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# *Oroxylum indicum* Vent Root Bark Extract Inhibits the Proliferation of Cancer Cells and Induce Apoptotic Cell Death

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**Abstract:** *Oroxylum indicum* Vent is a medium-sized deciduous tree that belongs to the family Bignoniaceae. The roots of this tree are used as one of the ten ingredients to prepare the Dasamula formulation in the Ayurvedic system of medicine in India. Although traditional uses are attributed to the medicinal plant, there are limited scientific data on its potential. The present study thus analyzed the cytotoxic and apoptotic potential of the plant against different cancer cells. MTT assay was used to determine cytotoxicity using HeLa, HCT 15, and MDA-MB-231 cells, with the IC50 values, revealed at concentrations of 92.43, 133.0, and 112.84 µg/mL respectively. However, the extract was less toxic to non-cancer cells. HeLa cells further treated with OIM were subjected to flow cytometric analysis for studying the cell cycle stages. When untreated cells at G1 phase were found at a relative percentage of 71.9%, it increased to 79.3 and 86% with OIM treatment at concentrations of 50 and 100 µg/mL; cells in the S phase decreased from 10.3 to 8.2 and 7.5%, concluding the arrest of the cell cycle at G1 phase. With further study of apoptotic morphology with dual acridine orange–ethidium bromide staining and Annexin–Hoechst staining, cells at early and late apoptotic stages were observed with OIM treatment at 100 µg/mL concentration. Although such effects were noticed with OIM treatment, it could not be concluded that the extract had remarkable anti-proliferative effects, since the small changes noticed in cell cycle arrest and apoptotic induction were attained at a high concentration of OIM 100 µg/mL. The biological activities of plants and their extracts are attributed to the presence of multifarious compounds present in them. LC-MS Q-TOF analysis confirmed the presence of biochanin A and baicalein in OIM. HPLC-based quantification of baicalein and chrysin was shown to be 3.36 and 1.11 mg/gram dry weight. To conclude, the above results suggest that the root bark of *O. indicum* has a broad spectrum of biological activities, including anticancer and apoptotic properties.

**Keywords:** *Oroxylum indicum*; cytotoxicity; apoptosis; morphological change; MTT assay



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## 1. Introduction

Drugs developed from plant origin are gaining accelerated demand for cancer therapy, mostly due to cytotoxicity, inhibition of cancer cell proliferation, and apoptosis induction [1]. Cancer prevention, suppression, and treatment using natural plant products are also practiced in Ayurveda, the oldest indigenous medicine system in India. It has been summed up from statistical data that plants with basic Ayurvedic characteristics exert significantly high anticancer properties [2]. *Oroxylum indicum* Vent., a member of the family Bignoniaceae, is known for its use in the preparation of the Ayurvedic formulation [3,4].

Besides, these plants are also utilized in folk medicines for the management of various illnesses [5–7].

The most remarkable property of cancer cells is their rapid proliferative ability; hence, cytotoxicity assays are used as a presumptive way of screening for anti-cancer activities of drugs [8–10]. Except for a report of the cytotoxic effect of root bark in MCF-7 cells [11], the in vitro cytotoxic and anti-proliferative activities or the underlying anti-cancer mechanism of *O. indicum* root bark remain unexplored. Hence, the study was focused on the short-term cytotoxic and long-term anti-proliferative activities of *O. indicum* root bark on DLA/EAC cell lines and HeLa/HCT15/Hep G2/MDA-MB-231 cell lines, respectively. The trypan blue dye exclusion method and MTT assay were used for the evaluation of cytotoxicity and anti-proliferation, respectively. The study also attempted to elucidate the mechanism of proliferative inhibition of cancer cells through analysis of cell cycle arrest and observation of apoptotic morphology in HeLa cells, in the presence of the extract. The studies of cell cycle arrest and apoptotic morphology were conducted using flow cytometric technique and microscopic examination (EtBr–acridine orange staining and Annexin–Hoechst staining), respectively.

As a forerunning step towards new drug discovery from plants, the separation of medicinally active parts from inert parts was done using extraction with suitable solvents. One way of selecting the appropriate solvent is based on the solubility and polarity of the target compounds to be extracted, which, in turn, depends on the phytochemical profile of the plant [12]. An alternate approach for selection is the biological activity-based screening of extracts prepared using different solvents, ranging from non-polar solvents (petroleum ether, dichloromethane, acetone) to polar solvents (methanol, water) [13–15]. Hence, to arrive at a suitable solvent system for the whole study, a preliminary screening of different extracts of *O. indicum* root bark was carried out. Apart from cytotoxicity, an interrelationship has been established between the antioxidant and anticancer activities of drugs, since free radical-induced damage is one of the postulated mechanisms of cancer development [16,17].

