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Anticancer Potential of The Leaf Extracts of *Breonadia salicina* Hepper and J. R. I. Wood (Rubiaceae) On Ht-29 Cancer Cell Line

Uwaisu Iliyasu*[1], Sani Shehu[1], YahayaYakubu[2], Ja'afaru Sani and Hammada Sani[4]

[1]Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences Kaduna State University, Kaduna-Nigeria
[2]Department of Pure and Applied Chemistry, Faculty of Physical Science, Kaduna State University, Kaduna-Nigeria
[3]Department of Biochemistry, Faculty of Life Science, Kaduna State University, Kaduna-Nigeria
[4]Department of Community Medicine, Faculty of Clinical Sciences, Kaduna State University, Kaduna-Nigeria

Abstract

Breonardia salicina is an evergreen plant commonly used across Nigeria and various African countries to treat ailments such as cancer, gastrointestinal disorders, fever, headache, arthritis, diabetes, inflamed wounds, and ulcers. This study investigated the anticancer properties of the leaf extracts of *B. salicina*. A 300 g portion of the dried, powdered leaves was extracted using 1 L of 95% ethanol through maceration. The resulting extract was filtered, concentrated to dryness, and fractionated using n-hexane, ethyl acetate, and n-butanol. The cytotoxic effects of the crude extract and its fractions on HT-29 cancer cells were assessed using MTT assay. Further analyses included fluorescence microscopy, apoptosis detection, and cell cycle profiling of the treated cells. The half-maximal inhibitory concentration (IC50) values for the extract and its fractions ranged from 26 to 374.78 µg/mL, demonstrating selective cytotoxicity against HT-29 cells. Microscopic images indicated a dose-dependent occurrence of early and late apoptosis and necrosis. Flow cytometry confirmed the induction of apoptosis, and cell cycle analysis revealed altered distributions among the G0/G1, S, G2/M, and sub-G1 phases of the cell cycle. These findings suggest that *B. salicina* leaf extract triggers apoptosis in HT-29 cells.

Keywords: Apoptosis, Cell cycle analysis, MTT assay, HT-29, *Breonardia salicina*

*Corresponding author:
uwaisu.iliyasu@kasu.edu.ng
+234 7039762897

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Introduction

Breonardia salicina is an evergreen tree that can reach a height of up to 20 m (Al-Qurainy *et al*., 2013). It is commonly known as Matumi or African Tears (Sani *et al*., 2018). In Northern Nigeria, Fulani speakers refer to it as Leggel, whereas in Hausa, it is called Kadanyar rafi (Bello and Ahmad, 2013) or Kadanyar kurmi (Sani *et al*., 2018). Among the Zulu people of South Africa, the plant is known as Umfomfo (Protected Trees, 2013). *B. salicina* is widely distributed across tropical and subtropical regions, including Saudi Arabia, Yemen, Madagascar, Ethiopia (Gabayi, 2017), and various parts of Nigeria (Bello and Ahmad, 2013).

Traditionally, the plant is used for a variety of medicinal purposes, including the treatment of cancer, gastrointestinal disorders, fever, headache, arthritis, diabetes, inflamed wounds, ulcers, and diarrhea (Sibandze *et al*., 2010). Its powdered bark is applied to fresh wounds (Neuwinger and Hans, 1994; Venter and Venter, 2002; Amusan *et al*., 2002), and decoctions made from the root are used to manage heart conditions (Arnold and Gulumian, 1984). In Tanzania, a root decoction serves as a purgative to ease constipation (Martins & Nunez, 2015), whereas in Malawi, a leaf infusion is used to treat stomachaches and diarrhea (Bisi-Johnson *et al*., 2010). In South Africa, the bark is used to treat gastric ailments (Mahlo *et al*., 2013). In Nigeria, leaf decoctions are used in bathing rituals to manage yellow fever (Olanipekun and Babajide, 2016). Among the Tiv people of North Central Nigeria, the plant is used to address sleeping sickness and respiratory issues (Nvau *et al*., 2019), and in Fulani communities, it is used against trypanosomiasis and parasitic worm infections (Sani *et al*., 2018).

