Evaluation of *Cucurbita maxima* Extract against Scopolamine-Induced Amnesia in Rats: Implication of Tumour Necrosis Factor Alpha

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Z. Naturforsch. **69c**, 407 – 417 (2014) / DOI: 10.5560/ZNC.2014-0003
Received January 7 / September 8, 2014 / published online November 12, 2014

Cucurbita maxima (CM) seed oil is commonly used in Indian folk medicine to treat various ailments. We have investigated the effect of CM seed oil on memory impairment induced by scopolamine in rats. Male adult Wistar rats were administered scopolamine 1 mg/kg body weight, i.p. or 1.25 mg/kg body weight, s.c. to induce memory impairment. The nootropic agent piracetam 100 mg/kg body weight, i.p. and CM seed oil 100 and 200 mg/kg body weight, p.o. were administered daily for five consecutive days. The memory function was evaluated in the Morris water maze (MWM) test, the social recognition test (SRT), the elevated plus maze (EPM) test, and the pole climbing test (PCT). Acetylcholinesterase (AChE) activity and oxidative stress parameters were estimated in the cortex, hippocampus, and cerebellum of the brains after completion of the behavioural studies. The effects of scopolamine on the levels of the tumour necrosis factor alpha (TNF-α) transcript were also investigated. Scopolamine caused memory impairment in all the behavioural paradigms along with a significant increase in the AChE activity and oxidative stress in the brain. Scopolamine also caused a significant increase in the expression of TNF-α in the hippocampus. CM seed oil exhibited antiamnesic activity as indicated by a significant reduction in the latency time in the MWM test and decreased social interaction during trial 2 in the SRT. Further, treatment with CM seed oil significantly decreased the AChE activity and malondialdehyde levels and increased the glutathione level in brain regions. CM seed oil also significantly decreased the expression of TNF-α in the hippocampus. The effect of CM seed oil on behavioural and biochemical parameters was comparable to that observed in rats treated with piracetam. These results indicate that CM seed oil may exert antiamnesic activity which may be attributed to the inhibition of AChE and inflammation as well as its antioxidant activity in the brain.

Key words: Cucurbita maxima, Memory Impairment, Tumour Necrosis Factor Alpha

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder clinically characterized by progressive loss of cognitive abilities such as memory, speaking, and problem solving. People over the age of 65 are most frequently affected by AD (Jewart *et al.*, 2005). The pathophysiology of AD is complex and is characterized by the deposition of excessive amounts of amyloid β plaques and neurofibrillary tangles in the brain (Mullane and Williams, 2013). Increasing evidence suggests that the loss of basal forebrain cholinergic neurons accompanied by a reduced acetylcholine (ACh) level is responsible for key symptoms of AD (Wang *et al.*, 2006). It is known that ACh is an important neurotransmitter related to learning and memory (Wang *et al.*, 2006; Tota *et al.*, 2012a). Therefore, acetylcholinesterase (AChE) inhibitors which decrease the ACh degradation have been in clinical use for the symptomatic treatment of AD. However, limited effi-

cacy, poor bioavailability, peripheral cholinergic side effects, and, most importantly, no significant effect on disease progression are the limitations to the success of currently available AChE inhibitors (Bores *et al.*, 1996; Ishola *et al.*, 2012) Therefore, it is an urgent medical need to search for novel therapeutic options for the treatment of various cognitive disorders.

The scopolamine model is based on cholinergic hypofunction and is widely used as experimental model of memory impairment in antidementic drug development. Scopolamine is a muscarinic receptor antagonist which interferes with the memory in animals and humans (Duka *et al.*, 1992; Ishola *et al.*, 2012; Pachauri *et al.*, 2012; Tota *et al.*, 2009). It has been reported to affect acquisition, consolidation, and recalling of memory in animals (Agarwal *et al.*, 2009). Scopolamine-induced memory impairment is associated with a significant increase in AChE activity and malondialdehyde (MDA) levels and a reduced antioxidant, *i. e.* glutathione (GSH), status in the rodent brain (Ishola *et al.*, 2012; Pachauri *et al.*, 2012).

Cucurbita maxima Duchesne (CM), family Cucurbitaceae, is a highly reputed medicinal plant, commonly known as pumpkin. Raw or roasted pumpkin seeds are used as a snack food for human consumption in many cultures all over the world. Pumpkin seed extract has been reported to have antidiabetic, antitumour, antibacterial, anticancer, and antioxidant activities (Villasenor *et al.*, 1995; Caili *et al.*, 2006; Yoshinari *et al.*, 2009; Saha *et al.*, 2011; Nawirska-Olszanska *et al.*, 2013). It has also been found to have strong hypotriglyceridemic and serum cholesterol-lowering effects (Caili *et al.*, 2006). The health benefits of pumpkin seeds are attributed to their macro- and microconstituent composition. They are a rich natural source of proteins, triterpenes, lignans, phytosterols, polyunsaturated fatty acids, antioxidative phenolic compounds, carotenoids, tocopherol, and minerals (Caili *et al.*, 2006). Pumpkin seeds possess valuable dietary and medicinal qualities besides being the source of good-quality edible oils. The present study was therefore designed to assess the effect of CM seed oil on scopolamine-induced amnesia in rats (Caili *et al.*, 2006).

