

Evaluation of Anxiolytic Activity of Vitex negundo Leaf Extract Using Elevated plus Maze Model in Rats

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Abstract:

Background: Vitex negundo has been traditionally used for several medicinal purposes, and experimental studies have suggested potential anxiolytic-like properties of its extracts. The elevated plus maze (EPM) is a validated behavioural model for assessing anxiety-like behaviour in rodents, in which increased open-arm exploration is generally interpreted as an anxiolytic-like response.

Aim: To evaluate the anxiolytic-like activity of Vitex negundo leaf extract at 100 and 200 mg/kg using the elevated plus maze in rats.

Materials and Methods: Male Wistar albino rats weighing 150–250 g were divided into four groups of six animals each: control, diazepam (1 mg/kg), Vitex negundo leaf extract 100 mg/kg (VNE100), and Vitex negundo leaf extract 200 mg/kg (VNE200). The leaf extract was prepared using 95% methanol. Following treatment, animals were evaluated in the elevated plus maze. Percentage of open-arm entries and time spent in the open arms were recorded. Data were expressed as mean $\pm$ standard deviation. Appropriate statistical analysis with post-hoc multiple comparisons was performed, with $p < 0.05$ considered statistically significant.

Results: The percentage of open-arm entries was $2.38 \pm 5.83\%$ in the control group, $47.82 \pm 28.68\%$ with diazepam, $8.33 \pm 20.41\%$ with VNE100, and $12.50 \pm 20.92\%$ with VNE200. Diazepam produced a statistically significant increase compared with control ($p = 0.0166$), whereas VNE100 and VNE200 did not show significant differences ($p > 0.9999$ for both). Time spent in the open arms was 4.33 ± 10.61 , 20.67 ± 12.58 , 7.67 ± 18.78 , and 8.00 ± 12.79 seconds in the control, diazepam, VNE100, and VNE200 groups, respectively. None of the comparisons for this parameter reached statistical significance.

Conclusion: Under the conditions of the present study, Vitex negundo leaf extract at 100 and 200 mg/kg produced numerical increases in EPM open-arm exploration but did not demonstrate statistically significant anxiolytic-like activity. The findings provide preliminary evidence warranting further investigation using larger sample sizes, standardized extracts and additional validated behavioural models.

Keywords: Vitex Negundo; Anxiolytic-Like Activity; Elevated Plus Maze; Diazepam; Anxiety; Wistar Rats.

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Introduction

Anxiety is characterized by excessive apprehension, fear and avoidance in situations perceived as threatening or uncertain. Because the underlying emotional states cannot be directly measured in experimental animals, preclinical studies assess anxiety-related behaviour through observable behavioural responses elicited by standardized environmental challenges.

Rodent behavioural tests are therefore widely used for the preliminary evaluation of compounds with potential anxiolytic activity. [1,2] Among the

commonly used behavioural paradigms, the Elevated Plus Maze (EPM) is an established test for assessing anxiety-like behaviour in rodents. The apparatus consists of two open and two enclosed arms arranged in a plus-shaped configuration and elevated above the floor.

The test is based on the natural exploratory tendency of rodents and their aversion to elevated, exposed and open spaces. This creates an approach-avoidance conflict in which the animal must balance exploration against avoidance of

potentially threatening open areas. [3,4] The principal behavioural measures in the EPM include the percentage of entries into the open arms and the time spent in the open arms. Untreated rodents generally show a preference for the enclosed arms, whereas anxiolytic-like treatments may increase exploration of the open arms. Therefore, an increase in open-arm entries and/or open-arm time is commonly interpreted as an anxiolytic-like behavioural response. [3-5] However, these parameters should be interpreted in the context of overall animal behaviour because locomotor activity, environmental conditions, prior exposure to the maze, strain, lighting and other experimental factors can influence EPM performance. [2,4]

