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ORIGINAL RESEARCH article

Effect of lignin-rich *Vitex negundo* leaf extract on antioxidant, thrombolytic, antiproliferative, antidepressant, and cytotoxic activities in mice

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Abstract: In several counties, *Vitex negundo* have been claimed to have anti-inflammatory, analgesic, and antioxidant activities. It's frequently used in folk medicine for the treatment of various pain disorders. The methanol extract of *Vitex negundo* leaves was studied for its biological effects. To investigate different biological exertions of the methanol extract of *Vitex negundo*, the leaf extracts were obtained with pure methanol. *In-vitro* anti-oxidant activity was resolved using a DPPH radical scavenging assay. A clot lysis test was used to assess the thrombolytic activity. Antiproliferative effects of DMSO *Vitex negundo* in cell lines acquired from human cervix adenocarcinoma (HeLa cells). Using the brine shrimp lethality bioassay method, the plant extracts were evaluated for cytotoxic action. LC_{50} values were determined through probit analysis of mortality percentages. In hole cross-test and open-field test, neuropharmacological activities of mice were assessed in mice. The findings revealed that *Vitex negundo* leaf extract possessed antioxidant effects, streptokinase exposed significant clot lysis and human cervix adenocarcinoma cells demonstrated mild cervical antiproliferative activity. Significant neuropharmacological action (antidepressant and anxiolytic) and cytotoxic action were shown by the methanol extract of *Vitex negundo* leaves.

Introduction

Several biological activities of natural phytochemicals, such as antioxidant, antibacterial, and anti-inflammatory qualities, have been documented [1, 2]. From the ancient era to till, people have frequently used natural remedies for every disease while they are facing old or new health problems or other illnesses [3]. A tiny tree or shrub called *Vitex negundo* is used in traditional Chinese medicine to treat a wide range of ailments [4]. The presence of lignins, saponins, phenols, glycosides, alkaloids, sterols, and tannins in *Vitex negundo* leaves extracts (VNLE) is responsible for its diverse biological activities [5]. *Vitex negundo* displays numerous types of pharmacological properties due to lignans, iridoid glycosides, and terpenoids which demonstrated antifungal, antibacterial, anti-inflammatory, antioxidant, and anticancer activities [6]. Vitex negundin, vitexilactone, vitetrifolin D, artemetin, vitexicarpin, and penduletin chemicals are among the labdane-type

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diterpenoids found in *V. negundo* leaves (VNL). These compounds can suppress the growth of malignant cell lines, specifically K562 Lu1, KB, and LNCaP [7]. Nevertheless, how cytotoxic labdane-type diterpenoids are against liver and breast malignancies has not yet been explained [8]. Leaves have antihistamine, analgesic, and anti-inflammatory qualities. Leprosy, dyspepsia, rheumatism, and piles are all treated with roots [9]. The flowers are used for cholera. Fruits are used as an anthelmintic [10]. The entire plant is used as a diuretic, antiseptic, antipyretic, and for inflammations [11, 12]. With the potential medicinal application, the objective of the current study is to observe the phytochemical constituents of VNL, as well as its antioxidant, thrombolytic, anticancer, cytotoxic, and antidepressant properties to offer a solid scientific backing for useful clinical use and traditional utilization.

Materials and methods

Plant collection: The plant sample was collected from Chittagong District, Bangladesh during April and May 2023. It was authenticated at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Science and Technology Chittagong with a voucher specimen no. PUSTC: 0223. VNL was properly cleaned to remove any remaining sand or dust. The 10.0 kg leaves were obtained by carefully separating the bough. After that, for two weeks, the leaves were dried in the absence of direct sunlight; through shade drying. The leaves were ground to get powder via a grinder. The resultant fine powder was 800 g stored in an airtight container and kept cool, dry, and dark until used.

Preparation of methanol extract: 800 g of VNL powder and 2.0 L of methanol were kept in two cleaned beakers. The bottles were kept tightly closed for 14 days with occasional shaking. By this time, a major portion of the powder had dissolved in methanol, which was further filtered by using cotton wool [13]. The residual methanol was removed by a rotary evaporator at 45°C and reduced pressure. The 176 g of methanol extract that resulted was stored in a refrigerator until used. The percentage yield of methanol extract was calculated [14-16].

Qualitative phytochemical screening

Test for flavonoids: A few drops of concentrated hydrochloric acid were added to a small amount of VNLE. Flavonoids are detected by the immediate development of red colour [17].

