment of neural antioxidant enzyme profile

The prevalence of the superoxide dismutase enzyme (SOD) activity in the supernatant of brain tissue was estimated as per the standard established spectrometric protocol.[25] Change in sample and reference absorbance was read at 480 nm. Estimation of catalase (CAT) was done as per Pinho *et al.* method, and the rate of decomposition of hydrogen peroxide was measured spectrophotometrically at 240 nm.[26] Glutathione peroxidase (GPx) estimation was carried out using conversion technique (NADPH to nicotinamide adenine dinucleotide phosphate), and the respective absorption changes were noted at 340 nm wavelength scale.[27] Glutathione reductase enzyme threshold was projected in unit of nanomoles of NADPH oxidized per minute by spectroscopic measurement at 340 nm.[28] Lipid peroxidation, an precise index of oxidative stress, was estimated by thiobarbituric acid (TBA) method at the wavelength of 532 nm.[29]

Estimation metabolic enzymes and neurotransmitter level in brain neural tissue

Brian level AChE was estimated by Elman method.[30] The monoamine oxidase (MAO)-B activity was estimated by Charles and McEwen method.[31] Estimation of dopamine was made as per the method described by Margret *et al.*[32] Serotonin was estimated as per the procedure described by Balamurugan *et al.*[33] Glutamate was estimated as per the procedure described by Sowerby and Ottaway.[34,35]

Molecular gene expression analysis by quantitative polymerase chain reaction

m-RNA expressions of inflammatory cytokine such as IL-1, IL-6, TNF-α, and nuclear factor-κ beta (NF-κβ) were analyzed by "Applied Biosystem Step one" instrument using Clonetech SYBR Premix with the following polymerase chain reaction (PCR) conditions: initial denaturation at 203°F for 60 s followed by forty denaturation cycles proceeded at the same temperature for about 30 s time, annealing with 137.3°F for 45 s, and further extension with 161.6°F for 45 min.[36] ACTB was used as an internal control, and the assay was performed in duplicates with 20 µl reaction. Quantitative polymerase chain reaction (QPCR) data were quantitatively analyzed by using the formation of $2^{-}\Delta\Delta^{Ct}$.

Special staining – metachromatic staining of isolated mouse brain

Isolated mouse brain tissue was sufficiently dehydrated, and the samples were processed as per the standard operating protocol[37] and optimized in house for metachromatic (toluidine blue) special staining purpose. In the next phase, the processed samples were incubated with toluidine blue stain at the concentration of 0.5% (w/v) and then heated to 56°C for 20 min. Soon after dehydration, clearing, and mounting, each slide was subjected to pathological investigation under Leica microscope. Neuromorphological characterization of dentate gyrus (DG) and CA zones of hippocampal slices was observed for further interpretation.

Statistical analysis

GraphPad prism software (San Diego, CA, USA) was utilized for statistical analysis. Significant data variability that exists among the groups was statistically correlated using one-way analysis of variance and was distinctively expressed as the average of mean ± standard error. Scale of probability (P) < 0.05 was considerably taken as level of significance.

RESULTS

Effect of ethanol extract of *Morinda tinctoria* on memory retention in mouse by step-down latency and object recognition task

Step-down latency (SDL) is a technical index to measure memory (long and short) retention in small animals. Latency period of amyloid-insulted group (II) was observed to hit steep declination ($P < 0.05$) than normal control group (I). Repeated treatment with EEMT at both dose levels demonstrated a considerable improvement in SDL time ($P < 0.05$) similar to standard donepezil (group V) as tabulated in Table 1. Result analysis of object recognition task clearly signified the decrease ($P < 0.05$) in exploration time toward newer object and increased ($P < 0.05$) exploration toward familiar object in amyloid-intoxicated Group II. EEMT (200 and 400 mg/kg) and donepezil (5 mg/kg)-supplemented mouse revealed significant improvement in memory retention observed by increased ($P < 0.05$) exploration toward newer object and decreased ($P < 0.05$) exploration toward familiar object. The results are depicted in Table 1.

