



Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd



Document heading

doi:10.1016/S2222-1808(13)60090-0

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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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PEER REVIEW

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Comments

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. This preliminary study on thrombolytic and membrane stabilizing activity of *V. negundo* bark is really appreciable. The obtained data related to anti-oxidant, antibacterial, anticancer activity of the bark extract of this plant is promising and may play an important role in discovering of effective drugs.

Details on Page 399

ABSTRACT

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 (AQSF) (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 AQSF (IC₅₀=7.78 µg/mL). The antimicrobial screening of the bark of *V. negundo* exhibited mild to moderate activity in test microorganisms. The chloroform 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 chloroform soluble fractions were found 3.7136 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 AQSF 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

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Foundation Project: Financially supported by the State University of Bangladesh Graduate Research Fund (Grant No. PG 14–03–11–034).

Article history:

Received 16 Jul 2013

Received in revised form 20 Jul, 2nd revised form 24 Jul, 3rd revised form 29 Jul 2013

Accepted 20 Aug 2013

Available online 28 Oct 2013

conditions, research has revealed that anti-oxidants 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 Wagenen *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 spectrophotometer.

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

$$I\% = (1 - A_{\text{sample}}/A_{\text{blank}}) \times 100$$

where, A_{blank} is the absorbance of the control reaction (containing all reagents except the test material). Extract concentration providing 50% inhibition (IC_{50}) 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 mg 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, 1.5625, 0.78125 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 (LC_{50}) 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 supernatant was measured at 540 nm using Shimadzu UV spectrophotometer. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation:

$$\% \text{ Inhibition of Haemolysis} = 100 \times [(OD_1 - OD_2) / OD_1]$$

Where, OD_1 =Optical density of hypotonic-buffered saline solution alone (control) and OD_2 =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 μ L (30000 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-weighed 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-weighed 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 μ L aqueous solution of different fractions of the crude extract was added to each pre-weighed micro centrifuge tube containing clot. A total of 100 μ L of distilled water was added to the control tubes as a positive control of 100 μ L of streptokinase and as a negative control of non thrombolytic. 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:

$$\text{Clot lysis (\%)} = (\text{wt of released clot} / \text{clot wt}) \times 100$$

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 LC_{50} of the test samples were obtained after 24 h in a plot of percentage of the shrimps died against the

