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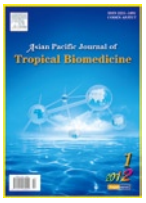

Document heading

Effect of hydroalcoholic extract of *Vitex negundo* Linn. leaves on learning and memory in normal and cognitive deficit mice

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ABSTRACT

Objective: To demonstrate the improvement in learning and memory by hydroalcoholic extract of *Vitex negundo* Linn. leaves (HEVN). **Methods:** The leaves were macerated and percolated using 70 % ethanol to obtain dark green colored semisolid mass of HEVN. Effects of HEVN were evaluated in normal as well as in scopolamine- induced cognitive deficit mice paradigms using elevated plus maze (EPM) and object recognition test (ORT). Additionally, the effect of HEVN on acetyl-cholinesterase level (AchE) and oxidative stress in mice brain and in sodium nitrite induced respiratory arrest in mice was evaluated. **Results:** Preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, proteins, and carbohydrates in the HEVN. Administration of HEVN (250 and 500 mg/kg/day, p.o.) for 8 days significantly increased inflexion ratio in EPM, discrimination index in ORT, and decreased brain AchE in both paradigms and prolonged the onset of time of death in sodium nitrite induced respiratory arrest in mice. Furthermore, HEVN (250, 500, and 1 000 mg/kg/day, p.o.) decreased brain lipid peroxidation and HEVN (500 and 1000 mg/kg/day, p.o.) increased brain reduced glutathione in scopolamine- induce cognitive deficit mice. **Conclusions:** The present study revealed the effectiveness of HEVN in improving learning and memory processes in both paradigms. The effect might be due to AchE inhibition, antioxidant effect, and/or increase in cholinergic transmission.

1. Introduction

Vitex negundo Linn. (Family: Verbenaceae) (VN) is commonly known as five leaved chaste tree (English), Nirgudi (Marathi), Nirgundi (Hindi), Indrani (Sanskrit)[1]. VN is large, aromatic, shrub or a small, slender tree with an irregular trunk growing up to 4.5 m in height. Its stem and branches are covered with thin, grey bark, which becomes almost black and scaly when old. It occurs wild in most parts of India near moist places[2]. Although all parts VN are used as medicine in the indigenous system of medicine, the leaves are the most potent for medicinal use[3]. It has been employed in Indian traditional medicinal system for the treatment of various ailments including brain tonic and to improve memory[4,5]. The effectiveness of VN has been scientifically reported for various activities such as anti-inflammatory[6]; gastroprotective[7]; anti-cancer[3]; antioxidant[8,9]; central nervous system (CNS) depressant[10]; anticonvulsant[11]; etc. VN leaves contains monoterpenoids iridoids (2-p-hydroxybenzoyl mussaenosidic; nishindaside; negundoside), triterpenoids (betulinic acid; ursolic acid), flavonoids (gardenin A; gardenin B; corymbosin; vitexicarpin; 5-hydroxy-3,6,7,3,4-penta-metoxyflavone; 3,5-dihydroxy-6,7,3,4 tetramethoxyflavanol), phenolic acid (p-hydroxybenzoic acid; 3,4-dihydroxybenzoic acid), and essential oil (sabinene, 4-terpineol, β-caryophyllene, and viridiflorol)[2]. Lignans, one class of natural compounds present in VN, showed anti-cholinesterase *in vitro* activity[12].

Recently, Kanwal *et al.* was demonstrated the improvement in learning and memory tasks in the shuttle-box by using the scopolamine- induced dementia with the aqueous herbal extract of plant VN at a dose of 300 mg/kg through inhibiting lipid peroxidation (LP), augmenting endogenous antioxidant enzymes and decreasing brain acetyl-cholinesterase (AChE) activity[12]. The higher concentration of most of the phytoconstituents, could be extracted using aqueous ethanol hence we used hydroalcoholic extract of VN leaves (HEVN).

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Our study was aimed to demonstrate the improvement of learning and memory by HEVN in normal as well as in scopolamine- induced cognitive deficit mice.