The EPM has been pharmacologically validated using established anxiolytic agents. Pellow et al. demonstrated that the distribution of open- and closed-arm exploration can be used to assess anxiety-related behaviour in rats, while subsequent work showed that anxiolytic and anxiogenic drugs can modify exploratory activity in the maze. [3,5] Thus, the EPM provides a relatively simple and rapid experimental paradigm for screening potential anxiety-modulating compounds. The model is particularly useful when interpreted using both open-arm entry and open-arm duration rather than relying on a single behavioural endpoint. [2,4] *Vitex negundo* L. is a medicinal plant belonging to the genus *Vitex* and has a long history of traditional medicinal use in the Indian subcontinent. Phytochemical investigations of *Vitex* species have identified several classes of biologically active constituents, including flavonoids, iridoid glycosides, diterpenoids, triterpenoids and other secondary metabolites. The genus has been investigated for several pharmacological activities, providing a scientific basis for further evaluation of individual *Vitex* species and their extracts. [6]

Previous experimental studies have provided preliminary evidence regarding the possible anxiolytic-like properties of *Vitex negundo*. Adnaik et al. reported anxiolytic activity of an ethanolic root extract of *Vitex negundo* in mice using the elevated plus maze and light-dark exploration tests.⁷ In another study, Aswar et al. specifically evaluated an ethanolic leaf extract of *Vitex negundo* using the elevated plus maze and reported anxiolytic-like effects in mice. [8] The latter finding is particularly relevant to the present investigation because the present study also evaluates a leaf extract of *V. negundo* using the EPM. The earlier study, however, was conducted in mice and used experimental conditions that differ from those of the present study; therefore, its findings provide a rationale for further investigation rather than direct confirmation

of the present results. The Aswar et al. article is reported as a CC BY publication and has DOI 10.18311/jnr/2012/268.

Despite these preliminary findings, the behavioural effects of *Vitex negundo* leaf extract require further evaluation using standardized experimental conditions and different animal populations. In particular, examination of both percentage open-arm entries and time spent in open arms can provide complementary information about the behavioural response to the extract. The present study was therefore undertaken to evaluate the anxiolytic-like activity of *V. negundo* leaf extract at 100 and 200 mg/kg in male Wistar albino rats using the Elevated Plus Maze, with diazepam 1 mg/kg included as the standard anxiolytic comparator.

Materials and Methods

An experimental, randomized, comparative animal study was conducted using male Wistar albino rats weighing 150–250 g. The study was commenced after IAEC approval. The animals were divided into four groups of six rats each. The control group received double-distilled water (0.5 mL), while the treatment groups received *Vitex negundo* leaf extract at doses of 100 mg/kg (VNE100) and 200 mg/kg (VNE200), respectively. The fourth group received diazepam 1 mg/kg and served as the standard drug group. The *Vitex negundo* leaves were shade-dried, powdered and exhaustively extracted with 95% methanol using a Soxhlet apparatus. The concentrated extract was used for oral administration.

Behavioural assessment was performed using the Open Field Test (OFT) and Elevated Plus Maze (EPM). In the OFT, rats were individually placed in a walled open-field apparatus measuring 19 × 19 inches, with a wall height of 15 inches. The floor was divided into 16 symmetrical squares, comprising four central and 12 peripheral squares. Behaviour was recorded using a video camera, and the number of central squares crossed, percentage of central squares crossed and central-to-peripheral square ratio were assessed.

In the EPM, rats were observed individually for 3 minutes in an apparatus consisting of two open and two closed arms. Percentage of open-arm entries and time spent in the open arms were recorded. Data were expressed as Mean ± SD. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test or Kruskal-Wallis test followed by Dunn's multiple comparisons test, as appropriate. A *p* value <0.05 was considered statistically significant.

