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Anxiolytic like effects of leaves extract of *Vitex negundo* (L) (fam:-verbaceae) in elevated plus maze test

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Abstract

Pathological anxiety is one of the most common emotional disorders and treatment of phobias or panic attacks is still not trivial. To our knowledge, no scientific data is available concerning the efficacy of ethanolic extract of leaves of *Vitex negundo* (VNE) in animal tests of anxiety. This study was carried out to compare the anxiolytic potential of ethanolic extract of leaves of *Vitex negundo* and compared with standard diazepam (3 mg/kg, i.p.). Acute effects of diazepam and a ethanolic extract of leaves of *Vitex negundo* preparation for anxiolytic activity compared to their respective controls, were examined in mice using the elevated plus maze (X-maze). The time spent on open arms, open-arm visits, distance travelled in open and closed arms, sleep induction, motor co-ordination and locomotion parameters describing the risk assessment were evaluated. VNE (200, 300 mg/kg, i.p.) affected the behavior measured in the X-maze test, inducing an anxiolytic like behavior similar to diazepam. These data support the use of ethanolic extract of leaves of *Vitex negundo* in the treatment of anxiety and could be useful in primary medical care. Further study need to be done to characterize active phytoconstituents.

***Keywords*:** Anxiolytic; Nirgudi; Sleep induction; *Vitex negundo*.

1. Introduction

Anxiety is a common emotion and is typically a response to perceived threat, such as an impending operation. Like depression, anxiety is usually seen in any patients and occurs in a similar proportion. It may be transient persistent, episodic or limited to specific situation. Anxiety affects one-eighth of the total population worldwide and has become a very important area of research interest in psychopharmacology during this decade. There are various ways of explaining the mechanisms of action of anti-anxiety agents because of the involvement of many CNS chemical mediators [1]. Benzodiazepines are still the most frequently used drugs for the treatment of generalized anxiety disorder despite their undesirable side effects such as muscle relaxation, sedation, physical dependence, memory disturbance, and

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interaction with other drugs. Nevertheless, there is considerable interest in the development of new anxiolytics [2, 3]. New synthesized compounds as well as drugs derived from traditional herbs may have a possible therapeutic relevance in the treatment of anxiety [4]. Especially, the use of "mild", "natural" and tolerable phytopharmaceuticals are in public favour for this purpose [5]. *Vitex negundo* Linn. is a large shrub with thin bark, grey branches. The plant is having pungent, bitter, acrid taste. This plant is prevalent in the north-western Himalayan region and has been used for various medicinal purposes in the ayurvedic and unani systems of medicine. Leaves are aromatic tonic and vermifuge. Decoction of nirgudi is given along with pepper in catarrhal fewer. Juice of leaves is said to have property of removing worms from ulcers. Oil is applied to sinuses [6]. Leaves are useful in dispersing swelling similarly leaves were found to be useful as an anti allergic [7, 8]. Dried leaves are smoked for headache. Almost all the parts are employed, but the leaves and the roots are important as drugs. Analgesic and anti-inflammatory actions of *Vitex negundo* seeds and fruit have been reviewed thoroughly. Petroleum ether extract of *Vitex negundo* leaves has shown significant analgesic activity and the anticonvulsant activity against strychnine and leptazole [9-11]. Dried leaves powder of *Vitex negundo* showed anti-arthritic activity in rats [12]. Preliminary evaluation of the ethanolic extract revealed that the extract inhibited passive peritoneal anaphylaxis and mast cell degranulation in rats in a dose-related manner [13]. The X-maze, based on the Y-maze [14], is a well-established test for anxiety-like behaviors that has been used in rats [15], mice [16] and in guinea pigs [17]. Additionally, the X-maze test is now the most popular and widely used animal test for anxiety and results obtained can be compared with the literature or rated more easily [18]. Aim of the present study was the determination of anxiolytic-related behavior on acute administration of ethanolic extract of leaves of *Vitex negundo*.

