DEVELOPMENT OF ISOLATION METHOD, CHARACTERIZATION, PHYTOCHEMICAL SCREENING, AND COMPUTATIONAL ANALYSIS OF *Vitex negundo* FOR ANTI-BREAST CANCER ACTIVITY USING LC-MS ANALYSIS, MOLECULAR DOCKING AND DFT STUDIES

B. S. Lakshmi¹, H. G. Anilkumar¹, Jayanna K. Bidarur² and B. S. Ravindranath³,●

¹Department of Science and Humanities, PES University, BSK III stage, Bengaluru 560085.
²Department of Chemistry, B. N. M. Institute of Technology, Bengaluru- 560070
³Department of Biotechnology, Manipal Institute of Technology, Manipal, Manipal Academy of Higher Education, Manipal-576104, Karnataka, India.

●Corresponding Author: ravindranath.bs@manipal.edu

ABSTRACT

Phytotherapeutic chemicals help to control, inhibit, or alleviate symptoms of diseased cells. The purpose of this research is to use LC-MS analysis to assess the chemotherapeutic efficacy of plant-derived compounds in inhibiting tumorigenesis. The current study is to evaluate the functionally bioactive phytocompounds of *Vitex negundo* for anticancer activity and execute an *in silico* molecular docking assessment of *V. negundo*. HPLC, LC-MS analysis, and DFT studies were used to evaluate bioactive phytocompounds, Casticin, and Luteolin. Further in-silico molecular docking studies of Casticin and Luteolin were performed against breast cancer targets to study the anticancer activities. Subsequently, these phytochemicals were tested for therapeutic activity through the ADMET evaluation using SwissADME and admetSAR prediction tools. ADMET prediction results revealed that Luteolin and Casticin might have the maximum anticancer activity against the various drug target proteins among all the phytochemicals identified in the LC-MS analysis and further, the significant compounds characterized by LC-MS analysis were subjected to molecular docking analysis to orient the ligand-protein interaction and their binding energy. Further, the chemical stability and chemical reactivity of compounds with optimized structures were ascertained with the DFT study. These results suggested the effectiveness of the pipeline adapted to extract and isolate the phytochemicals with pharmacological significance and targeted activity.

Keywords HPLC, LC-MS Analysis, DFT Studies, Molecular Docking, ADMET Evaluation.

INTRODUCTION

Cancer is the unbounded growth of aberrant cells in the human body, it causes major deaths all over the globe, in recent decades almost 9.6 million deaths every year have been due to cancer. Breast cancer is highly prevalent in women causes millions of deaths, and is the most common type of malignant neoplasm caused in women in recent years.¹ Every year, around two million new cases are investigated with breast cancer in patients, also >0.6 million women die due to breast cancer across the world. At an early stage diagnosis and treatment can reduce the rate of mortality in patients suffering from breast cancer.² This carcinogenesis is mainly due to the damage of genetic cells, which results in the abnormal division of cells and mutations.³ Breast cancer is a disease caused due to hormonal changes, in which the glandular tissues present in the breasts undergo hormonal changes because of high sensitivity, causing abnormal mutations and uncontrolled growth of cells.⁴ So, breast cancer is also known as hormonal cancer. These malignant cells can penetrate, nomadize, and spread all over the body. Phytochemicals, the bioactive chemical compounds, act as strong anti-cancer agents with greater benefits than synthetic compounds, with fewer side effects and toxicity. Easily available for extraction, these natural compounds are present in different portions of plants from roots to fruits.⁵,⁶ Classification of phytochemicals into different classes is based on their chemical structure and characteristics such as phenolics, terpenoids, lipids, alkaloids, carbohydrates,
and nitrogen-containing compounds. These secondary metabolites show various mechanisms to slow down or stop cancer growth by suppressing the proliferation of cells, and progression by reducing oxidative stress, stopping the angiogenic process, and creating the programmed cell death and detention of the cell cycle.

