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Research Article

NEUROPROTECTIVE AND COGNITIVE ENHANCING EFFECT OF METHANOLIC MORUS ALBA LEAF FRACTION IN U87MG CELL LINES AND EXPERIMENTAL RAT MODEL

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ABSTRACT

Objective: The present study aims to investigate the protective effect of methanol fraction of *Morus alba* (MEMA) leaves against hydrogen peroxide (H_2O_2)-induced U87MG cell toxicity and aluminum fluoride (ALF)-induced rat toxicity.

Methods: The study was divided into *in vitro* and *in vivo* sections. U87MG cell lines were pre-treated with different fractions of MEMA for 20 h and further tested against 1000 µM of H_2O_2. The best fraction from *in vitro* studies was used to study the protective effects against ALF-induced neurotoxicity. Rats were divided i nto five different groups, and MEMA (200 and 400 mg/kg p.o) was administered for 14 days to the animals with α-tocopherol as the standard drug treatment. Behavioral studies were assessed using Barnes maze. The major biochemical measurements included catalase, superoxide dismutase and glutathione reductase, lipid peroxidation (LPO), and acetylcholinesterase (AchE) levels.

Results: *In vitro* studies indicated MEMA as a potential candidate followed by AQMA and ethyl acetate. The MEMA fraction was able to ameliorate ALF-induced neurotoxicity in the behavioral assessment. The higher antioxidant content in the fraction decreased the LPO levels from 250±4.07 to 115±3.22 as well as elevated the levels of most of the endogenous antioxidant enzyme levels. AchE levels were also decreased to 33.89±0.71 from 38.94±0.64.

Conclusion: Although the results obtained indicate that MEMA could significantly suppress oxidative stress-induced central neuronal damage both *in vitro* and *in vivo*, further mechanistic studies are required to delineate its neuroprotective pathway.

Keywords: *Morus alba*, Methanol fraction of *Morus alba*, Neuroprotective, Oxidative stress.

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INTRODUCTION

Neurodegenerative diseases pose serious public health concerns with the most prevalent being Alzheimer's disease (AD) followed by Parkinson's disease (PD) [1]. About 5% of individuals aged 65 or older are affected by AD, whereas 1% of the individuals aged 60 years are affected by PD [2,3]. The evidence further suggests that AD will pose major neurological health concerns in the future [4]. AD accounts for 70% of cases of dementia, which displays progressive memory loss and cognitive dysfunction [5,6]. In 2010, the global cost of dementia was estimated at the United States (US) $604 billion and it crossed the US $1 trillion in 2018 [7].

Growing evidence points out oxidative stress and its associated consequences as one of the major risk factors for AD [8]. Oxidative stress describes a state of imbalance in the production of free radicals and antioxidant defenses [8,9]. Oxidative stress is implicated in the vicious circle of amyloid-beta production/accumulation, which is linked with AD development [10]. The primary reactive oxygen species (ROS) scavenging system is associated with oxidative stress, and in the brain, the system constitutes of the antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GSH). Any imbalance to this system could pave ways to amassing of ROS in the system and disrupt the normal poise required to protect the neurons from oxidative injury. Thus, one promising preventive or therapeutic measure for neuroprotection could be to suppress or attenuate the ROS production/accumulation through the exogenous supply of antioxidants and regain the disrupted endogenous oxidative balance [6].

Hydrogen peroxide (H_2O_2) plays dual roles of ROS by itself and an initiator/precursor for other ROS. Increased utilization of oxygen in the tissues is associated with the higher production of H_2O_2 in the brain, which increases the risk of oxidative insult that can lead to apoptosis in various cell types [11]. Upon neurodegeneration, the brain undergoes extensive oxidative stress and rapid biogenic amine metabolism, all of which leads to exacerbation of H_2O_2 and other potential toxin accumulations [12]. Likewise, the neurotoxic role of aluminum fluoride (ALF) inducing the formation of the amyloid-beta has gained significance in many debates in the recent past within the western community. The complex is found to cause neurodegeneration mainly through excitotoxicity by activation of protein kinase C leading to erosion in the animal's cognitive abilities.

