Research Article

Vitex negundo: An Important Traditional Medicinal Herb with Multiple Curative Properties

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ABSTRACT

The study is focussed on the in vitro antioxidant, antimicrobial, anti-hemolytic and anti-proliferative activity of *Vitex negundo* leaf, extracted with four different solvents based on polarity. The extracted compounds were analyzed for the qualitative phytochemicals by different phytochemical methods. *Vitex negundo* leaves arefound to be rich in proteins, carbohydrates, alkaloids, flavonoids, tannin, terpenoids, etc. The antioxidant activity of *Vitex negundo* extracts were analyzed by DPPH method and it was observed that all the extracts has good antioxidant activity but out of these hexane and ethyl acetate extract is showing very good results. Antimicrobial activity was done by agar plate diffusion method against *Staphylococus aureus* and the hexane extract showed more zone of inhibition. From the anti-hemolytic assay it was found that hexane extract and petroleum ether extract has more activity for hemolysis inhibition. The anti-proliferative activity of different extracts of *Vitex negundo* was performed by MTT assay in HeLa cell lines and all the extracts showed very good inhibition activity. On comparison, the ethyl acetate extract was found to be the most effective inhibitor in HeLa cells.

Keywords: Antioxidant, Antimicrobial, Anti-hemolytic, Anti-proliferative, HeLa cell line, MTT assay.

INTRODUCTION

Medicinal properties of plant are an attractive area of research because of their immense potential and traditional practices to cure diseases naturally. The property of medicinal plants depend on the presence of various phytoconstituents, nutritive elements etc. [1] Drugs derived from natural resources play a significant role in the prevention and as well as treatment of disease. [2]

Vitex negundo belongs to the family Verbanaceae which comprises of 5 Genera and nearly 250 species; commonly known as five leaved chaste tree. [3] It is a woody aromatic shrub which has bitter, pungent, astringent taste. Commonly found throughout the Indian subcontinent and riverbanks, moist localities and deciduous forests. The shrubs grow about 2-4m in height. *Vitex negundo* used as folk medicine in most of the states of India, Bangladesh, and South East Asia. [4] The leaves are most potent for medicinal use. *Vitex negundo* has been used to several ailment such as ; inflammation, eye disease, toothache, ulcers, fever, asthma, headache, digestion problems, sinuses, bronchitis, antibacterial, antipyretic, antihistaminic, analgesic, insecticidal, antidote for snake bite, etc. The leaves of these plants have been shown mosquito repellent effects as well as ant. The plant also found to have anticancer, rheumatoid arthritis healing and hepatoprotective potentials.[5] The leaves are used for treatment inflammation, skin-ulcers, gonorrhoea, and bronchitis. They are also used as tonics, vermifuge to treat catarrhal fever. Oral administration of the leaves claims to have antihyperglycemic, antibacterial, antipyretic, antihistaminic agents, anti-implantation activity. [6, 7]

The present study investigated the in vitro antimicrobial, antioxidant, antihemolytic, anti-proliferative potential of *Vitex negundo* which was collected from a village in Rangiya 26.47°N 91.63°E, Assam, India. The villagers commonly use this plant in treating liver problems and wound healing. In Assamese language, it is commonly known as "Posotia". The plant is used for both external application in the form of paste/oil and also for oral administration in the form of powder, juice extract and water decoction. In some areas of Assam, it is an important part of traditional cuisine.

MATERIALS AND METHODS

Collection and identification of Plant Material

Fresh plant leaves were collected from healthy tree of *Vitex negundo* from Rangiya and Kamalpur of Kamrup (Rural) District, Assam. The plant was identified as *Vitex negundo* L. by Department of Botany, Gauhati University, Guwahati. (Accession No.GUBH 185000).

Preparation of extracts

The plant leaves were washed and shade dried for 7days. The dried leaves were grinded to fine powder. 100gms of finely powdered leaves was extracted by using two methods; one is the maceration process and another is the Soxhlet extraction.