## 2. Materials and Methods

### 2.1. Cell Lines and Plant Materials

The *Oroxylum indicum* Vent roots were collected from Peechi, Thrissur, India (10.4632° N, 76.2439° E), collected from Thrissur, and authenticated by Dr. P Sujanapal, Taxonomist and Principal Scientist, Department of Silviculture, Sustainable Forest Management Division, Kerala Forest Research Institute, and a voucher specimen was deposited (KFRI/SILVA/GEN/06/11).

The human endometrial cancer cell—HeLa (passage number 27), human colorectal cancer cell—HCT15 (passage number 39), human triple-negative breast cancer cell—MDA-MB-231 (passage number 22), and African green monkey kidney cell-Vero (passage number 41) were obtained from the Repository of National Centre for Cell Science (NCCS), Pune (Department of Biotechnology, Government of India). The cells were of human origin, and they are established cell lines by original producer American Type Culture Collection. These cell lines were authenticated and approved by the NCCS, Pune, for their specificity and purity.

### 2.2. Collection and Preparation of Plant Extract

The air-dried, powdered root bark of *Oroxylum indicum* Vent was subjected to Soxhlet extraction using 70% methanol as solvent, followed by its evaporation to dry residue (OIM extract). This was dissolved in distilled water and diluted to the required concentrations for in vivo studies.

### 2.3. Extraction and Quantitative Phytocharacterization

The peeled bark was dried at 45–50 °C for a week in a hot air oven, powdered, and subjected to extraction using methanol in the Soxhlet apparatus. The total phenolic

content [18] and flavonoid determination [19] were carried out by previously described protocols. The LCMS analysis was carried out and MS/MS fragmentation reports were used to identify the compounds according to our study [20].

### 2.4. Cytotoxicity Analysis

HeLa, MDA-MB-231, Hep G2, HCT 15, and Vero cell lines were maintained with filter-sterilized Dulbecco's Modified Eagle Media (DMEM) and supplemented with 10% heat-inactivated fetal bovine serum (FBS) in 25 mL culture flasks under 37 °C incubation with 5% $CO_2$. Aseptic conditions were maintained throughout the study. The culture flasks were monitored regularly for any contamination or color change of medium or morphological changes of cells, with the renewal of the medium either periodically or on the appearance of yellow coloration, whichever came earlier. The cells were subcultured on the attainment of 70–80% confluence. Before this, fresh media, PBS, and trypsin were allowed to reach a temperature of 37 °C. After aspirating out the spent medium, the cells were washed thrice with PBS to remove any trace of media. Trypsinization for cell detachment was carried out at 37 °C using trypsin/EDTA solution and monitored under an inverted microscope. Following that, 1 mL of fresh medium was then added for recovering the effect of trypsin. The cell suspension was mixed gently and thoroughly using a sterile pipette to prevent any clumping of cells, and thereafter equally dispensed into five new culture flasks.

The long-term cytotoxicity of the extract was determined by MTT assay [21]. The assay reflects the number of live cells based on the metabolic activity of the cell. The viable cells were seeded in a 48-well plate and treated with different concentrations of the extract for 48 h. To assess the cell viability, 100 µL of MTT (5 mg/mL PBS) was added to each well, and incubation was continued for another 4 h. The insoluble formazan crystals formed in the assay system were dissolved in 1 mL of solubilizing solution (10% Triton X-100, 0.1 N HCl, 50 mL isopropanol) by repeated aspiration. The absorbance was read at 570 nm and the percentage of cell viability was calculated.

### 2.5. Analysis of Cell Cycle Arrest Using Flow Cytometry

The study of cell cycle stages in HeLa cells in the presence of OIM extract was conducted using flow cytometry. The cells were incubated with the extract at two concentrations (50 and 100 µg/mL) for 24 h at 37 °C. Thereafter, they were harvested by trypsinization and centrifuged at 5000 rpm for 10 min at 4 °C in a cold centrifuge. The pellet was resuspended in 250 µL ice-cold PBS and centrifuged again at 10,000 rpm for 5 min. The resultant pellet was resuspended in 300 µL PBS. Cells were fixed by the dropwise addition of 700 µL ice-cold 70% ethanol and occasional stirring in a vortex. The sample was incubated on ice for 30–60 min and centrifuged at 5000 rpm for 10 min at room temperature. The pellet collected was resuspended in PBS and centrifuged at 5000 rpm for 10 min at room temperature again. RNase (5µL) at a concentration of 10 mg/mL was added to the resuspended pellet (in 250 µL of PBS) and incubated for 1 hr at 37 °C. Following this, propidium iodide (PI) (10 µL at concentration 1 mg/mL) was added to the suspension and filtered (40 µm pore size membrane filter). The cell suspended was stored in the dark at 4 °C until it was analyzed. Flow cytometry was used to reveal the percentage of cells in different stages based on DNA concentration with propidium iodide staining, with reference to 10,000 events in each sample. Data were analyzed in Coulter Elite 4.5 Multicycle software 20.