Material and Methods

Plant material

Seeds of the winter squash variety of *Cucurbita maxima* Duchesne (CM) were obtained from the local botanical garden in Lucknow, India, and were identified by taxonomists from the National Botanical Research Institute, Lucknow, India. A voucher specimen (HIPER/HERB/14) has been deposited in our college.

Preparation of extract

The sun-dried seeds of CM were ground and powdered, and 500 g thereof were extracted with *n*-hexane (1 l) at 95 °C for 10 h in a Soxhlet extractor. The extract was filtered and the solvent evaporated under reduced pressure. A light green oil was obtained, which was kept in sealed bottles under refrigeration for analysis.

Chemicals

Unless mentioned otherwise, chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Trizol, Revert Aid first-strand cDNA synthesis kit, and Maxima SYBR Green were obtained from Thermo Fisher Scientific (Mumbai, India). Piracetam (Nootropil®) was purchased from UCB India (Mumbai, India).

Animals

The experiments were carried out with 8- to 9-week-old adult male Wistar rats, weighing 225 – 250 g, obtained from the Laboratory Animal Services Division of the Central Drug Research Institute, Lucknow, India. Research on experimental animals was conducted in accordance with the internationally accepted principles for laboratory animal use and care (1088/07/CPCSEA). They were kept in polyacrylic cages (22.5 cm × 37.5 cm) and were maintained under standard housing conditions (room temperature of 24 – 27 °C and humidity of 60 – 65 %) with a 12-h light/12-h dark cycle. Food and water were available *ad libitum*. The experimental protocols had been approved by the Institutional Animal Ethics Committee.

Experimental design and administration of CM seed oil to rats

Animals were divided into five groups with six rats each. The CM seed oil was administered at a dose of 100 and 200 mg/kg body weight (b.w.) orally (p.o.) for 5 d. Piracetam (100 mg/kg b.w.), a positive control, was administered intraperitoneally (i.p.) for five successive days (Alikatte *et al.*, 2012; Joshi and Parle,

2006a). Scopolamine (1 mg/kg b.w., i.p.) was administered 5 min prior to commencement of day 1 trial (session 1) to induce memory impairment in the Morris water maze (MWM) test (Alikatte *et al.*, 2012). Scopolamine (1.25 mg/kg b.w.) was injected subcutaneously (s.c.) 30 min prior to trial 1 of the social recognition test (SRT) to induce memory impairment (Loiseau *et al.*, 2008). The CM seed oil was mixed with 1 % carboxymethylcellulose (CMC) dissolved in water and administered p.o. One group received vehicle of CM seed oil (1 % CMC, p.o.) for 5 d and i.p. administration of normal saline 1 h prior to day 1 trial and served as control. The control group subjected to the SRT received an s.c. injection of normal saline 30 min prior to the commencement of trial 1.

Assessment of memory in rats

Morris water maze (MWM) test

The MWM consists of a circular pool (45 cm in diameter, 26 cm in height), filled with water [(26 ± 1) °C] to the depth of 20 cm. The pool was divided into four hypothetical quadrants labeled N, S, E, W. The water was made opaque by the addition of semi-skimmed milk. An escape platform was placed in one of the four maze quadrants (the target quadrant) and submerged 1 cm below the water surface. The platform was located in the SW quadrant and was not moved throughout the experiment. The rats were required to find the platform using only distal spatial cues available in the testing room. Cues were maintained constant throughout testing. Four different starting points – N, E, SE, NW – were placed around the perimeter of the pool. On each of the five training days, all four start points were used once in a pseudorandom sequence. The trial began by placing the animals in the water facing the wall of the pool at one of the starting points. If an animal failed to escape onto the platform within 120 s, it was gently placed there and allowed to stay for 30 s. Each animal was subjected to a daily session of four trials for five consecutive days. Escape latency time (*ELT*) to locate the hidden platform in the water maze was noted as an index of learning (Tota *et al.*, 2009, 2012b).

Social recognition test (SRT)

Rats for assessment of the effect of CM seed oil on scopolamine-induced paradigm of social recognition memory impairment were housed individually, and all testing was carried out in their home cage. An unfamiliar (no previous contact) juvenile (50 – 60 g), serving as social stimulus, was introduced, and the time spent by the adult rat in overall social interaction with the juvenile was recorded for a 5-min interval ($T1$). The juvenile rat was then removed to its home cage. After 30 min, the same juvenile was reintroduced, and overall social interaction was again recorded during a second 5-min interval ($T2$). A significant reduction in social interaction time in $T2$ in comparison to $T1$ was considered as successful learning. The recognition index ($RI = T2/T1$) was calculated according to Sujith *et al.* (2012).