Soxhlet extraction

80gms of dried leaves were extracted with soxhlet using different solvents fraction (nonpolar to polar solvents)

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successively with petroleum ether, hexane, ethyl acetate and then ethanol. The obtained extracts were evaporated to semi solid mass and stored at 4°C for further use.

Maceration process

10gm of dried powdered samples were mixed with 100ml of ethyl acetate, ethanol and hexane and petroleum ether in different conical flask and kept in shaker for 24 hours and then vortex for 10/15 minutes. After that it was allowed to stand for another three/four days in room temperature for settle down the plant materials. The solution obtained was filtered and filtrate was evaporated to semi solid mass at 30°C using Hot plate. The obtained extracts were kept in airtight bottles and stored at freezer for further studies.

Phytochemical analysis

The extracted plant compound obtained by maceration process was used for the qualitative phytochemical analysis by standard phytochemical methods. [8]

Determination of antioxidant activity

DPPH assay

Free radical scavenging activity was measured by the DPPH method. DPPH (2, 2-diphenyl picryl hydrazyl), is a commercially available stable free radical whose colour is purple. When antioxidant molecules present in the test samples reacts with DPPH in incubation time where the purple colour converts into yellow colour.

0.002gm of DPPH was dissolved in 50ml of methanol for stock solution. The preparation of test solution with a different concentration of the plant extract (10µl- 160µl) and making total volume up to 1ml adding distilled water accordingly. Then 2ml of methanolic DPPH solution was added in each test tube and final volume made to 3ml. The mixture was kept for incubation at dark for 30minutes and covers the test tubes with foil so that no light can pass through it. The absorbance was read at 517nm against a blank solution containing 3ml of methanol. Methanol and DPPH solution was used as control.

The scavenging activity of the leaf extract was calculated by using the following formula and IC_{50} was calculated using standard graph.

Scavenging Activity % = [(Absorbance control – Absorbance test)/Absorbance control] × 100

Antimicrobial/ antibacterial activity testing

Microorganism

Staphylococcus aureus was obtained from Hi media, India.

Antimicrobial activity screening using the plant extract

10mg of powdered extract was dissolved in 1ml of saline water i.e., 10mg/ml and kept for use. The test was performed agar well diffusion method. Bacterial suspension was enriched in Brain Heart infusion broth for overnight. 100µl of bacterial culture was spread aseptically over the Muller Hinton agar plates using a sterile cotton swab. Using sterile 100µl tip (Micropipette tips) agar gel was punctured to create well. Diluted plant extract was pipetted into those wells and Saline water used as control. The plates were kept at 4°C for few minutes for diffusion of extracts and after that incubated at 37°C for 24 hours. The zone of inhibition was measured.

Anti-hemolytic activity

Anti-hemolytic activity of extract was inspected by ELISA plate reader method. [9,10] 5ml of blood from a healthy person was collected in a EDTA vial and the vials are centrifuged for 5minutes at 1000×g.Supernatant (serum) was removed and pellet was washed 3times with PBS (0.2M, pH 7.4).The pellet was suspended in PBS solution to make RBC suspension. 0.5ml of different concentration of extract (0.2mg/ml, 0.4mg/ml, 0.6mg/ml, and 0.8mg/ml in PBS) was dispensed to 1ml of RBC suspension and incubated at room temperature for 20 minutes. After that 0.5ml of 2% H_2O_2 solution made in buffer solution was added. RBC suspension treated with only H_2O_2 solution was taken as negative control. For positive control, RBC suspension with phosphate buffer. The samples were then centrifuged at 1000×g for 10minutes and the absorbance of the supernatant was read at 570nm in ELISA reader.

The percentage of inhibition of hemolysis was calculated by the following formula.