### 2.6. Study of Apoptotic Morphology

The HeLa cells treated with OIM extract were studied to observe any characteristic morphological changes associated with apoptosis.

### 2.7. Dual Acridine Orange/Ethidium Bromide Staining (Ao/Eb)

HeLa cells (count 2 × $10^6$ cells/mL) were seeded in a 6-well plate and incubated at 37 °C until attachment. The cells were treated with two concentrations of the extract (50

and 100 µg/mL) and incubated for 16 h. They were then washed with PBS, harvested by trypsinization, and centrifuged at 2000 rpm for 10 min. The pellet was resuspended in 50 µL PBS. The cells were stained by mixing equal volumes of the suspension with Ao/Eb stain solution and subjected to fluorescent microscopy using a blue excitation filter (480 nm) and photographed.

### *2.8. Annexin V–Hoechst Staining*

HeLa cells (count 2 × $10^4$ cells/mL) were seeded on 96 well-glass bottom plates. At 50 % confluence, the cells were treated with two concentrations of the extract (50 and 100 µg/mL) and incubated for 16 h. Cells were harvested and washed with Annexing binding buffer two times and incubated with Annexin V Conjugate for 30 min at 1:100 dilution in binding buffer. Cells were then subjected to Hoechst33342 (Molecular probes H3570) staining and imaged with an epifluorescent microscope Nikon TiE using Alexa 647 Filter sets from Semrock USA. Images were collected using a CCD camera Retiga Exi (Q IMAGING) that was controlled through NIS element software Ver. 4.00 (Nikon).

### *2.9. Statistical Analysis*

The results are represented as the mean of individual parameters from six animals along with the standard deviation. One-way ANOVA, along with the Dunnett test, was used for statistical comparison (Graph Pad Prism 7.0).

## 3. Results

### *3.1. Phytochemical Analysis*

The *O. indicum* UPLC-Q-TOF-MS analysis is shown in Figure 1 as its total ion chromatogram; further, the individual compounds are listed in Table S1. Demethoxycentaureidin 7-O-rutinoside, Isorhamnetin-3-O-rutinoside, Baicalein-7-O-glucuronide, 5,6,7-trihydroxy flavone, 3-Hydroxy- 3′,4′,5′- trimethoxy flavone, 5,7-hydroxy -3-(4-methoxy phenyl) chromen-4-one, 4′-Hydroxy- 5,7-dimethoxy flavanone, and 6-Ethoxy -3(4′-hydroxy phenyl)-4-methyl coumarin are the compounds present in the *O. indicum* root extract.

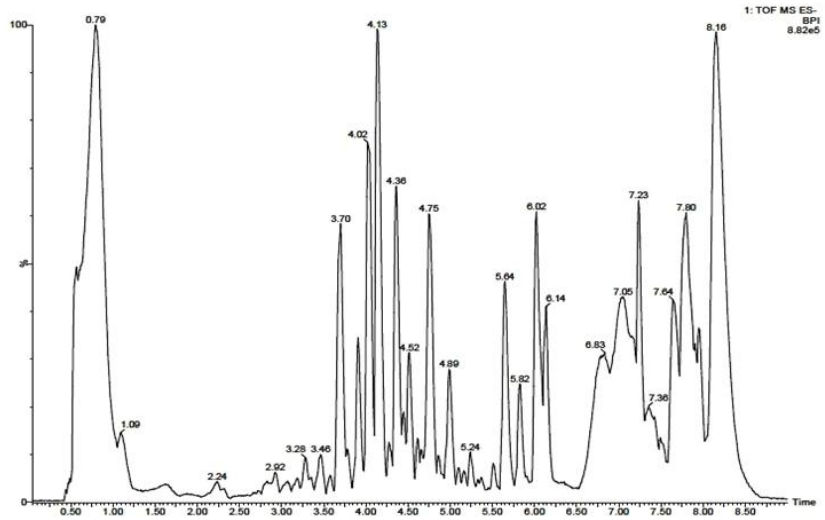


**Figure 1.** Total ion chromatogram of *O. indicum* Vent. Wild root bark (OIM) extract subjected to UPLC-Q-TOF-MS analysis (Data from own publication https://doi.org/10.3390/molecules27238459).