Morus alba (MA) or the mulberry plant, whose biological name is MA Linn., is largely being cultivated in the areas of silk harvesting as a food for the silkworms. The medicinal values of the plant, mainly attributed its phenolic content [13], have come into light in a span of a few years. Our in-house data suggest that the methanol fraction of MA (MEMA) leaves could be used as a protective agent owing to its hydroxyl radical scavenging and anticlastogenic activity [14]. Furthermore, our unpublished in-house data, the leaf extracts have shown protective effects in amyloid-beta and scopolamine-induced animal models by elevating the endogenous antioxidant enzyme levels providing a base to the present work [15]. The present work aims to identify the potent neuroprotective effect of MA leaf fraction by subjecting them to *in vitro* and *in vivo* antioxidant tests.

METHODS

Drugs and chemicals

Dulbecco's Modified Eagle media (DMEM) and fetal bovine serum (FBS) were purchased from Gibco, Invitrogen. H_2O_2, ALF, sulforhodamine

(SRB) dye, dichlorodihydrofluorescein diacetate (DCFH-DA), dimethyl sulfoxide (DMSO), acridine orange (AO), and ethidium bromide (EB) were procured from Sigma Aldrich. Thiobarbituric acid and trichloroacetic acid (TCA) were purchased from Hi-Media, Mumbai, India. All solvents (hexane [HEMA], petroleum ether [PEMA], ethyl acetate [ETMA], and methanol) used for extraction were of high-performance liquid chromatography (HPLC) grade procured from Sigma Aldrich.

Plant collection and preparation of fractions

Fresh leaves were collected from Srirangapatna, Mysuru, India (12.4216° N, 76.6931° E), and the sample specimen was confirmed by Dr. Naganandini M.N, Department of Pharmacognosy, Jagadguru Sri Shivarathreeshwara College of Pharmacy, Mysuru, India. The leaves were sun dried for 2 days and powdered using a mechanical grinder. Exhaustive Soxhletion method was used for extraction. Coarse powder of the leaves was loaded in the thimble made of Whatman filter paper in the Soxhlet apparatus successively extracted with the solvents in the increasing order of polarity, namely n-HEMA, PEMA, ETMA, MEMA, and water (AQMA). 500 ml of each solvent was used for the extraction. Each of the fraction, i.e., HEMA, PEMA, ETMA, MEMA, and AQMA, respectively, were filtered, concentrated using flash rotary evaporator, dried, and stored in a vacuum desiccator at room temperature till use.

Cell lines

U87MG glioblastoma cell lines were procured from NCCS, Pune, and maintained in Jagadguru Sri Shivarathreeshwara Medical College. The cell lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin at 37°C and 5% CO_2.

Animals

All the experimental and animal handling procedures were approved by the Institutional Animal and Ethics Committee (IAEC) of Jagadguru Sri Shivarathreeshwara College of Pharmacy, Mysuru, India (IAEC approval number 153/2014). 30 Wistar rats of either sex weighing 250±50 g at the start of the experiment were used. The animals were housed in a temperature and light-controlled room (22°C, a 12 h dark and 12 h light cycle), fed, and allowed to drink AQMA *ad libitum*.

Drug administration

In *in vitro* studies, all the fractions were initially dissolved in DMSO and later diluted with the DMEM media. From pilot studies, we found that 10, 5, and 2.5 µg/ml of all the fractions were non-toxic to the cells. Hence, the non-toxic doses were used to perform the cell viability assays, and the best out of these test doses were selected for further assays. H_2O_2 in 1000 µM at 6h was found to exert maximum toxicity (50%) from prior cytotoxic dose (62.5-1000 µM) and time dependent (2,4 and 6h) studies.

In *in vivo* studies, ALF was administered at 600 ppm p.o to the animals from the 7th day of the 14 days experiment design. α-Tocopherol (100 mg/kg) and MEMA (200 and 400 mg/kg) were administered orally continuously for 14 days.