Inhibition of Hemolysis (%)=(Absorbance of test extract/Absorbance of positive control)×100

Determination of anti-proliferative effect

HeLa cell line (ATCC origin) at a passage level of P-61 was obtained from Indian Institute of Technology, Guwahati, India. The cells were maintained in Dulbecco minimal Eagle's medium (DMEM) supplemented with 100 ml/L fetal bovine serum (FBS), 2mM/L glutamine, 1.5 g/L sodium bicarbonate, 1.0mM/L nonessential amino acids, 1.0mmol/L sodium pyruvate and incubated at 37 ºC in a humidified atmosphere containing 5% CO_2. HeLa cell were seeded at the concentration 10^5 cell in 96 well plates and incubated overnight at 37^0C with 5% CO_2 for 24 hours before addition of plant extracts. The cells were treated with 50 µl of each plant extract (two-fold serial dilution between 8mg/ml to 0.625mg/ml in serum free media) and were incubated at 37^0C for 48 hours. The cells were also visually examined for morphological changes at different time intervals. MTT reagent (20µl) was added at the concentration of 5 mg/ml and incubated for 4 hours. The supernatants were removed and DMSO (100µl/well) was added and kept for 15minutes incubation at 37°C. The optical density (OD) was measured at 570 nm with an ELISA reader (Lab systems Multiskan Plus, Thermo Fisher Scientific, USA).Curcumin was used as standard. A HeLa cell without treating with plant extracts was used as

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blank/control. The growth inhibition rate was calculated using the following equation. (Shiwoto et al., 2017).[11]

Growth inhibition rate= (Mean optical density of controls- Mean optical density of treatment)/ (Mean optical density of controls) × 100

IC_{50} value was calculated by standard graph.

RESULTS AND DISCUSSION

Phytochemical analysis

Table 1: Qualitative phytochemical analysis of different solvent extracts of *Vitex negundo* leaf

Phytochemicals	Extracts			
	Hexane	Ethyl acetate	Ethanol	Petroleum ether
Alkaloid	+	+	-	+
Carbohydrate	+	+	+	-
Flavonoid	+	+	-	+
Glycosides	-	+	+	+
Phenol	-	+	+	+
Protein	+	-	+	+
Saponin	-	+	-	-
Steroid	+	+	+	+
Tannin	+	+	+	-
Terpenoid	+	+	+	+

DPPH assay

DPPH scavenging activity was carried out with all four extracts of *Vitex negundo*, i.e. Hexane extract, Ethyl acetate extract, Ethanol extract and Petroleum ether extract. The scavenging activity was increased as the extract concentration was increased. All the extracts showed good antioxidant activity but Hexane and Ethyl acetate extract have more antioxidant activity followed by Ethanol and Petroleum ether extract (Fig.1). From the IC_{50} value calculation thepolar ethyl acetate extract is found to have lowest IC_{50} value 328.470µg/ml followed by ethanol extract (413.471µg/ml), hexane extract 431.271 µg/ml**)** and petroleum ether extract (541.70 µg/ml**)** respectively. Nyeem et al (2017), [12] have reported that polar fractions of the plant extract have potent antioxidant properties. All the fractions of the plant extract exhibited a potent scavenging activity. Antioxidant properties of *V negundo* has been reported by several workers including Tandon and Gupta [13], Kumar et al [14], Kulkarni et al [15], Devi et al [16] have reported antioxidant properties of *Vitex negundo*.

Antimicrobial Activity

Antimicrobial activity of *Vitex negundo* leaf extracts with different solvents against *Staphylococcus aureus* is shown in the Table 4. All the extracts showed effective results but the non-polar hexane extract has shown highest zone of inhibition of 11mm against *Staphylococcus aureus*. Ethanol extract showed 8mm zone of inhibition. While ethyl acetate and Petroleum ether extract showed less zone of inhibition, i.e.; 5mm and 4mm respectively. Deograde et al (2016), [17] reported *Vitex negundo* possesses significant antimicrobial activity against *S. auresus*. Devi et al (2008), [18] reported antibacterial activity of fresh and aqueous extracts of leaves in various dilutions of water, chloroform and methanolic leaf extracts of *Vitex negundo* against *E Coli*, *S aureus* and *K pneumonia.*

