

PHYTOCHEMICAL PROFILING, ANTIOXIDANT, ANTI-INFLAMMATORY AND ANTICANCER ACTIVITIES OF *VACCINIUM NEILGHERRENSE* WIGHT. LEAF EXTRACT: *IN VITRO* AND MOLECULAR DOCKING INVESTIGATIONS

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Abstract: The plant species, *Vaccinium neilgherrense* is medicinally important with little information on the study of phytochemical ingredients and bioactivities. The present study evaluated the phytochemical constituents and *in vitro* biological properties of *V. neilgherrense* leaf extract, together with *in silico* assessment of a major phytochemical constituent. Quantitative analysis in dry weight revealed that the total phenolic content was 62.3 ± 0.6 mg GAE/g and flavonoid content was 38.9 ± 6.4 mg RE/g. GC-MS identified six major phytoconstituents, while FTIR revealed diverse functional groups. The extract exhibited antibacterial activity, with the largest zone of inhibition being 15 ± 0.33 mm against *Bacillus subtilis*. The antioxidant potential was evaluated through four distinct assays at 500 μ g/mL, revealing peak performance levels of 56.36%, 59.72%, 59.20%, and 55.77% for DPPH, ABTS, hydroxyl radical scavenging, and FRAP, respectively. Their IC₅₀ values were 426.50, 420.78, 422.55, and 251.18 μ g/mL, respectively. Anti-inflammatory activity was observed in HRBC membrane stabilization and albumin denaturation assays, with maximum inhibition of 57.45% and 62.50% respectively, at 250 μ g/mL. Cytotoxicity was observed against A375, A431, and MCF-7 cancer cell lines, exhibiting IC₅₀ values of 84.35, 35.23, and 93.90 μ g/mL, respectively; A431 cells showed the highest sensitivity. Molecular docking of β -amyrin against the EGFR tyrosine kinase domain (PDB ID: 1M17) yielded a binding score of -9.1 kcal/mol. Overall, the extract has quantifiable antioxidant, antibacterial, anti-inflammatory, and cytotoxic properties, and docking shows early signs of β -amyrin-EGFR interaction. Further *in vivo*, toxicological studies are warranted to validate these activities, clarify underlying molecular mechanisms, and establish pharmacological relevance and safety and assess its potential as a therapeutic candidate in future pharmacological study.

Keywords: Phytochemical screening, Antibacterial, Antioxidants, Anti-cancer, Molecular docking

1. INTRODUCTION

The genus *Vaccinium* L. (family Ericaceae) comprises numerous shrubs and small trees distributed across temperate, subtropical, and tropical regions, with several species valued for their edible fruits, nutritional properties, and medicinal potential (Cedillo-Cortezano et al 2024), (Sundarrajan and Bhagtaney 2024). The abundance of phenolic acids, flavonoids, anthocyanins, proanthocyanidins, terpenoids, and other secondary metabolites in *Vaccinium* species is responsible for their antioxidant, antibacterial, anti-inflammatory, and cytoprotective properties (Sharma et al 2024), (Docrat et al 2024). In particular, phenolic and flavonoid constituents have been associated by way of showing the ability to scavenge reactive oxygen, nitrogen species and modulate biological pathways incorporated with oxidative stress and inflammation (Rajkumar et al 2025), (Sabarathinam and Satheesh, 2025). However, the phytochemical profiles and biological activities of *Vaccinium* plants may differ significantly based on the species, their geographical distribution, plant parts, environmental conditions, and extraction methods (Fekadu et al 2024).

Vaccinium neilgherrense Wight is a comparatively underexplored member of the Ericaceae family associated with the Western Ghats of South India, a region recognized for its rich plant diversity and endemic flora. It occurs as a shrub or small tree and is locally known by vernacular names such as Vanji and Kalavu. Its ripe fruits are traditionally consumed by local and indigenous communities, including the Paniya community, indicating its nutritional and ethnobotanical importance (Jerka et al 2024) (Summer et al 2024). Despite this traditional significance, scientific information concerning the phytochemical composition and pharmacological properties of *V. neilgherrense* remains limited (Bhol et al 2024). In particular, its leaves have received considerably less attention than the fruits of other extensively studied *Vaccinium* species. This represents an important knowledge gap because leaves may contain diverse phenolic, flavonoid, terpenoid, and other secondary metabolites with potential biological activities (Gayathiri et al 2025).

A systematic investigation of *V. neilgherrense* leaves is therefore warranted to establish their chemical composition and biological potential. Screening of major phytochemical constituents can initially identify key chemical classes such as flavonoids and phenolic compounds, tannins, and saponins. While GC-MS and FTIR investigations can further characterise the individual chemical constituents and their associated functional groups, (Ostoot et al 2024). Complementary biological assays are also necessary to determine whether the identified phytochemicals have the ability of showing antioxidant, antimicrobial, anti-inflammatory, and anticancer activities. In particular, studies conducted on MCF-7, A375, and A431 cancer cell lines provide a basis for the preliminary assessment of the leaf extract's cytotoxic potential. Furthermore, as Epidermal Growth Factor Receptor (EGFR) signalling controls the cellular proliferation, survival, differentiation, and migration, the (EGFR) tyrosine kinase domain is a crucial target in cancer biology (Ahmedah et al 2024). Molecular docking can give preliminary mechanistic insight into the potential interaction of identified phytochemicals and EGFR (Johariya et al 2024) (Ahmedah et al 2024).

Therefore, the primary objective of this study is to comprehensively investigate and evaluate the phytochemical properties and biological activity of *V. neilgherrense* leaf extract. The study was performed on the preliminary phytochemical screening, estimation of total flavonoid and phenolic contents, characterization by GC-MS and FTIR, antioxidant, antimicrobial, anti-inflammatory and cytotoxic activities. (Tundis et al 2025) (Becker et al 2024). Moreover, molecular docking was performed to investigate the interaction of the bioactive compound β -amyryn with the EGFR tyrosine kinase domain. The study had shown its novelty through the integrated investigation of an underexplored *Vaccinium* species, particularly its leaves, by combining phytochemical characterization with multiple complementary biological assays and molecular docking analysis (Scărlătescu et al 2017). The study therefore moves beyond a general assessment of antioxidant activity and provides a wider estimation of the chemical and pharmacological potential of *V. neilgherrense*. The obtained results are expected to add to the scientific characterization of this lesser-known species and afford a preliminary basis for further

studies in the further could involve the compound isolation, mechanistic validation, pharmacological evaluation, and toxicological assessment. Such investigations may ultimately clarify the potential value of *V. neilgherrense* be a source of naturally derived biologically active molecules for applications in the following fields viz. pharmaceutical, nutraceutical and biomedical (Lohvina et al 2021).

2. MATERIALS AND METHODS

2.1. Collection of Plant Material and Extraction

Leaf samples of the species *Vaccinium neilgherrense* were collected from the Shevaroy Hills of the Eastern Ghats located in the Salem district of Tamil Nadu, India at coordinates 11.816921° North latitude and 78.205491° East longitude. The plant specimens had excised from the tree species *V. neilgherrense* and carried out in conformity with local and national biodiversity conservation rule. The plant species was identified and authenticated by the experts with the help of Botanical Survey of India (BSI), Coimbatore, Tamil Nadu. A voucher specimen was deposited with the assigned number BSI/SRC/5/23/202/Tech/650. To remove soil particles and other surface contaminants adhering to the freshly collected leaves, they were first thoroughly washed in running tap water and subsequently in distilled water. At the room temperature 25-30 °C for two weeks the leaves were dried under the shade until a constant weight was achieved and then the dried leaves were grounded into fine powder using a mechanical grinder. 30 g of the leaf powder was added with 300 mL of analytical-grade ethanol and extraction process was started with Soxhlet apparatus at 40°C for 3 h. Following the extraction process, a rota-evaporator was operated at 50°C to remove the solvent from the obtained extract under reduced pressure and to concentrate the extract. The obtained crude extract was further vacuum-dried to obtain a semi-solid mass and preserved in sterile, airtight containers for subsequent analyses at 4 °C.

2.2 Determination of Extraction Yield analysis

The extraction yield was calculated to evaluate the efficiency of ethanol in isolating bioactive compounds from the leaf powder. The yield percentage was determined using the standard formula and compared across extraction techniques and solvents (Lohvina et al 2021).

$$\text{Extract yield (\%)} = (W_1 / W_2) \times 100,$$

W1 -Weight of Crude extract

W2 -Weight of Initial Sample

2.3 Qualitative Phytochemical Analysis

An initial phytochemical analysis was carried out in the powdered leaf of *Vaccinium neilgherrense* by using ethanol with standard procedures (Doss 2009). The existence of major bioactive phytochemicals, such as saponins, flavonoids, phenolics, alkaloids, tannins, terpenoids, steroids and glycosides, were evaluated to provide a qualitative profile of the extracts.

2.4 Quantitative Analysis

2.4.1 Total Phenolic Content (TPC) analysis

The overall phenolic content of the extract had been evaluated by the Folin-Ciocalteu (FC) colorimetric method (Do et al 2014). Briefly, 0.5 mL of FC reagent was added, followed by 200 µL of extract solution (1 mg/mL) and 3 mL of distilled water were mixed. After 3 minutes two milliliters of a 20% (w/v) solution of sodium carbonate were added. After 60 minutes of incubation in the dark at room temperature then the absorbance was measured at 650 nm in the spectrophotometer. The total phenolic content was expressed as mg GAE per gram of dry weight.

2.4.2 Total Flavonoid Content (TFC) analysis

By using aluminium chloride colorimetric method the total flavonoid content was evaluated. (Washington 1970). Briefly, An aliquot of 50 μL from the extract (concentration: 1 mg/ mL) was diluted to 1 mL with solvent, then mixed with 4 mL of distilled water and subsequently reacted with 0.3 mL of a 5% NaNO_2 solution. After five minutes, 0.3 mL of a 10% AlCl_3 solution was added, and the reaction was allowed to continue for six more minutes. After that, 2 mL of 1 M NaOH was added, and double-distilled water was used to increase the volume to 10 mL. After 15 min incubation, absorbance was measured at 510 nm. Total flavonoid content was measured in milligrams of rutin equivalent per gram of dry weight (mg RE/g d.w.).

2.5 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The ethanolic extract was subjected to GC-MS analysis using a Perkin Elmer Clarus 680 GC equipped with an Elite-5 MS column (5% biphenyl, 95% dimethylpolysiloxane, 30 m $\times$ 0.25 mm ID $\times$ 250 μm df). Helium served as the carrier gas at a constant flow rate of 1 mL/min. The injector's temperature was maintained at 260°C, ramped to 300°C at 10°C/min, and held for 6 minutes. The mass detector and ion source were maintained at 240°C, with electron impact ionization at 70 eV. The retention times and mass spectra of the extract components were compared with the NIST database using Turbo Mass software (version 5.4.0) for compound identification.

2.6 Fourier Transform Infrared (FTIR) Spectroscopy analysis

FTIR analysis was conducted to find out the functional groups in the dried ethanolic extract. Approximately 0.2-0.4 g of extract was mixed with potassium bromide (KBr) to form a pellet, which was scanned over a wavelength range of 400-4000 cm^{-1} at a resolution of 4 cm^{-1} using a Perkin Elmer FTIR spectrometer.

