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RESEARCH ARTICLE

Antiamnesic effect of *Mesua ferrea* (L.) flowers on scopolamine-induced memory impairment and oxidative stress in rats

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Abstract

Mesua ferrea Linn. flowers have been used in Ayurveda as a brain tonic and as an ingredient in memory-enhancing formulations such as *Brahma Rasayan* and *Chyawanprash*. However, this ethnomedicinal use has not been investigated scientifically. This study evaluated the effect of the ethanolic extract of *Mesua ferrea* flowers (MFE) on memory in scopolamine-induced models of cognitive dysfunction. MFE was administered to rats (100, 200 and 400 mg/kg, *p.o*) for a period of 14 days, after which amnesia was induced by giving scopolamine (1 mg/kg, *s.c*) on the 14th day. Piracetam (200 mg/kg, *p.o*) was given as a positive control. The models employed to assess memory in the rats were the T-maze continuous alternation task (T-CAT) and novel object recognition test (NORT). Pretreatment with MFE ameliorated the memory deficit caused by scopolamine; which was evidenced by a significantly greater relative proportion of spontaneous alternation percentage in the T-CAT, and a significant increase of discrimination index in the NORT. Further, MFE significantly inhibited anticholinesterase activity in the brain, elevated the levels of reduced glutathione and catalase, and decreased malondialdehyde and nitrite levels in the brain. The results of this study show that MFE exhibited significant anticholinesterase and antioxidant activities in scopolamine treated rats, which could be the possible underlying mechanism of its memory-enhancing activity and of its ethnomedicinal use as a brain tonic.

Keywords *Mesua ferrea* · Scopolamine · Acetylcholinesterase · Antioxidant · t-maze · Novel object recognition test

Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ANOVA	Analysis of variance
ATC	Acetylthiocholine iodide
CAT	Catalase
CPCSEA	Committee for purpose of control and supervision of experiments on animals
DI	Discrimination Index
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTNB	5,5'-dithio-bis (2-nitrobenzoic acid)
GAE	Gallic acid equivalents
GSH	Glutathione
IAEC	Institutional Animal Ethics Committee
LTP	Long-term potentiation
MDA	Malondialdehyde
MFE	*Mesua ferrea* flowers ethanolic extract
NORT	Novel object recognition test
PIR	Piracetam
RE	Rutin equivalents
ROS	Reactive oxygen species
SEM	Standard error of mean
SCOP	Scopolamine
TBARS	Thiobarbituric acid
TCA	Trichloroacetic acid
T-CAT	T-maze continuous alternation task
TFC	Total flavonoid content
TPC	Total phenolic content

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Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by a gradual loss of cognitive abilities, affecting nearly 47.5 million people worldwide (Pahwa and Goel 2016). It is an accelerating health crisis in developing countries with an increasing population of the elderly (Mathuranath et al. 2012). Many factors contribute to the etiology of AD, such as amyloid-beta plaque formation, hyperphosphorylation of tau protein, variation in cholinergic transmission, formation of reactive oxygen species (ROS) due to oxidative stress, inflammation, monoaminergic disturbances, and apoptotic processes (Odubanjo et al. 2018).

The extent of cognitive decline and memory impairment in an Alzheimer's patient is directly related to the loss of cholinergic function (Jeong et al. 2009). It has been proven that patients with AD exhibit a decrease in acetylcholine (ACh) and cholineacetyltransferase and an increase in acetylcholinesterase (AChE) levels (Rubio et al. 2007). Additionally, Keller et al. (2005) have demonstrated the presence of oxidative damage in the initial stages of AD. Reactive oxygen species (ROS) cause neurodegeneration by initiating lipid peroxidation in the central cholinergic system (Ngoupaye et al. 2017). This shows that oxidative stress coupled with cholinergic hypofunction might be one of the underlying causes of cognitive dysfunction in Alzheimer's disease.

The therapeutic agents currently used in the management of AD are AChE inhibitors such as donepezil, galantamine, rivastigmine, and tacrine. However, these drugs have limited efficacy, poor bioavailability, narrow therapeutic windows, and cause adverse effects like cholinergic side effects in the periphery and hepatoxicity; thus restricting their usage (Ghumatkar et al. 2015). There is a need to search for alternative therapy, as conventional anti-AD agents offer only symptomatic relief. There is a growing interest in herbal drugs as they are considered to be more 'natural' and 'gentle' alternatives to synthetic drugs (Ishola et al. 2013), and are easily available and cost effective (Foyet et al. 2015). Herbal drugs are also preferred as they have a 'built-in poly-pharmacology' due to the presence of numerous secondary metabolites in a single plant, and synergism among those phytoconstituents could provide a remedy to the multifactorial pathogenesis of AD (Ngoupaye et al. 2017; Dey et al. 2017).

Mesua ferrea Linn. (Calophyllaceae) is a perennial tree which is indigenous to parts of India, Sri Lanka, southern Nepal, Thailand, and a few other South-Asian countries (Lim 2016). In the Indian system of medicine, the plant, commonly known as *Nagakesara*, is traditionally used for its antiemetic, anthelminthic, aphrodisiac and diuretic effects, and as an antidote (Anandakumar et al. 1986). *Nagakesara* has been used as a brain tonic in Ayurveda (Anandakumar et al. 1986), and is an ingredient of ayurvedic formulations such as *Brahma Rasayana* and *Chyawanprash* (Chahar et al. 2012), which are manufactured commercially till date. *Brahma Rasayana* is primarily used for brain-specific disorders in the elderly. Daily consumption of *Brahma Rasayana* is believed to improve memory, learning and cognition (Baliga et al. 2013). *Chyawanprash* is a generic household cure used widely in India. It has been used as a health food in the country for over 2000 years, which aims at the maintenance of the physique and vigor, while delaying ageing. It rejuvenates and nourishes the brain cells, thereby enhancing memory and learning (Vasudevan and Parle 2007). *Nagakesara* is also used in the preparation of *Kalyanaka Ghrita*, an ayurvedic ghee-based formulation which is given to improve memory and concentration. This preparation is used in the treatment of disorders wherein the mental capabilities and intellect of a patient are weakened (Rajput 2018).