Previous studies by Mahlo *et al.* (2013) revealed the notable cytotoxic effects of crude extracts and several isolated compounds on Vero monkey kidney cells. The present study aimed to evaluate the anticancer properties of *B. salicina* leaf extracts and their respective fractions.

Collection and Identification of Plant Material

B. salicina was initially identified in the field based on its morphological characteristics in Kudingi Village, Giwa Local Government Area, Kaduna State, Nigeria. A sample of the plant's leaves along with its flowers was collected in September 2021 and taken to the Herbarium Unit of the Department of Botany, Ahmadu Bello University (ABU), Zaria, for authentication. The plant was identified by Mr. Namadi Musa, a taxonomist in the Department, and assigned a voucher specimen number ABU900383.

A sufficient quantity of leaves was harvested, cleaned of extraneous materials, and air-dried in the shade for two weeks. The dried leaves were then ground into a fine powder using a pestle and mortar and stored in an airtight container for future use.

Extraction Procedure

A total of 300 g of dried powdered leaves was macerated using 1 L of 95% ethanol. After 72 h, the extract was filtered through Whatman No. 1 filter paper and concentrated to dryness using a rotary evaporator at 27°C. The percentage yield of the extract was calculated, and the dried extract was stored in a desiccator for subsequent use."

Fractionating of the Crude Ethanol Extract

A 10 g sample of the leaf extract was suspended in 250 mL of distilled water and sonicated at 20°C for 10 min. The mixture was then partitioned with n-hexane using a separation funnel, and the hexane fraction was collected. This process was repeated several times until thorough partitioning was achieved. The combined n-hexane fraction was then evaporated to dryness using a rotary evaporator. The remaining aqueous layer was subsequently partitioned with ethyl acetate, and the extraction was repeated until the aqueous layer became colorless. The ethyl acetate fraction was collected and evaporated to dryness for further analysis. Finally, the residual aqueous layer was partitioned with n-butanol, and the process was repeated several times until exhaustive extraction was achieved. Both n-butanol and final aqueous fractions were collected separately, evaporated to dryness, and stored in a desiccator for future use.

Cytotoxicity Studies

Sourcing of the Cell lines (HT-29)

The HT-29 human colorectal cancer cell line was obtained from the Molecular Medicine Laboratory, Institute of Bioscience, at University of Putra Malaysia. The cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified incubator with 5% CO_2 and 95% air.

Cell Treatment Procedures

The cytotoxic effects of *B. salicina* leaf extracts and their fractions on HT-29 cells were assessed using the method described by Mosmann (1983) and modified by Olaru et al. (2015). Monolayer cultures were detached using trypsin-EDTA to obtain single-cell suspensions, and viable cells were counted. A total of 100 µL containing 5×10^3 HT-29 cells per well was seeded into a 96-well plate and incubated for 24 h at 37°C in a humidified atmosphere with 5% CO_2. After incubation, the supernatant was removed, and the cells were washed with phosphate-buffered saline (PBS). The extracts and fractions were then added to the respective wells at concentrations ranging from 0 to 1600 µg/mL and incubated for 24 h. Wells containing only culture medium served as negative controls, and all treatments were performed in triplicate.

Cytotoxicity and Cell Viability Assay of HT-29 on B. Salicina

Cytotoxicity testing on HT-29 cells was performed using the method outlined by Monks *et al.* (1991), with modifications described by Ismail *et al*. (2014). After 24 h of incubation with the test compounds, 20 µL of MTT solution (5 mg/mL in phosphate-buffered saline) was added to each well, followed by a 4-hour incubation at 37°C with 5% CO_2. The MTT-containing medium was then removed, and the resulting formazan crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using a Synergy H1 microplate reader (Biotek). Formazan production is directly correlated with the number of viable cells. Cell viability was assessed by calculating the percentage of cell growth inhibition using the following formula:

% Cell Growth Inhibition = 100 – [(OD of treated cells / OD of control cells) × 100]

A nonlinear regression curve was generated by plotting the percentage of cell inhibition against the logarithm of the extract concentration, from which the IC_{50} values were determined.