Pole climbing test (PCT)

The effect of CM seed oil on scopolamine-induced memory impairment was studied by using Cook's PCT. The apparatus consisted of a 25 cm × 25 cm × 40 cm chamber enclosed in a dimly lit, sound attenuated box. An electric shock was delivered to the grid floor of the chamber. A stainless steel pole, 2.5 cm in diameter, was suspended through a hole in the upper centre of the chamber. When an electrical stimulus was given, the rat had to jump onto the pole (shock-free zone) to avoid foot shock. Jumping on the pole terminated the shock, and this was classified as an escape, while jumping prior to the onset of the shock (after a buzzer sound) was considered as avoidance. The session was terminated after completion of ten trials with intervals of 30 s. After completion of the training, the animals were given the respective treatment and again subjected to the test procedure for five consecutive days. A significant reduction in escape latency time was considered as successful retention of avoidance memory (Goverdhan *et al.*, 2012).

Elevated plus maze (EPM) test

The EPM served as the exteroceptive behavioural model to evaluate learning and memory in mice. The apparatus consisted of two open arms (16 cm × 5 cm) and two covered arms (16 cm × 5 cm × 12 cm). The arms extended from a central platform (5 cm × 5 cm), and the maze was elevated to a height of 25 cm from the floor. On the first day, each animal was placed at the end of an open arm, facing away from the central platform. Transfer latency (*TL*) was taken as the time taken by an animal to move into one of the covered arms with all its four legs. *TL* was recorded on the first

day. If the animal did not enter into one of the covered arms within 90 s, it was gently pushed into one of the two covered arms, and the *TL* was assigned as 90 s. The animal was allowed to explore the maze for 10 s and was then returned to its home cage. Memory retention was examined 24 h after the first day trial (Kulkarni *et al.*, 2010).

Locomotor activity

After a period of 15 min for acclimatization, animals were placed individually in the activity cage for 10 min, and their activity was monitored. The photocell counts were noted, and decrease or increase in locomotor activity was calculated.

Brain tissue collection and preparation of brain homogenates

After completion of the behavioural studies on day 5, rats were sacrificed by anaesthesia with an overdose of diethyl ether. Brains were removed from all rats, kept on an ice-cold plate, and then dissected into cortex, cerebellum, and hippocampus. The brain tissue samples were washed, and 10% (w/v) homogenate of brain samples was prepared in ice-cold 0.1 M sodium phosphate buffer, pH 7.4, and used to measure the MDA and GSH levels, and the AChE activity (Tota *et al.*, 2012a).

Malondialdehyde (MDA)

To a volume of 0.5 ml of tissue homogenate, 0.5 ml distilled water and 1.0 ml 10% trichloroacetic acid (TCA) were added, mixed well, and centrifuged at $3000 \times g$ for 10 min. To 0.2 ml supernatant, 0.1 ml thiobarbituric acid (TBA) (2%, w/v) was added. The total solution was placed in a water bath at 80 °C for 40 min and then cooled to room temperature. The absorbance of the clear supernatant was measured at 532 nm. The MDA level was expressed as nmol/mg protein (Awasthi *et al.*, 2012).

Glutathione (GSH)

The GSH level was measured by its reaction with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (Ellman reagent) to yield a yellow chromophore whose absorbance was measured spectrophotometrically. The brain homogenate was mixed with an equal amount of 10% TCA and centrifuged at $200 \times g$ for 10 min at 4 °C. The supernatant was used for GSH estimation. To 0.1 ml of processed tissue sample, 2 ml of sodium phosphate buffer (pH 8.4), 0.5 ml of DTNB (0.2% in 0.1 M sodium phosphate buffer, pH 8.4), and 0.4 ml of double-distilled water were added, and the mixture was shaken vigorously on a vortex. The absorbance was read at 412 nm within 15 min. The GSH level was expressed as μg/mg protein (Awasthi *et al.*, 2012).

Acetylcholinesterase (AChE) activity

The homogenate of rat brain regions (500 μl) was mixed with an equal volume of 1% Triton X-100 and centrifuged at $10{,}000 \times g$ at 4 °C for 60 min. The supernatant was collected and used for determination of the AChE activity. The assay mixture contained 0.4 ml of supernatant, 2.4 ml of sodium phosphate buffer (pH 8.0), 20 μl of acetylthiocholine iodide (154.4 mM in 0.1 M sodium phosphate buffer, pH 8.0), and 100 μl of DTNB (0.2% in 0.1 M sodium phosphate buffer, pH 8.4). The change in absorbance was measured at 412 nm for 10 min at 2-min intervals. The activity of AChE was expressed as μmol/(min mg protein) (Awasthi *et al.*, 2012).

