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CYTOTOXIC AND APOPTOTIC EFFECTS OF NOVEL SYNTHESIZED FERULIC ACID DERIVATIVES ON LEUKEMIA CELL LINES

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ABSTRACT

Ferulic acid (FA), or 4-hydroxy-3-methoxycinnamic acid, is a phenolic compound that can be used in many pharmacological applications, especially in the area of cancer. In this study, four new FA-quinoline esters were successfully synthesized and characterized using ^{1}H-NMR, ^{13}C-NMR, HR-Mass, Elemental Analysis, and FT-IR techniques. Synthesized compounds were tested on leukemia cell lines (HL-60 and K562) with WST-1 assay and Annexin V-PI assay via flow cytometry. The WST-1 assay of compounds in HL-60 and K562 leukemia cells yielded IC50 124.4 and 205.4 μM/mL, respectively. According to the results, both the cytotoxic effect and apoptotic effects of 2c increased on HL-60 cell.

Keywords: Ferulic acid, Leukemia, Apoptosis, Cytotoxicity

1. INTRODUCTION

Phenolic compounds are secondary metabolites and can be found in many plant species, including vegetables and fruits and their extracts. In the numerous scientific studies conducted on phenolic compounds, many of them have been shown to have promising effects on several diseases, such as cancer [1], and to have anti-aging [2], anti-inflammatory [3], and anti-obesity [4] properties. Ellagic acid, gallic acid, and cinnamic acid are among the most commonly seen phenolic compounds in several plants.

Ferulic acid (FA), or 4-hydroxy-3-methoxycinnamic acid, is a phenolic compound which was first isolated from the Ferula foetida plant in 1866. It can be found abundantly in cereal grains, certain herbs, like broccoli, and in tomatoes, potatoes and fruits. The concentration of FA can reach up to 2g/kg in some plants at dry weight. In plants, FA is responsible for growth regulation, the uptake of minerals, and protection against insects, fungus and other damaging agents [5]. FA is a derivative of hydroxycinnamic acid, and the antioxidant property of FA comes from its hydroxyl and methoxy group located on the benzene part of the molecule [6]. According to their positive biological effects, FA and its derivatives have been reported to have many health benefitting properties, including anti-cancer [7], anti-microbial [8, 9], anti-inflammatory [10, 11], and anti-diabetic [12, 13].

Leukemia is one of the most aggressive hematologic malignancies. To date, except for acute promyelocytic leukemia and chronic myeloid leukemia, there are still no effective drugs for most types of leukemia [14]. In the past several years, certain phenolic compounds (e.g. FA, gallic acid, curcumin) have been found to inhibit the proliferation and induce apoptosis of leukemia cells [9].

In this study, we newly synthesized and characterized four different FA-hydroxyquinoline derivatives to study their anti-cancer and apoptotic activities. The 4-hydroxy-3-methoxycinnamic acid reacted with 2-hydroxyquinoline, 4-hydroxyquinoline, 6-hydroxyquinoline and 8-hydroxyquinoline to obtain four new FA esters. Characterized compounds anti-cancer and apoptotic activities were tested on HL60 and K562 cell lines.

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2. MATERIALS AND METHODS

2.1. Chemical

Reagents were commercially obtained from chemical companies and used without any purification. FA derivatives were synthesized under nitrogen atmosphere by applying conventional synthesis techniques with magnetic stirrer. Compounds were purified via recrystallization technique with suitable solvents. Proton and carbon nuclear magnetic resonance spectra were recorded on a Bruker 400 MHz spectrometer; FT-IR spectra were recorded with a Perkin Elmer Spectrum 100 Spectrometer; and mass spectra were recorded on a Shimadzu LCMS-IT-TOF spectrometer. A Stuart SMP-30 melting point apparatus was used to measure melting points.