Extracts of *Mesua ferrea* flowers are known to contain fractions of bioactive compounds including a biflavonone (mesuaferrone-b), triterpenoid (β-amyrin), sterol lipids (β-sitosterol), cyclohexadione compound (mesuaferrol), and eighteen 4-phenyl-5,7-dihydroxycoumarins which include mesuol, mammeisin, and mesuagin (Raju et al. 2016, Dennis and Kumar 1988, Verotta et al. 2004). Moreover, *Mesua ferrea* flowers have been reported to demonstrate antioxidant activity in ABTS (Wessapan et al. 2007) and DPPH assays (Makchuchit et al. 2010). However, no studies have justified the use of *Mesua ferrea* as a brain tonic or delved into its effects on memory and learning.

Scopolamine, a well-known anticholinergic, is often used in preclinical studies of new drugs which are designed to treat cognitive dysfunction. Studies have shown that scopolamine also causes oxidative stress through its actions on the cholinergic system, leading to memory impairment (Klinkenberg and Blokland 2010). This model was chosen as it is a quick and simplified method to test the nootropic potential of drugs. *Nagakesara* is mostly attributed to the stamens or flowers of *Mesua ferrea* (Anandakumar et al. 1986), and therefore the flowers of this tree were selected for the study. Based on the traditional claim and reported in-vitro antioxidant effects of *Mesua ferrea*, the aim of this study was to investigate the pharmacological effects of the ethanolic extract of its flowers on learning and memory in scopolamine-induced memory deficit models of disease. Piracetam, an important nootropic agent, acts by improving cholinergic function, stimulating oxidative glycolysis, and increasing cerebral oxygen consumption (Hitzenberger 1998). Since the interest of this experiment was to determine if

the herbal extract had anticholinesterase and antioxidant properties, piracetam was chosen as the standard drug for comparison.

Materials and methods

Plant collection, extract preparation and phytochemical analysis

Mesua ferrea flowers were collected from Ratnagiri, Maharashtra, India. The flowers were identified and authenticated at the Agharkar Research Institute, Pune. The ethanolic extract (Batch No.: 11/2016, Ref.: SE/EQ/2016-17/11) of *Mesua ferrea* flowers (MFE) was prepared by M/S Shamantak Enterprises (B/II/293/0206776, Pune, Maharashtra, India). A voucher specimen (No. 4/2016) was stored for future reference. The extraction procedure was carried out as described previously by Garg et al. (2009) with slight modifications. Dried and powdered flowers of *Mesua ferrea* (500 gms) were extracted with 90% ethanol (5 L) for 72 hours using a Soxhlet apparatus (temp: 55 °C). The residual solvent in the crude extract was then evaporated under vacuum to produce MFE (yield 23.7% w/w). The extract was an aromatic, viscous, chocolate brown paste with 10.2% w/w moisture content and 3.02% w/w ash content, the latter complying with the Indian Pharmacopoieal standards (Quality Report No. II 19). The presence of various phytochemical constituents were analysed using standard procedures (Khandelwal 2008). MFE tested positive for the presence of phenolics, tannins, flavonoids, saponins and alkaloids.

Quantitative phytochemical estimation

Total phenolic content (TPC) and total flavonoid content (TFC)

Folin-Ciocalteu reagent was used to determine the total phenolic content of the plant extract. The plant extract (0.1 ml) was mixed with 0.5 ml of Folin-Ciocalteu reagent and 1.5 ml of 20% w/v sodium carbonate. The solution was then mixed thoroughly and the volume was made up to 10 ml with distilled water. After allowing the mixture to stand for 2 hours, the absorbance was read at 765 nm. The data was used to estimate the total phenolic content using a standard curve obtained for various concentrations of gallic acid (Abozed et al. 2014). The total flavonoid content was determined using the procedure described in Kumaran and Joel Karunakaran (2007). 1 ml of plant extract in methanol (10 mg/ml) was added to 1 ml aluminum trichloride in ethanol (20 mg/ml) followed by a drop of acetic acid. This mixture was then diluted with ethanol to 25 ml. The absorption was recorded at 415 nm after 40 minutes. For the blank samples, 1 ml of plant extract was mixed with a drop of acetic acid, and diluted to 25 ml with ethanol. The standard used was rutin solution (0.5 mg/ml) in ethanol, and the absorption was measured at the same conditions mentioned above. All the determinations were performed in duplicates. The amount of flavonoids in the extract in rutin equivalents (RE) was calculated using the formula:

$X = \left(A \times m_0\right)/(A_0 \times m)$where X is the flavonoid content, mg/mg plant extract in rutin equivalents, A is the absorption of the plant extract solution, A_0 is the absorption of the standard rutin solution, m is the weight of the plant extract (mg) and m_0 is the weight of rutin in the solution (mg).

Drugs and chemicals

Scopolamine hydrobromide and Griess' reagent were purchased from Sigma-Aldrich, USA. Piracetam (Nootropil ®) was a product of Dr. Reddy's Laboratories, India. Acetylthiocholine iodide (ATC), trichloroacetic acid (TCA) and thiobarbituric acid (TBARS) were manufactured by Loba Chemie Pvt. Ltd., India. 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) and all other reagents were of analytical grade and were obtained from Analab Fine Chemicals, India.

Experimental animals

Thirty-six male Wistar rats, weighing about 180-220 g were used for the nootropic study. The animals were kept under ambient conditions at a temperature of 25 ± 2 °C, 45-55% relative humidity and a 12-hour light/dark cycle. Animals were fed with standard diet (Amrut Feed, Chakan Oil Mills, Pune) and water *ad libitum*. The research protocols used in this study complied with the requirements of Institutional Animal Ethics Committee (IAEC) of AISSMS College of Pharmacy, Pune, constituted under the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), approval no. CPCSEA/IAEC/PC-01/02-2K16. All efforts were taken to reduce animal suffering and to minimize the number of animals used in the experimental tasks.