Qualitative Apoptosis

Cultured cells were detached using trypsin-EDTA, collected, and centrifuged at 1000 RCF for 5 min. The resulting cell pellets were washed twice with phosphate-buffered saline (PBS), resuspended in PBS, and fixed with 4% methanol-free formaldehyde at room temperature for 15 min. Following fixation, the cells were incubated on ice for 30 min. For staining, 10 µL of propidium iodide (1 mg/mL) and 1 µL of acridine orange (10 mg/mL) were added to 10 µL of cell suspension. The mixture was incubated in the dark at room temperature for 15 min and then mounted onto a glass slide. Stained cells were observed using an inverted fluorescent microscope (Zeiss Axio Vert A1, Germany) equipped with an image acquisition system (AxioCam MRm, Germany). Multiple images of the cells were captured (Karthikeyan *et al*., 2011).

Quantitative Apoptosis

HT-29 cells were detached using trypsin-EDTA, transferred to a centrifuge tube, and centrifuged at 1000 RCF for 5 min. The resulting pellet was washed twice, resuspended in 100 µL of 1X binding buffer, and transferred to Eppendorf tubes for analysis. To each sample, 5 µL of Annexin-V-FITC and 10 µL of propidium iodide were added, with the exception of the single dye and the unstained control cells. The tubes were incubated at room temperature in the dark for 15 min. After incubation, the cell-dye mixture was gently vortexed until well mixed, followed by the addition of 400 µL of 1X binding buffer. The final mixture was transferred to flow cytometry tubes for analysis using a flow cytometer.

Cell Cycle Analysis

HT-29 cells were detached using trypsin-EDTA, collected, and centrifuged at 1000 RCF for 5 min. The resulting pellet was washed twice, resuspended in 500 µL of component A-diluted dye, and incubated for 1 h. After incubation, the cells were centrifuged again at 1000 RCF for 5 min, followed by three washes with complete growth medium. The washed cells were then resuspended in 500 µL of component B assay buffer. The final cell-buffer mixture was transferred into flow cytometry tubes and analyzed using a flow cytometer.

Statistical Analysis

For the cytotoxicity studies, the results were analyzed using a non-linear regression. A One-Way Analysis of Variance (ANOVA) was performed, followed by a post hoc Dunnett test, using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Cytotoxicity of the Extract and Fractions

MTT assay results demonstrated that HT-29 cell viability decreased in a concentration-dependent manner. Both the extract and its fractions significantly ($P < 0.01$) inhibited HT-29 cell growth,

with the ethyl acetate fraction exhibiting the most pronounced inhibitory effect. At concentrations of 100, 200, 400, 800, and 1600 µg/mL, the percentage inhibition of the ethyl acetate fraction was 72.4%, 82.9%, 83.9%, 87.1%, and 93.9%, respectively. The ethanol extract followed with percentage inhibition of 62.2%, 66.7%, 69.0%, 81.8%, and 88.8% at the same concentrations (Tables 1 and 2).

Qualitative Apoptosis of the EAF Using Microscopy

Morphological Studies by Monochromatic Light

The images in figure 1A show the normal morphology of untreated HT-29 cells, which served as the control group. In contrast, figure 2B (cells treated with EAF) displays the typical features of apoptosis. These include the formation of apoptotic bodies, membrane blebbing, and a noticeable decrease in cell population. Figure 1B further illustrates the apoptotic effects of the extract on the HT-29 cells.

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Quantitative Apoptosis of the EAF by Flow Cytometry

The proportion of viable cells was 31.1%, whereas cells undergoing early apoptosis, late apoptosis, and necrosis accounted for 22.5%, 19.9%, and 2.5%, respectively (Figure 4).

Cell Cycle of the HT-29 Cells Treated with EAF

Flow cytometry analysis of the cell cycle in the control group revealed a relatively even distribution across all four phases: 16% in Sub-G0, 36.8% in G0/G1, 26.5% in S, and 6% in G2/M, suggesting normal cell viability and cycle progression. In contrast, cells treated with the extract exhibited significant disruptions in cell cycle distribution, with 65.45% in Sub-G0, 10.9% in G0/G1, 8.1% in S, and 2.5% in G2/M. These alterations strongly suggest the induction of cell cycle arrest in these cells.

Table 1: Percentage inhibition of extracts and fractions on HT-29 cells.

