

Research Article

Antioxidant and Antiproliferative Potentials of *Curcuma neilgherrensis* Leaf Extracts, With Emphasis on Phytochemical Profiling and Apoptosis Induction in Colorectal Carcinoma HCT116 Cells by the Chloroform Extract

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Zingiberaceae family of plants are ethnomedicinally well known. One of the less-studied endemic species from this family, *Curcuma neilgherrensis*, and its leaves have been used as a folklore medicine by tribes of Western Ghats of India since ancient times. The aim of the study is to investigate in vitro antioxidant and antiproliferative potentials of organic solvent extracts prepared from *C. neilgherrensis* leaves as well as phytochemical profiling of the selected extract capable of apoptotic induction in cancer cells. In vitro antioxidant activity was evaluated by the estimation of total phenolics and flavanoid contents, DPPH, ABTS radical scavenging, and lipid peroxidation inhibition assays. Cytotoxic/antiproliferative potential was assessed employing human cancer cell lines (K562, HCT116, MDA-MB-231, and HL60) and normal lymphocyte cultures by MTT and trypan blue dye exclusion assays. Fluorescence microscopy, flow cytometry, DNA fragmentation, and caspase activity assays were utilized to study apoptosis induction by the extract in HCT116 cells. HR-ESI-MS/MS was used to identify chemical constituents present in the extract. *C. neilgherrensis* leaves extracts were found to contain varying amounts of total phenolics and flavanoid contents as well as antioxidant activity, with the methanolic extract (CNLM) displaying maximal activity. Higher antiproliferative potential was shown by the chloroform extract (CNLC) for a 48 h treatment period, against colorectal carcinoma HCT116 cells ($IC_{50} = 8.3 \pm 0.8 \mu\text{g/mL}$). CNLC treatment caused cytomorphological alternations, phosphatidylserine externalization, reduction in mitochondrial membrane potential, increase in intracellular ROS, sub-G1 phase cell population, and DNA fragmentation, as well as caspase 3 and 9 activities, confirming apoptosis induction in HCT116 cells. Phytochemical profiling of the CNLC extract by HR-ESI-MS/MS enabled identification of bioactive compounds, previously reported with anticancer potential such as rutin, tamarixetin, embelin, ginkgolide B, traumatic acid, and rosmarinic acid. To our knowledge, this is the first report demonstrating in vitro antioxidant and antiproliferative potentials of *C. neilgherrensis* leaf extracts. The results presented reveal that *C. neilgherrensis* leaves are indeed a potent bioresource for chemotherapeutic candidate drugs for future use.

Keywords: antioxidant potential; antiproliferative activity; apoptosis; *Curcuma neilgherrensis*; phytochemicals

1. Introduction

Zingiberaceae is one of the largest families of the plant kingdom with enormous ethnomedicinal importance. The family comprising 52 genera and over 1300 species is distributed widely throughout the tropics, particularly in

Southeast Asia [1]. Significant ethnobotanical diversity of Zingiberaceae species have been found in various regions of India including Western Ghats. Plant parts, such as rhizomes and seeds of these species have been extensively used in India since ancient times as ingredients of Ayurvedic preparations and flavorings for food items [2, 3].

Zingiberaceae plants are well known for their anticancer activity. Various extracts and pure compounds reported from *Curcuma*, *Zingiber*, *Kaempferia*, *Alpinia*, *Amomum*, and *Hedychium* genera reportedly possess antineoplastic activity as evidenced by in vitro and in vivo studies. Curcumin/curcuminoids (*Curcuma longa*), zerumbone (*Zingiber zerumbet*), gingerol, and shogaol (*Zingiber officinale*) are few amongst many others [4–9]. Despite an impressive array of such compounds, a large number of hitherto unexplored potential drug candidates are yet to be isolated and analyzed from endemic members of the family. *Curcuma neilgherrensis* Wight (Nilgiri turmeric) is one among such endemic species found in Western Ghats of India [1].

C. neilgherrensis is a folklore medicinal plant widely used by the tribes of Western Ghats for the management of diabetes mellitus. Traditional medical practitioners of Kodagu District of Karnataka State have identified its usage in diabetes mellitus whilst the Palliyar tribes of Tamil Nadu State have been using its tuber for edible purposes [10, 11]. Although benefits have been claimed for this plant, scientific studies available in the literature remain scant. Phytochemical screening of this plant has revealed the presence of various secondary metabolites lending credence to its medicinal usages [10–13]. Alcoholic extracts of its rhizome have also been shown to possess antifungal activity against *Candida albicans* and *Aspergillus niger* [14]. Both aqueous and alcoholic extracts of *C. neilgherrensis* rhizome and leaves are reported to possess antibacterial effects against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* [15]. The methanolic extract of *C. neilgherrensis* rhizome have been found to have anti-inflammatory activity in an in vivo model system [16]. Antiproliferative activity induced by *C. neilgherrensis* rhizome extracts against leukemic K562, REH, Nalm6, and breast cancer MCF7 cell lines have also been reported [17]. Despite its traditional use, no report exists on bioactivities of the plant, including its anticancer potential. Therefore, the present study was undertaken mainly to explore antioxidant and antiproliferative activities of organic solvent extracts prepared from *C. neilgherrensis* leaves. Furthermore, apoptosis induction in colorectal HCT116 cancer cells by the extract, which exhibited the most potent cytotoxicity, was also investigated including its chemical composition.

2. Materials and Methods

2.1. Chemicals. Tris base, acridine orange, ethidium bromide, Folin–Ciocalteu’s reagent, ABTS (2,2’-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)), gallic acid, quercetin, dimethylsulphoxide (DMSO), petroleum ether, chloroform, acetone, and methanol (all solvents of HPLC grade) were procured from SRL Pvt. Ltd., Mumbai, India. The 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), Dulbecco’s modified Eagle’s medium (DMEM), Roswell Park Memorial Institute (RPMI)-1640 medium, penicillin, streptomycin, HiKaryo XL RPMI medium, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from HiMedia Laboratories Pvt. Ltd, Mumbai, India. The 2’,7’-Dichlorodihydrofluorescein diacetate (DCFH-DA),

Rhodamine-123, Hoechst 33258, and caspase –3 and –9 activity assay (calorimetric) kit were purchased from Sigma-Aldrich, USA. Fetal bovine serum (FBS), phosphate buffer saline (PBS), propidium iodide, trypsin-EDTA solution, and Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit were purchased from Invitrogen, USA. All other chemicals and reagents used were of analytical grade.

2.2. Plant Material and Extraction. *C. neilgherrensis* was collected from Neliyampathi (Palakkad district), Kerala State, India. The plants were authenticated by Prof. M. Sabu, Department of Botany, University of Calicut, and a voucher specimen (Accession no. 6855 CALI) was deposited in the Calicut University Herbarium. The collected plant leaves were washed well with running water, cut into small pieces, shade-dried, powdered using a grinder, and then subjected to serial extractions with organic solvents (20 g in 100 mL) of increasing polarities such as petroleum ether, chloroform, acetone, and methanol by gentle mixing on an orbital shaker for 24 h. The resulting extracts were filtered through Whatman no. 1 filter paper, and the filtrates were evaporated to remove the solvents. The remaining extract was reconstituted in DMSO to create a stock solution, which was then stored at –20°C for future use.

2.3. In Vitro Antioxidant Activity

2.3.1. Estimation of Total Phenolic Content (TPC). TPC of each extract was determined by Folin–Ciocalteu method using gallic acid as standard [18]. In brief, 200 µL of Folin–Ciocalteu’s reagent was mixed with 2.0 mL of each extract (1.0 mg/mL) and allowed to react for 10 min at room temperature. Subsequently, 300 µL of 15% Na₂CO₃ solution was added, and the mixtures were left to incubate at 25°C for 2 h. The absorbance was then measured at 765 nm using a UV-visible spectrophotometer (Lambda 25, Perkin Elmer, UK). TPC of the extracts was calculated and expressed as gallic acid equivalents (GAEs).

2.3.2. Estimation of Total Flavonoid Content (TFC). The TFC of individual plant extracts was determined as described previously in [19]. In brief, 0.25 mL of each extract (1.0 mg/mL, dissolved in the respective solvent) and quercetin standard solution (ranging from 0.005 to 1.0 mg/mL) were mixed with 0.25 mL of distilled water in a test tube. To this, 75 µL of 5% (w/v) sodium nitrite solution was added. After 6 min, 150 µL of 10% (w/v) aluminum chloride solution was introduced, and the mixture was allowed to sit for another 5 min. Then, 0.5 mL of 1 M NaOH was added, and the volume was adjusted to 2.0 mL with distilled water. The mixture was thoroughly mixed, and the absorbance was measured immediately at 510 nm. The TFC of each extract was calculated and expressed as quercetin equivalents (QEs).

2.3.3. DPPH Scavenging Activity. The DPPH radical scavenging activity of the extracts was assessed using ascorbic acid as the positive control, following a method outlined in

a previous study [18]. In brief, 1.0 mL of a 100 mM methanolic DPPH solution was combined with an equal volume of each extract (concentrations ranging from 0.05 to 4.0 mg/mL). The mixture was incubated at 25°C for 30 min in the

dark. Absorbance was then measured at 517 nm using a spectrophotometer (Lambda 25, Perkin Elmer, UK). The radical scavenging activity was calculated using the following formula:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100. \quad (1)$$

2.3.4. ABTS Radical Scavenging Activity. ABTS cation (ABTS^{•+}) scavenging activity was adapted from Khan et al. [19] and Nitha et al. [20]. The ABTS^{•+} stock solution was prepared by combining equal volumes of a 7 mM ABTS solution and a 2.45 mM potassium persulfate solution, followed by 12 h of incubation in the dark at room temperature to generate the dark-colored ABTS^{•+}. A working solution was freshly prepared before each test by diluting the stock with 50% methanol to achieve an initial absorbance of approximately 0.700 (±0.02) at 745 nm, maintained at 30°C.

