Screening and evaluation of antioxidant, antimicrobial, cytotoxic, thrombolytic and membrane stabilizing properties of the methanolic extract and solvent–solvent partitioning effect of *Vitex negundo* Bark

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**Objective:** To investigate different biological activities of the methanolic extract and solvent–solvent partitioning of *Vitex negundo* (*V. negundo*) bark.

**Methods:** *In–vitro* anti–oxidant study was determined using total phenolic compound analysis and DPPH radical scavenging assay. *In vitro* antimicrobial study was measured by observing zone of inhibition. The cytotoxic activity was studied using brine shrimp lethality bioassay and thrombolytic activity by clot disruption method.

**Results:** In the evaluation of anti–oxidant activity, the amount of total phenolic content differed in different extractives as from 12.94 mg of GAE/g of extractives to 54.19 mg of GAE/g of extractives of *V. negundo*. Among all extractives, the highest phenolic content was found in *n*–hexane fraction (N–HXN) (54.19 mg of GAE/g of extractives). Significant amount of phenolic compounds were present in methanol extract (ME) (35.19 mg of GAE/g of extractives) and aqueous soluble fraction (AQSFR (12.94 mg of GAE/g of extractives). The free radical scavenging activity of ME and its different partitions were assessed using DPPH free radicals assay. The highest free radical scavenging activity was found in AQSFR (IC50=7.78 µg/mL). The antimicrobial screening of the bark of *V. negundo* exhibited mild to moderate activity in test microorganisms. The chloriform soluble fraction demonstrated the highest inhibition against microbial growth having zone of inhibition as 8–12 mm. In the brine shrimp lethality bioassay, The LC50 values of *n*–hexane and chloriform soluble fractions were found 3.7736 µg/mL and 0.910 µg/mL respectively while the LC50 values of standard Vincristine sulphate was 0.408 µg/mL. The methanol extract and their different organic soluble of *V. negundo* bark inhibited hypotonic solution–induced haemolysis of erythrocytes in *in–vitro* membrane stabilizing activity test. The AQSFR and N–HXN showed inhibition of haemolysis as 55.05% and 29.89% that were lower than 71.9% of aspirin (0.10 mg/mL). Methanol extract of *V. negundo* and all of its different partitions exhibited moderate thrombolytic activity of 36.95%–22.98%.

**Conclusions:** These experiments were able to show the several biological activity of methanolic extract and its soluble fractions of *V. negundo* at a time.

**KEYWORDS**

*Vitex negundo*, Antioxidant, Antimicrobial, Cytotoxic, Thrombolytic

1. Introduction

Anti–oxidants benefit our wellness by cleaning toxins out of our blood vessels. They can even secure our epidermis from sun damage, and decrease the occurrence of sun burn. Although anti–oxidants aren’t proven to cure any
conditions, research has revealed that antioxidants have also been suggested as a factor in the avoidance of a number of degenerative, age-related illness, such as cancer, cardiac arrest, intellectual incapacity, defense malfunction, cataracts, macular damage, and Alzheimer’s[1]. Microorganisms are living cells so small that most can only be seen under a microscope. Many microbes are helpful; some are not. They cause infectious diseases. Antimicrobial agents are used against microbes[2]. Cytotoxic agents inhibit the growth and proliferation of cells and neoplasms. They are also continually known as chemotherapeutic medication[3]. Thrombolytic agents are used to lyse already established blood clotting in medical configurations where ischemia may be critical (serious myocardial infarction, lung embolism, ischemic action, and arterial thrombosis)[4]. Membrane stabilizing agents are useful to reduce the hyper-excitability of nerves[5].

