Learning and memory promoting effects of crude garlic extract

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Chronic administration of aged garlic extract has been shown to prevent memory impairment in mice. Acute and chronic (21 days) effects of marketed formulation of crude garlic extract (Lasuna) were evaluated on learning and memory in mice using step down latency (SDL) by passive avoidance response and transfer latency (TL) using elevated plus maze. Scopolamine (0.4 mg/kg, ip) was used to induce amnesia in mice and piracetam (200 mg/kg, ip) served as positive control. In the acute study, Lasuna (65 mg/kg, po) partially reversed the scopolamine-induced amnesia but failed to improve learning and memory in untreated animals. Chronic administration of Lasuna (40 mg/kg/day for 21 days) significantly improved learning both in control and scopolamine induced amnesic animals. Influence of Lasuna on central cholinergic activity and its antioxidant properties were also studied by estimating the cortical acetylcholinesterase (AchE) activity and reduced glutathione (GSH) levels respectively. Chronic administration of Lasuna inhibited AchE, while increasing GSH levels. Thus the results indicate that long-term administration of crude garlic extract may improve learning and memory in mice while the underlying mechanism of action may be attributed to the anti-AchE activity and anti-oxidant property of garlic.

Keywords: Cholinergic system, Garlic, Learning, Memory

People exposed to environmental factors like psychosocial stress/environmental pollutants or having genetic predisposition are at increased risk for various neurodegenerative/neurocognitive disorders including Alzheimer's disease (AD), anxiety disorder, depression, multi infarct dementia, senile dementia, attention and concentrations deficit disorders which eventually leads to learning and memory impairment[1].

AD is a progressive neurodegenerative disorder, common to aged individuals which results from neurodegeneration, characterized by the deposition of beta amyloid plaques, development of neurofibrillary tangles, inflammation, and loss of neurons loss at specific regions of the basal forebrain. The most notable symptom of Alzheimer's disease is progressive memory loss followed by a general cognitive decline due to cholinergic deficiency in cortical regions[2]. Neuronal loss in the basal forebrain particularly within the septohippocampal cholinergic systems, involved in learning and memory processes

is well-defined characteristics of AD[2]. Acetylcolinesterase (AChE) inhibitors that increase the availability of acetylcholine (ACh) at cholinergic synapses is a popular treatment strategy for AD[3]. Herbal compounds such as gallantamine, rivastigmine antagonize the negative effects of scopolamine on spatial memory in various behavioural tests and are the first line AChE inhibitors for treatment of Alzheimer's related dementia[3]. A special class of drugs known as nootropics like piracetam, anaracetam which selectively increases the telencephalic integrative functions by modulation of brain metabolism and increase in cerebral blood flow are marketed for cerebrovascular related dementia[4].

Scopolamine, a muscarinic antagonist that induces central cholinergic blockade, produces a reversible impairment in both maintaining attention and processing of information with the acquisition of new knowledge in rodents and human. This cognitive impairment resembles the memory disturbances observed in AD and age related dementia[5]. Moreover it has also been reported that scopolamine induced amnesia mice model resembles the oxidative stress associated neurodegenerative disorder in human[6]. Hence, the scopolamine induced amnesic mouse is widely used as an animal model for AD[7].

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Garlic is a popular herb, which has its utility from cooking recipies to treatment for many diseases. Among its various physiological activities, it has been reported to increase cerebral blood flow in ischemic zones of rabbit brain[8], which suggests that it may be beneficial in neurological impairment associated with altered cerebrovasculature like ischemic stroke. Garlic has also been reported to have potent angiotensin-converting enzyme (ACE) inhibitory effect, which is thought to be responsible for its blood pressure lowering property[9] while ACE inhibitors have also been associated with memory enhancing properties[10].

Aged garlic extract has been reported to be beneficial in preventing neurodegenaration in AD, restricting several pathological cascades related to the synaptic degeneration and neuroinflammatory pathways due to its active ingredients, S allyl-L-cysteine[11]. Administration of aged garlic extract prevented memory impairment in Alzheimer's transgenic mice, suggesting that aged garlic extract has a potential for preventing AD progression[12]. Other reports suggest that aged garlic in the senescence-accelerated mouse improved longevity and learning performance by preventing cerebral atrophy[13]. However there is lack of evidence about role of crude garlic extract or fresh garlic, which is more readily available and may be used as home remedy, on learning and memory. Keeping these information in view the memory enhancing property of crude garlic extract (Lasuna®, Himalaya Drug Company, Baddi, India) has been evaluated following acute and chronic administration in mice.