To measure free radical scavenging, 100 µL of various extract concentrations (0.05–4.0 mg/mL) was added to 1.0 mL of the ABTS working solution. The reduction in absorbance was recorded at 745 nm (Lambda 25, Perkin Elmer, UK) precisely 1.0 min after mixing, with the final reading taken up to 6 min later. Ascorbic acid served as the positive control. The scavenging activity was then calculated based on the percentage of ABTS radicals neutralized using the following formula:

$$\text{ABTS}^{\bullet+} \text{ scavenging activity (\%)} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100. \quad (2)$$

2.3.5. Lipid Peroxidation Activity. A modified thiobarbituric acid-reactive substances (TBARSs) assay was used to quantify lipid peroxides, utilizing egg yolk homogenates as a lipid-rich medium [21]. In this process, malondialdehyde (MDA), a byproduct of polyunsaturated fatty acid (PUFA) oxidation, reacts with two thiobarbituric acid (TBA) molecules, forming a pink chromogen with a peak absorbance at 532 nm. Egg yolk homogenate (0.25 mL), prepared as 10% v/v in distilled water, was mixed with 50 µL of the extract and made up to 500 µL with distilled water. To this, 25 µL of 0.07 M FeSO₄ was added and the mixture was incubated for

30 min to induce lipid peroxidation. Afterward, 750 µL of 20% acetic acid (pH 3.5), 0.8% TBA (in 1.1% sodium dodecyl sulfate), and 25 µL of 20% trichloroacetic acid were added, thoroughly mixed, and heated in a boiling water bath for 1 h. Once cooled, 3.0 mL of 1-butanol was introduced to each sample and centrifuged at 3000 rpm for 10 min. Absorbance of the upper organic phase was then measured at 532 nm against a 3.0 mL butanol blank. In the negative control, distilled water (50 µL) replaced the extract. Lipid peroxide inhibition was determined by calculating the percentage decrease using the following formula:

$$\text{Lipid peroxidation inhibition (\%)} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100. \quad (3)$$

2.4. Cell Lines and Culture Conditions. The cell lines used in the present study were chronic myelogenous leukemia-K562, acute promyelocytic leukemia-HL60, colorectal carcinoma-HCT116, and breast cancer-MDA-MB-231 purchased from the National Center for Cell Science (NCCS), Pune, India. Suspension type K562 and HL60 cells were maintained and propagated in RPMI 1640 medium, whilst adherent HCT116 and MDA-MB-231 cells were cultured in DMEM. Both media types were supplemented with 10% (v/v) FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin. Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. For the leukemic cell lines, K562 and HL60, propagation involved centrifuging the culture and then resuspending approximately 1 × 10⁵ viable cells/mL

in fresh medium. Adherent cells were subcultured upon reaching 80% confluence, after detaching with a 0.25% (w/v) trypsin-0.53 mM EDTA solution.

2.5. Isolation and Culturing of Human Peripheral Blood Lymphocytes (hPBLs). The hPBLs, which can be cultured inexpensively, have routinely been used as a model system to assess the cytotoxicity of various chemical and physical agents including potential drugs. In brief, the procedure for isolating and culturing hPBLs included the following steps: 5.0 mL of human blood was mixed with an equal volume of phosphate-buffered saline (PBS) and carefully layered over 6.0 mL of Hi-Sep lymphocyte separation medium (LSM) in a centrifuge tube. This mixture was then centrifuged at

400 ×g for 30 min at room temperature. The buffy coat, found at the interface, was collected with a sterile Pasteur pipette and washed twice with PBS. Finally, the cells were cultured by transferring them into HiKaryo XL RPMI medium and incubating at 37°C to promote growth.

2.6. Ethical Statement. The blood samples used for the hemolysis assay and for isolating lymphocytes in the lymphocyte culture and MTT assay were voluntarily donated by the first author (Soumya Tumbath) and the second author (Lakshmipriya T.) of this manuscript. According to the National Ethical Guidelines for Biomedical and Health Research Involving Human Participants, published by the Indian Council of Medical Research (ICMR), New Delhi, India, ethical approval was not required for the use of self-donated blood by researchers. The guidelines state that research involving less than minimal risk is exempt from ethical review.

2.7. Cytotoxicity Assessments

2.7.1. MTT Assay. Cytotoxicity/antiproliferative activity determination was based on the reduction of yellow colored, water soluble dye, MTT. In this assay, MTT is reduced by

mitochondrial dehydrogenases to purple colored formazan crystals solubilized in DMSO and analyzed spectrophotometrically [22]. In brief, for K562 and HL60, cells were seeded at a density of 2×10^4 /well, whilst 1×10^4 cells/well were taken for each of HCT116 and MDA-MB-231 cell types into 96-well microtiter plates and incubated for overnight. hPBLs, which served as the normal control cells, were seeded at a density of 1×10^5 cells/mL into 5.0 mL HiKaryo XL RPMI medium and incubated at 37°C for 48 h. Both normal and cancer cell lines were treated with various concentrations of the extracts (ranging from 2.5 to 100 µg/mL) prepared from a stock solution in DMSO. The cells were incubated with these extracts for 24, 48, and 72 h. For the control group, appropriate amounts of DMSO (vehicle control) were added to match the conditions. After each treatment period, MTT was added to each well of a 96-well microplate, reaching a final concentration of 500 µg/mL. The plates were then incubated in the dark at 37°C for 3–4 h. Following incubation, the MTT solution was removed, and the formazan crystals, dissolved in DMSO, were measured for absorbance at 570 nm using a microplate reader (Multiscan EX, Thermo Scientific, USA). Cell viability was determined by comparing the absorbance of treated cells to that of untreated control cells using the following formula:

$$\text{Percentage cell viability} = \frac{(\text{absorbance}_{\text{sample}} - \text{absorbance}_{\text{blank}})}{(\text{absorbance}_{\text{control}} - \text{absorbance}_{\text{blank}})} \times 100. \quad (4)$$

2.7.2. Trypan Blue Dye Exclusion Assay. In addition to the cytotoxicity evaluation by the MTT assay, the viability of cells treated with the extract(s) was also determined using the trypan blue dye exclusion method. For this, 1×10^5 cells/mL of each cell line, K562, HL60, HCT116, and MDA-MB-231, were seeded into 24-well plates and cultured overnight. Cells were then treated with the individual extracts for 24, 48, and 72 h. After each treatment period, the cells were collected, rinsed with PBS (pH 7.4), stained with 0.4% trypan blue solution, and counted using a hemocytometer. Live cells with intact cell membranes exclude trypan blue dye, whereas dead cells lacking the same are stained blue [23].

2.8. Clonogenic Assay. HCT116 cells were exposed to the CNLC extract for 48 h, then collected by trypsinization, rinsed with PBS (pH 7.4), and counted. Aliquots of 500 cells were plated in 6-well plates with complete medium. After 10 days of incubation, the colonies were stained with 0.5% (w/v) crystal violet and manually counted [24].

2.9. Cytomorphological Observations. Cytomorphological changes induced by the CNLC extract on HCT116 cells, if any, were observed and photographed using light (phase contrast), fluorescence, and scanning electron microscopy. HCT116 cells were seeded in a 12-well plate at a density of

1×10^5 cells/mL, grown overnight, and then treated with different concentrations: sublethal (5 µg/mL), IC_{50} (8.3 µg/mL), and overlethal (25 µg/mL) of the CNLC extract for 48 h. The cells were then harvested and stained accordingly. For field-emission scanning electron microscopy, the control and treated cells were collected following trypsinization, washed with ice cold PBS, fixed in 4% glutaraldehyde prepared in PBS, centrifuged and washed twice with ice cold PBS. They were then dehydrated by passing through graded ascending acetone series (75%–85%–100%), and observed under a CARL-Zeiss Gemini 300 scanning electron microscope as described by Naqvi et al. [25].

2.9.1. Hoechst 33258 Staining. Nuclear condensation in apoptotic cells can be distinguished from the intact nuclear morphology of healthy cells by virtue of staining with fluorescent, DNA binding, Hoechst 33258 dye [26, 27]. The HCT116 control and treated cells were trypsinized, washed twice with PBS, and resuspended in Hoechst 33258 staining solution (1.0 mg/mL). After 10 min incubation at room temperature in the dark, the cells were examined for nuclear morphology using a fluorescence microscope.

2.9.2. Acridine Orange-Ethidium Bromide (AO-EB) Dual Staining. Morphological changes associated with apoptosis induction were assessed by dual staining with AO-EB [28].

Cells were collected, rinsed with ice-cold PBS (pH 7.4), and adjusted to a density of 1×10^5 cells/mL in PBS. AO-EB solution (1:1, v/v) was then added to the cell suspension at a final concentration of 100 $\mu\text{g/mL}$, and cytomorphological changes were observed under a fluorescence microscope [27].

2.10. Apoptosis Assays

2.10.1. Assessment of Phosphatidylserine (PS) Externalization. PS, which is normally found on the inner leaflet of the plasma membrane bilayer, is translocated to the cell surface at the onset of apoptosis [29]. This externalization of PS in treated cells was assessed by staining with Alexa Fluor 488-conjugated annexin V and propidium iodide (PI). In brief, the harvested control and treated HCT116 cells were washed with ice cold PBS, resuspended in 1x annexin V-binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; pH 7.4), followed by staining with 5 μL of annexin V and 10 μL of PI solution, and incubation for 10 min at room temperature in the dark. The cells were then mounted on slides, and the images were captured under fluorescence microscope. The result was also analyzed using BD FACS ARIA II flow cytometer and BD FACS Diva software 6.1.3.

2.10.2. Assessment of Intracellular Reactive Oxygen Species (ROS). Intracellular ROS levels induced by extract treatment were studied by using the cell permeable fluorescent probe, DCFH-DA [30]. In brief, control and treated cells were incubated with 10 μM DCFH-DA for 30 min at 37°C, followed by two washes with PBS [27, 31]. ROS levels were subsequently measured using a fluorescence microscope and a BD FACS ARIA II flow cytometer equipped with BD FACS Diva software Version 6.1.3.

2.10.3. Assessment of Mitochondrial Membrane Potential ($\Delta\psi\text{M}$). Early apoptosis is accompanied with a rapid collapse in the electrochemical gradient across the mitochondrial membrane which affects the $\Delta\psi\text{M}$. The $\Delta\psi\text{M}$ in both control and treated cells was assessed by staining with the lipophilic cationic dye rhodamine 123 (5 $\mu\text{g/mL}$) for 30 min at 37°C [32]. Following staining, the cells were rinsed with PBS, centrifuged, and then resuspended in PBS prior to being placed on slides for fluorescence microscopy. In addition, flow cytometric analysis of rhodamine 123-stained cells was conducted utilizing BD FACS ARIA II flow cytometer equipped with BD FACS Diva software Version 6.1.3.

2.11. Cell Cycle Analysis. Cell cycle distribution was quantified based on the DNA content, identifying the percentage of cells in sub-G0/G1, G0/G1, S, and G2/M phases. For cell cycle analysis, HCT116 cells treated with the extract, along with untreated controls, were harvested separately via trypsinization, washed twice with PBS, fixed in 70% ice-cold ethanol, and stored at -20°C until further use. Prior to data acquisition BD FACS-ARIA II cytometer with BD FACS

Diva software Version 6.1.3, the cells were stained with 20 $\mu\text{g/mL}$ PI solution containing 200 $\mu\text{g/mL}$ RNase A (DNase-free) for 30 min [33].

2.12. DNA Fragmentation Assay. The treated and untreated cells were washed with PBS, resuspended in lysis buffer (20 mM Tris; pH 8.0, 10 mM EDTA, and 0.2% Triton X-100), and incubated for 15 min at room temperature. The cell lysates were centrifuged, treated with proteinase K (200.0 $\mu\text{g/mL}$), and incubated for 90 min at 37°C. Later, RNase A (0.5 mg/mL) was added followed by incubation for 2 h at 37°C. DNA was then extracted with phenol: chloroform: isoamyl alcohol (25:24:1) and precipitated by 3 volumes of ice-cold ethanol containing 0.3 M sodium acetate at -20°C overnight. The DNA pellet was washed with 70% alcohol, dissolved in Tris-EDTA buffer (pH 8.0), and electrophoresed on 1.8% agarose gel at 50 V. The ethidium bromide-stained DNA profiling was carried out on the AlphaImager 2200 gel documentation system using AlphaEaseFC software.