***In vitro* cell culture studies**

Cell viability by SRB assay

The cytoprotective potential of MA extracts on U87MG glioblastoma cells was assessed using SRB assay [16]. A fixed number of cells (5000 cells/well) taken in a volume of 100 µL were added to each well of a flat-bottomed 96-well plate and kept in a CO_2 incubator until 80% confluence of the cells for 48 h. The cells were treated with 100 µL all the fractions, namely HEMA, PEMA, ETMA, ALMA, and AQMA at concentrations of 10, 5, and 2.5 µg/ml for 20 h followed by 100 µL of 1000 µM of H_2O_2 for 6 h. At the end of the 26th h, the cells were fixed with 50 µl of cold 10% (w/v) TCA for 1 h, washed with deionized AQMA to remove excess of TCA, dried, and stained with 100 µl of 0.4% (w/v) SRB dye solution for 30 min. The unbound SRB was then removed by washing with 1% acetic acid solution, and 100 µl of 10 mM aqueous tris base buffer was added to dissolve the dye. After thorough shaking, the optical density of the plate was read at 490 nm. The percentage viability was calculated by the formula shown in Equation 1:

$$Percentage\,Viability = \left(\frac{Avg - Avg(MB)}{Avg(VC) - Avg(MB)} \right) \times 100 \qquad \text{Equation 1}$$

Where,
Avg - Average of cells in each treatment wells
Avg (MB) - Average of the media blank (only media)
Avg (VC) - Average of cells in vehicle control.

ROS measurement by DCFH-DA

Fixed number of U87MG cells (5000 cells/well) was seeded in 96-well plate. Upon 80% confluence, cells were treated with 2.5 µg/ml of MEMA. Briefly, the cells were pre-treated with 2.5 µg/ml of methanol extract for 20 h. The media was removed and washed once with PBS and incubated with DCFH-DA solution (20 µM/well) for 30 min. The cells were then treated with 1000 µM of H_2O_2 for 6 h. Finally, at the end of the 6th h, the fluorescence intensity (FI) of DCF was measured in a microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 535 nm [17].

Fluorescent imaging by dual staining

Dual staining with EB and AO was performed [18]. Fixed number of U87MG cells (5000 cells) were treated with MEMA (2.5 µg/ml) and 1000 µM of H_2O_2. Upon completion of the treatment, the cells were trypsinized and suspended in PBS (25 µL) from each group and were mixed with 1 µL AO/EB for 15 min just before microscopic evaluation. A thin smear of stained cells was prepared on microscopic slides. Images were captured using a fluorescent microscope.

***In vivo* neuroprotective study**

Experimental

The rats were divided into five groups of six each: (1) Control group received saline treatment (0.9% NaCl), (2) standard group received α-tocopherol pre-treatment followed by ALF (600 ppm) treatment for 7 days, (3) ALF (600 ppm) alone-treated group, (4) MEMA leaves pre-treated group received ALF treatment for 7 days (MEMA1 [200 mg/kg] + ALF), and (5) MEMA leaves (MEMA2 [400 mg/kg] + ALF) pre-treated group received ALF treatment for 7 days. Control and ALF alone-treated groups were caged in the same conditions but in the absence of the treatment.

Spatial learning and memory-Barnes maze task

Spatial learning and memory of the experimental animals were tested by the Barnes maze [15,19]. The apparatus consisted of a clear circular platform (13 cm×29 cm×14 cm) with 12 equally spaced holes (10 cm diameter) located 2 cm from the border. The circular platform was virtually divided into four zones (including the target quadrant with the escape hole and the opposite quadrant). Each trial began by placing the animal in a black cylinder at the center of the platform that was removed after 10 s, allowing mice to freely explore the apparatus. The spatial acquisition was organized in four training sessions (day 1 and 2–2 trails/day). Rats that failed to find the target box within 3 min were gently guided to its location, and for those rats, 180 s were recorded as the escape latency. All animals remained in the target box for 60 s after entering.

