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Research Article

PRO-CHOLINERGIC, HYPO-CHOLESTEROLEMIC AND MEMORY IMPROVING EFFECTS OF CLOVE

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ABSTRACT
Clove is found to possess useful medicinal properties, such as anti-microbial, anti-inflammatory, anti-diabetic and anti-oxidant. The present study was undertaken to investigate the effects of *Syzygium aromaticum* (Clove) on cognitive functions in mice. Clove powder was administered orally along with diet in three doses (400, 800, 1600mg/kg) for seven successive days. 250 Swiss young mice divided in 50 groups and 100 aged mice divided in 20 groups were employed in the present study. The learning and memory parameters were assessed using elevated plus maze, passive avoidance apparatus and Hebb-Williams maze. Clove showed significant improvement in the memory of young and aged animals as reflected by decreased TL as well as TRC and increased SDL values. It also reversed the amnesia caused by ethanol (1.0 g/kg, i.p.) and diazepam (1mg/kg, i.p.). Furthermore, Clove reduced significantly the brain cholinesterase activity in young mice by 50.5 % and aged mice by 21.25 % at the dose of 800 mg/kg. Clove also showed remarkable reduction to the extent of 33% and 66.32 % in the total cholesterol levels of young and aged mice at the dose of 800 mg/kg. Diminished cholinergic transmission and high cholesterol levels appear to be responsible for the development of dementia in Alzheimer patients. Since Clove powder enhanced Ach levels and lowered cholesterol levels in the present study; it appears to be a promising candidate for improving memory. Thus it would be worthwhile to explore the potential of this spice (Clove) clinically in the management of Alzheimer's disease.
KEYWORDS: *Syzygium aromaticum, Eugenia caryophyllata*, Clove, Laung

INTRODUCTION

Cloves are the aromatic dried buds of a tree (*Syzygium aromaticum*, Family: Myrtaceae) used as a spice in virtually all the world's cuisine. Clove is a medicinally powerful herb with solid traditional heritage and history. Clove is looked upon as a champion of all the antioxidants known till date.[1] A drop of Clove oil is 400 times more powerful as an anti-oxidant than wolf berries or blueberries. The ORAC (Oxygen Radical Absorption Capacity test) score, of Clove is over 10 million. Clove represents one of the Mother Nature's premier antiseptic. Clove is used to treat broad spectrum of ailments as it possesses anti-fungal, anti-viral, anti-microbial, anti-diabetic, antithrombotic, anesthetic, anti-stress, pain reliving and insect repellent properties.[2-5]. Clove contains eugenol (primary component of its volatile oil) and flavonoids, which contribute to its anti-inflammatory activity. Medicinal uses of clove also reveal that it enhances memory retention and is good for relieving brain fog, lethargy and depressive state of mind. Therefore, we were motivated to investigate the potential of Clove in the management of memory deficits, total cholesterol levels and cholinesterase activity in mice.

MATERIALS AND METHODS

Plant material

Buds of Clove were collected during the month of April 2010 from the local market of Hisar, (Haryana), India. Buds were dried under shade and pulverized using mechanical grinder. The powdered form of Clove was stored in an air tight container. This powder was used in further experiments.

Animals

All the experiments were carried out using Swiss mice of either sex procured from disease free small animal house of CCS Haryana Agricultural University, Hisar, (Haryana), India. Young (2-3 months old) mice weighing around 15g and Aged (9-10 months old) mice weighing around 25g were used in the present study. The animals had free access to food and water, and they were housed in a natural (12h each) light-dark cycle. Food given to mice consisted of wheat in the form of dahlia boiled in water with small amount of refined oil. The animals were acclimatized for at least 5 days to the laboratory

conditions before behavioral experiments. The experimental protocol was approved by the Institutional Animals Ethics Committee and the care of laboratory animals was taken as per the guidelines of CPCSEA, Ministry of Forests and Environment, Government of India (Registration number - 0436). Volume of oral administration and i.p. injection was 0.1ml/10g of mouse.

Acute Toxicity Studies

Acute oral toxicity studies were performed according to OECD-423 guidelines (acute toxic class method). Swiss mice (n=3) of either sex selected by random sampling technique were employed in this study. The animals were fasted for 4 h with free access to water only. The Clove powder was administered orally in a single daily dose for 3 days in different concentrations (250, 500, 1000, 2000 mg/kg) and mortality was observed. If the mortality was observed in 2/3 or 3/3 animals, then the dose administered was considered as a toxic dose. However, if the mortality was observed in only one mouse out of three animals then the same dose was repeated again to confirm the toxic effect. If mortality was not observed, the procedure was then repeated with higher doses, such as 500, 1000 and 2000 mg/kg.