2. Materials and methods

2.1. The plant material and extraction

The fresh leaves of VN were collected from Bhor region (Dist. Pune, India) in the month of September 2009 and were authenticated and specimen was deposited at the Botanical Survey of India, Pune (Voucher Specimen No. BSI/WRA/Tech/545). The leaves were shade dried, coarsely powdered, and the powder material (200 g) was macerated for 24 h and percolated using 70% ethanol and the menstrum collected was concentrated till dry to obtain 48 g dark green coloured semisolid mass of HEVN (Yield 24%). HEVN was suspended in 1% gum acacia in distilled water[13,14].

2.2. Drug and chemicals

Piracetam (Nootropil® suspension, UCB Pvt. Ltd., India); scopolamine (Buscopan® injection, Cadila Pharma, India); acetylthiocholine iodide (Himedia, India); and other chemicals of analytical grade were procured from the local vendors of Pune, India. Nootropil® was suspended in 1 % gum acacia in distilled water; Buscopan® was diluted with distilled water; and sodium nitrite was dissolved in distilled water to prepare appropriate respective doses. All solutions were prepared freshly.

2.3. Animals

Adult Swiss albino mice (18-25 g) of either sex (Grade II), procured from National Toxicology Center, Pune, India were used for the studies. Animals were housed in groups of 5-6 in standard polypropylene cages with wire mesh top at standard environmental condition of temperature (25 ±2) ℃ and relative humidity of 45%-55% under 12 h: 12 h light: dark cycle in the institutional animal house. Animals had free access to standard pellet rodent diet (Lipton India Ltd., Mumbai, India) and water was provided ad libitum. All experiments were carried out between 08:00 to 16:00. The experimental protocol was approved by the Institutional Animal Ethics Committee of Rajgad Dnyanpeeth's College of Pharmacy, Bhor, India constituted as per the rules and guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Chennai, India (Approval No. RDCOP/ IAEC/12/09).

2.4. Dose selection

All drugs and vehicle (1% gum acacia in distilled water) were administered in the volume of 5 mL/kg and doses were calculated according to the body weight of animals. The doses of HEVN selected in the present study were according to the previously reported LD_{50} (7580 mg/kg, p.o.)[15] and that were used in the previous study[11].

2.5. Preliminary phytochemical analysis of HEVN

The preliminary phytochemical analysis was performed using various standard phytochemical tests for the qualitative estimation of presence of various phytochemicals in HEVN[16,17].

2.6. Experimental design

Swiss albino mice (18-25 g) of either sex were divided into 10 groups (n=6) and treated for 8 days as follows- Group I (Normal control): vehicle (5 mL/kg/day, p.o.); group II (Piracetam): piracetam (200 mg/kg/day, p.o.); group III, IV, and V (HEVN 250, 500, and 1000): HEVN (250, 500, and 1000 mg/kg/day, p.o.) respectively; group VI (Control): vehicle (5 ml/kg/day, p.o.); group VII (Piracetam+scop): piracetam (200 mg/kg/day, p.o.); group VIII, IX, and X (HEVN 250+scop, 500+scop, and 1000+scop): HEVN (250, 500, and 1000 mg/kg/day, p.o.) respectively. Additionally, on 8th day, in EPM test 45 minutes after the respective treatments and in ORT 30 minutes before the respective treatments, scopolamine (0.4 mg/kg, i.p.) was administered to groups VI, VII, VIII, IX, and X to induce cognitive deficit in mice. To evaluate the effect on learning and memory in normal mice (without inducing cognitive deficit) groups I, II, III, IV, and V were used and that in scopolamine- induced cognitive deficit mice groups VI, VII, VIII, IX, and X were used.

2.7. Elevated plus maze (EPM) test

The apparatus consisted of two open arms (16 cm× 5 cm) and two enclosed 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 8th day after the last treatment, each mouse was placed at the end of an open arm, facing away from the central platform. The transfer latency (TL), the time taken by mouse with all its four legs to move into one of the enclosed arm, was recorded as L0. If the animal did not enter into one of the enclosed arms within 90 s, it was gently pushed into one of the two enclosed arms and the TL was assigned as 90 s. The mouse was allowed to explore the maze for another 10 s and then returned to its home cage. The retention of this learned task was examined 24 h after the 8th day trial i.e. on 9th day and the TL was recorded as L1. The effect on TL was expressed by inflexion ratio (IR). Increase in IR after 24 h indicated improved retention of learned task. IR was calculated using the formula:

$IR = (L0 - L1) / L1$

Where L0 = initial TL (s) on 8th and L1 = TL after 24 hr of first day trial i.e. on 9th day[18-20].