The following parameters were scored during all training trials: Latency to escape and a total number of errors. Escape latency was defined as the time taken by animals to completely enter the target box (all four paws out of the platform). Total errors were defined as the total number of holes visited during the trial other than the target hole. A hole was considered visited when rats tilted their head over it (nose poke) or introduced their paws into the hole.

On day 14, reference short-term memory was evaluated by a probe trial (90 s) during which the target box was removed. The latency to find

the target hole (without box) was determined. Furthermore, rats were once again submitted to the acquisition trial in the same conditions to evaluate long-term retention. No training occurred between days 7 and 14.

Estimation of the endogenous antioxidant enzymes and lipid peroxidation (LPO)

The rats were decapitated under anesthesia (ketamine) [20]. The skull was cut open, and the brain was exposed from its dorsal side. The whole brain was quickly removed and cleaned with chilled 0.1 M sodium phosphate buffer (pH 7.4). A 10% (w/v) homogenate of brain samples (0.1 M sodium phosphate buffer, pH 7.4) were prepared using homogenizer and then centrifuged at 4000 rpm for 10 min at 4°C. The supernatant homogenate was used to measure, total protein content (Spinreact), and the activities of CAT, SOD, GSH, and LPO based on the standard methods [21-24].

Determination of acetylcholinesterase (AchE) activity

The AchE enzymatic assay was determined by spectrophotometric method [25]. The reaction mixture (2 ml final volume) contained 100 mM potassium phosphate buffer, pH 7.5 and 1 mM 5,5′-dithiobisnitrobenzoic acid. The method is based on the formation of the yellow anion, 5,5′-dithio-bis-acidnitrobenzoic, measured by absorbance at 412 nm during 2-min incubation at 25°C. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide. All samples were run in triplicate, and change in absorbance per minute is recorded in the kinetic mode of a spectrophotometer for 3 min at 412 nm.

Statistical analysis

Data are shown as means ± standard error of the mean (SEM). Statistical analysis was performed using Prism 5 (GraphPad). One-way and two-way repeated measures ANOVA followed by Tukey's and Bonferroni post hoc analysis were used for probe day and training trials data, respectively. The level of significance was set at $p<0.05$.

RESULTS

Cytoprotective activity of MA fractions against H_2O_2-induced cytotoxicity

U87MG cells were pre-treated with 10, 5, and 2.5 µg/ml of all the fractions for 20 h, and cytotoxicity was induced using H_2O_2 for 6 h. All fractions except PEMA reduced the cytotoxicity induced by H_2O_2, but pre-treatment with MEMA at 2.5 µg/ml significantly increased the cell viability compared to H_2O_2-treated cells and as well as the control (Table 1).

ROS generation by dichlorodihydrofluorescein diacetate

Cells were pre-treated with MEMA for 20 h and further treated with H_2O_2 for 6 h. The conversion of non-fluorescent DCFH-DA to fluorescent DCF in the presence of ROS was fluorometrically detected. MEMA (2.5 µg/ml) being the most effective dose was used to determine the ROS scavenging activity for providing evidence to the prior *in vitro* antioxidant results as well as a base to the dose selection for *in vivo* studies. MEMA (2.5 µg/ml) could scavenge the H_2O_2 generated ROS compared to the H_2O_2-treated group but could not bring back to the normal levels (Table 2).

Fluorescent imagining by dual staining

Fluorescent staining of U87MG cells was performed using AO/EB for qualitative analysis. MEMA pre-treated cells showed significant cell viability (green color) compared to the H_2O_2 treatment, whereas H_2O_2-treated cells showed significant cell death compared to control. The images were captured using fluorescent microscope (Olympus) (Fig. 1).