Drug Treatment

In the present investigation, the mice were divided into 70 different groups for subjecting to interoceptive and exteroceptive models and for biochemical estimations. Each group comprised of a minimum of five animals. Clove powder (400, 800, 1600 mg/kg) was administered orally along with diet for seven successive days to young and aged animals. After 90 min of the administration of the last dose (on seventh day), mice were exposed to elevated plus maze, passive avoidance paradigm and Hebb-Williams maze apparatus. Amnesia was induced in separate groups (interoceptive models) of young mice by ethanol (1g/kg, i.p.) or diazepam (1mg/kg, i.p.) after 90min of the last dose of the Clove powder (400, 800, 1600 mg/kg) administration on the seventh day. Piracetam (400 mg/kg, i.p.) was used as an established nootropic agent and was injected for 7days to positive control groups. Clove powder (400, 800, 1600 mg/kg) was administered orally along with diet for seven successive days to separate groups of young and aged mice for biochemical estimations. Donepezil (1mg/kg, i.p., 60 min before dissecting brain) served as the positive control for the comparison of brain cholinesterase activities. Simvastatin (5mg/kg, p.o., for 7day) served as the positive control for comparing total serum cholesterol levels. All control Group (young and aged) animals received dahlia diet for seven consecutive days.

Elevated Plus Maze

Elevated plus maze served as the exteroceptive behavioral model to evaluate learning and memory in mice. The procedure, technique and end point for testing learning and memory was followed as per the parameters described by the investigators working in the area of psychopharmacology[6,7]. The elevated plus maze for mice consisted of two open arms (16×5 $cm^{2)}$ and two covered arms (16×5×12 cm^3) extended from a central platform (5×5 cm^2), and the maze was elevated to the height of 25cm from the floor. [8] On the first day, each mouse was placed at the end of an open arm, facing away from the central platform. Transfer latency was defined as the time (in sec) taken by the animal to move from the open arm in to one of the covered arms with all its four legs. TL was recorded on the first day (training session) for each animal. The animal was allowed to explore the maze for another 2 minutes and then returned to its home cage. Retention of this learned task (memory) was examined 24 h after the first day trial. Significant reduction in TL value indicated improvement in memory.

Passive Avoidance Paradigm

Passive avoidance behavior based on the negative reinforcement was used to examine the long term memory.[9] The apparatus consisted of a box (27× 27× 27 cm^3) having three walls of wood and one wall of plexiglass, featuring a grid floor (made up of 3mm stainless steel rods set 8mm apart), with a wooden platform (10 ×7× 1.7 cm^3) in the centre of the grid floor. The box was illuminated with a 15W bulb during the experimental period. Electric shock (20V, A.C.) was delivered to the grid floor.[10] Training was carried out in two similar sessions. Each mouse was gently placed on the wooden platform set in the centre of the grid floor, when the mouse stepped down placing all his paws on the grid floor, shocks were delivered for 15 s and the step down latency (SDL) was recorded. SDL was defined as the time (in seconds) taken by the mouse to step down from the wooden platform with all its paws on the grid floor. Animals showing SDL in the range of 2-15 s 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. During second session, if the animals stepped down before 60 s, electric shocks were delivered once again for 15 s. During the second test, animals were removed from shock free zone, if they did not step down for a period of 60 s and were subjected to retention test. Retention (memory) was tested after 24h

in a similar manner, except that the electric shocks were not applied to the grid floor observing the upper cut off time of 300 s.[11]

Hebb- Williams Maze

It is an incentive based exteroceptive behavioral model useful for measuring spatial and working memory of mice.[10] It consists of mainly three components. Animal chamber (or Start box), which is attached to the middle chamber (or exploratory area) and a reward chamber at the other end of the maze in which the reward (food) is kept. All the three components were provided with guillotine removable doors. Each mouse was placed in animal chamber (Start box) and door was opened to facilitate the entry of animal into the next chamber. The door of start box was closed immediately after the animal moved in to the next chamber so as to prevent its back entry. Time taken in seconds by the animal to reach reward chamber (TRC) from the start box was noted for each animal. Each animal was allowed to explore the maze for additional 20 seconds, with all its doors opened before returning to its home cage. A fall in TRC on subsequent maze exposures was taken as an index of successful retention.