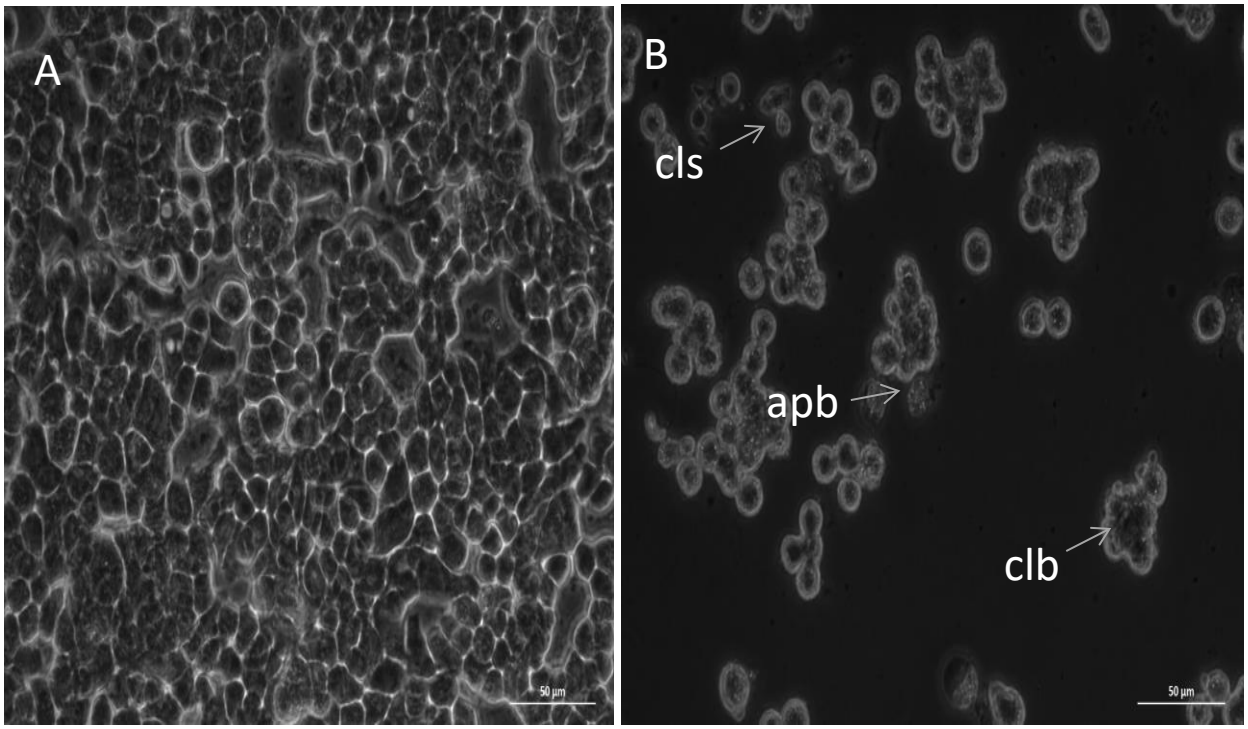
	Percentage inhibitions ± SEM						
Con µg/ml	0	100	200	400	800	1600	P value*
EAF	0.0	72.4 ± 0.9*	82.9 ± 0.1*	83.9 ± 2.3*	87.1 ± 0.5*	93.9 ± 0.0*	<0.01
EE	0.0	62.2 ± 0.2*	66.7 ± 0.1*	69.0 ± 0.3*	81.8 ± 0.8*	88.8 ± 0.1*	<0.01
NBF	0.0	47.7 ± 0.2	58.2 ± 0.1*	65.0 ± 0.3*	71.6 ± 1.1*	84.1 ± 0.2*	<0.01
AQF	0.0	57.2 ± 0.2*	60.5 ± 0.1*	66.2 ± 0.3*	69.5 ± 0.6*	85.6 ± 0.2*	<0.01

Key: Values are means ± SEM of three replicates. EE = 95% Leaf Ethanol Extract, EAF = Ethyl acetate Fraction, NBF = n-Butanol Fraction, AQF = Aqueous Fraction *P < 0.01

Table 2: Median Inhibitory Concentrations (IC_{50}) for the Extracts and Fractions

Fraction	IC50 (ug/ml)
EEF	200.75
EAF	26.74
NBF	374.78
AQF	313.28

Key: EE = 95% Ethanol Extract, EAF = Ethyl acetate Fraction, NBF = n-butanol Fraction, AQF = Aqueous Fraction.