Result

Table 1: Effect of Vitex negundo leaf extract on percentage of open-arm entries in the Elevated Plus Maze

Group	Control	Diazepam	VNE100	VNE200
Mean $\pm$ SD (%)	2.38 $\pm$ 5.83	47.82 $\pm$ 28.68	8.33 $\pm$ 20.41	12.50 $\pm$ 20.92

Dunn's multiple comparisons test

Comparison	Mean rank difference	Significant?	Adjusted P value
Control vs. Diazepam	-9.833*	Yes	0.0166
Control vs. VNE100	-0.6667	NS	>0.9999
Control vs. VNE200	-2.167	NS	>0.9999

*P < 0.05; NS = Not significant.

Table 2: Effect of Vitex negundo leaf extract on time spent in open arms in the Elevated Plus Maze

Group	Control	Diazepam	VNE100	VNE200
Mean $\pm$ SD (seconds)	4.33 $\pm$ 10.61	20.67 $\pm$ 12.58	7.67 $\pm$ 18.78	8.00 $\pm$ 12.79

Dunn's multiple comparisons test

Comparison	Mean rank difference	Significant?	Adjusted P value
Control vs. Diazepam	-7.583	No	0.0980
Control vs. VNE100	-0.6667	No	>0.9999
Control vs. VNE200	-1.750	No	>0.9999

NS = Not significant; P < 0.05 was considered statistically significant.

These values correspond directly to the EPM tables in the uploaded Article 2 document.

Table 3: Percentage change in Elevated Plus Maze parameters compared with control

Parameter	Diazepam (%)	VNE100 (%)	VNE200 (%)
Percentage of open-arm entries	+1909.24	+250.42	+425.21
Time spent in open arms	+376.92	+76.99	+84.73

The percentage of open-arm entries was 2.38 $\pm$ 5.83% in the control group, 47.82 $\pm$ 28.68% in the diazepam group, 8.33 $\pm$ 20.41% in the VNE100 group and 12.50 $\pm$ 20.92% in the VNE200 group. Thus, there was a marked numerical increase in open-arm entry percentage following diazepam treatment, while the increases with VNE100 and VNE200 were comparatively smaller. Compared with the control group, the percentage of open-arm entries increased descriptively by 1909.24% with diazepam, 250.42% with VNE100 and 425.21% with VNE200. These percentage changes are descriptive and should not be interpreted as evidence of statistical significance.

Dunn's multiple comparisons test demonstrated a statistically significant difference between the control and diazepam groups for percentage of open-arm entries, with a mean-rank difference of -9.833 and an adjusted P = 0.0166. This indicates that diazepam significantly increased open-arm entry behaviour compared with control. In contrast, the comparison between control and VNE100 showed a mean-rank difference of -0.6667 with an adjusted P > 0.9999, indicating no statistically significant difference. Similarly, the control versus VNE200 comparison showed a mean-rank difference of -2.167 with an adjusted P > 0.9999, also indicating no significant difference. Therefore, although both VNE doses showed numerically higher open-arm entry percentages than control, neither dose produced a statistically significant effect on this EPM parameter. The time spent in

open arms was 4.33 $\pm$ 10.61 seconds in the control group, 20.67 $\pm$ 12.58 seconds in the diazepam group, 7.67 $\pm$ 18.78 seconds in the VNE100 group and 8.00 $\pm$ 12.79 seconds in the VNE200 group. Descriptively, the diazepam group showed the greatest increase in time spent in open arms compared with control, while VNE100 and VNE200 showed relatively smaller increases. The corresponding percentage increases over control were 376.92% for diazepam, 76.99% for VNE100 and 84.73% for VNE200. Again, these percentages represent descriptive changes and do not by themselves establish statistical significance.