Collection of blood and brain samples

The animals were sacrificed by cervical decapitation under light anesthesia on the seventh day 90 min after administration of last doses of clove powder or standard drug or diet. Immediately after the decapitation, the trunk blood was collected. Then whole brain was carefully removed from the skull. The collected blood was centrifuged at 3000 rpm for 15 min so as to separate serum. The serum was used for estimation of cholesterol levels. For preparation of homogenate, the whole brain was weighed and transferred to glass homogenizer and homogenized in an ice bath after adding 10 volumes of 0.9% sodium chloride solution. The homogenate was centrifuged at 3000 rpm for 10 min and the resultant cloudy supernatant liquid was used for estimation of brain cholinesterase activities.

Estimation of brain cholinesterase

In this method 10 µl of test sample was pipetted into the reaction vessel using a micro-pipette and to it 1000 µl of working reagent 1 and 30 µl of working reagent 2 were mixed. The absorbance of this mixture was read at 405 nm using auto analyzer.

Estimation of serum total cholesterol

CHOD–PAP method was used for the estimation of serum total cholesterol.[12] In this method, test samples and working reagent were pipetted into the respective reaction vessels using a micro – pipette. For this 10 µl test sample and 1000 µl of working reagent were mixed. These mixtures were incubated for 5 min at 37° C. The absorbance was read at 500nm by using auto analyzer.

Statistical Analysis

All the results were expressed as mean ± standard error (SEM). Data was analyzed using one-way ANOVA followed by Dunett's t- test. P-values <0.05 were considered as statistically significant.

RESULTS

Acute Toxicity Studies

Clove powder did not produce any mortality even at the highest dose (2000 mg/kg, p.o). Both the doses of 250 mg/kg & 2000mg/kg were found to be non-toxic. The doses 400mg/kg, 800mg/kg & 1600mg/kg were selected for further psychopharmacological and biochemical studies.

Effect on TL using elevated plus maze

TL of Second day (8^{th} day of drug treatment) reflected retention of information or memory. Clove powder administered for seven days showed remarkable reduction ($P<0.01$) in TL of 8^{th} day at 400 mg/kg (4.6 ± 0.5 sec), 800 mg/kg (4.2 ± 0.3 sec) and 1600 mg/kg (3.8 ± 0.5 sec), when compared to the control group of young mice (8 ± 0.3) indicating significant improvement in memory. Clove powder 400 mg/kg (13.4 ± 1.3), 800mg/kg (11.8 ± 1.6 sec) & 1600 mg/kg (13.8 ± 2.1 sec) also exerted significant effect on TL ($P<0.01$) of aged mice as compared to the control (19 ± 1.8) group, when recorded on 8^{th} day (**Fig.1**). This remarkable reduction in the TL values indicated improvement in memory (retention capacity) of young and aged mice. Ethanol (1.0 g/kg, i.p.) & Diazepam (1mg/kg, i.p) injected before training (on 7^{th} day) significantly increased ($P<0.01$) TL (11± 1.2, 11.2± 0.5) of 8^{th} day indicating impairment in memory of young mice. All the three doses of Clove successfully reversed ($P<0.01$) memory deficits induced by ethanol (5.2 ± 0.2, 4± 0.5, 7± 0.6) & diazepam (5.6± 0.5, 4± 0.7, 8.6± 0.4) (**Fig. 2**). Piracetam (used as a positive control) at the dose of 400 mg/kg, i.p improved memory ($P< 0.01$) of both young mice and aged mice and reversed the amnesia induced by ethanol and diazepam as expected.

Effect on Step Down Latency (SDL) using passive avoidance paradigm

SDL on second day/8^{th} day of Clove treatment reflected long term memory of animals. Significant increase in the SDL value indicated improvement in the memory. Clove 400 mg, 800 mg & 1600 mg/kg exerted significant effect on SDL (177.8 ± 2.9, 198.8 ± 2.9, 269.4 ± 3.2sec) of young mice as compared to the control (25.8 ± 1.2sec) group. Clove 400 mg, 800 mg & 1600 mg/kg exerted significant effect on SDL (169.8 ± 2.1, 187.2 ± 3.6,

298.2 ± 1.1 sec) of aged mice as compared to the control (10.2 ± 0.86 sec) group. This remarkable enhancement of SDL values indicated improvement in memory (retention capacity) of young and aged mice (**Fig.3**). Whereas, Ethanol (1.0 g/kg, i.p.) and Diazepam (1 mg/kg, i.p.) significantly (P<0.01) decreased SDL (19.2 ±1.4, 13 ± 1.1sec) as compared to the control (25.8 ± 1.2sec) group of young mice indicating impairment in memory (amnesia). Clove (400 mg, 800 mg & 1600 mg/kg) administered for 7 days successfully reversed amnesia (**Fig.4**) induced by both ethanol (188.4 ± 1.8, 182.6 ± 2.6, 86.6 ± 2.8sec) and diazepam (SDL values were 25.6 ± 1.1, 33.4 ± 1.0, 31.2 ± 2.0sec). Piracetam (400 mg/kg, i.p.) administered for 7 successive days showed improvement in memory (P< 0.01) of both young and aged mice and reversed amnesia produced by ethanol and diazepam in young mice.