2.2. Synthesis of Ferulic Acid Derivatives

Compounds **1c, 2c, 3c** and **4c** were synthesized using the same three-step synthesis procedure (Figure 1). In the first step, FA (0.62 mmol, 120 mg) was dissolved in 10 mL of *N*,*N*-Dimethylformamide (DMF), and potassium carbonate (K_2CO_3) (2.48 mmol, 387 mg) was added to the first solution and then stirred for 15 min. Benzyl bromide (4.34 mmol, 742 mg) was added drop by drop to the first solution and stirred under nitrogen atmosphere at 80°C for 24 h. The reaction process was periodically checked with thin layer chromatography (TLC). At the end of the reaction, the solution was neutralized with 5% hydrochloric acid (HCl), quenched with 50 mL distilled water and extracted three times with ethyl acetate. Combined organic solutions were washed several times with sodium bicarbonate and brine. The organic phase was evaporated to dryness over magnesium sulfate ($MgSO_4$). Pure **1a** was obtained from recrystallization with ethyl acetate/hexane (2:1).

In the second step, 1a (0.40 mmol, 150 mg) was suspended in 10 mL of methanol: water (9:1), potassium hydroxide (KOH) (8 mmol, 450 mg) and tetrahydrofuran (THF) (1.5 mL) were added to the suspension before the reaction was left under nitrogen at 70°C for 12 h. At the end of the reaction, the solution acidified with 5% HCl and was extracted three times with ethyl acetate. The organic phase was dried and evaporated. Pure **1b** carboxylic acid was obtained.

In the last step, 1b (1.9 mmol, 545 mg) was suspended in 10 mL of benzene and heated up to 80 °C for 15 min. Thionyl chloride (1.5 mL) was added drop by drop to the hot solution and stirred for 4h under reflux. The enzyme, 2-hydroxyquinoline (2.8 mmol, 406 mg), was dissolved in a separate flask in 10 mL of THF. Sodium hydride (NaH) (3.4 mmol, 130 mg) was then carefully added in pieces to this solution and stirred at room temperature for 30 min. Finally, evaporated benzene solution was dissolved in 10 mL of THF and transferred to the hydroxyquinoline solution. The mixture was stirred under nitrogen at 45 °C for 12 h. After the reaction was completed, the solvent was evaporated and the residue was dissolved in ethyl acetate and extracted several times with 1N sodium hydroxide (NaOH) to remove excess unreacted hydroxyquinoline and by-products. The organic phase was dried to obtain a solid **1c** ester.