2.7 Antibacterial Activity

The bacterial strains used for the study were procured from the Department of Microbiology, Periyar University, Salem, Tamil Nadu, India. The collected bacterial strains were maintained under standard laboratory conditions using sub culturing method. We tested the antibacterial effects of the ethanolic extract of *V. neilgherrense* leaves against Gram-positive and Gram negative strains such as *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 2422), *Escherichia coli* and *Klebsiella pneumoniae* (MTCC 4030). The sub cultured bacterial strains were carefully inoculated on the Mueller Hinton agar plates (Washington 1970). Different concentrations of ethanolic leaf extract of *V. neilgherrense* (20, 40, and 60 $\mu\text{g/mL}$) were carefully placed on the sterile disc (6 mm) containing agar plates. For this study ethanol was used as negative control, while *Streptomycin* (30 μg) treated as a positive control. Then the agar plates were incubated at 37°C for 24 hours, and the diameters of the zones of inhibition were measured in millimetres (mm).

2.8 In Vitro Antioxidant Activity

2.8.1 DPPH (2, 2-Diphenyl-1-picrylhydrazyl) Assay

The DPPH assay for free radical scavenging activity of the leaf extract was evaluated by method described by (Dudonne et al 2009). Leaf extracts were prepared in the following concentrations thus obtained sample (1.0 mL) was mixed with 1.0 mL of 0.2 M DPPH solution and allowed to set for 5 minutes, then incubated for 30 minutes in the dark. Absorbance peak was measured at 517 nm, with Vitamin C used as the standard control. The IC_{50} , the required inhibitory concentration to cause 50% scavenging of DPPH molecules, was calculated as the mean value $\pm$ standard deviation (SD) of experiments performed in triplicate.

$$\% \text{ of inhibition} = (\text{Ac}-\text{As})/\text{Ac} \times 100$$

Here, Ac-represents absorbance of control; As-represents absorbance of sample

2.8.2 ABTS (2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) Radical Scavenging Assay

The ABTS radical scavenging activity of ethanol extract obtained from *Vaccinium neilgherrense* leaves was evaluated based on the previously described method (Dudonne et al 2009). Briefly, ABTS radicals were generated by mixing 7 mM ABTS solution with 2.45 mM potassium persulfate and allowing the mixture to react in the dark for 12–16 hours at room temperature to form a stable radical cation (ABTS⁺). For the assay, test tubes containing different concentrations of the extract (100, 200, 300, 400 and 500 µg/mL) were prepared. Each sample (1.0 mL) was added to 1.0 mL of the ABTS⁺ solution and incubated at room temperature for 10 minutes in the dark. The absorbance of the resulting mixture was measured at 734 nm using a UV-visible spectrophotometer. Vitamin C was used as the standard antioxidant control. The percentage inhibition of ABTS radicals was calculated and IC₅₀ values were determined to quantify the antioxidant potential of the extract.

$$\% \text{ of inhibition} = (\text{Ac}-\text{As})/\text{Ac} \times 100$$

Here, Ac-represents absorbance of control; As-represents absorbance of sample

2.8.3 Hydroxyl Radical Scavenging Assay

The hydroxyl radical (•OH) scavenging activity of the ethanolic leaf extract of *Vaccinium neilgherrense* was assessed following the method described by (Dudonne et al 2009). Hydroxyl radicals were generated through the Fenton reaction, and their scavenging by the extract was quantified spectrophotometrically. The extract was diluted to various concentrations (100, 200, 300, 400, and 500 µg/mL) using distilled water. For each concentration, 1.0 mL of 1.5 mM ferrous sulfate (FeSO₄) and 0.7 mL of 6 mM hydrogen peroxide (H₂O₂) were added to initiate hydroxyl radical formation. After incubating for 5 minutes at room temperature, 0.3 mL of 20 mM sodium salicylate solution was introduced as a probe to react with •OH radicals. The mixture was further incubated at 37°C for 30 minutes. The formation of hydroxylated products was measured by recording absorbance at 562 nm using a UV–visible spectrophotometer. Vitamin C was used as the standard antioxidant control. The percentage inhibition of hydroxyl radicals was calculated for each concentration, and IC₅₀ values were determined to evaluate the extract's radical scavenging efficiency.

$$\% \text{ of inhibition} = (\text{Ac}-\text{As})/\text{Ac} \times 100$$

Here, Ac-represents absorbance of control; As-represents absorbance of sample

2.8.4 Ferric Ion Reducing Antioxidant Power (FRAP) Assay

The ferric ion reducing power of the ethanolic leaf extract of *V. neilgherrense* was evaluated according to the method described by (Dudonne et al 2009). This assay measures the electron-donating ability of antioxidants in the extract, which reduces ferric ions (Fe³⁺) to ferrous ions (Fe²⁺). Different concentrations of the extract (100, 200, 300, 400, and 500 µg/mL) were prepared in distilled water. For each concentration, 2.5 mL of 0.2 M phosphate buffer saline (PBS, pH 6.6) and 2.5 mL of 1% potassium ferricyanide [K₃Fe (CN)₆] were added. The mixtures were incubated at 50°C for 20 minutes to allow reduction of ferric ions. Following incubation, 2.5 mL of 10% trichloroacetic acid (TCA) was added, and the samples were centrifuged at 3000 rpm for 10 minutes. The supernatant was collected, mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride (FeCl₃) solution, and the absorbance was recorded at 700 nm using a spectrophotometer. Vitamin C served as the standard reference. Higher absorbance values indicate greater reducing power, and results were used to determine the antioxidant potential of the extract.

$$\% \text{ of inhibition} = (\text{Ac}-\text{As})/\text{Ac} \times 100$$

Here, Ac-represents absorbance of control; As-represents absorbance of sample

2.9 In Vitro Anti-inflammatory Activity

2.9.1 Albumin Denaturation Assay

The anti-inflammatory potential of the ethanolic leaf extract of *V. neilgherrense* was evaluated using the albumin denaturation method as described by (Enechi et al 2020). This assay measures the ability of the extract to inhibit protein denaturation, a key process in inflammation. Briefly, a reaction mixture was prepared by combining 0.45 mL of distilled water with 0.5 mL of 5% aqueous bovine serum albumin (BSA) solution. The pH of the mixture was adjusted to 6.3 using 1 N HCl. Various concentrations of the ethanolic extract (50-250 µg/mL) were added to the mixture. The samples were incubated at 37°C for 20 minutes and then heated at 57°C for 5 minutes to induce protein denaturation. After cooling to room temperature, 2.5 mL of phosphate-buffered saline (PBS) was added, and the turbidity of the solution, indicating protein denaturation, was measured at 600 nm using a UV-Visible spectrophotometer (Systronics, India). The percentage inhibition of albumin denaturation was calculated for each concentration, and the IC₅₀ value, representing the concentration required to inhibit 50% of protein denaturation, was determined. Higher inhibition values indicate stronger anti-inflammatory activity of the extract.

$$\% \text{ of inhibition} = (\text{Ac}-\text{As})/\text{Ac} \times 100$$

Here, Ac-represents absorbance of control; As-represents absorbance of sample

2.9.2 Inhibition of Membrane Stabilization Assay

The membrane stabilizing activity of the ethanolic leaf extract of *V. neilgherrense* was assessed using the human red blood cell (HRBC) membrane stabilization method as described by (Anosike et al 2012). This assay evaluates the ability of the extract to protect erythrocyte membranes against hemolysis, which is considered analogous to the stabilization of lysosomal membranes during inflammatory conditions. Human blood samples used in this study were obtained from a certified medical laboratory and collected following standard ethical procedures with prior written informed consent from the volunteer donors. Fresh blood was mixed with Alsever's solution to prevent coagulation and centrifuged at 3000 rpm for 20 min. The packed erythrocytes were washed with isotonic saline (10% v/v) and re-suspended in the same solution to obtain a uniform RBC suspension. Different concentrations of the ethanolic extract (50-250 µg/mL) were prepared and mixed with the erythrocyte suspension, hypertonic saline, and phosphate buffer to induce hemolysis. The reaction mixtures were incubated at 37 °C for 30 min and then centrifuged at 3000 rpm for 10 min. The extent of hemolysis was quantified by measuring the hemoglobin content in the supernatant at 560 nm using a UV-Visible spectrophotometer (Systronics, India). Aspirin was used as the standard reference drug. The percentage inhibition of hemolysis was calculated for each concentration, and the IC₅₀ value at concentration required to produce 50% membrane stabilization was determined. Higher percentage inhibition indicates stronger anti-inflammatory potential of the extract.

$$\% \text{ of inhibition} = (\text{Ac}-\text{As})/\text{Ac} \times 100$$

Here, Ac-represents absorbance of control; As-represents absorbance of sample

2.10 In Vitro Cytotoxicity Assay

2.10.1 Cell Line Culture and Maintenance

Human cancer cell lines A375 (melanoma), A431 (epidermoid carcinoma), and MCF-7 (breast cancer) were obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% antimycotic solution. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cells were subcultured at 70–80% confluency and used during the exponential growth phase for the cytotoxicity assay (Sumantran 2011).

2.10.2 MTT Cell Viability Assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was handled to evaluate the cytotoxic potential of the ethanol based leaf extract of *Vaccinium neilgherrense* (Kumar et al 2018). The MTT assay was used to assess the presence of metabolically active cells, which was achieved by the reduction process of MTT into insoluble purple formazan crystals; therefore, the amount of formazan formed during the process is directly proportional to cell viability. Briefly, to encourage the cell attachment

in which the cells were seeded into 96-well plates for about 24 h at an appropriate cell density. Serial dilution was carried out by using the ethanolic leaf extract as a stock solution to achieve final concentration of 7.81, 15.62, 31.25, 62.5, and 125 µg/mL. The cells were treated with the respective concentrations for 48 h under standard culture conditions. Following treatment, 15 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 h at 37°C. The culture medium was then carefully removed, and 100 µL of DMSO was added to each well to dissolve the formed formazan crystals. Absorbance was measured at 570 nm using a microplate reader. Untreated cells served as the control, and all treatments were performed in triplicate. Cell viability was expressed as a percentage of the untreated control using the following equation:

$$\text{Cell viability (\%)} = (\text{Absorbance of treated cells} / \text{Absorbance of control cells}) \times 100$$

The IC₅₀ value was determined from the concentration–response curve using appropriate nonlinear regression analysis and represents the concentration of the extract required to reduce cell viability by 50%. The cytotoxicity assay was used to determine the concentration-dependent effects of the *V. neilgherrense* leaf extract on A375, A431, and MCF-7 cells (Almehdar et al 2012).