Effect on TRC using Hebb-Williams Maze

Clove powder (400 mg, 800 mg & 1600 mg/kg), when administered for seven days showed remarkable reduction (P<0.01) in TRC of 8th day in young (140.4 ± 1.5, 129 ± 1.8, 59.6 ± 2.4) and aged (76.2 ± 2.6, 81.6 ± 2.7, 52.8 ± 1.4) mice, when compared to their respective control (281 ± 1.09sec, 293 ± 2.2sec) groups indicating significant improvement in memory (**Fig. 5**). Ethanol (1.0 g/kg, i.p) & Diazepam (1 mg/kg, i.p) injected before training significantly increased (P<0.01) TRC (292.2 ± 3.0, 295.4 ± 1.9) indicating impairment in memory of young mice. All the three doses of Clove successfully reversed memory deficits induced by ethanol (117.6 ± 2.0, 83.8 ± 2.4, 73 ± 3.7) & diazepam (177.4 ± 1.8, 148.4 ± 1.8, 143.8 ± 3.3) (**Fig. 6**). Piracetam (used as a positive control) at the dose of 400 mg/kg, i.p improved memory (P< 0.01) of both young and aged mice and reversed the amnesia induced by ethanol and diazepam.

Effect on brain cholinesterase activity

Clove 400 mg/kg and 800 mg/kg produced marked reduction in brain cholinesterase activity in young and aged mice as compared to the control group. The percentage reduction in cholinesterase activity was 33.5 % and 50.5 % at the dose of 400 mg/kg and 800 mg/kg of Clove powder in young mice. The percentage reduction in cholinesterase activity was 11.08 % and 21.15 % at the dose of 400 mg /kg and 800 mg/kg of Clove powder in aged mice. Donepezil (1 mg/kg, i.p.), used as a standard drug showed reduction of brain cholinesterase activity in young mice by 41.7% and 59.4% in aged mice (**Fig. 7**).

Effect on total cholesterol level

The young and aged animals receiving Clove powder (400 mg/kg and 800 mg/kg) for seven successive days showed significant reduction in total cholesterol levels. The extent of reduction in total cholesterol levels of young mice were 10.91% (P<0.05) and 33% (P<0.01) at the doses of 400 mg and 800 mg/kg of Clove respectively. Similarly, the reductions were 66.21% and 66.32 % (P<0.01) at the doses of 400 mg/kg and 800 mg/kg in aged mice. The extent of reductions in total cholesterol levels with simvastatin (a standard cholesterol lowering agent) were 21.38% (P<0.01) in young animals and 66.49% (P<0.01) in aged animals (**Fig. 8**).

DISCUSSION

Alzheimer's disease is characterized by progressive memory loss, cognitive impairment and personality defects accompanied by diffuse structural abnormalities in the brain.[13, 14] Allopathic system of medicine is yet to provide a satisfactory remedy, despite the severity and high prevalence of this disease. Therefore, while looking for alternative therapies, we were interested to explore the potential of spice 'Clove' to manage this deadly disease (AD). In the present study, Clove when administered along with diet for 7 days successively improved the memory of mice as reflected by diminished TL as well as TRC and enhanced SDL values as compared to the respective control animals. Amnesia was induced in mice by intraperitoneal injection of ethanol or diazepam, in addition to ageing–induced amnesia (a natural process). Clove successfully reversed ethanol, diazepam or ageing- induced amnesia, when administered for 7 days. These findings suggest possible neuroprotective role for Clove. Acetylcholine is considered to be the most important neurotransmitter responsible for consolidating and maintaining long term memory.[15, 16] Selective loss of cholinergic neurons and increased acetyl cholinesterase (enzyme responsible for degradation of Ach) activity was also reported to be a characteristic feature of senile dementia of the Alzheimer's type.[17] The use of acetylcholinesterase (AChE) inhibitors reversed the amnesia produced by disruption of cholinergic system. In the present study, Clove showed elevation of acetylcholine levels by significant inhibition of acetylcholinesterase activity in the brains of young and aged mice.