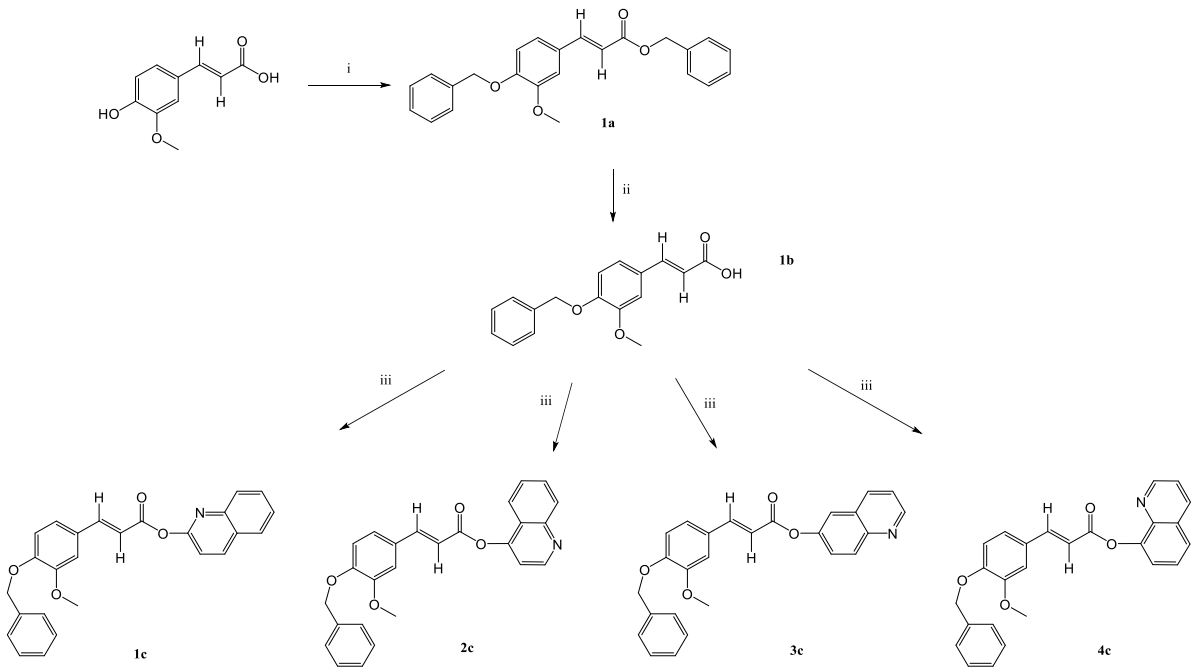

Figure 1. Synthesis procedure of compounds
Reagents and conditions: i) BnBr, DMF, K_2CO_3, 80°C for 24 h reflux; ii) MeOH:H_2O (9:1), THF, KOH, 70°C, 12 h, 5% HCl; iii) Benzene, 80 °C, 15 min., and THF, NaH, 45 °C for 12 h.

Quinolin-2-yl-(E)-3-(4-benzyloxy-3-methoxyphenyl) acrylate, 1c

White solid (421 mg, 54%), m.p.: 125 °C, FT-IR (KBr) cm^{-1}: 3068-3004, 1707, 1273, 1185, 1138, Anal. Calc. for $C_{26}H_{21}NO_4$ C=75.9; H=5.1; N=3.4; O=15.6; Found: C= 76.2, H= 4.7, N= 3.2, O= 15.9, ^{1}H-NMR:(400 MHz, $CDCl_3$-*d*, δ ppm): 8.25 (d, J=8.8 Hz, 1H), 8.02 (d, J=8.4 Hz, 1H), 7.87 (t, J=7.6 Hz, 2H), 7.73 (t, J=6.8 Hz, 1H), 7.55 (t, J=7.2 Hz, 1H), 7.37 (m, 6H), 7.11 (d, J=12.8 Hz, 2H), 6.9 (d, J=5.6 Hz, 1H), 6.53 (d, J=16.4 Hz, 1H), 5.21 (s, 2H), 3.94 (s, 3H), ^{13}C-NMR:(400 MHz, $CDCl_3$-*d*, δ ppm): 165.1, 150.9, 149.7, 147.5, 146.6, 139.8, 136.4, 130.1, 128.7, 128.6, 128.1, 127.5, 127.3, 127.2, 127.1, 126.5, 122.9, 115.8, 114.4, 113.3, 110.3, 105.4, 70.8, 56.0, MS $[M+Na]^+$: m/z 434.1360

Quinolin-4-yl-(E)-3-(4-benzyloxy-3-methoxyphenyl) acrylate, 2c

Yellow solid (507 mg, 65%), m.p.: 141 °C, FT-IR (KBr) cm^{-1}: 3065-2997, 1723, 1270, 1204, 1147, Anal. Calc. for $C_{26}H_{21}NO_4$ C=75.9; H=5.1; N=3.4; O=15.6; Found: C= 75.7, H= 5.3, N= 3.3, O=15.7, ^{1}H-NMR:(400 MHz, $CDCl_3$-*d*, δ ppm): 8.93 (s, 1H), 8.14 (d, J=8 Hz, 1H), 8.02 (d, J=8.0 Hz, 1H), 7.91 (d, J=15.6 Hz, 1H), 7.75 (t, J=7.2 Hz, 1H), 7.57 (t, J=7.2 Hz, 1H), 7.38 (m, 8H), 7.15 (d, J=10.0 Hz, 2H), 6.92 (d, J=7.2 Hz, 1H), 6.61 (d, J=16.0 Hz, 1H), 5.22 (s, 2H), 3.96 (s, 3H), ^{13}C-NMR:(400 MHz, $CDCl_3$-*d*, δ ppm): 164.3, 154.3, 151.0, 150.9, 149.8, 148.1, 130.0, 129.5, 128.7, 128.1, 127.2, 127.1, 126.8, 123.2, 121.4, 113.7, 113.3, 112.9, 110.3, 70.8, 56.1, MS $[M+1]^+$: m/z 412.1523