2.11 Pharmacokinetic Evaluation and Molecular Docking Study of β-Amyrin

GC-MS analysis of *Vaccinium neilgherrense* leaf extract revealed β-Amyrin as one of the predominant bioactive constituents, prompting its selection for a detailed *in silico* pharmacokinetic and molecular interaction assessment (Oliveira et al 2022). To initiate the pharmacokinetic evaluation, the canonical SMILES notation of β-Amyrin was generated from its structural data sourced from the PubChem database. This SMILES string was subsequently imported into online ADMET prediction platforms to compute essential drug-likeness parameters, absorption efficiency, and bioavailability indices. Compliance with Lipinski Rule of Five, as well as Veber and Ghose filters, was assessed to determine its suitability for oral drug development. Additional parameters including gastrointestinal permeability, blood brain barrier (BBB) penetration, cytochrome P450 enzyme inhibition potential, plasma protein binding (PPB), renal clearance tendency, and hepatotoxicity were analyzed to establish a comprehensive ADMET profile. To evaluate its therapeutic relevance, particularly its anticancer potential, molecular docking analysis was performed targeting the Epidermal Growth Factor Receptor (EGFR), a key oncogenic protein highly expressed in A431 epidermoid carcinoma cells. The three dimensional crystal structure of EGFR (PDB ID: 1M17) was retrieved from the RCSB Protein Data Bank. Prior to docking, the protein structure was refined by eliminating co-crystallized ligands, bound water molecules, and extraneous

heteroatoms. Hydrogen atoms were added, and energy minimization was performed to stabilize the protein conformation and prepare it for ligand interaction. β -Amyrin 3D molecular structure was retrieved in SDF format, converted into PDB format, and geometrically optimized using the MMFF94 force field to obtain a stable low-energy conformation. Docking simulations were executed using AutoDockVina, where the grid box coordinates were strategically positioned around the active site pocket of EGFR to ensure accurate binding predictions. The docking affinity was quantified in terms of binding energy (kcal/mol), and docking poses exhibiting the lowest binding scores were selected for further interpretation. Key molecular interactions including hydrophobic contacts, hydrogen bonding, van der Waals forces, and π - π stacking were visualized and interpreted using PyMOL and Discovery Studio Visualizer. These insights were employed to understand the putative mechanism through which β -Amyrin may exert inhibitory effects on EGFR activity, thereby contributing to its anticancer action. The combination of ADMET profiling and docking results collectively supports the potential of β -Amyrin as a promising lead phytochemical for future anticancer drug development (Almehdar et al 2012) (Pintor et al 2020).

2.12 Statistical Analysis

All experimental assays were conducted in triplicate ($n = 3$) to ensure reproducibility and reliability. Graphical representations were created using Origin 8 SRO (v8.0725), and data were analyzed using GraphPad Prism software (v5.01, GraphPad Software Inc., USA). Statistical significance among groups was

assessed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test for multiple comparisons. Differences with a p -value < 0.05 were considered statistically significant.

3. RESULTS

3.1. Percentage Yield Analysis

The extraction yield of *Vaccinium neilgherrense* leaves was assessed using various solvents, among which the ethanolic extract produced the highest yield of 46.6%. This substantial extraction efficiency indicates that ethanol is highly effective in solubilizing a broad spectrum of bioactive compounds from the leaf matrix, making it an ideal solvent for subsequent phytochemical and pharmacological investigations.

3.2. Qualitative Phytochemical Analysis

Preliminary phytochemical screening revealed the presence of several key secondary metabolites in the leaf extracts, as summarized in Table 1.

Table 1. Preliminary phytochemical screening of *V. neilgherrense* ethanolic leaf extract

S. No.	Phytochemicals Names	Tests	Presence and absence of phytochemicals
1.	Alkaloids	Dragendorff's test	+
2.	Flavonoids	Lead acetate test	+
3.	Saponins	Foam test	-
4.	Tannins	Ferric chloride test	+
5.	Steroids	Salkowski test	-

6.	Cardiac glycosides	Keller-Killani test	+
7.	Anthraquinones	Borntrager's test	+
8.	Phenol	Ferric chloride test	+
9.	Terpenoids	Salkowski's test	-
10.	Protein	Biuret test	-
11.	Carbohydrate	Fehling's Test	+
12.	Fixed oils	Fixed oils	+

Presence of bioactive compounds (+), Absence of bioactive compounds (-)

The aqueous extract tested positive for alkaloids, flavonoids, tannins, phenols, carbohydrates, glycosides, and anthraquinones, whereas saponins, steroids, proteins, terpenoids, and fixed oils were not detected. The presence of these diverse phytochemicals suggests that the extract possesses a rich biochemical profile, which may contribute to its observed biological activities.

3.3. Quantitative Phytochemical Analysis

Quantitative analysis of the ethanolic extract showed a total phenolic content (TPC) of 62.3 ± 0.6 mg GAE/g dry weight and total flavonoid content (TFC) of 38.9 ± 6.4 mg RE/g dry weight. The high levels of these phenolic and flavonoid compounds indicate strong antioxidant potential and support the extract's possible anti-inflammatory and therapeutic properties.

3.4. GC-MS Analysis

GC-MS analysis of the ethanolic leaf extract of *Vaccinium neilgherrense* revealed a diverse profile of phytoconstituents representing hydrocarbons, alcohols, fatty acids, esters, and sterol/triterpenoid compounds (Fig. 1; Table 2).

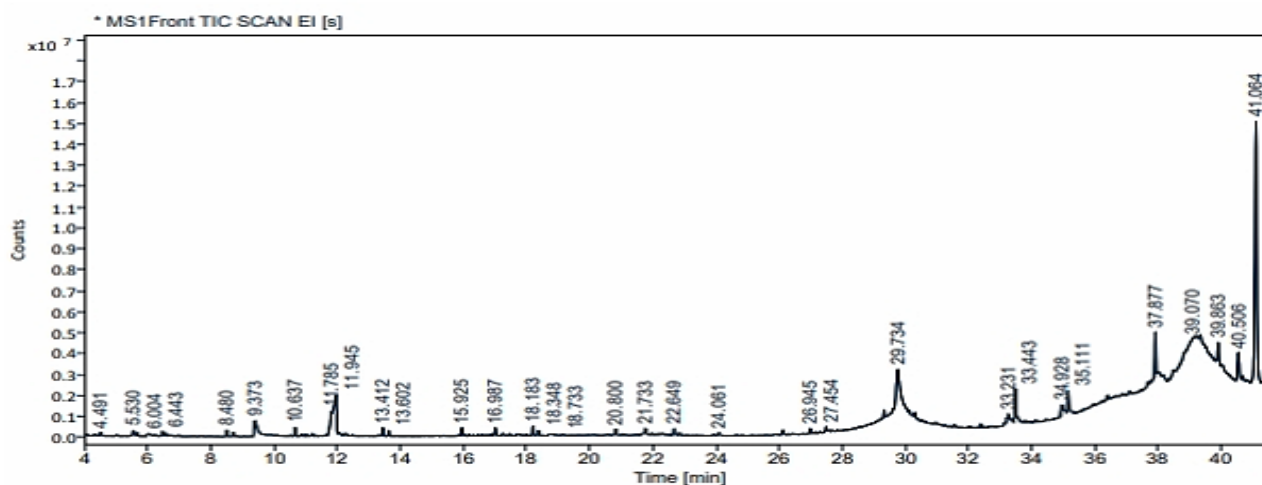
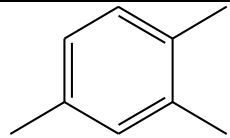
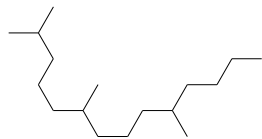
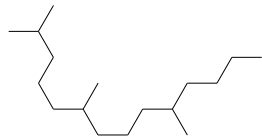
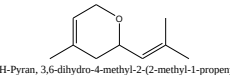
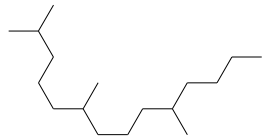
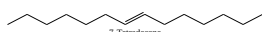
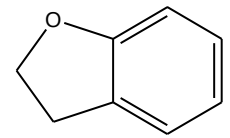
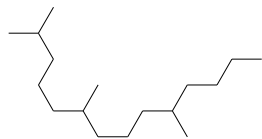
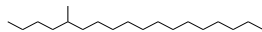
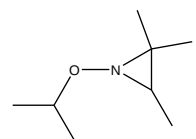
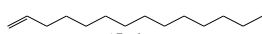
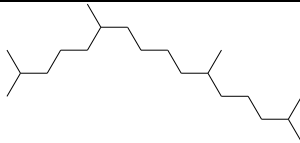
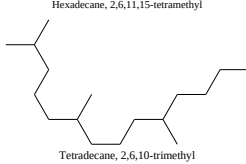
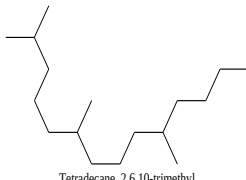
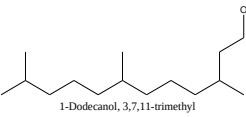
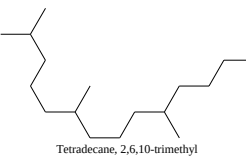
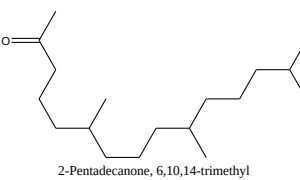
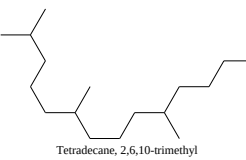
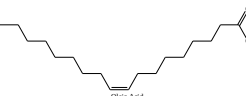
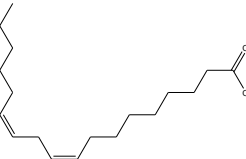
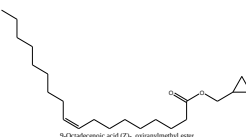


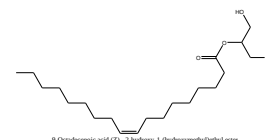
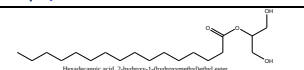
Fig. 1. GC-MS analysis of *V. neilgherrense* ethanolic leaf extract

S. no	R. Time	Compound name	Molecular formula	Area peak (%)	Compound structure
1.	4.494	Benzene, 1,2,4-trimethyl	C ₉ H ₁₂	14.38	 Benzene, 1,2,4-trimethyl
2.	5.531	Tetradecane, 2,6,10-trimethyl	C ₁₇ H ₃₆	18.3	 Tetradecane, 2,6,10-trimethyl
3.	6.006	Tetradecane, 2,6,10-trimethyl	C ₁₇ H ₃₆	43.68	 Tetradecane, 2,6,10-trimethyl
4.	4.489	2H-Pyran, 3,6-dihydro-4-methyl-2-(2-methyl-1-propenyl)-	C ₁₀ H ₁₆ O	10.7	 2H-Pyran, 3,6-dihydro-4-methyl-2-(2-methyl-1-propenyl)-
5.	6.225	Tetradecane, 2,6,10-trimethyl	C ₁₇ H ₃₆	4.61	 Tetradecane, 2,6,10-trimethyl
6.	6.444	7-Tetradecene	C ₁₄ H ₂₈	3.140	 7-Tetradecene
7.	8.482	Benzofuran, 2,3-dihydro	C ₈ H ₈ O	3.140	 Benzofuran, 2,3-dihydro
8.	9.376	Tetradecane, 2,6,10-trimethyl	C ₁₇ H ₃₆	2.967	 Tetradecane, 2,6,10-trimethyl
9.	10.632	Octadecane, 5-methyl	C ₁₉ H ₄₀	4.129	 Octadecane, 5-methyl
10.	11.783	1-Isopropoxy-2,2,3-trimethylaziridine	C ₈ H ₁₇ NO	37.94	 1-Isopropoxy-2,2,3-trimethylaziridine
11.	11.945	1-Tetradecene	C ₁₄ H ₂₈	7.68	 1-Tetradecene
12.	13.408	Tetradecane	C ₁₄ H ₂₈	6.45	 Tetradecane