2.8. Object recognition test (ORT)

The apparatus consisted of a white colored plywood box (70 × 60 × 30 cm) with a grid floor that could be easily cleaned with hydrogen peroxide after each trial. The apparatus was

illuminated by a 60 W lamp suspended 50 cm above the box. The object to be discriminated was also made of plywood in two different shapes of 8 cm height and coloured black. On the day before test, mice were allowed to explore the box (without any object) for 2 minutes. On the day of test in the first trial (T1), two identical objects were presented in two opposite corners of the box, and the amount of the 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 with nose. During the second trial (T2, 90 minutes after T1), a new object replaced one of the objects presented in T1, and mice were left individually in the box for 5 minutes. The time spent for exploring the familiar (F) and the new object (N) was recorded separately, and discrimination index (DI) was calculated as (N − F) / (N + F). Care was taken to avoid place preference and the influence of olfactory stimuli by randomly changing the role (familiar or new object) and the position of the two objects during T2 and cleaning the apparatus with hydrogen peroxide. The first trial (T1) was conducted 60 minutes after the last treatment on 8th day and second trial (T2) was 90 minutes after (T1)[21,22].

2.9. Biochemical estimations of brain

2.9.1. Supernatant preparation

On the 9th day immediately after the test animals from all groups were sacrificed by cervical dislocation and brain of individual animal was carefully isolated, placed on the petridish, over ice, and weighed. Whole brain samples were rinsed with ice cold normal saline. A 20 mg tissue/mL homogenate of brain samples were prepared by homogenizing in chilled phosphate buffer (pH 7.4) using glass Teflon homogenizer (RQ-127A, Remi motors; Mumbai, India). The homogenates were centrifuged at 800 g for 5 minutes at 4 ℃ to separate the nuclear debris. The supernatant thus obtained was centrifuged at 10 500 g for 20 minutes at 4 ℃ to get the supernatant. Supernatant was collected from individual animal from all groups and subjected to the estimation of AchE level and total protein; additionally supernatant collected from animal from group VI, VII, VIII, IX, and X was subjected to the estimation of lipid peroxidation (LP) and reduced glutathione (GSH)[23].

2.9.2. Estimation of brain AchE level

Cholinergic dysfunction was assessed by measuring AchE levels in whole brain according to the method of Ellman et al. (1961). Briefly, 0.4 ml supernatant was added to a cuvette containing 2.6 mL of sodium phosphate buffer (0.1 M, pH 7.2) to which a 100 μL of Ellaman′s reagent (0.5 mM, 19.8 mg DTNB and 0.1 M sodium phosphate, pH 7.2 to make 100 ml) was added and absorbance was measured spectrometrically (V-530, Jasco International Co. Ltd., Tokyo, Japan) at 412 nm till the increasing absorbance become stable. This stable absorbance was then set to zero and 20 μL of acetylthiocholine iodide (substrate) was added and changes in absorbance per minute were recorded for 10 minutes. The changes in absorbance were recorded spectrometrically. The change in absorbance per minutes was calculated. The rate was calculated by using following formula and AchE activity was measured as μM/L/min/g tissue[24].

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

Where, R is rate, in moles substrate hydrolyzed per minutes per g of tissue; A is change in absorbance per minutes; Co is original concentration of tissue i.e. 20 mg/mL.

2.9.3. Estimation of brain lipid peroxidation (LP)

The malondialdehyde (MDA) content, a measure of LP, was assayed in the form of thiobarbituric acid-reactive substances (TBARS) by the method of Wills. Briefly, 0.5 mL supernatant and 0.5 mL of Tris HCl were incubated at 37 ℃ for 2 h. After incubation 1 mL of 10 % trichloroacetic acid was added and centrifuged at 1000 g for 10 minutes. To 1 mL of supernatant, 1 mL of 0.67 % thiobarbituric acid was added and the tubes were kept in boiling water for 10 minutes. After cooling 1 mL double distilled water was added and absorbance was measured spectrometrically (V-530, Jasco International Co. Ltd., Tokyo, Japan) at 532 nm. The brain MDA content was expressed as nmol/mg protein[25].