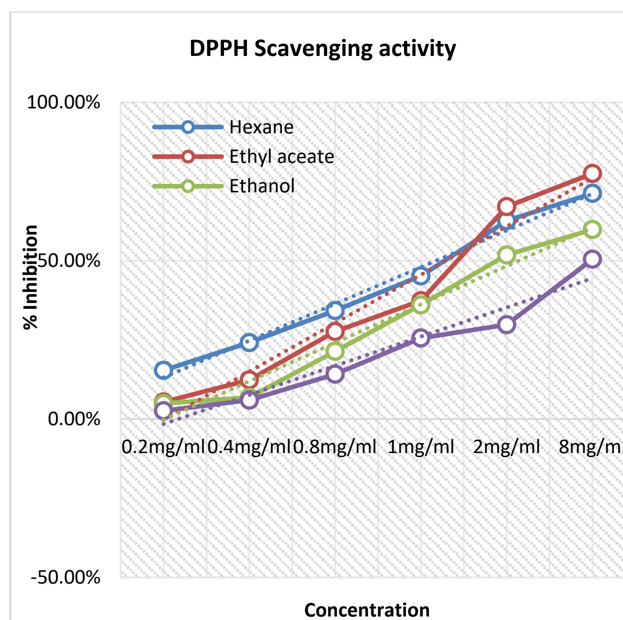

Figure 1: Graphical representation of DPPH scavenging assay

Anti-hemolytic activity

There are several factors responsible for hemolysis such as oxidative drugs, excess transmission of metals, radiation, etc. Our experiment revealed the anti-hemolytic effect of plant extract using hydrogen peroxide as control (haemolytic agent).The obtained results for % of inhibition of hemolysis are shown in the Table 5 and those results are represented graphically in the Fig 2.

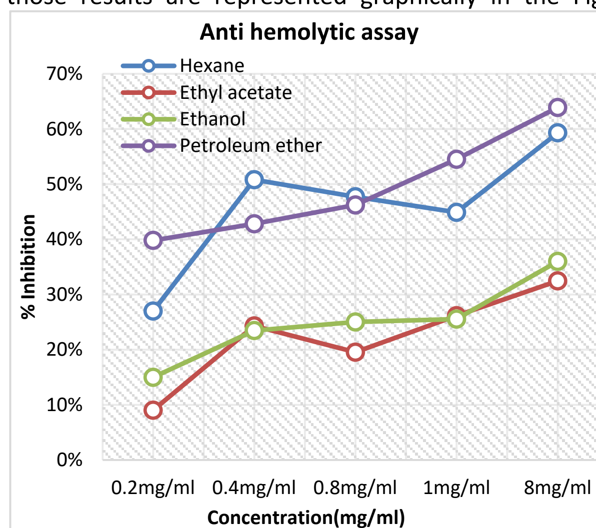

Figure 2: Anti hemolytic assay for *Vitex negundo*

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Anti-proliferative assay

The examination of morphological changes in the cells showed distinct variation in cells treated with the hexane, ethanol, ethyl acetate and petroleum ether extract at 48 hour (Fig. 3a-j). The result of the morphological changes was further confirmed by MTT assay. The percentage of inhibition after MTT assay is given in the Table 5 and represented graphically in Fig.12. Along with IC_{50} value is also calculated and given in the Table 2.