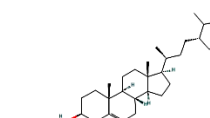
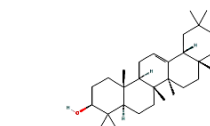
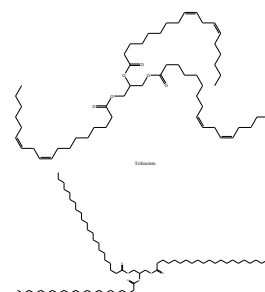
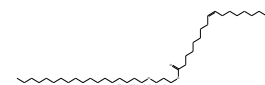
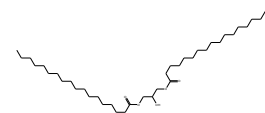
13	13.602	Hexadecane, 2,6,11,15-tetramethyl	$C_{20}H_{42}$	22.76	
14	15.927	Tetradecane, 2,6,10-trimethyl	$C_{17}H_{36}$	4.89	
15	16.990	1-Hexadecanol	$C_{16}H_{34}O$	28.7	
16	18.184	Tetradecane, 2,6,10-trimethyl	$C_{17}H_{36}$	6.23	
17	18.346	1-Dodecanol, 3,7,11-trimethyl	$C_{15}H_{32}O$	8.75	
18	18.734	Heptacosane	$C_{27}H_{56}$	4.31	
19	20.803	Tetradecane, 2,6,10-trimethyl	$C_{17}H_{36}$	20.31	
20	21.734	1-Eicosene	$C_{20}H_{40}$	32.88	
21	22.647	2-Pentadecanone, 6,10,14-trimethyl	$C_{18}H_{36}O$	7.34	
22	24.060	Tetradecane, 2,6,10-trimethyl	$C_{17}H_{36}$	16.20	
23	26.942	Oleic Acid	$C_{18}H_{34}O_2$	7.88	
24	27.454	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	38.33	
25	29.736	9-Octadecenoic acid (Z)-, oxiranylmethyl ester	$C_{21}H_{38}O_3$	11.89	

A
of 33

26	33.230	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O$	21.19
27	33.443	9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{21}H_{40}O_4$	37.02
28	34.930	Octadecanoic acid, 2-hydroxy-1,3-propanediyl di-ester	$C_{39}H_{76}O_5$	53.24
29	35.112	Oleic acid, 3-(octadecyloxy)propyl ester	$C_{39}H_{76}O_3$	68.2
30	39.069	Trilinolein	$C_{57}H_{98}O_6$	23.82
31	39.863	Docosanoic acid, 1,2,3-propanetriyl ester	$C_{69}H_{134}O_6$	18.23
32	40.506	β -Sitosterol	$C_{29}H_{50}O$	45.22
33	41.063	β -Amyrin	$C_{30}H_{50}O$	70.24



total



chromatographic entries were recorded based on their retention times and mass spectral library matching. Among the identified constituents, β -amyrin showed the highest reported peak area (70.24%), followed by oleic acid, 3-(octadecyloxy)propyl ester (68.20%), octadecanoic acid, 2-hydroxy-1,3-propanediyl di-ester (53.24%), β -sitosterol (45.22%), and tetradecane, 2,6,10-trimethyl- (43.68%). Other notable constituents included 1-isopropoxy-2,2,3-trimethylaziridine (37.94%), 9,12-octadecadienoic acid (Z,Z)- (38.33%), 9-octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester (37.02%), trilinolein (23.82%), hexadecane, 2,6,11,15-tetramethyl- (22.76%), hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (21.19%), and 1-eicosene (32.88%). Several other hydrocarbons and oxygenated compounds were detected at comparatively lower peak areas, including oleic acid (7.88%), 1-hexadecanol (28.70%), 1-dodecanol, 3,7,11-trimethyl- (8.75%), heptacosane (4.31%), benzofuran, 2,3-dihydro- (3.14%), and 7-tetradecene (3.14%). The GC-MS profile therefore demonstrated the presence of chemically diverse constituents, including fatty acids and their esters, long-chain hydrocarbons, alcohols, sterol, and triterpenoid compounds. Notably, β -amyrin and β -sitosterol were detected among the major constituents and may contribute to the biological properties observed for the leaf extract. However, the GC-MS peak-area percentages represent relative chromatographic abundance and should not be interpreted as absolute concentrations. Further isolation and structural confirmation using authentic standards and complementary spectroscopic techniques are required to establish the identity and biological contribution of individual constituents.

3.5. FTIR Analysis

FTIR spectroscopy revealed the presence of multiple functional groups within the ethanolic leaf extract (Fig. 2, Table 3). Significant peaks were observed at 3708.17 cm^{-1} (alcohols), 2979.08 cm^{-1} (alkynes), 1371.63 cm^{-1} (sulfonate), 1166.57 cm^{-1} (tertiary alcohol), and 960.57 cm^{-1} (alkanes).

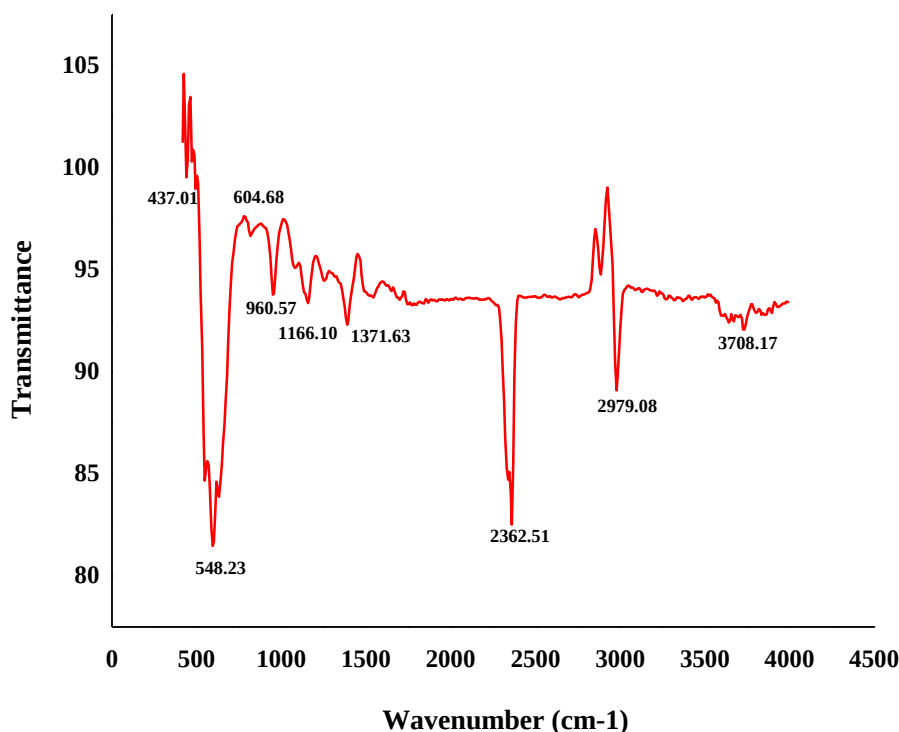


Fig. 2. FTIR analysis of *V. neilgherrense* ethanolic leaf extract

Table. 3. FTIR analysis of *V. neilgherrense* ethanolic leaf extract

S. No	Wave Number (cm ⁻¹)	Intensity	Bond responsible	Functional groups
1	437.01	Strong	Alkyl Halides	C-Br stretch
2	548.23	Medium	Alkene	C=C stretch
3	604.68	Strong	Halo Compound	C-I stretch
4	960.57	Medium	Alkene	C=C bending
5	1166.57	Strong	Tertiary Alcohol	C-O stretch
6	1371.63	Strong	Sulfonate	S-O stretch
7	2362.51	Strong	Alkane	C=C stretch
8	2979.08	Medium	Alkynes	C=C stretch
9	3708.17	Medium	Alcohol	C=C stretch

These functional groups are likely involved in the stabilization and capping of bioactive molecules and may contribute to the extract's pharmacological activities.

3.6. Antibacterial Activity

The antibacterial activity of the ethanolic leaf extract was assessed against selected Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*) using the disc diffusion assay. The extract demonstrated significant inhibitory effects on all tested bacterial strains, indicating a broad-spectrum antimicrobial potential. Among the organisms evaluated, *Bacillus subtilis* exhibited the highest susceptibility, showing a maximum zone of inhibition of 15 ± 0.33 mm, followed by *Klebsiella pneumoniae* with 13 ± 0.10 mm (Table 4, Fig. 3). The moderate inhibition observed against *Staphylococcus aureus* and *Escherichia coli* further supports the extract's ability to act against diverse bacterial groups.

Table. 4. Antibacterial activity of ethanolic leaf extract of *V. neilgherrense*

S. No.	Pathogenic microbes	Zone of Inhibition (mm) of ethanol leaf extract				
		Positive Control (<i>Streptomycin</i>)	Negative Control (ethanol)	Concentrations ($\mu\text{g/mL}$)		
				20	40	60
1	<i>Staphylococcus aureus</i>	$20 \pm 0.45\text{mm}$	-	$07 \pm 0.44\text{mm}$	$09 \pm 0.16\text{mm}$	$11 \pm 0.28\text{mm}$
2	<i>Klebsiella pneumoniae</i>	$21 \pm 0.23\text{mm}$	-	$09 \pm 0.22\text{mm}$	$10 \pm 0.62\text{mm}$	$13 \pm 0.10\text{mm}$
3	<i>Bacillus subtilis</i>	$20 \pm 0.45\text{mm}$	-	$08 \pm 0.31\text{mm}$	$09 \pm 0.43\text{mm}$	$15 \pm 0.33\text{mm}$
4	<i>Escherichia coli</i>	$22 \pm 0.21\text{mm}$	-	$06 \pm 0.35\text{mm}$	$08 \pm 0.55\text{mm}$	$10 \pm 0.12\text{mm}$

