Anticancer Activity and Apoptotic Induction of *C. Fruticosa* Leaf against HeLa cell Lines

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ABSTRACT

Cervical cancer remains one of the most prevalent cancers among women worldwide and continues to pose a major global health challenge. Despite advances in treatment, the limitations and adverse effects of conventional therapies emphasize the need for safer and more effective anticancer agents derived from natural sources. This study investigated the anticancer potential of *Cordyline fruticosa* leaf methanolic extract against cervical cancer (HeLa) cells by analyzing its phytochemical composition, cytotoxic activity, and ability to induce apoptosis. High-Performance Liquid Chromatography (HPLC) was used to identify the extract's flavonoid content. Five major flavonoids were detected: Rutin (35.2%), Luteolin (20.1%), Kaempferol-3-O-glucoside (17.8%), Apigenin (14.6%), and a Myricetin derivative (8.3%). These compounds are known for their antioxidant and anticancer properties, suggesting that *C. fruticosa* is a promising source of bioactive phytochemicals. Cytotoxic activity was evaluated using the MTT assay. HeLa cells treated with extract concentrations ranging from 0–100 µg/mL for 48 hours exhibited a dose-dependent reduction in cell viability, with an IC_{50} value of 21.58 µg/mL, indicating a high cytotoxicity. One-way ANOVA showed significant differences among treatment groups ($p < 0.001$), confirming the extract's inhibitory effect on cancer cell proliferation. To determine the mechanism underlying this cytotoxic effect, apoptosis was assessed using TUNEL and Annexin V/PI staining. The TUNEL assay revealed increased DNA fragmentation at higher extract concentrations. Flow cytometry analysis further demonstrated concentration-dependent apoptosis, with apoptotic cell percentages of 24.3%, 46.7%, and 71.2% at 25 µg/mL, 50 µg/mL, and 100 µg/mL, respectively, compared to 5.8% in untreated cells. These results demonstrate that the methanolic extract of *C. fruticosa* leaf not only inhibits cellular proliferation but also actively promotes programmed cell death, a desirable mechanism in anticancer therapy.

Keywords: MTT assay, Cytotoxicity, Flavonoids, TUNEL assay, Annexin V/PI staining, Apoptosis

INTRODUCTION

The human body is composed of trillions of cells that normally grow, divide, and undergo programmed cell death, known as apoptosis; when this regulation is disrupted and cells proliferate uncontrollably while evading apoptosis, cancer develops.[1] In 2020, approximately 19.3 million new cancer cases were reported worldwide.[2] In the Philippines, cervical cancer is the second most common cancer among women, with about 7,277 new cases and 3,807 deaths annually.[3] Chemotherapy remains a primary treatment modality for cancer, targeting rapidly dividing cells. Doxorubicin, for example, inhibits enzymes essential for cancer cell proliferation.[4] However, because chemotherapy also affects healthy rapidly dividing cells in the bone marrow, hair follicles, and gastrointestinal tract, patients frequently experience adverse effects such as fatigue, hair loss, nausea, mucositis, and fertility complications.[5] Despite continuing advances in treatment, a definitive cure for cancer remains elusive, prompting increasing interest in plant-derived therapeutic alternatives.

Despite extensive efforts to enhance cancer treatment and improve patient survival, a complete cure has yet to be achieved, leading to growing interest in plant-derived therapeutic options. In developing countries, about 80% of the population relies on traditional medicines, most of which originate from plants, increasing both their demand and the need for sustainable management.[6–7] *Cordyline fruticosa* (Ti Plant), native to Southeast Asia and Papua New Guinea, contains various bioactive compounds, including glycosides, steroids, terpenoids, flavonoids, phenolics, and saponins.[8] Its methanolic crude extract, defined as the mixture of plant compounds obtained using methanol as a solvent, has demonstrated cytotoxic activity in brine shrimp lethality assays.[9] However, limited studies have investigated its effects on human cervical cancer (HeLa) cells—an immortal cell line widely used in biomedical research—particularly in relation to apoptosis induction.

The MTT assay, a colorimetric method that measures cellular metabolic activity as an indicator of viability and cytotoxicity, is commonly used to determine the half-maximal inhibitory concentration (IC_{50}), or the concentration required to inhibit 50% of cell viability.[10] Nevertheless, because metabolic activity does not fully confirm programmed cell death, complementary assays such as TUNEL, which detects DNA fragmentation, and Annexin V/PI staining, which differentiates viable, early apoptotic, late apoptotic, and necrotic cells, are necessary. Plant-derived compounds, including flavonoids such as ginsenoside Rh2, have been reported to induce apoptosis in cancer cells, highlighting their therapeutic potential.[11,12]

In this context, the present study evaluates the anticancer activity and apoptotic effects of *Cordyline fruticosa* methanolic leaf crude extract against HeLa cells. It examines the specific flavonoids and bioactive compounds present in the extract and determines its percent inhibition at concentrations of 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, and 100 µg/mL using the MTT assay, from which the IC_{50} value is calculated. Furthermore, it assesses whether significant differences exist in inhibitory activity across concentrations and evaluates the extract's ability to induce apoptosis at 25, 50, and 100 µg/mL through TUNEL assay and Annexin V/PI staining, determining whether variations in apoptotic effects occur among the tested concentrations. Guided by these objectives, the study tests the null hypotheses that there is no significant difference in IC_{50} values among concentrations of the extract and that the extract does not produce a significant apoptotic effect on HeLa cells. The study also aims to provide insights for healthcare professionals and patients regarding the potential of medicinal plants, specifically *Cordyline fruticosa*, in cancer treatment.

MATERIALS AND METHODS

Collection and Identification of Plant Materials

Mature, healthy leaves of *Cordyline fruticosa* (Ti Plant) were collected in Delhi, India, during the dry season. The samples were placed in clean, sealed polyethylene bags to prevent contamination and reduce moisture loss during transport. Plant identification and authentication were conducted by specialists at ACME Research Solutions, and voucher specimens were deposited for future reference.

Preparation of plant extract

The collected *C. fruticosa* leaves were air-dried in a shaded, well-ventilated area for ten days and then ground into a fine powder using a mechanical grinder. Ten grams of the powdered leaves were macerated in 100 mL of methanol at a 1:10 (w/v) ratio and allowed to stand at room temperature for 24 to 72 hours to obtain the extract.

Rotary Evaporation

The filtered extract was transferred to a round-bottom flask and concentrated using a rotary evaporator at 45°C under reduced pressure, equipped with a water bath and condenser. This method allowed gradual solvent removal without subjecting the extract to excessive heat, thereby preserving the integrity of the phytochemicals. The resulting semi-solid residue was stored in amber vials at 4°C to protect it from light and oxidation. This approach minimized thermal degradation and maintain the stability of key phytochemicals, including flavonoids.[13]

Cell Lines and Culture Conditions

HeLa cells were obtained from the National Center for Cell Science (NCCS, Pune, India) that were used for all cytotoxicity and apoptosis assays. Cells were routinely screened and confirmed to be mycoplasma-free before use. They were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Prior to assays, cells were counted using a hemocytometer, and appropriate dilutions were prepared to achieve the desired seeding density.