Table 1: Results of step down latency and object recognition task in all experimental groups

Treatment	Step down inhibitory avoidance task		Object recognition task	
	STM (s)	LTM (s)	Exploration time in sec (new object)	Exploration time in sec (familiar object)
Control	143.3±1.68	163.8±1.37	31.67±1.38	10.33±1.17
Aβ peptide (25-35)	77.67±1.14^{a,*}	110.7±1.45^{a,*}	6.16±0.79^{a,*}	22.83±1.07^{a,*}
Aβ peptide + EEMT (200 mg/kg)	94±1.0^{b,*}	111±2.42^{b,*}	13.67±0.76^{b,*}	17.33±1.05^{b,*}
Aβ peptide + EEMT (400 mg/kg)	121.7±2.43^{b,*}	125.20±1.19^{b,*}	16.5±0.88^{b,**}	13±1.15^{b,*}
Aβ peptide + donepezil (5 mg/kg)	146.8±2.89^{b,*}	135.20±1.13^{b,**}	18.17±0.94^{b,*}	12±1.12^{b,*}

Values are expressed as mean±SEM (n=8), comparisons were made between: aGroup I (control) versus Group II (negative control), bGroup II (negative control) versus Group III (EEMT 200 mg/kg), IV (EEMT 400 mg/kg) and V Donepezil (5 mg/kg). Symbols represent statistical significance: *P<0.05, **P<0.01. Aβ: Amyloid beta (25-35); EEMT: Ethanol extract of *Morinda tinctoria*; STM: Short-term memory; LTM: Long-term memory; SEM: Standard error of mean

Effect of ethanol extract of *Morinda tinctoria* on neurobehavioral alteration and working memory task

Water maze (Morris) task extrapolated two identical learning memories such as spatial learning and visual discrimination learning. The results observed in Group II, in comparison with Group I, revealed a statistically significant increase ($P < 0.05$) in escape latency time onto the hidden platform. Indeed, EEMT treatment with 200 and 400 mg/kg to Groups III, IV, and donepezil to Group V (Standard) revealed steady declination in escape latency time ($P < 0.05$) [Table 2]. The elevated Y-maze is one of the widely used *in vivo* memory tasks for adjudicating the percentage alterations in the neuronal behavior. Rodents have tendency to explore newer arms in the open field by repeatedly memorizing the already-visited arm of the Y-Maze. Due to impaired cognitive ability, Group II mice have shown decreased ($P < 0.05$) percentage alteration in Y-maze. In contrast, mouse belongs to Group III and IV that received EEMT (200 and 400 mg/kg) had demonstrated typically higher level of behavioral alterations ($P < 0.05$) in Y-maze task. Similar observations were recorded in Group V that received standard donepezil [Table 2].

Effect of ethanol extract of *Morinda tinctoria* on brain metabolic enzymes acetylcholinesterase enzyme and monoamine oxidase-B

In the present investigation, AChE activity was estimated to realize the enzyme inhibition potential of the EEMT. ICV injection of Aβ statistically significantly ($P < 0.01$) elevated the level of AChE in the brain homogenates of stress-induced mouse (Group II) than normal control (Group I). Dosing with EEMT (200 and 400 mg/kg) and donepezil (5 mg/kg) revealed statistically significant decrease ($P < 0.05$) in AChE level in relative to Group II (amyloid challenge). The level of MAO-B in amyloid-injected mouse (Group II), seems much higher ($P < 0.05$) than Group I. Mitochondrial fractions of EEMT (200 and 400 mg/kg) and donepezil (5 mg/kg) treatment groups showed pronounced decrease ($P < 0.05$) in MAO-B level in relative to Group II [Table 3].