2. Materials and Methods

2.1. Plant Material

The leaves of *Vitex Negundo* were collected from Khadakwasla region of Pune, Maharashtra and were authentified by Dr. A.S. Upadhye, S cientist, Plant Drug Authentification Service, Botany Group, Agarkar Research Institute, Agarkar Road, Pune. The Voucher specimens (Auth. 10/102) have been deposited in the Herbarium of Dept. of Pharmacognosy of Sinhgad Institute of Pharmacy, Narhe, Pune.

2.2. Preparation of Extract

The leaves of *Vitex Negundo* (500 g) were shade dried and grinded to coarse powder and then passed through sieve no. 22#. The dried finely powdered material was then exhaustively extracted with 95% ethanol by soxhletion, concentrated under controlled temperature (yield 1%) and was stored in refrigerator until it was used for the phytochemical and pharmacological investigation. [19]

2.3. Apparatus

Elevated plus-maze apparatus consisting of two open arms (25 cm ×5 cm) and two enclosed arms (15 cm×5 cm×25 cm), extending from a central platform (5 cm×5 cm) and raised 50 cm above floor level [20].

2.4. Drugs

Diazepam (Watson Pharmaceuticals, India) was used as standard drug. VNE is freely soluble in distilled water. Solutions of extracts were prepared by dissolving the extracts in double

distilled water and were administered at different doses (200 and 300 mg/kg, i.p.). All solutions were prepared freshly on test days and administered intraperitoneally in a volume of 0.1 ml/10 g body weight of mice.

2.5. Animals

Swiss albino mice of either sex were obtained from National Toxicological Center, Pune and housed at the Institute Animal House in groups of six animals per cage at standard laboratory conditions at a temperature of 25 ± 10°C, relative humidity of 45–55% and 12:12 h dark and light cycle. The animal experimental protocol of this study was approved by the Institutional Animal Ethical Committee (IAEC) of Sinhgad Institute of Pharmacy, Pune, India constituted as per guidelines of "Committee for Purpose of Control and Supervision of Experimental Animals" (CPCSEA), India (Ref: SIOP/IAEC/2011-04).

2.6. Phytochemical Investigation of Extract

The extracts were tested for the presence of proteins, amino acids, alkaloids, flavanoids, glycosides, saponins and tannins using standard procedures [21].

2.7. Pharmacological Assays

Mice weighing between 22–25 g of either sex were selected for experiments. Animals had free access to food (Standard chaw pellet, Chakan oil mills, Sangli) and water *ad libitum*. All the experiments were carried out between 10:00 and 17:00 h. One hour prior the administration of the drugs, mice were moved into the laboratory to habituate to the experimental environment. Each animal was used only once.

2.7.1. Acute Toxicity

The acute toxicity study was performed as per the OECD guidelines 425 at a limit dose of 2000 mg/kg procedure. Animals were observed individually at least once during the first 30 min. after dosing, periodically during the first 24 h (with special attention given during the first 4 h), and daily thereafter, for a total of 14 days for mortality if any.

2.7.2. Assessment of Anxiolytic activity

The animals were placed in the middle of the X-maze facing a corner of the centre platform (equal choice of entering an open or closed arm) 60 min after receiving vehicle, VNE (200, 300 mg/kg, i.p.) or diazepam (3 mg/kg, i.p.). The experiments were performed for 5 min [22, 23]. All test sessions were video recorded with a camera mounted vertically above the maze. (Maze Master, VJ Instruments, India). A computerized measurement of the distance travelled in each arm was included. Each group was having 6 animals. The behavioral parameters measured were entries into the open arms in percent of the total entries into all arms, time spent on the open arms and the number of returning to the protecting closed arms (c-returns) as measures of anxiety-related activity and the total number of entries into all arms, the number of entries into the closed arms and the distance travelled in each arm as measures of locomotor activity. Four paws onto and two paws off of an arm constitute an arm entry and exit. The behavioral experiments took place under quiet conditions and low light (50 lux) and were carried between 13:30 and 16:30 h.

2.7.3. Spontaneous activity (SA) test

Mice were randomly assigned to different groups (n=6). The mouse's autonomic activity was measured with a locomotor-monitoring apparatus (Actophotometer, INCO, India). The counts of horizontal locomotion and rearing behavior of mouse in a locomotor monitoring cage were digitally recorded for 5 min. Each mouse was individually placed in the chamber at different times, namely, just before the dose administration and 30 min after the

administration. After each measurement, the locomotor-monitoring cages were carefully cleaned with wet tissue paper (10% ethanol solution).