High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) was employed to separate, detect, and preliminarily identify the major flavonoid constituents present in the crude methanolic extract of *Cordyline fruticosa* leaf. The extract was dissolved in HPLC-grade methanol (1 g/mL), sonicated for 15 min, filtered through a 0.45 µm syringe filter, and diluted (1:5–1:10, v/v) prior to injection. Chromatographic separation was carried out using a Shimadzu Prominence HPLC system with a photodiode array (PDA) detector and a reversed-phase C18 column (Phenomenex Luna, 250 × 4.6 mm, 5 µm) maintained at 30 °C. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B), delivered at 1.0 mL/min under a gradient program (10–100% B over 20 min). The injection volume was 20 µL. The calculation for concentration of each compound is given by this formula:

$$Concentration = \frac{(Peak\ Area\ -\ Intercept)}{Slope}$$

MTT Assay

After 24 hours of incubation to allow cell attachment, the 100 µL of complete medium was aspirated and replaced with fresh medium of the same concentration. HeLa cells were treated with serially diluted *C. fruticosa* methanolic leaf extract (100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 µg/mL) prepared from a 20 mg/mL stock solution. Treatments were applied in triplicate (200 µL/well). Doxorubicin (0.5–10 µM) served as the positive control, while DMEM-treated cells served as the negative control. After 24 h, MTT reagent (0.5 mg/mL final concentration) was added and incubated for 4 h. Formazan crystals were dissolved in ethanol, and absorbance was read at 570 nm with a reference wavelength of 630–690 nm. Cell viability and percent inhibition were calculated relative to the negative control. The calculation of percentage inhibition of cancer cells is carried out by a formula was adopted from the study of Fithrotunnisa *et al.* (2020) on the In Vitro Cytotoxicity of *Hibiscus sabdariffa Linn* Extracts on A549 Lung Cancer Cell Line:[21]

$$\%\ Inhibition = \frac{(absorbance\ of\ negative\ control\ -\ absorbance\ of\ treatment)}{absorbance\ of\ negative\ control}\ x\ 100\%$$

TUNEL Assay

Following incubation, cells were treated with the flavonoid-rich fraction of *Cordyline fruticosa* at final concentrations of 25, 50, and 100 µg/mL. The negative control group received vehicles only (0.1% DMSO), while the positive control group was treated with 1 µM staurosporine to induce apoptosis. All treatments were carried out for 24 hours under standard culture conditions. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and labeled using a commercial TUNEL assay kit according to the manufacturer's protocol. Nuclei were counterstained with DAPI, and fluorescence images were captured at 20× magnification. TUNEL-positive cells were quantified from three random fields per well.

Annexin V/PI Staining

Cells were treated with crude methanolic extract of *Cordyline fruticosa* leaf at final concentrations of 25, 50, and 100 µg/mL, prepared by serial dilution from a 1000 µg/mL stock solution in methanol. Vehicle-treated cells served as the negative control, while staurosporine (1 µM, 4 h) was used as the positive control. Extract treatments were applied for 24 h. After treatment, both adherent and floating cells were harvested by gentle trypsinization, pooled with media, centrifuged at 300 × g for 5 minutes, and washed twice with cold PBS. Cells were resuspended in 100 µL of binding buffer, stained with 5 µL Annexin V-FITC and 5 µL PI, and incubated for 15 minutes in the dark at room temperature. Subsequently, 400 µL of binding buffer was added, and samples were analyzed immediately using a flow cytometer (488 nm excitation, 530 nm emission for FITC, 617 nm for PI). A minimum of 10,000 events per sample were recorded, and compensation was applied using single-stained controls.

Statistical Analysis

A completely randomized experimental sampling technique was employed. All treatments were performed in triplicate, and cells were randomly assigned to treatment and control groups. Quantitative data on flavonoid content were performed using external standard calibration, in which the calibration curves were generated by plotting peak area (mAU·s) against known flavonoid concentrations (µg/mL). The concentrations of unknown samples were determined using the regression equation obtained from the standard curve in GraphPad Prism. Cytotoxicity data from the MTT assay were analyzed using one-way ANOVA to assess differences among the various concentrations of the *Cordyline fruticosa* leaf methanolic extract tested on HeLa cells. The IC_{50} value was determined through Non-linear Regression. Apoptosis assay results were also analyzed with one-way ANOVA followed by Tukey's post hoc test to compare treatment groups. Flow cytometry data from Annexin V/PI staining were processed in FlowJo software, and apoptosis was expressed as the percentage of Annexin V-positive cells. A significance level of $P < 0.05$ was applied for all statistical evaluations.

RESULTS AND DISCUSSION

High Performance Liquid Chromatography (HPLC) was performed to determine the major bioactive compounds present in *C. fruticosa* leaf methanolic extract. The following results are summarized in Table 1, including its retention time, peak area, and calculated concentrations in the said plant extract.

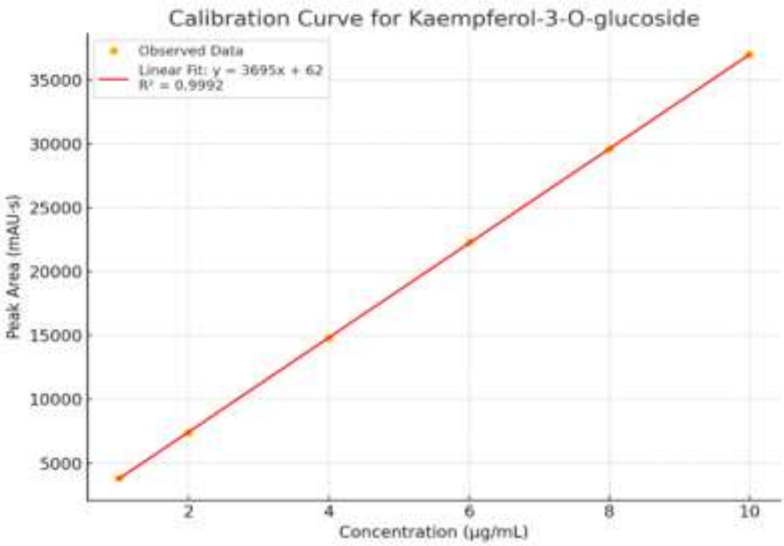
Table 1. Quantitative Analysis of Flavonoid Compounds in *C. fruticosa* Leaf Methanolic Crude Extract with Standard Solutions of 1 - 10 µg/ml by HPLC

Peak	**Compound**	**Retention Time (Min)**	**Peak Area (%)**	**R^2 value**	**Calc. Conc. (µg/ml)**
1	Kaempferol-3-O-glucoside	8	17.8	0.9992	4.2
2	Rutin (Quercetin glycoside)	9.3	35.2	0.9992	8.5
3	Luteolin	10.8	20.1	0.9993	5
4	Apigenin	13.5	14.6	0.9991	3.2
5	Myricetin derivative (minor)	15	8.3	0.9990	1.8

R^2 value = coefficient of determination for calibration curve linearity

Results from Table 1 reveal Rutin (Quercetin glycoside) is the most abundant flavonoid present in *C. fruticosa* leaf methanolic extract, accounting for 35.2% of the total peak area. The said bioactive compount was then followed by Luteolin (20.1%), Kaempferol-3-O-glucoside (17.8%), Apigenin (14.6%), and Myricetin derivative with the least amount (8.3%). Rutin being the most abundant in terms of the total flavonoid-associated peak area can be attributed to its strong solubility in methanol and structural stability during both extraction and analysis processes. These findings align with reports indicating Rutin (Quercetin glycoside) as the most stable and water-soluble bioactive compound commonly found in plants.[14]