Test for saponins: The crude extract and 5.0 mL of distilled water were combined in a test tube and shaken firmly. It is believed that the production of steady foam indicated the presence of saponin [18].

Test for tannins: Test with ferric chloride, 5.0-10.0 mL of filtered water was mixed with 0.5 g of leaf extract, and then 5.0% ferric chloride solution were added. The green precipitate is a sign that tannins exist [19].

Test for steroid: In Salkowski's test, 1.0 ml of chloroform was combined with 2.0 mg of the extract, and a few drops of H_2SO_4 that was concentrated were included presence of steroids was identified by a red hue formed in the bottom chloroform layer.

Liebermann-Burchard's test: After dissolving 2.0 mg of dry extract in acetic anhydride, heating the mixture to boiling, cooling it down, and then adding 1.0 mL of concentrated H_2SO_4 along the test tube's sides. Steroid presence was identified by the production of a green hue.

Test for glycosides: Salkowski's evaluation, 2.0 mL chloroform was combined with the crude extract. After that, 2.0 mL of concentrated H_2SO_4 was added and gently shaken. The glycone part of the glycoside, or steroidal ring, was visible as a reddish-brown tint.

Test for anthraquinones glycosides: 1.0 g VNL powder was added to a test tube, followed by the addition of 20 mL chloroform. For 5.0 min, it was heated in a steam bath. Filter the extract while it's still warm and let it cool

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afterwards. The filtrate was mixed with an equal volume of 10.0% ammonia solution. When the test tube was shaken, the top layer of bright pink coloration became visible, signifying the manifestation of anthraquinones. 10.0 mL of 10.0% ammonia and 5.0 mL chloroform were added to the test tube for control tests [20].

Test for reducing sugar: In Fehling's test, 2.0 mg of dry extract was dissolved in 1.0 mL of distilled water before adding Fehling's (A+B) solution. The test is validated by the formation of a brick-red precipitate [21].

Benedict's test: A test tube containing 5.0 mL of Benedict's reagent and 0.5 mL of the extract was heated in a water bath for 5.0 to 10.0 min. The solution's colour changes from green, yellow, or red to indicate the presence of the reducing sugar depending on the reducing sugar amount present in the test solution [22].

Antioxidant activity: The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to perform the VNL extract *in vitro* [23]. Thus, 10.0 mg of extract was dissolved in 10.0 mL of methanol, yielding a stock solution with 1000 µg/mL. Three solutions of 1000, 500, and 250 µg/mL were obtained by serial dilutions from this. DPPH was prepared without a sample using the sample preparation method, and ascorbic acid (1000, 500, and 250 µg/mL) was used as a standard 0.004%w/v DPPH solution was made by dissolving 4.0 mg of DPPH in 100 mL of methanol and was kept at 20°C. Following a thorough shake and 30 min at room temperature in the dark, 2.0 mL of extract or standard in various concentrations was added to 3.0 mL of DPPH solution. The absorbance of the studied samples was measured using a UV-vis spectrophotometer with methanol serving as a blank [24, 25].

Thrombolytic activity: Streptokinase (SK) with 15,000,000,000 I. U. was used, and 5.0 mL of sterile distilled water was added and thoroughly mixed to perform thrombolysis *in-vitro*, suspension's stock was made of 100 µl (30,000 I. U.) [26].

Human specimen: 20 healthy human volunteers who had never used oral contraceptives or anticoagulant medication before provided the 4.0 mL whole blood sample. The three weighed microcentrifuge tubes were used to transfer 0.5 mL of blood to form clots.

Herbal preparation: 10.0 mL of distilled water was mixed with 100 mg of VNLE using a vortex mixer. The soluble supernatant was extracted from the suspension after it had been drained overnight and passed through a syringe filter with a pore size of 0.22 microns. By adding 100 µl of the herb's aqueous preparation to the microcentrifuge tubes, the clot samples were examined for thrombolytic activity [27].

Experimental methods: Venous blood sample was extracted from healthy individuals and placed into separate, sterile, pre-weighed Alpine tubes (500 µl). After that, the tubes were incubated at 37°C for 45 min. Following clot formation, serum was expelled (aspirated out without disturbing the formed clot). Every tube that held a clot was weighed once more to determine the clot's weight. The clot weight was determined by weighing each tube containing a clot once more. A correct marking of 100 µl of plant extract was added to each Alpine tube containing a clot. Each tube was kept at 37ºC for 90 min while the clot lysis was observed the use of SK and water was for positive and negative (no thrombolytic) controls.