Quinolin-6-yl-(E)-3-(4-benzyloxy-3-methoxyphenyl) acrylate, 3c

Light brown solid (336 mg, 43%), m.p.: 142 °C, FT-IR (KBr) cm^{-1}: 3065-3000, 1715, 1274, 1205, 1140, Anal. Calc. for $C_{26}H_{21}NO_4$ C=75.9; H=5.1; N=3.4; O=15.6; Found: C= 76.0, H= 4.9, N= 3.3, O=15.8, ^{1}H-NMR:(400 MHz, $CDCl_3$-*d*, δ ppm): 8.9 (s, 1H), 8.13 (d, J=8.8 Hz, 2H), 7.83 (d, J=16.4 Hz, 1H), 7.64 (s, 1H), 7.53 (d, J=9.6 Hz, 1H), 7.37 (m, 6H), 7.12 (d, J=6.0 Hz, 2H), 6.90 (d, J=8.0Hz, 1H), 6.53 (d, J=16.0 Hz, 1H), 5.21 (s, 2H), 3.94 (s, 3H), ^{13}C-NMR:(400 MHz, $CDCl_3$-*d*, δ ppm): 165.6, 150.7, 150.1, 149.7, 148.7, 147.0, 146.2, 136.4, 135.9, 130.9, 128.7, 128.6, 128.1, 127.3, 127.2, 124.9, 122.9, 121.6, 118.4, 114.5, 113.3, 110.2, 70.8, 56.0, MS $[M+1]^+$: m/z 412.1536

Quinolin-8-yl-(E)-3-(4-benzyloxy-3-methoxyphenyl) acrylate, 4c

Light brown solid (374 mg, 48%), m.p.: 139 °C, FT-IR (KBr) cm^{-1}: 3065-2997, 1713, 1270, 1226, 1128, Anal. Calc. for $C_{26}H_{21}NO_4$ C=75.9; H=5.1; N=3.4; O=15.6; Found: C= 76.1, H= 5.2, N= 3.3, O= 15.4, ^{1}H-NMR:(400 MHz, DMSO-d_6, δ ppm): 8.86 (s, 1H), 8.43 (d, J=8.4 Hz, 1H), 7.90 (d, J=7.6 Hz, 1H), 7.81 (d, J=15.6 Hz, 1H), 7.59 (m, 3H), 7.51 (s, 1H), 7.38 (m, 6H), 7.09 (d, J=8.0 Hz, 1H), 6.94 (d, J=16.4 Hz, 1H), 5.14 (s, 2H), 3.83 (s, 3H), ^{13}C-NMR:(400 MHz, DMSO-d_6, δ ppm): 165.7, 150.9, 150.7, 149.7, 147.6, 147.0, 141.1, 137.1, 136.6, 129.5, 128.9, 128.4, 128.3, 127.5, 126.8, 126.4, 123.7, 122.5, 122.1, 115.2, 113.4, 111.3, 70.2, 56.1, MS $[M+1]^+$: m/z 412.1523

2.2 Cell Culture

The compounds 1c, 2c, 3c and 4c were subjected to WST-1 assay to determine the cytotoxic activity. The compounds were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 25, 50, 100, 200, and 400 μM. HL-60 human acute promyelocytic leukemia (ATCC® CCL-240™) cells, K562 human chronic myelogenous leukemia (ATCC® CCL-243™) cells were grown in DMEM with heat in activated 10% FBS, 100 mg/mL penicillin, 100 mg/mL streptomycin, and 1% L-glutamine. Cells were grown in a humidified atmosphere of 95% air/5% CO_2 at 37°C.