Figure 1. Calibration Curve for Kaempferol-3-O-glucoside



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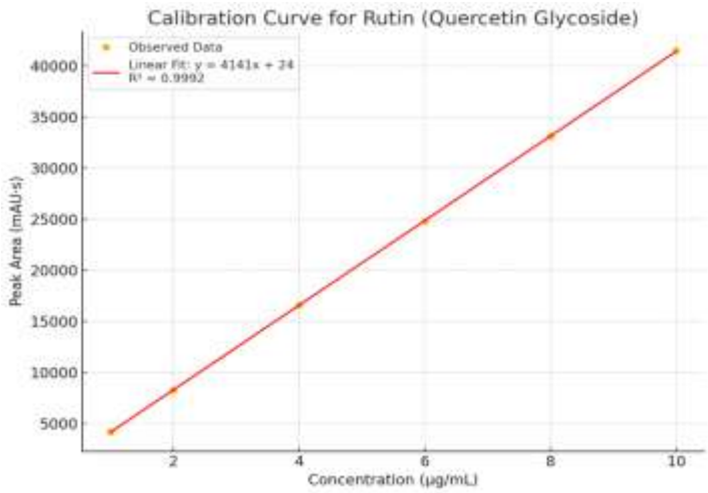
The calibration curve for Quercetin-3-O-glucoside, as depicted in Figure 1, shows a strong linear correlation between absorbance and flavonoid concentration, with an excellent coefficient of determination ($R^2 = 0.9992$), confirming that the detector response is directly proportional to the concentration across the tested range. This high degree of linearity establishes the robustness and reliability of the analytical method, thereby validating its suitability for precise quantitative determination of Quercetin-3-O-glucoside. Furthermore, the slope of 3695 reflects the sensitivity of the detector, indicating that each incremental increase of 1 µg/mL, corresponds to an approximate rise of 3695 mAU·s in the peak area.

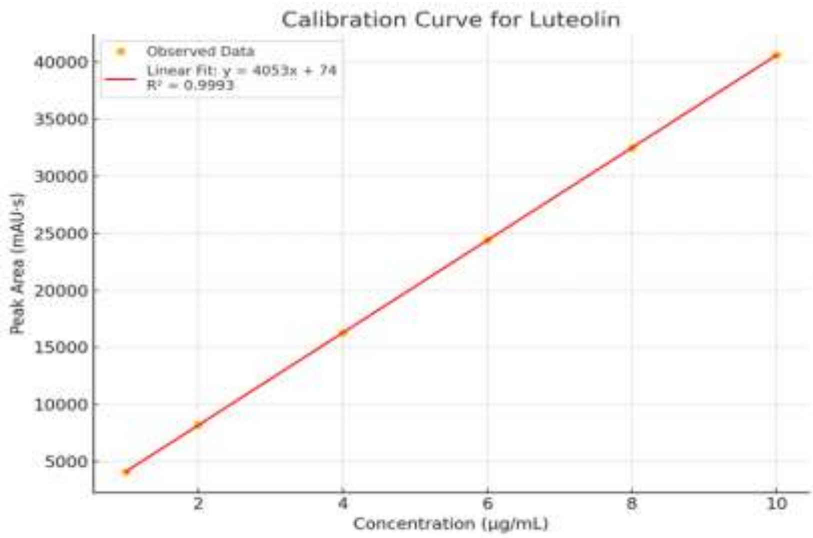
Figure 2. Calibration Curve for Rutin (*Quercetin glycoside)*



The calibration curve for Rutin *(Quercetin glycoside)*, as shown in Figure 2, exhibits a highly linear relationship between concentrations and detector response, with a correlation coefficient ($R^2 = 0.9992$) confirming the proportionality and reliability of the method across the tested range. The slope of 4141 reflects the detector's sensitivity, indicating that each incremental increase of 1 µg/mL in Rutin concentration corresponds to an approximate rise of 4141 mAU·s in peak area, thereby underscoring the method's precision in quantification.

Furthermore, Rutin accounted for 35.2% of the total flavonoid-associated peak area, establishing it as the most abundant flavonoid in the extract. This predominance highlights the compound's biological significance, as Rutin is well-documented for its diverse pharmacological properties, including anticancer, antioxidant, anti-inflammatory, and other therapeutic activities, thereby reinforcing the relevance of its quantification in phytochemical and biomedical research.

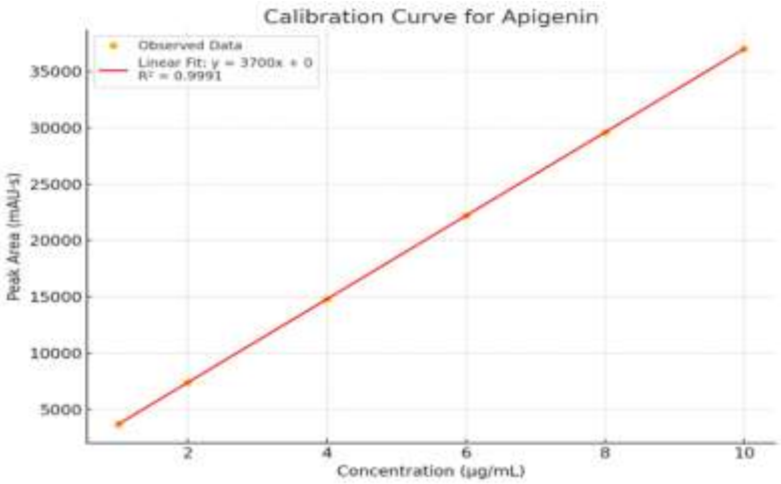
Figure 3. Calibration Curve for Luteolin



The calibration curve for Luteolin, as presented in Figure 3, demonstrates a direct and highly linear relationship between peak area and concentration, with an excellent correlation coefficient ($R^2 = 0.9993$) confirming the accuracy and reproducibility of the method across the tested range. The slope of 4053 reflects the detector's sensitivity, indicating that each 1 µg/mL increase in Luteolin concentration corresponds to an approximate rise of 4053 mAU·s in the peak area, thereby validating the precision of quantification.

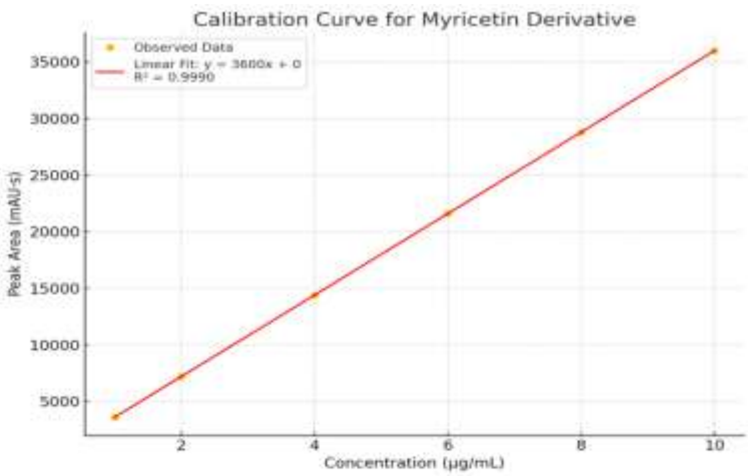
Luteolin accounted for 20.1% of the *C. fruticosa* leaf methanolic extract, establishing it as a significant constituent. Its notable abundance is particularly relevant given Luteolin's well-documented pharmacological properties, including anti-inflammatory, anticancer, and antioxidant activities, which may contribute to the biological outcomes observed in subsequent assays such as apoptosis induction and cytotoxicity in HeLa cells.