2.13. Caspase 3 and 9 Activity Assay. Caspase 3 and 9 activities were assessed using the colorimetric assay kit (Sigma-Aldrich, USA) according to manufacturer's protocol. In brief, control and treated cells were harvested and centrifuged at 600 $\times g$ and the pellets were incubated in ice-cold lysis buffer (250 mM HEPES, pH 7.4, 25 mM CHAPS, and 25 mM DTT) for 15 min, then they were centrifuged at 16,000 $\times g$ for 20 min. Supernatants were retrieved and aliquots corresponding to 50 μg total protein were incubated along with acetylated tetrapeptide Ac-DEVD-p-NA substrate (2 mM), for caspase 3 assay, or Ac-LEHD-p-NA substrate (2 mM), for caspase 9 assay (substrates labeled with the chromophore p-nitroaniline [p-NA]), in the presence of each caspase assay buffer in a 96-well microtiter plate. In the presence of active caspase 3 and 9 in the supernatant, cleavage, and release of p-NA from the substrate occurs. Free p-NA produces a yellow color detected spectrophotometrically at 405 nm against a blank containing assay buffer (200 mM HEPES, pH 7.4, 1% CHAPS, 50 mM DTT, and 20 mM EDTA) and substrate but without cell lysates/supernatant. A standard curve was utilized to determine the correspondence between absorbance and p-NA concentration and results were then expressed as caspase 3 and caspase 9 specific activity (μmol p-NA per min/mL protein) calculated as indicated by the manufacturer.

2.14. High Resolution-Liquid Chromatography-Mass Spectrometry (HR-ESI-MS/MS) Analysis. Liquid chromatography followed by HR-ESI-MS/MS of the CNLC extract was performed at SAIF, IIT Bombay, India. The analysis was done using the 1290 Infinity UHPLC system, 1260 infinity Nano HPLC with Chipcube with 6550 iFunnel Q-TOF (Agilent Technologies, USA). ZORBAX C-18 column (2.1 $\times$ 50 mm, 1.8 μm) was used to carry out chromatographic separation analysis. The mobile phases consisted of 0.1% formic acid in water (A) and 90% acetonitrile, 10%

water with 0.1% formic acid (B) with a binary gradient elution of 5%–100% B at 0–30 min at a flow rate of 0.3 mL/min. Electrospray ionization (ESI) was used as a source in operating positive mode and m/z scan range as 60–1000 Da. Data acquisition and analysis were performed using Agilent LC-MS-QTOF Mass-Hunter data acquisition software Version B.03.01.

2.15. Statistical Analysis. All experimental data are expressed as the mean $\pm$ SD from three biological replicates. Results were analyzed for significance by one-way ANOVA using SPSS software Version 16.0.

3. Result and Discussion

3.1. In Vitro Antioxidant Potential. The high reactivity of most oxygen containing free radicals, ROS, are capable of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids, thereby causing homeostatic imbalance, oxidative stress, cellular injury, and pathogenesis including cancer [34].

Plant phenolics and flavanoids are important secondary metabolites, which can provide health benefits by conferring protection against ROS action by scavenging these harmful free radicals [35]. TPC and TFC of *C. neilgherrensis* leaf extracts are shown in Table 1. CNLM was found to possess maximum phenolics (53.70 ± 1.27 mg GAE/g extract) and flavanoid (42.17 ± 1.76 mg QE/g extract) contents, whilst CNLP possessed lower amounts. TPC and TFC of various extracts, in the descending order, were found to be CNLM > CNLA > CNLC > CNLP with significant ($p < 0.05$) differences amongst themselves. There are several reports on TPC, TFC, as well as antioxidant potential of leaf extracts of various Zingiberaceae species [36–38]. TPC of methanolic extracts of *C. longa* leaves were found to be 20.13 ± 0.05 mg GAE/g extract [39] whilst that of aqueous extracts were reported with TPC of 3.65 ± 0.02 mg GAE/g extract and TFC of 4.99 ± 0.19 mg QE/g extract [40]. Atiqur Rahman et al. [41] reported that among the organic solvent extracts of *C. zedoaria* leaves, TPC was higher in the ethyl acetate extract (141.31 ± 1.5 GAE mg/g extract), followed by methanol and chloroform and the lowest value was exhibited by the nonpolar hexane extract. Similarly, TPC and TFC of ethanolic extracts prepared from *C. zedoaria* leaves were found to be 125.75 ± 0.17 mg of GAE and 6.12 ± 0.23 mg QE/g of extract, respectively [42]. Rhizome extracts of the common Zingiberaceae species such as *C. longa* and *Z. officinale* were also reported to possess varying amounts of phenolic and flavanoids, which in turn is dependent on factors such as the geographical location of the plant as well as the methodology of extraction [43, 44].

Plant-based antioxidants have attracted global attention as they have a multitude of applications in areas of food chemistry, food biology, natural plant chemistry, medicinal plants, and biochemistry. There are different types of antioxidant assays, each with its own specific target, advantages, and disadvantages. It is imperative to quantitate the antioxidant potential of each sample by employing more than

one method so as to rule out any ambiguity in the results obtained [45]. This is the first report on in vitro antioxidant potential of *C. neilgherrensis* leaf extracts, which was determined using three different methods: DPPH, ABTS, and lipid peroxidation inhibition assays.

In our study, all extracts showed DPPH and ABTS radical scavenging activities in a dose-dependent manner, which varied among different extracts. The results are expressed in IC_{50} values (Table 1). The highest DPPH scavenging activity was shown by the CNLM extract with an IC_{50} value of 0.188 mg/mL, followed by that of CNLA, CNLC, and CNLP. Highest ABTS radical scavenging activity was observed with CNLA (IC_{50} —0.347 mg/mL), followed by CNLM, CNLC, and CNLP. Here, it is relevant to distinguish between the ABTS and DPPH methods in that the former—based on generation of a blue/green $ABTS^{+}$ (detected at 734 nm far from the visible region)—is applicable to both hydrophilic and lipophilic antioxidant systems, whereas the latter uses DPPH radical absorbing at 517 nm soluble in organic media is applicable only to hydrophobic systems [46]. Hence, it would be logical to conclude that the CNLA extract contains more of hydrophilic antioxidants, whilst CNLM is enriched with hydrophobic compounds.

Overexpression of ROS can cause damage to esterified PUFAs in biomembranes. The products of this lipid degradation and decomposition are conjugated dienes and aldehyde end-products, which can cause oxidative stress to the cells [47]. MDA is one among such end product of lipid peroxidation and its reaction with TBA yields an intensely chromogenic, fluorescent, red adduct, which can be used for evaluation of oxidative degradation of lipids [48]. A modified TBARS-based method was used to measure lipid peroxidation inhibition by *C. neilgherrensis* leaf extracts; the results expressed in IC_{50} values are given in Table 1. The IC_{50} values revealed that the methanolic (0.993 ± 0.004 mg/mL) and chloroform (1 ± 0.001 mg/mL) extracts possessed the highest lipid peroxidation inhibition potential whilst acetone and petroleum ether extracts showed the least activity.

Later, HPTLC analysis of all these four extracts was also carried out to generate a chromatographic fingerprint of phytochemicals in these extracts and to enable the quantification of chemical markers present in them (Supporting Figure 1 and Supporting Table 1).

3.2. Antiproliferative Activities. Cytotoxic/antiproliferative activity of different extracts of *C. neilgherrensis* on K562, HCT116, MDA-MB-231, and HL60 cancer cell lines were evaluated using MTT assay. The cells were exposed to the extracts for 24, 48, and 72 h duration and results expressed in IC_{50} values are given in Table 2. CNLC and CNLA extracts were found to possess higher antiproliferative activity against different cancer cell lines. CNLM was also found to inhibit proliferation of HCT116 and HL60 cells, whilst CNLP was found to be ineffective ($IC_{50} > 100$ μ g/mL) against all cell lines tested. Colorectal HCT116 cancer cells were found to be the most sensitive cell line with lowest IC_{50} values for CNLC (8.3 ± 0.8 μ g/mL) and CNLA (9.8 ± 0.1 μ g/

TABLE 1: Percentage yield, total phenolic content, total flavanoid content, and antioxidant potential of *C. neilgherrensis* leaf extracts.

Extracts	Yield (w/w)	Total phenolic content (mg GAE/g extract)	Total flavanoid content (mg QE/g extract)	Antioxidant activity: IC ₅₀ values (mg/mL)		
				DPPH scavenging activity	ABTS scavenging activity	Lipid peroxidation inhibition activity
<i>C. neilgherrensis</i> leaf petroleum ether (CNLP)	0.46	7.93 ± 0.90	13.10 ± 0.79	> 4	3.106 ± 0.005	> 4
<i>C. neilgherrensis</i> leaf chloroform (CNLC)	0.24	11.70 ± 1.25	32.17 ± 1.89	1.9 ± 0.004	0.817 ± 0.004	1 ± 0.001
<i>C. neilgherrensis</i> leaf acetone (CNLA)	1.59	43.17 ± 2.47	33.07 ± 0.90	0.404 ± 0.004	0.347 ± 0.003	3.997 ± 0.002
<i>C. neilgherrensis</i> leaf methanol (CNLM)	2.78	53.70 ± 1.27	42.17 ± 1.76	0.188 ± 0.004	0.370 ± 0.002	0.993 ± 0.004

Note: Data expressed as the mean of triplicates ± SD (*p* < 0.05).
Abbreviations: GAE, gallic acid equivalent; QE, quercetin equivalent.

TABLE 2: IC₅₀ values for *C. neilgherrensis* leaf extracts against different human cancer cell lines for 24, 48, and 72 h treatment periods by MTT assay.

Cell lines	Time period (h)	IC ₅₀ values obtained for <i>C. neilgherrensis</i> leaf extract (μg/mL)			
		CNLP	CNLC	CNLA	CNLM
K562	24	> 100	50.0 ± 0.3	55.0 ± 1.3	> 100
	48	> 100	46.3 ± 1.2	43.8 ± 0.8	> 100
	72	> 100	38.0 ± 2.9	38.5 ± 0.8	> 100
HCT116	24	> 100	83.0 ± 3.3	25.0 ± 4.2	> 100
	48	> 100	8.3 ± 0.8	9.8 ± 0.1	23.5 ± 1.28
	72	> 100	24.8 ± 1.0	26.0 ± 2.2	70.6 ± 0.8
MDA-MB-231	24	> 100	> 100	> 100	> 100
	48	> 100	29.8 ± 3.9	17.4 ± 0.7	> 100
	72	> 100	21.0 ± 0.9	8.4 ± 0.8	> 100
HL60	24	> 100	30.3 ± 0.8	38.5 ± 1.26	80.0 ± 1.14
	48	> 100	24.8 ± 0.5	39.3 ± 1.9	80.0 ± 2.25
	72	> 100	38.1 ± 1.0	22.0 ± 0.8	> 100

Note: Data expressed as the mean of triplicates ± SD (three biological replicates). Values highlighted in bold font qualify NCI criteria for plant-derived anticancer agents (IC₅₀ < 30 μg/mL).

mL) for a 48 h treatment period followed by cell lines, MDA-MB-231 > HL60 > K562. Breast cancer cell line MDA-MB-231 was also found to more sensitive toward CNLA extract with an IC₅₀ value of 8.4 ± 0.8 μg/mL for the 72 h treatment period. Time-dependent cytotoxicity was less evident in CNLC-treated HCT116 cells. Since *C. neilgherrensis* extracts displayed higher cytotoxic potential at 48 h of treatment period, and trypan blue dye exclusion assay was also carried out for 48 h against all four cell lines tested. IC₅₀ values determined are given in Table 3, which corroborated well with the results of the MTT assay. In accordance with the guidelines of American National Cancer Institute (NCI), an IC₅₀ < 30 μg/mL of crude extract defines the concentration for a potential anticancer agent [49]. Here, cytotoxicity assays revealed that the *C. neilgherrensis* leaf extracts, specifically chloroform and acetone extracts, possessed antiproliferative potential against various cancer cell lines and can be harnessed as a future source of potent chemotherapeutic agents. To our knowledge, this is the first report on cytotoxic/antiproliferative potential of *C. neilgherrensis* leaf extracts. Based on the results of initial cytotoxicity assays, CNLC, which showed the highest activity against colorectal carcinoma HCT116 cells, was selected for further experiments to better understand the molecular mechanisms underlying cancer cell death induced by this extract. In addition, according to this initial cytotoxicity assessment (Supporting Figure 2), concentration ranges of 5 μg/mL (sublethal), 8.3 μg/mL (IC₅₀ concentration obtained from the MTT assay), and 25 μg/mL (overly lethal) with a 48 h treatment regimen were chosen for all experiments.