### *3.2. Anti-Proliferative Activity of OIM*

The study revealed that the OIM extract inhibited the proliferation of 50% of HeLa, HCT15, and MDA-MB-231 cells in the system at concentrations within 150 µg/mL (92.43, 133.0, and 112.84 µg/mL, respectively), with maximum activity in HeLa cell lines (Figure 2). The morphology of HeLa, HCT15, and MDA-MB-231 on treatment with OIM extract at

various concentrations is given in Figure S1. However, the extract could not induce 50% inhibition of Hep G2 cell growth up to a concentration of 200 µg/mL, indicating that it was less toxic to this cell type. No signs of toxicity were found in normal Vero cell lines (Figure S2).

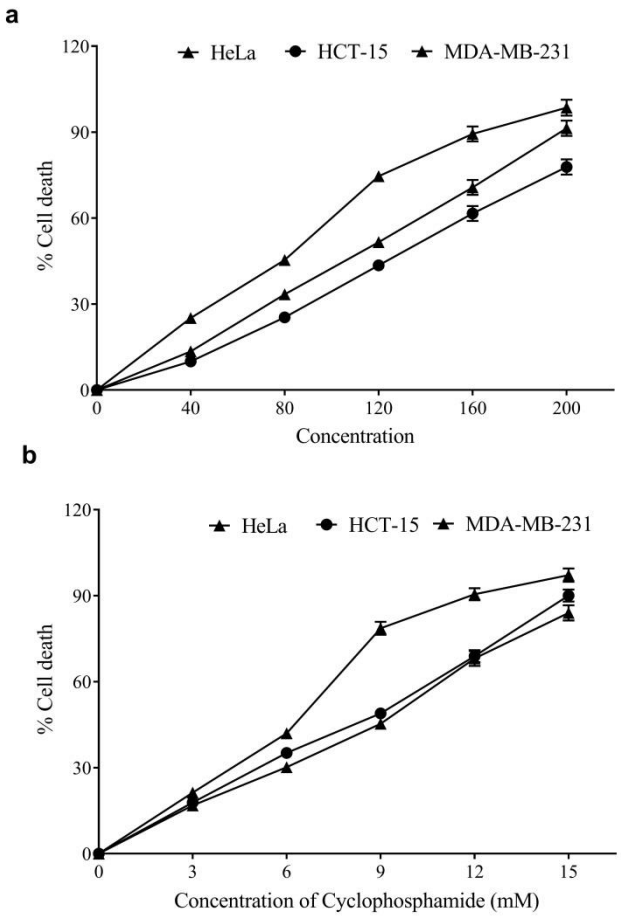


**Figure 2.** Anti-proliferative activity of OIM extract (**a**) and standard cyclophosphamide (**b**) on HeLa, HCT15, and MDA-MB-231 cells.

### *3.3. Analysis of Cell Cycle Stages*

As evident from the peaks (Figure 3) obtained from flow cytometric analysis based on cellular DNA concentration with PI staining, OIM extract was to arrest the HeLa cell cycle at the G1 phase. On treatment with OIM extract at concentrations of 50 and 100 µg/mL, respectively, 79.7 and 86.0% of cells remained in the $G_1$ phase of the cell cycle, compared to the untreated cells with only 71.9% of cells in $G_1$. Thus, OIM treatment brought about an increase in the relative percentage of $G_1$ cells by 7.8 and 14.1% in a dose-dependent manner, suggesting cell accumulation in the $G_1$/S transition phase. In support of the above finding, it was also observed that the relative percentage of cells in the S phase was lesser (7.5%) in the presence of 100 µg/mL OIM, compared to the untreated cells (10.5%). The concomitant result was obtained for cells in $G_2$, which maintained a relative population of 8.2 and 4.6% on treatment with 50 and 100 µg/mL OIM, respectively, compared to untreated cells (16.7%). A higher proportion of cells—1.8 and 1.9%—in the sub-$G_0$ phase was observed in cell lines treated with 50 and 100 µg/mL OIM, but the relative percentage was 0.3% in the untreated control. The relative cell percentage at various cell cycle stages is also depicted in Figure 3.