Effect of ethanol extract of *Morinda tinctoria* on quantification of brain level dopamine, serotonin, and glutamate

It was observed that the level of biogenic amines (dopamine and serotonin) significantly ($P < 0.05$) reduced in amyloid-challenged group (II), whereas treatment with EEMT at both the dose levels and standard donepezil (5 mg/kg) revealed significant restoration of the neurotransmitter level ($P < 0.05$) almost to that of the normal [Table 4]. Glutamate is an excitatory neurotransmitter which mediates critical neural signaling pertaining to memory and learning. *Ex vivo* estimation of brain glutamate revealed substantial increase ($P < 0.05$) in amyloid-challenged Group II, when compared with that of normal control group (I). It was further observed that there was a statistically significant ($P < 0.05$) declination in brain glutamate level in animals belongs to Group III (EEMT 200 mg/kg), IV (EEMT 400 mg/kg), and V (donepezil 5 mg/kg). The results are shown in Table 4.

Table 2: Results of water maze and Y-maze task in all experimental groups

Treatment	Morris water maze task escape latency time (s)	Y-maze task percentage alternation (%)
Control	12±0.73	77.98±2.13
Aβ peptide (25-35)	67.83±1.49a,*	33.2±1.08a,*
Aβ peptide + EEMT (200 mg/kg)	52.33±0.84b,*	52.67±1.15*, b
Aβ peptide + EEMT (400 mg/kg)	47.17±1.53b,*	63.09±0.92*, b
Aβ peptide + Donepezil (5 mg/kg)	38.67±2.02b,*	72.2±1.25b,*

Values are expressed as mean±SEM (n=8), comparisons were made between: [a]Group I (control) versus Group II (negative control), [b]Group II (negative control) versus Group III (EEMT 200 mg/kg), IV (EEMT 400 mg/kg) and V donepezil (5 mg/kg). Symbols represent statistical significance: *P<0.05, **P<0.01. Aβ: Amyloid beta (25-35); EEMT: Ethanol extract of *Morinda tinctoria*; SEM: Standard error of mean

Table 3: Results on quantification of brain level acetylcholinesterase and monoamine oxidase-B

Treatment	*Acetylcholinesterase* (micro moles/min/mg protein)	Monoamine oxidase-B (nano mol/mg protein)
Control	17.0±1.35	20.24±0.33
Aβ peptide (25-35)	32.96±1.80[a,**]	28.78±0.62[a,*]
Aβ peptide + EEMT (200 mg/kg)	24.83±1.24[b,*]	26.14±0.31[b,*]
Aβ peptide + EEMT (400 mg/kg)	22.02±1.19[b,*]	25.59±0.30[b,*]
Aβ peptide + donepezil (5 mg/kg)	18.02±0.42[b,*]	22.60±0.40[b,*]

Values are expressed as mean±SEM (n=8), comparisons were made between: aGroup I (control) versus Group II (negative control), bGroup II (negative control) versus Group III (EEMT 200 mg/kg), IV (EEMT 400 mg/kg) and V donepezil (5mg/kg). Symbols represent statistical significance: *P<0.05, **P<0.01. Aβ: Amyloid beta (25-35); EEMT: Ethanol extract of *Morinda tinctoria*; SEM: Standard error of mean

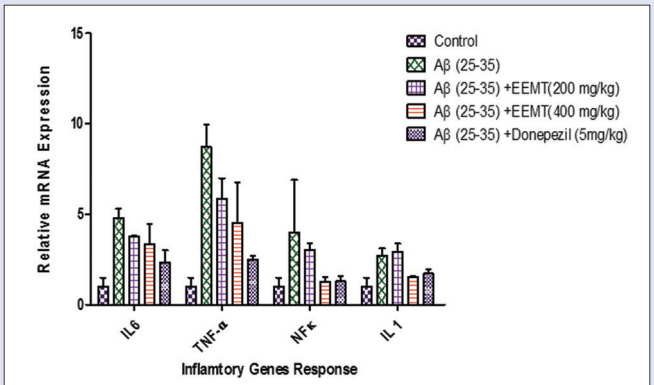
Table 4: Results on quantification of brain level dopamine, serotonin, and glutamate