A B

Figure 1A, B: Morphology of the untreated and treated unstained HT-29 cells Viewed Against dark background at Magnification X400. A = untreated cells, B = cells treated with the EAL fraction, apb = apoptotic bodies, clb = cell blebbing, and cls = cell shrinkage.

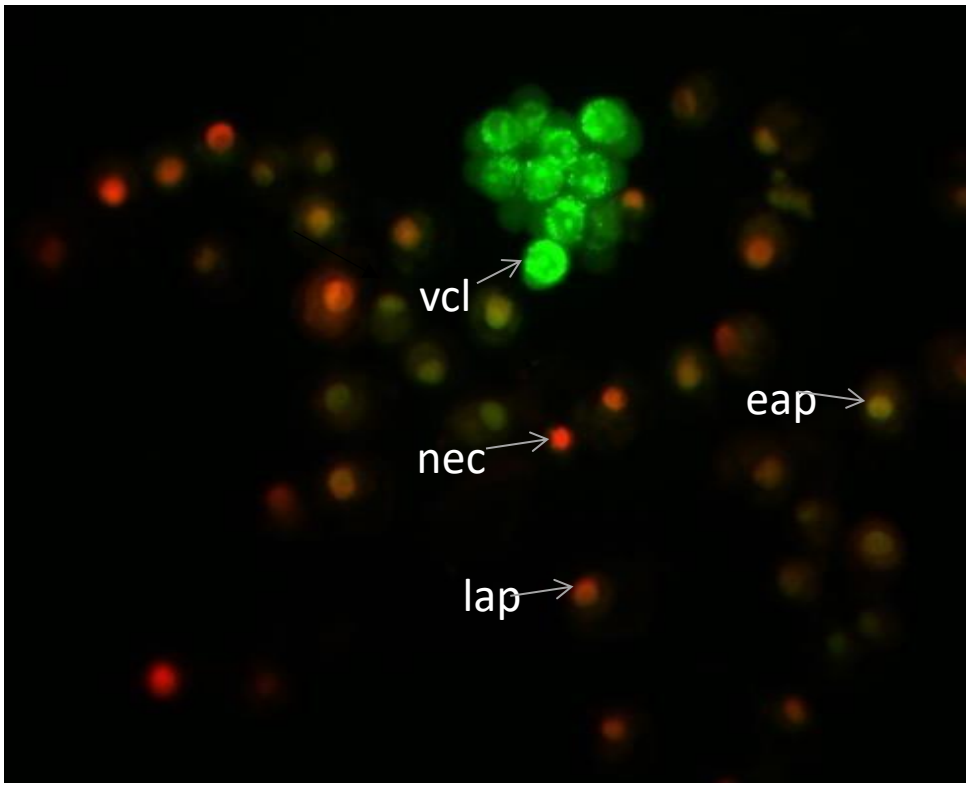


Figure 3: of the Morphology of HT-29 cells treated with EAF stained with AO/PI staining reagents and viewed under Fluorescent Microscope, magnification X400. eap = early apoptosis, lap = late apoptosis, nec = necrotic cells and vcl = viable cell