Anticancer activity: HeLa is a human cervical cancer cell line that is maintained in Dulbecco's Modified Eagle's Medium (DMEM), which contains 1.0% penicillin-streptomycin, 0.2% gentamycin, and 10.0% foetal bovine serum. Following a 24-hour incubation period at 37°C with 5.0% CO_2, cells were seeded at 2.0x10% 100 ml onto a 96-well plate. Two days later, each well received a 50 µl sample. Each well received a 50 µl sample the next day. An inverted light microscope was used to examine cytotoxicity after 48 hrs of incubation. Methanol extracts of VNL were put to the test against HeLa cell lines [28].

Brine shrimp cytotoxicity activity: To perform the cytotoxicity analysis on brine shrimp nauplii, the Meyer method was utilized. The lethality bioassay for brine shrimp was conducted using DMSO as a negative control (cytotoxic test). An aquarium store in Chittagong, Bangladesh was the source of the dried cyst of brine shrimp.

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The development of mature shrimp known as nauplii from dried brine shrimp cysts was made possible by the use of synthetic seawater, specifically an artificial seawater solution consisting of 3.8% NaCI, and vigorous aeration. Vincristine sulphate (2.0 mg/vial) served as a positive control. The serial concentration was then ascertained by dilution at 125, 250, 500, and 1000 µg/mL. Using a magnifying glass, the number of nauplii brine shrimp that survived each vial was counted after 24 hours period [29, 30].

Neuropharmacological screening

Testing groups: Utilizing the hole cross-test and open-field test, the neuropharmacological properties of the VNLE were assessed. Four groups of five mice in each group were used in each experiment: **Group 1:** Control: 0.5 mL per mouse, 1.0% Tween 80, **Group 2:** Positive control: 1.0 mg/kg of diazepam, **Group 3:** Test-1: VNL-200 mg/kg, and **Group 4:** Test-2: VNL-400 mg/kg.

Hole-cross test: A wooden box measuring 30cmx20cmx14cm was used to conduct the hole-cross test. A partitioning wall with a hole diameter of 3.0cm was used to construct a hole-cross apparatus, which was positioned 7.5cm above the floor. After giving each mouse an oral dose of the test agent, they were promptly placed on one side of the designated device. The mice were seen to move freely through the hole and between chambers for three minutes. At 0, 30, 60, 90, and 120 min, the inspection was conducted [31].

Open field test: There were three mice in all groups, which were split up into test, positive, and control groups. The test group was provided 200 and 400 mg/kg of VNLE orally, incidentally, the control group was given 1.0% Tween. 1.0 mg/kg diazepam was administered to the positive control group. The equipment stood 40cm tall, and the open-field floor was covered in squares that alternated between black and white. After the mice were given the oral intake, the number of squares they explored was recorded for three minutes at 0, 30, 60, 90, and 120 min [32].

Ethical approval: This study was approved by the University of Sciences and Technology Chittagong. Based on the National Statement on Ethical Conduct in Animal Research, the committee has approved the protocol number: USTC/AEAC/23/024.

Statistical analysis: One-way analysis of variance (ANOVA) was used to evaluate the data among the groups, reporting the results with mean±SEM. Tukey's multitudinous assessment tests were used to find significant differences between the means of the groups at $p<0.05$ using IBM SPSS Statistics for Windows, Version 25.0.

Results

Percentage yield and phytochemical profile of VNLE: The bioactive profile of the methanol extract of VNL is displayed in **Table 1**. The bioactive percentage yield of the VNL methanol extract was 22.0%.

Table 1: VNLE bioactive constituent's observation

Secondary metabolite	Name of the test	Observation	Findings
Flavonoids	Hydrochloric acid test	Orange to red to crimson	++
Saponins	Foam test	Stable foam	++
Tannins	Ferric chloride test	Greenish black precipitate	++
Steroid	Salkowski's test	The lower layer of chloroform is red	++
	Liebermann Burchard's test	The green color at the junction	++
Anthraquinones glycosides	Test for free anthraquinone	Red color	++
Glycosides	Salkowski's test	Orange-reddish color	++
Reducing Sugar	Benedict's test	Brick red precipitate	++
	Fehling's test	Precipitation that is brick red or red	++