Treatment	Dopamine (nano g/mg protein)	Serotonin (nano g/mg protein)	Glutamate (μmoles/g wet tissue)
Control	47.16±1.07	34.75±0.95	60.17±4.66
Aβ peptide (25-35)	28.72±1.65[a,**]	16.64±0.50[a,*]	86.60±4.39[a,*]
Aβ peptide + EEMT (200 mg/kg)	33.98±1.10[b,*]	21.08±1.40[b,*]	80.59±2.29[b,*]
Aβ peptide + EEMT (400 mg/kg)	36.64±0.76[b,*]	26.30±0.60[b,*]	78.66±1.87[b,*]
Aβ peptide + donepezil (5 mg/kg)	43.87±0.81[b,*]	27.40±0.38[b,*]	65.67±1.97[b,**]

Values are expressed as mean±SEM (n=8), comparisons were made between: [a]Group I (control) versus Group II (negative control), [b]Group II (negative control) versus Group III (EEMT 200 mg/kg), IV (EEMT 400 mg/kg) and V donepezil (5 mg/kg). Symbols represent statistical significance: *P<0.05, **P<0.01. Aβ: Amyloid beta (25-35); EEMT: Ethanol extract of *Morinda tinctoria*; SEM: Standard error of mean

Effect of ethanol extract of *Morinda tinctoria* on brain antioxidant enzyme profile

Pretreatment with EEMT at 200 mg/kg and at 400 mg/kg dose dependently ameliorated the oxidative stress level by reverting back the enzymatic profile (SOD, CAT, GR, and GPx) of mouse brain almost to that of the normal when compared to the disease control Group II. Similar types of activity were observed in mouse treated with donepezil (5 mg/kg). The results are shown in Table 5. Further there was a significant ($P < 0.05$) increase in the level of TBA (oxidative stress marker) were observed in amyloid-injected mouse (Group II). Administration with EEMT (200 and 400 mg/kg) and donepezil (5 mg/kg) has shown substantial declination in TBA derivative level ($P < 0.05$) on brain homogenates belonging to Groups III, IV, and V. The corresponding data's and figures were tabulated in Table 5.



Gene	Primers Sequence	Size (bp)	Accession No
ACTB	Forward Primer 5'GGACTCTGGTGATGGTGTGA3' Reverse Primer 5' GCTCGGTCAGGATCTTCAT3'	129	AF057040.1
NFKB	Forward Primer 5'AGCCATCATCCACCTTCA3' Reverse Primer 5'CCTCCACCACATCTTCCT3'	152	NM_001276711.1
IL-1 Beta	Forward Primer 5'CATTGTGGCTGTGGAGAAG3' Reverse Primer 5'CTAGCAGGTCGTCATCATC3'	143	NM_031512.2
TNFα	Forward Primer 5'AGCATGATCCGAGATGTG3' Reverse Primer 5'ACGAGCAGGAATGAGAAG3'	110	NM_012675.3
IL-6	Forward Primer 5'CAGAGTCATTCAGAGCAATAC3' Reverse Primer 5'GATGGTCTTGGTCCTTAGC'	154	NM_012589.2

Figure 1: QPCR gene expression analysis of inflammatory cytokines. QPCR: Quantitative polymerase chain reaction; IL-6: Interleukin 6; TNF-α: Tumor necrosis factor; IL-1: Interleukin-1; NF-κ: Nuclear factor-κ; Aβ: Amyloid beta (25–35); EEMT: Ethanol extract of *Morinda tinctoria*

Effect of ethanol extract of *Morinda tinctoria* on gene expression analysis of inflammatory cytokines

Molecular estimation of pro-inflammatory cytokine implicates nearly five to ten fold increases in relative m-RNA expression of TNF-α, IL-1, and NF-κ level as observed in amyloid-insulted group (II) than normal control group (I). Further, EEMT (200 and 400 mg/kg) and donepezil (5 mg/kg)-treated groups significantly attenuated the expression of TNF-α, IL-1, and NF-κ level, thereby expecting to control the progression of neuroinflammation, as shown in Figure 1.