Vitex negundo (V. negundo), a large, aromatic deciduous shrub with quadrangular, densely whitish tomentose, branchlets, up to 4.5 m in height or sometimes a small tree distributed particularly in South and Southeast Asia. It is used as folk medicine for asthma, cough, mosquitoes bite, tonic, febrifuge, expectorant, dyspepsia, worms, leprosy, rheumatism, diarrhea, cholera, liver diseases, cardiac difficulties, paralyses, limb-pain, weakness, angina, and rheumatic problems in Bangladesh and other region of the Asia. An extract from the leaves showed anticancer activity against Ehrlich ascites tumour-cells. The earlier study of this plant shows that it has anti-oxidant, antimicrobial, cytotoxic effect, but we have found that there is no study on thrombolytic and membrane stabilizing activity[6,7]. Thrombolytic drugs are widely used in the management of cerebral venous sinus thrombosis (CVST) patients. Thrombolytic drugs such as tissue plasminogen activator (t-PA), urokinase, and streptokinase, play a crucial role for the management of patients with CVST. Streptokinase and urokinase have many adverse effects because they might cause serious bleeding complications along with reocclusion and reinfarction[8-10]. So search for alternative thrombolytic agents from plant source might be beneficial. So we conducted the investigation on methanolic extract and solvent–solvent partitioning effect of V. negundo bark to find out bioactivities at a time.

2. Materials and methods

2.1. Plant material

The bark of V. negundo was collected from the village Chapitola in Muradnagar–Comilla, Bangladesh. The bark was sun dried for several d and then oven dried for 24 h at temperature 400 °C for better grinding. The dried bark was then ground to a coarse powder (350 g) using high–capacity grinding machine.

2.2. Drugs and chemicals

Streptokinase was collected from Beacon pharmaceutical Ltd., Bangladesh. Aspirin was bought from Incepta Pharmaceuticals Ltd., Bangladesh. Artemia salina leach (brine shrimp eggs), sea salt (NaCl), n–hexane (N–HXN), carbon tetra chloride, chloroform (CSF), methanol, ascorbic acid, tert–butyl–1–hydroxytoluene, Na2CO3 solution (7.5%), Folin–Ciocalteu reagent (10 fold diluted), nutrient agar medium, and ethanol were obtained from Merck, Germany and were in analytical grade. RBCs were collected from the human male (45 kg) brown complexion and free from diseases. The collected RBC was kept in a test tube with an anticoagulant EDTA under standard conditions of temperature (28±2) °C and relative humidity (65±10)%.

2.3. Experimental

2.3.1. Extraction

The powdered material (350 g) was taken in a cleaned, amber bottle (5 L) and soaked in 2.0 L of methanol. The container with its content was sealed by bottle cap and kept for a period of 10 d accompanying occasional shaking and stirring. The whole mixture was then filtered through a fresh cotton plug and finally with a Whatman No.1 filters paper. The volume of the filtrate was then allowed to evaporate at ambient temperature until approximately 70% solvent was evaporated.

2.3.2. Solvent–solvent partitioning

Solvent–solvent partitioning was done using the protocol designed by Kupchan and modified by Van Wagener et al. The crude extract (5 g) was dissolved in 10% aqueous methanol. It was extracted with N–HXN, carbon tetrachloride (CTCSF) and CSF. All fractions were evaporated to dryness and were used for further analysis.

2.4. Anti-oxidant test

2.4.1. Total phenolic compound analysis

Total phenolic content of V. negundo bark extract was determined using the method described by Skerget et al. involving Folin–Ciocalteu reagent as oxidizing agent and gallic acid as standard[11,12]. A total of 2 mg of the extract was taken and dissolved in the distilled water to get a sample concentration of 2 mg/mL in every case. About 2.5 mL of Folin–Ciocalteu reagent (diluted 10 times with water) and 2.0 mL of Na2CO3, (7.5% w/v) solution was added to prepare 0.5 mL of extract solution (conc. 2 mg/mL). The mixture was incubated for 20 min at room temperature. After
20 min, the absorbance was measured at 760 nm by UV–
spectrophotometer (Veego, India) and using the standard
curve prepared from gallic acid solution with different
concentration, the total phenols content to the sample
was measured. The phenolic contents of the sample were
expressed as mg of gallic acid equivalent (GAE)/g of the
extract.

