IN VITRO AND IN SILICO ANTICANCER ACTIVITY OF NEGUNDOSIDE ISOLATED FROM LEAVES OF VITEX NEGUNDO LINN

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ABSTRACT

Vitex negundo Linn. [Verbenaceae], commonly known as Five-leaved Chaste tree or Monk’s Pepper (Hindi — Sambhali, Nirgundi) is used as medicine fairly throughout the greater part of India. The aim of present study is to isolate negundoside and evaluate anticancer activity by in vitro and in silico method. Negundoside was isolated by column chromatography from ethyl acetate fractionation of methanol extract of leaves of V. negundo. Negundoside was characterized by UV, IR, 1H-NMR, 13C-NMR and Mass spectrometry. Standardization of negundoside was done by HPTLC fingerprinting. In vitro anticancer activity was done using HeLa cell lines by MTT assay at different concentrations ranging from 20-100 µg/ml and in silico docking studies using enzyme EGFR tyrosine kinase. Fingerprinting of isolated negundoside were done by HPTLC method. The IC50 value was found to be 62.69 µg/ml in vitro anticancer activity in HeLa Cell lines. Negundoside was subjected to molecular docking studies for the inhibition of the enzyme EGFR tyrosine kinase, which is one of the targets for inhibition of cancer cells. It has shown -7.32 kJ mol⁻¹ binding and -11.32 kJ mol⁻¹ docking energy with five hydrogen bonds. Negundoside has shown to possess anticancer activity both in vitro and in silico studies.

KEYWORDS: In vitro anticancer activity; In silico docking studies; Isolation; Negundoside; Vitex negundo.

INTRODUCTION

Cancer is one of the highest impacting diseases worldwide with significant morbidity and mortality rates. The current known therapies are based on radio and chemotherapies and although in many cases, the patients have their health re-established, the treatment is very painful since their immunological system is severely compromised, because these procedures are not cells selective.[1] Substantial advances have been made in understanding the key roles of receptor tyrosine kinase (RTK) in the signalling pathways that govern fundamental cellular processes, such as proliferation, migration, metabolism, differentiation and survival. In the normal cells RTK activity is tightly controlled. When they are mutated or structurally altered, they become potent oncoproteins which leads to abnormal activation of RTKs in transformed cells has been shown to be causally involved in the development and progression of many human cancers.[2] The cost of treatment is very high and with lot of side effects. In order to find new natural sources that are biologically active substances from plants have acquired immense attention. A number of studies have been carried out on various plants, vegetables and fruits because they are rich sources of phytoconstituents which prevent free radical damage thereby reducing risk of chronic diseases viz., cancer, cardiovascular diseases etc. This beneficial role of plants has led to increase in the search for newer plant based sources for the treatment of diseases like cancer. One such plant is Vitex negundo Linn.

Vitex negundo Linn., commonly known as Five-leaved Chaste tree or Monk’s Pepper (Hindi — Sambhali, Nirgundi) is used as medicine fairly throughout the greater part of India and found mostly at warmer zones and ascending to an altitude of 1500m in outer Western Himalayas.[3,4] In traditional system of medicine, the plant is used as bitter, acrid, astringent, cephalic, stomachic, antiseptic, alterant, thermogenic, deputative, rejuvenating, ophthalmic, anti-gonorrhoeic, antiinflammatory, antipyretic and useful in bronchitis, asthma and enlargement of spleen. Leaves are aromatic, bitter, acrid, astringent, anodyne, antiinflammatory, antipyretic or febrifuge, tranquillizer, bronchial smooth muscle relaxant, anti-arthritic, antihelminitic and vermifuge.[5] It is reported to possess analgesic, anti-inflammatory, antioxidant, anticonvulsant, antimalarial, anti-filarial,
hepatoprotective, antibacterial and antifertility activities.\(^{(5)}\)

The aim of the present study is to isolate negundoside from dried leaves of *Vitex negundo* and perform *in vitro* MTT assay and *in silico* activity to prove its anticancer activity.

**MATERIAL AND METHODS**

**Plant Material**

The dried leaves of *Vitex negundo* (Verbenaceae) were collected, identified and authenticated by Dr Shiddamallayya N (SMUP/ADRI/BNG/345) at National Ayurveda Dietetics Research Institute, Bengaluru, Karnataka. A voucher specimen was deposited in the Herbarium of Department of Pharmacognosy, The Oxford College of Pharmacy, Bangalore. The leaves were dried under normal environmental conditions. The dried leaves were powdered and stored in a closed container for further use.