EXPERIMENTAL

Collection of Vitex negundo Plants and Extraction of Plant Sample
V. negundo plants were collected from the Hilly regions of Western Ghats in the Shimoga district of Karnataka during the winter season, from December to February. Authentication was done by a Botanist at Kuvempu University. The fresh leaves were collected from the V. negundo plant, washed and cleaned with distilled water, and dried in a shady area (around 30°C for 20 days). The dried plant leaves were ground and extracted with Absolute ethanol of HPLC grade using the Soxhlet apparatus. The plant material was extracted continuously for 72 hours. The sample extracts were collected at regular intervals of time. The crude ethanol extract was sieved using a 0.2 µm membrane filter and then concentrated by evaporation of the solvent under reduced pressure/vacuum.

HPLC Analysis (High-Performance Liquid Chromatography)
The plant extract was analyzed using the Analytical method, the HPLC system of Shimadzu. The Shimadzu HPLC system consists of pump LC-10ADVp, Detector: SPD-10AVp, Autosampler: SIL HTA, Degasser: DGU-12A, Column Oven: CTO-10AVp. The plant sample of 100mg was taken in a conical flask along with the inclusion of 50ml of Ethanol. The mixture of plant sample and Ethanol was ultrasonicated in the water bath for 5 minutes and sieved using a 0.45µm membrane filter. HPLC was used for the Chromatographic separation of phytochemicals from plant samples. The detector at 25°C. HPLC analysis was performed using a C18 column (50 X 4.6mm, 3.5µ particle size). The elusion was carried out with Acetonitrile: 0.1% of Formic acid in Water used as a mobile phase and the flow rate was 1.2 ml/minute. The volume injected was 20µL. The observation was detected at two different wavelengths of 225 and 254 nm respectively using an ultra-violet (UV) detector.

LC-MS Studies (Liquid Chromatography-Mass Spectrometry)
LC-MS study was executed on a WATERS XBridge, 50 X 4.6mm 3.5µ, the separation was done using a C18 column. Gradient elution was performed using Solvent A: 0.1% Formic acid in distilled water and Solvent B: Acetonitrile MS grade at a constant rate of flow is 1.2ml/min. Diluent: Methanol, Acetonitrile, Water, 0.1% Formic acid, TFA. The intensity of Temperature at the column was kept constant at 30°C till the flow time of 15 minutes. The volume injected was 20µL. The mass range m/z 50 – m/z 1000 was used to record the electrospray mass spectra data on both the positive and negative ionization modes. MS spectra of WATERS, Model: Micromass Quattro micro, Software: MassLynx. Version: V4.1SCN805. MS condition: Triple Quadrupole (QqQ) MSMS. In both positive and negative ionization modes, MS spectra were obtained using the electrospray ionization (ESI) source. The capillary voltage of 3.45(kV) was utilized in both ionization modes with a desolvation of 800(L/hr.) for collision gas flow. The Cone was 50(L/hr.) and source temperature of 110°C for both, and the temperature of desolvation was 350°C. Further, the assessment was performed using an external instrument.

Screening of Ligands Based on Activity
Prioritizing the ligands based on the specific activity is an essential step in drug design and development. The chemical-gene and gene-disease interactions database like the Comparative Toxicogenomics Database (CTD) provides robust manually curated information on the types of interaction along with the disease types. In the current study, all the phytochemicals identified from V. negundo through LC-MS were subjected to CTD to analyze the plant metabolites for their association with a specific cancer activity.