2.7.4. Pentobarbitone-induced sleep

Mice were pretreated intraperitoneally with either vehicle or VNE (200, 300 mg/kg) 30 min before pentobarbitone (40 mg/kg, i.p.). The onset and duration of sleeping time was measured as the period that mice lost the righting reflex after receiving the hypnotic drug.

2.7. Neuromuscular coordination – Rotarod

The effect of the VNE (200, 300 mg/kg) on coordinated motor movements was assessed using the rotarod test. A day before the test, mice were trained to stay on the rotating wheel (3 cm in diameter, 20 rpm) for more than 1 min. On the test day, mice were tested on the rotarod (model 7600, INCO, India) before and 30 min after the administration of saline, diazepam or the VNE. The number of seconds each mouse remained on the rotating wheel was recorded for a maximum of 300 seconds.

2.8. Statistical analysis

Values are expressed as mean ± S.E.M. Data were analyzed by one-way and two-way analysis of variance (ANOVA) followed by Dunnett's'*t*' test and Bonferroni post tests for multiple comparisons. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Phytochemical Investigations

The extract was found to show the sugars, glycosides, alkaloids and flavanoids.

3.2. Acute Toxicity Study

Ethanolic extract of leaves of *Vitex negundo* was found to be safe in the doses used and there were no mortality up to a dose of 2000 mg/kg, P.O. upto fourteen days observation.

3.3. Assessment of Anxiolytic activity

The vehicle-treated mice spent 4.33 ± 0.42 s in open arm and 37.29 ± 0.57 s in closed arm with 2.16 ± 0.30 entries in open arm and 15.67 ± 0.84 entries into the enclosed arm and total distance traveled on each arm is 52.36 ±1.48 sec. Diazepam (3 mg/kg) and VNE (300 mg/kg) showed significant ($P < 0.05$) increase in the occupancy in open arm and decrease in occupancy in closed arm whereas VNE, 200 mg/kg showed insignificant decrease in the time spent in closed arm. The animals treated with diazepam and VNE showed decreased preference to the closed-arm entries and open-arm entries were significantly increased by VNE.