Acute oral toxicity study

The determination of acute toxicity was conducted in accordance to the Organization for Economic Cooperation and Development (OECD) guideline 420 (OECD 2001). Female rats were used in this study as females are generally more sensitive to toxic effects as compared to males (OECD 2000). Six rats, aged 8-10 weeks were divided into two groups comprising three animals each. The animals were fasted overnight with access to water, weighed and marked prior to administration. The first group received the MFE at a dose of 2000 mg/kg and the second group received only

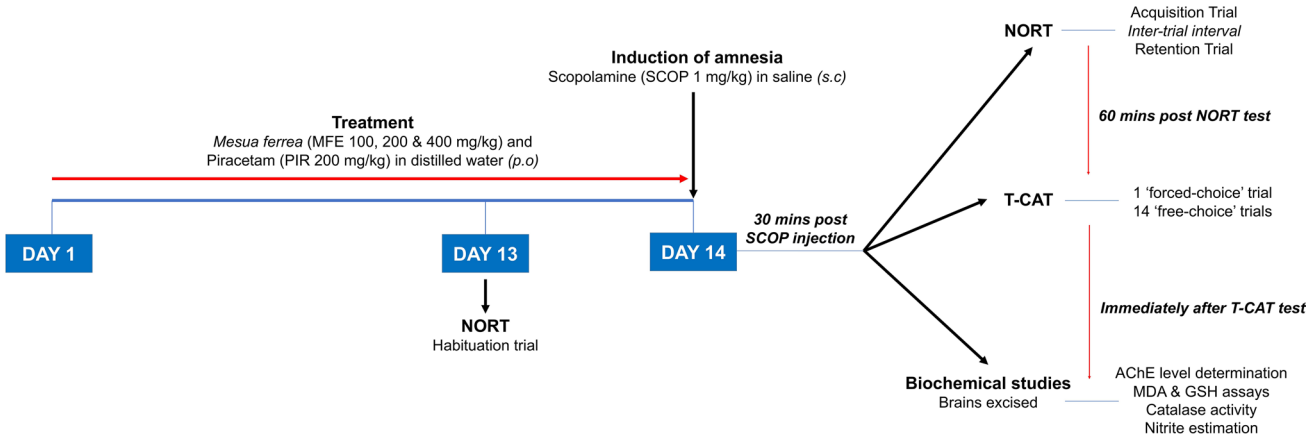

Fig. 1 Experimental timeline and dosing regimen in rats. NORT, novel object recognition test; T-CAT, T-maze continuous alternation task; AChE, acetylcholinesterase; MDA, malondialdehyde; GSH, reduced glutathione

distilled water orally. The rats were observed for individually for 24 h for signs of toxicity and death. For the remaining 14 days, the animals were monitored daily for any behavioral changes or clinical signs of toxicity and the weekly body weight was recorded. On the last day, the animals were sacrificed by cervical dislocation under inhaled diethyl ether anesthesia and the organs and tissues were macroscopically examined for any toxic abnormalities. The administration of a single dose of MFE (2000 mg/kg) did not induce mortality or any toxic effects in the animals after treatment. The changes in body weights were normal and there were no visible abnormalities in the tissues examined.

Experimental groups and drug treatments

The animals were randomly divided into groups (n=6) and treated as follows for 14 days:

- Group I (Control) - normal control group (vehicle; *p.o.*)
- Group II (SCOP) - amnesic control (vehicle, *p.o.*)
- Groups III-V (MFE groups) - test groups (MFE - 100, 200 and 400 mg/kg respectively, *p.o.*)
- Group VI (PIR) - standard group (Piracetam - 200 mg/kg, *p.o.*)

Freshly prepared solutions of MFE and piracetam in distilled water, and scopolamine in saline were administered to the rats. Fig. 1 depicts the pictorial representation of the experimental paradigm. On the 14th day, scopolamine was administered subcutaneously at a dose of 1 mg/kg (Bhattamisra et al. 2012, Shin et al. 2018, Kumar et al. 2000) to groups II-VI, one hour after the administration of distilled water/MFE/piracetam. Cognitive tests were conducted 30 minutes after scopolamine injection on the 14th day. The animals were examined daily for any signs of inactivity or lethargy. All the experiments were conducted during the diurnal phase (9:00 am to 6:00 pm). The animals were shifted to the laboratory one hour prior to the experiment to allow habituation and were deprived of food 12 hours before the behavioral testing. However, all the animals were allowed to drink water *ad libitum*. On the 13th day, the rats were subjected to a habituation trial for the NORT, wherein they were allowed to explore the open area freely for 2 minutes. The same set of animals were subjected to the T-CAT and NORT. The T-CAT was conducted 1 hour after the NORT, and the rats were sacrificed immediately after the behavioral tests to collect the brains for the assessment of biochemical parameters. During the experiments, the odor cues were moderated by cleaning the maze with a damp cloth dipped in 70% alcohol before each new animal started its session.

Behavioral cognition tests

T-maze continuous alternation task (T-CAT)

The T-maze (VJ Instruments, India) made of black colored plywood, consisted of two open arms (40 cm × 10 cm × 20 cm) and the starting arm (70 cm × 10 cm), elevated to a height of 25 cm. The experiment was conducted according to the procedure described in Spowart-Manning and van der Staay (2004) with slight modifications. Briefly, the experiment protocol consisted of one single session, which started with one 'forced-choice' trial, followed by 14 'free-choice' trials. In the 'forced-choice' trial, entry to either of the maze arms were obstructed by a black-colored guillotine door. After a 5 s confinement period in the start box, the rat would navigate the maze, in due course enter the open goal arm, and return back. For the subsequent 14 'free-choice' trials, post confinement, the rat was free to choose between the

left and right open goal arms (no guillotine doors lowered). After the rat entered either one open goal arm, the other arm was closed. The rat then eventually returned to the start box, and the next free-choice trial started 5 s after restriction in the start box. A session was termed completed as soon as 14 free-choice trials were performed or 10 minutes had passed, whichever occurred first. Spontaneous alternations are defined as entry in a different arm of the T-maze over successive trials (i.e., left-right-left, etc.). The percentage of alternations over the free 14 free-choice trials were determined for each rat accordingly:

$$\textit{Percent spontaneous alternations} = (\textit{No. of spontaneous alternations}/\textit{Total number of free choice trials}) \times 100$$

The total time elapsed until completion of each session was also recorded. Throughout the sessions, the animals were not once handled by the experimenter, which is a key modification of the usual T-maze task. Continuous handling of the animals might induce fear, and the anxious animal would react either by long freezing responses or refusal to run in the maze. This would cause disruptions in the continuation of the experiment, and also increase the duration of the trials (Gerlai 1998; Wu et al. 2018).