Effect of MEMA on spatial learning and memory by Barnes maze

The ALF-treated group significantly increased mean escape latencies throughout the training days when compared with normal. α-tocopherol significantly reduced the escape latencies and number of error compared to ALF-treated group and significantly increased

Table 1: Effect of *Morus alba* leaf fractions on H_2O_2-induced cell death in U87MG cell lines (n = 3)

Group	Treatment	% viability
1	Control	87.89 ± 0.97
2	H_2O_2	49 ± 2.13[a]
3	HEMA (10 µg/ml)	63.21 ± 3.24[b]
4	HEMA (5 µg/ml)	59.21 ± 5.81[a,b]
5	HEMA (2.5 µg/ml)	50.30 ± 6.94[a]
6	PEMA (10 µg/ml)	57.13 ± 4.7[a, b]
7	PEMA (5 µg/ml)	50.12 ± 3.99[a]
8	PEMA (2.5 µg/ml)	48.98 ± 4.5[a]
9	ETMA (10 µg/ml)	77.01 ± 7.12[b]
10	ETMA (5 µg/ml)	71.31 ± 6.87[b]
11	ETMA (2.5 µg/ml)	80.42 ± 4.94[b]
12	MEMA (10 µg/ml)	88.17 ± 5.92[b]
13	MEMA (5 µg/ml)	91.53 ± 3.36[b]
14	MEMA (2.5 µg/ml)	97.04 ± 5.55[b]
15	AQMA (10 µg/ml)	79.17 ± 3.73[b]
16	AQMA (5 µg/ml)	83.87 ± 6.82[b]
17	AQMA (2.5 µg/ml)	86.96 ± 5.91[b]

Data were analyzed by one-way ANOVA followed by post hoc Tukey's multiple comparison test. [a]$p < 0.05$ compared to control blank and [b]$p < 0.05$ compared to H_2O_2. H_2O_2: Hydrogen peroxide, HEMA: Hexane, PEMA: Petroleum ether, ETMA: Ethyl acetate, MEMA: Methanol fraction of *Morus alba*

Table 2: Effect of MEMA on H_2O_2-induced ROS generation in U87MG cell lines (n=3)

Group	Fluorescence intensity (mean)
Control	109.25
H_2O_2	159[a]
MEMA (2.5 µg/ml)	111.38[b]
MEMA (2.5 µg/ml)+ H_2O_2	129.25[a,b]

Data were analyzed by one-way ANOVA followed by post hoc Tukey's multiple comparison test. [a]$p<0.05$ compared to control blank and [b]$p<0.05$ compared to H_2O_2. H_2O_2: Hydrogen peroxide, ROS: Reactive oxygen species, MEMA: Methanol fraction of *Morus alba*

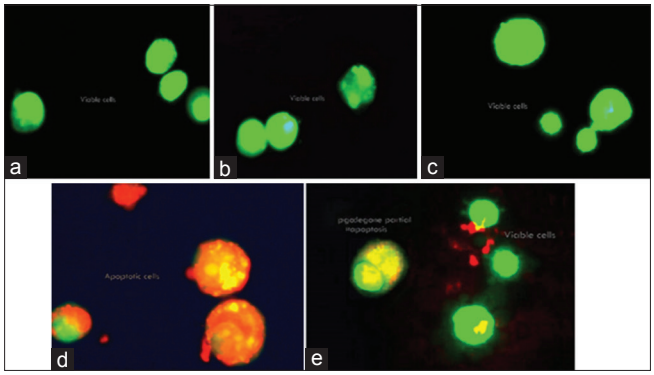

Fig. 1: Dual-stained images of treated and control cells under fluorescent microscope. Figures represent the different treated cells and the cell blank after dual staining viewed under fluorescent microscope. (a) Cell blank, (b) vehicle control (dimethyl sulfoxide), (c) methanol fraction of *Morus alba* (MEMA) (2.5 µg/ml), (d) hydrogen peroxide (H_2O_2) (1000 µM for 6 h), and (e) MEMA (2.5 µg/ml for 20 h) + H_2O_2 (1000 µM for 6 h)

the time spent in escape quadrant. MEMA also at both the doses significantly succeeded in shortening the escape latencies prolonged by ALF treatment as well as number of errors made. In the probe trial, MEMA1 (Table 3) (200 mg/kg) pre-treated group significantly increased the time spent in the escape quadrant as compared to the standard treatment (Fig. 2).