The results are shown below in Fig.1 and Fig.2

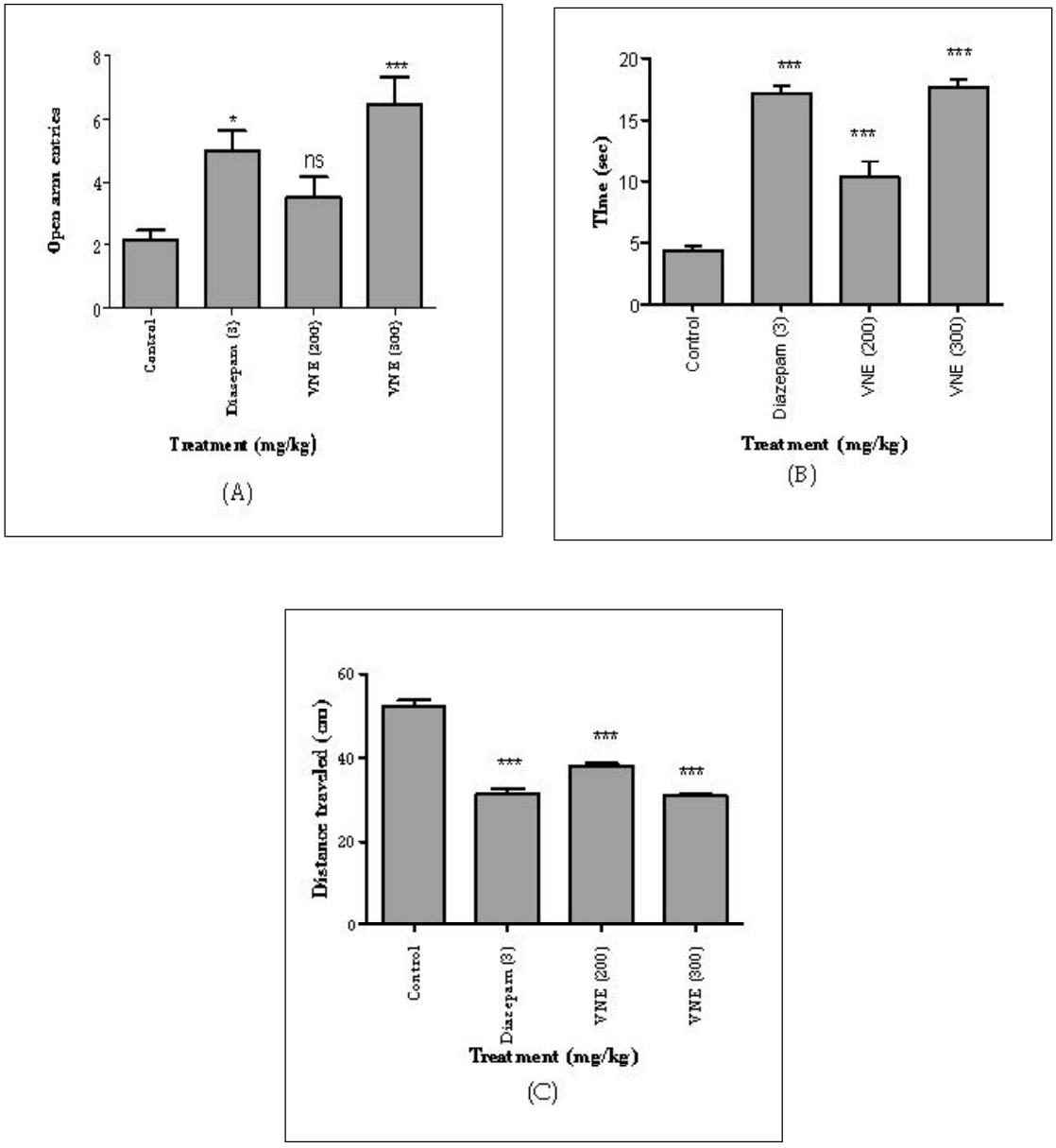

Fig 1. VNE (200, 300 mg/kg, i.p.; n = 6) induced an ''anxiolytic'' behavior on exposure to the X-maze with increased entries (A) into the open arms and more time spent (B) on the open arms compared to the control (n = 6). Total distance travelled (C) in open arms is also decreased.
* $P < 0.05$, one-way ANOVA followed by Dunnett's test. Data presented as means ± S.E.M., n=6