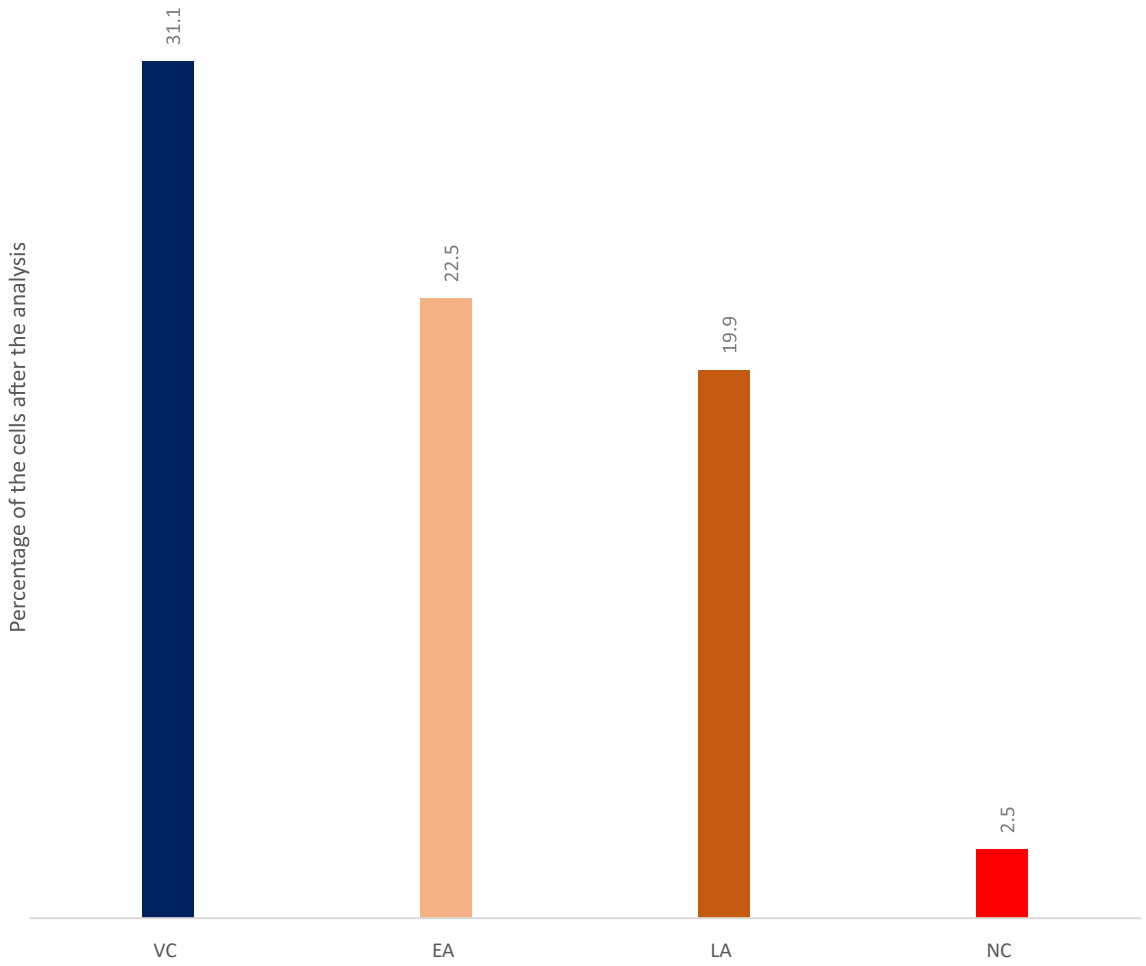


Figure 4: Apoptotic activity of EAF in HT-29 cells. VC = Viable cells, EA = Early apoptosis, LA = Late apoptosis, NC = Necrotic cells.