**Drugs and Chemicals**

DMEM medium (GIBCO), heat-inactivated fetal bovine serum (FBS), trypsin, ethylene-diaminetetraacetic acid (EDTA), PBS and MTT were purchased from Hi media and Sigma Chemicals. All chemicals and reagents used in this study were at least of analytical grade.

**Extraction and Isolation Procedure**

The dried leaves of *V. negundo* (350 g) was refluxed with methanol (1.5 l) for 1 h. Filter and repeat the process of reflux by adding methanol(1.5 l) to the marc. Distill the combined methanol extract to remove the solvent and dry the concentrated residue under vacuum to get a thick green paste(35 g). Dissolve the extractive in 1 litre of water and carry out partitioning (liquid-liquid) with ethyl acetate and water (1:1) three times. Concentrate the ethyl acetate layer under vacuum. Ethyl acetate fraction was subjected to column chromatography over silica gel (60-120 mesh) using n-hexane with increasing percentage of chloroform and methanol. The fractions eluted with 20-30 % methanol in chloroform were collected and concentrated under vacuum to get a residue (1 g) which was further chromatographed on a polystyrene gel (Diona HP-20) (60 g) column. Elute the column by gradient elution using water with increasing percentage of acetone. The fractions eluted with 10 to 15 % acetone in water were combined and concentrate under vacuum to get enriched fraction of negundoside (0.2g). Crystallize the fraction in methanol to get negundoside (100mg).

**Charcterization of Negundoside**

The structure of Negundoside was characterized by UV, IR, NMR, Mass spectrum. HPTLC fingerprinting was done to confirm the presence and purity of negundoside.

**Chromatographic Finger Printing of the Dried Leaf of V. negundo using Negundoside**

**TLC Identity Test**

Weigh 2 g of coarsely powdered drug and transfer to a 250-ml conical flask. Extract with 50 ml of methanol by refluxing for about 20 min and filter. Repeat the process 4 to 5 times till the raw material is completely exhausted or till the extract is colourless. Combine the extracts and concentrate to a volume of about 100 ml, cool to room temperature. Use the solution for TLC profiling. Standard solution was prepared by dissolving 5 mg of negundoside in 10 ml of methanol. Solvent system used was Ethyl acetate : Methanol : Water : Acetic acid (7:1.2:0.7:0.3). Apply 20 μl of test solution and 5 μl of standard solution separately on a precoated silica gel 60F\(_{254}\)TLC plate (E. Merck) of uniform thickness (0.2 mm). Develop the plate in the solvent system till the solvent rises to a distance of 8 cm. Visualization was done after spraying with anisaldehyde-sulphuric acid reagent and followed by heating at 105°C for 5 to 10 min. The \(R\_f\) value and colour of the resolved bands were noted.

**In vitro anticancer activity using HeLA cell lines by MTT assay**

**Cell culture**

HeLa cell line was maintained in DMEM medium (GIBCO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% antibiotic solution (penicillin 100 U/ml and streptomycin 100 μg/ml) at 37°C in a humidified atmosphere of 95% air/5% CO\(_2\). The medium was changed every second day, and cells were subcultured when confluency reach to 95% by 0.25% trypsin containing 0.02% ethylene-diaminetetraacetic acid (EDTA) in PBS for 3 min at 37°C.

**MTT Assay**

The MTT assay was carried out as described previously to measure cell viability\(^{6}\). Ten thousand cells in 100μL of DMEM media were seeded in the wells of a 96-well plate. After 24 h, existing media was removed and 100 μL of various concentrations of compound was added and incubated for 48 h at 37°C in a CO\(_2\) incubator. Control cells were supplemented with 0.05% DMSO vehicle. At the 48h hour of incubation, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide- supplied from Sigma, 10μL of 5 mg/mL) was added to the plate. The contents of the plate were pipetted out carefully, the formazan crystals formed were dissolved in 100 μL of DMSO, and the absorbance was measured at 550 nm in a microplate reader (Tecan, infinite F200 Pro). Experiments were performed in triplicate, and the results were expressed as mean of percentage inhibition. A graph of the concentration versus percentage growth inhibition was plotted, and the concentration at which 50% cell death occurred was considered as the IC\(_{50}\) value. Before adding MTT, bright field images (Olympus 1X81, cellSens...
In silico activity: Molecular Docking studies