(+) = Presence of phytochemical

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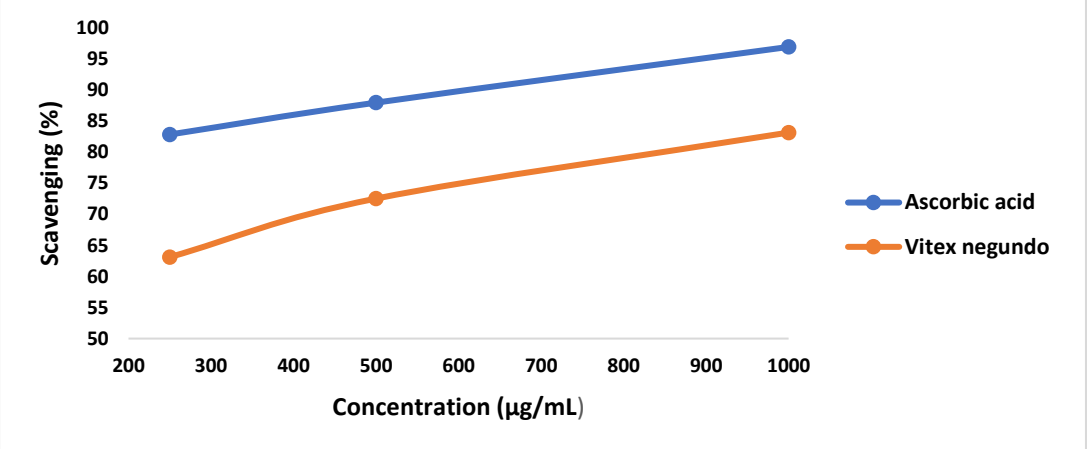
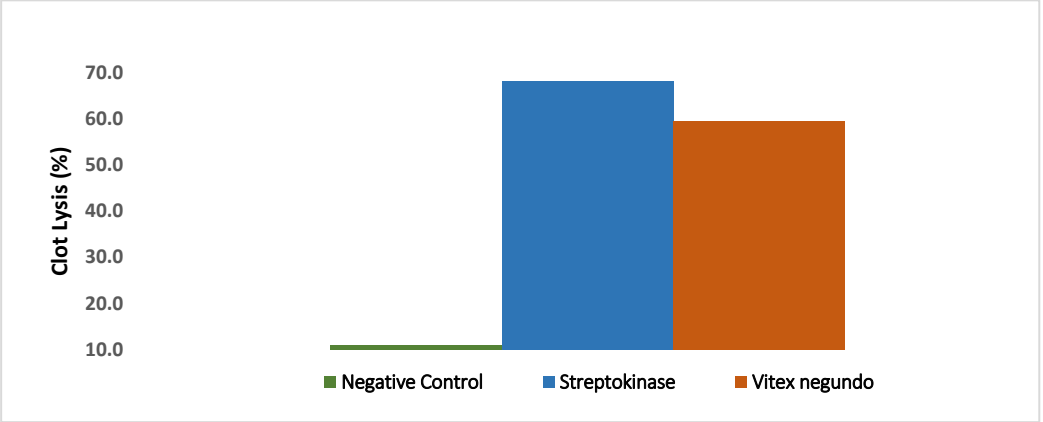
Antioxidant test (DPPH scavenging): The antioxidant capacity of VNLE, determined via the DPPH assay, was contrasted with ascorbic acid, an established antioxidant. The extract elicited a negligibly significant level of DPPH scavenging activity in tests with IC_{50} =691.423 µg/mL contrasted to standard ascorbic acid (536.75 µg/mL). In both of these tests, the extract showed dose-dependent activity. **Figure 1** illustrates the DPPH scavenging activity of VNL methanol extract and ascorbic acid respectively.

Figure 1: The DPPH-scavenging properties of ascorbic acid and VNL methanol extract



In-vitro thrombolytic activity: **Figure 2** displays the *in-vitro* thrombolytic activity results and their means. Thus, 10 mg/mL of VNL triggered clot lysis with a 59.5% efficiency after treatment with 100 µl, whereas, 100 µl of SK (30,000 IU) caused 68.3% of the clots to lysis compared to the negative control (10.9%) clot lysis.

Figure 2: *In-vitro* thrombolytic activity of VNL methanol extract and Streptokinase



In-vitro antiproliferative activity: The VNLE on HeLa cell viability was decreased to 70-80 concentration of reduction in HeLa cells was observed at 50 µg/mL (**Table 2**). The HeLa cells gradually separated post-VNLE treatment. The change in morphology of HeLa cells is depicted in **Figure 3**.