Novel object recognition test (NORT)

The object recognition apparatus (VJ Instruments, India) was made of an enclosed plexiglass box with a black colored floor (70 × 60 × 30 cm). The experiment was performed according to the procedure described in Ozarowski et al. (2013). Post the habituation trial on the 13th day, the test conducted the next day was divided into three phases – acquisition, inter-trial interval and retention trials. In the acquisition trial on the test day, two identical objects (A1 and A2; biologically inert substance – plastic, sufficiently weighted to stay put) were placed in two opposite corners of the box. The animals were allowed to explore the objects for a period of 3 minutes. The behavior was termed as exploration when the animals' nose was directed towards the object at a distance less than 2 cm and/or touching the object with the nose or sniffing at the object. However, leaning against, standing/sitting on the object and turning round were not noted as exploratory behavior. The animals were then returned to their cages for a period of 30 minutes. At the retention trial, one of the objects used in the acquisition trial was replaced with a novel object (B), and the rats were then allowed to explore the box individually for 3 minutes. The time spent in exploring the familiar (A) and novel (B) object were recorded separately, and the discrimination index (DI) was calculated as (B − A) − /(B + A). A higher DI is reflective of a higher memory capacity.

Biochemical assays

Preparation of tissue homogenates

On completion of the behavioral tests on the 14th day, the rats were anesthetized and sacrificed by cervical dislocation. The brain tissue was rapidly excised and weighed. The tissue samples were then rinsed with cold normal saline, and then homogenized in 0.03 M sodium phosphate buffer (pH 7.4). The homogenates were used in the determination of acetylcholinesterase (AChE) and in the antioxidant assays of malondialdehyde (MDA), reduced glutathione (GSH), catalase (CAT) and nitrite estimation.

Estimation of brain AChE level

The quantitative measurement of AChE levels in the brain was performed according to the method of Ellman et al. (1961). The assay mixture containing 0.4 ml of brain homogenate, 2.6 ml of phosphate buffer (0.1 M, pH 8.0) and 100 µl DTNB was mixed by bubbling air and placing it in a spectrophotometer. Once the reaction mixture was stable, absorbance was noted at 412 nm for the basal reading followed by addition of 5.2 µl of acetylcholine iodide to the cuvette. Any change in absorbance was recorded from zero time until 10 minutes at 25 °C. The rates were calculated as follows:

$$R = (5.74 \times 10^{-4} \times \Delta A)/C_0$$

where R is rate, in moles substrate hydrolyzed per minutes per g of tissue; ΔA is change in absorbance per minute; C_0 is the original concentration of the tissue (mg/ml). Acetylcholinesterase activity was expressed as nmol of acetylthiocholine iodide hydrolyzed/min/mg of tissue.

Determination of MDA

Malondialdehyde levels were estimated using the thiobarbituric acid (TBARS) assay procedure. The supernatant (200 µl) was briefly mixed with 1 ml of 50% trichloroacetic acid in 0.1 M HCl and 1 ml of 26 mM thiobarbituric acid. After mixing on a vortex, the samples were maintained at 95 °C for 20 minutes, after which they were centrifuged at 960×*g* for 10 minutes. The supernatants recovered after centrifugation were read at 532 nm. The results were expressed as U/mg protein (Ishola et al. 2018).

Determination of GSH

GSH levels were determined using the method described by Ellman (1959). Equal amounts of the brain homogenate and 10% TCA were mixed and centrifuged at 2000×*g* for 10 minutes at 4 °C. The supernatant was collected, and to 300 µl of the supernatant, 0.5 ml of phosphate buffer (0.1 M, pH 8.4) and 0.2 ml of DTNB were added. This mixture was shaken vigorously on a mixer, after which the absorbance was read at 412 nm within 15 minutes. The results were expressed as U/mg protein.

Determination of catalase activity

The measurement of catalase activity in the brain was performed according to the colorimetric assay described by Sinha (1972). 100 µl of the brain homogenate was mixed with 2 ml of 0.01 M phosphate buffer (pH 7.0) and 0.4 ml of 0.2 M hydrogen peroxide. The reaction was stopped by addition of 2 ml of dichromate acetic acid reagent. The decomposition of hydrogen peroxide was directly estimated by the net decrease in absorbance at 570 nm. The results were expressed as U/mg protein.

Nitrite estimation

Nitrite estimation was performed using Griess' reagent which is an indicator of nitric oxide production. Briefly, 100 µl of Griess' reagent was mixed with equal volume of the supernatant and vortexed. The absorbance was read at 542 nm. Nitrite concentration was calculated using standard curve for sodium nitrite (0.01–0.1 mg/ml) (Ishola et al. 2018).

Statistical analysis

All statistical analysis were performed using GraphPad Prism Version 7 (GraphPad Software, CA, USA). The results were expressed as mean ± SEM (n=6). Two-way ANOVA followed by Bonferroni's multiple comparisons test was used to determine the difference in exploration time between groups in the NORT. One-way ANOVA followed by Tukey's post hoc multiple comparisons test was used to determine the discrimination index in the NORT, percent spontaneous alternations and mean session durations in the T-CAT, and the biochemical assay results. A $P < 0.05$ was considered statistically significant.

