Targeting Serotonergic Pathway for Anti-amnesic Activity by *Morus alba* L.

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**ABSTRACT**

The present study aimed to evaluate the anti-amnesic potential of ethyl acetate soluble fraction of methanolic extract of *Morus alba* L (EASF). The effect of EASF of *Morus alba* on learning and memory was evaluated by using object recognition test (ORT), elevated plus maze (EPM) and water maze test (WMT). EASF was administrated in doses of 25, 50 and 100 mg/kg p.o. Scopolamine (1 mg/kg, i.p.) was used to induce impairment in memory. The effect of EASF was also studied on serotonin-induced contractions of isolated rat fundus. EASF significantly improved discrimination index (DI) in ORT. Pretreatment with EASF significantly increased transfer latency (TL) and swimming time in target quadrant in EPM and WMT respectively. The result of the *in-vitro* study showed that EASF inhibited serotonin-induced contractions on rat fundus, possessing anti-serotonergic activity. Thus, the present study revealed that EASF of *M. alba* has significantly improved learning and memory through its anti-serotonergic mechanism. *Morus alba* may be useful for the treatment of dementia and other cognitive disorders.

**Keywords:** Elevated plus maze, transfer latency, water maze test.

**INTRODUCTION**

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder which occurs gradually leading to memory loss, change in behavior, and changes in personality and resulted into death.[1] In this disorder disturbance occurs in different cortical functions e.g learning and memory, judgment.[2] Worldwide about 26 million population sufferers from Alzheimer’s disease and it is expected to quadruple by the year 2050 as an unfortunate by product of increasing life expectancy. [3] Clinically, AD is characterized by progressive cognitive decline which is associated with impairment in activities of daily living and behavioral symptoms such as depression, agitation, psychosis, and aggression that can vary in severity according to the stage of the disease. The causes of AD are still rather poorly understood, and it is now considered that AD is of heterogenous origin, with various etiologies leading to the hallmark plaque and tangle pathology and profound neuronal loss. At the neurochemical level, some 20 to 30 years ago AD was thought to be primarily a disorder of cortical cholinergic innervation the so-called cholinergic hypothesis of aging and AD.[4] The complexity and diversity of the cognitive and behavioral abnormalities observed in AD, however, it is highly likely that dysfunction of multiple neurotransmitter systems is at play. The cholinesterase inhibitors designed specifically to boost residual cholinergic transmission, are at best and only partially effective in treating the clinical manifestations of AD.[5] More recently, deficits in the serotonergic, GABAergic, noradrenergic, dopaminergic, and glutamatergic pathways have all
been described to track, to a greater or lesser extent, with cognitive and/or behavioral changes in AD. From a therapeutic perspective, the serotonergic system appears to be a particularly attractive target, because it has been implicated not only in cognitive processes but also in depression, psychosis, and aggression. A decline in cholinergic neurotransmission may be a major factor in CNS deterioration of cognitive abilities. The cholinergic hypothesis is based on specific dysfunctions in cholinergic markers in the brains of patients suffering from age-related memory loss, the induction of behavioural impairments in animals following lesion of central cholinergic systems. Along with cholinergic neurotransmission, serotonergic neurotransmission play an important role in cognitive function. For example, there is considerable evidence that acetylcholine release is under an inhibitory 5-hydroxytryptaminergic tone. Thus, systemically administered 5-HT agonists and 5-methoxy-N, N-dimethyltryptamine increase striatal acetylcholine levels suggesting reduced release, and in in-vitro experiments 5-HT agonists reduce acetylcholine release from striatal slices. Conversely, 5-HT synthesis inhibition or destruction of 5-HT cells in the dorsal raphe nucleus can potentiate acetylcholine release and turnover in the striatum, cortex and hippocampus. The effects in the cortex and hippocampus may be particularly relevant to an understanding of changes in cognitive performance, and it has been concluded that the inhibitory action of a 5-HT pathway on hippocampal cholinergic activity may be relevant to memory. Therefore, it could be hypothesized that the actions of 5-HT to reduce acetylcholine release may afford a novel site of drug action to influence cholinergic function and cognition. It is reported that 5-HT3 receptors mediate the inhibitory effects of 5-HT on acetylcholine release. Ayurveda is a real holistic Indian medicinal system of diagnosis and treatment of diseases and disorders preached since pre-ancient period. In recent years, there has been an exclusive increase in the interest of the scientific community to explore the pharmacological actions of several herbs of Ayurveda. Morus alba L. (Moraceae) is 3-6 meters high tree and commonly found in India, China and Japan. The common name of Morus alba is known as white mulberry. The leaves of white mulberry are used as the main food source for the silkworms. In Chinese medicine mulberry has long history of medicinal use. Traditionally, the mulberry fruit has been used as a medicinal agent to nourish the blood, benefit the kidneys and treat weakness, fatigue, anemia and premature graying of hair. The medicinal uses of the plant reported so far include analgesic, antiasthmatic, antirheumatic, antitussive, astringent, diaphoretic, diuretic, emollient and expectorant, hypnotensive and brain tonic. Morus alba have been extensively studied for its anti-HIV activity, anti-diabetic activity, antibacterial activity, antianaphylactic activity, neuroprotective activity, antioxidative activity, hypolipidemic activity and anti-anxiety activities. The phytoconstituents present in the plant are tannins, phytosterols, sitosterols, saponins, triterpenes, flavanoids, benzofuran derivatives, morusimic acid, anthocyanins, anthroquinones, glycosides and oleancolic acid as the main active principles. Although several medicinal uses have been reported for Morus alba, no investigative report pertaining to its an anti-amnesic potential through serotonergic mechanism exists. Hence, an attempt has been made to evaluate the anti-amnesic activity of Morus alba.