In addition, a hemolysis assay was conducted for all extracts. Erythrocyte membrane damage leads to hemoglobin leakage, indicating cytotoxicity, and can be measured spectrophotometrically as a percentage of hemolysis. Hemolysis below 10% is considered nonhemolytic, while values above 25% are hemolytic, making the hemolysis assay a useful safety indicator for parenteral formulations [50].

TABLE 3: IC₅₀ values for *C. neilgherrensis* leaf extracts against different human cancer cell lines for 48 h treatment period by trypan blue dye exclusion assay.

Cell lines	IC ₅₀ values obtained for <i>C. neilgherrensis</i> leaf extract (μg/mL)			
	CNLP	CNLC	CNLA	CNLM
K562	> 100	52 ± 2.1	47.5 ± 1.3	> 100
HCT116	> 100	9.3 ± 0.5	10.5 ± 1.5	25.8 ± 2.3
MDA-MB-231	> 100	31.3 ± 0.8	17 ± 2.0	> 100
HL60	> 100	24 ± 1.3	38.5 ± 0.5	84.5 ± 3.5

Note: Data expressed as the mean of triplicates ± SD. Values highlighted in bold font qualify NCI criteria for plant-derived anticancer agents (IC₅₀ < 30 μg/mL).

Interestingly, all extracts showed negligible hemolysis (< 10%) up to a concentration of 30 μg/mL, which meet the NCI criteria for anticancer drug development. However, at the highest concentration tested (100 μg/mL), all extracts except CNLC (8%) showed hemolysis above 10%, in the decreasing order: CNLM > CNLP > CNLA > CNLC, indicating the relative safety of the CNLC extract (Supporting Figure 3).

hPBLs is an easily available normal cell, which can be cultured inexpensively and have been routinely used as a model system to assess the cytotoxicity of various chemical and physical agents including potential drugs [51]. MTT assay performed with cultured hPBLs, following treatment with CNLC at different concentrations (5, 10, and 25 μg/mL), revealed absence of cytotoxicity on normal hPBLs (Figure 1), thereby attesting to selective killing potential of CNLC extract against cancer cells.

Clonogenic or colony formation assay was used to determine the loss of cell's capability to form a colony by reproduction following treatment with ionic radiations/cytotoxic agents [24]. CNLC-treated HCT116 cells exhibited a concentration dependent reduction in colony number compared with the untreated and vehicle (0.5% DMSO)-treated control cells (Figure 2), which was clearly indicative of the extract's ability to affect cell's reproductive ability.

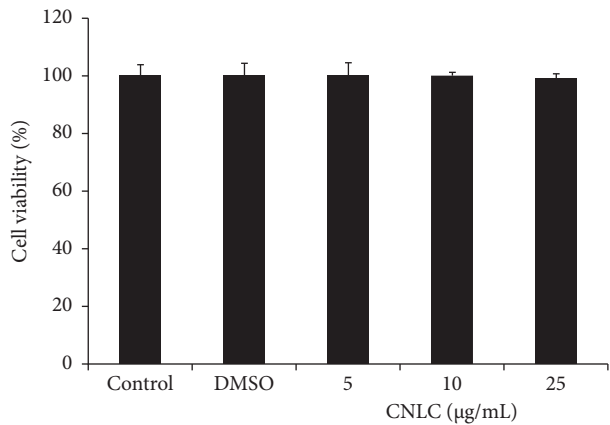
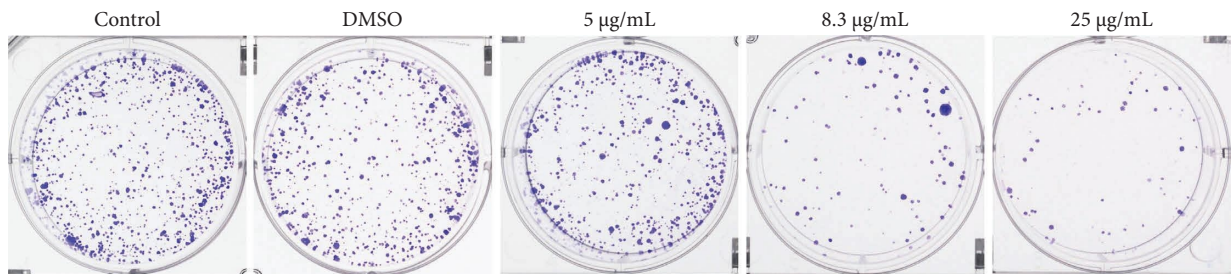
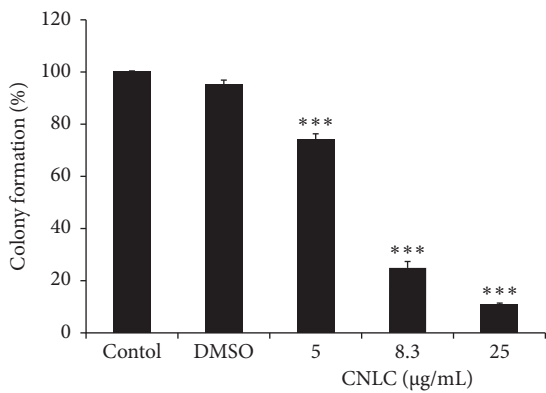


FIGURE 1: Cytotoxicity assessment of CNLC extract against normal hPBLs by MTT assay. Values represent the mean ± SD of three biological replicates; $p < 0.05$.

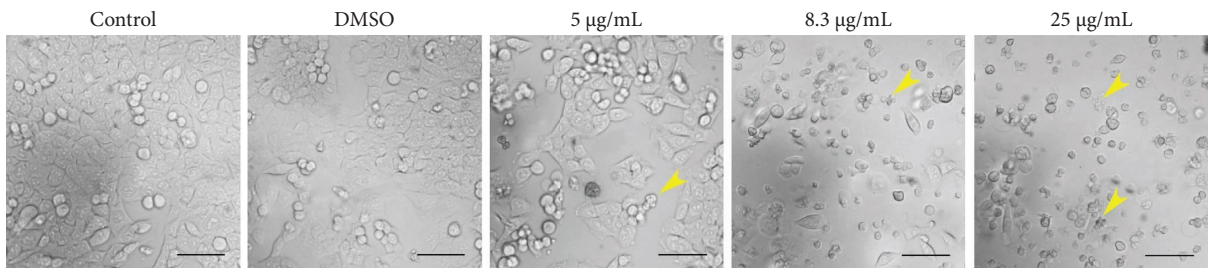


(a)



(b)

FIGURE 2: Clonogenic assay of HCT116 cells treated with CNLC extract. (a) Colonies stained with crystal violet and (b) histogram showing significant percentage reduction in number of colonies compared with untreated and vehicle (DMSO)-treated controls. Data represent the mean ± SD of three biological replicates, *** $p < 0.001$.



(a)

FIGURE 3: Continued.

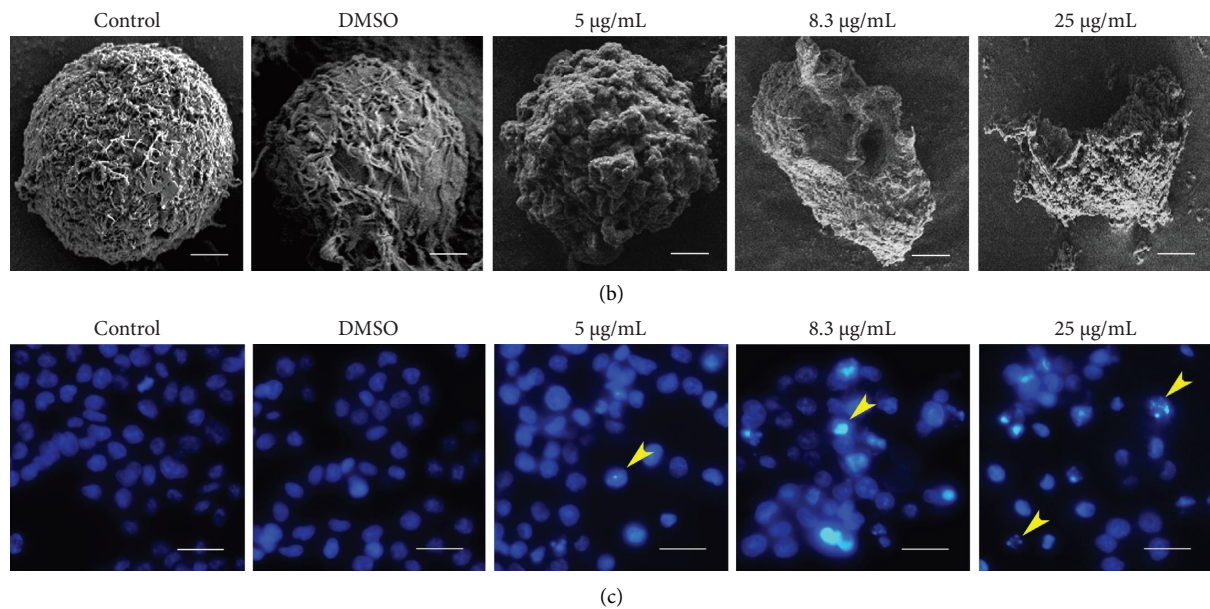


FIGURE 3: Cytomorphological changes in HCT116 cancer cells following 48 h treatment with CNLC extract. (a) Light microscopic images, (b) field-emission scanning electron microscopic images, and (c) fluorescence microscopic images of Hoechst 33258 stained cells (arrow heads indicate cells undergoing apoptosis). Scale bar: 20 μm for HCT116 cells for all images except for SEM (scale bar: 2 μm).