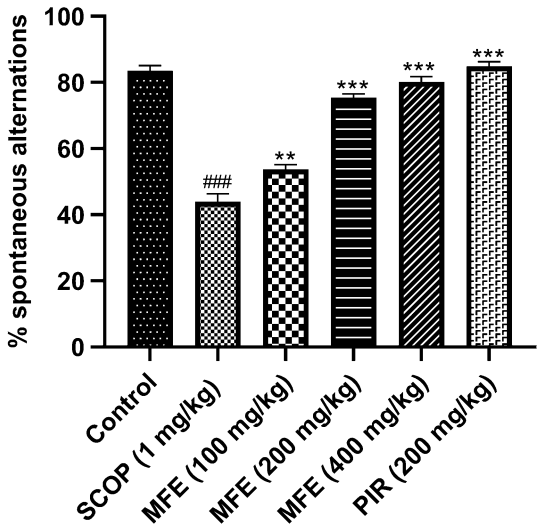

Fig. 2 Effect of MFE on percent spontaneous alternations in the T-CAT (n = 6). $^{###}P < 0.001$ when compared to control, $^{**}P < 0.01$; $^{***}P < 0.001$ when compared to SCOP (vehicle-scopolamine) group; one-way ANOVA followed by post-hoc Tukey's multiple comparison test. SCOP, scopolamine; MFE, *Mesua ferrea* ethanolic extract; PIR, Piracetam

Results

Quantitative phytochemical estimation

Total phenolic content (TPC) and total flavonoid content (TFC)

The total phenolic content of MFE obtained from the calibration curve *(y= R^2= 0.9973)* was 118.35 ± 2.08 mg/g GAE (gallic acid equivalents). The total flavonoid content of MFE was found to be 29 ± 3.46 mg/g RE (Rutin equivalent). The values were taken in triplicates.

Behavioral cognition tests

T-maze continuous alternation task (T-CAT)

The effects of MFE on spatial memory were investigated by assessing the spontaneous alternation behavior exhibited in the T-maze task (Fig. 2). One way ANOVA showed a significant difference between the treatment groups ($P <$ 0.001). Post hoc test demonstrated the significant deficit in percent spontaneous alternations caused by scopolamine in the amnesic control group, when compared with the control group ($P < 0.001$). However, pre-treatment with MFE and piracetam ameliorated the effect of scopolamine on spontaneous alternations. The lowest dose of MFE (100 mg/kg) significantly ($P < 0.01$) alleviated the memory impairment caused by scopolamine when compared with the amnesic

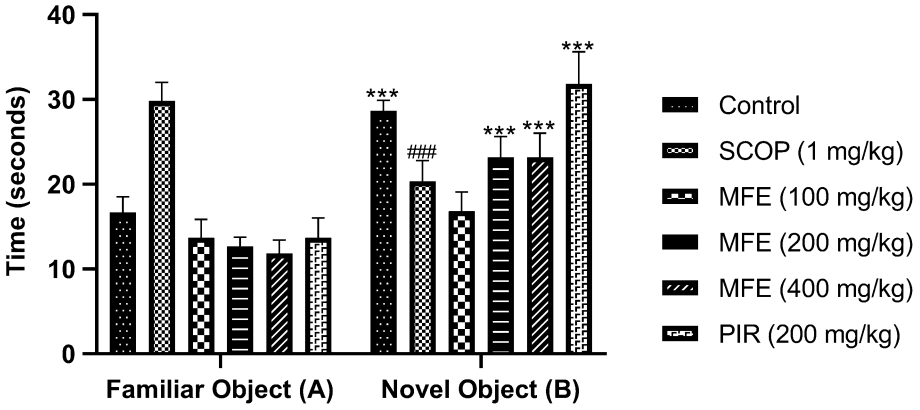

Fig. 3 Effect of MFE on exploration time in the NORT (n = 6). The graph represents the exploration time in seconds of the familiar (A*) and novel (B) objects within 3 min of the retention trial. Each animal serves as its own control. $^{###}P < 0.001$ scopolamine group when compared with exploration time of the familiar object; $^{***}P < 0.001$ when compared with exploration time of the familiar object; two-way ANOVA followed by post-hoc Bonferroni's test. SCOP, scopolamine; MFE, *Mesua ferrea* ethanolic extract; PIR, Piracetam

control group. The other groups, i.e., MFE (200 and 400 mg/kg) and piracetam (200 mg/kg) also showed a significantly higher percent of spontaneous alternation behavior when compared to the amnesic control group ($P < 0.001$). The highest dose of MFE (400 mg/kg) produced similar effects as that of the standard piracetam group. However, there was no visible difference seen in the time taken to complete the sessions between each of the groups (data not shown). The difference in mean session duration between the groups was statistically insignificant ($P < 0.05$).

Novel object recognition test (NORT)

The effects of MFE on non-spatial memory were assessed using the novel object recognition test. Fig. 3 depicts the results of the exploration time of the groups between the novel (B) and familiar (A) object. The control group spent a significantly longer time ($P < 0.001$) exploring the novel object (B). However, the amnesic control group (vehicle + scopolamine) spent lesser time exploring the novel object and explored the familiar object (A) for a significant amount of time ($P < 0.001$), suggesting cognitive impairment. The rats in this group had lost the ability to distinguish between the familiar and novel objects. The group treated with the lowest dose of MFE (100 mg/kg) did spend more time exploring the novel object, however, this was statistically insignificant when compared to the time spent exploring the familiar object. The other groups, treated with MFE (200, 400 mg/kg) and piracetam (200 mg/kg) showed a higher preference toward novel object exploration, and spent a significant time ($P < 0.001$) exploring the same, when compared to the amnesic control (vehicle + scopolamine) group.