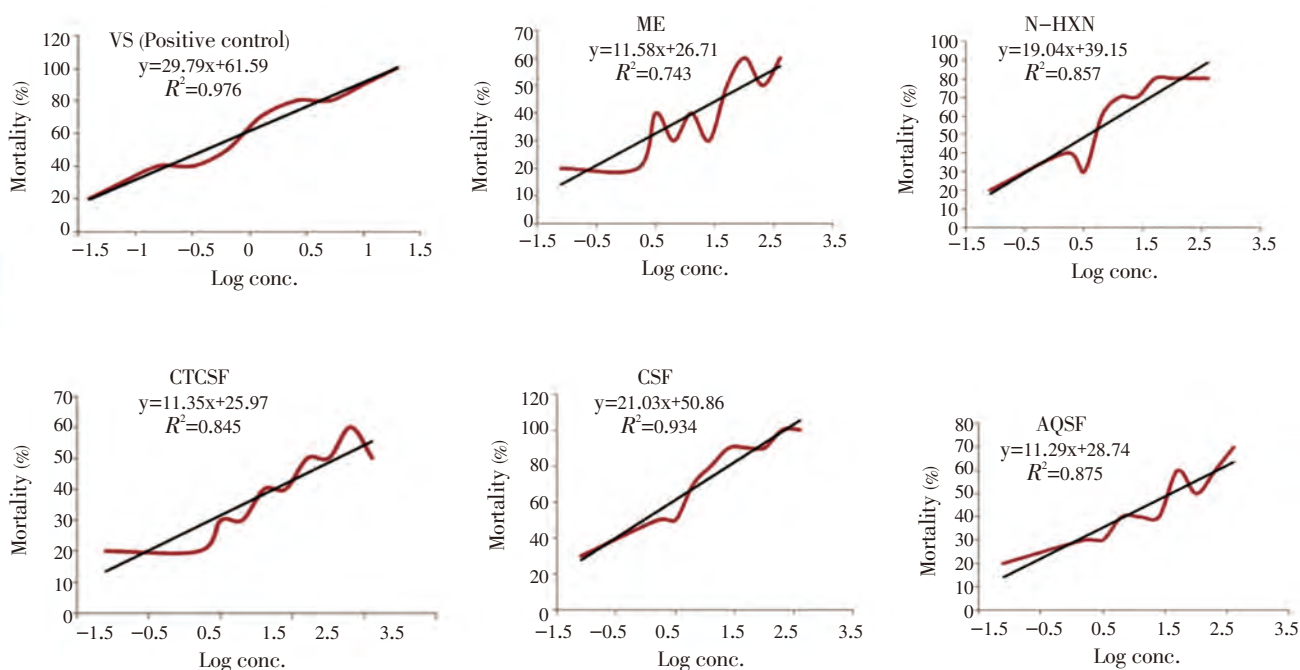


Figure 1. Cytotoxicity activity of *V. negundo* bark on shrimps nauplii (VS=Vincristine sulphate, ME=Methanol extract of *V. negundo*, N-HXN=n-hexane soluble fraction of the Methanol extract of *V. negundo*, CTCSF=Carbon tetrachloride soluble fraction, CSF=Dichloromethane soluble fraction, AQSF=Aqueous 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, 3.7136, 130.97, 0.910, and 39.78 µg/mL for ME, N-HXN, CTCSF, CSF, and AQSF respectively ($P<0.001$) (Figure 2).

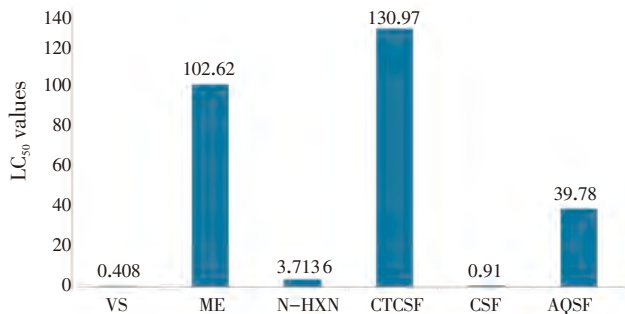


Figure 2. LC₅₀ values of vincristine sulphate and *V. negundo* bark extract and fractions on shrimp nauplii.

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).

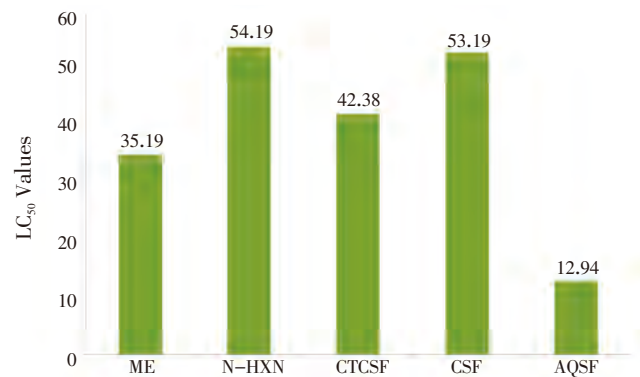


Figure 3. Total phenolic content activity of the standard and fractions of *V. negundo* bark (2 mg/mL).

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).

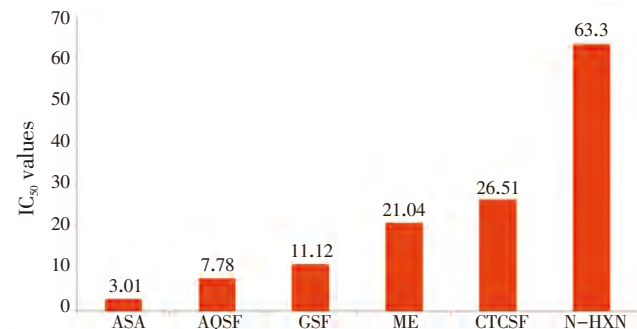


Figure 4. DPPH free radical scavenging activity of the standard and fractions of *V. negundo* bark. ASA: Ascorbic acid (standard), AQSF: Aqueous soluble fraction of methanolic extract of *V. negundo*, CSF: Chloroform soluble fraction of methanolic extract.