Histopathological analysis by toluidine blue staining

Histopathological investigation reveals distribution of pyknotic neurons with marginal derangement in CA1 and CA2 zones of hippocampal arc in Aβ injected mice. Hippocampi of mouse dosed with EEMT (200 and 400 mg/kg) and donepezil (5 mg/kg) revealed significant reduction in perineural space followed by dense network of pyramidal cell layer with increased granular cell mass in DG region, as shown in Figure 2a-e.

DISCUSSION

Step-down inhibitory avoidance task ascertains the conditioned learning pattern by activation of hippocampus and amygdala that potentiate two distinct types of memory (short-term memory and long-term memory) in rodents.[38] ICV injection of Aβ (25–35) causes severe cognitive impairment evident by marked decrease in SDL latency time of mice exposed to passive avoidance test. EEMT- and donepezil-treated mice have shown significant increase in SDL latency time, an index of both short-term and long-term memory retention.

Performance on water maze task relies on spatial and reference memory mediated by hippocampus NMDA receptors. Navigation towards hidden platform reciprocates the intensity of working memory and optical discrimination learning in rodents.[39] EEMT at both the dose levels has shown a remarkable decrement in escape latency time ($P < 0.05$) as observed in water maze task.

Y maze task used for witnessing the percentage alteration in behavior of the experimental animals in order to ascertain the potential of spatial working memory. Mice with severe cognitive defect have less preference toward arm exploration, which results in decreased alteration and decreased number of entries in Y maze task.[40] Treatment with EEMT and donepezil significantly ($P < 0.05$) increases the percentage alteration behavior in mice subjected to Y maze task; these results clearly justify the memory retention potential of the *Morinda tinctoria* (MTR).

Object recognition task provokes the exploratory tendency of mice toward newer and familiar objects. Parameters such as shape, color, location, and spatial orientation of the objects were utilized for understanding the pattern of recognition memory.[41] In the present study, object recognition

Table 5: Results on estimation of antioxidant enzyme profile

Treatment	Superoxide dismutase (units/mg protein)	Catalase (units/mg protein)	Glutathione peroxidase (units/mg protein)	Glutathione reductase (units/mg protein)	TBARS (nano mole of MDA/mg protein)
Control	9.47±0.13	6.45±0.29	4.30±0.07	2.83±0.05	2.69±0.05
Aβ peptide (25-35)	3.71±0.23^{a,*}	2.87±0.29^{a,*}	2.20±0.08^{a,*}	1.31±0.04^{a,*}	6.079±0.09^{a,*}
Aβ peptide + EEMT (200 mg/kg)	5.78±0.15^{b,*}	3.56±0.22^{b,*}	2.92±0.04^{b,*}	1.68±0.09^{b,*}	5.347±0.15^{b,*}
Aβ peptide + EEMT (400 mg/kg)	6.41±0.11^{b,*}	4.38±0.35^{b,*}	3.22±0.03^{b,*}	1.93±0.02^{b,*}	4.856±0.13^{b,*}
Aβ peptide + donepezil (5 mg/kg)	7.31±0.13^{b,*}	5.72±0.34^{b,*}	3.71±0.15^{b,*}	2.19±0.07^{b,**}	3.045±0.04^{b,*}

Values are expressed as mean±SEM (n=8), comparisons were made between: ^{a}Group I (control) versus Group II (negative control), ^{b}Group II (negative control) versus Group III (EEMT 200 mg/kg), IV (EEMT 400 mg/kg) and V donepezil (5 mg/kg). Symbols represent statistical significance: *P<0.05, **P<0.01. Aβ: Amyloid beta (25-35); EEMT: Ethanol extract of *Morinda tinctoria*; SEM: Standard error of mean; TBARS: Thiobarbituric acid reactive substances, MDA: Malonyldialdehyde