2.9.4. Estimation of brain reduced glutathione (GSH)

One ml of supernatant was precipitated with 1 mL of sulphosalicylic acid (4 %). The samples were kept at 4 ℃ for at least 1 h and then centrifuged at 1 200 g for 15 minutes at 4 ℃. The assay mixture contained 0.1 mL supernatant, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 mL Ellman′s reagent, 0.1 mM, pH 8.0 in a total volume of 3.0 mL. The yellow color developed was read immediately spectrometrically (V-530, Jasco International Co. Ltd., Tokyo, Japan) at 412 nm. The GSH concentrations of the samples were derived from the standard curve prepared using known amounts of GSH and expressed as μg/mg protein[26,27].

2.9.5. Estimation of brain total protein

Brain total protein was estimated by the Biuret method using test kit (Coral clinical system, India) on a semi-autoanalyser (Lablife chemmaster, India)[28].

2.10. Sodium nitrite induced respiratory arrest in mice

Swiss albino mice (18-25 g) of either sex were divided into 4 groups (n=6) and treated as group I (Control): vehicle (5 ml/kg/day, p.o.); group II, III, and IV (HEVN 250, 500, and 1000): HEVN (250, 500, and 1 000 mg/kg/day, p.o.) respectively at specific time daily for 8 days. On 8th day, 60 minutes after respective treatments, sodium nitrite (250 mg/kg, i.p.) was administered to the animals from all groups and the time between injection of sodium nitrite and death was recorded[22,29].

2.11. Statistical analysis

Data was expressed as mean±SEM (n=6) for each experimental group. The results were analyzed for statistical significance using Student′s t- test or one-way analysis of variance (ANOVA) followed by Dunnett′s test. P<0.05,

$P<0.01$, and $P<0.001$ was considered to be significant.

3. Results

3.1. Preliminary phytochemical analysis of HEVN

Preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, proteins, and carbohydrates in the HEVN.

3.2. Elevated plus maze (EPM) test

In normal mice, administration of HEVN (250 and 500 mg/kg) showed significant increase in the IR as compared with normal control group ($P<0.01$ and $P<0.05$). Whereas HEVN 1 000 mg/kg showed insignificant effects in this regard. Furthermore, administration of piracetam 200 mg/kg in normal animals showed significant increase in IR as compared with normal control group ($P<0.01$) (Table 1).

Administration of scopolamine 0.4 mg/kg showed significant decrease in IR in control group as compared with normal control group ($P<0.01$). This revealed the induction of cognitive deficit in control group. In scopolamine- induced cognitive deficit mice, administration of HEVN (250 and 500 mg/kg) showed significant increase in IR as compared with control group ($P<0.01$ and $P<0.05$). Whereas HEVN 1 000 mg/kg showed insignificant effects in this regard. Furthermore, administration of piracetam 200 mg/kg showed significant increase in IR as compared with control group ($P<0.01$) (Table 1).

Table 1
Effect of HEVN in normal mice and scopolamine induced cognitive deficit mice in EPM test (Mean±SEM) (n=6).

Group No.	Groups	Transfer latency (Seconds)		Inflexion ratio
		8th day	9th day	
I	Normal control	35.500±2.078	29.330±2.246	0.210±0.034
II	Piracetam	26.670±1.229	19.000 ±0.894[b]	0.380 ±0.015[b]
III	HEVN 250	32.000±1.932	23.33 0±1.838[a]	0.380 ±0.034[b]
IV	HEVN 500	36.330±2.333	27.170±1.833	0.340 ±0.016[a]
V	HEVN 1000	36.170±1.276	28.000±1.033	0.250±0.035
VI	Control	46.330±2.721	41.170±2.301[a]	0.110±0.010[b]
VII	Piracetam+Scop	34.830±1.990	27.830±1.558[*]	0.240±0.017[**]
VIII	HEVN 250+Scop	39.000±2.793	32.000±2.463[*]	0.210±0.013[**]
IX	HEVN 500+Scop	40.500±4.440	34.170±3.928[*]	0.170±0.019[*]
X	HEVN 1000+Scop	43.830±3.487	38.330±2.836	0.130±0.010

[b]: $P<0.01$, [c]: $P<0.001$ as compared with normal control group; *: $P<0.05$, **: $P<0.01$ as compared with control group; Scop: scopolamine.