2.4.2. DPPH assay

dPPH was used to evaluate the free radical scavenging
activity[13,14]. Ascorbic acid and tert–butyl–1-hydroxytoluene
was used as positive control. A total of 20 mg DPPH powder
was weighed and dissolved in methanol to get a DPPH
solution having a concentration 20 μg/mL. The solution was
prepared in the amber reagent bottle and kept in the light
proof box. About 2.0 mL of a methanol solution of the sample
(extractives/control) at different concentrations (500–0.977 µg/
ml) were mixed with 3.0 mL of a DPPH methanol solution (20
µg/mL). After 30 min reaction period at room temperature in
dark place, the absorbance was measured at 517 nm against
methanol as blank by UV spetrophotometer.

Inhibition of free radical DPPH in percent (I%) was
calculated as follows:

\[ I\% = (1 - \frac{A_{sample}}{A_{blank}}) \times 100 \]

where, \( A_{blank} \) is the absorbance of the control reaction
(containing all reagents except the test material). Extract
concentration providing 50% inhibition (IC50) was calculated
from the graph plotted inhibition percentage against extract
concentration.

2.5. In–vitro microbiological test

The slants were used for preparation of bacterial and
fungal culture for sensitivity study. The medium of 10 mL
and 5 mL (pH 7.2–7.6 at 25 °C) was sterilized by autoclaving
at 15 lbs pressure and 121 °C for 20 min. The test organisms
were transferred to the agar slants through a transfer loop.
The inoculated strains were then incubated for 24 h at 37
°C for their optimum growth. These fresh cultures were
used for the sensitivity test. About 10 mL of melted and
sterilized agar medium was taken in test tubes and shaken
continuously for uniform suspension of the organisms.
The bacterial and fungal suspension was immediately
transferred to the sterilized petridishes. The petridishes
were rotated clockwise and anticlockwise for homogenous
distribution of the test organisms in the media. About 8.0
mg of each test sample was dissolved in specific volume
of solvent (CSF or methanol) to obtain the 400 µg/disc
concentrations in an aseptic condition. Sterilized metrical
(BBL, Cocksville, USA) filter paper discs were taken in a
blank Petri dish under the laminar hood. Then discs were
soaked with solutions of test samples and dried. Standard
ciprofloxacin (30 µg/disc) discs were used as positive control
of standard antibiotic agent. Blank discs were used as
negative controls, which ensure that the residual solvents
(left over the discs even after air-drying) and inactive filter
papers. Sample discs, standard antibiotic discs and control
discs were placed gently on the previously marked zones
in the agar plates pre–inoculated with test bacteria and
fungi. After that, they kept in a refrigerator at 40 °C for 24 h
to allow sufficient diffusion from the discs to agar medium.
The plates were then inverted and kept in an incubator at
37 °C for 24 h. The antimicrobial potency of the test agents
were measured by observing clear zone of inhibition. After
incubation, the antimicrobial activities of the test samples
were determined by measuring the diameter of the zones
of inhibition in millimeter.

2.6. Brine shrimp lethality bioassay

Brine shrimp lethality bioassay was performed[15,16],
Artemia salina leach (brine shrimp eggs) was used as the
test organism. Seawater (38 g NaCl) was taken in the small
tank and shrimp eggs were added to one side of the tank
and covered. One day was allowed to hatch the shrimp
and to be matured as nauplii. Constant oxygen supply was
carried out through the hatching time. The hatched shrimps
were attracted to the lamp through the perforated dam and
they were taken for experiment. With the help of a pasteur
pipette, 10 living shrimps were added to each of the test tubes
containing 5 mL of seawater. About 4 µg of each extractive
was taken in vials and dissolved in 100 µL of pure dimethyl
sulfoxide (DMSO) to get stock solutions. Then 50 µL of solution
was taken in the first test tube containing 5 mL of seawater and
10 shrimp nauplii. Thus, final concentration of the
prepared solution in the first test tube was 400 µg/mL. Then
a series of solutions of varying concentrations (400, 20, 100,
50, 25, 12.5, 6.25, 3.125, 1.562 5, 0.781 25 µg/mL) were prepared
from the stock solution using 50 µL samples and fresh 50
µL DMSO. Usually, two types of control groups are used. In
the present study, vincristine sulphate (VS) was used as the
positive control. Measured amount of the VS was dissolved in
DMSO. Then the positive control solutions were added to the
premarked vials containing 10 living brine shrimp nauplii in
5 mL sea water to get the positive control groups. A total of
100 µL of DMSO was added to each of three premarked glass
vials containing 5 mL of sea water and 10 shrimp nauplii as
control groups. If the brine shrimps in these vials show a
rapid mortality rate, then the test is considered as invalid
as the nauplii died due to cytotoxicity of the compounds.
After 24 h, the vials were inspected using a magnifying glass
and the number of survivors were counted. The percent (%)
mortality was calculated for test samples. The concentration–
mortality data were analyzed using linear regression. The
concentration–mortality relationship of plant product
expressed as a median lethal concentration (LC50) value.
2.7 Membrane stabilizing activity