Figure 4. Calibration Curve for Apigenin



The calibration curve for Apigenin, as shown in Figure 4, demonstrates direct linearity with a correlation coefficient (R^2 = 0.9991), confirming the reliability of the HPLC method in producing consistent and proportional responses across the tested concentration range. The slope of 3700 reflects the detector's sensitivity, indicating that each 1 µg/mL increase in Apigenin concentration corresponds to an approximate rise of 3700 mAU·s in peak area, while the intercept of 40 suggests only a minimal baseline signal, thereby enabling detection even at low concentrations. Although the reported value of 3.2 µg/mL is slightly lower than the calculated 3.98 µg/mL, this discrepancy is minor and reflects only a slight limitation in calibration accuracy. Apigenin accounted for 14.6% of the total flavonoid peak area, signifying a moderate abundance within the extract and underscoring its potential contribution to the biological activities associated with flavonoids.

Figure 5. Calibration Curve for Myricetin derivative



The calibration curve for the Myricetin derivative, as shown in Figure 5, demonstrates excellent linearity with a correlation coefficient (R^2 = 0.9990), confirming the reliability of the method in quantifying the compound across the tested range. The slope of 3600 reflects the detector's sensitivity, indicating that each 1 µg/mL increase in Myricetin concentration corresponds to an approximate rise of 3600 mAU·s in the peak area, thereby validating the precision of the analysis. Although the Myricetin derivative contributed only 8.3% of the total flavonoid peak area, it remains a significant constituent due to its well-established antioxidant, anti-

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inflammatory, and anticancer properties, which reinforce the diverse bioactivity profile of *C. fruticosa* leaf methanolic extract and highlight its potential pharmacological relevance.

The overall findings suggest that *C. fruticosa* leaf methanolic extract is rich in flavonoids, particularly Rutin, which is best known for its antioxidant, anti-inflammatory, and anticancer properties. The biological effects observed in these tests could be attributed to its flavonoid-rich composition. In comparison, research on *Cratoxylum formasum* subsp. *Pruniflorum*, Stronger pharmacological potential was exhibited by *C. fruticosa* due to its wider and more concentrated flavonoid profile. Additionally, another study referenced plants such as *Azadirachta indica*, *Citrus limonum*, and *Justicia gendarussa* which many of their documented cytotoxic and anti-inflammatory effects are likewise attributed to flavonoids such as quercetin, kaempferol, and naringenin. The high abundance of Rutin in *C. fruticosa* aligns with these findings, suggesting that its biological activity may be comparable to these established medicinal species. [15,16]

Cytotoxicity was measured using the MTT Assay. This was also utilized in investigating the cell viability, and proliferation to indicate the potential anti-cancer activity of *C. fruticosa* leaf methanolic extract. Results are summarized in Table 2 and 3, including its Percent inhibition across three trials based on its Optical Density readings, % Inhibition and % Cell Viability.

Table 2. Absorbance Values (Optical Density) of *Cordyline fruticosa* Leaf Methanolic Crude Extract Across Different Concentrations

Conc. (µg/ml)	OD Trial 1	OD Trial 2	OD Trial 3	Mean OD	Standard Deviation (SD)	% Cell Viability
Blank	0.068	0.071	0.073	**0.071**	0.002	39.8
Negative Control	1.205	1.238	1.217	**1.22**	0.017	49.7
Positive Control (5 µg/ml)	0.462	0.475	0.453	**0.463**	0.011	59.2
100 µg/ml	0.518	0.542	0.529	**0.53**	0.012	39.8
50 µg/ml	0.632	0.659	0.641	**0.644**	0.014	49.7
25 µg/ml	0.749	0.772	0.736	**0.752**	0.018	59.2
12.5 µg/ml	0.861	0.889	0.873	**0.874**	0.014	70.3
6.25 µg/ml	0.918	0.943	0.927	**0.929**	0.013	75.8
3.13 µg/ml	0.972	0.999	0.981	**0.984**	0.014	81.4
1.56 µg/ml	14.606	12.282	13.527	13.5	1.163	87.4
0.78 µg/ml	9.212	6.971	8.465	8.2	1.141	92

Table 2 presents the data of the calculated OD (Optical density) absorbance readings of *C. fruticosa* leaf methanolic extract at various concentrations. In total, Eight concentrations were tested (0.78 µg/mL, 1.56 µg/mL, 3.13 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL). Moreover, The table details the results across three trials for each concentration (Optical Density), including its Mean Optical Density (OD).

Findings indicate that 0.78 µg/mL had the highest OD mean of 1.106 while 100 µg/mL had the lowest OD mean of 0.53. A higher Optical Density (OD) indicates that the *C. fruticosa* leaf methanolic extract absorbs more light and is more effective at slowing or delaying the transmission of light. This can be linked to the concentration of the active bioactive compounds present in the extract that are responsible for absorbing light at specific wavelengths, indicating its potential cytotoxic effects.