It has been observed that elderly patients suffering from Alzheimer's disease showed reduction in symptoms upon chronic use of anti-inflammatory drugs.[18] Epidemiological studies have almost confirmed that non-steroidal anti-inflammatory drugs reduce the incidence of AD.[19] Clove has been shown to be an effective anti-inflammatory agent,[3] which might protect from the development of inflammatory lesions in brain. Oxygen

free radicals and other by products of oxidative metabolism have been shown to be neurotoxic. Oxygen free radicals are implicated in the process of age related cognitive decline and may be responsible for the development of AD in elderly persons.[20] Clove may be looked upon as a champion of all the antioxidants known till date.[1, 21] by virtue of which susceptible brain cells get exposed to less oxidative stress resulting in reduced brain damage and improved neuronal function. Thus, improvement of memory by Clove may be due to its anti-oxidant and anti-inflammatory property. The main histological features of AD include deposition of inter neuronal Aβ plaques and intraneuronal neuro fibrillary tangles.[22, 23] Abnormal accumulation of cholesterol levels increase Aβ in cellular and most animal models of AD; and the drugs that inhibit cholesterol synthesis lower Aβ deposits in these models.[24] Several studies link high levels of cholesterol and progression of Alzheimer's disease.[25] Interestingly, the animals, which were treated with Clove showed reduction in total cholesterol levels. Hypocholesterolemic activity exhibited by Clove in the present study may be preventing the accumulation of β-amyloid plaques and intraneuronal neuro fibrillary tangles. Thus, the memory enhancing effects shown by Clove in the present study could be related to its diverse beneficial effects viz., anti-cholinesterase activity, cholesterol lowering property, anti-oxidant activity and the anti-inflammatory action.

CONCLUSION

In the present study, Clove reversed the memory deficits induced by ethanol and diazepam in young mice. Furthermore, Clove improved the retention capacity of aged mice, when administered for 7- days along with diet. In addition, clove inhibited AChE activity and lowered cholesterol levels of mice. These behavioral and biochemical effects of clove can prove helpful in the management of cognitive dysfunctions seen in Alzheimer patients.

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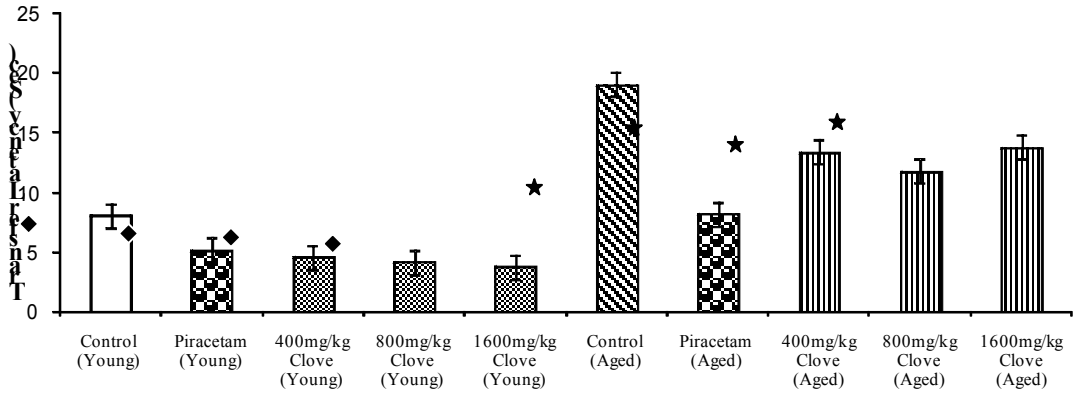

Fig 1: Effect of Various concentrations of Clove powder administered along with diet for seven successive days on transfer latency (TL) of Young and Aged mice.

Piracetam (400 mg/kg) was used as a standard drug. Values are in mean ± SEM (n = 5). Denotes p<0.01 as compared to control group of young mice. Denotes p<0.01 as compared to control group of aged mice. One – way ANOVA followed by Dunnett's *t*-test.