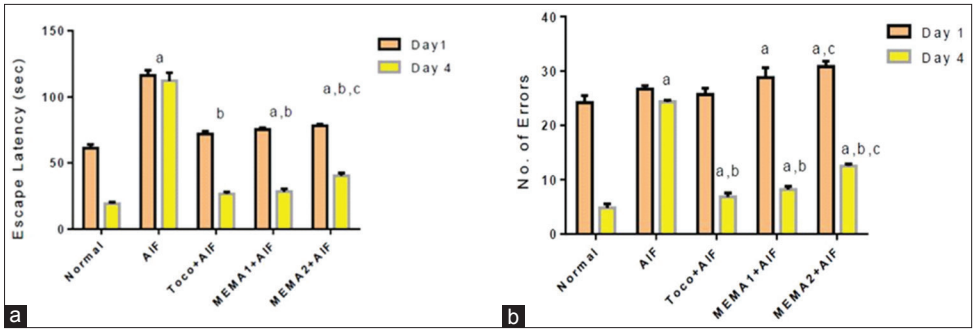

Fig. 2: Effect of methanol fraction of *Morus alba* (MEMA) on spatial learning and memory by Barnes maze. (a) Effect of MEMA on escape latencies in Barnes maze and (b) effect of MEMA on total number of errors made in Barnes maze. All values were expressed as mean ± standard error of the mean, n=6. Data were analyzed by two-way ANOVA followed by post hoc Bonferroni's multiple comparison test.

Effect of MEMA on endogenous antioxidant enzymes

Reports indicate that the endogenous antioxidant enzyme levels are found least in the brain compared to other organs [26]. Hence, it is important to check the levels of these enzymes pre- and post-treatment to confirm the antioxidant efficacy of the fraction. After ALF treatment, rat brains showed a marked decrease in SOD, GSH, and CAT levels when compared with normal. α-tocopherol (100 mg/kg i.p) significantly increased the decline in ALF-induced brain endogenous enzymes while the treatment with MEMA at both doses (200 and 400 mg/kg) significantly reversed the dropped brain enzymes level (Table 4).

Effect of MEMA on brain AchE activity

Acetylcholine is considered to be very important in proper brain functions especially memory and related cognitive domains. AchE enzyme causes breakdown of Ach leading to its loss of activity. The direct levels of Ach measurement are a bit tedious due to the highly sensitive and unstable nature of the neurotransmitter, and hence, the levels of AchE are estimated to indirectly give a measurement of Ach levels in the brain [27]. AchE activity in normal animals was found to be 27.72±1.77 μM/min/mg protein. AchE activity was significantly increased in animals treated with ALF (38.94±0.64) when compared with normal. MEMA at 200 and 400 mg/kg and standard significantly reversed the increase in AchE activity treated by ALF (Table 5).

Effect of MEMA on LPO

The LPO levels are one of the most important markers of oxidative stress. Lipids when reacting with free radicals undergo peroxidation to form lipid peroxides indirectly indicating the free radical density [28]. The MEMA1 treatment not only significantly reduced the malondialdehyde (MDA) formed due to ALF-induced stress compared to the control group but also the values were analogous the standard treatment (Table 6).

DISCUSSION

Ample evidence from preclinical and clinical studies prove the role of oxidative stress in the pathogenesis of neurodegenerative diseases. The oxygen-free radicals are found to play an important role in the decline of the health status of the elderly [29]. Finding novel and effective treatment agents that modify the disease course through neuroprotective therapy either by slowing down disease progression or promoting the genesis of new neurons are an unmet clinical need [30]. Exogenous administration of antioxidants to scavenge the generated free radicals and strengthen the endogenous antioxidant enzymes is one such treatment strategy. Plants and its products are splendid sources of antioxidants and hence have been used for its therapeutic benefits and to increase the effectiveness of treatment. The MEMA leaves are found to exert beneficial effects comparable to *Ginkgo biloba* for AD in addition to its anti-Aβ aggregation effect in primary hippocampal cell cultures at a concentration of 30 mg/ml [31]. With this aim, the present study was selected to explore the possible neuroprotective activity of leaves of MA against H_2O_2 cytotoxicity and ALF-induced neurotoxicity.