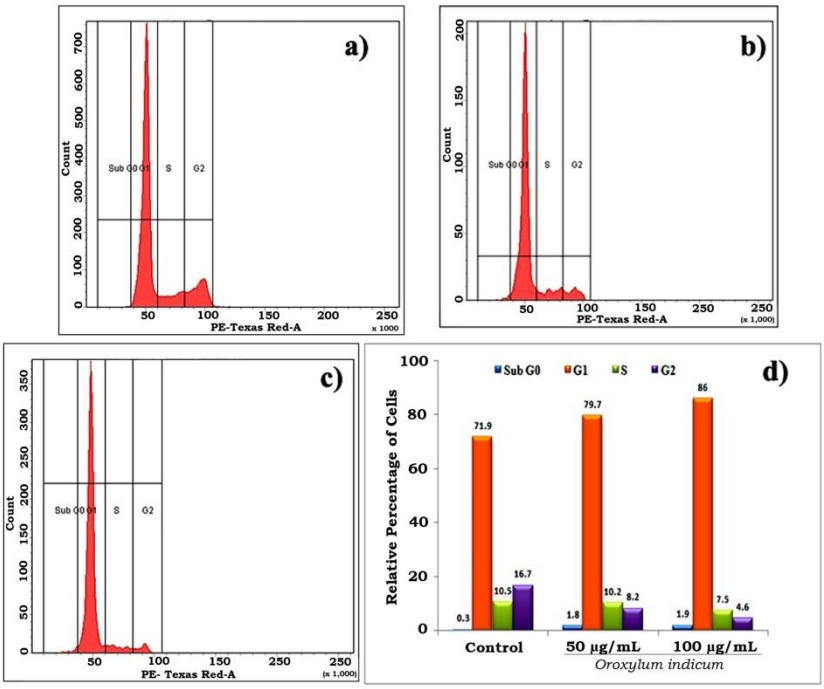


**Figure 3.** Flow cytometric analysis showing cell cycle stages in (**a**) untreated HeLa cells, (**b**,**c**) HeLa cells treated with OIM 50 and 100 µg/mL, respectively, and (**d**) a relative percentage of cells at various phases of cell cycle.

### *3.4. Study of Apoptotic Morphology*

#### 3.4.1. Dual Ao/Eb Staining

Fluorescent microscopic observation revealed that HeLa cell sample incubated in the presence of 100 µg/mL OIM showed early and late apoptotic stages under Ao/Eb staining. The untreated cells showed live morphology and were uniformly stained green. The early apoptotic cells in the OIM-treated sample also appeared green (in Ao nuclear stain), with bright green dots inside, characteristic of nuclear fragmentation. The late apoptotic cells with EtBr stain incorporated in nuclei appeared orange but were with fragmented nuclei (Figure 4).

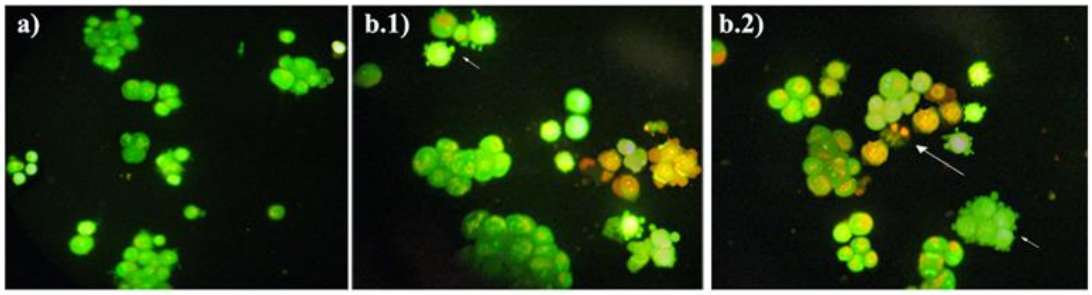


**Figure 4.** Morphology of HeLa cells with EtBr and acridine orange staining, revealing apoptotic stages. (**a**) Untreated HeLa cells. (**b**) Cells treated with 100 µg/mL OIM showed early apoptotic (small arrow—**b.1** and **b.2**) and late apoptotic (long arrow—**b.2**) stages.

#### 3.4.2. Annexin–Hoechst Staining

As revealed through the fluorescent microscopic images (Figure 5), the untreated cells stained negative for Annexin V, whereas HeLa cells treated with OIM showed Annexin V positive staining. This indicated that no cell death occurred in the untreated sample, but Annexin V positive treated cells revealed death morphology. Hoechst staining exposed nuclear fragmentation in OIM-treated samples, but no such noticeable nuclear morphology

was present in untreated samples. In a merged view of Annexin–Hoechst-stained samples, it was observed that many of the Annexin V positive cells of the treated sample correspondingly revealed nuclear fragmentation (evident from Hoechst staining). This is suggestive of apoptotic death of the cells in the presence of OIM.