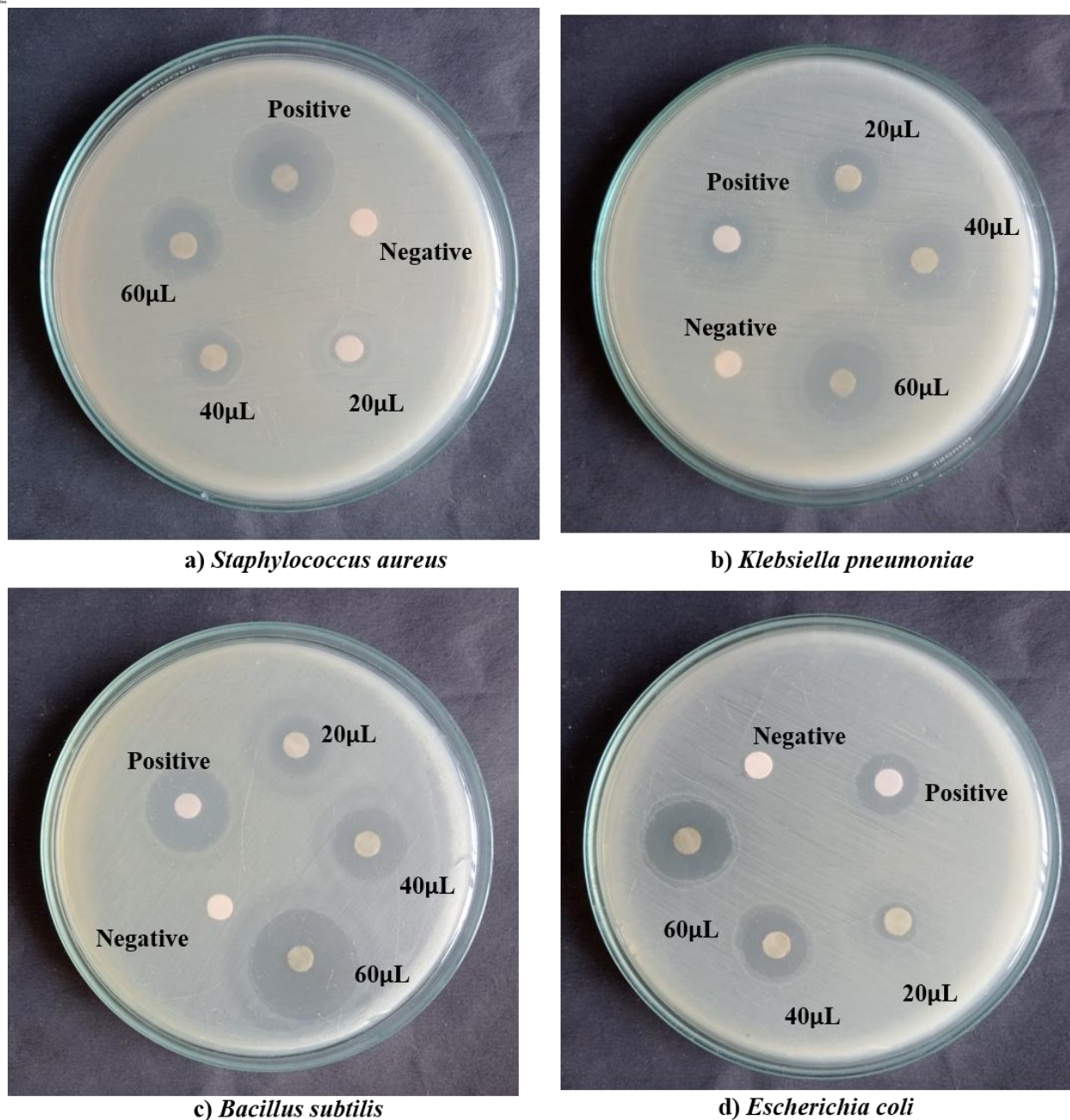


Fig. 3. Antibacterial activity of *V. neilgherrense* ethanolic leaf extract a) *Staphylococcus aureus* b) *Klebsiella pneumoniae* c) *Bacillus subtilis* d) *Escherichia coli* (Positive control- *Streptomycin*, Negative control-ethanol)

These findings suggest that the phytochemicals present in *Vaccinium neilgherrense* may contribute synergistically to its antibacterial efficacy, highlighting its potential application in developing natural antimicrobial agents.

3.7. In-vitro Antioxidant Activity

3.7.1. DPPH Radical Scavenging Activity

The DPPH assay measures the extract's ability to neutralize the stable DPPH free radical through hydrogen atom donation. The ethanolic extract exhibited a concentration-dependent increase in radical scavenging, achieving a maximum inhibition of 56.36% at 500 µg/mL. The calculated IC₅₀ value of 426.50 µg/mL reflects

moderate antioxidant potential. This capacity indicates that the extract contains compounds capable of effectively quenching free radicals, thus preventing oxidative stress.

3.7.2. ABTS Radical Scavenging Activity

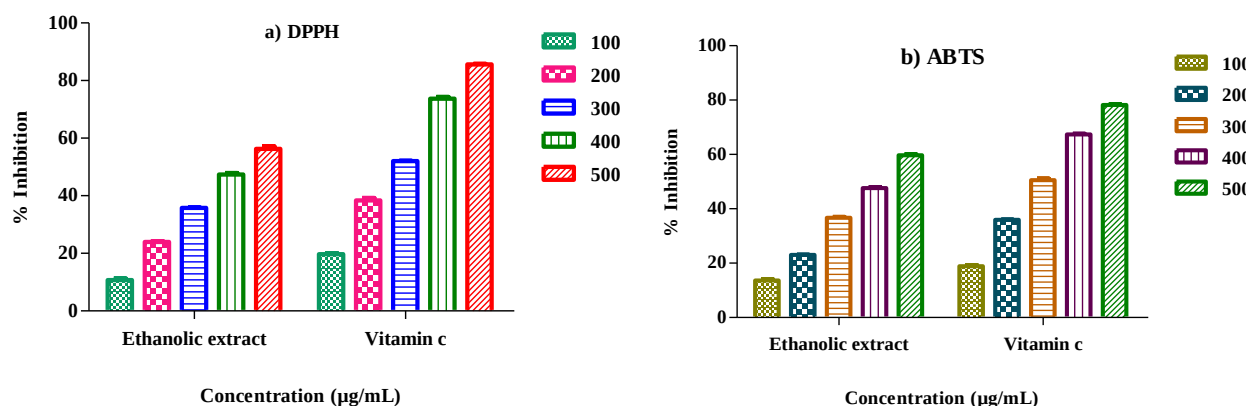
The ABTS assay evaluates both hydrophilic and lipophilic antioxidant components, providing a broader assessment of radical scavenging ability. The extract showed slightly higher antioxidant efficiency in this assay compared to DPPH, with 59.72% inhibition at 500 $\mu\text{g/mL}$ and an IC_{50} of 420.78 $\mu\text{g/mL}$. This suggests that the extract contains bioactive molecules that interact efficiently with the ABTS^+ radical cation, indicating strong electron- and hydrogen-donating potential.

3.7.3. Hydroxyl radical scavenging capacity

The hydroxyl radical scavenging capacity of the extract showed a clear concentration-dependent increase, demonstrating its ability to counteract one of the most reactive and damaging free radicals. At the highest tested concentration (500 $\mu\text{g/mL}$), the extract achieved 59.20% inhibition, reflecting substantial scavenging activity. The corresponding IC_{50} value of 422.55 $\mu\text{g/mL}$ further suggests that a moderate amount of the extract is required to neutralize 50% of hydroxyl radicals (Fig. 4c). These findings indicate that the extract possesses a moderate but significant ability to quench hydroxyl radicals, contributing to its overall antioxidant potential.

3.7.4. Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay measures the extract's reducing ability by converting ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions. The ethanolic extract showed a robust and concentration-dependent increase in reducing power, reaching 55.77% activity at 500 $\mu\text{g/mL}$. Notably, the FRAP assay produced the lowest IC_{50} value of 251.18 $\mu\text{g/mL}$, indicating that the extract possesses strong electron-donating antioxidants capable of reducing oxidized intermediates.



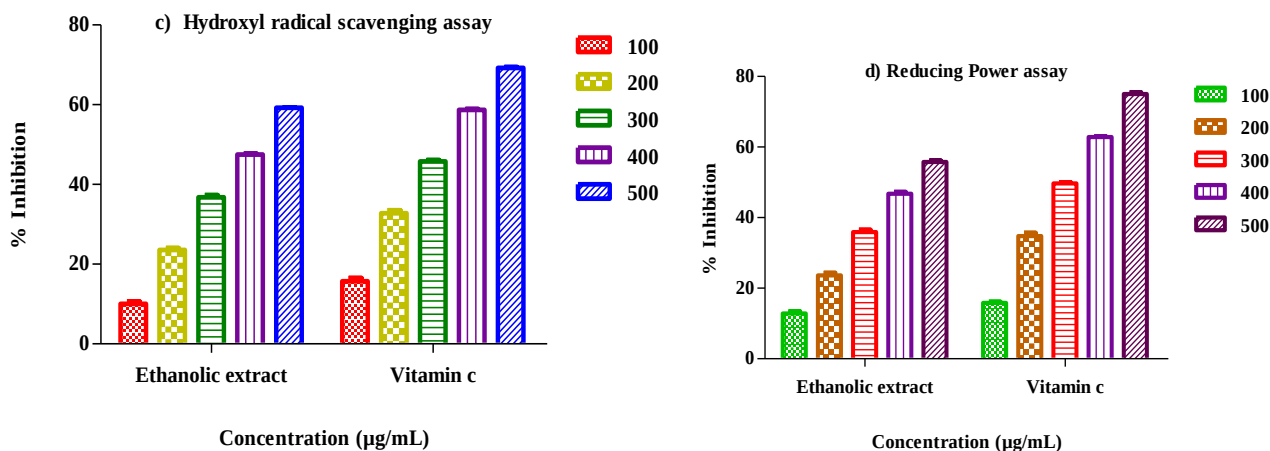


Fig. 4. *In-vitro* Antioxidant potential of *V. neilgherrense* ethanolic leaf extract a) DPPH b) ABTS⁺ c) Hydroxyl scavenging activity d) Ferric ion reducing power activity; The values expressed as Mean $\pm$ SD values, analyzed by One-Way analysis of Variance (ANOVA).

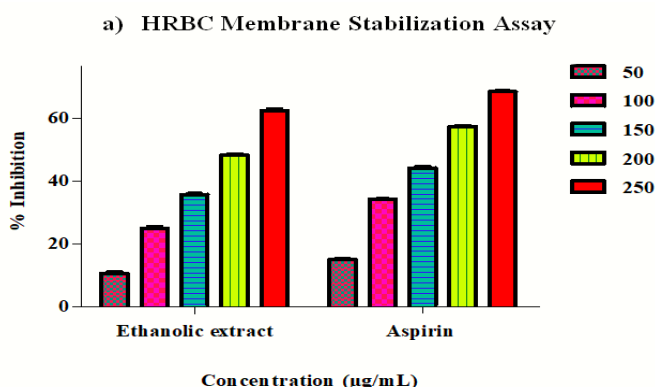
3.8. *In-vitro* Anti-inflammatory Activity

3.8.1. HRBC Membrane Stabilization Assay

During inflammation, lysosomal enzymes released from neutrophils and other immune cells contribute significantly to tissue damage and inflammatory progression. The HRBC membrane is structurally and functionally similar to the lysosomal membrane; therefore, its stabilization reflects the ability of a substance to inhibit the release of such inflammatory mediators. In this study, the ethanolic extract demonstrated a clear concentration-dependent membrane-protective effect.,(Fig.5a) At the highest tested concentration (250 µg/mL), a membrane stabilization percentage of 57.45% was recorded, with an IC₅₀ value calculated as 211.30 µg/mL. This ability to prevent hypotonicity-induced hemolysis indicates that bioactive constituents within the extract are capable of reinforcing membrane integrity, thereby reducing erythrocyte lysis and inhibiting downstream inflammatory signaling. The results collectively suggest that the extract mimics the action of non-steroidal anti-inflammatory drugs (NSAIDs), which also exert effects through membrane stabilization.

3.8.2. Albumin Denaturation Assay

Protein denaturation is a key biochemical process associated with inflammation, particularly in disorders such as arthritis where denatured proteins can act as autoantigens and trigger immune responses. In this study, the ethanolic leaf extract of *Vaccinium neilgherrense* exhibited a significant dose-dependent inhibition of heat-induced albumin denaturation. At the highest tested concentration (250 µg/mL), the extract showed 62.50% inhibition, with an IC₅₀ value of 206.17 µg/mL. This protein-protective effect suggests that bioactive phytochemicals such as phenolics and flavonoids may interact with albumin to maintain its structural stability and prevent unfolding under stress(Fig.5b). The observed mechanism is comparable to that of standard anti-inflammatory agents that mitigate inflammation by inhibiting protein denaturation. Overall, these results, along with HRBC membrane stabilization findings, confirm that the extract demonstrates strong anti-inflammatory potential. The dual action membrane stabilization and protein protection may be attributed to antioxidant-rich constituents capable of scavenging free radicals and



preserving cellular and molecular integrity. Considering the central role of inflammation in chronic diseases such as cancer, cardiovascular disorders, and arthritis, these findings highlight the extract therapeutic promise and justify *further in-vivo* validation and mechanistic studies.