Materials and Methods

Experimental animals—Swiss albino mice (25-30 g) of either sex were obtained from animal house of Gupta College of Technological Sciences, Asansol, West Bengal, India. The animals were housed under standard conditions of temperature (24±2 °C), relative humidity (55±10%) and 12:12 h light:dark cycle. Animals were fed with commercial pellet diet with water *ad libitum*. All animal experiments were carried out in accordance with the guidelines of CPCSEA and the protocol was approved by Institutional Animal Ethics Committee.

Materials—Crude garlic extract was obtained by the capsule fills of marketed garlic extract (Lasuna®, Himalaya Drug Company, Baddi, India) containing 250 mg of extract per capsules. The capsule fills were opened and content was dissolved in water for injection (WFI) before administration. Piracetam (Nootropil®, UCB India Pvt. Ltd., Vapi, Gujarat) a standard nootropic agent was used as a positive control and scopolamine hydrobromide (Sigma Aldrich, USA) was used as amnesic agent. Both these were diluted in water for injection and administered intraperitoneally. Bovine serum albumin (BSA), acetylthiocholine iodide, 5, 5'-dithiobis(2-nitro-benzoic acid) (DTNB), reduced glutathione (GSH) and Bradfoards reagent were obtained from (Sigma-Aldrich, USA).

Experimental design—For oral administration of crude garlic extract, content from the capsule fills were dissolved in WFI and administered at 10 mL/kg body weight. To study the acute effect the crude garlic extract was administered at a dose of 65 mg/kg, po and for chronic study 40 mg/kg was administered for 21 days. Separate sets of animals were used for elevated plus maze and passive avoidance study. Animals were randomly divided into five groups with 6-8 animals/group for behavioural studies.

Chronic study—Animals were divided into following five groups

Gr. I: control mice were treated with water for injection for 21 days,

Gr. II: mice were orally fed with crude garlic extract 40 mg/kg for 21 days,

Gr. III: mice were injected with scopolamine 0.4 mg/kg, ip on day 21,

Gr. IV: mice were injected with piracetam 200 mg/kg, ip from day 14 to day 21 and scopolamine was administered (0.4 mg/kg ip) after 45 min of piracetam injection on day 21 and

Gr V: mice were orally fed with crude garlic extract 40 mg/kg for 21 days & scopolamine was administered (0.4 mg/kg, ip) after 60 min of garlic administration on day 21.

All behavioural tests were conducted 45 min after scopolamine injection on day 21. While the biochemical studies on cortical brain lysates were performed on the same animals after the behavioural studies.

Acute study—For the acute study similar protocol was followed as mentioned under chronic study; however the dose of garlic extract used was 65 mg/kg for 1 hr.

Measurement of cognitive functions using elevated plus maze (EPM)—The elevated plus maze served as the exteroceptive behavioural model (wherein the

stimulus existed outside the body) to evaluate learning and memory in mice. The apparatus consisted of two open arms (16 cm × 5 cm) and two closed arms (16 cm × 5 cm × 12 cm). The arms extended from a central platform (5 cm × 5 cm), and 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 taken as the time taken by the mouse to move into any 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 closed arms within 90 sec, it was gently pushed into one of the two covered arms and the TL was assigned as 90 sec. The mouse was allowed to explore the maze for 10 sec and then returned to its home cage. Memory retention was examined[14,15].

Measurement of cognitive functions using passive avoidance paradigm—Passive avoidance behaviour based on negative reinforcement was recorded to examine long-term memory. The apparatus consisted of a box (27 × 27 × 27 cm) having three walls of wood and one wall of Plexiglas, featuring a grid floor (3 mm stainless steel rods set 8 mm apart), with a wooden platform (10 × 7 × 1.7 cm) in the center of the grid floor. The box was illuminated with a 15 W bulb during the experimental period. Electric shock (20 V AC) was delivered to the grid floor. Training was carried out in two similar sessions. Each mouse was gently placed on the wooden platform set in the center of the grid floor. When the mouse stepped down and placed all its paws on the grid floor, shocks were delivered for 15 sec and the step-down latency (SDL) was recorded. SDL was defined as the time taken by the mouse to step down from wooden platform to grid floor with its entire paw on the grid floor. Animals showing SDL in the range (2-15 sec) during the first test were used for the second session and the retention test. The second-session was carried out 90 min after the first test. When the animals stepped down before 60 sec, electric shock was delivered for 15 sec. During the second test, animals were removed from shock free zone if they did not step down for a period of 60 sec. Retention was tested after 24 h in a similar manner, except that the electric shocks were not applied to the grid floor. Each mouse was again placed on the platform, and the SDL was recorded with an upper cut off time of 300 sec[16,17].