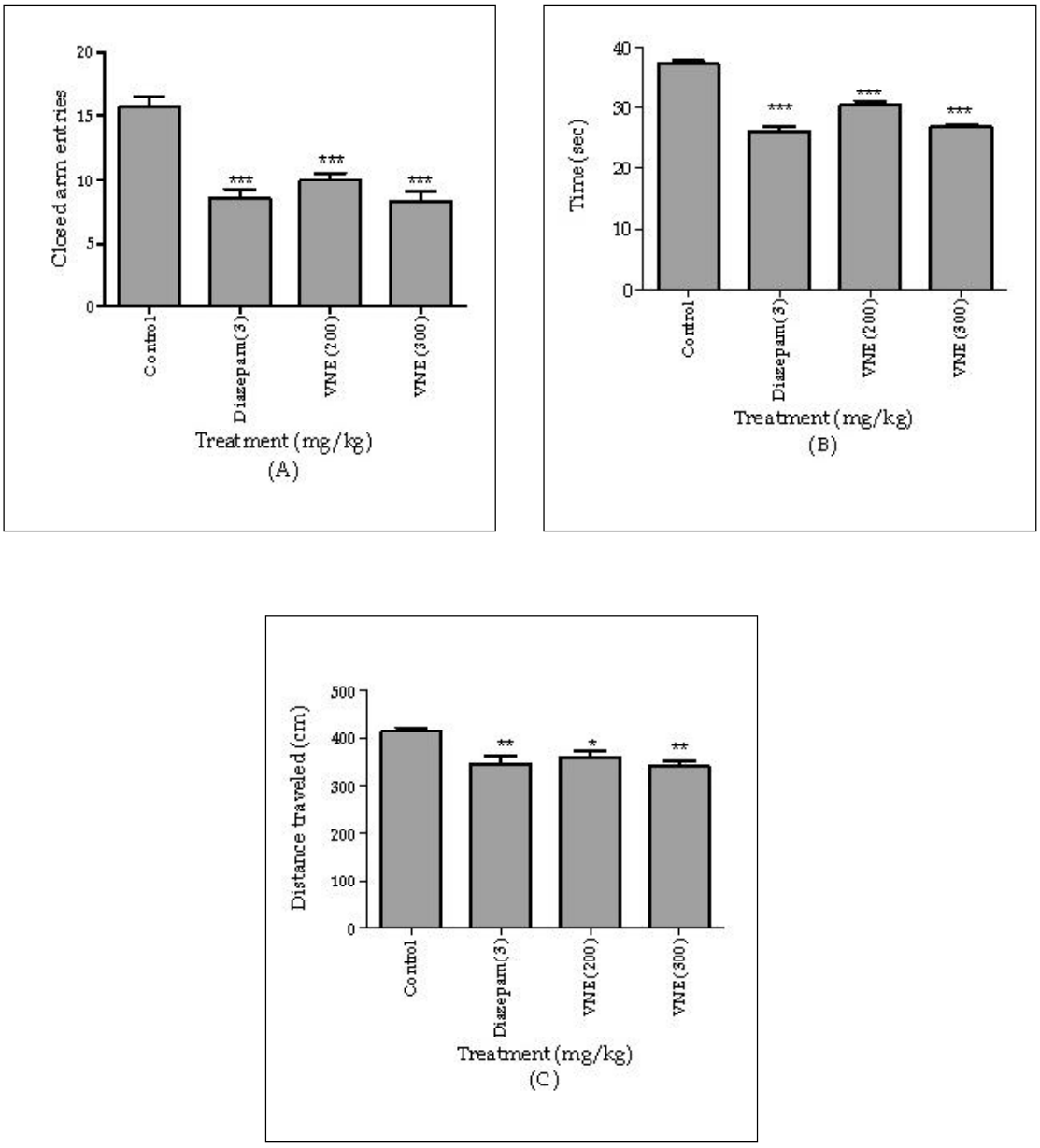

Fig 2. VNE (200, 300 mg/kg, i.p.; n = 6) induced an ''anxiolytic'' behavior on exposure to the X-maze with decreased entries (A) into the closed arms and less time spent (B) on the closed arms compared to the control (n = 6). Total distance travelled (C) in closed arms is also decreased.
* $P < 0.05$, one-way ANOVA followed by Dunnett's test. Data presented as means ± S.E.M., n=6

3.4. Spontaneous activity (SA) test

Dunnett *t* post hoc comparison revealed that diazepam at 3 mg/kg and the VNE (200, 300 mg/kg) caused a significant reduction in spontaneous activities, compared to control group. VNE (200, 300 mg/kg) decreased locomotion significantly from 565.7 to 301.7 and 292.0 respectively. The results are shown in Fig.3.

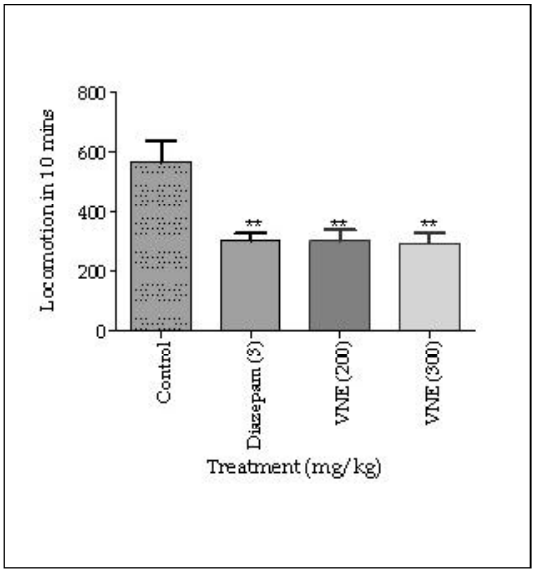

Fig.3. Mean (±S.E.M. n=6) spontaneous activity counts in mice accumulated at 5-min following administration of Saline, Diazepam (3 mg/kg) and VNE (200, 300mg/kg) significantly reduced activity compared to saline group ($P < 0.05$). One way ANOVA followed by Dunnett's test.