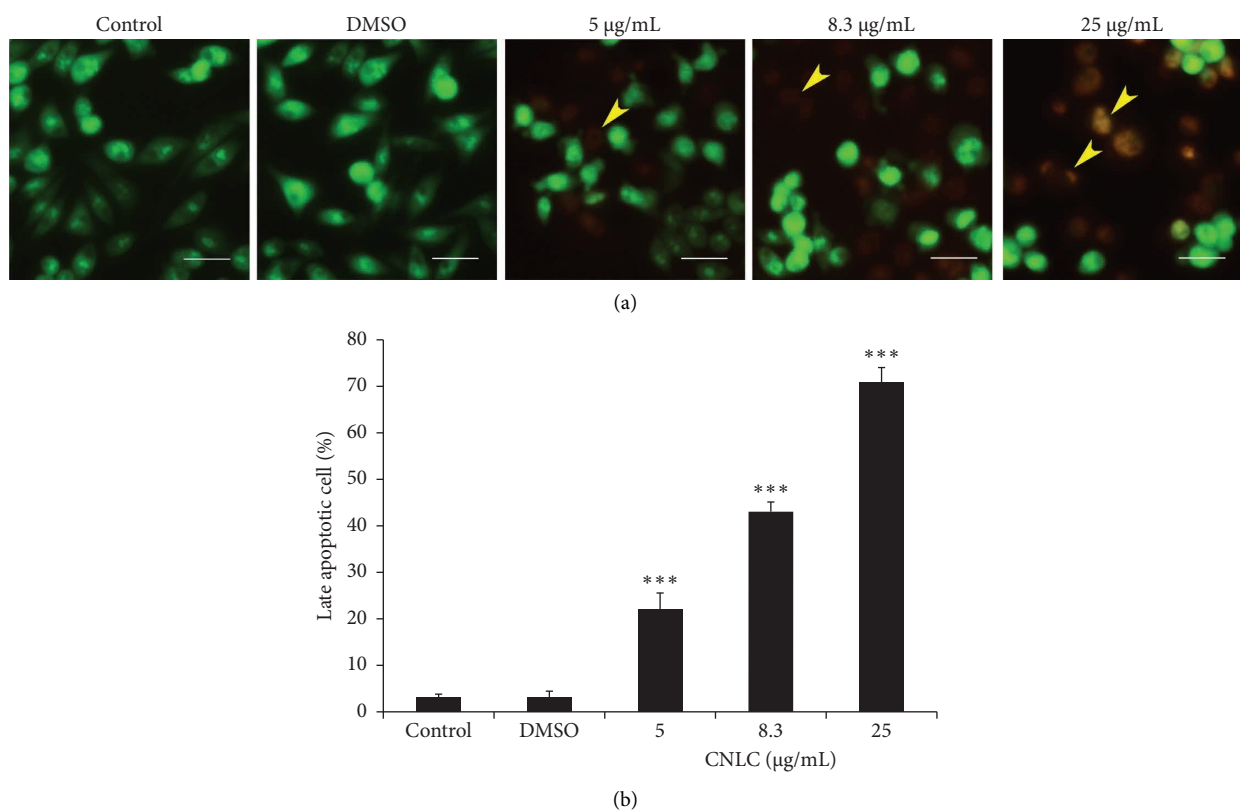


FIGURE 4: Cytomorphological alternations CNLC extract on HCT116 cells analyzed by acridine orange-ethidium bromide dual staining. (a) Fluorescence microscopy images and (b) quantitative analysis of apoptotic cells. Data represent the mean $\pm$ SD of three biological replicates, *** $p < 0.001$.

3.3. CNLC Extract Induces Apoptotic Cell Death in HCT116 Cells. HCT116 cells exposed to the CNLC extract for 48 h were observed for cytomorphological alternations by light

and scanning electron microscopy techniques. Confluent aggregates of polygonal HCT116 cells in untreated control showed conspicuous alternations into more rounded,

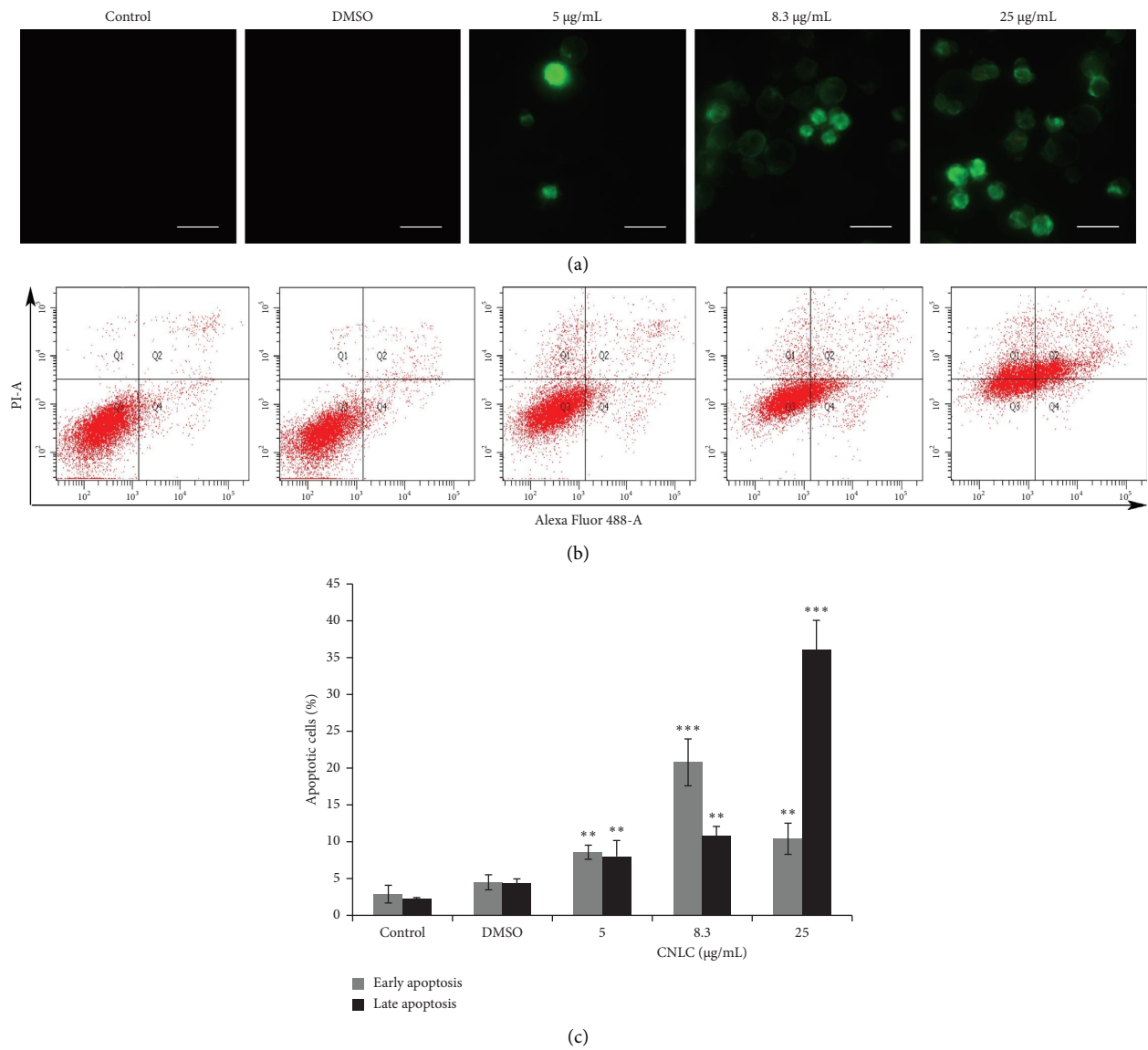


FIGURE 5: Apoptosis induction in HCT116 cells stained with Alexa Fluor 488 annexin V/PI following 48 h treatment with CNLC extract. (a) Fluorescence microscopy images, (b) flow cytometric data, and (c) histogram showing percentages of early and late apoptotic cells. Data represent the mean $\pm$ SD of three biological replicates, ** $p < 0.01$ and *** $p < 0.001$.

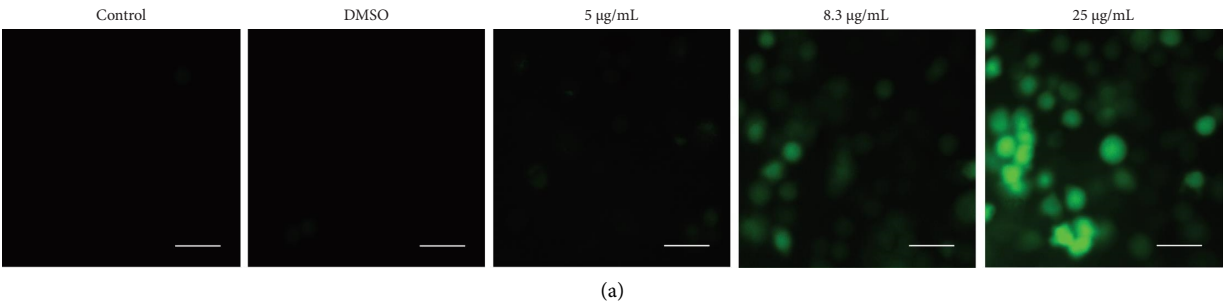


FIGURE 6: Continued.

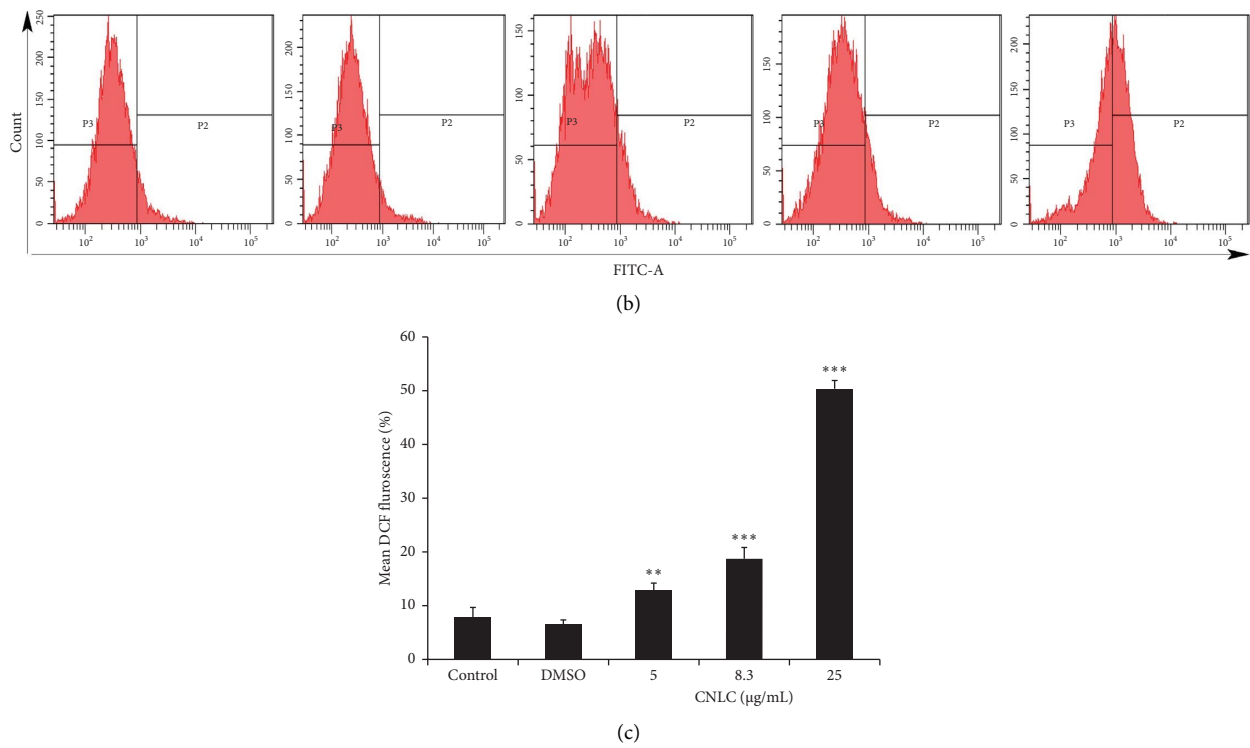


FIGURE 6: Measurement of intracellular ROS in HCT116 cells using DCFH-DA following 48 h of CNLC treatment. (a) Flow cytometric data, and (b) histogram showing mean fluorescence intensity of HCT116 cells stained with ROS indicator DCFH-DA. Data represent the mean $\pm$ SD of three biological replicates, ** $p < 0.01$ and *** $p < 0.001$.