2.3. Determination of Cytotoxicity by WST-1 Method

WST-1 (4- [3- (4-Iodophenyl) -2- (4-nitrophenyl) -2H-5-tetrazolio] -1, 3-benzene disulfonate) is a tetrazolium salt which specifically binds to the succinate-dehydrogenase enzyme in the mitochondria of living cells and converts to water-insoluble formazan salts. The absorbance value measured spectrophotometrically in the WST-1 method indicates the metabolic activities of the cells in culture, and this value is related to the number of living cells. As the proliferation increases, the absorbance increases with the formation of formazan salt [15]. K562 and HL-60 cells were treated through decreasing concentrations (25, 50, 100, 200,400 μM) after 24 h of incubation with an equal number of (5×10^3) cells [16]. They were left to incubate for 24 hours. At the end of the 24-hour incubation period, 20 μL of WST-1 reactivity was added to the cells in each well, and the cells were incubated for 3 hours in the incubator. At the end of the incubation period, absorbance values at 420 nm, determined using the Cytation 3 Cell Imaging Multi-Mode Reader, were read as 7 replicates (7 wells, 1 blind) for each concentration.

2.4. Annexin V-PI Apoptosis Assay by Flow Cytometry

Propidium iodide (PI) and Annexin V are used to detect the viability of cells from differences in the integrity and permeability of plasma membranes of apoptotic and necrotic cells. PI is used more often than other core dyes due to its stability and economy and its capacity of being a good indicator for cell viability. The release of phosphatidylserine from apoptotic cells inside the healthy cell membranes via disintegration of the cell membrane can be observed with Annexin V-PI to show late stages of cell death, or necrotic cells [17]. To carry out this study, the protocol of the Annexin V FITC Apoptosis Detection Kit (catalogue no: 556547, BD) was applied. HL-60 and K562 cells were seeded in medium with 6 well plates (1×10^5 cells in each well). IC50 concentrations calculated for FA derivatives according to the results of WST-1 were prepared and given to the cells. Cells in the control group were treated with 0.1% DMSO-containing nutrient. Plates were then allowed to incubate for 24 hours. At the end of the incubation period, the cells in each of the 6 wells were removed and centrifuged at 1200 rpm for 5 minutes. The supernatant was then removed and kit procedure instructions were applied. Samples were analyzed on a flow cytometer (Becton-Dickinson Acuri C6).

3. RESULTS AND DISCUSSION

3.1. Characterization of Compounds

The synthetic pathway of the four new FA esters is depicted in Scheme 1. Synthesized molecules are stable and in a solid state at room temperature conditions. The yield, melting point, infrared spectroscopy, elemental analysis, nuclear magnetic resonance and high-resolution mass values of the synthesized compounds are given in the experimental section. Elemental analysis results and high-resolution mass spectroscopy measurements were in agreement with suggested structures.

According to the infrared spectroscopy of the four FA derivatives, aromatic hydrogen (C_{Ar-H}) and carbonyl bond (C=O) vibrational peaks were present between 3068-2997 cm^{-1} and between 1723-1707 cm^{-1}, respectively. The ester bond (O=C-O-) and pyridine part (C-N) bond vibrational peaks were observed around 1270 cm^{-1} and 1100 cm^{-1}. The ether bonds vibrational peaks observed around 1128 cm^{-1} verify the suggested structures of FA esters.

In the ^{1}H-NMR spectra of compounds, the methoxy group (-OCH_3) and the methylene (-CH_2-) part of the benzyl group appeared as a singlet around 3.8 and 5 ppm; the aromatic ring protons were observed between 9-6 ppm. Ethylene protons coupling constants found to be around 16 Hz indicated that compounds have trans isomerism. ^{13}C-NMR spectrum of four compounds exhibited twenty-four signals around 165 to 56 ppm. Carbonyl groups (C=O) signals observed around 165 ppm. Signals observed around 70 and 56 ppm are related to methylene (-CH_2-) and methyl (CH_3-) groups proving that the molecules had the expected structures.

Due to the isomeric nature of four synthesized compounds, their high resolution mass results were obtained at the same values for 2c, 3c and 4c around *m/z*=412.15 and *m/z*=434.13 for 1c (due to the sodium ionization).