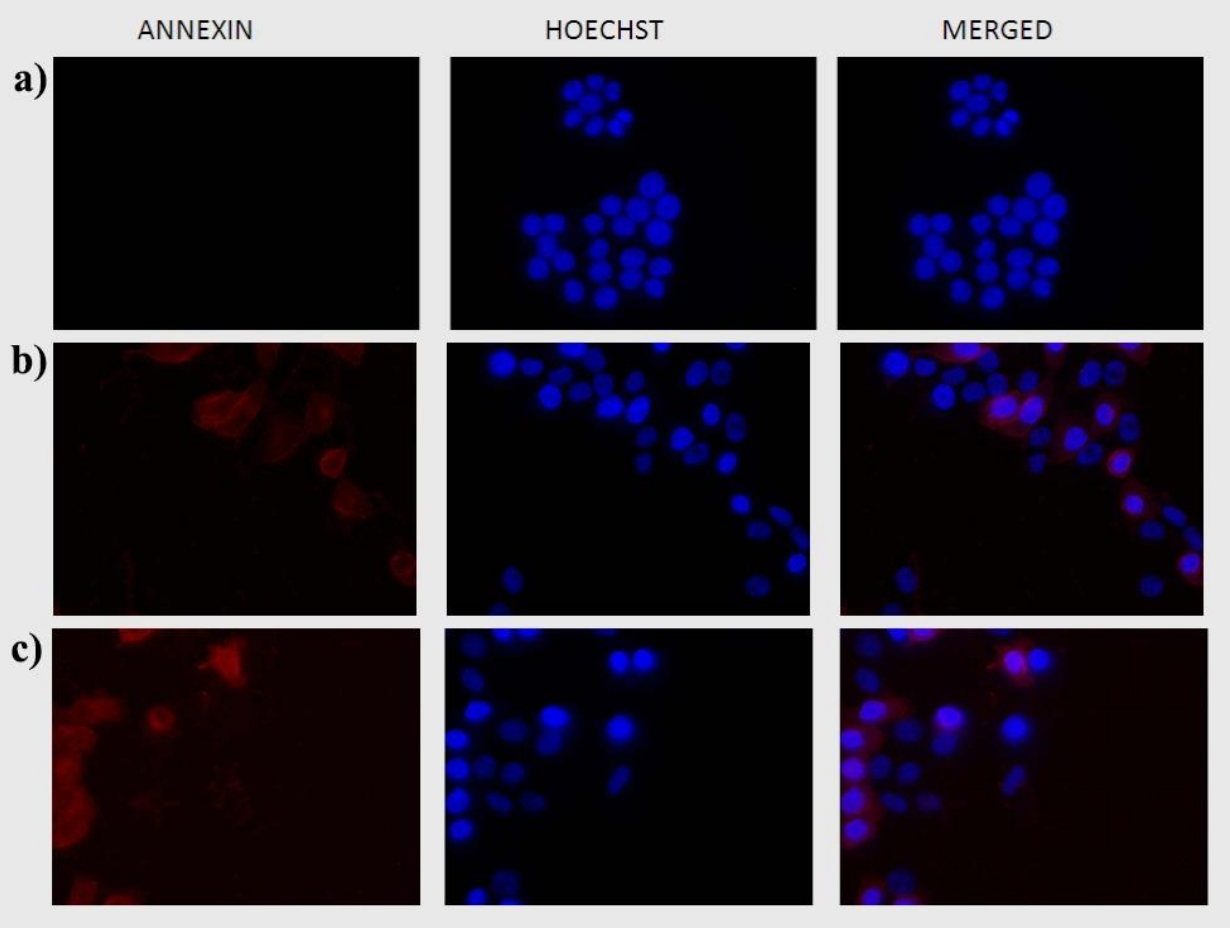


**Figure 5.** Morphology of HeLa cells with Annexin–Hoechst staining; (**a**) Untreated cells, (**b**,**c**) HeLa cells treated with OIM 50 and 100 μg/mL, respectively.