Table 2: *In vitro* antiproliferative effect by observing the cell viability against HeLa cells

Group	Cell viability
Control (solvent)	100%
DMSO (2.5%) & Solvent	>95%
VNLE (50 µg/mL)	70-80%

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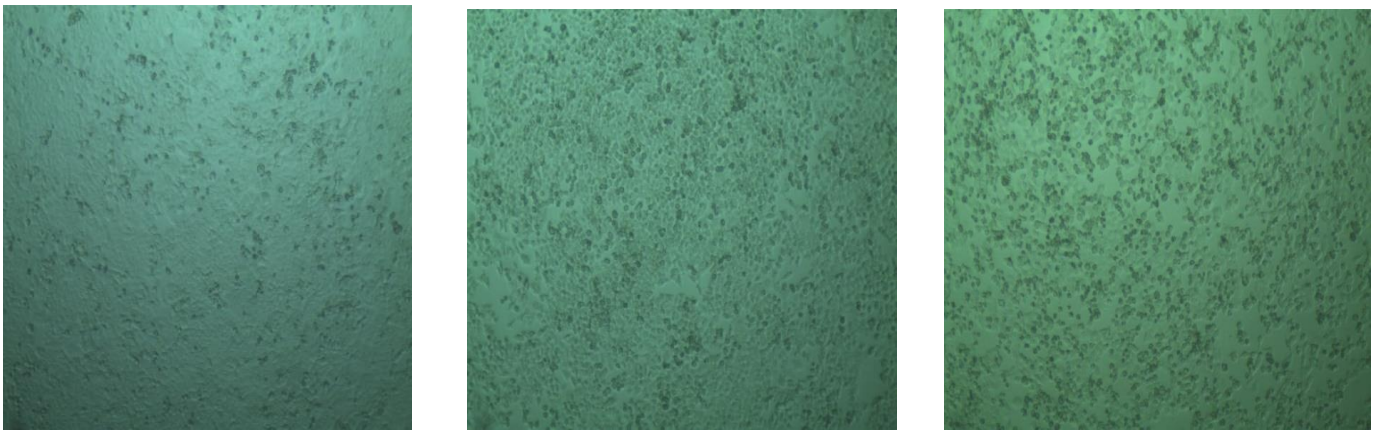
Figure 3: *In vitro* anticancer activity of VNLE and DMSO on human cervical carcinoma cell line



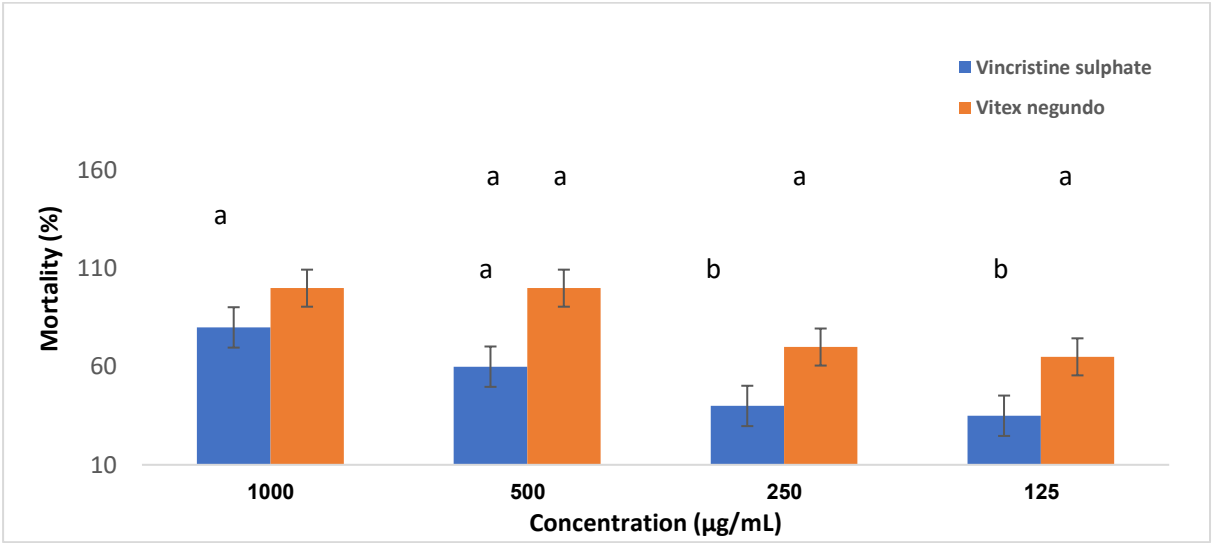
Solvent (-) Solvent (+) *V. negundo* leaves