MATERIALS AND METHODS
Plant material
The collection of leaves of Morus alba were done in the month of August from local area of Nashik and authenticated at the Botanical Survey of India, Pune, where a voucher specimen was submitted (Voucher no. NVMA-2).

Extract preparation
The leaves were shade dried and reduced to coarse powder. The powdered M. alba leaves were defatted with Petroleum ether (60 - 80°C) under Soxhlet extraction. The defatted marc was air-dried and put for exclusive extraction under Soxhlet using methanol. The extract was then filtered and evaporated to dryness under reduced pressure (yield 5.2% w/w). The methanolic extract was exhaustively extracted with ethyl acetate to give ethyl acetate soluble (EASF 2.1% w/w) and ethyl acetate insoluble fractions (EAF 1.9% w/w). Suspensions of EASF was prepared in distilled water using Tween 80 (0.2% v/v) as suspending agent. The extract was administered in doses of 25, 50 and 100 mg/kg per orally (p.o.). Control group was given only vehicle (0.2% v/v, Tween 80) in volume equivalent to that of the plant extract.

Experimental animals
Male Swiss albino mice (22 - 25 g) and wistar rats (120-150 g) were used for the study. Animals were housed in polypropylene cages and maintained under the standard laboratory environmental conditions; temperature 25± 2°C, 12: 12 h L: D cycle and 50 ± 5% RH with free access to food and water ad libitum. Animals were acclimatized to laboratory conditions before the test. Each group consisted of five (n = 5) animals. All the experiments were carried out during the light period (08:00-16:00 h). The studies were carried out in accordance with the guidelines given by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi (India). The Institutional Animal Ethical Committee of M.V.P.S College of Pharmacy, Nashik approved the protocol of the study (IAEC/2011/08).

Drugs and chemical
Scopolamine (Sigma- Aldrich, USA) and Ondansetron (Alkem, India) were used for study. All the chemicals
used were of analytical grade and purchased from standard manufacturers.