3.5. Pentobarbitone-induced sleep

The pre-treatment with intraperitoneal administration of 200 and 300 mg/kg of VNE significantly potentiated pentobarbital-induced sleep with decreased onset and prolonged duration of sleep. The VNE (200,300 mg/kg i.p.) increased sleeping time from 33.83 min to 67.66 and 78.66 respectively min.

The results are shown in Fig. 4.

3.6. Neuromuscular coordination – Rotarod

Diazepam at 3 mg/kg and the VNE at doses of 200 and 300 mg/kg, i.p. caused significant reduction in the time spent on the rotarod, compared to control group. In control animals the fall off time was 32 seconds which is significantly reduced to 17, 13 and 17 seconds respectively after treatment with Diazepam, VNE (200 and 300 mg/kg).

The results are shown in Fig. 5.

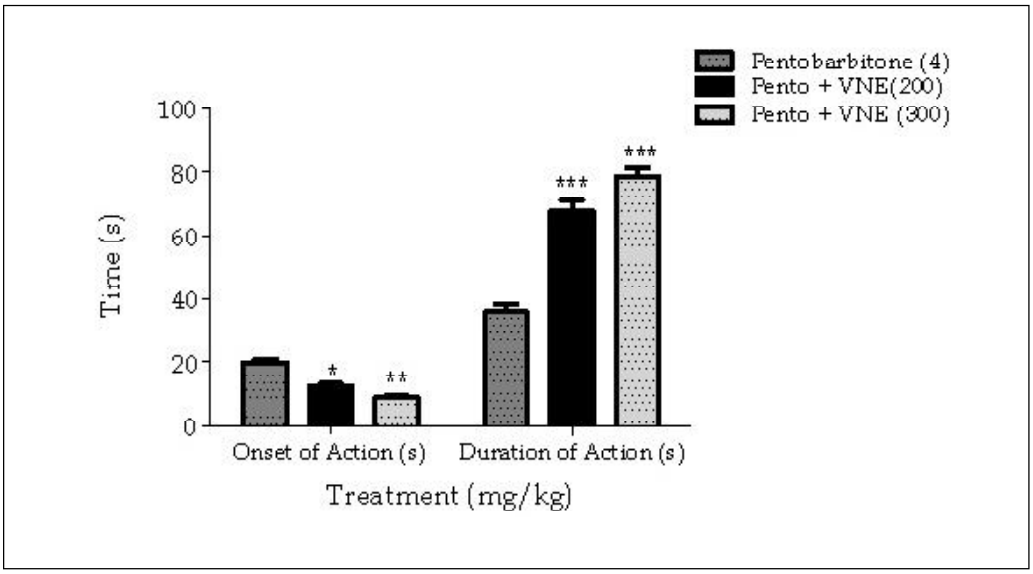

Fig 4. Pretreatment with VNE (200, 300 mg/kg) significantly decreases the onset of sleep and increases duration of action of sleep induced by Pentobarbitone (40 mg/kg). Two way ANOVA followed by Bonferroni post tests. Data presented as means ± S.E.M., n=6