Protein

Protein contents of brain homogenates for MDA and GSH determinations were measured as described by Lowry *et al.* (1951), and in extracts for AChE activity measurements by the method of Wang and Smith (1975) using bovine serum albumin (BSA) as standard.

Total RNA preparation and reverse transcriptase-polymerase chain reaction (RT-PCR)

After decapitation, the brain was quickly removed and the hippocampus was isolated. Total RNA was isolated from the hippocampus using the Trizol procedure as described previously (Singh *et al.*, 2013). Complementary DNA was synthesized from total RNA with a Revert Aid first-strand kit. Tumour necrosis factor alpha (TNF-α) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript levels were determined by semi-quantitative RT-PCR. Primer sequences used were, for TNF-α: CACCACGCTCTTCTGTCTACTGAAC (sense), CCGGACTCCGTGATGTCTAAGTACT (antisense); and for GAPDH: CACGGCAAGTTCAATGGCACA (sense), GAATTGTGAGGGAGAGTGCTC (antisense). Amplification conditions were similar to those described previously

(Sun *et al.*, 2004). The PCR products were analysed on agarose gels and quantified using a calibrated BioRad densitometer (Berkeley, CA, USA).

Statistical analysis

Results are expressed as mean ± S.E.M. The statistical significance of differences between the groups was determined by one-way ANOVA, followed by Bonferroni's post hoc test using the software GraphPad Prism 5 (San Diego, CA, USA) and $P < 0.05$ was considered statistically significant.

Results

Phytochemical investigations

A preliminary phytochemical screening for the presence of flavonoids, steroids, alkaloids, and triterpenoids was done by using specific tests including the Shinoda test, the Salkowski reaction, the Liebermann-Burchard reaction, as well as Wagner's, Mayer's, and Hager's test, respectively, which collectively indicated the presence of flavonoids, steroids, traces of alkaloids, and phytosterols. The following properties of CM seed oil were determined by standard techniques: ash value (4.89%), loss on drying by using a muffle furnace (7%), acid-insoluble ash (0.74%), and water-soluble ash (3.2%). In addition, the following parameters were determined: saponification value (190.34 ± 1.07), iodine value (102.34 ± 1.32), acid value (0.98 ± 0.07), and refractive index (0.92 ± 0.001).

Acute toxicity study

The CM seed oil was safe for use over a wide range of concentrations, as no gross behavioural changes and cases of death were recorded for up to 2000 mg/kg b.w. after oral administration in an acute toxicity study with mice carried out as described previously (Joshi and Parle, 2006b).

Effect of CM seed oil on scopolamine-induced memory impairment in the Morris water maze (MWM) test

In control groups, a significant decrease ($P < 0.01$) in latency time during the 4th and 5th sessions was observed in comparison to the first session. Administration of scopolamine caused memory impairment as there was no significant change in the latency time ($P > 0.05$) throughout all water maze sessions (Fig. 1). Treatment with the standard nootropic drug piracetam prevented scopolamine-induced amnesia as indicated by a significant reduction ($P < 0.01$) in the latency time during the 3rd, 4th, and 5th sessions in comparison to the first session. The CM seed oil ameliorated

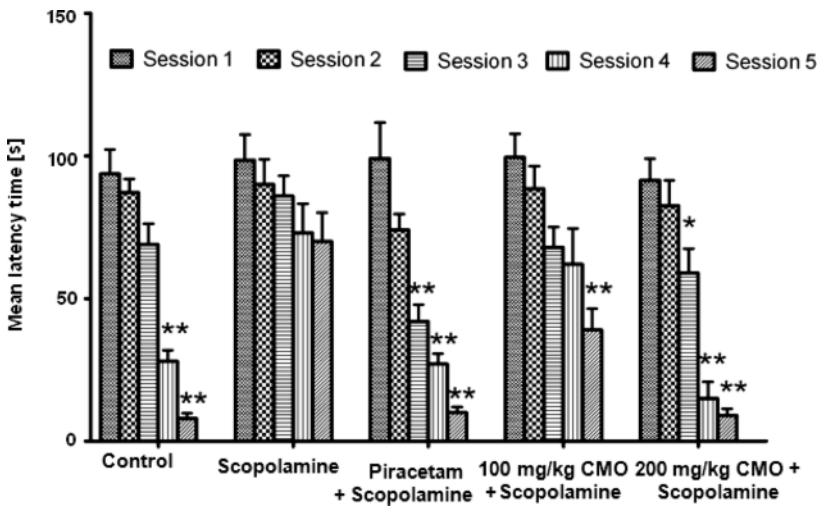

Fig. 1. Effect of CM seed oil (CMO) on scopolamine-induced memory impairment in rats using the Morris water maze (MWM) test. Data are expressed as mean escape latency time (s) ± S.E.M. * Significant difference ($^{*}P < 0.05$ and $^{**}P < 0.01$) in comparison to session 1 of the respective groups.