**Treatment schedule**
The animals were divided into six groups, each containing five mice. Group I - Control (Vehicle for EASF (0.2% v/v Tween 80 in distilled water, 10 ml/kg, p.o.), Group II - Scopolamine - (Vehicle 0.2% v/v Tween 80 in distilled water (10 ml/kg, p.o.) + Scopolamine (1 mg/kg, i.p.), Group III - Ondansetron (Ondansetron 0.5 mg/kg, i.p.) + Scopolamine (1 mg/kg, i.p.), Group IV - EASF - 25 (EASF of M. alba extract 25 mg/kg, p.o.) + Scopolamine (1 mg/kg, i.p.), Group V - EASF - 50 (EASF of M. alba extract 50 mg/kg, p.o.) + Scopolamine (1 mg/kg, i.p.) Group VI - EASF- 100 (EASF of M. alba extract 100 mg/kg, p.o.) + Scopolamine (1 mg/kg, i.p.)

**Phytochemical screening**
For preliminary phytochemical screening, the EASF of methanolic extract was tested for the presence of alkaloids, glycosides, tannins, flavonoids, triterpenes and steroids using the standard procedures. [17]

**Experimental methods**

**Object recognition test**
The object recognition test (ORT) is a behavioral test that is widely used to examine animal’s memory performance. Memory performance in the ORT is based on the natural tendency of animals to explore novel objects. An important advantage of this task is that no aversive/stressful stimuli need to be applied. The apparatus consists of an open white colored plywood box (70 × 60 × 30 cm) with a well-furnished floor. The apparatus was illuminated by a 60 W lamp suspended 50 cm above the box. The object to be discriminated were made of plywood in two different shapes of 8 cm and coloured black. The day before test, mice were given a habituation session where they were left to freely exploring the box for 2 min. No object was placed in the box during the habituation trial. On the day of test, two identical objects were presented in two opposite corner of the box during first trial (T1), and the amount of time taken by each mouse to complete 20 s of object exploration was recorded. Exploration was considered as directing the nose at a distance less than 2 cm to the object and/or touching it with nose and/or forepaw. Turning around or sitting on the object was not considered as an exploratory behavior. During second trial (T2), 90 min after T1, one of the objects presented in T1 (i.e., familiar object) was replaced by new object and mice was left in box for 5 min. The time spent (s) for exploration of the familiar (F) and new (N) object were recorded separately and discrimination index (D) was calculated by using formula DI = N - F/ N + F; whereas DI= discrimination index, N = exploration of the new object, F= exploration of the familiar object. Scopolamine (1 mg/kg) was injected i.p. after 45 min of administration EASF of M. alba (25, 50, 100 mg/kg) or Ondansetron (0.5 mg/kg) or vehicle and first trial was given 45 min of injection of scopolamine. [18-19]

**Elevated plus maze**
Elevated plus-maze served as the exteroceptive behavior model to evaluate learning and memory in mice. The apparatus consisted of two open arms (16 × 5 cm) and two enclosed arms (16 × 5 × 12 cm). The arms extended from a central platform (5 × 5 cm) and the maze was elevated to a height of 25 cm from the floor. On the first day, each mouse was placed at the end of an open arm, facing away from the central platform. Transfer latency (TL) was the time taken by mouse with all its four legs to move into one of the enclosed arms. TL was recorded on the first day. If the animal did not enter into one of the enclosed arms within 120 s, it was gently pushed into one of the two enclosed arms and the TL was assigned as 120 s. The mouse was allowed to explore the maze for another 10 s and then returned to its home cage. Retention of this learned-task was examined 24 h after the first day trial. EASF of M. alba (25, 50, 100 mg/kg) or Ondansetron (0.5 mg/kg) or vehicle were administered orally for 8 days. Scopolamine (1 mg/kg) was injected i.p. after 90 min of administration of EASF or Ondansetron or vehicle and TL was noted after 45 min of administration of last dose on 8th day and again after 24 h i.e on 9th day. [20-21]