In silico Molecular Docking Simulation
In-silico Molecular docking simulation was executed to know the best attainable orientation of the prepared ligands with the protein targets. In the current study, we have imported the proteins from the PDB (PubChem database) of greater resolution. The docking was performed with the phytocompounds as ligands selected from the LC-MS data. The protein targets of breast cancer were evaluated based on the binding affinities. The value of binding affinities is calculated as binding energies. The acceptable PDB
formats and the techniques required for ligand preparation are done by using different tools such as Protein Data Bank, AutoDock vina software, MGL Tools, PyRx, PyMol, Open Babel, and Discovery Studio software.¹⁹ In the present study, HER2 (3RCD) and EGFR (3W2O) protein targets of breast cancer were docked with the ligands selected from LC-MS data. The binding energies were calculated to know the best possible orientation and the best-predicted poses of the ligand-receptor complex see Table-1. It is analyzed that the lower binding energy ligands form the favorable ligand-receptor complex.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Phytochemicals</th>
<th>Binding affinity (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HER2(3RCD)</td>
<td>EGFR(3W2O)</td>
</tr>
<tr>
<td>1.</td>
<td>Artemetin</td>
<td>-8.2</td>
</tr>
<tr>
<td>2.</td>
<td>Terpinen-4-ol</td>
<td>-5.9</td>
</tr>
<tr>
<td>3.</td>
<td>d-guaiene</td>
<td>-7.7</td>
</tr>
<tr>
<td>4.</td>
<td>Nerolidol</td>
<td>-6.4</td>
</tr>
<tr>
<td>5.</td>
<td>Luteolin</td>
<td>-8.5</td>
</tr>
<tr>
<td>6.</td>
<td>α-cedrene</td>
<td>-6.4</td>
</tr>
<tr>
<td>7.</td>
<td>Valencene</td>
<td>-7.0</td>
</tr>
<tr>
<td>8.</td>
<td>Quercetin</td>
<td>-8.2</td>
</tr>
<tr>
<td>9.</td>
<td>Casticin</td>
<td>-8.3</td>
</tr>
<tr>
<td>10.</td>
<td>Sabinene</td>
<td>-5.4</td>
</tr>
<tr>
<td>11.</td>
<td>Gamma-terpinene</td>
<td>-5.7</td>
</tr>
<tr>
<td>12.</td>
<td>Catechin</td>
<td>-8.2</td>
</tr>
<tr>
<td>13.</td>
<td>Epicatechin</td>
<td>-8.2</td>
</tr>
<tr>
<td>14.</td>
<td>Monoolein</td>
<td>-6.0</td>
</tr>
<tr>
<td>15.</td>
<td>a-selinene</td>
<td>-7.2</td>
</tr>
<tr>
<td>16.</td>
<td>12-dien-28-oic acid</td>
<td>-6.5</td>
</tr>
<tr>
<td>17.</td>
<td>Oxy isophthalic acid</td>
<td>-6.5</td>
</tr>
</tbody>
</table>

ADMET Analysis and Drug Likeness
The ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) attributes are very important to analyze during drug discovery. The ADMET properties can be predicted before the drug discovery so that the drug failure can be eliminated and only potential drug candidates can be focused on during the process of drug discovery. The ADMET analysis replaces the external and internal living organism studies and prior identification of ADMET properties helps us in cost-effective drug discovery. The ADMET evaluation gives the toxicity behavior of phytochemicals in the human body. In all the pharmaceutically substantial compounds, the structures were transformed into canonical smiles. These phytochemicals are submitted to admetSAR (http://lmmd.ecust.edu.cn) and SwissADME (http://www.swissadme.ch) to analyze in silico pharmacokinetics. ADMET properties help in analyzing Lipinski's rule.²⁰

RESULTS AND DISCUSSION

Primary Phytochemical Screening
The phytochemical study of dried leaf extracts of the plant, *V. negundo* in ethanol solvent, showed an array of phytochemicals.

Chemical Composition
The chemical composition of ethanolic extracts from *V. negundo* was detected by LC-MS study. The positive and negative ion chromatograms of ethanolic leaf extracts of *V. negundo* in LC-MS analysis are shown in Fig.-1. The Soxhlet extraction was performed using ethanol solvent. Further analyzed by HPLC and LC-MS analysis to acquire 78 essential bioactive phytochemicals. A total of 17 Phytochemicals were obtained using the Ethanolic plant extract.

Molecular Docking Analysis
LC-MS study revealed that the leaf extract of *V. negundo* contained 78 bioactive phytochemicals. The phytochemicals were analyzed for activities against the cancer protein targets. Out of 78 phytochemicals,
the top screened phytoligands from LC-MS analysis and CTD analysis were docked with two anticancer drug targets related protein structures.