Dose selection of crude garlic extract—As recommended human dose by the manufacturer was 500 mg/day so the mice dose was selected by the Paget Burner table method on the basis of body surface area ratio with reference to suggested human doses[18,19].

Conversion formula: Human dose × 0.0026 (conversion factor for 20 g mice) = (therapeutic effective dose) mg/kg for 20 g of mice.

For acute study absolute human dose of 500 mg was converted to 65 mg/kg and for chronic study 40 mg/kg/day were used.

Acute toxicity study—The contents from the capsules was dissolved in water for injection and administered orally at 500, 1000 and 2000 mg/kg to different groups of male swiss albino mice, as per OECD test guidelines (425) for oral acute toxicity study and mortality was observed for up to 7 days.

Brain tissue preparation—After performing the task performances 6 mice per group were sacrificed by cervical dislocation under mild ether anesthesia and brains were quickly removed and cleaned with ice cold saline in ice. Tissues were weighed and homogenized (0.03 *M* sodium phosphate buffer pH 7.4) to make 10% (w/v) homogenate in ultra turrax T-25 homogenizer at 9500 rpm. The homogenized tissue preparation was used for estimation of GSH and AChE activity. All the biochemical studies were performed at the end of behavioural test.

Estimation of brain AchE activity—The brain homogenate was centrifuged at 100,000 g at 4 °C in a Beckman Ultracentrifuge (LE 80, USA), using a fixed angle rotor (80 ti) for 60 min. Supernatant was collected and stored at 4 °C for AchE estimation by Ellman's method with slight modification[20,21]. An aliquot (0.4 mL) of the supernatant was added to a cuvette containing 2.6 mL phosphate buffer (0.1M, pH 8) and 100 μL of 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB). The contents of the cuvette were mixed thoroughly and absorbance was measured at 412 nm in a UV-visible spectrophotometer (Shimadzu, USA). When absorbance reached a stable value, it was recorded as the basal reading and 20μL of substrate i.e., Ach iodide was added and change in absorbance was recorded for 10 min at an interval of 30 sec. Change in absorbance per min was calculated. One unit of AchE activity was defined as the number of micromoles of Ach iodide hydrolyzed/min/mg of protein. The specific activity of AchE was expressed in μ moles/min/mg protein.

Calculation of enzyme activity was done using the following formula:

$$R = \frac{\delta A \times \text{total reaction volume (3.12 mL)}}{13.33 \text{ cm}/\mu \text{ mol} \times 1 \text{ cm} \times \text{sample volume (0.4 mL)} \times \text{mg of protein}}$$

where, R = rate in μ moles of substrate hydrolyzed/min/mg of protein; δ A = change in absorbance/min; path length = 1 cm. Absorption coefficient for thionitrobenzoic acid at 412 nm = 13.33 cm/μ mol.

Estimation of brain reduced glutathione (GSH) levels—GSH was determined by its reaction with DTNB to yield a yellow chromophore, which was measured spectrophotometrically[22]. The brain homogenate was mixed with an equal amount of 10% TCA and centrifuged at 2000 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 phosphate buffer (*p*H 8.4), 0.5 mL of DTNB and 0.4 mL of double-distilled water were added and the mixture was shaken vigorously on vortex. The absorbance was measured at 412 nm in a UV-visible spectrophotometer (Shimadzu, USA). GSH concentration was calculated by using standard curve prepared with reduced glutathione and expressed as μg/mg protein.

Estimation of total protein in brain samples—Protein was estimated in all the supernatant of brain samples by the Bradford's method where various concentrations of bovine serum albumin (0.01 - 0.1 mg/mL) were used as standard[23].

Statistical analysis—All values were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey's post hoc analysis. P<0.05 was considered to be statistically significant. All statistical analysis was carried out using Graph Pad Prism 4.0 software (GraphPad Software, Inc, San Diego CA).

Results

Acute Toxicity Study—Lasuna (crude garlic extract) did not show any mortality up to 7 days at the maximum dose of 2 g/kg in mice.

Acute administration of crude garlic extract and SDL in passive avoidance test—Increase in memory leads to increase in step down latency (SDL) i.e., mice tend to stay on the shock free zone (SFZ) for a longer period of time. Here scopolamine at a dose of 0.4 mg/kg acted as an amnesic agent-reducing SDL

significantly i.e., the mice did not remember the task & thus stepped down from SFZ to shock zone. Piracetam at a dose of 200 mg/kg acted as a standard memory-enhancing drug (nootropic), which reversed the SDL back to normal. Also garlic at a single dose of 65 mg/kg partially reversed this scopalamine-induced amnesia. But under normal conditions application of garlic alone did not show any significant improvementin SDL. The above results suggest that single dose of garlic may prevent scopolamine-induced amnesia to some extent (Fig. 1a).