3.2. Cytotoxic and Apoptotic Activities

All synthesized compounds were evaluated for their *in vitro* cytotoxic activity against human myelocytic leukemia cell lines HL-60 and K562. The compounds (1c-4c) were submitted to WST-1 assay to determine cytotoxic activity. The results are given in Table 1. Doxorubicin was used as a positive control. Doxorubicin is a widely used anticancer drug in cell lines related to leukemia [18].

It was used as a positive control because it is a drug in our laboratory.

Table 1: Cytotoxicity of compounds 1c, 2c, 3c, 4c against HL60 and K562 cells as IC50 values (μM/mL).

Compound	HL60 cell line	K562 cell line
1c	387.2±4.08	422.8±2.45
2c	124.4±2.11	205.4±6.39
3c	275.1±1.87	488.9±2.77
4c	298.4±3.56	436.1±3.61
Doxorubicin	102.4±8.54	419.4±5.48

FA regulates cell growth and proliferation, stimulates cytoprotective enzymes, and inhibits cytotoxic systems in both *in vitro* and *in vivo* experimental models [19-20]. The apoptotic effects of 1c, 2c, 3c and 4c IC50 concentrations, which were analyzed for HL60 and K562 cells based on Annexin V-PI binding capacities in flow cytometry, are depicted in Fig 2-3. Following the flow cytometric analyses, apoptotic effects (early and late apoptotic effects) of 1c, 2c, 3c and 4c IC50 concentrations were calculated as 2.1, 27.5, 12.9 and 14.8 percent, respectively, while their necrotic effects were calculated as 0.1, 1.3, 14.1 and 3.9 percent, respectively. As seen in Fig. 3 and Table 2, the apoptotic effects of 2c especially were found to have increased. According to these findings, 2c increase on HL-60 cells apoptotic effects.