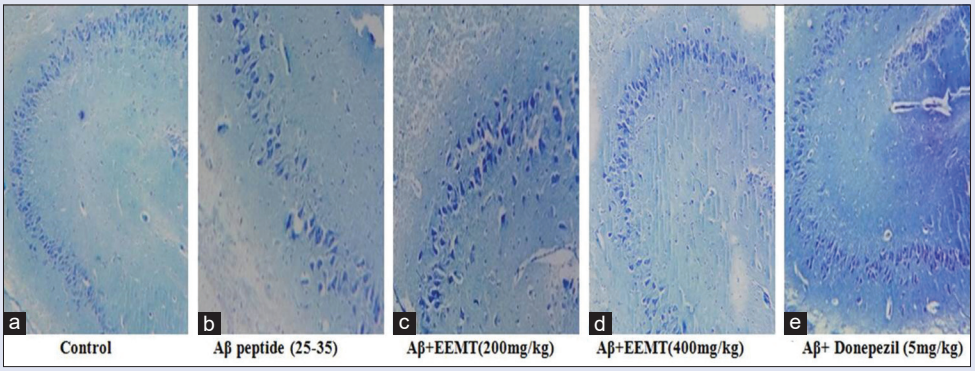


Figure 2: (a-e) Histomorphological changes on hippocampi zonal arc of control, amyloid, and treatment groups. Amyloid beta-induced group (b) reveals the presence of shrunken nuclei and deranged neuronal structure signs of apoptosis observed in hippocampi region. EEMT (c and d)- and donepezil (e)-supplemented group restored the regular neural morphology with densely packed pyramidal cells properly aligned on CA zones of hippocampus Aβ: Amyloid beta (25–35); EEMT: Ethanol extract of *Morinda tinctoria*

task was used as a functional model for measuring exploration time and memory retention in mice. EEMT (200 and 400 mg/kg)- and donepezil (5 mg/kg)-supplemented group revealed significant improvement in memory retention observed by increased ($P < 0.05$) exploration toward newer object and decreased ($P < 0.05$) exploration toward familiar object.

ACh induced cholinergic innervation on frontal and hippocampi neural circuits predominantly mediates the memory and learning responses in the brain. Immunogenic deposition of amyloid protein substantially increases the level of AChE enzyme, which tends to hydrolyze the biologically active acetylcholine, ultimately resulting in cognitive impairment. Inhibition of AChE enzyme activity has dual advantages of enhancing the cholinergic nerve transmission and also preventing the aggregation of neurotoxic amyloid fibril.[42,43] The present investigation clearly evidents that supplementation with EEMT at both the dose levels and donepezil has shown significant decrease in AChE enzyme level in the brain homogenates, which may be expected to elevated level of endogenous ACh that would potentiate the cholinergic neural transmission and boost up the memory.

Neurotransmitters such as dopamine and serotonin propose extensive role in learning, working memory, reasoning, motor coordination, decision-making, and motivational behavior.[44] MAO is a catabolizing enzyme that exists in two isoforms (MAO-A and MAO-B) in which the isoform B has spectacular distribution, especially in ganglia and in serotonergic neuronal pathway. It is evident from the literature that increased expression of the enzyme MAO B in neocortex, parietal, and occipital cortex regions of the brain are majorly responsible for depletion of monoamines (dopamine and serotonin).[45,46] Results of biochemical estimation of the present study reveal that EEMT (200 and 400 mg/kg)- and standard donepezil (5 mg/kg)-treated group have shown notable declination in MAO-B level ($P < 0.05$) in comparison with mouse belongs to amyloid insult group. Further estimation of biogenic amines on subcortical region (including the striatum) of brain justifies that EEMT and donepezil significantly ($P < 0.05$) restored the level of both dopamine and serotonin almost to that of the normal in experimental animals.