The results depicting the discrimination index (DI) data are shown in Fig. 4. The amnesic control rats showed impaired cognitive ability as evident by the decreased

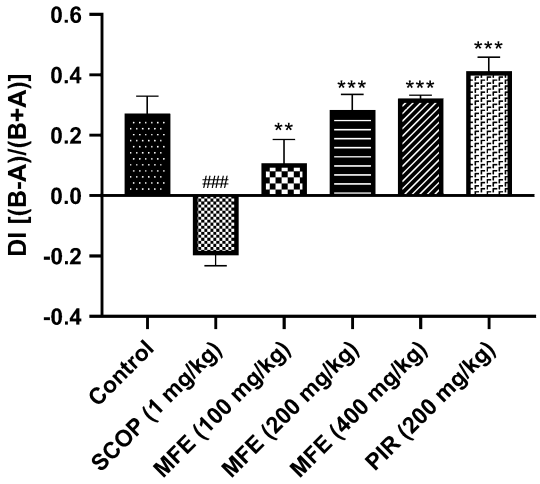

Fig. 4 Effect of MFE on discrimination index in the NORT (n = 6). $^{###}P < 0.001$ when compared to control, $^{**}P < 0.01$; $^{***}P < 0.001$ when compared to SCOP (vehicle-scopolamine) group; one-way ANOVA followed by post-hoc Tukey's multiple comparison test. SCOP, scopolamine; MFE, *Mesua ferrea* ethanolic extract; PIR, Piracetam

discrimination index ($P < 0.001$) as compared to the control group. Administration of the lowest dose of MFE (100 mg/kg) significantly ($P < 0.01$) prevented the memory impairment caused by scopolamine, based on the DI value when compared with the amnesic control group. Post hoc analysis demonstrated that the groups treated with MFE (200 and 400 mg/kg) and piracetam (200 mg/kg) remembered the familiar object better compared to the amnesic control group ($P < 0.001$) as evidenced by their preference to explore the novel object. This indicates amelioration of memory impairment caused by scopolamine and improved cognitive ability in the above-mentioned groups.

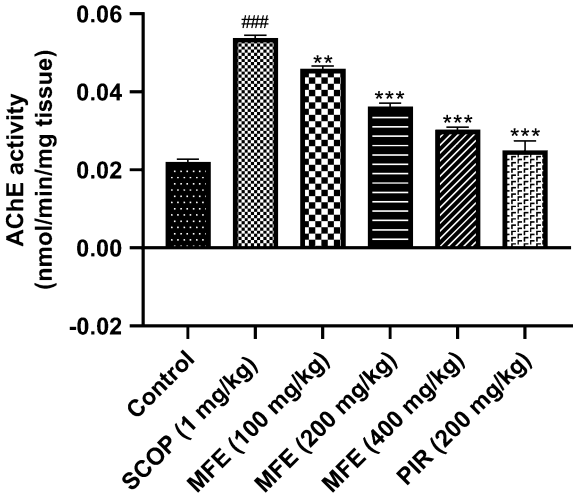

Fig. 5 Effect of MFE on acetylcholinesterase (AChE) activity in the rat brains ($n = 6$). $^{###}P < 0.001$ when compared to control, $^{**}P < 0.01$; $^{***}P < 0.001$ when compared to SCOP (vehicle-scopolamine) group; one-way ANOVA followed by post-hoc Tukey's multiple comparison test. SCOP, scopolamine; MFE, *Mesua ferrea* ethanolic extract; PIR, Piracetam

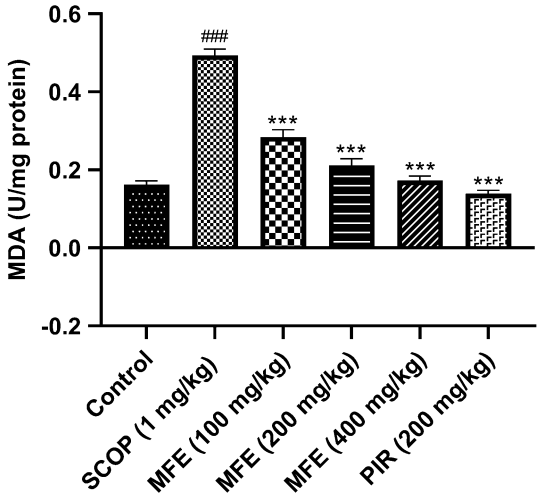

Fig. 6 Effect of MFE on concentration of malondialdehyde (MDA) in the rat brains ($n = 6$). $^{###}P < 0.001$ when compared to control; $^{***}P < 0.001$ when compared to SCOP (vehicle-scopolamine) group; one-way ANOVA followed by post-hoc Tukey's multiple comparison test. SCOP, scopolamine; MFE, *Mesua ferrea* ethanolic extract; PIR, Piracetam

Biochemical assays

Estimation of brain AChE level

Administration of scopolamine (1 mg/kg) produced a significant increase ($P < 0.001$) in brain AChE levels as compared to the control group. Administration of MFE (100, 200 and 400 mg/kg) produced a significant decrease ($P < 0.01$, $P < 0.001$) in AChE levels when compared to the amnesic control group. Furthermore, piracetam (200 mg/kg) showed a significant decrease ($P < 0.001$) in the brain AChE levels when compared with the amnesic control group (Fig. 5).

Determination of MDA

Scopolamine administration (1 mg/kg) significantly increased ($P < 0.001$) malondialdehyde (MDA) content when compared with the control group. Pretreatment of the rats with MFE and piracetam for 14 days significantly decreased ($P < 0.001$) MDA levels when compared to amnesic control (Fig. 6).

Determination of GSH

As revealed in Fig. 7, scopolamine injection (1 mg/kg) produced a significant decrease ($P < 0.001$) in GSH levels, as compared to the control group. The lowest dose of MFE (100 mg/kg) significantly ($P < 0.05$) mitigated this effect compared with the amnesic control group. Pretreatment with MFE (200 and 400 mg/kg) and piracetam (200 mg/kg) prior to scopolamine administration significantly increased ($P < 0.001$) GSH levels in the brain.

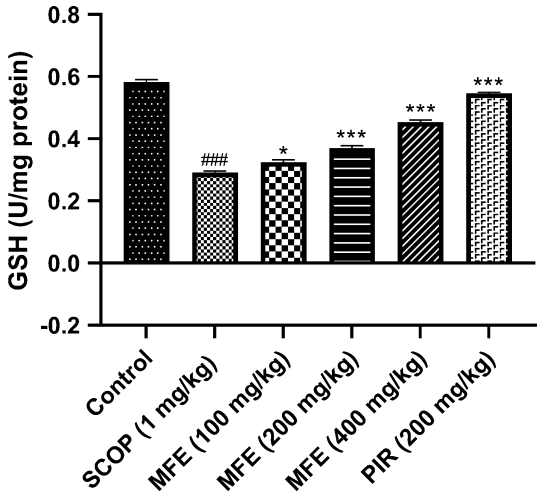

Fig. 7 Effect of MFE on concentration of reduced glutathione (GSH) in the rat brains ($n = 6$). $^{###}P < 0.001$ when compared to control, $^{*}P < 0.05$; $^{***}P < 0.001$ when compared to SCOP (vehicle-scopolamine) group; one-way ANOVA followed by post-hoc Tukey's multiple comparison test. SCOP, scopolamine; MFE, *Mesua ferrea* ethanolic extract; PIR, Piracetam