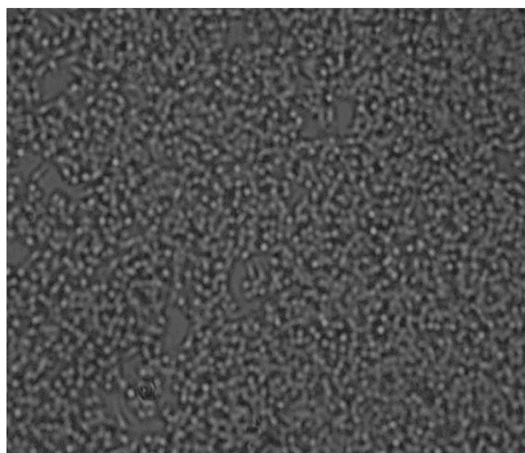

Figure 3(a): Control (without treatment)

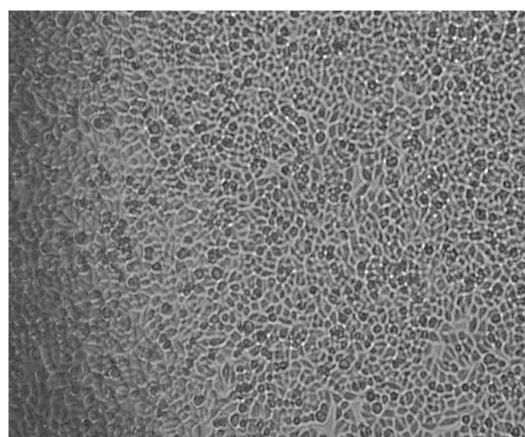

Figure 3(b): Treatment with hexane at 0hr

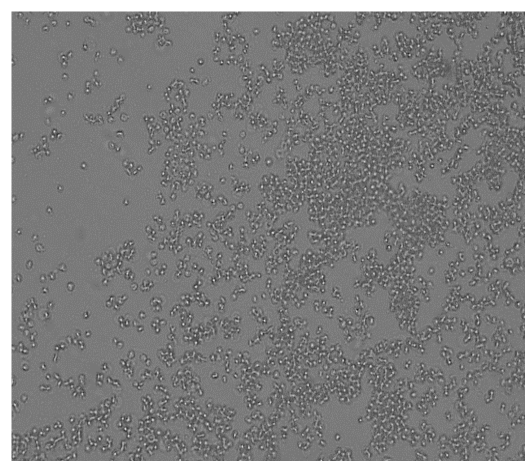

Figure 3(c): Treatment with hexane at 48hr

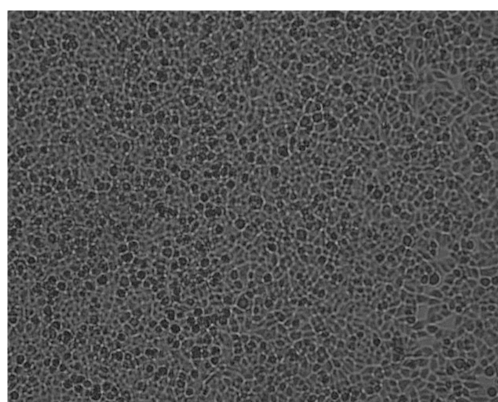

Figure 3(d): Treatment with ethyl acetate at 0hr

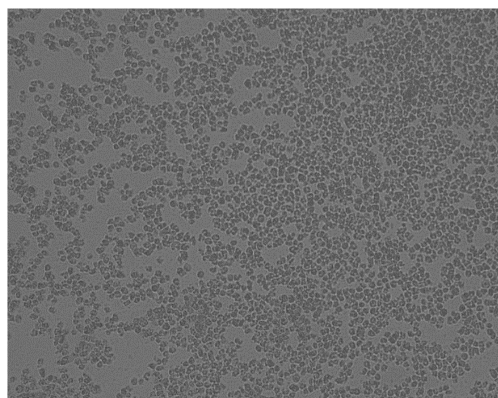

Figure 3(e): Treatment with ethyl acetate at 48hr

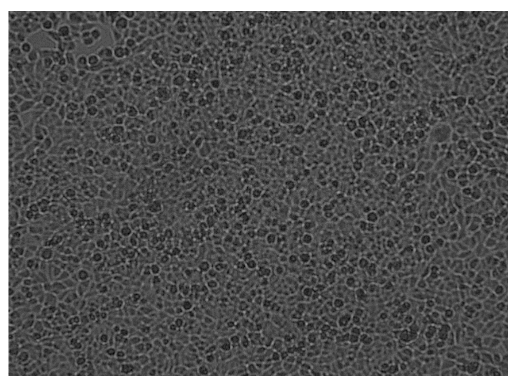

Figure 3(f): Treatment with ethanol at 0hr

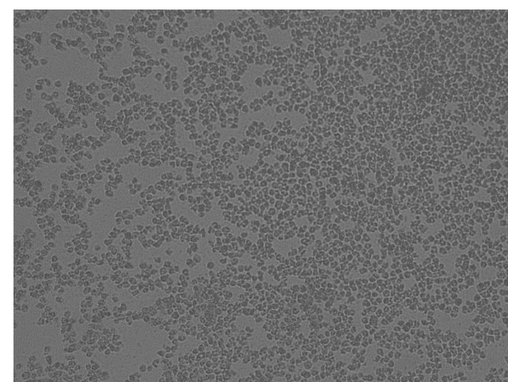

Figure 3(g): Treatment with ethanol at 48hr

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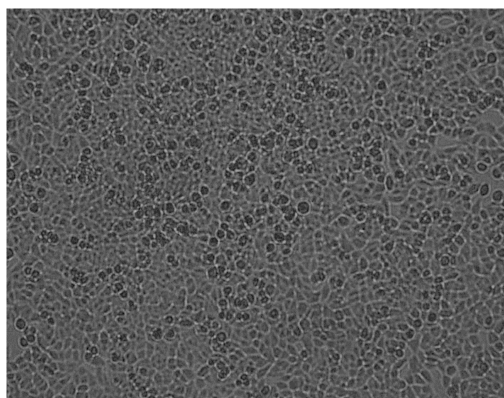

Figure 3(h): Treatment with petroleum ether at 0hr

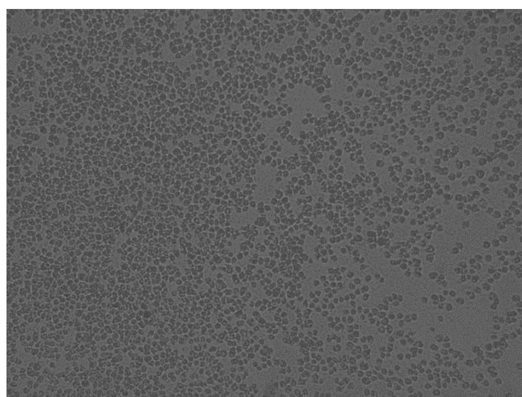

Figure 3(i): Treatment with ethyl acetate at 48hr

Table 2: IC_{50} value for treatment

Treatment	C_{50}
Vitex negundo Hexane extract (VNHE	**1.16**
Vitex negundo ethyl acetate extract (VNEE)	**<0.312**
Vitex negundo ethanol extract (VnolE)	**0.866**
Vitex negundo petroleum ether extract (VNPEE)	**0.247**

The present study showed that various extracts of *Vitex negundo* inhibitedproliferation of HeLa cells. On comparing the various extracts, it was found that IC_{50} of the ethyl acetate extract was the lowest (< 0.312 mg/ml), followed by petroleum ether (0.247 mg/ml), ethanol (0.866 mg/ml) and hexane extract (1.16 mg/ml). Hence various extract of *Vitex negundo* possess potency to inhibit the HeLa cells.

Kadir et al, (2013) [19] reported PASS-predicted (software) *V negundo* activity which highlighted antioxidant and antiproliferative properties on human hepatoma cells.

Badgujar et al [20] reported the anti-lung cancer activity by inhibiting the growth and apoptosis of A-549 cells.