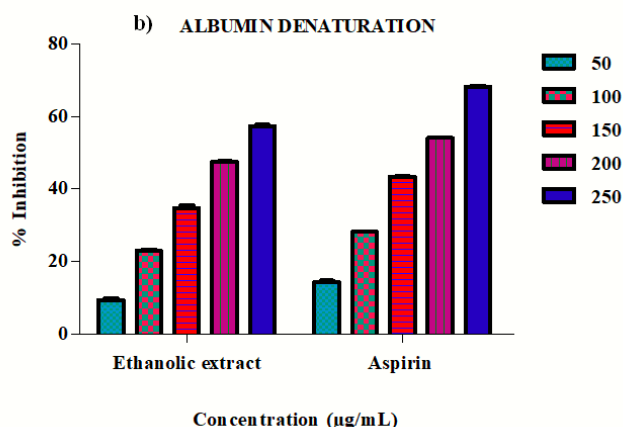


Fig. 5. *In-vitro* Anti-inflammatory activity of *V. neilgherrense* ethanolic leaf extract a) HRBC membrane stabilization assay; b) Albumin denaturation assay; the values expressed as Mean $\pm$ SD values, analyzed by One-Way analysis of Variance (ANOVA).

3.9. *In-vitro* Anticancer Activity

The cytotoxic activity of the ethanolic leaf extract of *Vaccinium neilgherrense* was evaluated against A375 (melanoma), A431 (epidermoid carcinoma), and MCF-7 (breast cancer) cell lines. The extract showed a concentration-dependent reduction in cell viability in the tested cell lines. In A375 cells, treatment with 125 µg/mL extract reduced cell viability to 74.09%, with an IC_{50} value of 84.35 µg/mL (Fig. 6 and Fig.7).

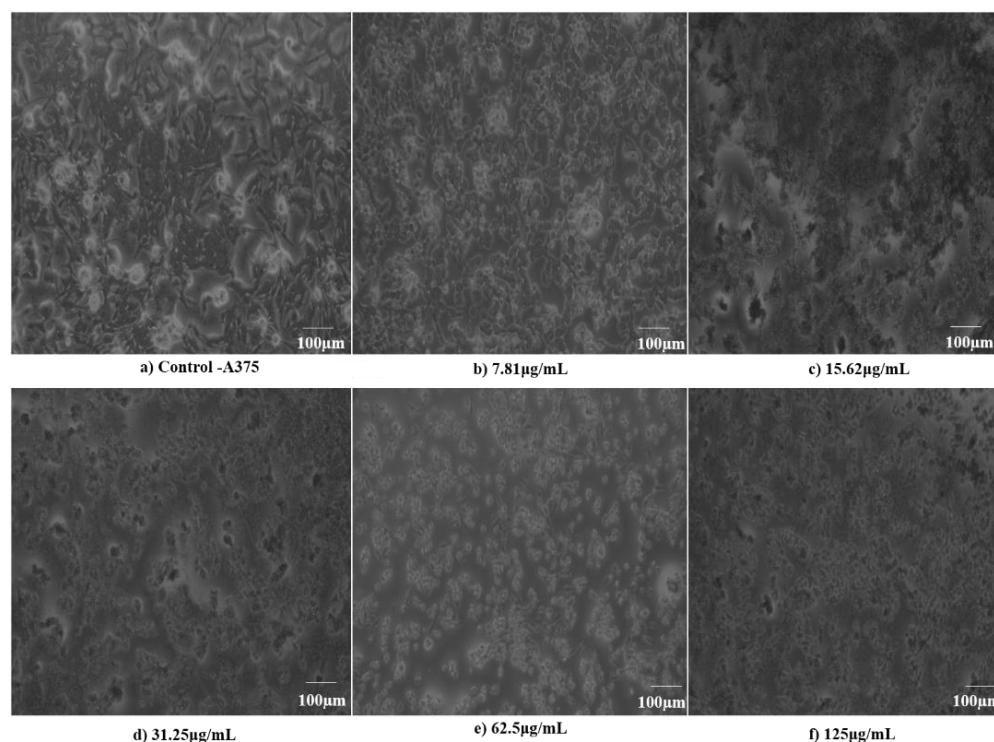


Fig.6. *In-vitro* Anti-proliferative activity of *V. neilgherrense* ethanolic leaf extract in A375 cell line using confocal microscope 340 pixels observed in treated and untreated cells for different concentrations a) Control b) 7.81 c) 15.62 d) 31.25 e) 62.51 and f) 125µg/mL and (Bar scale-100µm)

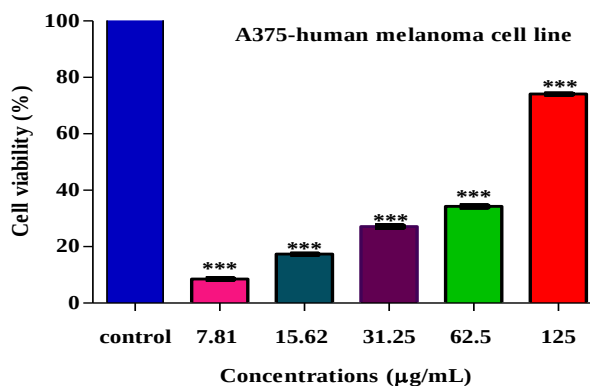


Fig. 7. MTT assay confirming the anti-proliferative activity of ethanolic leaf extract against A375 cancer cell line. The values are expressed as Mean $\pm$ SD values and analyzed by One-Way analysis of Variance (ANOVA). Asterisk indicates significant difference among treatments with respect compared to control (***P < 0.001).

In A431 cells, cell viability was reduced to 81.30% at 125 µg/mL (Fig. 8 and Fig.9).

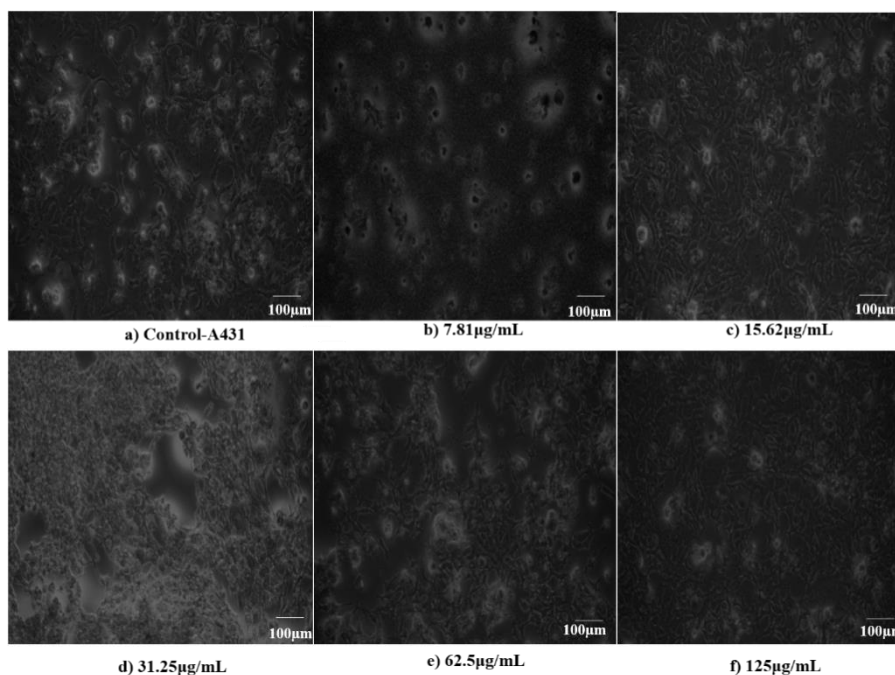


Fig.8. *In-vitro* Anti-proliferative activity of *V. neilgherrense* ethanolic leaf extract in A431 cell line using confocal microscope 340 pixels observed in treated and untreated cells for different concentrations a) Control b) 7.81 c) 15.62 d) 31.25 e) 62.51 and f) 125 µg/mL (Bar scale-100 µm)

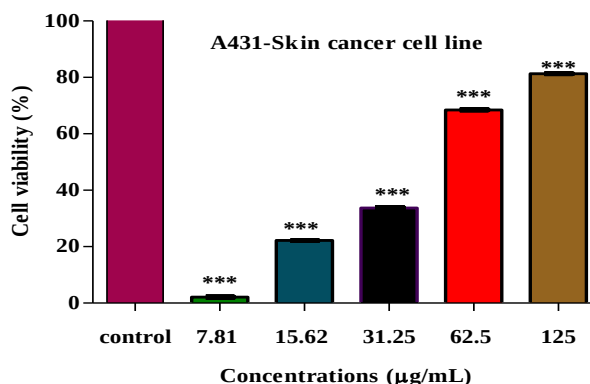


Fig. 9. MTT assay confirming the anti-proliferative activity of ethanolic leaf extract against A431 Skin cancer cell line. The values are expressed as Mean $\pm$ SD values and analyzed by One-Way analysis of Variance (ANOVA). Asterisk indicates significant difference among treatments with respect compared to control (***P < 0.001).

Because cell viability remained above 50% at the highest tested concentration, an IC_{50} value could not be reliably determined within the tested concentration range. In MCF-7 cells, treatment with 125 μ g/mL extract reduced cell viability to 66.51%, with an IC_{50} value of 93.90 μ g/mL (Figs. 10 and 11). Overall, the ethanolic leaf extract exhibited cytotoxic effects against the tested cancer cell lines, with the strongest reduction in cell viability observed in MCF-7 cells at the highest concentration tested. Further studies using a wider concentration range and appropriate concentration–response analysis are required to accurately determine the IC_{50} value for A431 cells.

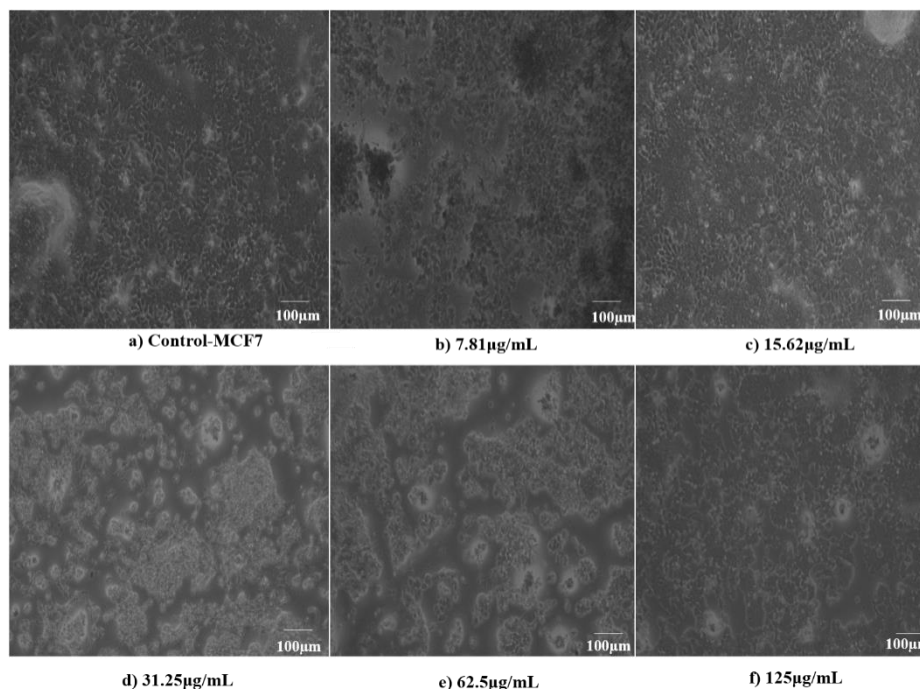


Fig. 10. *In-vitro* Anti-proliferative activity of *V. neilgherrense* ethanolic leaf extract in MCF-7 cell line using confocal microscope 340 pixels observed in treated and untreated cells for different concentrations a) Control b) 7.81 c) 15.62 d) 31.25 e) 62.51 and f) 125µg/mL (Bar scale-100µm).