Table 3: Effect of MEMA on probe trial in Barnes maze (n=6)

Groups	Latency to find target hole (s)
Normal	71.17±4.19
ALF	20.1±1.35[a]
α-tocopherol+ALF	67.67±2.60[b]
MEMA1+ALF	63±2.19[b]
MEMA2+ALF	50.17±2.32[a,b,c]

Data were analyzed by one-way ANOVA followed by post hoc Tukey's multiple comparison test where [a]p<0.05 compared to normal, [b]p<0.05 compared to ALF, [c]p<0.05 compared to standard. MEMA: Methanol fraction of *Morus alba*, ALF: Aluminum fluoride

As mentioned earlier, H_2O_2 is a generator as well as a propagator of ROS in the living system. An elevation in H_2O_2 levels correlates with the toxic consequences of AD and Aβ, and reports suggest that Aβ increases H_2O_2 accumulation in the cells indicating a "second messenger" role of H_2O_2 which induces apoptosis [32]. *In-vitro* studies have also demonstrated morphological and biochemical similarities between amyloid beta and H_2O_2 induced neurotoxicities [33]. Hence, we presumed in our study that levels of cytoprotection extended by the fractions against H_2O_2-induced toxicity could also provide an indirect mechanism for their neuroprotective activity against Aβ-induced toxicity.

Fractions were subjected to *in vitro* neuroprotective assay on the human glioblastoma cell lines (U87MG) against H_2O_2-induced cytotoxicity. The U87MG cells pre-treated with all the fractions followed by H_2O_2 (1000lo) treatment 6 h were found to resist death in most of the groups except for PEMA but actually increased the number of cells in the MEMA group possibly due to its higher antioxidants, especially phenolic content [14]. Due to the significant protective effect of the methanol fraction at 2.5 μg/ml, it was selected for further studies. As discussed earlier, hydroxyl radicals are identified to be one of the major contributors to aging and most of the chronic diseases. In addition, direct exposure of H_2O_2 has been reported to block glutathione biosynthesis and induce oxidative stress, resulting in apoptotic cell death [34]. Our previous in-house data indicated a prominent hydroxyl radical scavenging activity for the MEMA fraction. Hence, there was a requirement of reproducibility for the same effect in a living system for which FI produced on conversion of DCFH-DA to DCF in the presence of ROS (which also includes OH radicals) was estimated. MEMA 2.5 μg/ml readily scavenged H_2O_2 generated ROS and markedly decreased the FI compared to the H_2O_2. Further, fluorescent imaging was performed to qualitatively confirm the protective mechanism of MEMA at 2.5 μg/ml. The results supported the protective data obtained from the above assays. The significant antioxidant ability of MEMA might delay the occurrence of apoptosis or prevent it altogether.

From numerous neurotoxins, ALF particularly stands out due to the combination of two potent neurotoxins: ALF. Being individually toxic,

Table 4: Effect of MEMA on endogenous antioxidant enzymes (catalase, SOD, and GSH) (n=6)

Group	Catalase (μ/mg protein)	SOD (μ/mg protein)	GSH (μmole/g/tissue)
Normal	7.32±2.21	10.96±3.21	21.4±2.57
ALF	1.84±3.51[a]	0.992±5.41[a]	8.46±4.11[a]
α-tocopherol+ALF	5.82±5.27[a,b]	0.93±4.2[b]	20.21±3.77[b]
MEMA1+ALF	4.78±3.34[a,b]	7.98±2.03[a,b]	18.78±2.57[a,b]
MEMA2+ALF	3.73±3.34[a,b,c]	6.12±2.30[a,b]	15.11±4.47[a,b,c]

Data are expressed as mean±SEM. Data were analyzed by one-way ANOVA followed by post hoc Tukey's multiple comparison test. In Table 3, [a]p<0.05 compared to normal, [b]p<0.05 compared to ALF, and [c]p<0.05 compared to standard. SEM: Standard error of the mean, SOD: Superoxide dismutase, GSH: Glutathione reductase, MEMA: Methanol fraction of *Morus alba*