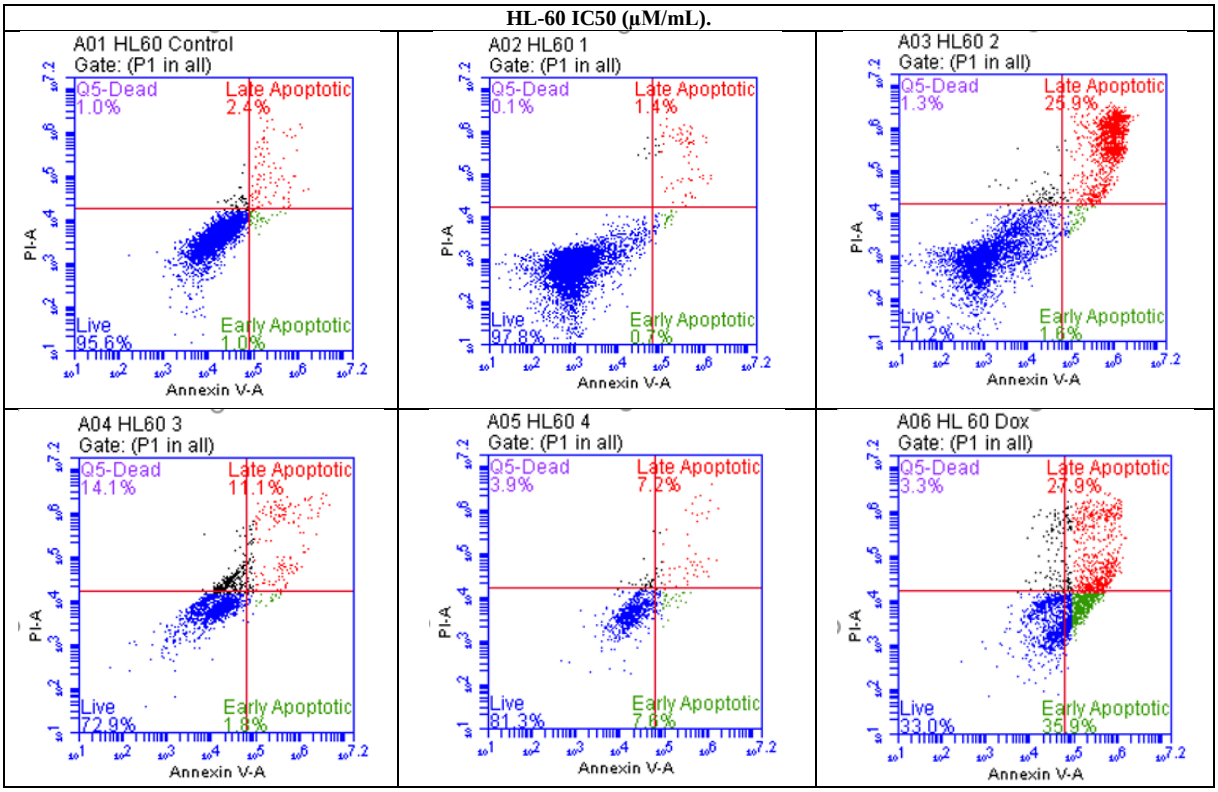

Figure 2. Flow cytometric results. Early and late apoptotic, living and dead cell percentage of HL-60 after 1=1c, 2=2c, 3=3c, 4=4c and doxorubicin treatment with IC50 values at the end of the incubation period.