Determination of catalase activity

Scopolamine (1 mg/kg) significantly decreased ($P < 0.001$) catalase levels in the amnesic control group, when compared

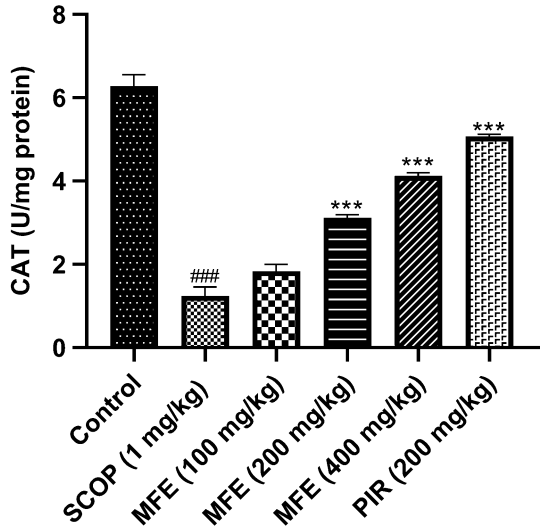

Fig. 8 Effect of MFE on catalase (CAT) activity in the rat brains (n = 6). $^{###}P < 0.001$ when compared to control; $^{***}P < 0.001$ when compared to SCOP (vehicle-scopolamine) group; one-way ANOVA followed by post-hoc Tukey's multiple comparison test. SCOP, scopolamine; MFE, *Mesua ferrea* ethanolic extract; PIR, Piracetam

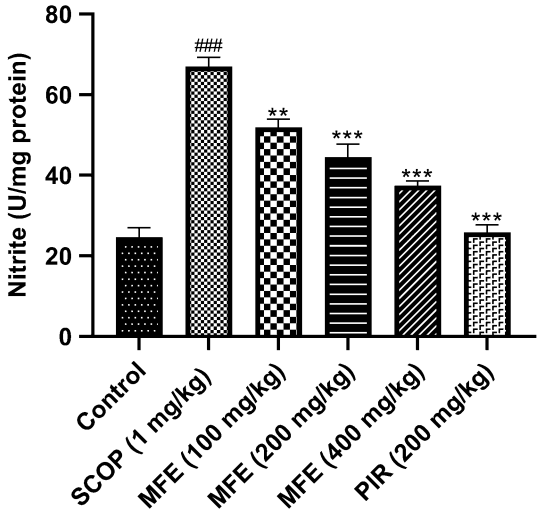

Fig. 9 Effect of MFE on nitrite levels in the rat brains (n = 6). $^{###}P < 0.001$ when compared to control, $^{**}P < 0.01$; $^{***}P < 0.001$ when compared to SCOP (vehicle-scopolamine) group; one-way ANOVA followed by post-hoc Tukey's multiple comparison test. SCOP, scopolamine; MFE, *Mesua ferrea* ethanolic extract; PIR, Piracetam

with control. MFE (200 and 400 mg/kg) and piracetam significantly increased ($P < 0.001$) the levels of catalase when compared with amnesic control group. However, pre-treatment with 100 mg/kg of MFE did not yield significant results (Fig. 8).

Nitrite estimation

Nitrite levels in the amnesic control group were significantly increased ($P < 0.001$) after scopolamine administration, when compared with control. The first group pre-treated with MFE (100 mg/kg) significantly ($P < 0.01$) decreased nitrite levels as compared to amnesic control. Pretreatment with MFE (200 and 400 mg/kg) and piracetam (200 mg/kg) significantly decreased ($P < 0.001$) nitrite levels in the brain (Fig. 9).

Discussion

This study was conducted to assess the nootropic effects of the ethanolic extract of *Mesua ferrea* flowers (MFE) in scopolamine-treated rats. We decided to explore the possible mechanisms of the reported ethnomedicinal claim, and have justified the use of this plant as a brain tonic scientifically. The results of this study proved that pre-treatment with MFE at the doses of 100, 200 and 400 mg/kg successfully ameliorated scopolamine-induced memory deficits.

According to the 'cholinergic hypothesis', deficiency of ACh levels in the brain is a significant factor in the prognosis of AD. Amelioration of AD, therefore, is focused on the enhancement of the cholinergic function of the brain (Yassin et al. 2013). Scopolamine, a non-selective muscarinic receptor antagonist, is used to cause cognitive disruption and is a widely-used paradigm for evaluating potential nootropic agents (Vasileva et al. 2016). Scopolamine produces cognitive impairment including lack of focusing attention, and processing and learning of new information; both in rodents and humans (Ozarowski et al. 2013). Studies have shown that scopolamine causes oxidative stress through its actions on the cholinergic system, leading to memory impairment. Oxidative stress is an imbalance between free radicals and antioxidative defense, both of which are important factors in age-related neurodegeneration (Rabiei et al. 2015). Increase in oxidative stress after scopolamine administration, coupled with disruption of cholinergic function, makes it a suitable model for testing potential nootropic agents (Alibabaei et al. 2014). This study was designed to investigate if MFE had a positive effect on cholinergic function, and also alter the oxidative stress indices caused by scopolamine administration.

The first model employed to test cognitive activity was the T-maze; a test which is highly sensitive to numerous pharmacological manipulations which affect memory formation (Andriambeloson et al. 2014). The role of ACh in spontaneous alternations has been previously established, and is used in assessment of drug effects. Scopolamine adversely effects alternation rate in rats and mice (Spowart-Manning and van der Staay 2004). In agreement with previous studies, scopolamine administration impaired spatial working memory, evidenced by the significant reduction in spontaneous alternation behavior. Pre-treatment of rats with MFE at all

the selected doses significantly improved the relative proportion of spontaneous alternation percentage when compared to the scopolamine-treated group. These results suggest that MFE could enhance short-term or working memory. On the contrast, scopolamine had no effect on the time taken by the rats to complete the sessions. This resonates with the findings of Thouvarecq et al. (2001), where subcutaneous administration of scopolamine hydrobromide did not affect motor skills of the animals. There was no significant difference noticed in the mean session durations between any of the groups.