Table 5: Effect of MEMA on AchE levels (n=6)

Group	AchE activity (μM/min/mg), Mean±SEM
Normal	27.72±1.77
ALF	38.94±0.64[a]
α-tocopherol+ALF	32.56±0.56[a,b]
MEMA1+ALF	33.18±0.60[a,b]
MEMA2+ALF	33.89±0.71[a,b]

Data were analyzed by one-way ANOVA followed by post hoc Tukey's multiple comparison test. In Table 4, [a]p<0.05 compared to normal while [b]p<0.05 compared to ALF. ALF: Aluminum fluoride, MEMA: Methanol fraction of *Morus alba,* AchE: Acetylcholinesterase

Table 6: Effect of MEMA on lipid peroxidation (n=6)

Group	Lipid peroxidation (nmol TBARS/min/mg tissue)
Normal	88±3.06
ALF	250±4.07[a]
α-tocopherol+ALF	104±2.14[a,b]
MEMA1+ALF	115±3.22[a,b]
MEMA2+ALF	130±2.60[a,b,c]

Data were analyzed by one-way ANOVA followed by post hoc Tukey's multiple comparison test. In Table 5, [a]p<0.05 compared to normal while [b]p<0.05 compared to ALF. ALF: Aluminum fluoride, MEMA: Methanol fraction of *Morus alba,* TBARS: Thiobarbituric acid reactive substances

their combination is found to be highly neuronal degrading since the complex interferes with most of all the neurochemical functions including impairing glutamate transporters and microglial activation all of which finally leading to excitotoxicity. The toxin is found to significantly increase the ROS/reactive nitrogen species and LPO products leading to mitochondrial dysfunction ultimately facilitating the accumulation of neurodegenerative products such as Aβ and tau. Al, being an abundant metal, and fluoride, a component of drinking water, possess many chances in our everyday lives to react and form the deadly "AlF duo" [12,35]. Hence, the model is important to be studied to predict its consequences likely to be posed to human society.

Previous investigations and *in vitro* studies carried out indicate methanol fraction to be more active compared to other fractions. Hence, the MEMA fractions at doses of 200 and 400 mg/kg were chosen for *in vivo* studies directed against ALF-induced neurotoxicity. The escape latency and number of errors were assessed using Barnes maze. MEMA at 200 and 400 mg/kg dose substantially shortened the escape latencies prolonged by ALF and also reduced the total number of errors made by the animals while acquisition. MEMA1 (200 mg/kg) had an effect comparable to the standard. The cognitive enhancement could be due to PDE4 inhibition by Moracin present in the leaves leading to the increased cyclic adenosine monophosphate levels in the hippocampus indicated in memory enhancement [36].

The evaluation of biochemical parameters was performed to support the data obtained from the behavioral studies. In comparison with control, the fraction at both the doses considerably decreased the AchE activity, but MEMA1 showed similar efficacy as that of the α-tocopherol group in the reduction MDA levels. The decreased AchE activity may be attributed to the antiserotonergic effect of MEMA fraction which assists in the enhanced release of Ach from the cortical area [37]. Both the doses of MEMA considerably increased the levels of CAT, SOD, and GSH, when compared to AlF treated group with MEMA1 exhibiting a slightly increased activity, correlating to our previous antioxidant activity reports.

CONCLUSION

The present study indicated that pre-exposure to MEMA leaves could effectively restore antioxidant brain status both *in vitro* and *in vivo* and may confer neuroprotection due to the alleviation of oxidative damage induced by two different toxins. Therefore, MEMA could be a potential candidate for the further preclinical study aimed at the moderation of dementia symptoms in neurodegenerative diseases.

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AUTHOR'S CONTRIBUTION

Miss Anjali Raj performed the *in vivo* studies while Mr. Sumit Dey performed the *in vitro* studies at JSS Medical College. Dr. Subbarao was instrumental in formulating and guiding the *in vitro* work while Dr. Manjula supervised the entire work. All the authors have contributed equally in reviewing the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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