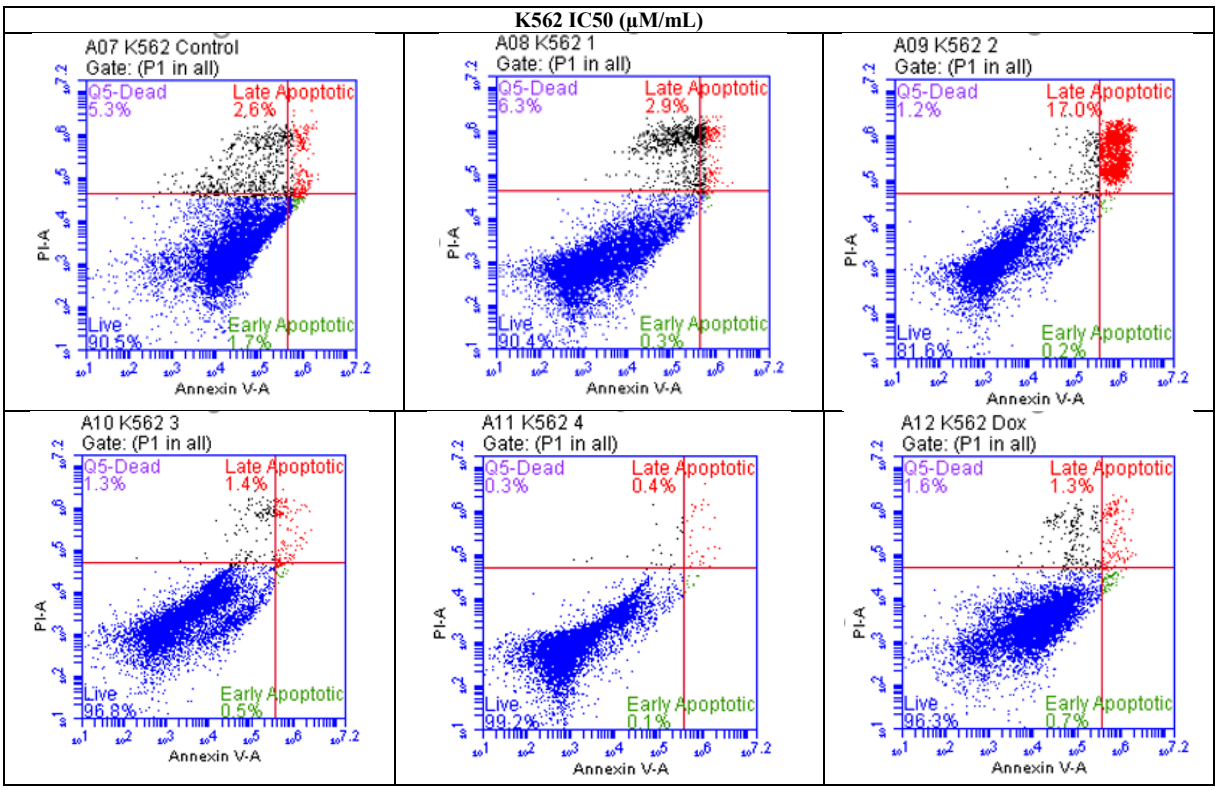

Figure 3. Flow cytometric results. Early and late apoptotic, living and dead cell percentage of K562 after 1=1c, 2=2c, 3=3c, 4=4c and doxorubicin treatment with IC50 values at the end of the incubation period.

Table 2: Comparison of percentages of 1c, 2c, 3c, 4c and doxorubicin viable, early apoptotic, late apoptotic, and necrotic cells on HL60 cell line stained with Annexin V-propidium iodide using flow cytometry.

HL60 cell line	(Live)%	(Dead)%	Apoptotic (Early and Late) %
1c	97.8±2.21	0.1±0	0.7+1.4=2.1±0.25
2c	71.2±3.05	1.3±0.01	1.6+25.9=**27.5**±2.5
3c	72.9±2.02	14.1±1.14	1.8+11.1=12.9±2.16
4c	81.3±3.09	3.9±0.5	7.6+7.2=14.8±1.85
Doxorubicin	33.0±2.26	3.3±0.4	35.9+27.9=63.8±3.69

According to flow cytometric analyses, the apoptotic effects (early and late apoptotic effects) of 1c, 2c, 3c and 4c IC50 concentrations were calculated as 3.2, 17.2, 1.9 and 2.0 percent, respectively. Doxorubicin was used as positive control of cytotoxic and apoptotic activities. Compound 2c was more active than doxorubicin on K562 cell line. The results are given in Table 3. To evaluate the extent of the apoptosis, annexin V-FITC/PI dual staining followed by FACS analysis was carried out. However, there are some studies that have failed to show this apoptotic activity. For example, Rajendran et al. [16] have shown that a series of ferulic acid compounds caused apoptosis in K562 and U937 cell lines associated with bcr-abl kinase domain inhibited expression compared different anticancer drug imatinib. This leads to apoptosis and the proliferation of the leukemia cells will be controlled. In the light of this work, it can be said that ferulic acid has anticancer and apoptotic potential.

Table 3: Comparison of percentages of 1c, 2c, 3c, 4c and doxorubicin viable, early apoptotic, late apoptotic, and necrotic cells on K562 cell line stained with Annexin V-propidium iodide using flow cytometry.

K562 cell line	(Live)%	(Dead)%	Apoptotic (Early and Late) %
1c	90.4±1.03	6.3±0.01	0.3+2.9=3.2±0.6
2c	81.6±2.08	1.2±0.8	0.2+17.0=**17.2**±2.5
3c	96.8±0.8	1.3±0.2	0.5+1.4=1.9±0.25
4c	99.2±0.2	0.3±0.05	0.1+0.4=0.5±0.37
Doxorubicin	96.3±1.01	1.6±0.4	0.7+1.3=2.0±1.02

We have synthesized four ferulic acid derivatives because of the difference in the quinoline is connected in different positions. The active compound 2c is bonded at 4 positions. Quinoline is a pharmacologically valuable scaffold that is a prevalent in a variety of biologically active synthetic and natural compound [21]. Quinolines has been the subject of intense study and different interesting bioactivity such as antibacterial, antimalarial and anticancer activities [22-23].

CONCLUSION

In conclusion, four new FA-quinoline esters were synthesized via esterification between FA and 2,4,6 and 8-hydroxyquinoline respectively. All of the compounds was obtained in good yields and characterized successfully with several spectroscopic techniques. The compounds were found to be effective against HL-60 and K562 cell lines. Especially, compound 2c exhibited highest anticancer activity than the others and twice as much doxorubicin on K562 cells. It was concluded that, anticancer activity directly influential with compounds stereochemistry.

CONFLICT OF INTEREST

There is no conflict of interest, financial or otherwise for the authors.

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