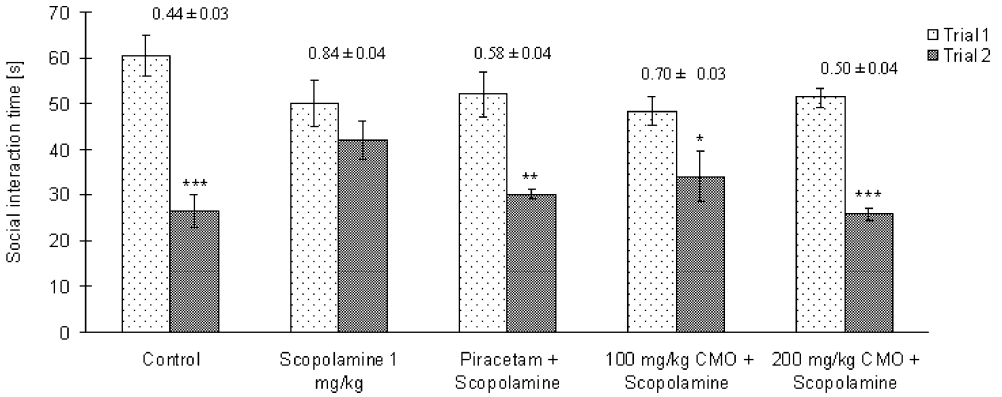

Fig. 2. Effect of CM seed oil (CMO) on scopolamine-induced memory impairment in rats using the social recognition test (SRT). Data are expressed as mean social interaction time (s) ± S.E.M. and mean recognition index ($RI = T2/T1$) ± S.E.M. (numbers above columns). *Significant difference ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$) in comparison to trial 1 of the respective group.

scopolamine-induced memory impairment as a function of its dose. At a dose of 100 mg/kg b.w., CM seed oil significantly decreased ($P < 0.05$) the latency time during the 5th session in comparison to session 1, while at 200 mg/kg b.w. the latency time was decreased significantly ($P < 0.01$) from the 3rd session onward. No significant change was observed between the latency times of session 1 ($P > 0.05$) and session 2 ($P > 0.05$) of all groups (Fig. 1).

Effect of CM seed oil on scopolamine-induced memory impairment in the social recognition test (SRT)

As shown in Fig. 2, in control rats there was a significant reduction ($P < 0.01$) in the social interaction time in trial 2 ($T2$) in comparison to trial 1 ($T1$) indicating successful learning. Scopolamine, administered 30 min prior to $T1$, caused impairment in the social recognition, as there was no significant difference ($P > 0.05$) in the social interaction times of $T1$ and $T2$. Scopolamine-induced impairment in the recognition memory was ameliorated by piracetam as indicated by a significant reduction ($P < 0.01$) in the social interaction time of $T2$ in comparison to $T1$. Administration of CM seed oil prevented scopolamine-induced memory impairment at both doses as shown by a significant decrease ($P < 0.05$) in the social interaction time of $T2$ in comparison to that of $T1$. Further, analysis of the recognition index (RI) showed that scopolamine caused a significant increase ($P < 0.01$) in the RI in comparison to the control group, and this effect was prevented both by piracetam and CM seed oil.

Effect of CM seed oil on scopolamine-induced memory impairment in the elevated plus maze (EPM) test

The effects of scopolamine, piracetam, and CM seed oil on rats in the EPM test were evaluated on days 7 and 8. Transfer latency on the 7th day of drug treatment reflected learning behaviour of the animals, whereas the transfer latency on the next day (day 8) reflected retention of memory. As shown in Fig. 3, the scopolamine group showed a significant increase ($P < 0.05$) in transfer latency values in the acquisition as well as

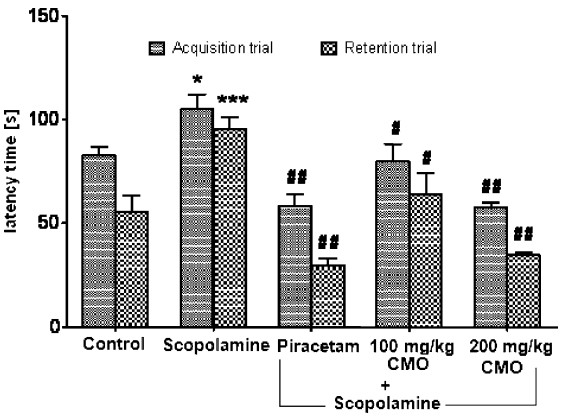

Fig. 3. Effect of CM seed oil (CMO) on scopolamine-induced memory impairment in rats using the elevated plus maze (EPM) test. Data are expressed as mean latency time (s) ± S.E.M. *Significant difference ($^{*}P < 0.05$ and $^{***}P < 0.001$) in comparison to the respective trial of the control group. #Significant difference ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) in comparison to the scopolamine group.