Hypotonic solution-induced haemolysis experiment was used to determine the membrane stabilizing activity. The experiment was carried out with hypotonic solution. The test sample consisted of stock erythrocyte (RBC) suspension (0.5 mL) with 5 mL of hypotonic solution (50 mmol/L NaCl) in 10 mmol/L sodium phosphate buffer saline (pH 7.4) containing either the different methanol extract (EM) (1.0 mg/mL) or acetyl salicylic acid (0.1 mg/mL). The acetyl salicylic acid or aspirin was used as a reference standard. The mixtures were incubated for 10 min at room temperature, centrifuged for 10 min at 3000 g and the absorbance (O.D.) of the supernated was measured at 540 nm using Shimadzu UV spectrophotometer. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation:

% Inhibition of Haemolysis = 100[(OD1- OD2)/ OD1]

Where, OD1=Optical density of hypotonic-buffered saline solution alone (control) and OD2=Optical density of test sample in hypotonic solution.

2.8 In-vivo thrombolytic activity test

The dry crude extract (100 mg) was suspended in 10 mL of distilled water and it was kept overnight. Then the soluble supernatant was decanted and filtered. Streptokinase (1 500 000 I.U.) suspension was used as a stock for 100 µL (30 000 I.U.) was used for in vitro thrombolysis. Blood (n=10) was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 1 mL of blood was transferred to pre-weighted micro centrifuge tubes allowed to clots. Aliquots (5 mL) of venous blood was drawn from a healthy volunteer which was distributed in five different pre-weighted sterile micro centrifuge tubes (1 mL/tube) and incubated at 37 °C for 45 min. Then serum was completely removed without disturbing the clot and clot was weighed (clot weight=weight of clot containing tube−weight of tube alone). About 100 µL aqueous solution of different fractions of the crude extract was added to each pre-weighted micro centrifuge tube containing clot. A total of 100 µL of distilled water was added to the control tubes as a positive control of 100 µL of streptokinase and as a negative control of non thromboytic. All the tubes were then incubated at 37 °C for 90 min and observed for clot lysis. After incubation, the released of fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. Weight taken before and after clot lysis was expressed as percentage of clot lysis as following:

Clot lysis (%) = (wt of released clot/clot wt)×100

3. Results

Cytotoxicity activity of methanol extract (ME) of V. negundo bark and its different organic soluble fractions of N–HXN fraction, CTCSF, CSF, and aqueous soluble fractions (AQSF) were tested using brine shrimp lethality bioassay. The lethal concentration LC50 of the test samples were obtained after 24 h in a plot of percentage of the shrimps died against the

Figure 1. Cytotoxicity activity of V. negundo bark on shrimp nauplii (VS=Vincristine sulphate, ME=Methanol extract of V. negundo, N–HXN=n-hexane soluble fraction of the Methanol extract of V. negundo, CTCSF=Carbon tetrachloride soluble fraction, CSF=Dichloromethane soluble fraction, AQSF=Aqueous soluble fraction).
log concentration (toxicant concentration) of the sample and the best-fit line was obtained from the curve of regression analysis (Figure 1). LC50 of VS was found 0.408 µg/mL, but negative control showed significant mortality rate of 102.62, 3.713 6, 130.97, 0.910, and 39.78 µg/mL for ME, N-HXN, CTCSF, CSF, and AQSF respectively (P<0.001) (Figure 2).