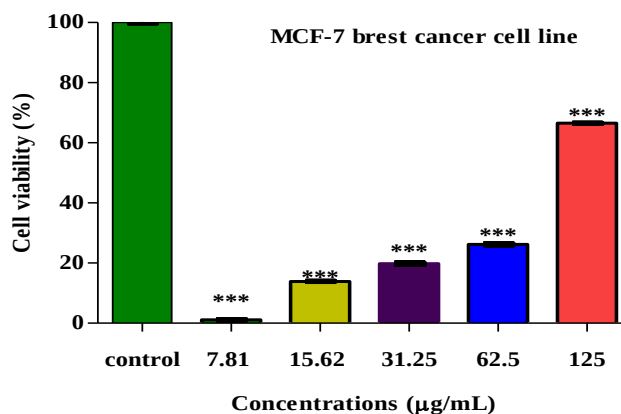


Fig. 11. MTT assay confirming the anti-proliferative activity of ethanolic leaf extract against MCF-7 cancer cell line. The values are expressed as Mean $\pm$ SD values and analyzed by One-Way analysis of Variance (ANOVA). Asterisk indicates significant difference among treatments with respect compared to control (***P < 0.001).

3.10. Pharmacokinetic Properties and Molecular Docking Study of β -Amyrin

GC-MS analysis of *Vaccinium neilgherrense* leaf extract identified β -Amyrin ($C_{30}H_{50}O$; MW 426.72 g/mol) as a major phytoconstituent, which was selected for detailed in silicoevaluation. β -Amyrin is a highly lipophilic triterpenoid with a high carbon saturation index (Fraction C_{sp^3} = 0.93), minimal rotatable bonds, and a compact structure, indicating strong molecular rigidity. The presence of a single hydroxyl group results in a low polar surface area (20.23 Å²), supporting good passive membrane permeability. These physicochemical features suggest that β -Amyrin can readily interact with biological membranes and may demonstrate selective tissue penetration. Pharmacokinetic (ADME) predictions showed low gastrointestinal (GI) absorption, implying limited oral bioavailability. Therefore, formulations such as nano encapsulation, liposomal delivery, or alternative routes may be required for optimal systemic uptake. Blood-brain barrier permeability was also predicted to be minimal, which lowers the likelihood of central nervous system-related toxicity favorable for peripherally targeted cancer therapies.

Importantly, β -Amyrin is predicted not to act as a substrate for P-glycoprotein (P-gp), suggesting minimal risk of transporter-mediated efflux. It also showed no significant inhibitory effects on key cytochrome P450 enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4), indicating a low probability of metabolic drug drug interactions. Toxicity prediction revealed a high LD₅₀ value (70,000 mg/kg), supporting a favorable safety profile. Bioavailability radar and toxicity features are illustrated in Fig. 12(a) and (b).

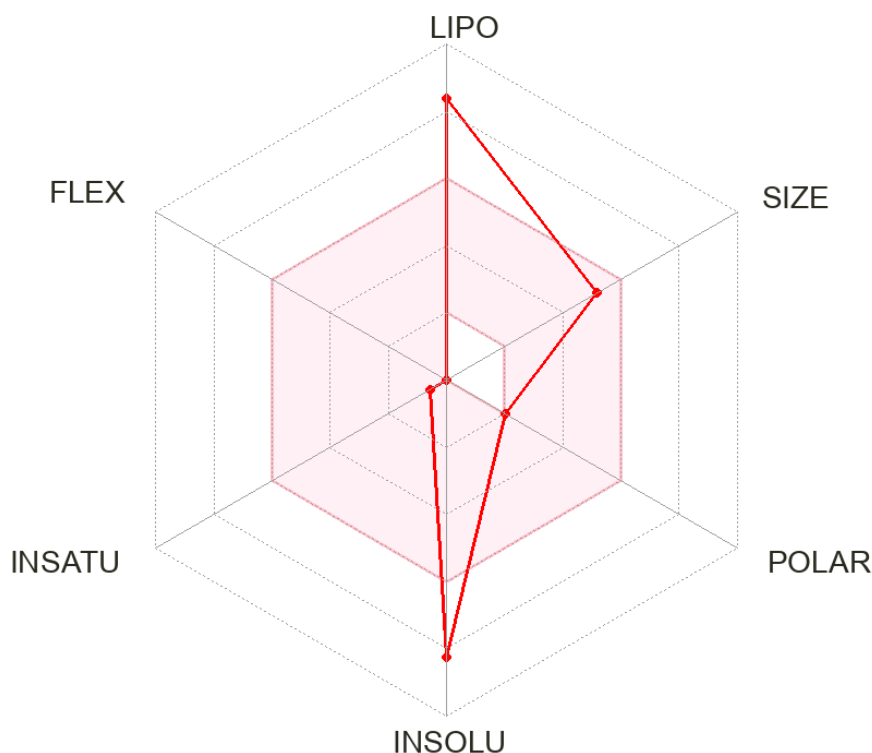


Fig. 12(a): Bioavailability Radar of Beta-amyryn (Number of red nodes within the pink region represent the high-drug likeness properties)

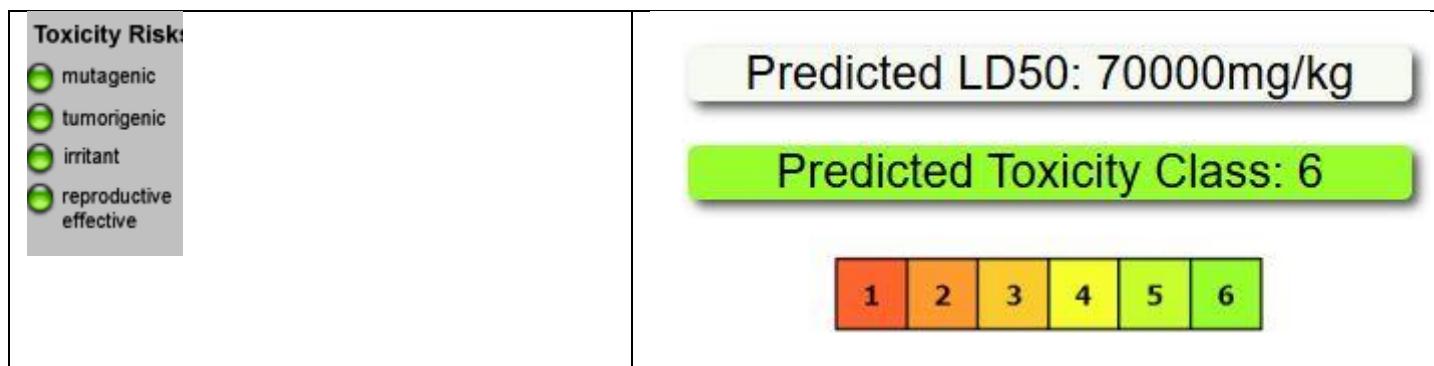


Fig 12(b): Toxicity profile of Beta-amyryn-Green color and color scale indicates -Toxic free

Molecular docking was performed to assess potential anticancer mechanisms. β -Amyrin demonstrated strong binding affinity toward the epidermal growth factor receptor (EGFR) tyrosine kinase domain (PDB ID: 1M17), with a docking score of -9.1 kcal/mol obtained using AutoDockVina. The compound formed multiple stabilizing interactions with key catalytic residues, including LEU694, GLY695, SER696, PHE699, VAL702, ALA719, LYS721, MET769, PHE771, ASP776, ARG817, ASN818, and THR830. These residues are functionally involved in EGFR-mediated signaling, suggesting that β -Amyrin may inhibit kinase activity and suppress proliferation in EGFR-overexpressing cancer cells, such as A431 epidermoid carcinoma. The docking conformation and interacting residues are presented in Fig. 13.

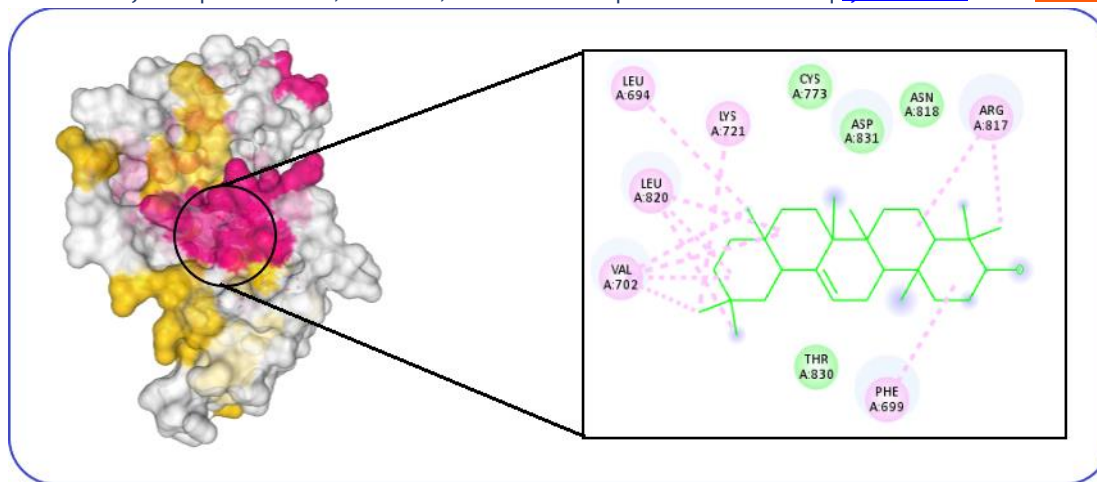


Fig. 13: Molecular docking image of Beta-amyrin towards the cancer pathway targets (PDB:1M17)

4. DISCUSSION

The genus *Vaccinium* is recognized as an important source of diverse secondary metabolites, including phenolic compounds, flavonoids, terpenoids, tannins, alkaloids, and glycosides, which may contribute to its nutritional and pharmacological properties (Pintor et al 2020). Several well-investigated species, including *Vaccinium macrocarpon* and *Vaccinium oxycoccos*, have been extensively studied for their phytochemical composition and biological activities, particularly antioxidant, antimicrobial, and cytoprotective effects (Martău et al 2023) (Oszmianański et al 2017). However, comparatively limited information is available regarding the phytochemical composition and biological potential of *V. neilgherrense*. Therefore, the present study provides useful preliminary information on the chemical profile and biological properties of its ethanolic leaf extract. The choice of extraction solvent is an important factor influencing the recovery of plant secondary metabolites. Ethanol is commonly used for extracting phenolic, flavonoid, and other moderately polar constituents because of its ability to penetrate plant tissues and solubilize a broad range of phytochemicals. Previous studies have reported substantial recovery of polyphenols and other bioactive constituents from methanolic extracts of *V. oxycoccos* and other medicinal plants (Smith 2002) (Česonienė and Daubaras 2016) (Loganathan et al 2021). In the present study, ethanol was used to obtain the leaf extract of *V. neilgherrense*, allowing the extraction of a chemically diverse group of constituents. The preliminary phytochemical screening confirmed the presence of several classes of secondary metabolites, supporting the potential contribution of these compounds to the biological activities observed in subsequent assays.