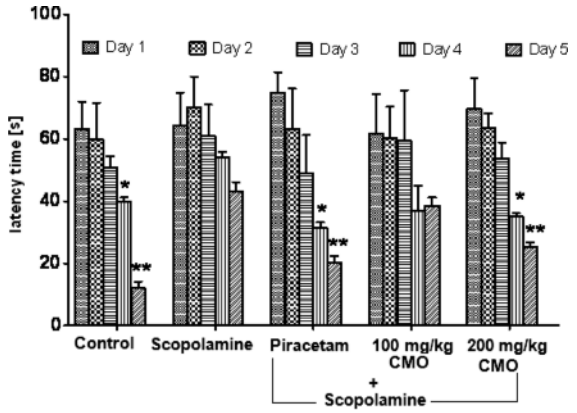

Fig. 4. Effect of CM seed oil (CMO) on scopolamine-induced memory impairment in rats using the pole climbing test (PCT). Data are expressed as mean latency time (s) ± S.E.M. *Significant difference ($^{*}P < 0.05$ and $^{**}P < 0.01$) in comparison to day 1 of the respective group.

the retention period over those of the vehicle control rats, indicating impairment in learning and memory. Preventive treatment with CM seed oil improved the memory in the EPM test in a dose-dependent manner. The CM seed oil caused a significant reduction ($P < 0.05$) in the acquisition and retention latency in comparison to the scopolamine-treated group.

Effect of CM seed oil on scopolamine-induced memory impairment in the pole climbing test (PCT)

There was a significant ($P < 0.05$) reduction in the latency time in the control group on days 4 and 5, whereas in the scopolamine group no significant ($P > 0.05$) reduction was seen (Fig. 4). Administration of piracetam or CM seed oil (200 mg/kg b.w.) significantly ($P < 0.05$) reduced the latency time, indicating prevention of scopolamine-induced amnesia. The lower dose of CM seed oil had no significant effect.

Effect of CM seed oil on locomotor activity

No significant change ($P > 0.05$) in the spontaneous locomotor activity was observed among different groups at both concentrations of the oil.

Effect of CM seed oil on acetylcholinesterase (AChE) activity

As shown in Fig. 5, there was a significant increase in the AChE activity in cortex ($P < 0.01$), hippocampus ($P < 0.01$), and cerebellum ($P < 0.05$) of scopolamine-treated rats as compared to the control, and piracetam significantly ($P < 0.05$) suppressed this increase. Preventive treatment with CM seed oil (200 mg/kg b.w.) significantly decreased the AChE activity in the cortex ($P < 0.01$), hippocampus ($P < 0.05$), and cerebellum ($P < 0.05$) of scopolamine-treated rats, but there was no such effect at 100 mg/kg b.w. CM seed oil.

Effect of CM seed oil on malondialdehyde (MDA) level

MDA levels were determined in rat brain regions after the completion of the behavioural studies. The MDA level was significantly increased in the cortex ($P < 0.05$), hippocampus ($P < 0.01$), and cerebellum ($P < 0.01$) of scopolamine-treated rats as compared to the control group, and this effect was significantly ($P < 0.05$) suppressed by piracetam. Preventive treatment with 200 mg/kg b.w. of CM seed oil significantly decreased the MDA levels in the cortex, hippocampus, and cerebellum ($P < 0.05$ in each case) of scopolamine-injected rats, whereas the lower dose was not effective ($P > 0.05$) (Fig. 6).

Effect of CM seed oil on glutathione (GSH) level

GSH levels were determined in the brain regions after the completion of the behavioural studies. As

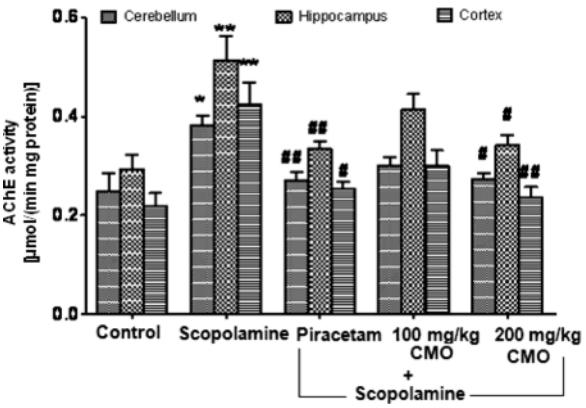

Fig. 5. Effect of CM seed oil (CMO) on acetylcholinesterase (AChE) activity in rat brain regions. Data are expressed as mean AChE activity [μmol/(min mg protein)] ± S.E.M. *Significant increase ($^{*}P < 0.05$ and $^{**}P < 0.01$) in comparison to the control. #Significant decrease ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) in comparison to the scopolamine group.