The three dimensional structure of target protein EGFR tyrosine kinase (PDB ID: 2J5F) was downloaded from PDB (www.rcsb.org/pdb) structural database. This file was then opened in SPDB viewer edited by removing the heteroatoms, adding C terminal oxygen. The active pockets on target protein molecule were found out using CASTp server. The ligands were drawn using ChemDraw Ultra 6.0 and assigned with proper 2D orientation (ChemOffice package). 3D coordinates were prepared using PRODRG server. Autodock V3.0 was used to perform Automated Molecular Docking in AMD Athlon (TM)2x2 215 at 2.70 GHz, with 1.75 GB of RAM. AutoDock 3.0 was compiled and run under Microsoft Windows XP service pack 3. For docking, grid map is required in AutoDock, the size of the grid box was set at 102, 126 and 118 Å (R, G, and B), and grid center -58.865, -8.115, -24.556 for x, y, and z-coordinates. All torsions were allowed to rotate during docking. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization, using default parameters.

RESULTS

The Characterization of the Negundoside

Physical data: Negundoside is an amorphous white powder, mp 156°, lit mp 155-159°.

\[ [\alpha]_2^D -128.24°, \text{lit}^a [\alpha] -119.2°. \] Soluble in chloroform and methanol; insoluble in water.

Molecular formula: C_{23}H_{28}O_{12}. Molecular weight: 496; Elemental composition: C 55.64%; H 5.68%; O 38.67%.

Spectral Data

UV-VIS spectrum shows absorption at 254 nm (ε = 1.8 x 10^3). The UV-VIS spectrum indicates the presence of chromophoric system in the molecule. The IR spectrum shows a broad band at 3309 cm^{-1} due to presence of hydroxyl group. A band at 1698 cm^{-1} indicates the carbonyl group in the molecule. The band at 1514 cm^{-1} is due to aromaticity in the molecule and band at 1273 cm^{-1} can be assigned to a stretching at C-O-C. 1H-NMR spectrum (DMSO-d_{6}, 300 MHz): The signal at δ 1.13 (3H, s, C_{4}H), 1.29 (1H, m, C_{6}H), 1.55 (2H, m, C_{6}H), 1.99 (1H, dd, C_{3}H, J=9.6, 3.3 Hz), 2.04 (1H, m, C_{6}H), 2.73 (1H, m, C_{6}H), 3.52 (1H, m, C_{6}H), 3.76 (1H, dd, C_{2}H, J=11.1, 1.5 Hz), 4.74 (1H, t, C_{2}H, J=9.0 Hz), 4.85 (1H, d, C_{1}H, J=8.1 Hz), 5.30 (1H, d, C_{1}H, J=3.0 Hz), 6.81 (2H, d, C_{1}H, C_{2}H, J=8.7 Hz), 7.04 (1H, s, C_{3}H), 7.73 (2H, d, C_{2}H, C_{6}H, J=8.4 Hz).

The 1H-NMR spectrum shows singlet at δ 1.13 due to methyl protons at C_{6}. Multiplets at δ 1.29 and 1.55 correspond to protons at C_{4} and C_{5}, respectively. Proton at C_{7a} appears as doublet of doublet at δ 1.99. Protons at C_{5b}, C_{4a}, and C_{6a} were observed as multiplets at 62.04, 2.73 and 3.52, respectively. Protons at C_{2b} and C_{2} are observed as doublet of doublet and triplet at δ 3.76 and 4.74, respectively. Protons at C_{1} and C_{1} appear as doublets at δ 68.85 and 5.30, respectively. Doublet at δ 68.1 corresponds to protons at C_{1} and C_{1}. Olefinic proton at C_{3} is observed as singlet at δ 6.04. The protons at C_{2} and C_{0} appear as doublet at δ 67.73.

13C NMR spectrum (CD_{2}OD - 300 MHz): the signals at δ 22.97, 28.79, 29.82, 39.87, 51.03, 61.38, 70.41, 73.57, 74.69, 77.12, 78.48, 93.71, 96.51, 112.40, 114.76, 120.86, 131.49, 149.76, 161.90, 165.93, were prepared using PRODRG server.

The HPTLC fingerprinting confirmed the presence of negundoside [Table No.2, 3 and Figure No. 23]. A grey and reddish-pink coloured bands are observed at (R_{f} 0.57) corresponding to negundoside is visible in both the test and standard solution tracks under UV at 254 nm and after derivatization.