Anti-oxidant activity of ME of V. negundo bark and its different organic soluble fractions of N–HXN, CTCSF, CSF, and AQSF were tested for total phenolic content. The amount of total phenolic content in different extractives was found 12.94–54.19 mg of GAE/g. Among all extractives of V. negundo bark, the highest phenolic content was found in CSF as 53.19 mg of GAE/g of extractives and in N–HXN as 54.19 mg of GAE/g of extractives. Significant amount of phenolic compounds were also present in ME as 35.19 mg of GAE/g of extractives, AQSF as 12.94 mg of GAE/g of extractives and CTCSF as 42.38 mg of GAE/g of extractives (Figure 3).

The free radical scavenging activity of ME and its soluble fraction of V. negundo bark was assessed using DPPH free radicals assay and found significant free radical scavenging activity at the dose of 1.5 µg/mL (P<0.001) (Figures 4 and 5).

**Figure 2.** LC50 values of vincristine sulphate and V. negundo bark extract and fractions on shrimp nauplii.

**Figure 3.** Total phenolic content activity of the standard and fractions of V. negundo bark (2 mg/mL).

**Figure 4.** DPPH free radical scavenging activity of the standard and fractions of V. negundo bark. ASA: Ascorbic acid standard, AQSF: Aqueous soluble fraction of methanolic extract of V. negundo, CSF: Chloroform soluble fraction of methanolic extract.

**Figure 5:** Free radical scavenging activity of V. negundo bark and ascorbic acid.
Antimicrobial screening of ME of V. negundo bark and its different organic soluble fractions of N-HXN, CTCSF, CSF and AQSF were performed at the dose of 400 µg/disc. CSF and CTCSF showed significant antimicrobial activity against the microorganisms (Figure 6). CSF and CTCSF showed the significant zone of inhibition ranged from 8-12 mm and 7-10 mm respectively (P<0.001).

Thrombolytic activity of streptokinase showed 66.77% lysis of clot. Distilled water was treated as negative control showed negligible percentages of 2.57% lysis clot. In this study, methanol extract of CSF of V. negundo bark and its different organic soluble fractions of N-HXN, CTCSF, CSF and AQSF were performed at the dose of 1.0 mg/mL were tested by hypotonic solution induced haemolysis. In hypotonic solution induced condition, the AQSF and n–hexane soluble fraction (N–HIXNSF) inhibited 55.05% and 29.89% of haemolysis of RBC respectively (P<0.01). Aspirin (0.10 mg/mL) showed 71.9%. CTCSF, CSF, and methanol extract showed significant haemolysis of RBC (Figure 7).

Cytotoxicity is good for malignant cells. V. negundo bark showed significant cytotoxicity level at the dose of 400.0–0.781 25 µg/mL (P<0.001) than the study of Thombre et al. found growth inhibition rate of (48.14±0.92) % against THP−1 cell lines, followed by ethanolic extract of V. negundo L. [(31.13±2.64)%]7,17–19. It might be used as chemotherapeutic agent after preparing well known dose on clinical trial. We should be careful to the use of V. negundo for the treatment of diseases as a folk medicine and avoid overdosing.

Free radicals are harmful for cells, heart disease, cancer and other diseases. Anti–oxidants protect these free radicals. Phenolic compounds are analyzed using Folin–Ciocalteu reagent for the anti–oxidant activity of V. negundo bark. Based on the absorbance values of the various extract solutions, the colorimetric analysis of the total phelnic of different extracts were determined and compared with the standard solutions of GAE. Total phelnic contents of the samples were expressed as mg of GAE/g of extractives. Therefore, anti–oxidant activity of V. negundo bark extracts is to be expected due to a high content of phelnic compounds. Previously published study had failed to show such significant results of methanol extract and its soluble fractions of V. negundo bark[6,20,21].