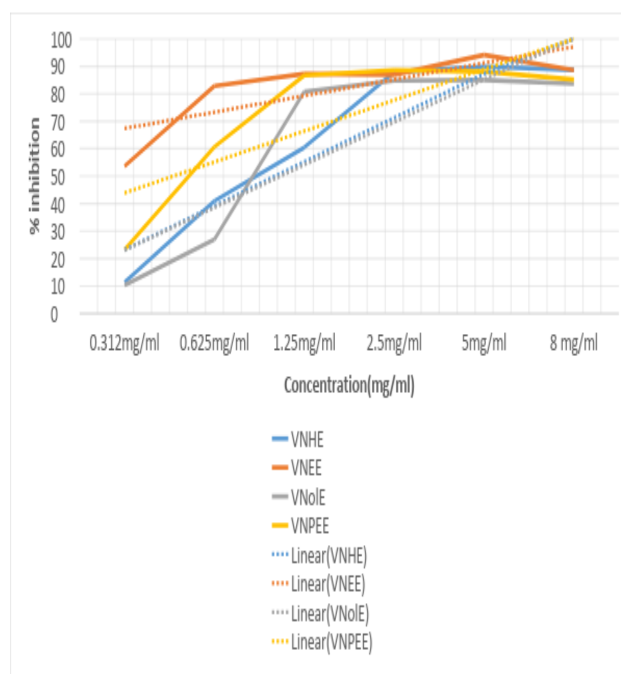

Figure 12: Percent inhibition of HeLa cells treated with the various extracts of *Vitex negundo*

*VNHE = *Vitex negundo* hexane extract.

*VNEE = *Vitex negundo* ethyl acetate extract.

*VNolE = *Vitex negundo* methanol extract.

*VNPEE = *Vitex negundo* petroleum ether extract.

CONCLUSION

The results of current experimental study provide that leaves of *Vitex negundo* used in the traditional medicine possess anti-hemolytic, antioxidant, antimicrobial property. The study also provides the evidences that the leaf is having a good antiproliferative activity.

In the present study, the phytochemical screening of different extracts of *Vitex negundo* showed positive results for alkaloid, carbohydrate, flavonoids, glycosides, phenol, protein, steroid, tannin. The ethyl acetate extract found to have saponin only (Table-1). These secondary metabolites are found to have curative activity against several human ailments and this could explain the use of traditional medicinal plant in various disease.The leaf found to have a good antioxidant activity. The ethyl acetate extract showed highest scavenging activity (IC_{50}; 328.47µg/ml) followed by ethanol extract, hexane extract and petroleum ether extract respectively.The antimicrobial activity of different extracts of *Vitex negundo* leaf is shown in the Table 4. The hexane extract showed the highest zone inhibition. This could be due to the presence of phytochemicals alkaloid, phenol, steroid, terpenoid, reducing sugars from phytochemical analysis.The anti-hemolytic activity of different extracts of *Vitex negundo* shown that petroleum ether extract has highest activity, followed by hexane extract, ethyl acetate and ethanol. This results proved that *Vitex negundo* has preventive activity to oxidative damage caused to

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erythrocyte membrane.The four extracts of *Vitex negundo* was tested for antiproliferative activity in HeLa cell line and the results showed that the ethyl acetate extract have lowest IC_{50} followed by petroleum ether, ethanol and hexane extract. Presence of alkaloid, flavonoids, saponin, glycosides or other secondary metabolites in natural plants including phenolic compounds have a crucial role in anticancer property either by inhibiting cancer cell activating proteins, enzymes or in signalling pathway.[21]

The phytochemical test of medicinal plant found to have many bioactive compounds which are potent source of anticancer property as well as several other ailments. The free radical scavenging activity of is one of the mechanisms of a plant to exhibit antioxidant activity. The polarity of compounds also plays a key role in their bioactivity. The exact compounds responsible for this antioxidant activity, antimicrobial, antiproliferative activity can be known by purifying the active compounds using various analytical techniques viz. TLC, column chromatography, HPLC, FTIR etc. And In vivo and clinical trials are needed to be performed for successful development and commercialization of drug with lesser side effects.

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