## 4. Discussion

The pharmacological applications of the *Oroxylum indicum* in traditional and folk medicines are yet to be discovered. Hence, the present study analyzed the antiproliferative effects of the extract in multiple cancer cells. There are preliminary studies available on the anticancer activity of the plant in the cell culture milieu; a study has indicated the cytotoxic effect of *Oroxylum indicum* against HL-60 cells [22]. Bioactive flavonoid baicalein has been reported in the root bark extract of the plant [23]. Besides, many compounds reportedly present in the root bark are reported to have an anti-proliferative activity such as chrysin [24], biochanin A [25], and ellagic acid [26], suggesting a correlation between the presence of cytotoxic compounds and the biological activity of the extract. The apoptosis in HeLa cells is known to be mediated through the modulation of microRNAs and cell signaling [27]. Meanwhile, the OIM extract was found to be less effective in inhibiting the proliferation of HepG2 (liver carcinoma) cell lines. The signaling pathways involved in cancer cell proliferation and proliferation inhibition are different [28]. The targeted signaling pathways of cytotoxic compounds are selective in different cancer cell types, as revealed earlier [29,30] from the differential cytotoxicity of OIM extract in cancer cell types. In the anti-proliferative assays, no evident toxic effect was produced by the extract on the non-cancerous cell line (Vero). These are aneuploid, immortalized, and continuous cell

lines used to confirm the safety of using cytotoxic anti-cancer agents in cancer-related drug discovery investigations [31,32]. The results of the current study also suggest that OIM extract is safe on normal cells, while being toxic to certain cancer cell types.

It was found that OIM extract was most effective in inhibiting the proliferation of HeLa cells ($IC_{50}$: 92.43 µg/mL), compared to other cell types used in the study. Hence, the investigation was extended to find out the cytological effects of OIM in HeLa cells using flow cytometric analysis and fluorescent microscopic examination. Cell cycle kinetics, as revealed through flow cytometric analysis, suggest that in the presence of 100 µg/mL OIM extract, there was an accumulation of cells in $G_1$ phase and concomitant depletion of cells in S phase and $G_2$ phase from the proliferative pool. It is therefore conclusive that OIM induces cell cycle arrest in the $G_1$/S transition phase. OIM treatment at this concentration developed a peak in the sub-$G_0$ phase, suggestive of proliferation arrest at this phase, a qualitative indicator of apoptosis [33]. However, the results were less evident in comparison with that of $G_1$/S phase arrest. The results obtained at this concentration of OIM can also be correlated to its $IC_{50}$ value (92.43 µg/mL) in the MTT assay. Cytotoxicity by anticancer agents is mediated through targeted mechanisms leading to cell cycle arrest and induction of apoptosis [34], and hence, regulation of cell cycle kinetics is correlated to the toxicity of many compounds in cancer cells [35]. Consistent with the findings of previous studies [36,37], the results of the present work also link to the proliferative inhibition of HeLa cells by OIM extract with its influence on mediating cell cycle arrest at $G_1$/S transition. Since there is a well-established link between the regulation of cell cycle events and apoptosis [38], the HeLa cells treated with 100 µg/mL OIM were subjected to the study of apoptotic morphology using Ao/Eb and Annexin V–Hoechst staining methods.

In the discovery of anti-cancer agents, apoptotic induction is a major hub because of the association between apoptotic dysregulation and cancer development [39,40]. Loss of cell viability, fragmentation of DNA, and condensation of DNA are apoptotic cell features that can be identified with their inability to exclude vital dyes, electrophoretic patterns, and nuclear staining. Basic morphological changes in apoptosis can be studied using dual Ao/Eb fluorescent staining, which reveals differences among normal, early, late apoptotic and necrotic cells. Only late apoptotic and dead cells with damaged membranes are permeated by Eb, emitting orange-red fluorescence. Necrotic cells also take up Eb and stain uniformly orange, and fragmented apoptotic bodies are not seen in them [41,42]. In Ao/EtBr staining of cells treated with 100 µg/mL OIM, early and late apoptosis-related morphological peculiarities were observed.

Phosphatidyl serine (PS) is a membrane phospholipid seen on the cytoplasmic side of the membrane in normal cells that externalized in apoptotic cells to facilitate phagocytic recognition. This occurs before the nuclear changes in apoptosis. Annexin V can specifically bind to PS because of its high affinity to the latter, and hence is a positive indicator of apoptosis. In apoptotic cells, it reveals a brightly stained appearance with condensed chromatin in the early stages but shows fragmented nuclei, discernible as blue beads in the late stages. Hence, in double staining, Annexin V positive cells indicate death (apoptosis and necrosis) and Hoechst stain reveals nuclear fragmentation, if any, characteristic of only apoptotic cells, but not necrotic cells [43]. In the results of the present work, fluorescent microscopic observation revealed corresponding apoptotic changes as above, but the number of cells revealing apoptotic morphology was less in both concentrations of OIM used. The morphological changes induced by the OIM in HeLa cells are suggestive of apoptotic death. Further, the cell cycle analysis confirms the cell cycle arrest at the $G_1$/S phase.