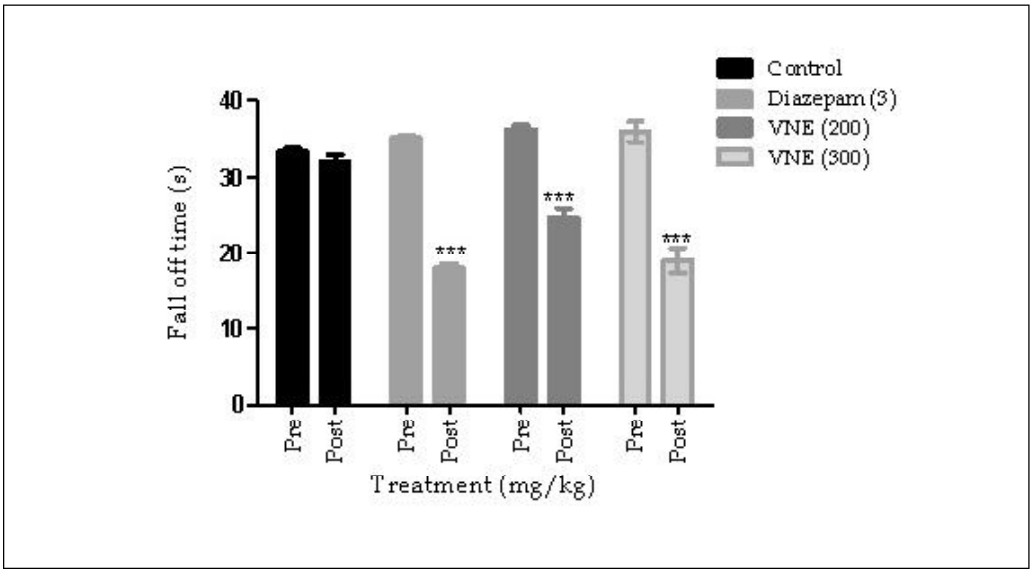

Fig 5. Vehicle treated group showed no change in the fall off time from rotarod whereas
Diazepam (3 mg/kg), VNE (200, 300 mg/kg) significantly decreases fall of time when mice were placed on rotarod. Two way ANOVA followed by Bonferroni post tests. Data presented as means ± S.E.M., n=6.

4. Discussion

Anxiety is also associated with augmented autonomic activity resulting in increased defecation and urination. Increase of the time spent in the central area as well as the ratio of central/total locomotion or decreases of latency to enter the central are indications of anxiolysis in an elevated plus maze. The elevated plus-maze is a well-established animal model for testing anxiolytic drugs [24, 25]. Diazepam, a standard anxiolytic used clinically, is also employed in behavioral pharmacology as a reference compound for inducing anxiolytic-like effects, even when the compound being screened does not act via benzodiazepine receptors. The effect of most of the anxiolytic agents is to enhance the response to GABA, by facilitating the opening of GABA-activated chloride channels. GABA-A receptors were involved in anxiety and their direct activation would have an anxiolytic effect. Anxiolytic drugs have also been shown to act on limbic system, hypothalamus, and the brain stem reticular system [1].

In agreement with previously published reports, diazepam increased the number of open arm entries and the time spent in the open arms [26-28], confirming its anxiolytic effects. In the present study, we found that the ethanolic extract of VNE leaves increased the percentage of open arm entries and time spent in open arms and thus showed anxiolytic effects in this model. The anxiolytic effects of drugs such as benzodiazepines are accompanied by decreased locomotor activity, sleep induction, skeletal muscle relaxation. VNE (200, 300 mg/kg) inhibited locomotion, decreases the onset of sleep with prolonged duration and also relaxes the skeletal muscle. The effect of most of the anxiolytic agents is to enhance the response to GABA, by facilitating the opening of GABA-activated chloride channels. GABA A receptors was involved in anxiety and their direct activation would have an anxiolytic effect [29]. These data seem to be in good agreement with our results. It is well documented that pentylenetetrazole-induced convulsions are produced due to diminution of GABA level in brain [30, 31]. A recent study showed that VNE possesses anticonvulsant activity particularly against pentylenetetrazole-induced convulsion. It is likely that VNE might possibly be producing anticonvulsant action by increasing the level of GABA, an inhibitory neurotransmitter in the central nervous system [32]

In conclusion, the action of ethanolic extract of leaves of *Vitex nigundo* produced anxiolytic-like actions in mice subjected to the elevated plus-maze test are in accord with the traditional use of VNE and could be useful in primary medical care. In the same way, identification of compound(s) responsible for biological activity could be used as prototype(s) to design new substances with anxiolytic activity. Although further major active components and precise anxiolytic mechanisms need to be identified.

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