3.3. Object recognition test (ORT)

In normal mice, administration of HEVN (250 and 500 mg/kg) showed significant increase in DI as compared with normal control group ($P<0.001$ and $P<0.01$). Whereas HEVN 1 000 mg/kg showed insignificant effects in this regard. Furthermore, administration of piracetam 200 mg/kg in normal animals showed significant increase in DI as compared with normal control group ($P<0.001$) (Table 2).

Administration of scopolamine 0.4 mg/kg induced cognitive deficit in control group, as evident from significant decrease in DI as compared with normal control group ($P<0.001$). In scopolamine- induced cognitive deficit mice, administration of HEVN (250 and 500 mg/kg) showed significant increase in DI as compared with control group ($P<0.01$ and $P<0.05$). Whereas HEVN 1000 mg/kg showed insignificant effects in this regard. Furthermore, administration of piracetam 200 mg/kg showed significant increase in DI as compared with control group ($P<0.01$) (Table 2).

Table 2
Effect of HEVN in normal mice and scopolamine induced cognitive deficit mice in ORT (Mean±SEM) (n=6).

Group No.	Groups	Exploration time (Seconds)			Discrimination index
		T1 session	T2 session		
			N	F	
I	Normal control	384.5±24.9	152.7±24.5	93.00±15.8	0.24±0.02
II	Piracetam	347.8±24.1	127.3±22.0	42.33±11.4	0.52 ±0.04[c]
III	HEVN 250	406.7±43.3	154.8±17.7	51.83±4.91	0.48±0.03[c]
IV	HEVN 500	372.0±41.0	171.8±21.5	67.50±8.60	0.43±0.01[b]
V	HEVN 1000	382.5±37.0	174.0±25.6	96.17±17.70	0.27±0.01
VI	Control	548.0±42.7	137.5±21.2	117.20±15.90	0.06±0.01[c]
VII	Piracetam +Scop	349.0±36.0	133.5±11.4	88.50±8.83	0.20±0.02[**]
VIII	HEVN 250+Scop	312.5±27.6	146.7±9.1	103.00±7.12	0.17±0.02[**]
IX	HEVN 500+Scop	392.2±39.1	159.5±15.5	117.80±14.50	0.15±0.01[*]
X	HEVN 1000+Scop	388.3±30.1	175.3±19.6	146.30±18.30	0.08±0.01

[b]: $P<0.01$, [c]: $P<0.001$ as compared with normal control group; [*]: $P<0.05$, **: $P<0.01$ as compared with control group; N: Novel object; F: Familiar object; Scop: scopolamine.

3.4. Estimation of brain AchE level

In normal mice, administration of HEVN (250 and 500 mg/kg) showed significant decrease in brain AchE level as compared with normal control group ($P<0.01$ and $P<0.05$). Whereas HEVN 1 000 mg/kg showed insignificant effects in this regard. Furthermore, administration of piracetam 200 mg/kg showed significant decrease in brain AchE level as compared with normal control group ($P<0.01$) (Table 3).

Administration of scopolamine 0.4 mg/kg showed significant increase in brain AchE level in control group as comapred with normal control group ($P<0.01$). In scopolamine- induced cognitive deficit mice, administration of HEVN (250 and 500 mg/kg) showed significant decrease in AchE level as compared with control group ($P<0.01$ and $P<0.05$). Whereas HEVN 1 000 mg/kg showed insignificant effects in this regard. Furthermore, administration of piracetam 200 mg/kg showed significant decrease in brain

AchE level as comapred with control group (P<0.01) (Table 3).

Table 3
Effect of HEVN on brain AchE level in normal and scopolamine induced cognitive deficit mice (Mean±SEM) (n=6).