**Morris water maze task**
The apparatus is a circular water tank filled to a depth of 20 cm with 25°C water containing 500 ml of milk. Four points equally distributed along the perimeter of the tank serves as starting locations. The tank was divided in four equal quadrants and a small platform (19 cm height) was located in the centre of one of the quadrants. The first experimental day was dedicated to swimming for 60 s in the absence of the platform. During the four subsequent days the mice was given two trial sessions per day with the platform in place. When mice located the platform, it was permitted to remain on it for 10 s. If the mouse did not locate the platform within 120 s, it was placed on the platform for 10 s. The time interval between trial sessions was 30 min. One day after the final training trial sessions, mice were individually subjected to a probe trial session in which the platform was removed from the pool, and mice were allowed to swim for 120 s to search for it. The time of swimming was recorded in the pool quadrant where the platform has been previously placed. EASF of M. alba (25, 50, 100 mg/kg) or Ondansetron (0.5 mg/kg) or vehicle were given for four days. Scopolamine (1 mg/kg) was injected i.p. after 45 min of administration of extract or standard drug and trial was given 45 min of injection of scopolamine for four days. [22]

**Effect of EASF on serotonin-induced contractions of isolated rat fundus**
Adult male Wistar rats were sacrificed by cervical dislocation and the fundus was removed and kept in Krebs solution. The dose- responses to serotonin (10, 20, 40, 80 and 160μg/ml) were recorded on the fundus. Dose-response to serotonin was later repeated in presence of EASF (0.5 ml of 25 mg/ml). The contact

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time between the serotonin and the tissue were maintained 60 s.\cite{23}

**Statistics analysis**
Results are expressed as mean ± SEM, and the statistical analysis of data was done using one-way analysis of variance (ANOVA) followed by Dunnett’s test. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Phytochemical screening**
Phytochemical screening of EASF of methanolic extract of *M. alba* had shown the presence of tannins, alkaloids, glycosides, and flavonoids.

**Object recognition test**
The discrimination index was significantly \( (P < 0.01) \) decreased in scopolamine treated group as compared to control. Pretreatment with EASF (25, 50 and 100 mg/kg) significantly \( (P < 0.01) \) increased discrimination index as compared to scopolamine group. Ondansetron (a 5HT\(_3\) antagonist) also significantly increased discrimination index showing its anti-serotonergic effect (Fig. 1).

**Elevated plus maze**
The mice were administered with EASF (25, 50, 100 mg/kg) or Ondansetron (0.5 mg/kg) or vehicle orally for 8 days. Scopolamine (1 mg/kg) was injected *i.p.* after 90 min of administration of EASF or Ondansetron or vehicle and each mouse was placed individually in the EPM. The TL was noted after 45 min of administration of last dose on 8\textsuperscript{th} day and again after 24 h i.e on 9\textsuperscript{th} day. Statistical significance indicates the dose 25 mg/kg of EASF reveals no significant decrease in transfer latency on 8\textsuperscript{th} day. The EASF at both the dose levels (50 and 100 mg/kg) decreased the transfer latency on 8\textsuperscript{th} day significantly \( (P < 0.01) \) and dose dependently. The transfer latency was also significantly \( (P < 0.01) \) decreased by EASF at all doses on 9\textsuperscript{th} day dose dependently. The reference standard Ondansetron treated group showed significant decrease in the transfer latency on both days \( (P < 0.01) \) (Fig. 2 a, b).

**Morris water maze task**
The effect of EASF on spatial learning was evaluated using the Morris water maze test. Scopolamine administered animals showed less swimming time in target quadrant where platform was placed on the 5\textsuperscript{th} day. Administration of EASF (50 and 100 mg/kg) significantly \( (P < 0.01) \) increased swimming time in target quadrant. Thus, the less swimming time within the platform quadrant induced by scopolamine was significantly reversed by EASF. The swimming time in target quadrant was increased by Ondansetron (a 5HT\(_3\) antagonist) (Fig. 3).