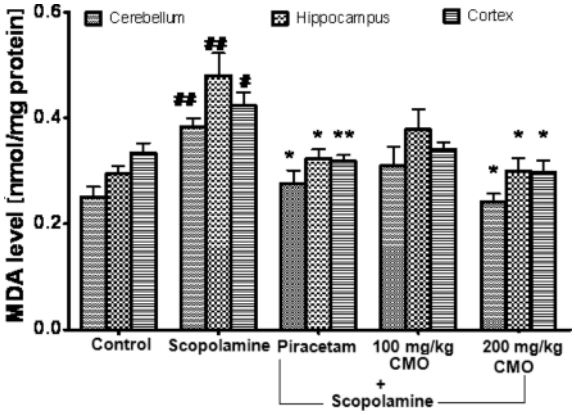

Fig. 6. Effect of CM seed oil (CMO) on malondialdehyde (MDA) levels in rat brain regions. Data are expressed as mean MDA level (nmol/mg protein) ± S.E.M. $^{\#}$Significant increase ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$) in comparison to the control. *Significant decrease ($^{*}P < 0.05$ and $^{**}P < 0.01$) in comparison to the scopolamine group.

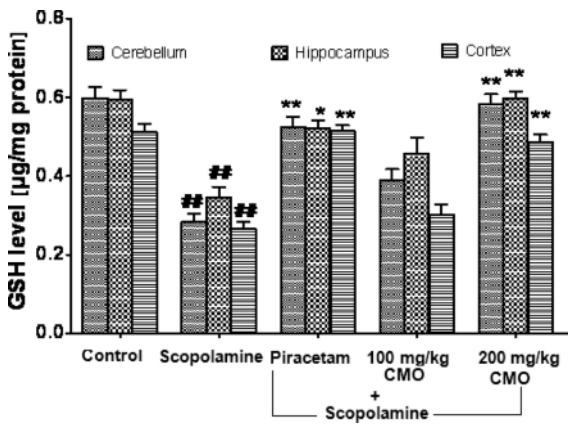

Fig. 7. Effect of CM seed oil (CMO) on the glutathione (GSH) levels in rat brain regions. Data are expressed as mean GSH level (μg/mg protein) ± S.E.M. $^{\#}$Significant decrease ($^{\#\#}P < 0.01$) in comparison to the control. *Significant increase ($^{*}P < 0.05$ and $^{**}P < 0.01$) in comparison to the scopolamine group.

shown in Fig. 7, a significant decline in the levels of GSH was observed in the cortex, hippocampus, and cerebellum ($P < 0.01$ in each case) of the scopolamine-treated animals as compared to the control, while there was a significant rise ($P > 0.01$) in the level of GSH in all three brain regions of the rats treated with 200 mg/kg b.w. CM seed oil in comparison to the scopolamine-treated group. However, the lower dose of CM seed oil had no significant ($P > 0.05$) effect on

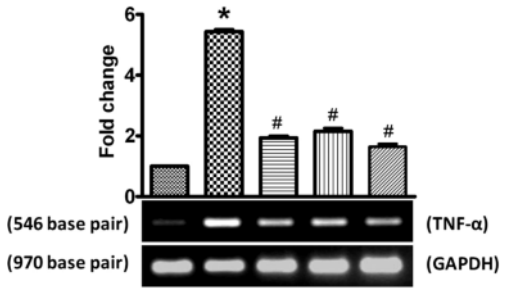

Fig. 8. Effect of CM seed oil (CMO) on TNF-α transcript abundance in rat brain regions. The bar diagram represents fold change in the transcript levels of TNF-α. The lower panel shows the expression profile according to the RT-PCR analysis. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$ in comparison to the control. $^{\#}P < 0.05$ in comparison to the scopolamine-treated groups.

the GSH level in the brains of scopolamine-injected rats (Fig. 7).

Expression of inflammatory mediators

TNF-α expression was measured in the hippocampus region of the rats. An approximately 5.5-fold increase in the abundance of the TNF-α transcript was observed in the scopolamine-treated group, and this increase was significantly suppressed in both the piracetam- and CM seed oil-treated groups (Fig. 8).

Discussion

In the present study, CM seed oil was administered orally, because it would be taken up this way when pumpkin seeds are consumed as a snack, whereas piracetam was given i.p., because this route of administration requires lesser amounts.