Among seventeen phytoligands docked against protein targets, Casticin and Luteolin ligands were acquired after ADME screening. Molecular docking was carried out employing AutoDock Vina to analyze the binding energy scores to find the better docking complex. The binding energies were estimated based on the bonding between the target protein and ligand, which is visualized in the Discovery studio visualizer. Casticin and Luteolin showed greater binding configurations with lesser binding energy values with the breast cancer target proteins.\(^{21,22}\) For Casticin binding energy obtained was (-8.3 kcal/mol) and for Luteolin the binding energy obtained was (-8.5 kcal/mol) (Table-2). According to previous studies, the lesser the binding energy, the stability of the ligand-protein bond is greater. The molecular docking results with target proteins such as HER2 and EGFR confirm that the binding affinity for ligand-protein is greater for HER2 see Fig.-2 & Fig.-3, which is key for anticancer activity.\(^{23}\) In comparison with Gefitinib, the extracted Luteolin and Casticin are natural phytocompounds, and comparatively showed significant docking results almost the same as a commercial anticancer drug.\(^{24}\) The binding affinities of screened phytochemicals as phytoligands with the protein targets are described in the Table-2.

### Table-2: The Binding Affinity of Selected Phytocompounds Against Breast Cancer Target Proteins

<table>
<thead>
<tr>
<th>Ligands</th>
<th>HER2(3RCD) kcal/mol</th>
<th>EGFR(3W2O) kcal/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luteolin</td>
<td>-8.5</td>
<td>-7.3</td>
</tr>
<tr>
<td>Casticin</td>
<td>-8.3</td>
<td>-7.7</td>
</tr>
</tbody>
</table>

**Density Functional Theory (DFT) studies**

DFT calculations are necessary for understanding the molecular structure of compounds, chemical reactivity, electron affinity, ionization potential, and many more quantum chemical properties of compounds. The computations of this study were performed using Gaussian 09. All the geometric molecular optimization calculations were done using the Hartree-Fock (HF).
Quantum Chemical Theories

Optimized Geometrical Analysis of Phytoconstituents

The geometrically optimized structure with 6-31G and B3LYP level of theory basis set of both the compounds Casticin and Luteolin with the numbering of atoms is present in Fig.-4. The geometrically optimized molecular structures were acquired using the 6-31G and B3LYP level set along with a dipole moment of 9.023 D for Casticin and a dipole moment of 4.864 D for Luteolin. The dihedral angle (C5-C6-C10-O18) of Casticin is 20.41. The dihedral angle (C21-C14-C12-O13) of Luteolin is -0.007 = 0.00. As there are no imaginary frequencies indicates that these optimized structures possess minimum energy at potential energy surfaces.

![Fig.-4: The Geometrically Optimized Structure of (a) Casticin and (b) Luteolin with Atom Numbering, Obtained at the 6-31G and B3LYP Level Basis Set](image)

Frontier Molecular Orbital Study

The Frontier molecular orbital theory gives the Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO) energies of Casticin and Luteolin molecules. HOMO and LUMO energies help us to analyze the molecular chemical reaction stability. In the present study, the HOMO and LUMO orbitals of Casticin and Luteolin are spread throughout the structure of both molecules due to the aromatic rings. HOMO and LUMO energy values are represented in Fig.-5. The energy gap of HOMO and LUMO with 6-31G and B3LYP level of theory basis set of Casticin is 6.7425 eV and Luteolin is 3.9623 eV respectively. So, the energy gap of Casticin is more compared to Luteolin which indicates that Casticin is more stable than the Luteolin molecule.

Molecular Electrostatic Potential (MEP) Surface

Electrostatic potential surface mapping is one of the important parameters in drug design. It helps in understanding the interactions among the molecules, electron density, and chemical properties of the molecules. The electrostatic potential surfaces are represented by the color coding. The greater electrostatic potential is represented with a blue color which indicates an absence of electrons or hydrogen donors and the lesser electrostatic potential (red) indicates the presence of an adequate number of electrons or hydrogen acceptors. The electrostatic potential of the Casticin ranges from -7.964 eV to +7.964 eV and Luteolin ranges from -8.549 eV to +8.549 eV (represented in specific colors) subsequently as shown in Fig.-5.