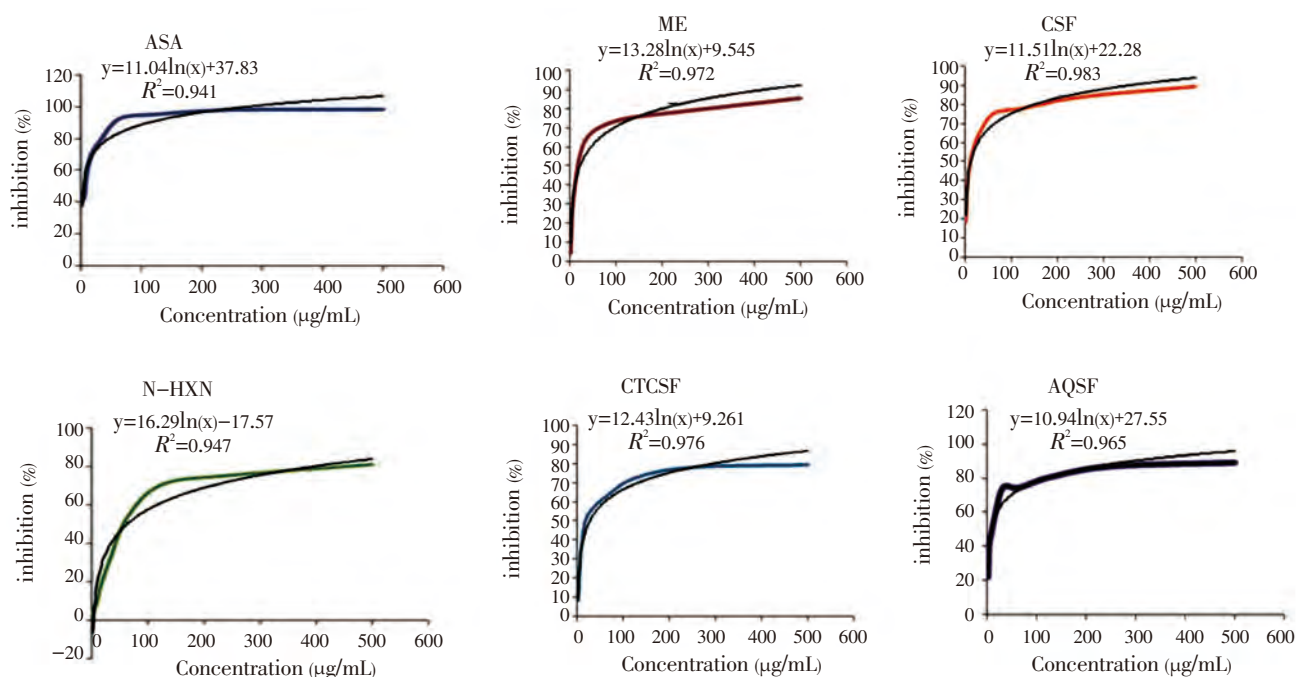


Figure 5. Free radical scavenging activity of *V. negundo* bark and ascorbic acid.

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 ($P < 0.001$).

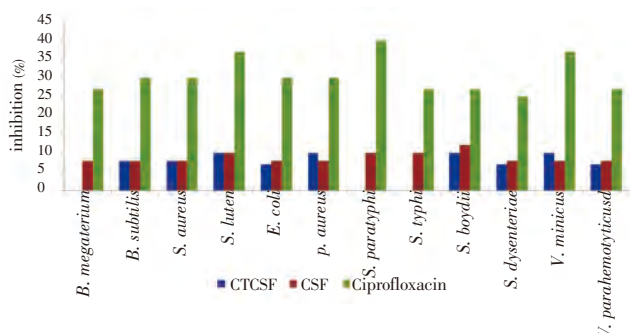


Figure 6. Antimicrobial activity of *V. negundo*.

Lysis activity in human erythrocyte membrane of ME of *V. negundo* bark and its different organic soluble fractions 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-HXNSF) 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).

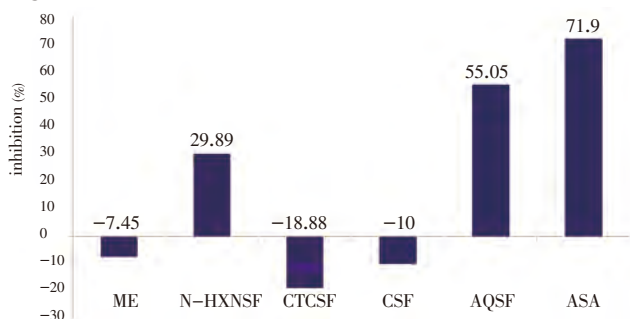


Figure 7. The inhibition of hypotonic solution induced haemolysis of different extracts of the bark of *V. negundo*.

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* demonstrated significant thrombolytic activity of 40.14% ($P < 0.01$) (Figure 8).

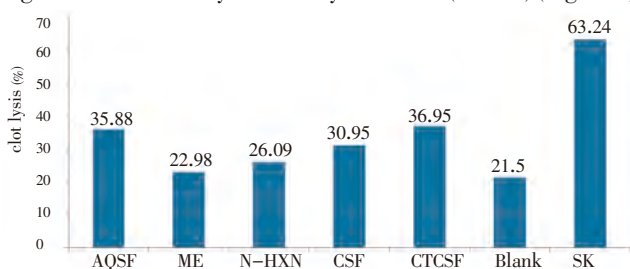


Figure 8. Thrombolytic activity methanol extract and different fractions of *V. negundo*.

4. Discussion

Cytotoxicity is good for malignant cells. *V. negundo* bark showed significant cytotoxicity level at the dose of 400.0–0.78125 µg/mL ($P < 0.001$) than the study of Thombre et al. found growth inhibition rate of $(48.14 \pm 0.92) \%$ against THP-1 cell lines, followed by ethanolic extract of *V. negundo* L. $[(31.13 \pm 2.64) \%]$ [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 phenolic of different extracts were determined and compared with the standard solutions of GAE. Total phenolic 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 phenolic 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 than other 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-vitro* 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.

Acknowledgements

The entire research work was supported by the State University of Bangladesh Graduate Research Fund (Grant No. PG 14–03–11–034). We are grateful to our Lab assistant and colleagues of State University of Bangladesh.

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.

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