Phenolic compounds and flavonoids are among the most important plant-derived antioxidants because they can participate in hydrogen-atom donation, electron transfer, and metal-ion chelation (Ayoub et al 2010) (Zeb 2020). The relatively high total phenolic and flavonoid contents observed in the present study provide a possible chemical basis for the antioxidant activity of the *V. neilgherrense* leaf extract. GC-MS analysis further demonstrated the presence of a diverse range of chemical constituents, including long-chain hydrocarbons, fatty acids, esters, alcohols, sterols, and triterpenoids. Similar chemical diversity has been reported in ethanolic extracts of several medicinal plants using GC-MS analysis (Olszowy 2019). FTIR analysis additionally indicated the presence of characteristic functional groups corresponding to different classes of organic compounds. Such spectroscopic information supports the presence of chemically diverse constituents in the leaf extract and complements the GC-MS findings (Adeoye-Isijola et al 2021) (Alex et al 2023).

The antioxidant activity of the *V. neilgherrense* leaf extract was evaluated using DPPH, ABTS, hydroxyl radical scavenging, and FRAP assays. The use of multiple antioxidant assays is important because each method is based on a different chemical mechanism and therefore provides complementary information regarding antioxidant capacity. The extract exhibited concentration-dependent antioxidant activity, with a DPPH IC₅₀ value of 378.14 µg/mL. This value was comparable to the reported DPPH IC₅₀ of 345.41 µg/mL for

Chaptalianutans (Zahra et al 2024), although some other plant extracts, including *Nephrolepisauriculata* and *Chaerophyllumbulbosum*, have demonstrated lower IC₅₀ values of 102.00 and 59.51 µg/mL, respectively (Bruck de Souza et al 2020) (Sureshkumar and Ayyanar 2022). These differences may be associated with variation in phytochemical composition, extraction conditions, plant material, and assay methodology. The ABTS assay also demonstrated concentration-dependent radical-scavenging activity, with inhibition ranging from 57.8% to 63.5%. The observed antioxidant effects may be partly related to the phenolic and flavonoid constituents detected in the extract.

The FRAP assay further supported the reducing capacity of the *V. neilgherrense* extract. This assay measures the ability of antioxidants to reduce Fe³⁺ to Fe²⁺, providing an indication of electron-donating capacity. Similarly, the hydroxyl radical scavenging assay demonstrated the ability of the extract to neutralize highly reactive hydroxyl radicals. Hydroxyl radicals are particularly damaging because of their ability to react rapidly with cellular macromolecules. Previous work with *Knoxia sumatrensis* reported a hydroxyl radical IC₅₀ of 430.58 µg/mL, illustrating the variability in radical-scavenging activity among plant extracts (Česonienė and Daubaras 2016). Taken together, the DPPH, ABTS, hydroxyl radical, and FRAP findings indicate that *V. neilgherrense* leaves possess measurable antioxidant activity. Nevertheless, antioxidant activity observed in chemical assays should not be directly interpreted as therapeutic antioxidant efficacy in biological systems and requires further cellular and *in-vivo* validation.

The antimicrobial activity observed in the present study further indicates that the ethanolic leaf extract contains constituents capable of inhibiting selected microorganisms. Plant-derived antimicrobial activity can vary considerably depending on microbial species and the chemical composition of the extract. Differences in bacterial cell-wall structure, membrane permeability, and cellular targets may contribute to differences in susceptibility between Gram-positive and Gram-negative organisms (Maduabuchi and Awucha 2020) (Singh et al 2021). The antibacterial activity observed against the tested organisms may therefore reflect interactions between multiple phytochemicals and microbial cellular components. Fatty acids, terpenoids, phenolics, and other constituents identified by GC-MS may contribute individually or synergistically to this activity. However, isolation of individual compounds and determination of their minimum inhibitory concentrations would be necessary to establish the specific compounds responsible for the observed antimicrobial effects.

The anti-inflammatory potential of the *V. neilgherrense* leaf extract was investigated using HRBC membrane stabilization and albumin denaturation assays. These *in-vitro* models are commonly used as preliminary approaches for assessing membrane-protective and protein-stabilizing effects of plant extracts. Stabilization of the erythrocyte membrane may indicate a protective effect against membrane disruption, whereas inhibition of albumin denaturation provides an indication of potential protein-stabilizing activity. Previous studies have demonstrated similar anti-inflammatory effects for extracts of *Peltophorum pterocarpum*, *Chromolaena odorata*, and other medicinal plants (Lawal et al 2022) (Ali et al 2019) (Okeke et al 2020). In the present study, the *V. neilgherrense* extract exhibited concentration-dependent anti-inflammatory activity in the tested models. This effect may be associated with phenolic, flavonoid, and terpenoid constituents that can interact with oxidative and inflammatory processes. However, these assays provide preliminary evidence and cannot by themselves establish inhibition of specific inflammatory mediators or signaling pathways.

The cytotoxic potential of the ethanolic leaf extract was evaluated against A375, A431, and MCF-7 cancer cell lines. The extract produced a concentration-dependent reduction in cell viability, although the magnitude of the response differed among the cell lines. A375 cells showed 74.09% viability at 125 µg/mL, with an IC₅₀ value of 84.35 µg/mL. In MCF-7 cells, viability decreased to 66.51% at the same concentration, with an IC₅₀ value of 93.90 µg/mL. For A431 cells, viability was 81.30% at 125 µg/mL. Because viability remained above 50% at the highest tested concentration, the IC₅₀ value should be interpreted cautiously and should not be reported as 35.23 µg/mL unless it is supported by recalculation from the complete raw concentration–response data. These findings indicate that the extract possesses measurable cytotoxic potential, but further studies are required to determine its selectivity toward cancer cells and its underlying mechanism of action.

The cytotoxic effects observed in the present study may be associated with the combined activity of several phytochemical constituents. Plant-derived phenolics, flavonoids, terpenoids, and other secondary metabolites have been reported to influence oxidative balance, mitochondrial function, cell-cycle regulation, apoptosis, and cancer-associated signaling pathways (Kyriakou et al 2023) (Kulak et al 2026) (Gül 2026) (Slimani et al 2023) (Shende et al 2015) (Rani et al 2023). The chemical diversity identified by GC-MS in *V. neilgherrense* leaves supports the possibility that multiple constituents contribute to the observed cellular effects. However, because the present study evaluated a crude extract, a direct relationship between an individual compound and cytotoxicity cannot be established. Bioassay-guided fractionation and testing of purified compounds will therefore be necessary to identify the principal cytotoxic constituents. Among the compounds identified by GC-MS, β -amyrin is of particular interest because it is a pentacyclotriterpenoid that has been investigated for several biological activities. Previous studies have reported antimicrobial and cytotoxic properties of β -amyrin and related triterpenoids (Gül et al 2026) (Yenigun et al 2026). In the present study, molecular docking analysis indicated a favorable interaction between β -amyrin and the EGFR tyrosine kinase domain. EGFR is an important signaling molecule involved in cellular proliferation, survival, differentiation, and migration, and dysregulation of EGFR signaling is associated with the progression of several cancers. The predicted interaction of β -amyrin with EGFR therefore provides a possible molecular hypothesis for the observed cytotoxic effects. However, molecular docking represents a computational prediction and does not demonstrate direct EGFR inhibition. Experimental validation using enzyme inhibition assays, EGFR phosphorylation studies, gene or protein-expression analysis, and apoptosis-related assays would be required to confirm this proposed mechanism.

The predicted pharmacokinetic characteristics of β -amyrin may also be relevant to its future development. Although computational ADMET analysis may suggest favorable characteristics for some metabolic and transporter-related parameters, poor gastrointestinal absorption and limited systemic bioavailability may restrict the therapeutic application of β -amyrin. Such limitations could potentially be addressed through appropriate formulation strategies, including nano-encapsulation, lipid-based delivery systems, or other advanced delivery approaches (KaurKohli et al 2020) (Manda et al 2009). Nevertheless, these approaches should be considered as future development strategies rather than established therapeutic solutions based solely on the present *in-vitro* and *in-silico* findings. Overall, the present study demonstrates that the ethanolic leaf extract of *V. neilgherrense* contains diverse phytochemical constituents and exhibits antioxidant, antimicrobial, anti-inflammatory, and cytotoxic activities in the experimental models investigated. The antioxidant findings are supported by the phenolic and flavonoid contents and the results of multiple complementary assays. The GC-MS profile further identified several potentially bioactive constituents, including β -amyrin and β -sitosterol. Molecular docking provided preliminary evidence for a possible interaction between β -amyrin and the EGFR tyrosine kinase domain. However, the findings should be considered preliminary because the study was primarily based on crude-extract *in-vitro* assays and computational analysis. Further investigations involving compound isolation, non-cancerous cell lines, mechanistic cellular assays, *in-vivo* studies, toxicological evaluation, and formulation optimization are necessary to establish the biological significance and therapeutic potential of *V. neilgherrense* (Stamos et al 2002).

5. CONCLUSION

The present study demonstrates that *Vaccinium neilgherrense* leaf extract possesses a diverse phytochemical profile and exhibits measurable biological activities. The extract contained appreciable levels of phenolic and flavonoid compounds, with total phenolic and flavonoid contents of 62.3 ± 0.6 mg GAE/g and 38.9 ± 6.4 mg RE/g dry weight, respectively. GC-MS analysis identified six major phytoconstituents, supporting the chemical richness of the extract. The extract showed antibacterial activity, particularly against *Bacillus subtilis*, with a maximum inhibition zone of 15 ± 0.33 mm. It also demonstrated concentration-dependent antioxidant activity in DPPH, ABTS, hydroxyl radical scavenging, and FRAP assays, with IC_{50} values of 426.50, 420.78, 422.55, and 251.18 μ g/mL, respectively. Anti-inflammatory activity was observed through HRBC membrane stabilization and albumin denaturation assays, with IC_{50} values of 211.30 and 206.17 μ g/mL, respectively. Cytotoxicity against A375, A431, and MCF-7 cancer cell lines yielded IC_{50} values of

84.35, 35.23, and 93.90 $\mu\text{g/mL}$, respectively, with A431 cells showing the greatest sensitivity. Molecular docking further indicated that β -amyrin interacted favorably with the EGFR tyrosine kinase domain, showing a binding score of -9.1 kcal/mol. However, this docking result represents preliminary computational evidence and does not confirm EGFR inhibition. Overall, the findings support the biological potential of *V. neilgherrense* leaf extract and provide a basis for further investigation. Future studies should include mechanistic validation, MIC/MBC determination, *in vivo* efficacy, compound-level characterization, and comprehensive toxicological evaluation to establish its pharmacological relevance and safety.

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