Group No.	Groups	AchE (μ M/L/min/g tissue)
I	Normal control	7.692±0.195
II	Piracetam	6.597±0.155[b]
III	HEVN 250	6.810±0.111[b]
IV	HEVN 500	7.144±0.174[a]
V	HEVN 1 000	7.621±0.150
VI	Control	9.370±0.145[b]
VII	Piracetam+Scop	7.864±0.154[**]
VIII	HEVN 250+Scop	8.232±0.142[**]
IX	HEVN 500+Scop	8.481±0.172[*]
X	HEVN 1000+Scop	9.129± 0.149

[a]: P<0.05, [b]: P<0.01, [c]: P<0.001 as compared with normal control group; *: P<0.05, **: P<0.01 as compared with control group; AchE: acetylcholine esterase; Scop: scopolamine.

3.5. Estimation of brain lipid peroxidation (LP)

Administration of scopolamine 0.4 mg/kg showed significant increase in brain MDA level in control group as compared with normal control group (P<0.001). In scopolamine- induced cognitive deficit mice, administration of HEVN (250, 500, and 1 000 mg/kg) showed significant decrease the brain MDA level as compared with control group (P<0.05 and P<0.01). Furthermore, administration of piracetam 200 mg/kg showed significant decreases in brain MDA level as compared with control group (P<0.01) (Figure 1).

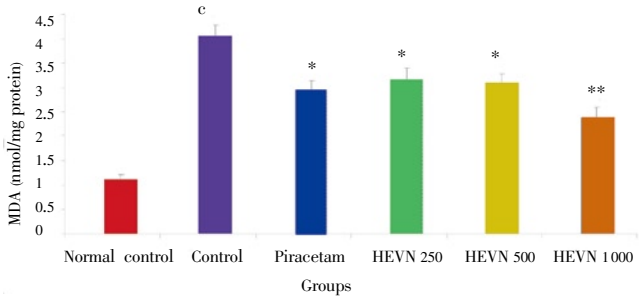

Figure 1. Effect of HEVN on brain LP in scopolamine induced cogntive deficit mice (Mean±SEM) (n=6).
[c]: P<0.001 as compared with normal control group; [*]: P<0.05, [**]: P<0.01 as compared with control group.

3.6. Estimation of brain reduced glutathione (GSH)

Administration of scopolamine 0.4 mg/kg showed significant decrease in brain GSH level in control group as compared with normal control group (P<0.01). In scopolamine- induced cognitive deficit mice, administration of HEVN (500 and 1000 mg/kg) showed significant increase in brain GSH level as compared with control group (P<0.05). Whereas HEVN 250 mg/kg showed insignificant effects in this regard. Furthermore, administration of piracetam 200 mg/kg showed significant increase in brain GSH level as compared with control group (P<0.01) (Figure 2).

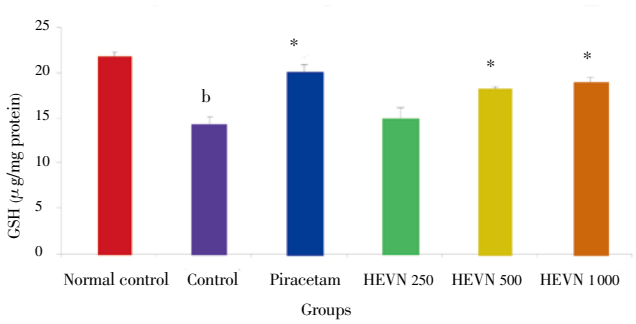

Figure 2. Effect of HEVN on brain GSH level in scopolamine induced cognitive deficit mice (Mean±SEM) (n=6).
b: P<0.01 as compared with normal control group (Student's t- test); *: P<0.05 as compared with control group.

3.7. Sodium nitrite induced respiratory arrest in mice

The mice, from control group, treated with sodium nitrite died after (15.8±0.92) minutes of its administration, whereas death occurred after (20.5±1.06), (19.4±0.67), and (16.3±0.71) minutes in the HEVN (250, 500, and 1000 mg/kg) treated mice. Administration of HEVN (250 and 500 mg/kg) were significantly prolonged the time of onset of the death due to sodium nitrite treatment (P<0.01), whereas that of HEVN 1000 mg/kg was insignificant in this regard as compared with control group (Figure 3).