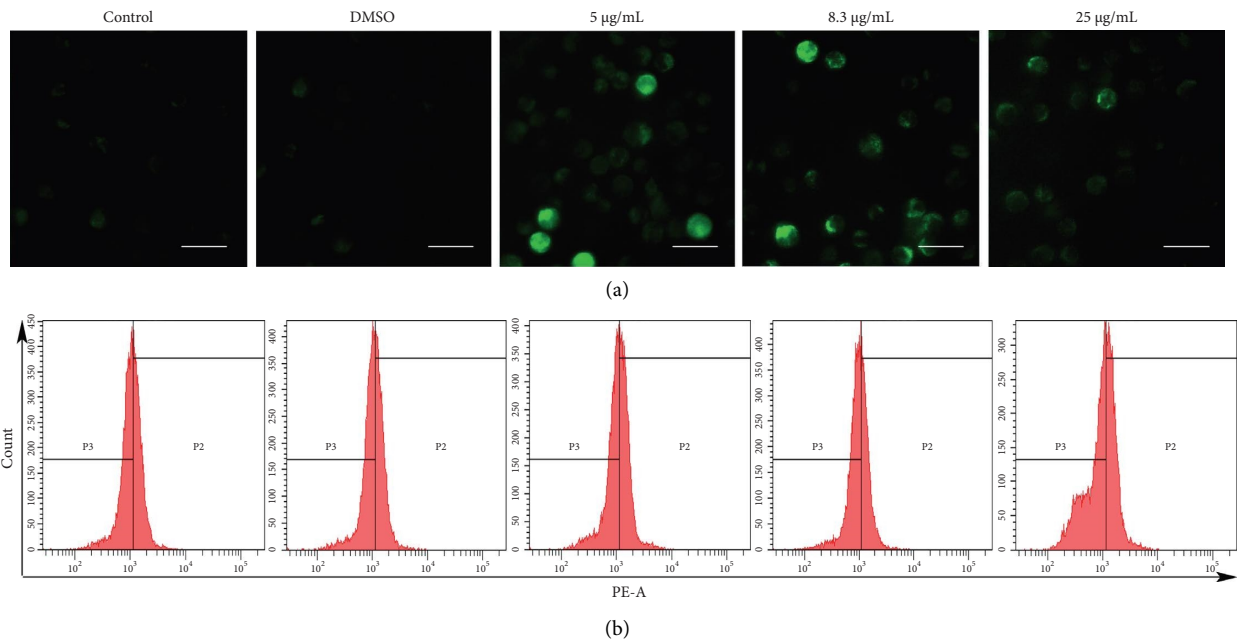
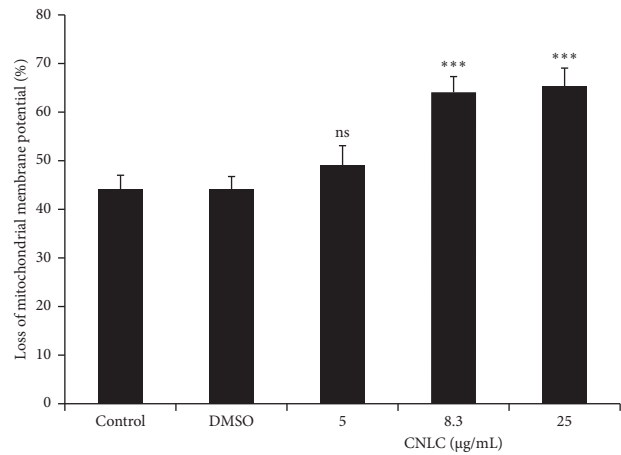
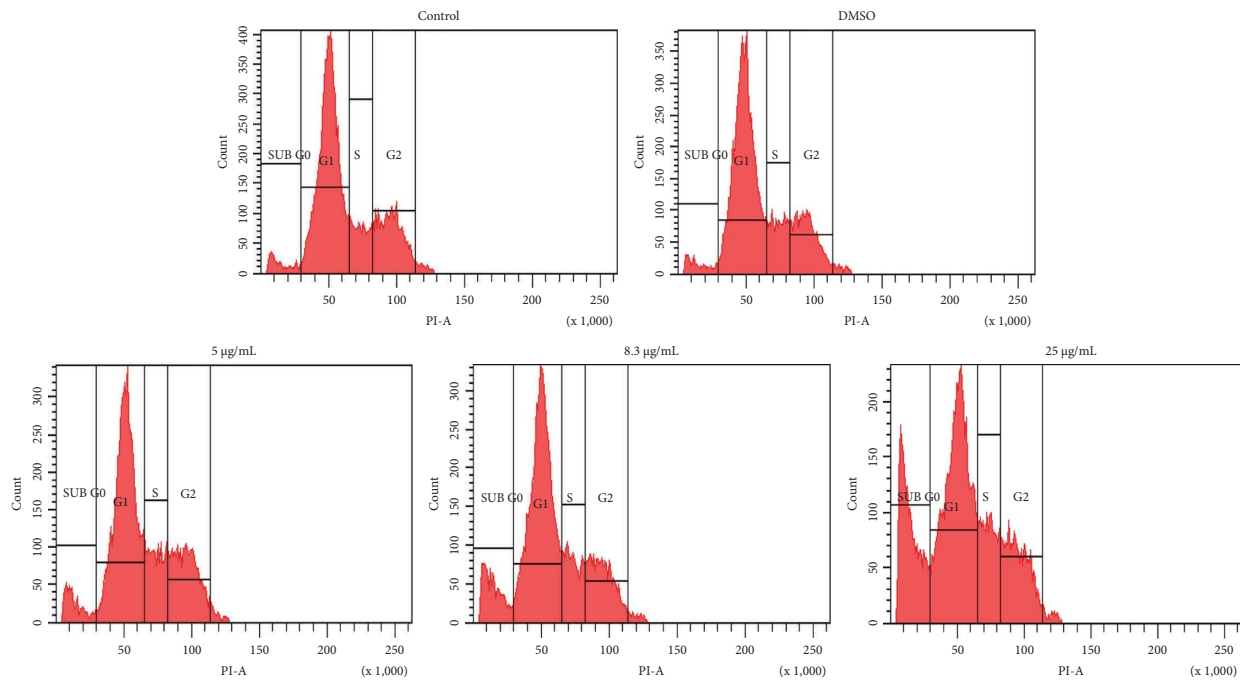


FIGURE 7: Continued.



(c)

FIGURE 7: Measurement of mitochondrial membrane potential in HCT116 cells using rhodamine 123, following 48 h treatment with CNLC. (a) Fluorescence microscopy images, (b) flow cytometric data, and (c) histogram showing mean fluorescence data. Data represent the mean $\pm$ SD of three biological replicates. ns: not significant, *** $p < 0.001$.



(a)

FIGURE 8: Continued.

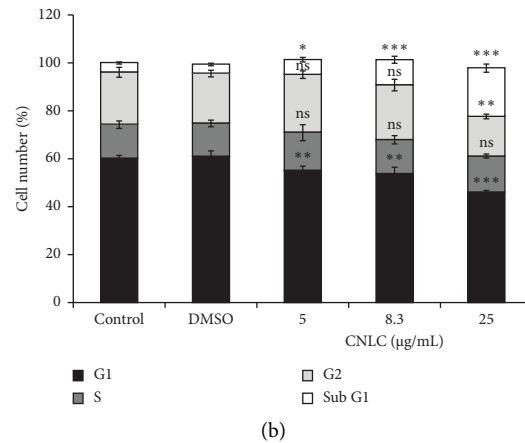


FIGURE 8: Effect of CNLC extract on cell cycle distribution of HCT116 cells. (a) Flow cytometric analysis following PI staining and (b) graphical representation of percentage of cells in different cell cycle phases. Data represent the mean $\pm$ SD of three biological replicates. ns: not significant, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

detached shrunken cells with membrane blebs, following treatment with extract in a dose dependent manner, indicative of apoptotic cell death (Figure 3). Apoptotic cells with condensed DNA and fragmented nuclei compared with the intact nuclei of normal healthy cells can be distinguished by staining with DNA binding, fluorescent dyes such as Hoechst 33258 [26]. Hoechst 33258-stained CNLC-treated HCT116 cells displayed a dose-dependent increase in cells with condensed DNA and fragmented nuclei, indicative of apoptotic cell death, compared with the intact nuclei of normal healthy cells in untreated and vehicle (DMSO) controls (Figure 3). Similarly, dual staining using AO and EB is a simple technique used to determine apoptosis qualitatively and quantitatively [28]. The untreated and vehicle (DMSO)-treated control cells were observed with only intact bright green nuclei stained with AO, which are permeable across intact cell membranes and stain live cells. Whilst a concentration-dependent increase in cells with orange fluorescence (apoptotic) was observed in CNLC extract treated cells, stained with DNA binding EB, which can enter into cells with disrupted membrane integrity (Figure 4). Cytomorphological alternations in CNLC-treated cells, as observed with light, scanning electron microscopy as well as Hoechst 33258 and AO-EB dual staining in fluorescence microscopy are in agreement with the previous reports on cytomorphological changes induced by plant extracts indicating apoptotic cell death [52, 53].