Dunn's multiple comparisons analysis showed that the increase in time spent in open arms in the diazepam group was not statistically significant compared with control, despite the considerable numerical difference. The mean-rank difference was -7.583, with an adjusted P = 0.0980. The VNE100 group showed a mean-rank difference of -0.6667 with an adjusted P > 0.9999, while VNE200 showed a mean-rank difference of -1.750 with an adjusted P > 0.9999. Thus, neither VNE100 nor VNE200 produced a statistically significant change in time spent in open arms. When both EPM parameters are considered together, the findings show that diazepam significantly increased percentage open-arm entries but did not significantly increase time spent in open arms. For Vitex negundo, neither 100 mg/kg nor 200 mg/kg produced a statistically significant change in either EPM parameter. Although VNE200 had a higher

mean percentage of open-arm entries than VNE100 (12.50% vs 8.33%) and a slightly higher mean time spent in open arms (8.00 vs 7.67 seconds), these differences were not directly tested against each other in the reported post-hoc analysis. Therefore, the present data do not establish a statistically significant dose-response relationship between VNE100 and VNE200. The very large descriptive percentage increase observed with diazepam for open-arm entries (1909.24%) should also be interpreted cautiously because the control mean was very low (2.38%). When the baseline value is small, even a moderate absolute increase can produce a very large percentage change. Therefore, the adjusted P value and the underlying group values are more appropriate for determining statistical significance than the percentage change alone.

Overall, the EPM findings indicate that the standard drug diazepam produced the expected significant increase in open-arm entry behaviour, demonstrating a measurable response in the experimental model. However, the *Vitex negundo* groups did not show statistically significant changes in either percentage of open-arm entries or time spent in open arms. Therefore, based strictly on the EPM data provided, the present study does not demonstrate statistically significant anxiolytic-like activity of VNE100 or VNE200 in the Elevated Plus Maze, although small numerical increases were observed in both parameters, particularly with VNE200 for percentage open-arm entries.

One important point for the final manuscript is that we should not write that VNE100 or VNE200 “significantly increased open-arm exploration”. The tables clearly show that this was not statistically significant. The appropriate wording is that *Vitex negundo* produced numerical increases without statistically significant differences from control. This will make the Results section completely consistent with your actual tables and images.

Discussion

The present study evaluated the anxiolytic-like effects of *Vitex negundo* leaf extract (VNE) at doses of 100 and 200 mg/kg using the elevated plus maze (EPM) in rats. The EPM is a well-established behavioural model based on the natural conflict between the exploratory drive of rodents and their avoidance of elevated, exposed spaces. The percentage of open-arm entries and the time spent in the open arms are commonly used behavioural indices, with increased open-arm exploration generally interpreted as reduced anxiety-like behaviour. [1-4] In the present study, percentage open-arm entries showed a statistically significant increase only in the diazepam-treated group compared with the control group ($47.82 \pm 28.68\%$

vs. $2.38 \pm 5.83\%$; adjusted $p = 0.0166$). This finding is consistent with the established pharmacological validation of the EPM, in which clinically used anxiolytic agents such as diazepam increase exploration of the open arms. [3-5] The significant response to diazepam therefore provides evidence that the behavioural model was capable of detecting an anxiolytic-like effect under the experimental conditions used. Although the VNE100 and VNE200 groups demonstrated higher mean percentages of open-arm entries than the control group ($8.33 \pm 20.41\%$ and $12.50 \pm 20.92\%$, respectively), neither comparison reached statistical significance ($p > 0.9999$ for both comparisons). Thus, the numerical increase should be interpreted as a descriptive trend rather than evidence of a statistically established anxiolytic-like effect. The relatively large variability within the VNE groups, reflected by the standard deviations, may also have contributed to the absence of statistical significance.

A similar pattern was observed for time spent in the open arms. The mean time increased from 4.33 ± 10.61 seconds in the control group to 20.67 ± 12.58 seconds with diazepam, 7.67 ± 18.78 seconds with VNE100, and 8.00 ± 12.79 seconds with VNE200. However, none of these comparisons was statistically significant, including the diazepam-control comparison ($p = 0.0980$). Since both open-arm entry and open-arm duration are complementary EPM measures, interpretation of the findings should consider both parameters rather than relying solely on percentage change. [1,3,4]

The descriptive percentage changes showed a substantial increase in open-arm entries with diazepam (+1909.24%), VNE100 (+250.42%) and VNE200 (+425.21%) compared with control. However, the very low control mean makes percentage changes particularly sensitive to the baseline value. Therefore, these large percentage increases should not be interpreted as equivalent evidence of efficacy. The inferential comparisons are more appropriate for determining statistical evidence of group differences.