Brine shrimp cytotoxicity activity: 293.494 µg/mL is the LC_{50} value for vincristine sulfate determined by equation (**Table 3**). When vincristine sulphate was present at 1000 µg/mL, the death rate was pragmatically 80.0% at 125 µg/mL, on the other hand, 35.0% of deaths were found. Using the equation, the LC_{50} value (100.53 µg/mL) is obtained and presented by VNLE along with the fatality percentage. **Figure 4** shows that at 1000 µg/mL and 125 µg/mL of VNLE, the mortality rates were 100% and 65.0%, respectively.

Table 3: The regression values of brine shrimp cytotoxic effect

Group	Regression value
Vincristine sulphate	$y = 1.39x + 1.57$
VNLE	$y = 2.588x - 0.182$

Figure 4: *In-vivo* cytotoxic activity of VNL methanol extract and vincristine sulphate



Statistical significance differences among numerous therapeutics are indicated by different alphabets

Neuropharmacological activity

Hole-cross test: The number of holes visited by the mice in the healthy control group did not change between 0 and 60 minutes (**Figure 5**). The 0-minute to 90-minute movement of the hole cross-test in VNLE-treated groups decreased compared to the initial value. A dose of 400 mg/kg exhibited a maximum suppression of locomotor activity which is similar to diazepam ($p<0.05$).

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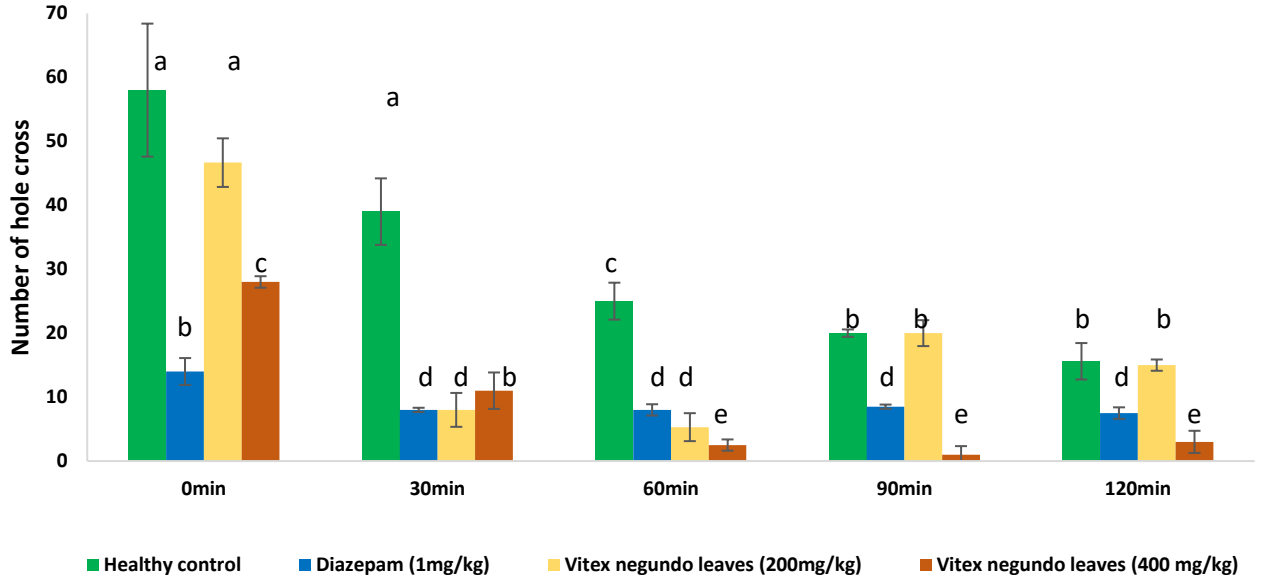
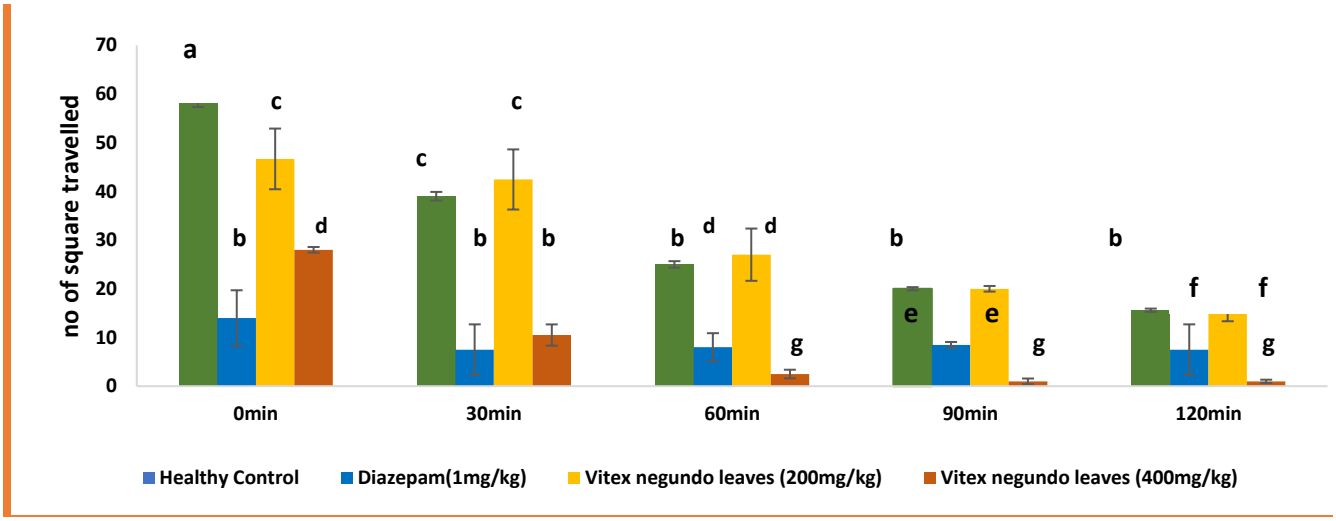
Figure 5: *Vitex negundo* methanolic extract's effect on exploratory behaviour