Infectious diseases are resulted from the infection, presence and growth of pathogenic biological agents in an individual host organism. Antimicrobials prevent or slow down the transmission of infectious diseases. V. negundo was investigated for antimicrobial activity, and showed the growth of inhibition of pathogenic microorganisms other than published studies[6,22]. The results showed that AQSF extracts was potent on human erythrocyte in hypotonic induced lyses. The activity was comparable to standard anti–inflammatory drug (aspirin). It has been reported that flavonoids have profound stabilizing effects on lysosomes both in–vivo and in–vivo experiments[23–25], while tannin and saponins have the ability to bind cations and able to stabilize erythrocyte membrane[26,27]. The present investigation suggests that the membrane stabilizing activity of V. negundo bark plays a significant role in anti–inflammatory activity may be due to its high flavonoids or tannin content. It can be concluded that some extractives of V. negundo showed significant clot lysis activity.

These herbal preparations may be incorporated as a thrombolytic agent for the improvement of the patients suffering from atherothrombotic diseases. This is a
preliminary study and the extract should thoroughly be investigated phytochemically and pharmacologically to exploit their medicinal and pharmaceutical potentialities.

Previously published study on *V. negundo* has been studied on its leaf and performed only single study, but our study was designed on investigation of multiple activities on bark extract at the same time. The ME of *V. negundo* bark and its different organic soluble fractions were used for evaluation of antioxidant activity (total phenolic content, DPPH assay), brine shrimp lethality bioassay, antimicrobial screening, membrane stabilizing activity and thrombolytic activity at a time. The investigation confirmed that *V. negundo* bark has a significant amount of phenolic content and mild to moderate antimicrobial activity to certain experimental organisms. The different extractives of *V. negundo* bark moderately protect the lysis of human erythrocyte membrane induced by hypotonic solutions which confirms that the plant has potent membrane stabilizing activity as it stabilized the membrane of RBCs. Therefore, considering the potential bioactivity, the plant materials can further be studied extensively to find out their unexplored efficacy and to rationalize their uses as traditional medicines.

**Conflict of interest statement**

We declare that we have no conflict of interest.

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**Comments**

**Background**

*V. negundo* (*Chaste tree*) is well-known for pharmaceutical use against a number of diseases. Every part of this plant is valuable. Several researches of *V. negundo* plant are still going on. The extraction of this plant will probably be used as a powerful anticancer drug near future. Chitra *et al.*, Dewade *et al.* and Arulvasu *et al.* showed this plant useful in tumor treatment. These are preliminary study and the studied extract of bark showed normal data while the present work described different extraction method using bark part of the plant.

**Research frontiers**

Research showed the beneficial effect of anti-oxidant, antibacterial, anticancer, atherothrombolytic and anti-inflammatory activity of solvent–solvent partitioning effect of *V. negundo* bark. These findings make valid the use of this plant as a folk medicine.

**Related reports**

No report has been found on the exact mechanism of action of this plant. This research work found significant results, but further study is needed to describe the mechanism of action why extract showed more fruitful results.

**Innovations & breakthroughs**

Data in the present study is important for its higher yield than previous studies of anti-oxidant, antibacterial, anticancer activity of leaves extract. This research showed the effect of atherothrombolytic and anti-inflammatory of *V. negundo* and employed the bark instead of leaves in solvent–solvent portioning extract system. As a result, more valuable data has been found in this study.

**Applications**

The usefulness of *V. negundo* is widely known for asthma, cold and heart diseases. The study of this project showed the effectiveness against these problems. However, a clinical trial on animal model is required using the isolated chemicals of this plant for the development of current drugs in the market.

**Peer review**

This is a very good preliminary study of thrombolytic and membrane stabilizing activity of *V. negundo* bark. The anti-oxidant, antibacterial, anticancer activity of leaves extract of this plant has been studied, but the authors found highly potential result using their method described. Overall the results are interesting.

**References**