Appearance of PS at cell surface, which is normally restricted to inner membrane layer, is considered as a hallmark of apoptosis. This can be detected by calcium-dependent binding of annexin V- fluorochrome tagged to PS using fluorescence microscopy and flow cytometry. Annexin V-PI costaining helps to detect late apoptotic/necrotic cells from early apoptotic cells, as PI can only enter into cell and interact with DNA when membrane integrity is lost [29, 54]. Dose-dependent increases in apoptotic cells were clearly visible in fluorescence microscopic images of CNLC-treated HT116 cells (Figure 5). Quantitative measurement of PS externalization by flow cytometry also showed that CNLC treatment for 48 h caused

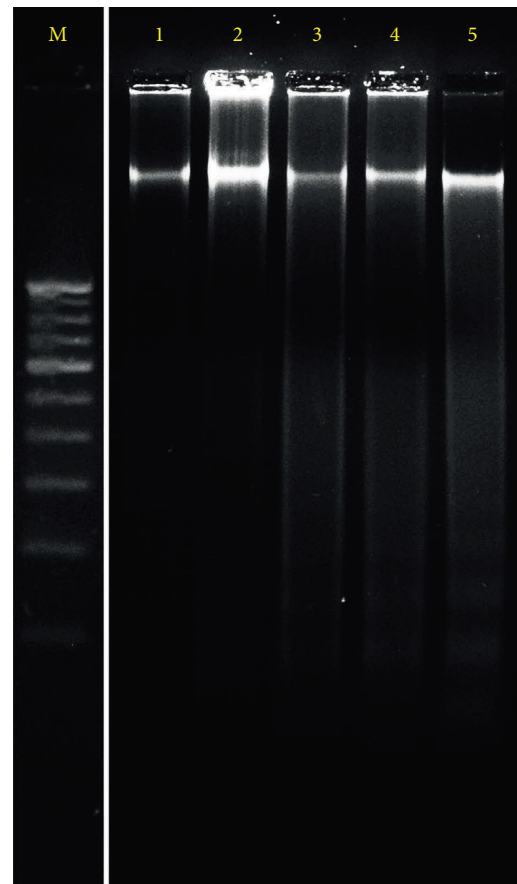


FIGURE 9: DNA fragmentation induced by CNLC in HCT116 cells- lane M 100 bp DNA ladder. Lane 1: DNA from untreated control cells, Lane 2: DNA from DMSO-treated cells, Lane 3: DNA from CNLC (5 µg/mL) treated cells, Lane 4: DNA from CNLC (8.3 µg/mL)-treated cells, and Lane 5: DNA from CNLC (25 µg/mL)-treated cells.

increase in apoptotic population. At the highest concentration (25 µg/mL) tested, late apoptotic (Annexin V⁺/PI⁺) cell population increases were up to 36% from 2% in untreated control.

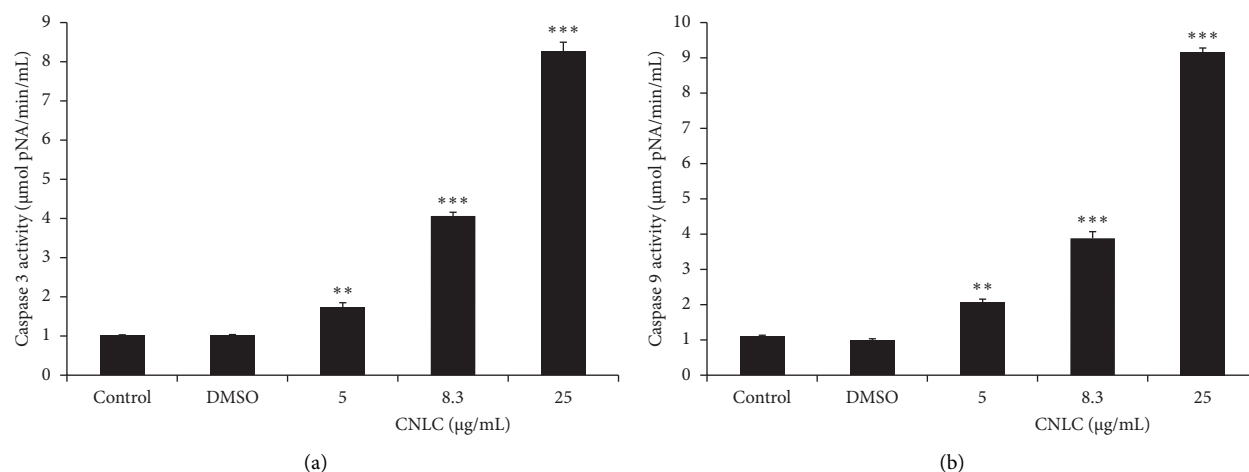


FIGURE 10: Effect of the CNLC extract on caspase activity in HCT116 cells. Histogram representation of (a) caspase 3 and (b) caspase 9 activity assays. Data represent the mean $\pm$ SD of three biological replicates. ** $p < 0.01$ and *** $p < 0.001$.

Excessive levels of ROS can cause damage of proteins, lipids, nucleic acids, membranes, and organelles leading to apoptosis induction [55]. DCFH-DA is a cell-permeable probe commonly used to detect intracellular ROS levels. Once inside the cell, cellular esterases convert DCFH-DA into H2DCF, which is membrane impermeable. This compound is subsequently oxidized by ROS to produce the highly fluorescent DCF. Therefore, increase in its fluorescence is directly proportional to the amount of intracellular ROS [56]. A dose-dependent increase in intracellular ROS was observed with fluorescence microscopic and flow cytometric analysis, following CNLC extract treatment for 48 h in HCT116 cells (Figure 6). About six-fold increase in mean DCF fluorescence of HCT116 cells treated with CNLC (25 $\mu\text{g/mL}$) was observed in comparison with that of both untreated and DMSO-treated controls by flow cytometry analysis. Many plant-derived extracts as well as compounds have been reported to induce oxidative stress by excessive ROS production, thereby causing induction of apoptosis [57, 58].

Lowering of mitochondrial membrane potential associated with the induction of cellular apoptosis. Rhodamine 123 is an extensively used, voltage-dependent, lipophilic, cell permeable, fluorochrome probe which rapidly equilibrates onto mitochondrial membranes and stains them proportionate to membrane potential [33, 59, 60]. In CNLC-treated HCT116 cells, the mitochondrial fluorescence intensity was observed to decrease in a dose-dependent manner as evidenced by fluorescence microscopy and flow cytometry, thereby indicating loss of mitochondrial membrane potential caused by the extract treatment (Figure 7).

Cell cycle analysis of HCT116 cells treated with CNLC extract showed a dose-dependent reduction in cells at G1, S, and G2/M phases, with a concomitant increase in sub-G1 phase (Figure 8), which represent cells undergoing apoptosis with the fractional DNA content [61]. At 8.3 $\mu\text{g/mL}$ and 25 $\mu\text{g/mL}$ of CNLC extract treatment, sub-G1 population was increased to 11% and 20%, respectively, from 4% in controls, indicating apoptotic cell death by the extract

treatment. Also, there is any cell cycle arrest was observed in G1, S, and G2/M phases of in HCT116 cells treated with CNLC extract for 48 h.

DNA fragmentation is considered as the hallmark of apoptosis. In apoptotic cells, chromosomal DNA is cleaved by CAD into internucleosomal 180-bp oligomers, which is detectable as a DNA ladder on agarose gels [62]. In the present study, DNA was extracted from control as well as the CNLC-treated HCT116 cells, electrophoresed on 1.8% agarose gel. In extract-treated samples, a distinct DNA laddering pattern with increasing DNA band intensity was observed, compared with high molecular weight single band of genomic DNA in controls (Figure 9). This observation clearly illustrates the occurrence of internucleosomal DNA fragmentation in CNLC-treated HCT116 cells, thereby confirming apoptotic induction by the extract.

Apoptosis is primarily executed by a family of cysteine protease known as caspases (cysteinyl, aspartate-specific proteases), and these proteases have a cysteine at their active site and cleave their target proteins at specific aspartate at C-terminal of the substrate protein [63, 64]. Following 48 h treatment of HCT116 cells with various concentrations of the CNLC extract, caspase 3 and 9 activities were measured and compared with those of control cells. As shown in Figure 10, treated cells showed a significant concentration-dependent increase of caspase 3 and 9 activities. At the highest concentration tested (25 $\mu\text{g/mL}$), caspase 3 and 9 activities were increased up to 8 and 9 folds, respectively, compared with the untreated control. Apoptosis may be triggered by two major mechanisms: the extrinsic death receptor pathway and intrinsic mitochondrial pathway. Caspase 3 is an executioner caspase involved in both pathways, while Caspase 9 is an initiator caspase which play major role in intrinsic apoptotic pathway [65]. As its name implies intrinsic pathway is initiated within the cell in response to internal stimuli such as irreparable genetic damage, hypoxia, extremely high concentrations of cytosolic Ca^{2+} and severe oxidative stress, which results in increased mitochondrial permeability and the release of proapoptotic

TABLE 4: Putative bioactive compounds found in chloroform extract from leaves of *C. neilgherrensis* (CNLC) by HR-ESI-MS/MS.

Sl. no.	RT (min)	m/z	MS/MS fragments	Possible compounds identified	Molecular formula	Molecular weight (gm/mol)	Class of identified compounds
<i>Positive mode (ESI⁺)</i>							
1.	9.31	633.1335 [M + Na] ⁺	186.0882, 242.2797, 303.0463	Rutin	C ₂₇ H ₃₀ O ₁₆	610.1464	Flavanoid
2.	10.37	317.0613 [M + H] ⁺	163.035, 325.1198	Tamarixetin	C ₁₆ H ₁₂ O ₇	316.054	Flavanoid
3.	11.99	317.1685 [M + Na] ⁺	145.0998, 211.1210	Embelin	C ₁₇ H ₂₆ O ₄	294.1792	Benzoquinone
4.	13.76	277.1746 [M + H] ⁺	277.1704	Cyclandelate	C ₁₇ H ₂₄ O ₃	276.1674	Ester
5.	13.85	351.2107 [M + Na] ⁺	279.0898, 301.0722, 351.2103	9S,11R,15S-trihydroxy-2,3-dinor-13E-prostaenoic acid cyclo [8S,12R]	C ₁₈ H ₃₂ O ₅	328.2215	Fatty acid
6.	14.14	425.1542 [M + H] ⁺	105.0657, 279.0881, 409.1548	Ginkgolide B	C ₂₀ H ₂₄ O ₁₀	424.1469	Diterpenoid
7.	15.55	251.1231 [M + Na] ⁺	128.0603	Traumatic acid	C ₁₂ H ₂₀ O ₄	228.1339	Dicarboxylic acid (cytokinin)
8.	15.75	289.1744 [M + Na] ⁺	145.0997, 289.1772	Juvenile hormone III	C ₁₆ H ₂₆ O ₃	266.1851	Sesquiterpenoid
9.	16.47	288.2867 [M + H] ⁺	288.2844	C17 Sphinganine	C ₁₇ H ₃₇ NO ₂	287.2793	Sphingolipid
10.	16.89	301.1923 [M + H] ⁺	173.1290, 301.1377	Ergoline-8-methanol, 10-methoxy-1,6-dimethyl-	C ₁₈ H ₂₄ N ₂ O ₂	300.1851	Alkaloid
11.	19.22	637.3004 [M + Na] ⁺	83.0470, 161.0564, 337.0202, 393.0798	Harderoporpyrinogen	C ₃₅ H ₄₂ N ₄ O ₆	614.3113	Porphyrin
12.	19.91	631.2468 [M + Na] ⁺	353.2628, 633.4418	Harderoporpyrin	C ₃₅ H ₃₆ N ₄ O ₆	608.2576	Porphyrin
13.	20.87	353.2639 [M + Na] ⁺	113.1044, 224.1207, 353.2629	1-Hexadecanoyl-sn-glycerol	C ₁₉ H ₃₈ O ₄	330.2748	Fatty acid
14.	21.42	599.424 [M + H] ⁺	159.1142, 279.0893, 381.2951, 513.7927	Mytiloxanthin	C ₄₀ H ₅₄ O ₄	598.4167	Carotenoid
15.	23.93	887.5606 [M + H] ⁺	174.1248, 279.0897, 420.2217, 503.2401	Glc-GP(18:0/20:4 (5Z,8Z,11Z,14Z))	C ₄₇ H ₈₃ O ₁₃ P	886.5534	Phosphatidyl glucose
16.	24.61	893.5481 [M + H] ⁺	279.0887, 539.2357	Zeaxanthin beta-D-digluconide	C ₅₂ H ₇₆ O ₁₂	892.5405	Carotenoid

TABLE 4: Continued.