CM seed oil prevented scopolamine-induced amnesia in rats in all of the behavioural tests, *i. e.* the MWM test, SRT, EPM test, and PCT. Further, the antiamnesic effect of CM seed oil was associated with a significant reduction in oxidative stress and AChE activity in rat brain regions.

It is well known that scopolamine impairs learning and memory in rodents and humans (Duka *et al.*, 1992; Ishola *et al.*, 2012; Pachauri *et al.*, 2012). Therefore, this model has been widely used in antidementia drug development (Agarwal *et al.*, 2009; Pachauri *et al.*, 2012; Tota *et al.*, 2012a). In the present study, systemic administration of scopolamine in rats 5 min prior to session 1 of the MWM test induced memory

impairment. There was no significant change in the latency time throughout all MWM sessions in the scopolamine-treated group. Further, scopolamine, when administered 30 min prior to trial 1, caused a social recognition memory deficit, as shown by the absence of a significant reduction in the social interaction time in trial 2 ($T2$) in comparison to trial 1 ($T1$). These observations are in agreement with a large number of previous studies reporting a memory impairing effect of scopolamine in rodents (see references cited above). As reported earlier, the clinically used nootropic agent piracetam ameliorated scopolamine-induced memory impairment in the two behavioural tests (Alikatte *et al.*, 2012; Joshi and Parle, 2006b).

Preventive administration of CM seed oil for five days ameliorated the scopolamine-induced memory impairment in a concentration-dependent manner. In the MWM test, CM seed oil caused a significant reduction in the latency time to reach the hidden platform in comparison to the scopolamine-treated group, thus indicating successful learning. These results suggest that the antiamnesic effect of CM seed oil on scopolamine-induced memory deficit may be mediated via facilitation of the central cholinergic nervous system.

To further confirm the antiamnesic effect of CM seed oil, rats were subjected to the SRT. Scopolamine-treated rats exhibited a significantly higher recognition index (RI) than control rats, indicating impairment in the social recognition memory as a result of scopolamine injection. In CM seed oil-treated rats, a dose-dependent reduction in the time spent in interaction with a juvenile rat was observed during $T2$ as compared to $T1$. Further, the scopolamine-induced elevation in *RI* was also significantly reduced by CM seed oil, thus substantiating the above findings. There was no significant change in the spontaneous locomotor activity in any group, suggesting that the behavioural effects are not associated with any change in this activity.

Scopolamine-induced memory impairment has been reported to be associated with cholinergic hypofunction and oxidative stress in the brain (Ishola *et al.*, 2012; Tota *et al.*, 2012a). To understand the possible mechanism of action of CM seed oil, AChE activities and antioxidant levels were investigated in three brain regions. Scopolamine caused a significant increase in the AChE activity in the cortex, hippocampus, and cerebellum, and this effect was counteracted by both CM seed oil and piracetam. The reduction of the AChE activity by these agents could lead to an increased ACh level in the brain which may be responsible for their antiamnesic effect.

Many preclinical and clinical studies have provided evidence that oxidative stress is involved in the pathogenesis of Alzheimer's disease. In the present study, MDA and GSH levels were determined in the brain regions as indicators of lipid peroxidation and the endogenous antioxidant status, respectively. An increased MDA level suggests neuronal degeneration. GSH is an endogenous antioxidant which plays a major role in the maintenance of the intracellular redox state. In the brain regions of scopolamine-treated rats, there was a significant increase in the MDA level, and a decrease in the GSH level, compared to the control, indicating elevated oxidative stress. The nootropic agent piracetam significantly decreased the MDA level and increased the GSH level in the rat brain, indicating an antioxidative action as reported previously (Alikatte *et al.*, 2012). Likewise, the preventive administration of CM seed oil reduced the scopolamine-induced oxidative stress as is evident from the significantly reduced MDA levels and elevated GSH levels. Since memory impairment is an inflammatory disorder and as TNF-α is a cytokine playing an important role in the progression of inflammatory diseases (Perry *et al.*, 2001), we evaluated the effect of CM seed oil on the scopolamine-induced elevation of the TNF-α transcript level. Prevention of this elevation by CM seed oil indicates its potent anti-inflammatory activity. Our study has revealed a correlation between the antiamnesic effect of CM seed oil and its effects on the antioxidant levels and AChE activity, thus in future studies, the component(s) of CM seed oil responsible for the reduction of oxidative stress and the prevention of the elevation of AChE activity should be identified and their neuroprotective effect be investigated in more detail.

Acknowledgement

The authors are grateful to the Hygia Institute of Pharmaceutical Education and Research, Lucknow, India, for providing necessary facilities to carry out this research. The authors would also like to thank the National Botanical Research Institute, Lucknow, for plant authentication and the Central Drug Research Institute, Lucknow, for providing animals. The study was supported by a financial grant to T. J. from the Hygia Institute of Pharmaceutical Education and Research. The funding agency had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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