Statistical significance among numerous therapeutics is indicated by different alphabets

Open-field test: During the open-field test, the VNL-treated groups (200 and 400 mg/kg), demonstrated a noteworthy and dose-dependent decline in movement between 0 minutes and 120 minutes at the baseline, as demonstrated in **Figure 6**. At the 400 mg/kg dose level, the number of squares the mice travelled decreased significantly from its initial value at 0 minutes to 90 minutes ($p<0.05$).

Figure 6: Effect of VNLE on mice's investigational behaviour in an open-field test



Variations in alphabets denote statistical significance among various treatments

Discussion

Plants naturally contain substances called phytochemicals that have a range of therapeutic uses [33, 34]. The diverse phytochemicals present in plants, including alkaloids, tannins, saponins, flavonoids, phenols, steroids, and carotenoids, all contribute to their protective benefits against inflammation, diabetes, aging, microbes, parasites, depression, cancer, oxidative damage, and wound healing [31, 35]. Flavonoids, saponins, tannins, alkaloids, steroids, anthraquinone glycosides, glycosides, and reducing sugars were found in VNL's methanol extracts. The capacity to donate hydrogen is what gives DPPH, a free radical with a reputation for being highly

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stable, its antioxidant effect on DPPH radical scavenging [36]. This assay is typically used to assess the phenolic components in plant extracts' antioxidant qualities [21]. In this assay, the prevalence of antioxidant components in plant extract is indicated by the change of DPPH solution to DPPH, which is yellow [37]. The antioxidant activity of VNLE assay using the DPPH method. The higher the percentage of the inhibitory effect, the greater the antioxidant capacity of the extracts. Conversely, the bigger the antioxidant activity of the extracts, the smaller the IC_{50} value. The scavenging effect of the reference standard ascorbic acid was 97.0% at the highest concentration whereas the IC_{50} value was much less (536.75 µg/mL) which showed its highly effective potential as a free radical scavenger. The negative control methanol also showed no antioxidant activity. VNLE showed a dose-dependent free radical scavenging effect compared to that of the reference.