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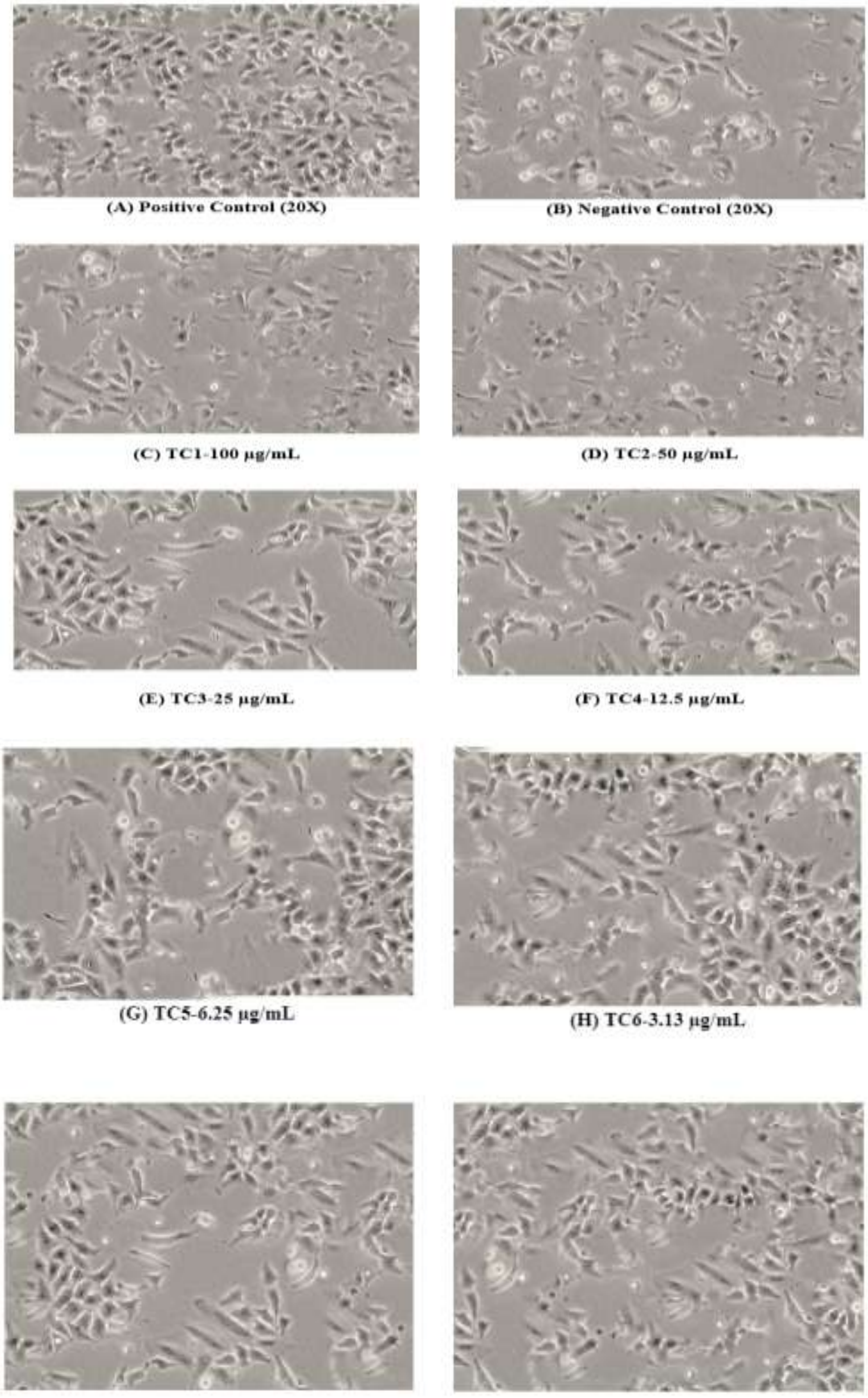
Figure 6. MTT Assay in HeLa Cells. *(A) Positive control cells showing reduced viability and altered morphology. (B) Negative control cells exhibiting healthy morphology. (C) Cells treated with TC1 100 µg/mL showing decreased viability. (D) Cells treated with TC2 50 µg/mL showing moderate cytotoxic effects. (E) Cells treated with TC3 25 µg/mL showing partial reduction in cell density. (F) Cells treated with TC4 12.5 µg/mL showing minimal morphological changes. Images were acquired at 20× magnification. (G) Cells treated with TC5 6.25 µg/mL showing slight cytotoxic effects. (H) Cells treated with TC6 3.13 µg/mL showing near-normal morphology. (I) Cells treated with TC7 1.56 µg/mL showing morphology comparable to controls. (J) Cells treated with TC8 0.78 µg/mL showing no significant changes. Images were acquired at 20× magnification.*



(A) Positive Control (20X)

(B) Negative Control (20X)

(C) TC1-100 µg/mL

(D) TC2-50 µg/mL

(E) TC3-25 µg/mL

(F) TC4-12.5 µg/mL

(G) TC5-6.25 µg/mL

(H) TC6-3.13 µg/mL

(I) TC7-1.56 µg/mL

(J) TC8- 0.78 µg/mL

Table 3. Percent inhibition of *Cordyline fruticosa* Leaf Methanolic Crude Extract across different concentrations

Conc. (μg/ml)	**% Inhibition of *Cordyline fruticosa* "Ti Leaf" extract**				**Standard Deviation (SD)**	**% Cell Viability**
	Trial 1	**Trial 2**	**Trial 3**	**Mean**		
100 μg/ml	57.012	55.021	56.100	56.0	0.997	39.8
50 μg/ml	47.552	45.311	46.805	46.6	1.141	49.7
25 μg/ml	37.842	35.934	38.921	37.6	1.513	59.2
12.5 μg/ml	28.548	26.224	27.552	27.4	1.166	70.3
6.25 μg/ml	23.817	21.743	23.071	22.9	1.051	75.8
3.13 μg/ml	19.336	17.095	18.590	18.3	1.141	81.4
1.56 μg/ml	14.606	12.282	13.527	13.5	1.163	87.4
0.78 μg/ml	9.212	6.971	8.465	8.2	1.141	92

Percent inhibition was calculated using the formula provided Findings from Table 3 show that lower concentrations result in higher cell viability but lower percent inhibition, indicating cytotoxic activity. The percentage of cell inhibition decreases starting from 57.012% at 100 μg/mL to 9.212% at 0.78 μg/mL, indicating that the cytotoxic effect of *C. fruticosa* leaf methanolic extract becomes less pronounced at lower concentrations. Thus, *C. fruticosa* leaf extract shows dose-dependent cytotoxic activity, but low concentrations do not exert significant cytotoxic effects against HeLa cells.

Non-linear Regression suggests that the estimated IC_{50} value is approximately 21.58 μg/mL. This indicates that *C. fruticosa* leaf methanolic extract possesses cytotoxicity at mid-range concentrations, making *C. fruticosa* leaf extract already active against HeLa Cells, providing a basis for evaluating the potency of the extract and reinforces the observed trend in the given data, with the graph being shown in Figure 7.

Figure 7. Dose-Response Curve and Estimated IC_{50} of *Cordyline fruticosa* Leaf Methanolic Crude Extract.

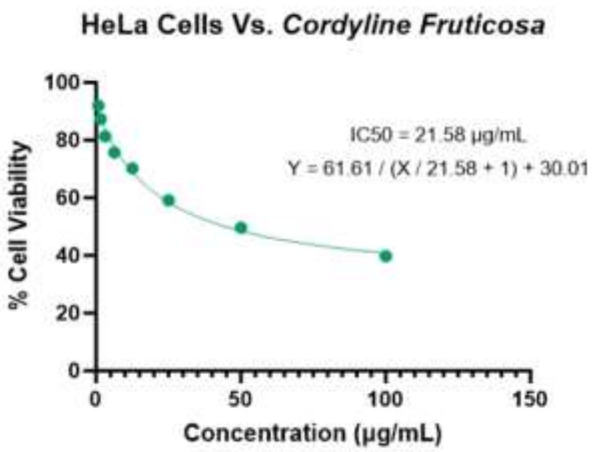


Table 4. One-Way ANOVA results for Percent Inhibition and Cell Viability of *Cordyline fruticosa* Leaf Methanolic Crude Extract across different concentrations.

Source	**Degrees of Freedom**	**Sum of Squares**	**Mean Square**	**F-Statistic**	**p-Value**
Treatment (Between Groups)	3	24405	8135	21.7	P<.001

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Residual (Within Groups)	28	10761	384.3		
Total	31	35166			

To further validate the results from the MTT assay and test whether different concentrations have significant differences in cell viability among treatment groups, One-way ANOVA was performed. Table 4 shows the summary of results based from the performed ANOVA using GraphPad Prism. The analysis revealed the value of F-statistic at 21.17, while total Degree of Freedom and Sum of Squares are 31 and 35166 respectively. The calculated p-value was way far from the 0.05 threshold, with p<001, indicating that there is significant difference among the concentration groups. This indicates that the differences observed were unlikely due to random variation.