In vitro anticancer activity on HeLa Cell Lines

The MTT values obtained demonstrated that Negundoside has good cytotoxic effect. The IC_{50} value was found to be 62.69 µg/ml. Microscopy images representing the cell death caused by the compounds are as seen in Figure No.4. It is very clear that it is very good cytotoxic agent.

In silico molecular docking studies

The tyrosine kinase receptors have multidomain extracellular Ligands for specific Ligand, a signal pass transmembrane hydrophobic helix and tyrosine kinase domain. The receptor tyrosine kinases are not only cell surfaces transmembrane receptors, but are also enzymes having kinase activity. In cancer, angiogenesis is an important step in which new capillaries develop and develop for supplying a vasculature to provide nutrient and removing waste material. So tyrosine kinase inhibitor as an anti-angiogenic agent is new cancer 4 therapy. Developing natural drugs and produgs as inhibitor is today’s trend. Low molecular weight substances, which inhibit tyrosine kinase phosphorylation block signaling pathway, initiated in the extracellular part of receptor[10]. Since, the type I receptor tyrosine kinase is a major regulator of several distinct and diverse cellular pathways we have evaluated it as a target.

Negundoside was taken and docked to get the best conformer. Results were compared for the binding energy, docking energy and number of hydrogen bonds formed. According to the docking results (Table No.4), 1H NMR, it has binding energy of: -7.32, with five hydrogen bonds formed.
Molecular docking with EGFR tyrosine kinase domain revealed that, our compound have inhibitory capability and thereby exhibiting interactions with one or the other amino acids in the active pockets as shown in Figure No.5. The topology of the active site of EGFR tyrosine kinase was similar in all synthesized molecules, which is lined by interacting amino acids as predicted from the ligplot (Figure No.5). In *in vitro* studies the molecule emerged as more active against the cell line used.

<table>
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<tr>
<th>Table No.1: $^{13}$C NMR Assignments of negundoside.</th>
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<p>| Table No.2: HPTLC Details of Test Solution of <em>Vitex negundo</em> Dried Leaf at 254 nm. |
|---|---|---|</p>
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<th>R$_f$ value</th>
<th>Colour of the band</th>
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<tr>
<td>0.52</td>
<td>Grey</td>
</tr>
<tr>
<td>0.57</td>
<td>Grey (Negundoside)</td>
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<tr>
<td>0.94</td>
<td>Grey</td>
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<p>| Table No.3: HPTLC Details of Test Solution of <em>Vitex negundo</em> dried Leaf after Derivatization. |
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<th>R$_f$ value</th>
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<tr>
<td>0.37</td>
<td>Light pink</td>
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<tr>
<td>0.52</td>
<td>Reddish-brown</td>
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<tr>
<td>0.57</td>
<td>Reddish-pink (Negundoside)</td>
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<p>| Table No.4: Molecular docking results of Negundoside with EGFR tyrosine kinase. |
|---|---|---|---|---|---|</p>
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<tr>
<th>Molecule</th>
<th>Binding energy</th>
<th>Docking energy</th>
<th>Inhibitory constant</th>
<th>Intermol energy</th>
<th>H-bonds</th>
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<tr>
<td>NGD</td>
<td>-7.32</td>
<td>-11.32</td>
<td>4.3e-006</td>
<td>-9.81</td>
<td>5</td>
<td>NGD::DRG::OAG::TK::A:ARG831::HH12 NGD::DRG::HBD::TK::A:ASP770::OD2 NGD::DRG::OB1::TK::A:ARG776::HE NGD::DRG::H22::TK::A:SER768::O NGD::DRG::HAR::TK::A:VAL769::O</td>
</tr>
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</table>
**CONCLUSION**

Negundoside has shown to possess anticancer activity both in vitro and in silico studies. The IC$_{50}$ value was found to be 62.69 µg/ml and in silico studies, it has more number of hydrogen bonds with minimum binding and docking energy and may be considered as inhibitor of EGFR tyrosine kinase. More experiments are required to understand the exact mechanism by which the cells are affected. It is important to correlate the structure of these compounds with their biological effect, which will be valuable to propose new lead compounds with better cytotoxic potential.

**ACKNOWLEDGEMENT**

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**Ethical Issues**

There is none to be applied.

**Conflict of Interest**

None to be declared.

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