UPLC-Q-TOF-MS fingerprinting of the root bark confirmed the presence of flavonoids such as Demethoxycentaureidin 7-O-rutinoside, Isorhamnetin- 3-O- rutinoside, Baicalein -7-O- glucuronide, 5,6,7- trihydroxy flavone, 3-Hydroxy -3′,4′,5′- trimethoxy flavone, 5,7-hydroxy -3-(4-methoxy phenyl) chromen-4-one, 4′-Hydroxy -5,7- dimethoxy flavanone, and 6-Ethoxy- 3(4′-hydroxy phenyl)-4-methyl coumarin in the *O. indicum* root extract. Previous studies have also indicated the presence of baicalein and biochanin A [23] in the roots of *O. indicum*. The compounds, which revealed their common presence in both

root bark, are attributed with anti-proliferative properties—biochanin A [25], baicalin [44], and baicalein [22] against cancer cell lines. The antioxidant properties of baicalein [45,46] and biochanin A [47] have been established previously and are also discussed in earlier chapters. Baicalin, the glucoronide of baicalein, is also an antioxidant [48]. Most of the compounds identified in the UPLC-Q-TOF-MS analysis of both extracts were flavonoids, which are already appreciated for their anti-inflammatory effects [49,50], and therefore, the alleviation of paw edema by OIM and OCM treatment discussed in previous chapters remains explainable. As per earlier reports [23], the root bark of *O. indicum* Vent. contains a biologically active flavonoid, chrysin, which has anti-proliferative [24], antioxidant, and anti-inflammatory properties [51]. HPLC analysis of OIM extract in this study, using acetonitrile: water (45:55) as a solvent system, revealed the presence of chrysin (retention time (RT): 17.15 min) by extending the run time to 20 min, reconfirming its presence. The callus extract also produced a feeble peak for chrysin at the same RT. Baicalein (RT: 8.65 min) was also detected in both extracts using HPLC analysis with a similar solvent system. A quantitative phytochemical estimation of these two compounds using HPLC revealed that the concentration of baicalein and chrysin was less in callus, as compared to root bark.

## 5. Conclusions

The *Oroxylum indicum* Vent roots extract (OIM) exhibited a dose-dependent cytotoxicity towards the colon, breast, and endometrial cancer cells. The study also indicated the possible mechanism of action of OIM is mediated through the cell cycle arrest at $G_1$/S phase. Induction of apoptosis in these cells was confirmed through double acridine orange-ethidium bromide staining/Annexin–Hoecsht staining and fluorescent microscopy. Further, the tested doses were found to be less toxic to the non-cancerous cell lines. Hence, the study concludes that the OIM can evolve as a source for future anticancer agents on bioassay-guided fractionation and purification.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr11010188/s1, Table S1. The compounds tentatively identified from O. indicum Vent. wild root bark (OIM) extract by UPLC-Q-TOF-MS analysis; Figure S1. Morphology of HeLa, HCT15 and MDA-MB-231 on treatment with OIM extract. (a) Untreated HeLa cells, (b) HeLa cells treated with 120 µg/mL OIM, (c) Untreated MDA-MB-231 cells, (d) MDA-MB-231 cells treated with 120 µg/mL OIM, (e) Untreated HCT15 cells and f) HCT15 treated with 120 µg/mL OIM; Figure S2. Cytotoxicity on non-cancer cell lines IEC-6 (rat intestinal cells) and vero (monkey kidney) cells.

**Author Contributions:** Conceptualization, S.M., J.J.A., H.H. and J.P.; methodology, S.M., J.J.A., H.H. and J.P.; software, A.N.; validation, S.M., S.E.M. and A.N.; formal analysis, S.M., J.J.A., H.H., J.P., L.L., S.E.M. and A.N.; investigation, S.M., J.J.A., H.H. and J.P.; resources, S.M., L.L., S.E.M. and A.N.; data curation, S.M, L.L., S.E.M. and A.N.; writing—original draft preparation, S.M., J.J.A., H.H. and J.P.; writing—review and editing, S.M., L.L., S.E.M. and A.N.; visualization, S.M., S.E.M. and A.N.; supervision, S.M. and A.N.; project administration, S.M., J.J.A., H.H. and J.P.; funding acquisition J.J.A. and H.H. All authors have read and agreed to the published version of the manuscript.

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