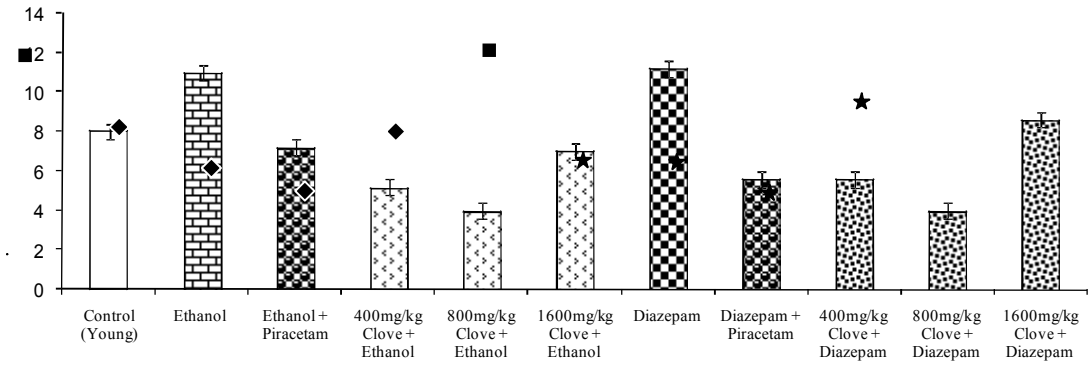

Fig. 2: Reversal of Ethanol or Diazepam induced amnesia by Clove powder in Young mice using elevated plus maze.
Piracetam (400 mg/kg) was used as a standard drug. Values are in mean ± SEM (n = 5). Denotes p<0.01 as compared to Ethanol alone group of young mice. Denotes p<0.01 as compared to Diazepam alone group of young mice. Denotes p<0.01 as compared to control group of young mice. One – way ANOVA followed by Dunnett's *t*-test.

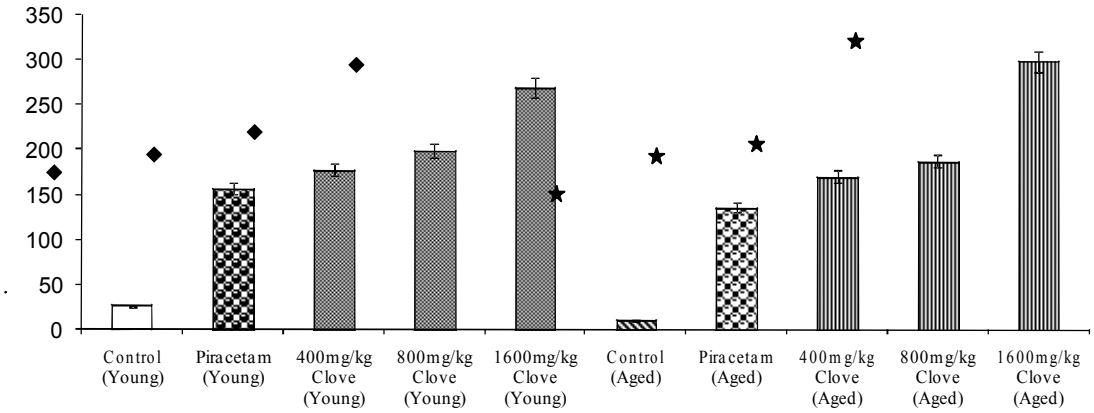

Fig. 3: Effect of Various Concentrations of Clove powder on Step-Down Latency (SDL) of Young and Aged mice.
Piracetam (400 mg/kg) was used as a standard drug. Values are in mean ± SEM (n = 5). Denotes p<0.01s as compared to control group of young mice. Denotes p<0.01 as compared to control group of aged mice. One – way ANOVA followed by Dunnett's *t*-test.