Sl. no.	RT (min)	m/z	MS/MS fragments	Possible compounds identified	Molecular formula	Molecular weight (gm/mol)	Class of identified compounds
<i>Negative mode (ESI⁻)</i>							
17.	1.43	191.0188 [M - H] ⁻	111.0085, 191.0183	2,3-Dioxogulonic acid	C ₆ H ₈ O ₇	192.026	Carboxylic acid
18.	6.68	179.0339 [M - H] ⁻	135.0441, 179.0338	3-(4 Hydroxyphenyl) pyruvic acid	C ₉ H ₈ O ₄	180.0411	Carboxylic acid
19.	10.13	161.0241 [M - H] ⁻	133.0290, 161.0241	o-Hydroxyphenylpyruvic acid lactone/3-hydroxycoumarin	C ₉ H ₆ O ₃	162.0313	Coumarin
20.	10.2	359.0759 [M - H] ⁻	72.9923, 161.0238, 311.0511	Rosmarinic acid	C ₁₈ H ₁₆ O ₈	360.0831	Phenolics
21.	11.37	207.0655 [M - H] ⁻	135.0439, 207.0650	Dimethyl caffeic acid	C ₁₁ H ₁₂ O ₄	208.0728	Phenolics

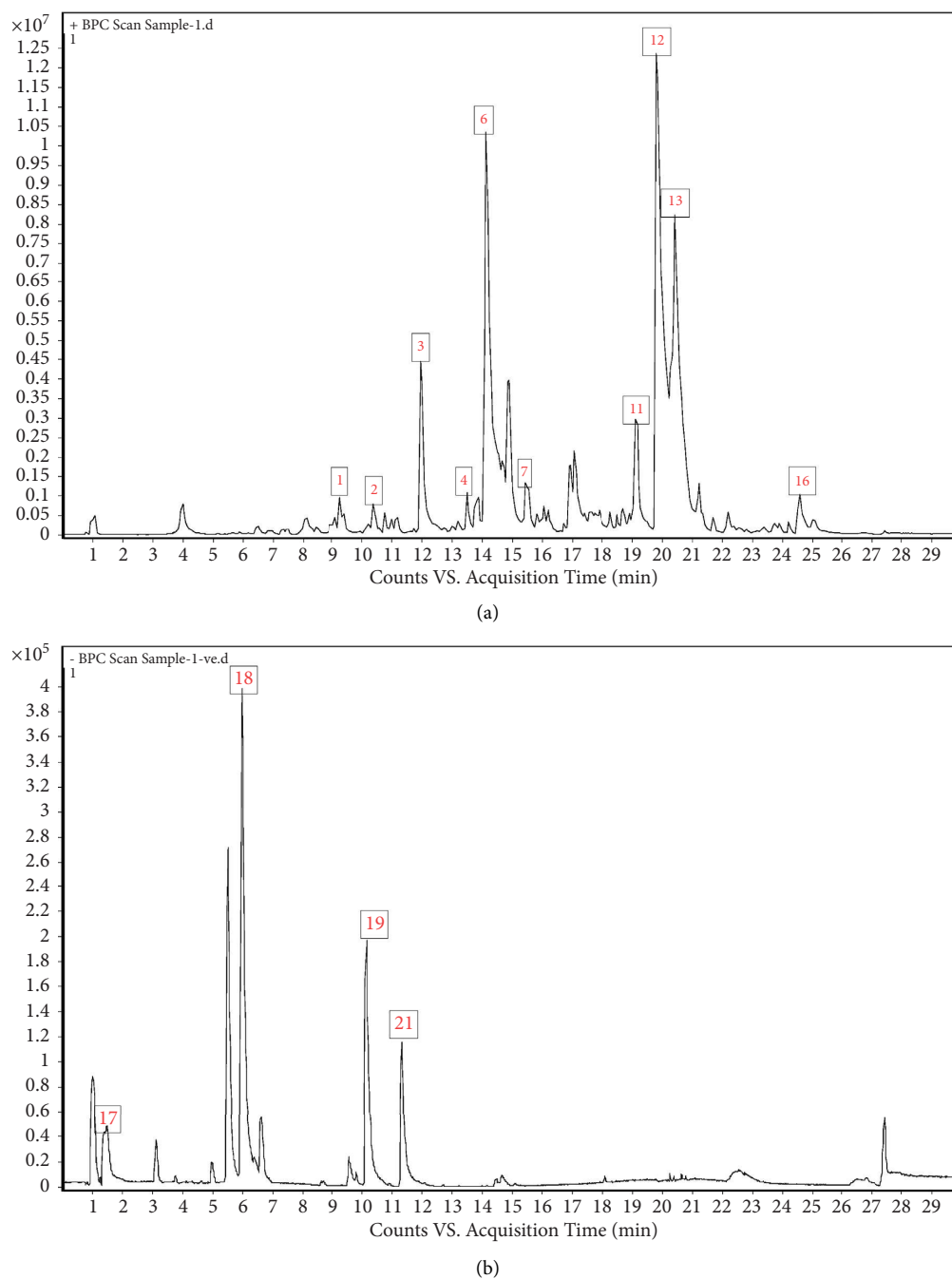


FIGURE 11: HPLC-MS chromatogram of chloroform extract from leaves of *C. neilgherrensis* (CNLC) obtained in (a) positive mode and (b) negative mode.

molecules such as cytochrome c into the cytoplasm. Cytochrome c molecules proceed to aggregate with Apaf-1 to form the seven spoked apoptosome which converts procaspase 9 into active caspase 9, which in turn, cleaves and activates executioner caspases including caspase 3 [66]. Caspase 3 can specifically cleave most of caspase related substrate proteins involved in apoptosis regulation such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP) and the inhibitor of caspase-activated deoxyribonuclease

(ICAD). Activation of caspase 3 also leads to the breakdown of various cellular structures such as cytoskeleton and chromatin, which can ultimately cause apoptotic cell death [67]. Results of caspase activity assay clearly indicate that the CNLC extract induces apoptosis in colorectal HCT116 cancer cells and may occur through the activation of common executors of apoptosis such as caspase 3 by the activation of caspase 9, which is involved in intrinsic or mitochondrial apoptotic pathway.

3.4. Chemical Profile of the CNLC Extract by HR-ESI-MS/MS. Phytochemical profile of the most cytotoxic extract, CNLC, was analyzed by HR-ESI-MS/MS. Figure 11 shows HPLC-MS chromatogram, displaying numerous peaks representing different phytochemicals present within the extract. High-resolution mass spectrometric analysis of these peaks mainly enabled identification of the putative 16 compounds in positive and 5 compounds in negative electrospray ionization ion modes. These compounds belong to different chemical families such as phenolics, flavanoids, alkaloids, terpenoids, carotenoids, fatty acids, carboxylic acids, and porphyrins (Table 4).

Among these, some have been previously reported with various bioactivities including potent cytotoxic/anticancer activity such as rutin [68], tamarixetin [69], embelin [70], ginkgolide B [71], traumatic acid [72], and rosmarinic acid [73]. In addition, derivatives of certain compounds such as C17 sphinganine, ergoline-8-methanol, 10-methoxy-1, and 6-dimethyl- were reported with anticancer potential [74, 75]. Porphyrins and carotenoids are compounds which also reportedly possess antitumor potential [76, 77]. Presumably, the combined or synergetic activity of these bioactive compounds lends credence to the potent cytotoxicity and apoptosis induction in HCT116 cancer cells by the CNLC extract as reported by us in this paper.

4. Conclusion

C. neilgherrensis leaves have been used as a traditional indigenous medicine for diabetes mellitus by tribes of Western Ghats of India since ancient times. The present study successfully demonstrates for the first time the in vitro antioxidant and anticancer potential of organic solvent extracts from *C. neilgherrensis* leaves. The chloroform extract, CNLC, was observed to be the most cytotoxic against colorectal HCT116 cancer cells, without affecting normal hPBLs.

The results clearly showed that the CNLC extract induced apoptosis through intrinsic or mitochondrial pathway in HCT116 cells as evidenced by cytomorphological changes, phosphatidylserine externalization, elevation in intracellular ROS, reduction in mitochondrial membrane potential, DNA fragmentation, and increase in sub-G1 phase cell population, as well as caspase 3 and 9 activity in the treated cells. Interestingly, HR-ESI-MS/MS analysis proved that the extract is a bioresource enriched with multipotent bioactive compounds, previously reported with cytotoxic/anticancer potential. In conclusion, *C. neilgherrensis* leaf is a promising candidate for future chemotherapeutic drug development.

Nomenclature

ABTS	2,2-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid)
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CNLA	<i>Curcuma neilgherrensis</i> leaf acetone extract
CNLC	<i>Curcuma neilgherrensis</i> leaf chloroform extract
CNLM	<i>Curcuma neilgherrensis</i> leaf methanol extract

CNLP	<i>Curcuma neilgherrensis</i> leaf petroleum ether extract
DCFH-DA	Dichloro-dihydro-fluorescent diacetate
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FeSO ₄	Ferrous sulfate
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
hPBLs	Human peripheral blood lymphocytes
HPTLC	High performance thin layer chromatography
HR-ESI-MS/MS	High-resolution electrospray ionization mass spectrometry with tandem mass spectrometry
<i>m/z</i>	Mass/charge
MTT	3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
Na ₂ CO ₃	Sodium carbonate
NaOH	Sodium hydroxide
PBS	Phosphate-buffered saline
PI	Propidium iodide
Q-TOF	Quadrupole time-of-flight
Rf	Retardation factor
ROS	Reactive oxygen species
Ac-DEVD-pNA	N-acetyl-L-α-aspartyl-L-α-glutamyl-L-valyl-N-(4-nitrophenyl)-L-α-asparagine
Ac-LEHD-pNA	(2S,5S,8S,11S)-5-((1H-imidazole-5-yl)methyl)-8-(2-carboxyethyl)
RPMI	Roswell Park Memorial Institute medium
SD	Standard deviation
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid-reactive species
Tris	2-amino-2 hydroxy methyl-propane-1-3- diol
HPLC-MS	High-performance liquid chromatography-mass spectrometry
UHPLC	Ultra high performance liquid chromatography

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

S.T., P.R.J., and P.R.M.K. designed the experiments. S.T. performed all experiments and analyzed the data. T.L. assisted in reagent preparation and data analysis. P.R.J. and P.R.M.K. supervised all experiments. S. T., P.R.J., and P.R.M.K. analyzed data and wrote and edited the manuscript.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. (*Supporting Information*)

Additional data from the HPTLC analysis (Supporting Figure 1 and Table 1), cell survival assay (Supporting Figure 2), and hemolytic assay (Supporting Figure 3) of the *C. neilgherrensis* extracts are included in the supporting information.

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