Acute administration of crude garlic extract and TL in elevated plus maze—Increase in memory reflects decrease in transfer latency (TL) i.e., mice take less time to reach the closed arm. Here scopolamine at a dose of 0.4 mg/kg acting as an amnesic agent increased TL significantly i.e., mice did not remember the task, hence took longer to travel from open to closed arm. Piracetam at a dose of 200 mg/kg acted as a standard memory-enhancing drug, which reversed the SDL back to normal thus ameliorating the amnesia. Garlic at a dose of 65 mg/kg also reversed scopolamine induced amnesia. However garlic alone did not show any significant improvement in learning over control animals under normal conditions.
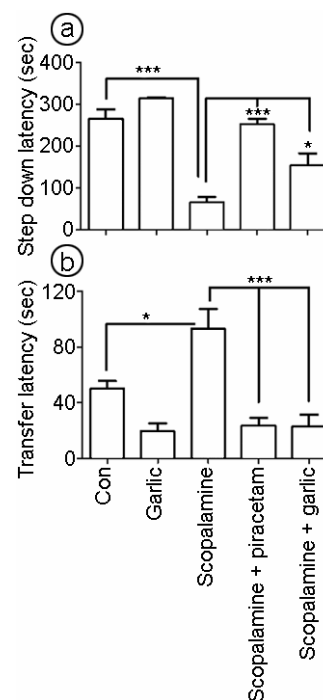

Fig. 1—Effect of acute garlic treatment on (a) step down latency and (b) transfer latency. [Values are mean ± SE from 6 animals in each group. *P* values: *<0.05; ***<0.001].

(Fig. 1b) Hence acute administration of garlic did not result in better learning over the control groups, but it prevented scopolamine induced amnesia to some extent.

Chronic administration of crude garlic extract and SDL in passive avoidance test—Garlic treatment (40 mg/kg/day for 21 days) showed increase in SDL over control animals. While 7 day pre-treatment of standard nootropic agent piracetam reversed the scopalamine mediated decrease in SDL, pretreatment with garlic at a dose of 40 mg/kg for 21 days also partially reversed the scopalamine mediated decrease in step down latency (Fig. 2a). Hence upon chronic application garlic not only ameliorated scopalamine-induced amnesia but also increased memory in control/scopolamine-untreated mice.

Chronic administration of crude garlic extract and TL in elevated plus maze—Here scopolamine at a dose of 0.4 mg/kg acting as an amnestic agent, increased TL significantly i.e., the mice did not remember the task and thus took more time to reach the closed arm. Piracetam at a dose of 200 mg/kg acted as a standard memory-enhancing drug, and reversed the SDL back to normal thus ameliorating the amnesia. Application of garlic for 21 days at a dose of 40 mg/kg/day also reversed scopalamine induced amnesia. Here garlic also showed significant
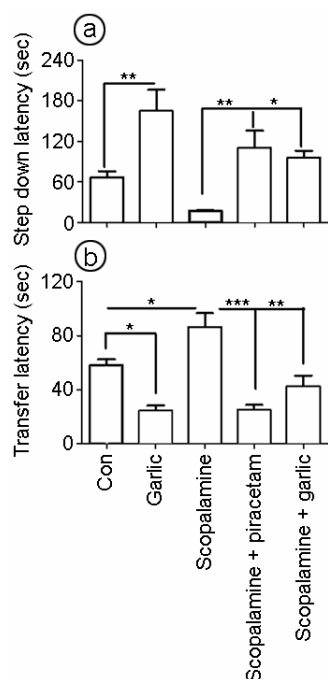
improvement in learning over control animals under normal conditions i.e., garlic treated group performed better in their learning and memory task compared to untreated animals (Fig. 2b). Thus it is evident that upon chronic administration garlic increased learning and memory and prevented scopolamine induced amnesia.

Chronic administration of crude garlic extract on brain AchE activity—Chronic administration of garlic at a dose of 40 mg/kg/day for 21 days showed reduced activity of brain AchE with respect to normal i.e. water for injection treated animals. Amnestic agent scopolamine significantly increased the activity of AchE. Standard drug piracetam reversed the amnesia by decreasing the activity of AchE. Garlic pre-treatment prevented the scopolamine induced decrease in AchE activity (Fig. 3). Thus it may be concluded that chronic administration of garlic reduced the cortical AchE activity leading to increase in Ach levels, which may be responsible for its memory enhancing properties.