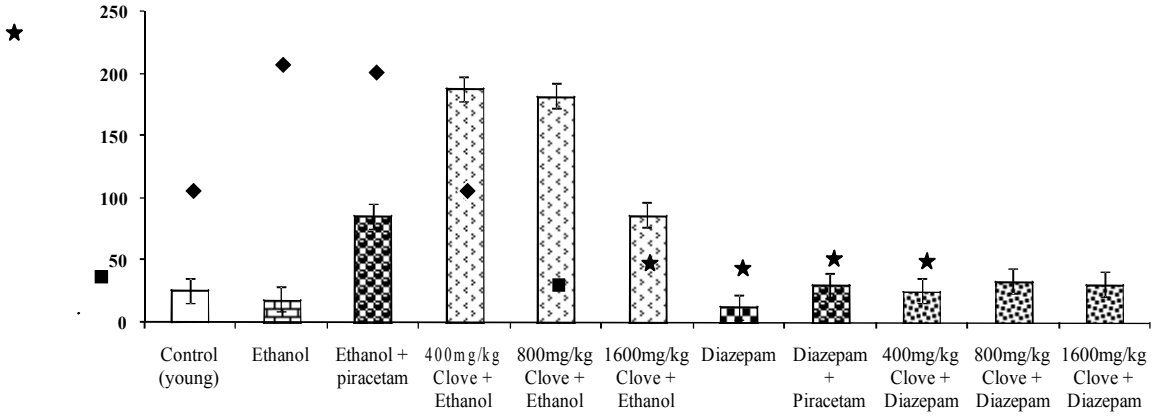

Fig. 4: Reversal of Ethanol or Diazepam induced amnesia by Clove powder in Young mice using Passive avoidance paradigm.
Piracetam (400 mg/kg) was used as a standard drug. Values are in mean ± SEM (n = 5). Denotes p<0.01 as compared to control group of young mice. Denotes p<0.01 as compared to Ethanol alone group of young mice. Denotes p<0.01 as compared to Diazepam alone group of young mice. One – way ANOVA followed by Dunnett's *t*-test.

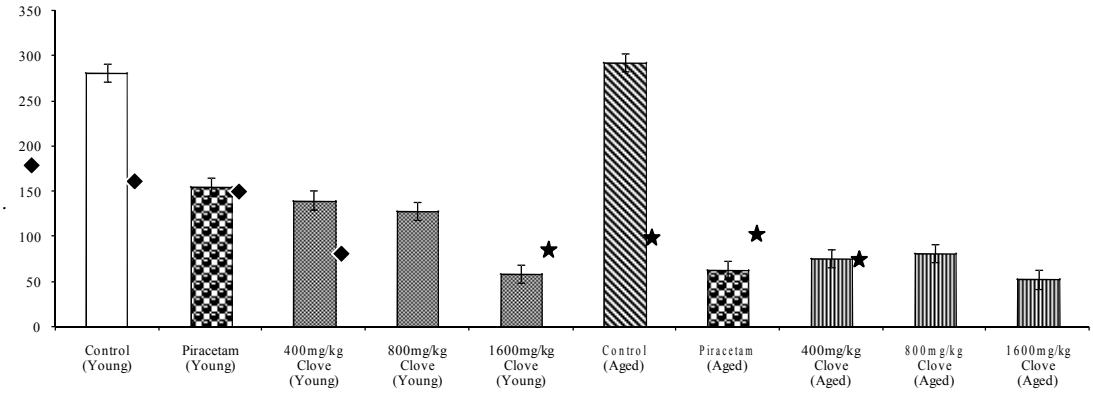

Fig. 5: Effect of Various concentrations of Clove powder administered along with diet for seven successive days on TRC of Young and Aged mice.
Piracetam (400 mg/kg) was used as a standard drug. Values are in mean ± SEM (n = 5). Denotes p<0.01 as compared to control group of young mice. Denotes p<0.01 as compared to control group of aged mice. One – way ANOVA followed by Dunnett's *t*-test.

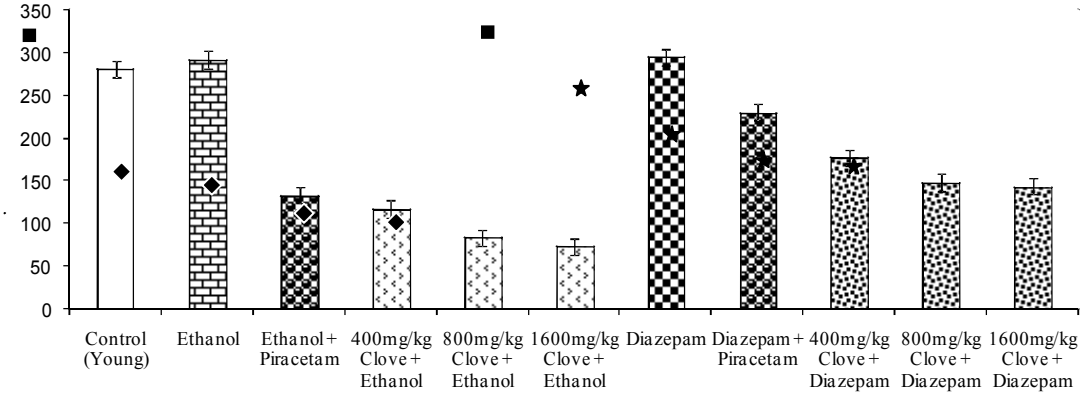

Fig. 6: Reversal of Ethanol or Diazepam induced amnesia by Clove powder in Young mice using Hebb-Williams maze.
Piracetam (400 mg/kg) was used as a standard drug. Values are in mean ± SEM (n = 5). Denotes p<0.01 as compared to Ethanol alone group of young mice. Denotes p<0.01 as compared Diazepam alone group of young mice. Denotes p<0.01 as compared to control group of young mice. One – way ANOVA followed by Dunnett's *t*-test.