Thrombolysis involves dissolving dangerous blood clots to maintain blood flow and protect tissues and organs from damage. An internal haemorrhage occurring due to blood accumulation poses a risk to vital organs. SK is a thrombolytic agent that dissolves blood clots. A thrombolytic mediator catalyses the formation of plasmin from plasminogen, which then liquefies blood clots. This proteolytic enzyme can prevent fibrin particles from cross-linking [38]. Herbs, leaves, fruits, and seeds are encompassed by sources that have fibrinolytic, anti-clotting, and anti-platelet characteristics. At 10 mg/mL, VNL's clot lysis capacity was significantly higher than that of the negative control (10.9%), with a value of 59.5%, which was significant compared with the negative control while the capability for clot lysis for SK was 68.3%. The VNLE contains flavonoids responsible for its thrombolytic properties. Hela cells were exposed to VNLE for cytotoxicity assessment. Hela cells' propagation and viability were decreased by the VNLE concentration range. Vinblastine, irinotecan, topotecan, and paclitaxel, derived from plants, have proven clinical efficacy as anticancer drugs. Flavonoids, phenolic compounds, and polyphenols undergo various biological interactions with enzymes and toxic proteins, resulting in cellular toxicity or growth inhibition [38]. Studies suggest that flavonoids found in natural plants are responsible for their cytotoxic and anticancer effects, significantly contributing to chemoprevention via influence on cell signalling and angiogenesis. Flavonoids, a type of polyphenolic compound, play a significant role in cancer prevention [40, 41]. Previous studies reported high levels of polyphenols in VNLE. Because of its chemotherapeutic qualities, VNLE may have killed Hela cells [42].

Brine shrimp lethality (BSL) assay is uncomplicated, as it does not necessitate aseptic conditions, completes within 24 hours, is cost-effective, and does not require any specialized equipment. BSL assay is an *in-vivo* monitor for discovering, screening, and monitoring bioactivities (cell-line toxicity, antitumor, cytotoxic, phototoxic, pesticidal, trypanocidal, enzyme inhibition, and ion regulation) within natural compounds. Cytotoxic properties in the BSL assay were determined as follows: safe within the range of and nontoxic in the range LC_{50}>1000 µg/mL; weak within the range 500≥LC_{50}≤1000 µg/mL; moderate within the range 100≥LC_{50}≤500 µg/mL; and strong within the range 0.0>LC_{50}<100 µg/mL. The crude methanol, petroleum ether, and carbon tetrachloride fractions, obtained from the VNL, were shown to have cytotoxic activity. The cytotoxic activity of agnuside and negundoside from VNL was demonstrated using a BDL assay. The bark of *V. negundo* verified significant cytotoxic activity against brine shrimp larvae [43]. The VNLE and vincristine sulphate caused brine shrimp larvae to die in a dose-dependent manner in our study. Higher cytotoxicity was observed in the case of VNLE [LC_{50} of 100.53 µg/mL] than that of vincristine sulphate [LC_{50} of 293.49 µg/mL). The VNLE, rich in phenolics and flavonoids, may account for the high shrimp mortality rate due to its anti-tumor properties [44]. The hole-cross test and open-field test are the typically used experiments for assessing an animal's exploratory tendencies [45, 46]. Diazepam is known to reduce animal exploration and locomotor activity in response to a new environment [47]. CNS depressant drugs, like diazepam, negatively impact animal performance on the hole cross-test and open-field test due to anxiolytic activity, decreased ambulatory activity, and sedation [46, 47]. At 200 and 400 mg/kg doses, VNLE significantly declined the number of animal holes crossed and the number of squares visited. Simultaneously, a dose of 1.0 mg/kg of

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diazepam also resulted in a comparable pattern of effects to that seen with VNLE across all experiments [48]. However, to recapitulate, more insightful scientific research is required to generate further reproducible outcomes in the sectors of antioxidant, thrombolytic, antiproliferative, neuropharmacological, and cytotoxic activities. Still, apart from these, some other activities must be evaluated so that the potentiality of the seeds of this medicinal plant can be assessed appropriately. The limitation of this study is that was performed mostly *in-vitro*. There are substantial differences in *in-vitro* and *in-vivo* test results. Additionally, crude methanol extract was utilized rather than the isolation of bioactive compounds available in that extract. Generally, the crude extract contains a mixture of different types of compounds. Therefore, it is possible that one compound's bioactivity can be antagonized by other compounds available. Hence, compound isolation and molecular docking approaches can allow us to know the real potential.

Conclusion: It is plausible that the methanol extract of *Vitex negundo* leaves like diazepam, reacts with the central nervous system, as evidenced by the animals' reduced motor coordination, anxiolytic exertion, and sedation.

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