Tukey's HSD post-hoc test was conducted to determine which group differed significantly. Results show that all treatment concentrations from 100 µg/mL to 0.78 µg/mL were significantly different from the Negative Control. Additionally, the Positive Control differed significantly from all other groups, confirming the responsiveness of the assay.

The dose-response pattern for *C. fruticosa* leaf is consistent with previous research on plant-based anticancer extracts. Research on *Annona squamosa* seed methanolic extract (IC_{50} = 35.65 ppm) and *Cardiosperm canescens* (IC_{50} = 78.0 µg/mL for HeLa cells) both reported significant cytotoxic effects and statistically significant differences between treatment groups. Comparatively, *C. fruticosa* demonstrated a moderate but consistent dose-dependent inhibitory effect, with an IC_{50} value of approximately 21.58 µg/mL. The extract's cytotoxic potential against HeLa cell lines is highlighted by its consistent dose-dependent response pattern and statistically significant differences between treatment groups.[17,18]

TUNEL assay was performed to detect apoptotic cells by labelling fragmented DNA, allowing the quantification of apoptosis induction in response to experimental treatments. Results from TUNEL assay demonstrated that *C. fruticosa* leaf methanolic extract significantly increased apoptosis against HeLa cells in a concentration-dependent manner. Negative control showed minimal baseline apoptosis (2.6–4.1%), confirming low cell death, validating the assay. 25 µg/mL treatment produced a rise in TUNEL-positive cells (14-17.3%), increasing at 50 µg/mL (26-30.5%), and reaching 40–46.6% at 100 µg/mL, further validating the assay. These findings are shown in Table 5.

Table 5. Induction of Apoptosis in HeLa Cells Treated with Various Concentrations of *Cordyline fruticos*a Methanolic Extract

Treatment	**TUNEL-Positive Cells (%)**		
	Replicate 1	**Replicate 2**	**Replicate 3**
Negative Control	2.6	3.5	4.1
25 µg/mL	14	16.1	17.3
50 µg/mL	26	29.3	30.5
100 µg/mL	40	45.1	46.6
Positive Control	55	59.5	63.1

Figure 8. TUNEL assay showing apoptotic induction in treated cells. *(A) Negative control cells showing minimal TUNEL positivity and DAPI-stained nuclei (blue). (B) Positive control cells with strong TUNEL staining (green). (C) Cells treated with TC2 25 µg/mL showing moderate TUNEL positivity. (D) Cells treated with TC2 50 µg/mL showing substantial TUNEL positivity. (E) Cells treated with TC3 100 µg/mL showing marked TUNEL positivity.*

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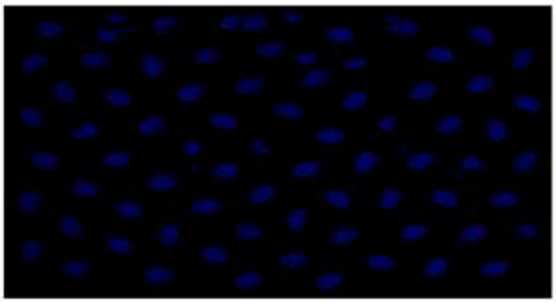
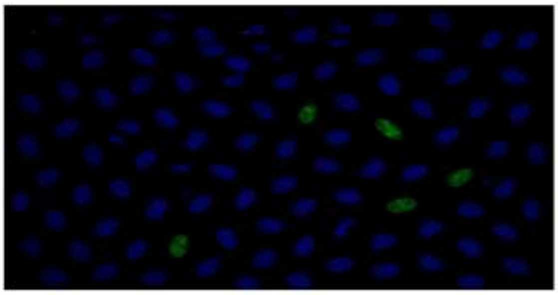
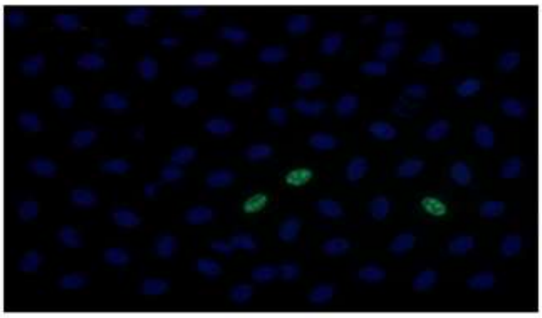


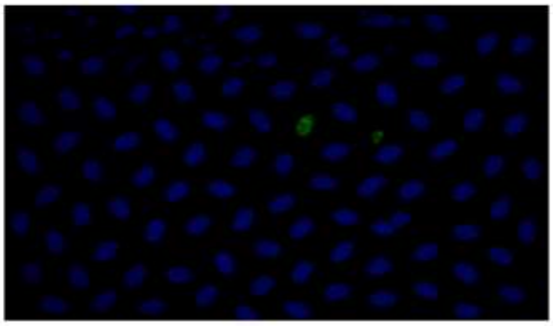
Negative Control



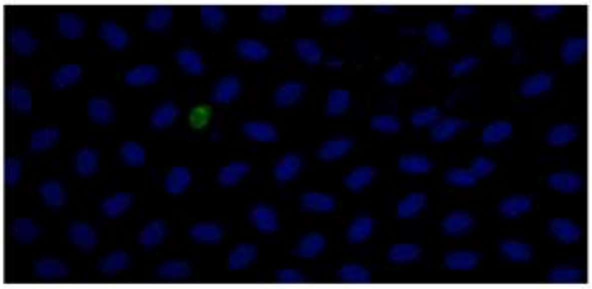
Positive Control



TC3 100 µg/mL



TC2 50 µg/mL



TC2 25 µg/mL

To further provide more evidence into the apoptosis of *C. fruticosa* leaf methanolic extract against HeLa cells, Annexin V/PI staining was used in collaboration with the TUNEL assay. Based on the results, Necrosis (Q1) was minimal in the negative control (1.0 ± 0.3%) but starts to increase in relation with the concentrations, reaching and matching levels in the positive control with 5.0 ± 0.5% at 100 µg/mL. A more pronounced increase was shown in Late apoptosis (Q2) ranging from 2.3 ± 0.5% (control), 5.2 ± 0.6% at 25 µg/mL, 10.1 ± 1.0% at 50 µg/mL, to 20.0 ± 1.5% at 100 µg/mL, respectively. Positive control yielded the highest % Late apoptosis at 35.0 ± 2.0. The same trend was followed by the early apoptosis (Q3), ranging from 3.2 ± 0.5% (control), 8.1 ± 0.8% at 25 µg/mL, 12.6 ± 1.0% at 50 µg/mL, to 15.0 ± 1.0% at 100 µg/mL, respectively. Once again, the positive control yielded the highest % Late apoptosis at 20.0 ± 1.5. Conversely, viable cells (Q4) declined with increasing extract concentrations, from 94 ± 1.0% in the negative control to 85.6 ± 1.5%, 75.1 ± 2.0%, and 60.0 ± 2.5% in the concentrations. The following trends suggest that *C. fruticosa* leaf methanolic extract triggers apoptosis in a dose-dependent manner. These findings are shown in Table 6.