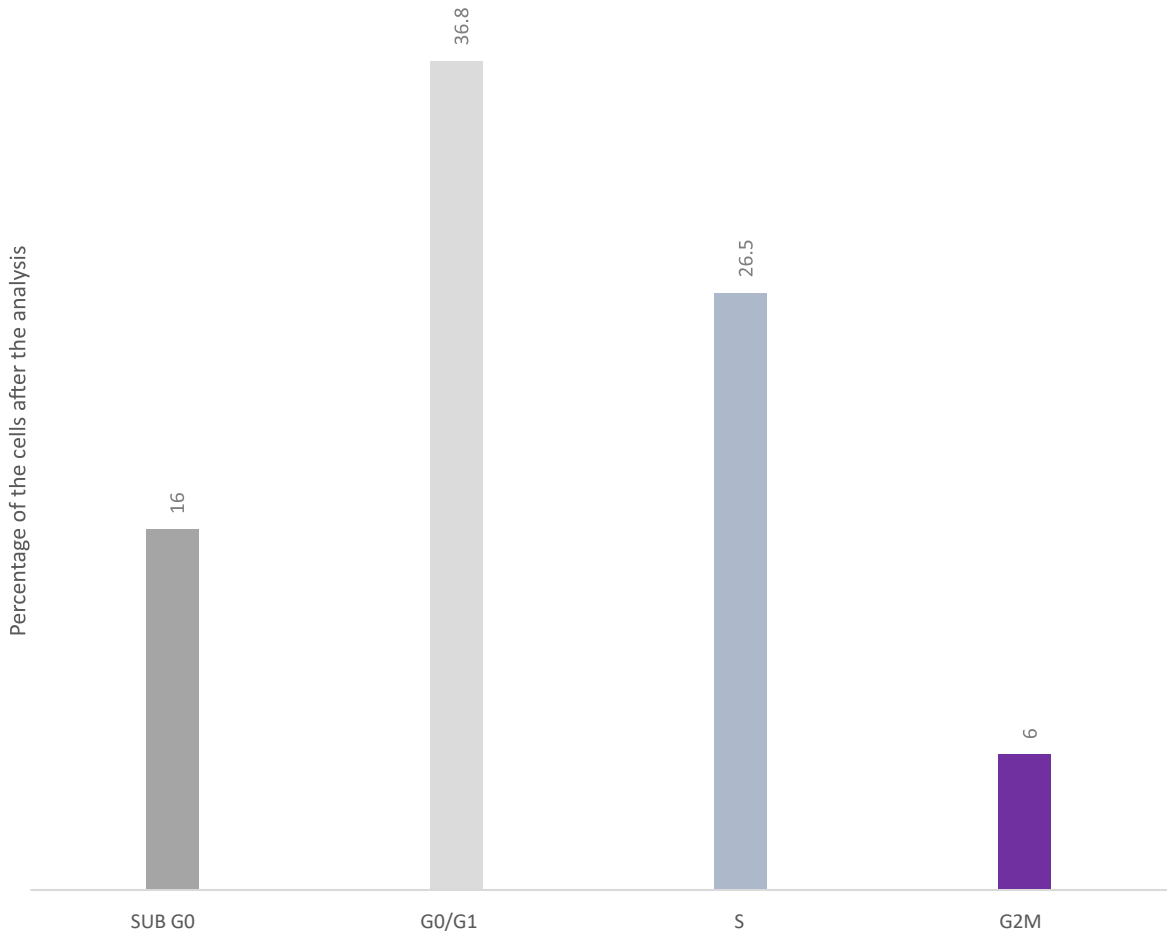


Figure 5: Cell Cycle Analysis of the Control Showing SUB G0, G0/G1, S and G2M Phases.