Chronic administration of crude garlic extract on brain GSH levels—Increase in brain glutathione (GSH) levels reflects resistance to oxidative and nitrosativestress by amnestic agents like scopolamine. Chronic administration (21 days) of garlic at a dose of 40 mg/kg/day increased the GSH levels significantly over control animals, which reflects its antioxidant property. Scopalamine treatment led to reduction in cortical GSH, due to oxidative stress, partially responsible for its amnestic properties, while animals pre-treated with piracetam and garlic followed by scopolamine administration showed increased GSH levels (Fig. 4). In the present study
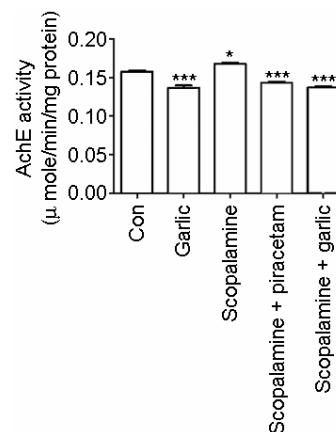

Fig. 2—Effect of chronic garlic treatment (a) step down latency and (b) transfer latency. [Values are mean ± SE from 6 animals in each group. *P* values: *<0.05; **<0.01; ***<0.001].

Fig. 3—Effect of chronic garlic treatment on cortical AchE activity. [Values are mean ± SE from 6 animals in each group. *P* values: *<0.05; ***<0.001]

garlic was found to be more efficient than piracetam in increasing GSH levels.

Discussion

In the present study acute administration of garlic did not result in better learning over control groups, but it prevented scopolamine induced amnesia to some extent. Chronic administration led to significant increase in learning in garlic treated group over the control groups. Chronic administration of garlic, led to increased cholinergic activity in mice cortex due to decreased AchE activity which was evident from the *in vivo* enzyme assay.

Previous human and animal studies have demonstrated that, learning and memory can be modified by drugs affecting the central cholinergic system[24]. Cholinergic transmission is terminated mainly by acetylcholine hydrolysis via the enzyme AchE. It is believed that the action of this enzyme could affect the progression of AD[25]. Consequently, AchE has been proposed as a potential target for treatment of AD. A clear correlation between reduced AchE levels with chronic administration of garlic was observed in the present study. Preliminary observations have shown that *in vitro* AchE inhibition of garlic was not significant (data unpublished) i.e., it may not be an inhibitor, but its chronic administration showed decreased activity of AchE, which may be due to weak inhibitory activity or garlic mediated decrease in AchE expression.

Strong correlation between oxidative stress and pathogenesis of AD has been reported[26]. In

these reports, the GSH levels were found to be elevated in AD brain. It is well established that, neurodegeneration may be prevented by free radical scavengers and antioxidants[27]. It is also reported that memory impairment induced by acute scopolamine administration in rats is associated with altered levels of GSH in the brain[28]. In the present experiment, scopolamine administration resulted in a significant decrease in GSH, an important marker for oxidative stress in the cortex and hippocampus of amnesic mice. The administration of garlic produced a significant increase in GSH, i.e., there was restoration of the GSH levels in mice cortex which got depleted after scopolamine treatment. It is suggested that the increased GSH levels by garlic might promote scavenging of free radicals. This observation demonstrates that garlic may possess potent antioxidant activity. They may be involved in scavenging reactive oxygen species (ROS) and exert a protective effect against oxidative damage induced by scopolamine by diminishing the levels of GSH. Thus chronic administration of crude garlic extract, increased cortical cholinergic activity and GSH levels in mice. This central cholinergic activity and high GSH, which quenches the free radicals, may be responsible for attenuation of scopolamine-induced amnesia.

In conclusion, chronic administration of garlic showed potent cognition-enhancing activity. This may be mediated by inhibition of AchE activity and or by prevention of oxidative insult to the CNS. If the results from the animal studies hold true for humans then it may be postulated that daily consumption of garlic as part of our diet may act as memory enhancer.
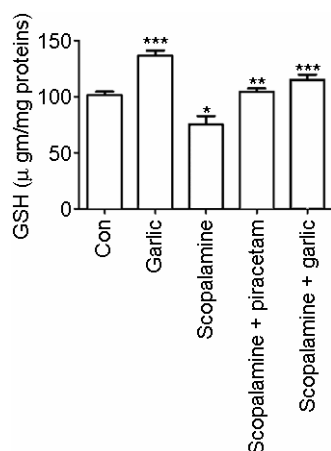
Fig. 4—Effect of chronic garlic treatment on cortical GSH levels. [Values are mean ± SE from 6 animals in each group. *P* values: *<0.05; **<0.01; ***<0.001].

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