Neurobiologically increased amyloid content may aggravate the production of reactive oxygen species (ROS). Repeated exposure of neurons to unstable oxygen and nitrogen moieties destabilize the neural membrane and was subjected to the condition of apoptosis. Hydroxyl radicals potentiate lipid peroxidation in the electron-rich nervous tissues and narrow down the molecular mechanism of DNA damage and cell death. Degenerated neurons fail to counteract the hyperactive radicals due to progressive declination in antioxidant enzyme production.[47]

In central nervous system, glutamate is an excitatory neurotransmitter which mediates several physiology of cognitive skills including learning and attention. Glutamine is a product of glutamate derived upon enzymatic action of glutamine synthetase. Glutamate excitotoxicity activates microglia and stimulates neuroinflammation.[48] Control of hyperactive glutamate is one of the golden standard treatments in AD. In our study, amyloid-challenged mice exhibit significantly higher level of glutamate ($P < 0.05$), whereas treatment with EEMT and donepezil showed significant ($P < 0.05$ and $P < 0.01$, respectively) control on glutamate-induced neuronal excitability.

Increased production of reactive oxygen, nitroxy, peroxy radical coupled with inadequate antioxidant enzymes causes accumulation of quenching radicals. Major antioxidant enzymes such as SOD, CAT, GPR, and GPx residing on mitochondrial segment of neural architecture are involved in scavenging the highly reactive species and convert them into oxygen and water.[49] Extracellular deposition of amyloid and intracellular tangles plays a primary role in shifting the balance between ROS production and the antioxidant defense system. This oxidative insult impairs the expression of brain level enzymatic chelators.[50,51]

Supplementation of herbal antioxidants surely uplifts the level of these biologically active enzymes and shall act as a better therapeutic moiety in managing the stress-induced neurodegeneration.[52] Findings of our current investigation strongly palliate the antioxidant property of the EEMT and donepezil in modulating oxidative stress by improving the brain antioxidant enzymes by downstreaming the level of lipid peroxidation. Therefore, regular intake of phytotherapeutics such as morinda may lower the risk and progression of AD.

Inflammation plays a prominent role in the neurophysiology of AD. Neural signal (mitogen-activated protein kinases) disruption and altered redox state provoke the release of a wide range of inflammatory mediators.[53] Compromised state of antioxidant defense system perhaps paves a way for increased production of reactive radicals, thereby increasing the expression of NF-κB, TNF-a and TNF-b, and IL-1b, IL-2, and IL-6. Research evidences have strongly suggested that expression of cytokines upsurges the state of oxidative stress mediated by NF-κB.[54,55]

In the present study, molecular estimation of pro-inflammatory cytokines was evaluated by using QPCR method. Results evident that nearly five to ten fold increases in the m-RNA expression of brain level TNF-α, IL-1, IL-6, and NF-κ in amyloid-intoxicated mouse in comparison with control group mouse. EEMT treatment (200 and 400 mg/kg) and donepezil

(5 mg/kg) has shown significant control on expression of cytokines. Hence, supplementation with EEMT potentially limits the secretion of cytokines and subsequently halts the progression of neuroinflammation triggered by amyloid injection.

Histomorphometric analysis of brain sample belonging to amyloid-induced group (II) reveals the presence of shrunken nuclei and deranged neuronal structure signs of apoptosis observed in hippocampi region. EEMT- and donepezil-supplemented group restored the regular neural morphology with densely packed pyramidal cells properly aligned on CA zones of hippocampus. Results of histological analysis further confirm that EEMT established high range of neuroprotection against amyloid-induced degeneration.

CONCLUSION

The present investigation provides an evidence-based data which clearly signify the promising neurotherapeutic potential of the herb *M. tinctoria* against amyloid-induced cognitive stress in mouse model. From the research perspective, it was further concluded that medicinal herb such as *M. tinctoria* may have wider therapeutic opportunity and shall better considered as potential lead in the management of AD and other neurodegenerative disorders in the near future.

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Conflicts of interest

There are no conflicts of interest.

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