**Effect of EASF on serotonin-induced contractions of isolated rat fundus**
The result of the *in-vitro* test indicated that EASF significantly inhibited serotonin-induced contractions on rat fundus. Thus, EASF possess anti-serotonergic activity (Fig. 4).
Scopolamine, the cholinergic muscarinic antagonist is most widely used to induce amnesia in experimental animals. Along with the cholinergic transmission serotoninergic transmission play an important role in cognitive function. Recently the role of serotonin in learning and memory has been focused. Serotonin regulates acetylcholine release from cortical area. The release of Acetylcholine is under inhibitory serotoninergic tone. 5-HT$_3$ receptor antagonist e.g Ondansetron has shown its effectiveness in enhancing cognitive performance.

The anti-amnesic effect of *Morus alba* was evaluated by using different models such as object recognition task, elevated plus maze, and water maze tests. Object recognition task (ORT) is a simple and quick method to test short term memory. Rodents are able to discriminate between a familiar object (previously exposed) and a novel object. Therefore, the time spent for exploring the new object will be more than for familiar object. Recognition of the object depends on innate exploratory behavior only. Scopolamine-induced amnesia served as the interceptive behavioral models. Pretreatment with EASF (25, 50 and 100 mg/kg) significantly increased discrimination index and attenuated scopolamine-induced memory impairment. Ondansetron (5HT$_3$ antagonist) also significantly increased discrimination index showing improvement in cognitive performance. EPM is the traditional tool in assessing learning and memory performance in laboratory animals. Originally designed to evaluate the anti-anxiety agents, EPM has also recently been extended to measure the cognitive performance, notably to evaluate the spatial long-term memory. The previous exposure of an animal to the elevated plane induces fear and to avoid the feeling of fear the animal occupies a safe position in the elevated plus maze. The latency to reach the central platform of the elevated plus maze is indicative of learning ability of an animal. The animal is said to have learnt if the latency to reach the central platform is reduced. The drugs impairing memory delay the entry of animal in the central platform. In this model, EASF at both the dose levels (50 and 100 mg/kg) decreased the transfer latency on 8th day significantly and dose dependently. The transfer latency was also significantly decreased by EASF at all doses on 9th day dose dependently. The reference standard Ondansetron (5-HT$_3$ antagonist) also inhibited this impairment by decreasing the transfer latency on both days.

The effect of EASF on spatial learning was evaluated using the Morris water maze test, which represents a more specific test of spatial memory. A task was developed where mice learn to swim in a water tank to find an escape platform hidden under the water. Learning is reflected on the shorter latencies to escape and the decrease on the length of the path to find the platform. The scopolamine-treated animals exhibited less swimming time in target quadrant where platform was placed on the 5th day. Treatment with EASF (50 and 100 mg/kg) significantly increased...
swimming time in target quadrant than the other quadrants in doing so animals crossed the target quadrant (quadrant which previously containing the platform) repeatedly than others. Thus, the shorter swimming time within the platform quadrant induced by scopolamine was significantly reversed by EASF. Ondansetron (5HT; antagonist) also significantly increased swimming time in target quadrant. The results indicated that EASF has significantly improved spatial memory.

It has been indicated that an increase in brain serotonergic transmission in the median raphe of mid brain will interfere with learning acquisition and memory consolidation. [29] In in-vitro study, EASF significantly inhibited serotonin-induced contractions on rat fundus, demonstrating its anti-serotonergic effect. Thus, the present study revealed that EASF of Morus alba significantly improved learning and memory. The results of the present study suggest that Morus alba has the ability to improve spatial long-term and working memory through its anti-serotonergic mechanism. Thus, Morus alba may be useful as anti-amnesic agent in the treatment of dementia and various cognitive disorders. Phytochemical tests of EASF showed presence of flavonoids, alkaloids and tannins, which might be responsible for anti-amnesic activity.

REFERENCES

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