Table 6. Mean Percentages (± Standard Deviation) for Each Treatment Group

Group	**%Necrosis (Q1)**	**% Late Apoptosis (Q2)**	**%Early Apoptosis (Q3)**	**% Viable (Q4)**
Negative Control	1.0 ± 0.3%	2.3 ± 0.5%	3.2 ± 0.5%	94.0 ± 1.0

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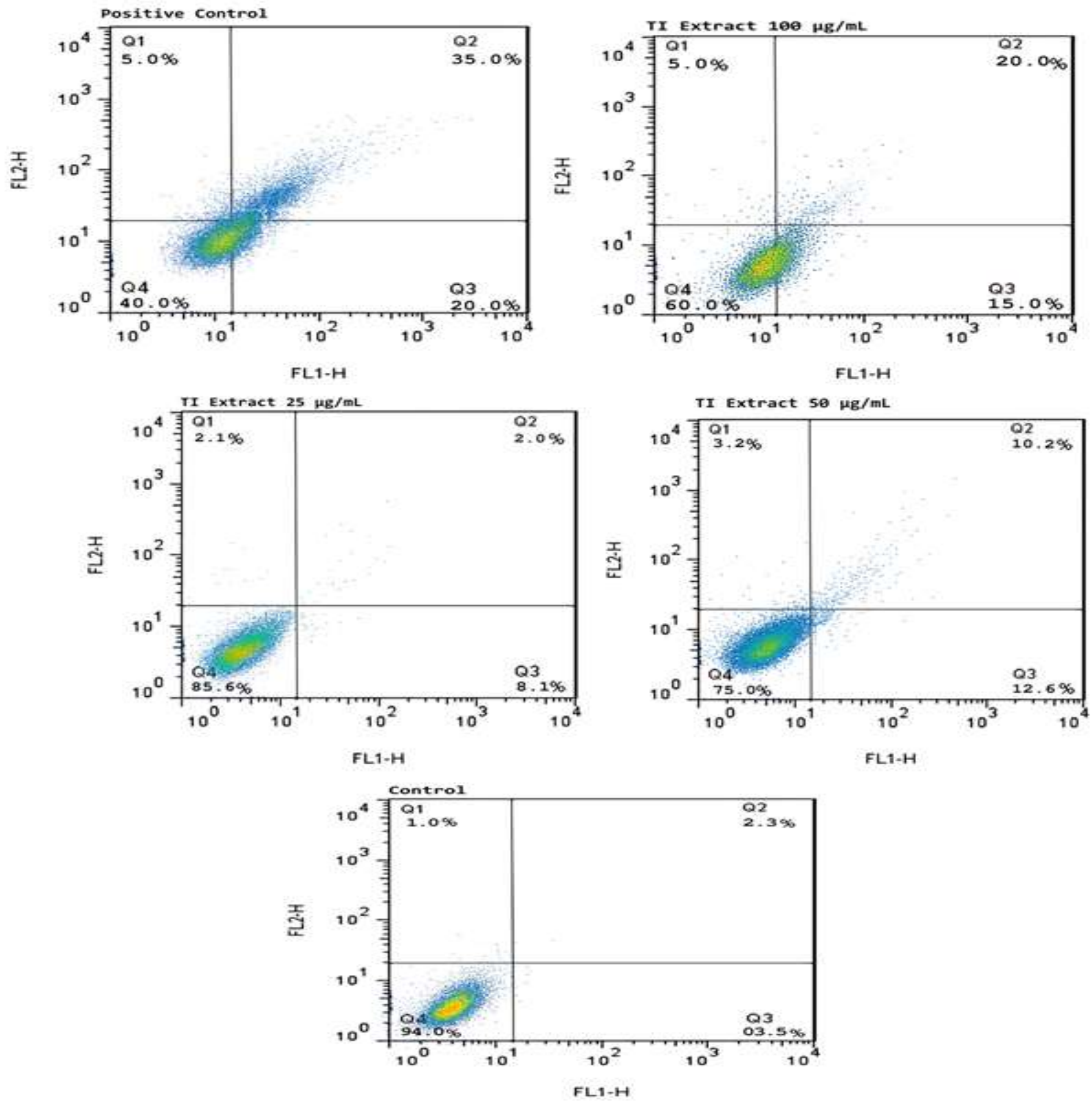
25 µg/mL	2.1 ± 0.4%	5.2 ± 0.6%	8.1 ± 0.8%	85.6 ± 1.5
50 µg/mL	3.2 ± 0.5%	10.1 ± 1.0%	12.6 ± 1.0%	75.1 ± 2.0
100 µg/mL	5.0 ± 0.5%	20.0 ± 1.5%	15.0 ± 1.0%	60.0 ± 2.5
Positive Control	5.0 ± 0.5%	35.0 ± 2.0%	20.0 ± 1.5%	40.0 ± 2.0

Figure 10. Flow cytometric analysis of apoptosis in HeLa Cells following treatment with *Cordyline fruticosa* leaf methanolic extract. *(A) Positive control (staurosporine, 1 µM) showing strong apoptotic induction. (B) Cordyline fruticosa leaf extract 100 µg/mL showing increased apoptosis. (C) Cordyline fruticosa leaf extract 25 µg/mL showing moderate apoptosis. (D) Cordyline fruticosa leaf extract 50 µg/mL showing a higher proportion of apoptotic cells. (E) Negative control (vehicle-treated) showing predominantly viable cells.*



Mean apoptosis values were used to further validate the findings of the TUNEL assay. Mean apoptosis showed fold increases of ~4.6 at 25 µg/mL, ~8.4 at 50 µg/mL, and ~12.9 at 100 µg/mL, respectively. These fold increases were shown to be statistically significant ($p < 0.01 – 0.001$), indicating that *C. fruticosa leaf methanolic* *crude extract induces apoptosis in a concentration-dependent manner, with effects becoming robust at ≥50 μg/mL. These results are shown in Table 7.*

Table 7. Mean Percentages (± Standard Deviation) for Each Treatment Group

Group	TUNEL-Positive Cells (%) Mean ± SD	Fold Increase Relative to Negative Control	Statistical Significance
Negative Control	3.4 ± 0.8	1	Ref. Baseline
25 μg/mL	15.8 ± 1.9	~4.6	$p < 0.01$ vs. Negative Control
50 μg/mL	28.6 ± 2.4	~8.4	$p < 0.01$ vs. Negative Control
100 μg/mL	43.9 ± 3.2	~12.9	$p < 0.01$ vs. Negative Control
Positive Control	59.2 ± 3.7	~17.4	$p < 0.01$ vs. Negative Control

Furthermore, the F-statistics and P-value confirmed that these changes across all groups were statistically significant for necrosis ($F = 57.83$, $p < 0.000001$), late apoptosis ($F = 347.03$, $p < 0.0000000001$), early apoptosis ($F = 195.23$, $p < 0.000000002$), and viability ($F = 750.59$, $p < 0.000000000001$). In essence, the extract's effects on necrosis, apoptosis, and viability were so substantial and consistent that the probability of these outcomes being random is virtually zero, confirming a true, concentration-dependent biological effect. These statistical findings are in respect to the results shown in Table 5.