The novel object recognition test is a task based on a non-rewarded model, which is centered around the spontaneous exploratory behavior of rodents (Pitsikas 2015). The test employs both an exploratory behavior and a memory retention paradigm, as the animal must first spend considerable time exploring the familiar object in the acquisition trial, before it is able to distinguish between the familiar and novel objects in the retention trial (Foyet et al. 2015). MFE significantly increased the preference of the rats towards exploring the novel object as compared to the amnesic control group, and subsequently increased the discrimination index, inferring that MFE has a positive effect on recognition memory.

In order to understand the underlying mechanism of action of MFE, we measured various biochemical parameters, i.e., AChE and antioxidant enzyme levels in the brain. Evidence suggests an extensive loss of cholinergic function in AD, and alteration of AChE activity is a hallmark of AD pathophysiology. Therefore, AChE inhibitors have been a first choice of treatment of AD, with an aim to increase the endogenous acetylcholine levels and relieve symptoms, thereby reversing cognitive impairment (Lima et al. 2009; Khan 2012). In this study, pre-treatment with MFE showed a significant reduction in AChE activity, with the highest dose of MFE producing effects similar to that of the standard drug. Thus, it can be inferred that the nootropic effect of MFE could be due to the alteration of the cholinergic neuronal system.

Agents with more than one pharmacological activity can be more beneficial in the treatment of AD, since they can act on multiple targets simultaneously (Barai et al. 2018). Healthy neural cells contain high concentrations of enzymes such as GSH peroxidase, catalase; and small molecules such as glutathione and ascorbic acid. These substances act as antioxidant defenses which protect the cells from damage caused by ROS. Oxidative stress occurs due to an excessive production of ROS, loss of antioxidant defenses, or a combination of both (Schulz et al. 2000). Since the brain consumes a high amount of oxygen, it is therefore highly predisposed to the detrimental effects of oxidative stress (Ngoupaye et al. 2017). Lipid peroxidation is a process caused by ROS-mediated damage to cellular membranes, which usually produce a number of stable end-products like malondialdehyde (MDA); which can in turn be used as a biomarker to determine oxidative stress (Sultana et al. 2013). Increased nitrite levels are indicative of nitrosative stress in the brain (Ishola et al. 2016), and increased nitric oxide production is observed in neurodegenerative diseases (Ma et al. 2010). Scopolamine administration caused an increase in oxidative and nitrosative stress shown by elevated MDA and nitrite levels. Pre-treatment with MFE significantly lowered the elevated levels of MDA and nitrite in the brain. Glutathione is a tripeptide thiol antioxidant which is involved in cellular detoxification of ROS. Intracellular presence of GSH indicates oxidative stress. (Naik et al 2017; Rahman et al. 2007). Catalase is an enzyme which reduces hydrogen peroxide and prevents hydroxyl radical generation, thereby exerting a protective effect on cells. The activity of CAT is highly impacted by oxidative alterations (Khan 2012). Treatment of the amnesic rats with MFE significantly preserved the activities of GSH and catalase, which were adversely affected by scopolamine. These results denote that MFE possesses potent antioxidant activity by scavenging free radicals, thereby exerting a protective effect against SCOP-mediated oxidative damage.

Quantitative phytochemical estimation revealed MFE to be a rich source of phenolic compounds, primarily, of flavonoids. Reports have established a link between phenolic compounds and antioxidant activity (Habu and Ibeh 2015). Out of the 8000 naturally occurring phenolics, about a half of them are flavonoids; which are present in plants either in their free state or as glycosides. Flavonoids acts as antioxidants, an activity highly dependent on their free hydroxyl group (Sulaiman and Balachandran 2012). There is strong evidence that flavonoids have a positive impact on memory and learning with possible mechanisms including synaptic signal regulations, which affect synaptic plasticity and produce an elongated long-term potentiation (LTP) in the hippocampus (Rendeiro et al. 2012). The results obtained in this study confirm the flavonoid-rich extract of *Mesua ferrea* to possess significant nootropic activity.

Increase in cholinergic neurotransmission is achieved by inhibiting the metabolizing enzyme AChE, therefore making cholinesterase inhibitors an important therapeutic regimen in the treatment of AD (Saxena et al. 2007). This mechanism combined with an antioxidant effect of a drug, would effectively inhibit the progression of AD; therefore making *Mesua ferrea* a potential candidate for the treatment of dementia.

Conclusion

To our best knowledge, this is the first report with scientific evidence on the memory-enhancing effect of *Mesua ferrea* flowers. The extract improved scopolamine-induced spatial

and non-spatial memory deficits in the T-maze continuous alteration and novel object recognition paradigms/tasks/models, decreased brain acetylcholinesterase activity, and demonstrated potent antioxidant capability. However, this can be confirmed by further studies on the identification and isolation of the active constituent/s of this plant responsible for this activity.

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Author's contributions Pallavi Shirsat-John – planned and performed experiments and biochemical analysis, data collection, statistical analysis, and drafting of the article; Tina Saldanha – data analysis and interpretation, critical revision of the article; Swati Kolhe – project planning and supervision; Ziyaurrahman A.R. – supervision of the research work and in-charge of overall direction and planning.

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Availability of data and material To be made available on request.

Code availability Not applicable.

Declarations

Ethical statement The guidelines established by the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Govt. of India, were followed for the animal experiments. The protocols were approved by the Institutional Animal Ethics Committee of AISSMS College of Pharmacy, Pune (Reg. No. – 257/PO/ReBi/S/2000/CPCSEA).

Conflict of interest Shirsat-John Pallavi has no conflict of interest. Tina Saldanha has no conflict of interest. Swati Kolhe has no conflict of interest. A. R. Ziyaurrahman has no conflict of interest.

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