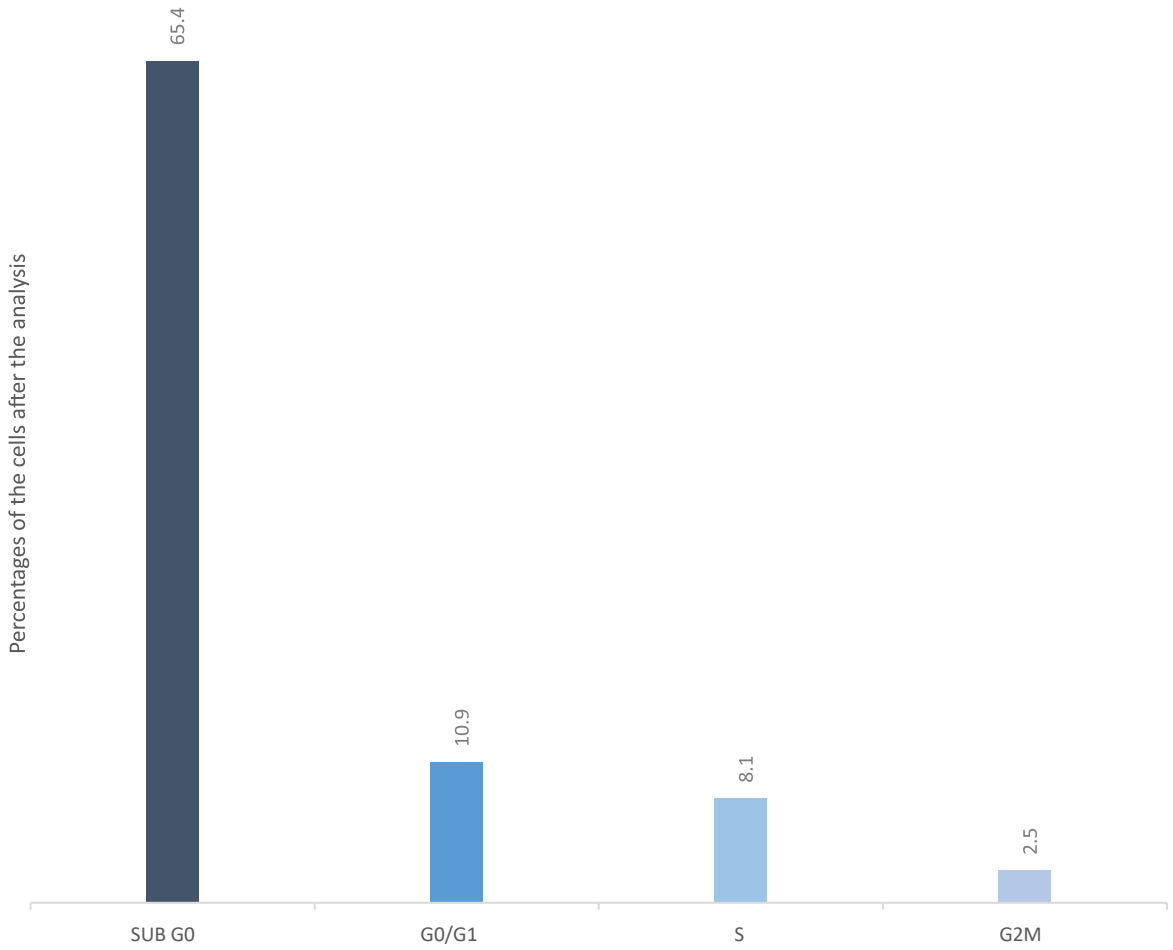


Figure 6: Cell Cycle Analysis of EAF against HT-29 cells Showing SUB G0, G0/G1, S and G2M Phases.

Discussion

The yellow, water-soluble salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) serves as a viability indicator in cytotoxicity assays. In living cells, the mitochondrial enzyme succinate dehydrogenase reduces MTT by cleaving its tetrazolium ring, resulting in the formation of insoluble purple-formazan crystals. The quantity of formazan produced is directly proportional to the number of viable cells, and cell viability was determined based on the absorbance of the crystals. According to the American National Cancer Institute classification, plant extracts exhibit cytotoxic activity against human cancer cell lines as follows: IC50 ≤ 20 µg/mL indicates strong anticancer potential, 21–100 µg/mL indicates active, 200–500 µg/mL denotes moderate activity, and IC50 ≥ 1000 µg/mL is considered inactive (Dehghan-Nayeri *et al*., 2020).

Apoptosis, a tightly regulated process of programmed cell death, serves as a critical endpoint in the screening of anti-cancer agents (Zhang *et al*., 1998). Prioritizing compounds that induce apoptosis helps eliminate those that cause nonspecific cytotoxicity, thereby improving drug discovery efficiency (Aigner, 2002). Numerous natural products with apoptosis-inducing properties have been identified, many of which are plant-derived and consumed regularly in small quantities (El Menshawi *et al*., 2010). Therefore, it is imperative to investigate such botanical sources for potential apoptotic inducers (Shiezadeh *et al*., 2013). In this study, the ethyl acetate fraction (EAF) was identified as the most active and was further examined for morphological indicators of apoptosis using both unstained and stained microscopy. Unstained images displayed classical apoptotic features, such as membrane blebbing, apoptotic body formation, and a reduced cell population. Fluorescent staining with

acridine orange (AO) and propidium iodide (PI) confirmed these findings. AO-stained viable cells were green, while PI-stained apoptotic and necrotic cells were red, with the intensity of red coloration reflecting the progression from early apoptosis to necrosis. These results indicate that the ethyl acetate fraction of the ethanol leaf extract exhibits notable anticancer activity against HT-29 human colorectal cancer cells, particularly by inducing apoptosis during the G0/G1 phase of the cell cycle. These findings are consistent with those reported by Jayalalitha and Natarajan (2018).

Conclusion

The study demonstrated that *Breonardia salicina* extract exerts a dose-dependent growth-inhibitory effect on HT-29 cell lines, with the ethyl acetate fraction showing the most potent anticancer activity.

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