![Fig.-5: Frontier Molecular Orbital Energy of (a) Casticin and (b) Luteolin Calculated at the B3LYP Level of Theory and 6-31G Basis Set, Positive Phase (Red) and Negative Phase (Green)](image)

The determined MEP surface indicates that the electrophilic attack is shown by negative regions and the nucleophilic attack is shown by the positive regions. The hydroxyl group in the Luteolin acts as a hydrogen donor while the methyl group in the Casticin shows a hydrogen acceptor behavior as shown in Fig.-6.
CONCLUSION

In the current study, we focused on identifying varied biologically active phytochemicals from the ethanolic leaf extracts of the *V. negundo* plant by HPLC and LC-MS studies. These biologically active phytochemicals are accountable for numerous pharmacological and therapeutical properties. This study provides evidence of *V. negundo* extracts containing anticancer and antioxidant potentials. The bioactive compounds' favorable binding conformation was confirmed for both the target proteins through docking simulation. Among these bioactive compounds, Casticin and Luteolin were selected as the best ligand-protein conformations based on the ADMET properties. From our studies, the plant may allow us to develop an efficient drug with reliable medicinal properties against various cancer diseases especially breast cancer revealed from molecular docking studies. Our study also reveals the time and cost-effective method for analyzing the bioactive compounds by ADMET studies, before going for the expensive analytical methods for the purification of unknown drug candidates. Based on the DFT studies, HOMO and LUMO energy levels, and energy gaps suggested that the Casticin compound is more stable than the Luteolin. Also, Casticin is less chemically reactive compared to Luteolin. The determined MEP surfaces suggested the nucleophilic and electrophilic behavior of these compounds. By adopting the above pipeline, the potential ligands with the highest binding potential were confined so that the target-specific compound could be precisely isolated based on specific activity among the metabolites (phytochemicals) from the whole phytometabolome. We anticipate that the above pipeline may critically augment and benefit the research community involved in the design and development of phytometabolome-based future therapeutics.

ACKNOWLEDGMENTS

Authors express their deep sense of gratitude for PES University, Bangalore, BNM Institute of Technology, and Manipal Academy of Higher Education, Manipal for the research facilities and support provided to carry out and disseminate the current research work.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

B. S. Lakshmi [http://orcid.org/0009-0005-4300-1534](http://orcid.org/0009-0005-4300-1534)

H.G. Anilkumar [http://orcid.org/0000-0002-9392-9630](http://orcid.org/0000-0002-9392-9630)

K.B. Jayanna [http://orcid.org/0000-0002-3957-0655](http://orcid.org/0000-0002-3957-0655)

B.S. Ravindranath [https://orcid.org/0000-0002-3713-3646](https://orcid.org/0000-0002-3713-3646)

Open Access: This article is distributed under the terms of the Creative Commons Attribution 4.0 International License ([http://creativecommons.org/licenses/by/4.0/](http://creativecommons.org/licenses/by/4.0/)), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.
REFERENCES

2. Basharat Ahmad Bhat, Wajahat Rashid Mir, Bashir Ahmad Sheikh, Mustafa Alkanani, and Manzoor Ahmad Mir, Scientific Reports,12,7296 (2022), https://doi.org/10.1038/s41598-022-10796-7
16. Kouadio Ibrahime Sinan , Stefano Dall’Acqua , Irene Ferrarese , Adriano Mollica, Azzurra Stefanucci, Jasmina Glamo olija , Marina Sokovic, Marija Nenadi, Abdurrahman Aktunsek and Gokhan Zengin, Antioxidants, 10(10), 1570(2021), https://doi.org/10.3390/antioxid10101570
18. Laldinfeli Ralte, Laldinliana Khiangte, Nurpen Meitei Thangjam, Awadhesh Kumar and Yengkhom Tunginba Singh, Scientific Reports, 12, 3395(2022), https://doi.org/10.1038/s41598-022-07320-2

[RJC-8661/2023]