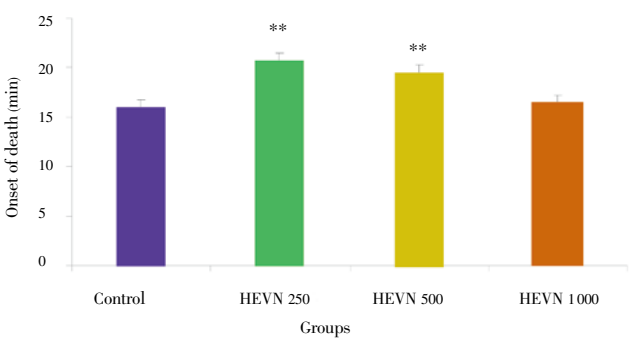

Figure 3. Effect of HEVN on sodium nitrite induced respiratory arrest in mice (Mean±SEM) (n=6).
**: P<0.01 as compared with control group.

4. Discussion

EPM is a neutral exteroceptive behavioral model to assess learning and short-term memory in mice[18,30]. The principle of the EPM test is based on the assumption that mice prefer the enclosed arms to the open arms. Animals spend more time in the enclosed arms than the open arms because they dislike the open arms. It has reported that the aversive quality of the open arms is not apparent until the rats enter them[19]. Moreover, ORT measures nonspatial working

memory with the characteristics of episodic memory lasting for at least 60 minutes[21,31]. ORT is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one. The choice to explore the novel object reflects the use of learning and recognition memory. Object recognition depends on the integrity of the cholinergic system as demonstrated by its impairment by scopolamine, lesions of the nucleus basalis, and aging. The impairment is restored by administration of oxiracetam and aniracetam with an inverse U-shaped dose-response curve[31,32].

The nootropic drugs belong to the category of psychotropic agents with selective facilitatory effect on intellectual performance, learning, and memory. The increase in the IR in EPM and DI in ORT by HEVN (250 and 500 mg/kg) per se proved that HEVN possessed nootropic activity. Thus, the HEVN meets a major criterion for nootropic activity, *i.e.* improvement of memory in absence of cognitive deficit[20,33,34]. These results could be further extrapolated for possible utility of HEVN in poor learner normal individuals to meet the competitive demands of life.

Management of cognitive deficit is another prime target for nootropics. The cholinergic pathways projecting to the cerebral cortex and hippocampus play a key role in mechanisms of learning and memory[31]. It has been suggested that cholinergic deficiency may account for some of the cognitive impairments in Alzheimer's disease (AD)[35]. Scopolamine, a nonselective muscarinic cholinergic receptor antagonist, is a well known centrally acting cholinergic probe that causes impairment of learning and memory in rodents and humans, especially the processes of learning acquisition and short-term memory[36]. An interoceptive behavioral model such as scopolamine- induced amnesia in experimental subjects is a widely cited model simulating human dementia in general and AD in particular[30,37]. The administration scopolamine produces transient memory deficit when given shortly before test. The ability of a range of different cholinergic agonist drugs to reverse the amnesic affects of scopolamine is now well documented in animals and human volunteers[12]. AchE inhibitors, which enhance the availability of acetylcholine (Ach) in the synaptic cleft, were able to reverse the scopolamine- induced cognitive deficit, indicating that the cognitive deficit is cholinergic in nature[37].

In present study, pretreatment with HEVN (250 and 500 mg/kg) for 8 days protected the animals from learning and memory impairment produced by scopolamine 0.4 mg/kg, as evident from significant increase in IR in EPM test and DI in ORT. The antagonistic action of HEVN against scopolamine- induced amnesia substantiates nootropic activity of HEVN. These findings suggested the possible utility of the HEVN towards wide spectrum of cognitive impairment associated with cholinergic transmission and modulation in CNS and thus suggested the possible neuroprotective role of HEVN.