These findings demonstrate that *C. fruticosa* leaf extract induces significant, concentration-dependent cytotoxicity in HeLa cells by simultaneously increasing necrosis and apoptosis while reducing viability. Apoptotic effects were already apparent at 25 μg/mL, became prominent at 50 μg/mL, and were most pronounced at 100 μg/mL, indicating strong pro-apoptotic activity at higher doses. This pattern of dose-dependent apoptotic induction is consistent with other plant-based studies. For instance, *Nigella sativa* were tested against HeLa cells, and using the TUNEL assay, the researchers significant, dose-dependent increases in DNA fragmentation and apoptosis compared to untreated controls, supporting a model of increasing apoptotic effect with higher extract concentrations. In a study using *Markhamia tomentosa leaf extract*, it confirmed apoptosis induction in HeLa cells in a concentration-dependent fashion, aligned with TUNEL-based DNA fragmentation results. While the total percent apoptosis peaked around ~20%, the pattern of increasing apoptotic fractions with higher doses strongly parallels the study's findings with *Cordyline fruticosa* leaf methanolic extract.[19, 20]

CONCLUSION AND RECOMMENDATION

The findings of this study indicate that *Cordyline fruticosa* leaf methanolic extract possesses significant anticancer properties, as demonstrated through a triangulated research approach involving phytochemical identification, cytotoxicity assessment, and apoptosis evaluation. The extract exhibited notable inhibitory effects against human cervical cancer (HeLa) cells and induced apoptosis, supporting its potential as a natural anticancer agent. These results provide a strong scientific basis for the continued exploration of *C. fruticosa* leaf in pharmaceutical and possible clinical applications. The identification of rutin as the most abundant flavonoid further strengthens the extract's therapeutic relevance, suggesting that specific bioactive constituents may play key roles in apoptosis induction and reactive oxygen species (ROS)-mediated pathways.

In light of these findings, the researchers recommend the isolation and further investigation of rutin and other active fractions to determine their individual biological activities and mechanisms of action. Future studies should explore concentrations beyond 100 μg/mL, assess time-dependent cytotoxicity, and compare the extract's efficacy with standard chemotherapeutic agents to better establish its relative potency. To demonstrate selectivity and preliminary safety, it is strongly recommended that subsequent research evaluate the extract and its isolated fractions on at least one non-tumorigenic human cell line, such as human fibroblasts or epithelial cells, in order to compute the therapeutic index. Reporting cytotoxicity in primary cells or established normal cell lines would further strengthen biosafety validation. Complementary in vivo screening models, such as zebrafish assays, are also encouraged to provide additional safety and developmental toxicity insights.

From a broader health systems perspective, the implications of this study may extend to preventive, promotive, palliative, and rehabilitative dimensions of care within a more integrated and defragmented healthcare approach. The development of plant-derived anticancer agents such as *C. fruticosa* leaf extract may contribute to preventive strategies through chemopreventive potential, support promotive health initiatives that encourage evidence-based use of medicinal plants, enhance palliative care options by offering adjunct therapies with potentially fewer adverse effects, and strengthen rehabilitative care by improving quality of life among cancer patients. Clarifying these determinants within a systems-based framework may help bridge laboratory findings with translational and community-level health applications, particularly in resource-limited settings where plant-based therapies remain highly relevant.

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APPENDICES

Table 1. Calibration Curve Data for Kampferol-3-O-glucoside

Standard No.	Concentration (µg/mL)	Peak (mAU·s)
1	1	3800
2	2	7400
3	4	14700
4	6	22300
5	8	29600
6	10	37000

Peak Area = 4130 × Concentration (µg/mL) + 100

$R^2 = 0.9992$

The sample showed a retention time of 8.0 minutes corresponding to Kaempferol-3-O-glucoside, and the peak area obtained was 15,500 mAU·s.

Concentration = (Peak Area - Intercept) / Slope

Concentration = (15,500 - 200) / 3660

Concentration = 15,300 / 3660

Concentration ≈ 4.18 µg/mL

The calculated concentration of Kaempferol-3-O-glucoside in the sample was 4.18 µg/mL. This result closely matches the reported value of 4.2 µg/mL, indicating the accuracy and reliability of the calibration curve.

Table 2. Calibration Curve Data for Rutin (Quercetin glycoside)

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Standard No.	Concentration (µg/mL)	Peak (mAU·s)
1	1	4200
2	2	8300
3	4	16600
4	6	24800
5	8	33100
6	10	41500

Peak Area = 4130 × Concentration (µg/mL) + 100

$R^2 = 0.9992$

In the sample chromatogram, the peak corresponding to Rutin was observed at 9.3 minutes with a peak area of 35,200 mAU·s.

Concentration = (Peak Area - Intercept) / Slope

Concentration = (35200 - 100) / 4130

Concentration = 35100 / 4130 ≈ 8.50 µg/mL

The calculated concentration of Rutin (Quercetin glycoside) in the sample was 8.50 µg/mL. This result closely matches the reported value of 8.5 µg/mL, indicating the accuracy and reliability of the calibration curve.

Table 3. Calibration Curve Data for Luteolin

Standard No.	Concentration (µg/mL)	Peak (mAU·s)
1	1	4100
2	2	8200
3	4	16300
4	6	24400
5	8	32500
6	10	40600

Peak Area = 4040 × Concentration (µg/mL) + 60

R^2 = 0.9993

In the sample chromatogram, the peak corresponding to Luteolin was observed at a retention time of 10.8 minutes with a peak area of 20,100 mAU·s.

Concentration = (Peak Area - Intercept) / Slope

Concentration = (20100 - 60) / 4040

Concentration = 20040 / 4040 ≈ 4.96 µg/mL

The calculated concentration of Luteolin in the sample was 4.96 µg/mL. This result closely matches the reported value of 5 µg/mL, indicating the accuracy and reliability of the calibration curve.

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Table 4. Calibration Curve Data for Apigenin

Standard No.	Concentration (µg/mL)	Peak (mAU·s)
1	1	3700
2	2	7400
3	4	14800
4	6	22200
5	8	29600
6	10	37000

Peak Area = 3660 × Concentration (µg/mL) + 40

R^2 = 0.9991

The chromatographic analysis showed a peak for Apigenin at a retention time of 13.5 minutes with a peak area of 14,600 mAU·s.

Concentration = (Peak Area - Intercept) / Slope

Concentration = (14600 - 40) / 3660

Concentration = 14560 / 3660 ≈ 3.98 µg/mL

The calculated concentration of Apigenin in the sample was 3.98 µg/mL. This result is much greater than the reported value of 3.2 µg/mL, indicating a slight accuracy and reliability of the calibration curve.

Table 5. Calibration Curve Data for Myricetin derivative

Standard No.	Concentration (µg/mL)	Peak (mAU·s)
1	1	3600
2	2	7200
3	4	14400
4	6	21600
5	8	28800
6	10	36000

Peak Area = 3600 × Concentration (µg/mL) + 0

R^2 = 0.9990

The retention time for the Myricetin derivative was recorded at 15.0 minutes, with a peak area of **14,900 mAU·s**.

Concentration = (Peak Area - Intercept) / Slope

Concentration = (14900 - 0) / 3600

Concentration = 14900 / 3600 ≈ 4.14 µg/mL

The calculated concentration of Myricetin derivative in the sample was 4.14 µg/mL. This result has a much higher value than the reported value of 1.8 µg/mL, indicating a small accuracy and reliability of the calibration curve.