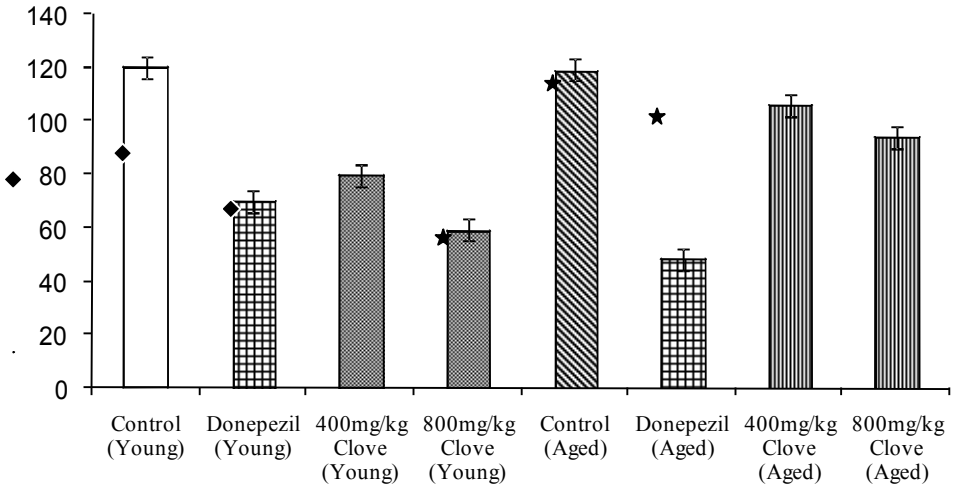

Fig. 7: Effect of Clove powder administered along with diet for seven successive Days on Brain Cholinesterase (AChE) Activity of Young and Aged mice.
Donepezil (1 mg/kg, i.p.) served as positive control. Values are in mean ± SEM (n = 5). Denotes p<0.01 as compared to control group of young mice. Denotes p<0.01 as compared to control group of aged mice. One – way ANOVA followed by Dunnett's *t*-test).

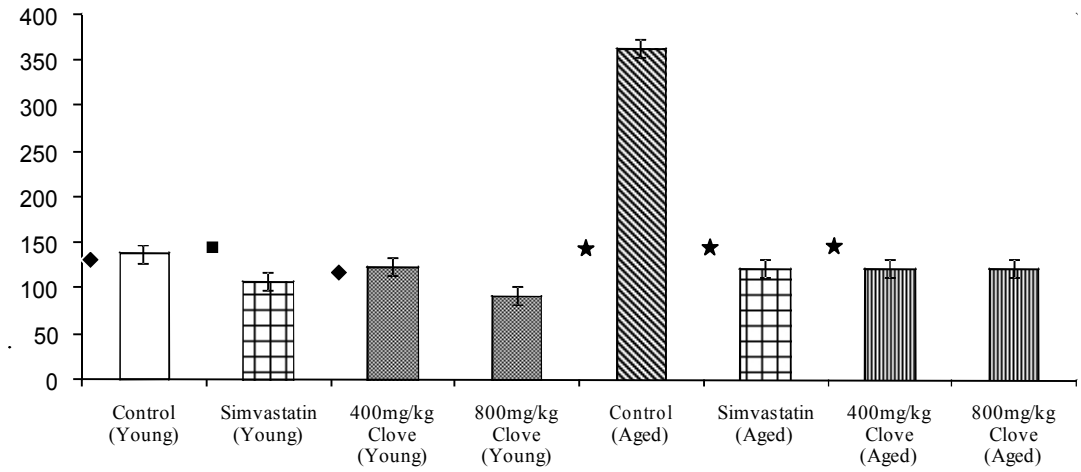

Fig. 8: Effect of Clove powder administered along with diet for seven successive Days on Serum Cholesterol Level of Young and Aged Mice.
Simvastatin (5mg/kg, p.o) served as positive control. Values are in mean ± SEM (n=5). Denotes p<0.01 as compared to control group of young mice. Denotes p<0.05 as compared to control group of young mice. Denotes p<0.01 as compared to control group of aged mice One – way ANOVA followed by Dunnett's *t*-test.