AchE enzyme is essential in maintaining the normal function of the nervous system, since it rapidly terminates the action of Ach released into the synapse, and it participates in the underlying processes in AD. Cholinergic interneurons in the striatum are even richer source of AChE[36]. One of the treatment strategies to enhance cholinergic functions is the use of AchE inhibitors that increase the availability of Ach in central cholinergic synapses and are used to treat AD[36,38]. In present study, administration of HEVN (250 and 500 mg/kg) significantly decreased brain AchE level in normal as well as in scopolamine treated mice.

Furthermore, Ach is involved in central respiratory control including central chemosensitivity[39] and particularly it is important in the central chemo sensitivity in the ventilatory response to H^+ / CO_2 stimulus. Inhibition of Ach synthesis lead to prompt reversible depressions of respiratory neuronal activity while enhanced Ach synthesis resulted in tonic activation of respiratory neuronal output[40,41]. Sodium nitrite is known to reduce the oxygen-carrying capacity of blood by converting hemoglobin to methemoglobin that induces chemical hypoxia. Sodium nitrite (250 mg/kg, i.p.) produces death due to respiratory arrest in mice. Drugs increasing central cholinergic transmission delay or prevent the onset of respiratory arrest[22]. In present study, administration of HEVN (250 and 500 mg/kg) was significantly prolonged the time of onset of death in sodium nitrite induced respiratory arrest in mice. The results of estimation of brain AchE level and sodium nitrite induced respiratory arrest in mice indicated that nootropic potential of HEVN may be due to inhibiting AchE or by increasing the cholinergic transmission in brain.

Additionally, it is reported that increasing incidences of cognitive deficit has largely dedicated to oxidative stress[42,43]. Recently it was reported that cognitive impairment in the scopolamine- induced cognitive deficit is associated with increased oxidative stress in brain[44,45]. Hence reduction of oxidative stress by virtue of antioxidants can be a good option for the treatment of cognitive deficit. Antioxidant enzyme like SOD and non enzymatic antioxidant like GSH are the first line of defense against LP due to oxidative stress[7]. The raised level of GSH, an endogenous antioxidant, was taken as an indicator of the antioxidant property of the test drug.

Tandon and Gupta demonstrated that HEVN produced significant reduction in MDA levels and a marginal rise of SOD (not significant) after 14-day treatment at only the higher dose (500 mg/kg, p.o.) in ethanol-induced oxidative stress in rats[46]. In present study HEVN (250, 500, and 1000 mg/kg) significantly decreased the MDA level and HEVN (500 and 1000 mg/kg) increased the GSH level. The results are consistent with the earlier reports of antioxidant activity of VN[7,8,12,47] confirming its antioxidant potential of HEVN.

Further the present study showed most significant effect of HEVN at lower dose suggesting potential nature of the extract with minimum chances of dose dependent toxicity.

Tandon and Gupta previously reported that HEVN significantly increased post-ictal depression at dose of 1 000 mg/kg, thereby indicated its CNS depressant action at this dose[11]. In present study HEVN 1 000 mg/kg showed lack of nootropic activity. This may be due to sluggishness induced by the depressant activity associated with this dose. This type of inverse U dose-response relationship typical of drugs acting on CNS particularly nootropic and anxiolytic agents has been reported previously[20,29]. The possible reason for this effect may be due to dose dependent modulation in various neurotransmitters involved in learning and memory which can be confirmed only after detailed neurochemical estimation.

In summary, in spite of the previous study by Kanwal *et al*. focused on the effect of aqueous extract of the plant *Vitex negundo* extract on scopolamine induced amnesia in rats[12], present study investigated and revealed the effectiveness of hydroalcoholic extract of Vitex negundo leaves in normal as well as in scopolamine- induced cognitive deficit mice and thus the possible utility of HEVN in poor learners to meet cognitive demand as well as in age, stress, emotions, etc. induced cognitive dysfunction. The results suggested that flavonoids, triterpenoids, phenolic acid, lignans etc. present in the extract might be responsible for the nootropic activity. The effect may be due to AchE inhibition, antioxidant effect, and/or increase in cholinergic transmission by the extract. However, this can be further confirmed by studies like isolation and characterization of active phytoconstituent(s) in the extract responsible for this activity.

Conflict of interest statement

We declare that we have no conflict of interest.

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