The present findings differ from some previous reports on *Vitex negundo*. Adnaik et al. reported that ethanolic root extract of *V. negundo* at 100 and 200 mg/kg significantly increased open-arm entries and percentage time spent in open arms in mice. [7] Similarly, Aswar et al. investigated ethanolic leaf extract of *V. negundo* in the EPM and reported anxiolytic-like effects in mice. [8] The difference between those findings and the present results may relate to several methodological factors, including plant part, extraction procedure, animal species, experimental conditions, dose, and behavioural variability. Therefore, findings obtained with root extract in mice or with a different preparation of

leaf extract cannot be assumed to be directly equivalent to the present study.

The pharmacological literature indicates that *Vitex* species contain several classes of biologically active constituents, including flavonoids, iridoid glycosides, diterpenoids, lignans and essential oils. [6] These phytochemical constituents provide a biological rationale for investigating *Vitex negundo* in behavioural models; however, the present study did not identify the specific constituents responsible for any behavioural changes. Consequently, mechanistic explanations should remain tentative until supported by phytochemical standardisation and receptor- or pathway-specific investigations. Importantly, the present study does not establish a statistically significant dose-response relationship between VNE100 and VNE200. Although the mean percentage of open-arm entries was numerically greater with VNE200 than VNE100 (12.50% vs. 8.33%), there was no direct statistical comparison between the two VNE doses. Therefore, the results can only indicate a numerical difference between doses and should not be described as demonstrating a confirmed dose-dependent anxiolytic effect. Another consideration is that behaviour in the EPM can be influenced by factors other than anxiety, including exploratory activity, locomotor behaviour, environmental conditions and handling. The EPM is therefore best regarded as a behavioural model of anxiety-like responses rather than a direct measure of human anxiety. [2,4] Overall, the present EPM findings provide clear evidence of an anxiolytic-like response to diazepam but do not demonstrate statistically significant anxiolytic-like activity of VNE100 or VNE200 for the measured EPM outcomes. The numerical increases observed with VNE, particularly at 200 mg/kg, warrant further investigation but should be considered preliminary. Future studies using larger sample sizes, standardised extracts, additional validated anxiety paradigms, phytochemical characterisation and direct dose-to-dose statistical comparisons would help determine whether *Vitex negundo* leaf extract possesses reproducible anxiolytic-like properties.

Conclusion

The present study evaluated the anxiolytic-like activity of *Vitex negundo* leaf extract at 100 and 200 mg/kg using the elevated plus maze in rats. The diazepam-treated group showed a statistically significant increase in percentage open-arm entries, supporting the responsiveness of the experimental model. Although VNE100 and VNE200 produced numerical increases in percentage open-arm entries and time spent in open arms compared with control, these differences were not statistically significant. Therefore, the present findings do not provide sufficient statistical evidence to establish significant anxiolytic-like activity of either

VNE100 or VNE200 in the EPM model. The higher numerical values observed with VNE200 should be considered a preliminary trend rather than proof of a dose-dependent effect, as no direct statistical comparison between VNE100 and VNE200 was performed. Overall, *Vitex negundo* leaf extract showed promising but inconclusive behavioural effects in the present EPM experiment. Further studies with larger sample sizes, standardized extracts, higher doses, additional validated anxiety models, phytochemical characterization and direct dose-to-dose comparisons are warranted to clarify its potential anxiolytic-like properties. The EPM remains a useful model for such investigations because increased open-arm exploration is commonly used as an indicator of reduced anxiety-like behaviour in rodents.

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